SMARCA4/BRG1-INACTIVATING MUTATIONS AS POTENTIAL PREDICTIVE MARKERS FOR AURORA KINASE A-TARGETED THERAPY IN NON-SMALL CELL LUNG CANCERS

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DEDICATION

In memory of my uncle, Mevlut Albayrak

Dedicated to all young scientists

SMARCA4/BRG1-INACTIVATING MUTATIONS AS POTENTIAL PREDICTIVE MARKERS FOR AURORA KINASE A-TARGETED THERAPY IN NON-SMALL CELL LUNG CANCERS

by

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SMARCA4/BRG1-INACTIVATION MUTATIONS AS POTENTIAL PREDICTIVE MARKERS FOR AURORA KINASE A-TARGETED THERAPY IN NON-SMALL CELL LUNG CANCERS

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SMARCA4 encodes a catalytic subunit of the SWI/SNF chromatin remodeling complex, BRG1. Frequent occurrence of SMARCA4/BRG1-inactivating mutations and their mutually exclusive nature from EGFR and ALK lesions create one of the largest subsets of Non-Small Cell Lung Cancers (NSCLCs). Since these mutations have been identified as bona fide tumor suppressors, efforts have focused on understanding the pathology of cancer caused by SMARCA4/BRG1 aberrations. However, no therapeutic agent has been identified as synthetically lethal with

SMARCA4/BRG1 loss. Utilizing genome-wide high-throughput small interfering RNA (siRNA)-based screening, we show here that Aurora kinase A (AURKA) activity is essential in NSCLCs carrying SMARCA4/BRG1-inactivating mutations. RNAi-mediated depletion or chemical inhibition of AURKA induces apoptosis and diminish cellular viability in SMARCA4/BRG1-mutant NSCLC cells in vitro and in mouse models. The relation between SMARCA4/BRG1 inactivation and increased requirement for AURKA appears to be due to the impairment of functional centrosomes. Thus, AURKA-centered, centrosome-independent, mitotic spindle assembly machinery becomes solely responsible for mitotic spindle formation and proper chromosome segregation during mitosis. DLG7, the only known protein specific to this centrosome-independent mitotic spindle assembly, is required for the survival and proliferation of cells with inactivated SMARCA4/BRG1. Depletion of DLG7 causes no effect in SMARCA4/BRG1-proficient cells, but significant decrease in cell viability occurs in SMARCA/BRG1-deficient NCI-H1819 cells and this cytotoxic effect can be rescued with the restoration of wild-type SMARCA4/BRG1 expression. Altogether, our findings identify AURKA inhibition with VX-680 as a candidate therapeutic strategy for biomarker-driven clinical studies to treat the NSCLCs harboring SMARCA4/BRG1 inactivation mutations, which account for approximately 35% of all NSCLC cases. Furthermore, these observations suggest a previously unrecognized concept of redundancy for mitotic

spindle assembly machinery that has a potential use for cancer therapeutics.

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LIST OF ABBREVIATIONS IN ALPHABETICAL ORDER

ABL – Abelson gene

ALK - Anaplastic lymphoma kinase

APC - Anaphase Promoting Complex

ATP - Adenosine triphosphate

AURKA - Aurora kinase A

BAF - Brahma Associated Factor

BCR - Break point cluster

BRG1 - Brahma-related gene 1

BRM - Brahma

CDK4 - Cyclin dependent kinase 4

CDKN2A - cyclin dependent kinase inhibitor 2A, p16INK4A

CML - Chronic myelogenous leukemia

DLG7 - Discs, large (Drosophila) homolog 7

DNA – Deoxyribonucleic acid

EGF - Epidermal growth factor

EGFR - Epidermal Growth Factor Receptor

FDA - Food and drug administration

GTP- Guanosine triphosphate

HBEC - Human bronchial epithelial cell

hTERT - Human telomerase reverse transcriptase

LKB1 - Liver kinase B

MTOC - Microtubule organizing center

nM - Nanomolar

NOD/SCID - Non-obese diabetic/

NSCLC - Non-Small Cell Lung Cancer

PAK1 - p21 protein (Cdc42/Rac)-activated kinase 1

PARP - Poly ADP ribose polymerase

PBS - Phosphate buffered saline

PEG-300 - Polyethylene glycol

PLK1 - Polo like kinase 1

QLQ - Glutamine/Leucine/Glutamine

RAN - RAS-related nuclear protein

Rb -Retinoblastoma

RNA - Ribonucleic acid

RNAi - RNA interference

siRNA - single interfering RNA

SMARCA - SWI/SNF-related matrix-associated actin-dependent

regulator of chromatin

SNF - Sucrose non-fermenting

Thr288 - Threonine 288

TP53 - Tumor protein p53

TPX2 - Targeting protein for Xklp2

uM – Micromolar

VEGFR - Vascular endothelial growth factor receptor

CHAPTER I

INTRODUCTION

A Brief History of Cancer Research and Therapeutics

In contrary to the common notion that cancer is a modern disease, cancer has existed since the evolution of complex life forms. Besides observing within the living fauna and flora, paleopathologic findings reveal that prehistorical animals and plants had tumors long before the evolution of homo sapiens (Hajdu, 2011a). However, the occurrence of cancer in humans gradually changed over time. Safe living environments, easy access to nutrient-rich diets and high-quality medication increased life standards and life span. Eventually, these adjustments raised the issue of inevitable degeneration of human tissues by age and made cancer one of the most common lethal diseases in the world today.

In the history of human disease, abnormal growths from the human body parts were observed and reported as early as the first writing systems were developed. The historical findings indicated that cancer was as old as the evolution of human beings and the very first records in medicine about the cases of cancer could be seen in the first written documents from Egypt, in around 3000 BCE (Breasted, 1930). Although the causes of

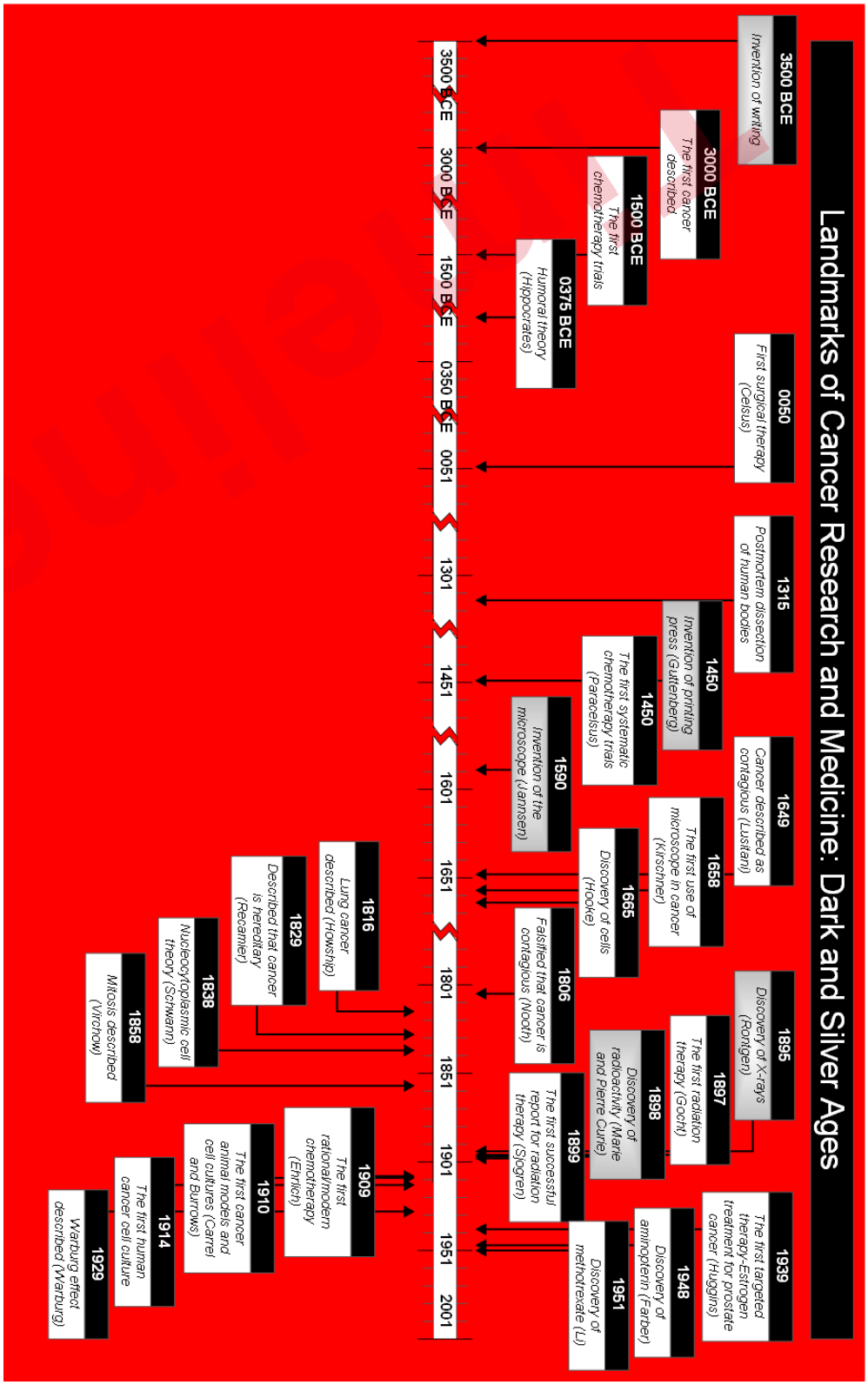


Figure 1-1. Timeline of the important events impacting the cancer research and therapeutics between 3500 BCE and 1953 (Scientific events are colored white, technological events are colored gray)

cancer remained completely mysterious until middle ages, historical records show that the very first trials to treat tumorigenesis started in 1500 BCE with primitive surgical methods such as cautery, removal with knives or the first chemotherapies by applying herbal, mineral or arsenic remedies (Ebbell, 1937). In the 300s BCE, Hippocrates, also the originator of the name “cancer” for the disease, made the first known description of cancer and revealed his thoughts about its initiation. He emphasized the role of natural causes rather than superstitious and supernatural explanations as the origin of cancer and his now discredited humoral theory was considered as the basis of all diseases including cancer until the discovery of cell theory in the 1800s CE (Virchow, 1858). Humoral theory suggested that a human body is a combination of bodily fluids including blood, phlegm, black and yellow bile. Excess or deficiency of any components caused a disorder and can be fixed only by balancing them (Hippocrates & Littré, 1839). For many centuries, this theory was adopted by other physicians in many different civilizations.

Failure in describing the causes of cancer did cause a delay in understanding the biology of cancer and therapies involving chemicals, but not operative techniques. In 50 CE, Celsus, a Roman physician, introduced the first rational surgical methods to remove tumors (Spencer,

1926). Since then, surgical therapy has been perfected and become an effective method for all type of cancers.

Despite some interest and attempt in understanding the basis of cancer, technical and informational limitations held the discoveries at the minimal level for almost a millennium. After this dark age, cancer research again gained a peak in 1300s, which coincides with the beginning of the anatomy era and the first post-mortem dissection of human bodies in 1315 in Italy (Hajdu, 2011b). Starting from this landmark event, in about 400 years the anatomy and physiology of human body were almost perfectly understood. These advances in medicine also overlapped with the introduction of the movable-type printing in 1450 by Guttenberg. Scientific findings were, in a rapid way, reported in the forms of written documents or books, providing the rapid delivery of new information to the scientists, physicians and surgeons of the era.

Subsequently, the first systematic use of chemicals against cancer was initiated in the 1500s. The primary chemotherapy used by Paracelsus, a Swiss physician-chemist, contained a special combination of mercury, lead, sulfur, iron, zinc, copper, arsenic, iodine and potassium. Paracelsus was also the first physician who described the poisonous/side effects of

the chemotherapy if not used at proper concentrations of each ingredient (Hajdu, 2005; Paracelsus, 1562).

In 1649 and 1652, two physicians shared the conclusions from their population studies and reported that cancers were contagious (Lusitani, 1649; Tulp, 1652). Although they were disproved later in the history for the majority of cancers, these reports are considered as the first expression of the idea that cancer is a genetic disease and inherited cancer genes cause familial cancers.

The Renaissance movement provided advances not only in art, but also in technology and medicine. Parallel to the developments in anatomy and physiology, following the invention of the microscope in 1590, the discovery of cells by Robert Hooke in 1665 affected the improvements in understanding the cancer disease at the cellular level and led to a tremendous amount of new discoveries in the discipline of pathology (Gest, 2004). Kirscher in 1658 introduced this technology to the cancer medicine (Hajdu, 2011b). Tumor pathology simultaneously became a routine for the detection of cancerous tissues. Overall, starting from the invention of the printing press and postmortem analysis of human bodies, human anatomy, physiology and pathology gained a great advance in the process of understanding and treating cancers.

These advances until 18th century prepared the ground to discovering the details of the macroscopic and microscopic nature of the cancers. All the discoveries in this and the next centuries increased the knowledge about the cellular biology of tumorigenesis. In 1806, James Nooth, a surgeon from England, disproved the earlier claim that cancer is a contagious disease by injecting himself with chopped breast cancer pieces. The failure with this experiment led him report that cancers do not spread similar to the contagious diseases such as bacterial infections (Nooth, 1806). Joseph Recamier in 1829 reported the hereditary nature of some cancers and indicated the first solid clues about the connection of cancer to genetics (Recamier, 1829). Another big discovery of this century was made by Theodor Schwann in 1838. He significantly improved the cell theory by demonstrating for the first time that tissues are composed of cells containing a nucleus and cytoplasm (Schwann, 1838). This novel description of the normal cells led to identifying abnormal microscopic features of cancers such as multi-nuclei or abnormal nucleus-to-cytoplasm ratio. After accumulated findings on the cellular structure of normal and cancerous cells, microscopic pathology was deliberately integrated into the diagnosis of cancer and John Bennett, an English pathologist, in 1849 stated that cancers could be detected with full certainty by utilizing the microscope (Bennett, 1849). Clearly, Bennett's statement transformed the

use of the microscope from its exclusive utilization for research to its use for diagnosis, which is still the most effective way to pinpoint the cancerous tissues in vital organs.

Further, advances in cellular microscopy in the 19th century led to another landmark discovery in human medicine. In 1858, Rudolph Virchow introduced his new report revealing that all cells come from existing cells (Virchow, 1858; Virchow & Chance, 1860). This cell doctrine opened a new era in the disciplines of biology and medicine by disproving the 2000-year-old 4 component humoral theory (Hajdu, 2012). In the path to discover DNA and understand the role of genetics in life and diseases, Walther Fleming, in 1882, made another big contribution by discovering chromatin (Flemming, 1882). In addition to the advances in pathology and cellular biology, lung cancer was also described for the first time in this period in 1816 by J. Howship, a British surgeon (Howship, 1816). Before entering the 20th century, science prepared the ground for more discoveries to understand cancer and novel approaches to cure it.

Throughout all this time, failure with internal and external use of home remedies as primitive chemotherapy and lack of any other curative options made the surgical approaches dominate cancer medicine. However, in 1896, Wilhelm Rontgen, a physicist from Germany, made a breakthrough

shift in almost all disciplines of sciences by discovering x-rays, namely radiation (Rontgen, 1896). This new technology was rapidly transformed into medicine and the anti-tumor features of x-rays were discovered in 1897 (Gocht, 1897-1898). 4 years after radiation therapy was introduced as a treatment option for cancers, the first reports showed successful treatment of carcinomas with radiation in 1899 (Sjogren, 1899). Almost simultaneously with Rontgen, in 1896 and 1898, Antoine Becquerel and the Curies discovered the first radioactive substances, uranium and radium, and made another immediate contribution to the improvement of cancer diagnosis and therapies (Becquerel, 1896; Pierre Curie, 1898).

In the same era, the first known rational chemotherapy was introduced by Paul Ehrlich, a German biochemist and immunologist. His expertise in microbiology made him become a strong advocate of the use of chemicals in all diseases including cancer. In this regard, he attempted to destroy cancer cells in rats by injecting pyocyanase and selenium, two chemicals also used against bacterial infections. These two drug candidates were withdrawn later due to the low efficacy and intolerable side effects (Ehrlich, 1909). Although Ehrlich didn't have potent chemicals to utilize, his observations with pyocyanase, selenium and some others revealed the first discovery of acquired chemoresistance and he reported in 1909 that cancers are heterogeneously composed of chemically sensitive and

resistant cells and therefore, a uniform response to treatment may not be expected (Ehrlich, 1909). His studies acknowledged two fundamental facts about cancer therapy, which is the potential curative effect of chemicals and the inevitable acquired resistance against them.

The discovery of the latter two therapy options alternative to surgery, radiation and chemo-therapy, in one decade attracted a great deal of interest. However, the technical difficulties with radiation and the scarcity of chemotherapy options did not change the dominance of surgical therapies for another 3 decades (Hajdu & Darvishian, 2013). In this period, cancer research gained a huge peak and important discoveries were made to understand the causation of cancer. The experiments of Theodor Boveri in 1914 demonstrated that cancer is initiated by chromosomal mutations (Boveri, 1914). The use of animals in cancer studies increased after Peyton Rous induced cancer in hens with cell-free filtrates of the original sarcoma in 1910 and in rats by exposure to x-rays, viruses or coal tar (Pierre Marie, 1910; Rous, 1910a, 1910b). In the same year, Alexis Carrel and Montrose Burrows from New York succeeded to establish the first tissue culture lines and grow tumor cells derived from the Rous hen sarcoma in vitro (Carrel, 1910). These two platforms, animal models and cell cultures, eased and revolutionized the research on cancer and are currently still used.

Otto Warburg, a biochemist from Germany, reported for the first time that oxygen is a prerequisite for the functional normal tissues and when oxygenation was lowered, the shift to fermentation may cause cancer. This phenomenon, now recognized as “Warburg effect”, explained the differences in ATP homeostasis of normal and cancerous tissues(Warburg, 1929). In parallel to the discoveries in cancer metabolism, the genetic origin of cancer was debated without any solid evidence for a significant period of years. Finally, Warthin reported the first proof on the heredity of cancer in 1913 (Warthin, 1913).

After the introduction of the concept that chemicals should be used in cancer therapies by Ehrlich at the end of 19th century, many pioneers of chemotherapy tested various chemicals such as alcohol, arsenic, jodoform, selenium, potassium chloride, osmium, pyoctanin and thallium in cancer medicine (Hajdu, 2005). Chemical approaches and advances in radiologic techniques gained a significant speed starting from the 20th century. This period also adopted the statistics as a side discipline and the first reports revealed that cancer was the second leading cause of death in the US and incidences of certain cancers such as lung carcinomas dramatically increased (Haggard, 1932). The exponential growth of knowledge in physiological and cancer biology contributed to the entry of the “Silver Age” of cancer research and the first half of the 20th century

harbored more research in the cancer field than the previous millenniums combined. The emergence of microscopic and other technological advances and perfection of the operative techniques dramatically accelerated the basic and clinical cancer research before entering the DNA era.

There have been two fundamental questions since the first description of cancer. What causes cancer and how to cure it remained to be asked and searched throughout the human history. After the increase in the use of animal models and tissue cultures, many carcinogens and cancer-promoting viruses were identified. In addition, anti-cancer chemicals were sought and these efforts created a new specialty, named medical oncology. The first chemotherapy agents showed promising results in the model systems. However, these chemicals such as urethane or nitrogen mustard also had intolerable side effects in humans (Berman & Axelrod, 1948; Jacobson, Spurr, & et al., 1946). Sidney Farber, an American pediatric pathologist, focused on anti-folates based on his observations correlating leukemia incidence rate with folic acid-rich diets. His first success came with the folic acid antagonist aminopterin in 1948 (Farber & Diamond, 1948). Although this therapy did not show significant survival advantages in the patients, his post-mortem examinations revealed that aminopterin significantly reduced the number of abnormal blood cells.

These observations inspired another scientist, Min Chiu Li to discover a more potent anti-folate agent, methotrexate, in 1951 (Hertz, Li, & Spencer, 1956). Methotrexate became one of the most potent chemotherapy agents and it is still in use for cancers including lung carcinoma.

In 1953, the breakthrough discovery in the life sciences was made by identifying the structure and constitution of DNA and the “Golden Age” of cancer research began (Franklin & Gosling, 1953; Watson & Crick, 1953). DNA and genetics not only explained how the codes of life are stored and inherited by the next generations, but also revealed the platform to understand the causes of many diseases, including cancer. In the post-DNA years, several influential advances were reported. Perhaps the most important was the complete sequence of the human genome in 2001 (Lander et al., 2001; Venter et al., 2001). The capability of sequencing the full-length DNA transformed the field of genetics into the field of genomics. This technique was rapidly adapted by all disciplines concerned with genetic contribution to the diseases and the first genome-scale discoveries for cancer were reported in 2008 for AML and lung carcinomas (Campbell et al., 2008; Ley et al., 2008).

In the latest half-century, after understanding the basic science behind cell division, research for alternative cytotoxic chemotherapy agents focused

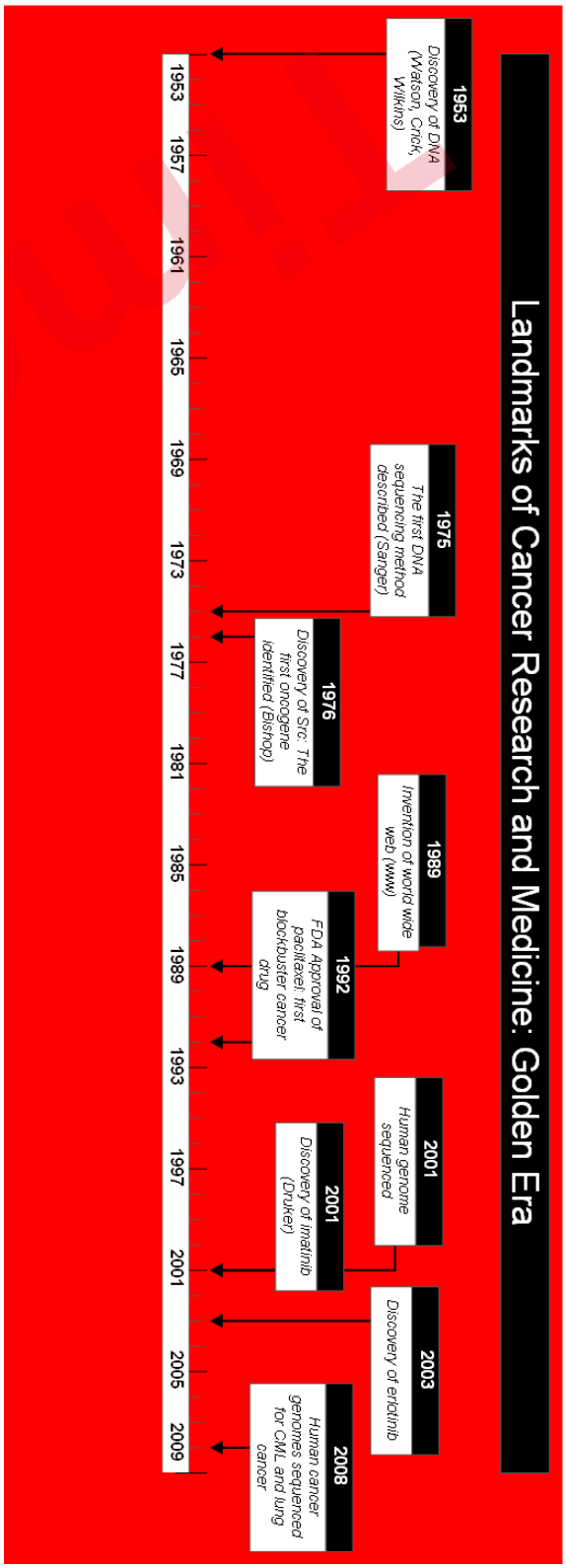


Figure 1-2. Timeline of the important events impacting the cancer research and therapeutics between 1953 and 2014 (Scientific events are colored white, technological events are colored gray)

on blocking the necessary mechanisms for progression through the cell cycle, such as DNA replication or microtubule assembly. Promising results with the early chemotherapy regimens like methotrexate, cisplatin, cyclophosphamide and fluorouracil were obtained (Chabner & Roberts, 2005). Paclitaxel, a microtubule poison, was approved by FDA in 1992 and rapidly became the first blockbuster anti-cancer drug (Chabner & Roberts, 2005). Post-DNA era studies also yielded applicable results at the onset of the latest millennium, and in 2001, Brian Druker provided elaborate evidence to get the first FDA-approved targeted therapy, imatinib, to inhibit the BCR-ABL fusions in chronic myelogenous leukemia (CML) (Druker, 2001). The success with imatinib paved the way for many other promising targeted therapies, such as erlotinib, an EGFR inhibitor, and bevacizumab, a VEGFR inhibitor, among many new targeted therapies developed to enrich the pool of cancer therapeutic options. Today, thanks to all these advances in the last 5000 years of known cancer research history, cancer is found to be a genetic disease and identified cancer driving machineries led the discoveries of targeted therapies as well as cytotoxic chemotherapies that are designed to block rapid growth of the cells. As of today, 45 targeted therapies (Appendix 1) and 40 chemotherapy agents (Appendix 2) are FDA-approved for use of cancer therapeutics (source: cancer.gov).

The Challenges in Cancer Medicine and The Need For Personalized Cancer Therapy

In the current clinical settings, oncologists need to overcome the challenge of effectively matching the correct treatment option with the patient. Successfully combating cancer requires powerful diagnostic techniques capable of categorizing patients correctly and predicting the most efficient drug among the existing pool of anti-cancer agents (Piquette-Miller & Grant, 2007). This approach appears to be the most rational strategy to improve the margin of success in the cancer medicine today.

Novel technologies make large-scale genomic, proteomic or metabolic profiling systems possible to get comprehensive information about the individual cancers. Among potential prognostic tools, pharmacogenomics provide the most promising capacity to identify individual patients with the highest probability of benefiting from an anti-cancer medicine due to the genetic origin of cancers. In the last two decades, the discoveries of targeted-therapies against mutated and activated oncogenes such as ABL, EGFR, ALK and so on, emphasized the importance of better understanding the genetic changes in the whole genome scale for cancer cells derived from patients.

Currently, in parallel to the search for novel drugs in clinical medicine to enrich the pool of anti-cancer agents, the goal of pharmacogenomics is to characterize new genetic alterations as predictive markers for either existing drugs or known drug targets. The growing need for genomic ways to match the individuals with the most beneficial drug demands more understanding of the genetic mutations in cancers and their strong association to the acquired targetable vulnerabilities.

The Role of SWI/SNF Chromatin Remodeling Complex in Normal and Cancer Physiology

The genes encoding the subunits of the SWI/SNF chromatin remodeling complex were identified initially in two independent yeast genetic screens to find genes that regulate either mating-type switching phenotype or sucrose metabolism, respectively (Carlson, Osmond, & Botstein, 1981; Stern, Jensen, & Herskowitz, 1984). These studies elucidated gene sets called mating-type SWItching (SWI) or Sucrose Non-Fermenting (SNF) genes. Because SWI genes significantly overlapped with the SNF gene set, all genes identified in both screens and others identified later were combined as SWI/SNF genes. Further studies revealed that the activity of SWI/SNF genes affects approximately 7-8% of all yeast transcriptome(Monahan et al., 2008; Sudarsanam, Iyer, Brown, &

Winston, 2000; Zraly, Middleton, & Dingwall, 2006). Two third of these genes are regulated by activation and the remaining subgroup is repressed via SWI/SNF-mediated activity (Sudarsanam et al., 2000).

The SWI/SNF chromatin remodeling complex controls a large number of cellular processes including embryonic development, cell differentiation, proliferation, DNA replication and repair. Initially, how SWI/SNF complex functions was demonstrated in *Drosophila melanogaster* by characterizing the role of Brahma (BRM) on the transcriptional activation of Hox genes, which regulate the body plan of embryos (Tamkun, 1995). Further studies showed that BRM is involved in chromatin remodeling and it is homologous to the yeast SWI/SNF gene SNF2 (Elfring et al., 1998; Papoulas et al., 1998). More specifically, BRM functions in the body segmentation process during the development of fruit flies through regulating Hox genes. Elucidation of the functions of other SWI/SNF complex components in the same mechanism suggested a potentially universal link between embryological development in multicellular organisms and SWI/SNF chromating remodeling activity.

In mammalian systems, two closely related BRM homologs were identified. In addition to BRM, Brahma-related gene-1 (BRG1) is expressed in higher vertebrates. Homology studies demonstrated that

BRG1 is 74% identical to BRM (Khavari, Peterson, Tamkun, Mendel, & Crabtree, 1993). However, their biological functions are mostly non-redundant and not interchangeable as shown with *in vitro* and *in vivo* models. Homozygous Brg1 knock-out in mouse models is embryonic lethal and in heterozygotes potentiates epithelial tumors whereas Brm knockouts are viable with no significant predisposition to cancer (Bultman et al., 2000; Reyes et al., 1998). Evolution of BRG1, in humans and other vertebrates suggest that organisms harboring BRG1 in their genome more likely have functionally more diverse SWI/SNF chromatin remodeling activity than lower eukaryotes.

No data, thus far, have revealed that BRG1 and BRM are both assembled in the same individual SWI/SNF complex, suggesting a mutually exclusive nature of these two subunits. Tremendous research has been conducted on the components of SWI/SNF complex since its first discovery and these studies revealed that SWI/SNF remodels the chromatin in an ATP-dependent manner. The energy for this process is provided by the catalytic activity of BRG1 or BRM for each complex, explaining why they are critical for the remodeling activity (Hassan, Neely, Vignali, Reese, & Workman, 2001; Muchardt & Yaniv, 1999).

BRG1 and BRM have high degree of homology in protein sequence as well as their DNA sequences with 6 out of 7 protein domains conserved among both. These include ATPase, Rb binding, BROMO, helicase/SANT-associated (HAS), proline-rich and QLQ domains. In addition to these, BRM has an N-terminal glutamine-rich region with an unknown function (Bourachot, Yaniv, & Muchardt, 1999; Dahiya, Gavin, Luo, & Dean, 2000; Martens & Winston, 2003; Muchardt & Yaniv, 1999).

The SWI/SNF complex is a master regulator of gene expression. Recent studies showed that up to 10% of the human transcriptome is dependent on its activity (Medina et al., 2005). Rather than having relationships to particular pathways, the SWI/SNF chromatin remodeling complex takes place as an essential regulator of a diverse and mostly unrelated group of cellular mechanisms. For that reason, the specific role is not yet defined of inactivating mutations in the genes encoding SWI/SNF complex that are frequently found in cancer. However, BRG1 is closely linked with the constant proliferation and self-renewal capabilities whereas BRM is predominantly expressed after differentiation (Reisman, Sciarrotta, Bouldin, Weissman, & Funkhouser, 2005). The first evidence about the involvement of the components of the SWI/SNF complex in cancer demonstrated that SNF5 is frequently mutated in pediatric rhabdoid tumors, which arise mainly in kidney and brain (Versteege et al., 1998). In

addition, heterozygous knock-outs of SNF5 in mice caused tumors with similar characteristics to human rhabdoid tumors (Roberts, Leroux, Fleming, & Orkin, 2002). These initial data confirmed that SNF5 and possibly other SWI/SNF components acted as bona fide tumor suppressors.

However, surprisingly, SNF5 mutations are not common in adult cancers. Instead, major lesions appear to be the inactivation of other SWI/SNF complex components including, BRG1, BAF180, BAF250a, BAF250b, BAF155, BAF60, BAF57 and BAF200 (Romero & Sanchez-Cespedes, 2013). Among those, BRG1 mutations were identified first in a variety of cancer cell lines (Wong et al., 2000) and lung primary tumors (Medina et al., 2004; Medina et al., 2008). Further large scale profiling studies confirmed that BRG1-inactivating mutations occur in more than 25% of non-small cell lung cancer lines. This finding made BRG1 one of the most commonly mutated genes with TP53, CDKN2A, LKB1 (Blanco et al., 2009; Ding et al., 2008). Overall, BRG1 and all other SWI/SNF mutations cover the largest subset of NSCLC among all mutations. The widespread existence of alterations at genes encoding chromatin remodeling regulators including SWI/SNF complex, histone deacetylases or DNA methylases reveal the epigenetic deregulation as a novel hallmark of cancer (Ding et al., 2008).

Mitotic Spindle Assembly Machinery: Its Regulation and Its Role on Normal Cell and Changes with Cancer

Mitotic spindles are tightly regulated and formed by centrosome-dependent and independent machineries in animal cells. The earliest descriptions of centrosomes were based on their localization during mitosis(Mitchison, Evans, Schulze, & Kirschner, 1986). Since they were found to be present at the polar regions of the mitotic spindles and supernumerary centrosomes resulted in multipolar cell division, centrosomes as the primary microtubule organizing centers (MTOCs) were thought to be solely responsible for mitotic spindle assembly (Brinkley, 2001; Nigg, 2002). Indeed, astral microtubules are generated by the centrosomes during mitosis and these microtubules actively search for the chromosomes to capture them at their kinetochore regions. After all chromosomes are connected to the microtubules aligned from both poles of the cell, the typical bipolar spindle shape is formed in normal somatic cells (Rieder, 1990). This “search-and-capture” model for the mitotic spindle assembly by centrosomes explained the accuracy of chromosome segregation in mitosis in most cells. However, plants and oocytes naturally lack centrosomes, creating a gap in full understanding of spindle formation machinery requiring another mechanism that might assist the centrosome-dependent pathway. The intrinsic capability of microtubules to form

spindles in acentrosomal cells was successfully shown in Xenopus eggs (Heald et al., 1996). Furthermore, the key regulators of this centrosome-independent spindle machinery, the small GTPase RAN and its effector TPX2, were found to localize to chromatin and trigger microtubule formation in the absence of centrosomes (Carazo-Salas et al., 1999; Ohba, Nakamura, Nishitani, & Nishimoto, 1999; Wilde & Zheng, 1999). Identification of the chromosome-mediated spindle assembly pathway provided an explanation for successful cell division of acentrosomal cells. In this pathway, the gradient of RAN in the vicinity of the chromosomes creates the platform for nucleation of new microtubules. The challenge of aligning these newly formed microtubules in one direction from the chromosomes to the poles is overcome by use of motor and microtubule bundling proteins(Wadsworth & Khodjakov, 2004).

Cell division and mitotic spindle assembly are highly complex machineries involving an orchestrated group of proteins. Among many components, Aurora kinase A is a major regulator of both centrosome-dependent and –independent spindle assembly pathways. The human Aurora-A gene maps to 20q13 and this region is frequently amplified in cancers (Bischoff et al., 1998; Zhou et al., 1998). Overexpression of Aurora kinase A causes centrosome abnormalities and chromosome instability, indicating its potential role in cancer progression (Anand, Penrhyn-Lowe, &

Venkitaraman, 2003; Kufer, Nigg, & Sillje, 2003; Kufer et al., 2002; Zhou et al., 1998). The expression of Aurora kinase A is strictly regulated during the cell cycle. Although its expression levels are low in G1 and S phases, a dramatic increase occurs starting from G2 and reaches the maximum level at the onset of M phase(Kimura et al., 1997; Zhou et al., 1998). At the late anaphase stage of mitosis, anaphase-promoting complex (APC) begins to degrade Aurora A so that it gradually reaches the basal levels in G1 phase (Honda et al., 2000; Taguchi et al., 2002).

Aurora kinase A activity is dependent on its autophosphorylation of Thr288 in its activation loop (Walter, Seghezzi, Korver, Sheung, & Lees, 2000). A conformational change occurs due to its binding to cofactors such as TPX2, BORA, AJUBA and so on (Eyers, Erikson, Chen, & Maller, 2003; Hirota et al., 2003; Hutterer et al., 2006). Upon activation, Aurora kinase A initiates a signaling cascade to nucleate new microtubules and regulate bipolar mitotic spindle assembly.

The evolutionarily conserved functions of Aurora kinase A are well established in different organisms including yeast, *C. elegans*, *D. melanogaster* and humans (Berdnik & Knoblich, 2002; Hannak, Kirkham, Hyman, & Oegema, 2001). In specific cell types, Aurora kinase A depletion causes significant failure in the nucleation of centrosomal

microtubule levels. However, some cell types like neuroblasts or S2 cells in fruit flies do not need Aurora A functions (Giet et al., 2002). These opposing observations raise a fundamental question about the role of Aurora kinase A.

CHAPTER II

SMARCA4/BRG1-INACTIVATING MUTATIONS AS POTENTIAL PREDICTIVE MARKERS FOR AURORA KINASE A-TARGETED THERAPY IN NON-SMALL CELL LUNG CANCERS

Introduction

The goal of personalized cancer medicine is to effectively match the correct therapy with the correct patient. However, due to lack of key prognostic methods, current therapies are incapable of guiding the right therapy option to the individual patient and unfortunately, yield little-to-no overall survival advantage as of today (Anguiano, Nevins, & Potti, 2008; Meric-Bernstam & Mills, 2012). Lung cancer is a prime example of marginal success in cancer therapeutics. Despite the discovery of 7 novel anti-cancer agents and a total of 12 FDA-approved drugs in use, the mortality associated with this disease has not changed significantly in the last quarter century (Anguiano et al., 2008). This is in part due to the fact that the current therapeutic strategies in patient care consider large patient populations as homogenous groups, regardless of inter-individual variations. It is becoming clear that the ability to identify susceptible patient subtypes for each of the existing therapeutic strategies is as essential as the discovery of new-generation cancer therapeutic agents.

Pharmacogenomics provides the potential to create rational strategies to appropriately categorize patients and predict sensitivity to therapy by identifying genetic changes in tumors that can be linked to “druggable” vulnerabilities. Therefore, current research in the practice of oncology attempts to build a reliable database of linkages between genetic repertoires of tumors and existing therapeutic strategies (Garraway, 2013).

In the last decade, the feasibility of the genomics-driven cancer therapeutics approach has been validated by successfully tailoring therapies to increase positive outcomes. One example is EGFR-targeted therapies in lung cancers with EGFR-activating mutations (Lynch et al., 2004; Paez et al., 2004). Another is the success in treating tumors expressing ALK fusion proteins with ALK-targeted therapies (Kwak et al., 2010). These successes suggest that research on genomic changes in cancers shall be conducted not only to understand the etiology of the disease, but also to identify concurrent targetable vulnerabilities for killing cancer cells, while sparing normal cells. In this regard, SMARCA4/BRG1(henceforth BRG1)-inactivating mutations are attracting interest as predictive genetic markers for acquired vulnerabilities, especially in NSCLCs. Somatic mutations in the gene encoding BRG1 protein occur in 15-35% NSCLC patients (Ding et al., 2008; Medina et al.,

2004; Medina et al., 2008). Accumulating evidence suggests that BRG1 has a tumor suppressor role for many cancer types (Romero & Sanchez-Cespedes, 2013). Its inactivation is predicted to interfere with the cellular functions of ATP-dependent SWI/SNF chromatin remodeling complex due to the fact that BRG1 drives the SWI/SNF complex activity as one of the two mutually exclusive ATPase subunits(Wilson & Roberts, 2011). SWI/SNF complexes have widespread and essential functions in controlling gene expression and regulate a large variety of cellular processes. SWI/SNF complex formation mobilizes histone octamers in an ATP-dependent manner to allow or repress gene transcription ((Saha, Wittmeyer, & Cairns, 2006a, 2006b). Loss of BRG1 creates changes in approximately 10% of mammalian transcriptome, increasing the likelihood for the loss of functions that might be redundant in normal cells and non-redundant in tumors lacking BRG1.

To identify targetable gene products related to BRG1-inactivating mutations, we developed and applied a high throughput, cell-based, one-well/one-gene screening platform with a genome wide library of chemically synthesized small interfering RNAs. Using this approach, among many hits identified, we found that Aurora kinase A, which concentrates on microtubule organizing centers (MTOCs) during mitosis and is required for

mitotic spindle assembly, is a potential therapeutic target for NSCLCs harboring BRG1-inactivating mutations (Barr & Gergely, 2007).

Results

To seek candidates for targeted therapy that are synthetically lethal to loss of BRG1 in NSCLCs, we conducted a whole-genome high-throughput screen of small interfering-RNAs (siRNAs) in a cell line from a panel of NSCLC-derived cell lines established and extensively characterized by Drs. John Minna and Adi Gazdar (Figure 2-1). At the onset of designing our primary screen, 16 NSCLC lines in this panel were known to harbor homozygous BRG1-inactivating mutations (Table 1). From these we chose NCI-H1819 (henceforth H1819) because it had no detectable wild-type BRG1 protein (Figure 2-2), lacked mutations in the three other genes, p53, EGFR and RAS, most commonly detected as mutant in NSCLCs, and might therefore produce a phenotype most reliant on loss of BRG1.

Small interfering RNA (siRNA) transfections were performed in triplicate with 50 nM of each pool of 4 individual siRNA duplexes targeting 21,124 genes and cell viability was measured after 96-hours. We identified 46 genes whose depletion inhibited the growth or survival of H1819 cells by more than 50% (Figure 2-3, Appendix 1). From this list, we excluded 8 genes that had been found to be toxic in a previous screen performed with

Table 1. Other Important Mutations in BRG1-mutant Non-Small Cell Lung Cancers

(✓ mutant, × wild-type)

Cell line	BRG1	TP53	KRAS	EGFR
H1819	✓	×	×	×
A427	✓	×	✓	×
A549	✓	×	✓	×
H2030	✓	✓	✓	×
H1299	✓	✓	×	×
H157	✓	✓	✓	×
H1703	✓	✓	×	×
H2126	✓	✓	×	×
H838	✓	✓	×	×
H841	✓	✓	×	×
H1693	✓	✓	×	×
H661	✓	✓	×	×
H1573	✓	✓	×	×
H23	✓	✓	×	×
H522	✓	✓	×	×
HCC15	✓	✓	×	×

a wild-type BRG1-expressing immortalized human bronchial epithelial cell line, HBEC30-KT (Figure 2-2, Appendix 1) ((Ward et al., 2012). Finally, we identified a set of 38 genes that are critical for the growth or survival of BRG1-mutant H1819 line. We performed a secondary validation assay with fresh pools of siRNAs targeting these genes and 22 out of 38 reiteratively confirmed the toxic phenotype when depleted (Figure 2-4). The hits were separated into high-priority and low-priority categories with 50% toxicity cut-off in the secondary validation screen. Due to higher significance, we focused on the high-priority hits in the next step of our tests.

To distinguish cytotoxic from cytostatic effects, we tested this high-priority hit group for a caspase 3/7 activity test and identified 7 siRNA pools that induced apoptosis in H1819 line after knock-down (Figure 2-5). The increased apoptosis with these hits was also confirmed with immunoblotting against cleaved PARP (Figure 2-6). Previous reports showed that inactivation of BRG1 in cells derived from non-cancerous tissue causes mitotic catastrophe as the primary defect (Bourgo et al., 2009). Among the 7 cytotoxic siRNA pools for H1819 cells, TPX2 was of immediate interest because it is directly linked to mitosis and Aurora kinase A (AURKA), for which inhibitors are already available in clinical trials (Figure 2-7) (Harrington et al., 2004; Kufer et al., 2002).

To exclude the possibility of off-target effects with the pooled siRNA duplexes in the library, we obtained individual siRNAs to confirm our screen results with TPX2 knock-down. We first checked TPX2 protein levels after transfection with these individual siRNAs (Figure 2-8). Three of four siRNAs successfully reduced the TPX2 protein and those siRNAs individually induced significant toxic effects (Figure 2-9). After confirming the results of our screening with an ATP-based viability assay, we also verified the induction of apoptosis in response to TPX2 depletion (Figure 2-10). To investigate the effect of TPX2 depletion on mitosis, we checked the phosphorylation levels of Histone H3, which is phosphorylated only during mitosis. In the unsynchronized cell population treated with a non-targeting siRNA, very low levels of phosphorylated H3 were observed, consistent with 2-3% of the cell population undergoing mitosis at any time point. However, cell samples treated with each of the three siRNAs targeting TPX2 showed a large increase in histone H3 phosphorylation. This suggested that lack of TPX2 resulted in greatly delayed exit from mitosis (Figure 2-10). Taken together, these results suggested that Aurora kinase A, the kinase that TPX2 binds and activates, may have a critical and differential role in the survival/proliferation of BRG1-mutant NSCLCs.

To test this hypothesis, we depleted AURKA protein with 4 individual siRNAs to identify the most efficient ones for further follow-up experiments

(Figure 2-11). Among 4 siRNAs, only 1 showed complete knock-down of AURKA whereas 2 of 4 resulted in partial depletion. Only the most efficient siRNA produced more than 50% reduction in cell growth indicating that quite low levels of AURKA support cell viability (Figure 2-12). Due to its higher efficacy, we utilized siRNA #28 against AURKA in the following experiments. We measured and confirmed the induction of apoptosis and arrest in mitosis after depletion of AURKA in H1819 cells (Figure 2-13). To understand whether sensitivity to AURKA depletion is causally linked with BRG1 loss, we created an H1819 cell line in which wild-type BRG1 expression was restored and we performed identical cell toxicity assays with both parental and BRG1-expressing H1819 cells. Expression of exogenous BRG1 expression significantly reduced the response to AURKA knock-down, suggesting that BRG1 loss may sensitize cells to AURKA-targeted therapies (Figure 2-14).

To confirm our observations chemically, H1819 cells were treated with Aurora kinase inhibitor, VX-680. VX-680 caused significant toxicity in H1819 cells with an EC50 of approximately 50 nM (Figure 2-15). To determine the degree to which BRG1 loss correlates with sensitivity to inhibition of AURKA, we tested a panel of NSCLC and HBEC-KT lines known to be either BRG1-wild-type or mutant and measured the sensitivity to VX-680. All mutant lines were hypersensitive to VX-680 compared to

NSCLC lines expressing wild-type BRG1 or immortalized human bronchial epithelial cells (HBECs) (Figure 2-16).

We next sought to understand the basis for the synthetic lethality resulting from AURKA inhibition in BRG1-inactivated cells. It has been previously shown that mitotic spindles can be formed by two different mechanisms (Barr & Gergely, 2007). Although centrosomes are the organelles responsible of proper mitotic spindle assembly in mammals, a centrosome-independent pathway also exists. Among these two distinct pathways, most of the components overlap. However, the centrosome-independent machinery requires the microtubule-bundling protein, DLG7, but centrosome-dependent mechanism does not (Koffa et al., 2006; Sillje, Nagel, Korner, & Nigg, 2006; Tsai et al., 2008). Depletion of DLG7 expression with specific siRNAs resulted in decreased viability in H1819 cells. However, H1819 cells with restored wild-type BRG1 were affected significantly less in the absence of DLG7 proteins (Figure 2-17). This indicates that mammalian cells with BRG1 loss may lose the centrosome-dependent mitotic spindle mechanism during the progression of cancer, but tolerate this aberration if the chromosome-dependent machinery is functional, or not inhibited.

Altogether, our data suggest that BRG1 has specific roles in the maintenance of the mitotic spindle machinery and , when deficient, cancer cells become addicted to AURKA.

Discussion

Non-small cell lung cancers (NSCLCs), as well as several other cancer types, frequently carry homozygous BRG1-inactivating mutations (Medina et al., 2004; Medina & Sanchez-Cespedes, 2008). The precise role of BRG1 loss in tumor initiation and/or progression is not known. However, the observation that loss of BRG1 function affects one tenth of mammalian transcriptome suggested that it might create unique vulnerabilities in tumors that could be exploited for therapy.

Knocking out BRG1 causes mitotic catastrophe in primary cells (Bourgo et al., 2009). Here we show that inhibition of AURKA, a mitotic kinase, preferentially reduces the viability in NSCLCs with BRG1-inactivating mutations. Aurora A, the “polar kinase”, is required for bipolar spindle assembly. The levels of Aurora A are often elevated in tumors and it maps to a locus (20q13) that is frequently amplified in cancers (Barr & Gergely, 2007). Mitosis has been an important target for anticancer therapy development. Anti-mitotics consist of both conventional chemotherapeutic agents that alter microtubule dynamics such as Vinca alkaloids, taxanes

or epothilones, as well as novel classes of anti-neoplastic drugs targeting the regulatory system that controls mitosis, such as Aurora or Polo-like kinases or Kinespondin inhibitors (Lapenna & Giordano, 2009; Sudakin & Yen, 2007). Due to severe side effects of cytotoxic microtubule poisons, therapies targeting the molecular regulators of mitotic spindle machinery offer an alternative and potentially safer way to treat patients.

There are two known major cellular mechanisms to assemble mitotic spindles. The centrosome-dependent mitotic spindle machinery is responsible of the majority of mammalian cell division (Wadsworth & Khodjakov, 2004). However, mitotic spindles can also be formed in a chromosome-oriented manner, in the absence of centrosomes naturally in plants and oocytes (Clayton, Black, & Lloyd, 1985; Karsenti, Newport, Hubble, & Kirschner, 1984), suggesting that mitotic spindle machinery might be redundant in most mammalian cells. Recently, some of the components of chromosome –dependent mitotic spindle machinery were identified (Barr & Gergely, 2007). All, but one overlapped with the centrosome-dependent system. DLG7 was shown to be a microtubule bundling protein that is necessary to arrange and align the microtubules properly from chromosomes to the poles of the cells (Koffa et al., 2006). DLG7 knock-out mice, unlike mice deficient in other mitotic spindle regulators, do not show any abnormal phenotype during development

except infertility in females, so it is clear that DLG7 is not required in centrosome-harboring mammalian somatic cells (Tsai et al., 2008). Additional research on these female mice lacking DLG7 gene expression demonstrated that oocytes, which lack centrosomes naturally, were not capable of dividing properly(Breuer et al., 2010), proving that DLG7 is critical for centrosome-independent mitotic spindle formation. Here we report that DLG7 was necessary for the viability of H1819 cells and this requirement was significantly reduced when BRG1 expression is restored. Although it is not known how BRG1 regulates centrosomes, these data suggest that BRG1 loss causes the centrosomes to weaken or lose their role on mitotic spindle assembly and makes cell division largely chromosome-dependent.

Besides the unique molecular pathology, BRG1-inactivated NSCLC lines in our panel have no overlap with therapeutically targetable genetic lesions such as EGFR, ALK, FGFR1 and DDR2 mutations (Kohno et al., 2012; Lovly & Carbone, 2011; Takeuchi et al., 2012; Weiss et al., 2010). Being excluded from the available molecular therapy options against these genetic alterations highlights the importance and urgency of discovering treatment strategies for NSCLC patients with BRG1-deficient tumors. The results of our study address this need in the practice of oncology and

provide the preclinical rationale for a potential success in treating patients in this subset.

Furthermore, besides TPX2 and AURKA, we report a validated collection of genes that potentially regulate the survival and/or proliferation of H1819 cells. While some of these have established links to drugs or inhibitors, others are less characterized, but offer a platform for detailed functional studies. In combination with the full genome sequence of H1819 cells, this list of selectively toxic genes, when depleted, may lead to novel vulnerabilities related to the status of BRG1 or other genes altered in H1819 line. Besides discovering new drug target candidates, this list also provide novel candidate genes to explore and better understand the molecular biology of the cancer.

Because BRG1 is mutated or deleted in other cancer types, AURKA interventions, specifically VX-680 treatments, might be beneficial for a larger patient population involving a variety of cancers. Our data indicate a novel biological concept to explain how BRG1 loss could create loss of redundancy in mitotic spindle assembly mechanisms and make cancers acquire a vulnerability and therapeutic opportunity against DLG7-involved Aurora kinase A activity.

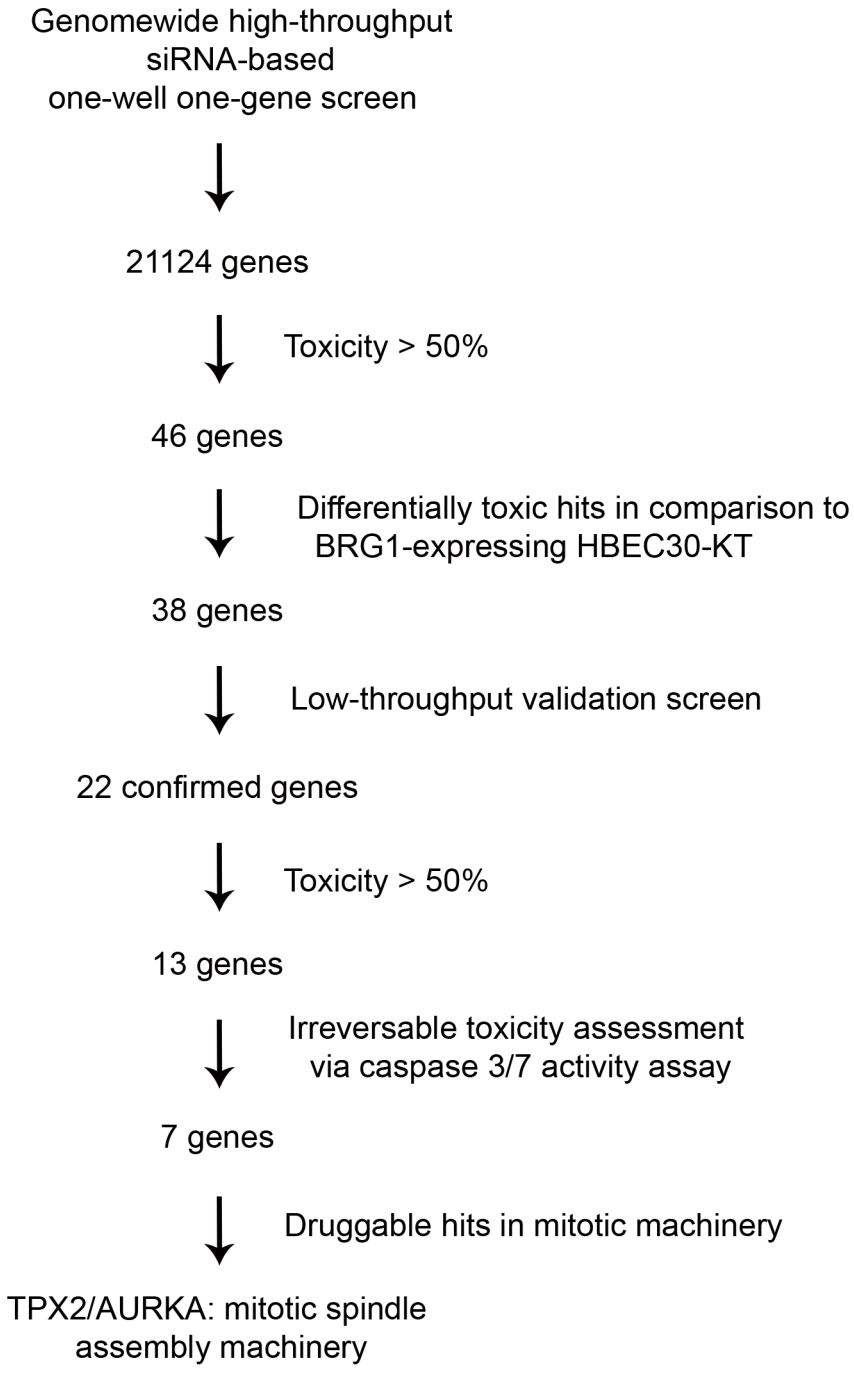
The goal of cancer therapeutics is to provide selective killing of tumor cells while sparing normal cells. To accomplish this, unique characteristics of cancer cells are the key to be identified and utilized. Among a limited number of methods, pharmacogenetics has the most promising future due to the fact that aberrations in the DNA of the cancer cells are one of the most reliable leads to understand the specific biology of each tumor and accurately guide the therapies (Garraway, 2013).

Overall, here, we propose that AURKA inhibition may prove useful in the treatment of human NSCLCs that harbor BRG1-inactivating mutations.

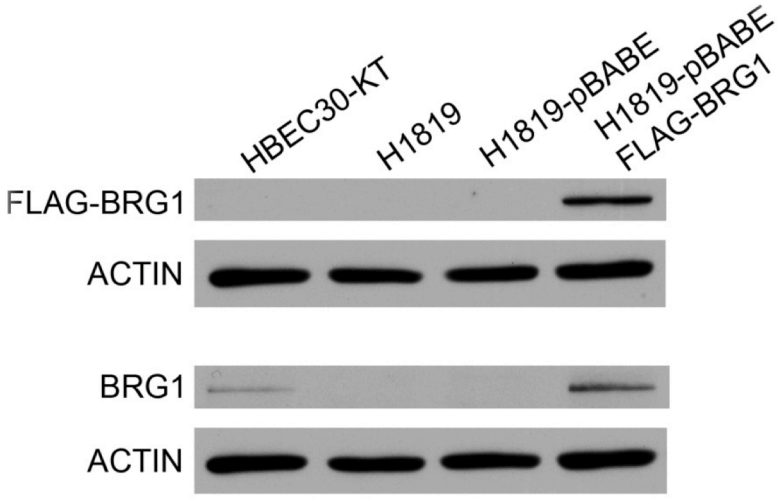
FIGURES OF CHAPTER I

SMARCA4/BRG1-Inactivating Mutations as Potential Predictive Markers for Aurora Kinase A-Targeted Therapy in NSCLCs

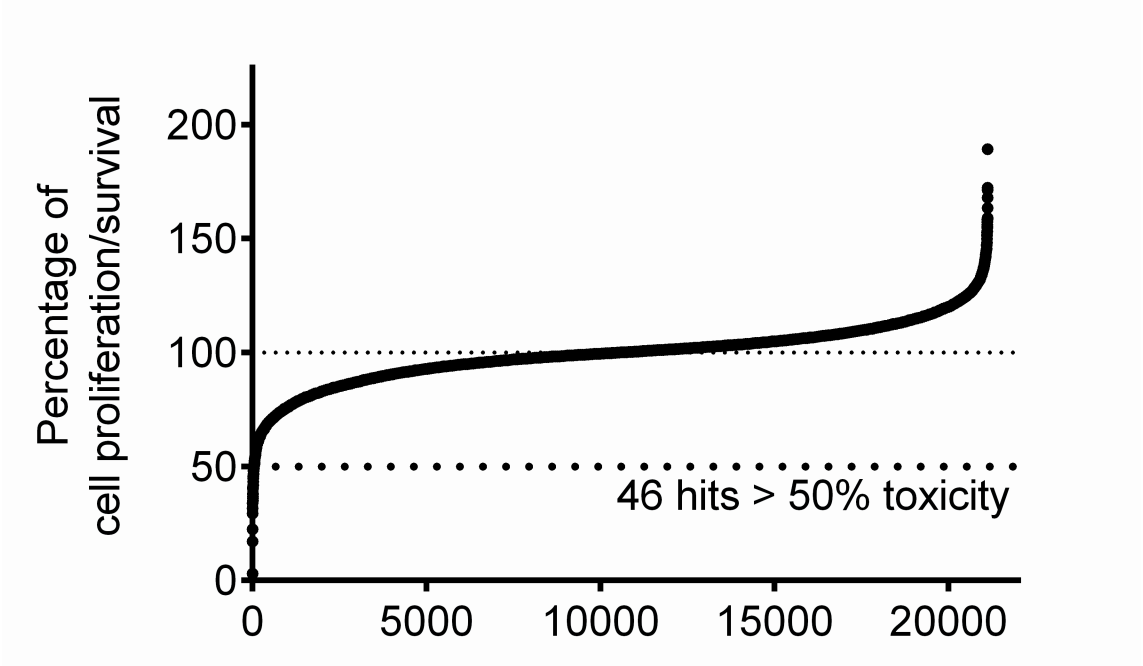
2-1



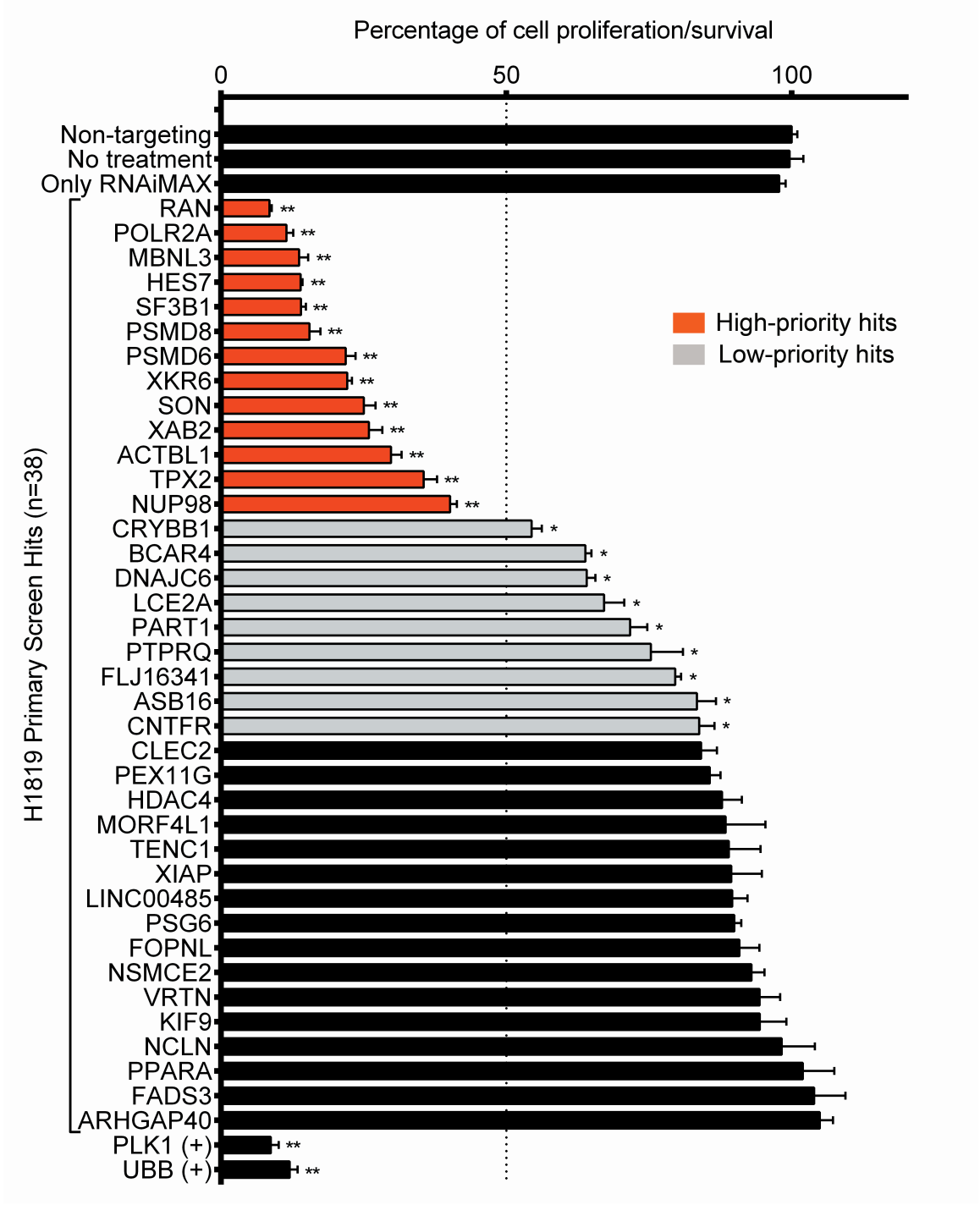
2-2


HBEC30-KT
H1819
H1819-pBABE
H1819-pBABE FLAG-BRG1
FLAG-BRG1
ACTIN
BRG1
ACTIN

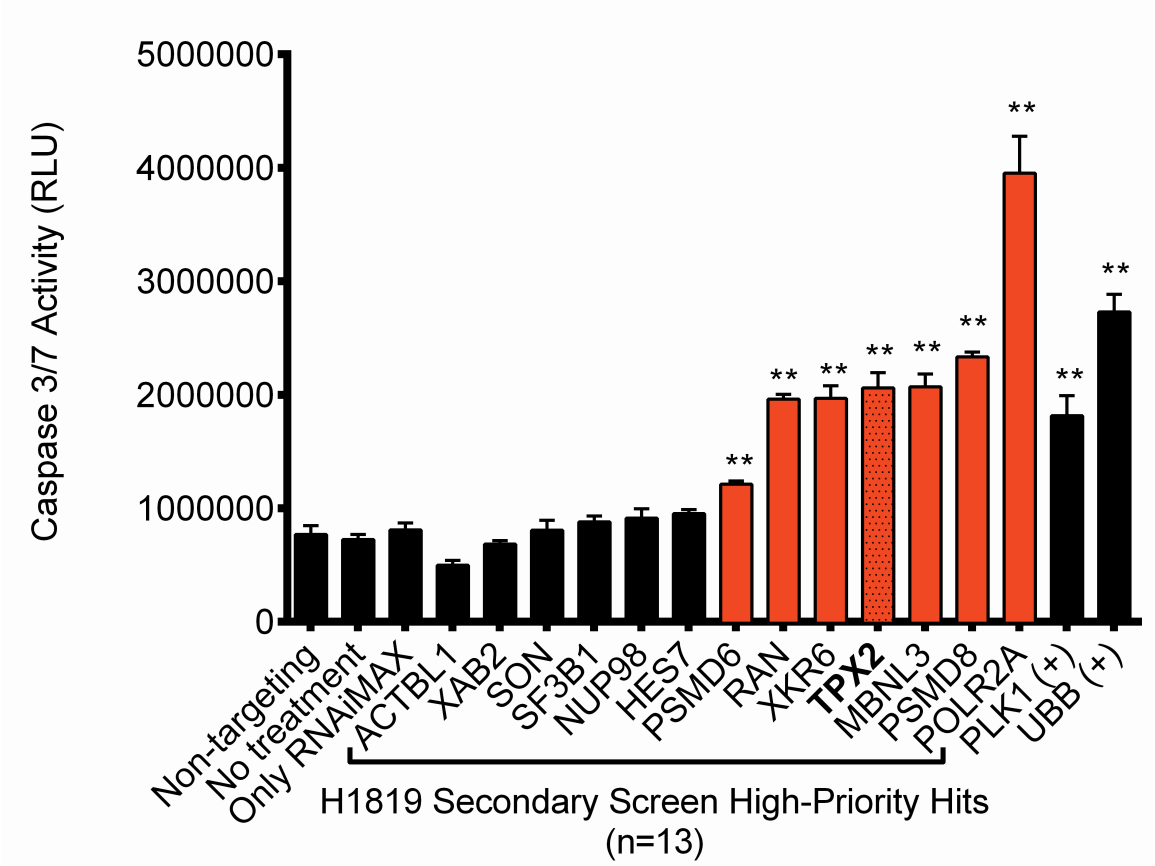
2-3


Percentage of cell proliferation/survival
200
150
100
50
0
0
5000
10000
15000
20000
46 hits > 50% toxicity

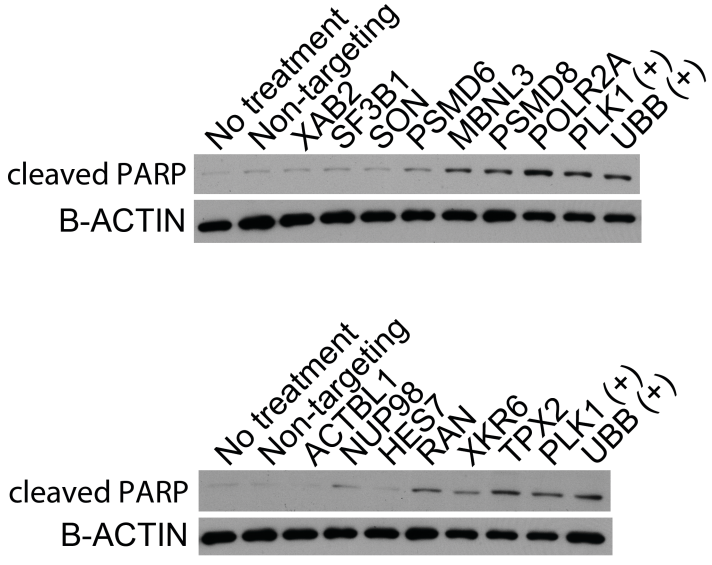
2-4


Percentage of cell proliferation/survival
0
50
100
Non-targeting
No treatment
Only RNAiMAX
RAN
POLR2A
MBNL3
HES7
SF3B1
PSMD8
PSMD6
XKR6
SON
XAB2
ACTBL1
TPX2
NUP98
CRYBB1
BCAR4
DNAJC6
LCE2A
PART1
PTPRQ
FLJ16341
ASB16
CNTFR
CLEC2
PEX11G
HDAC4
MORF4L1
TENC1
XIAP
LINC00485
PSG6
FOPNL
NSMCE2
VRTN
KIF9
NCLN
PPARA
FADS3
ARHGAP40
PLK1 (+)
UBB (+)
High-priority hits
Low-priority hits
H1819 Primary Screen Hits (n=38)

2-5

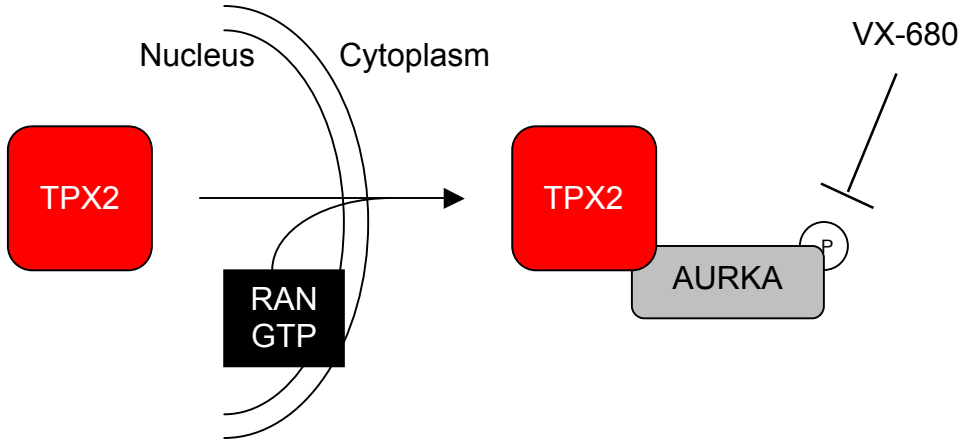

Caspase 3/7 Activity (RLU)
5000000
4000000
3000000
2000000
1000000
0
**
Non-targeting
No treatment
Only RNAiMAX
ACTBL1
XAB2
SON
SF3B1
NUP98
HES7
PSMD6
RAN
XKR6
TPX2
MBNL3
PSMD8
POLR2A
PLK1 (+)
UBB (+)
H1819 Secondary Screen High-Priority Hits
(n=13)

2-6

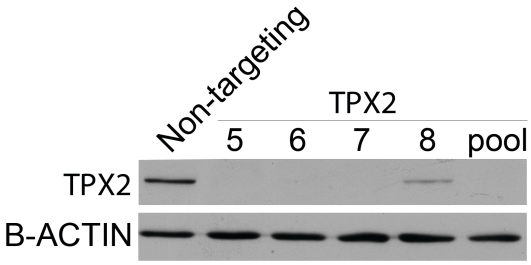

No treatment
Non-targeting
XAB2
SF3B1
SON
PSMD6
MBNL3
PSMD8
POLR2A
PLK1 (+)
UBB (+)
cleaved PARP
B-ACTIN
No treatment
Non-targeting
ACTBL1
NUP98
HES7
RAN
XKR6
TPX2
PLK1 (+)
UBB (+)
cleaved PARP
B-ACTIN

Figures 2-1/6: Genome-wide high-throughput siRNA-based screening in H1819 cell line provided the opportunity to identify an unbiased collection of potentially targetable gene products for NSCLCs harboring SMARCA4/BRG1-inactivating mutations. (2-1) Overview of the screen workflow. **(2-2)** Western blot analysis with monoclonal anti-BRG1, FLAG-BRG1 and B-ACTIN antibodies. The endogenous and ectopic expression levels of BRG1 and FLAG-BRG1 in HBEC30-KT, H1819 and H1819 infected with pBABE or pBABE-FLAG-BRG1 were shown. **(2-3)** Schematic representation of the results from screening 21,124 genes in the human genome. Genes were depleted singly in H1819 by reverse transfection of 50 nM of siRNA pools targeting the corresponding genes and cell viability after the knock-downs was measured via luciferase –based cell-titer glo assay. Mean viability for each triplicate were calculated and genes with viability below 50% were followed up as hits in the further analysis and experiments. **(2-4)** Secondary validation screen. Among 46 hit genes, 38 differentially toxic hits were re-analyzed to validate. 22 of 38 showed significant effects on the viability of H1819 cells. **(2-5)** 13 of the confirmed hits, high-priority hits, were followed up in the tertiary caspase3/7 activity screen with H1819 to investigate the induction of programmed cell death. **(2-6)** Western blot analysis with monoclonal cleaved PARP and B-ACTIN antibodies. The increase in apoptotic activity was measured by western blotting.

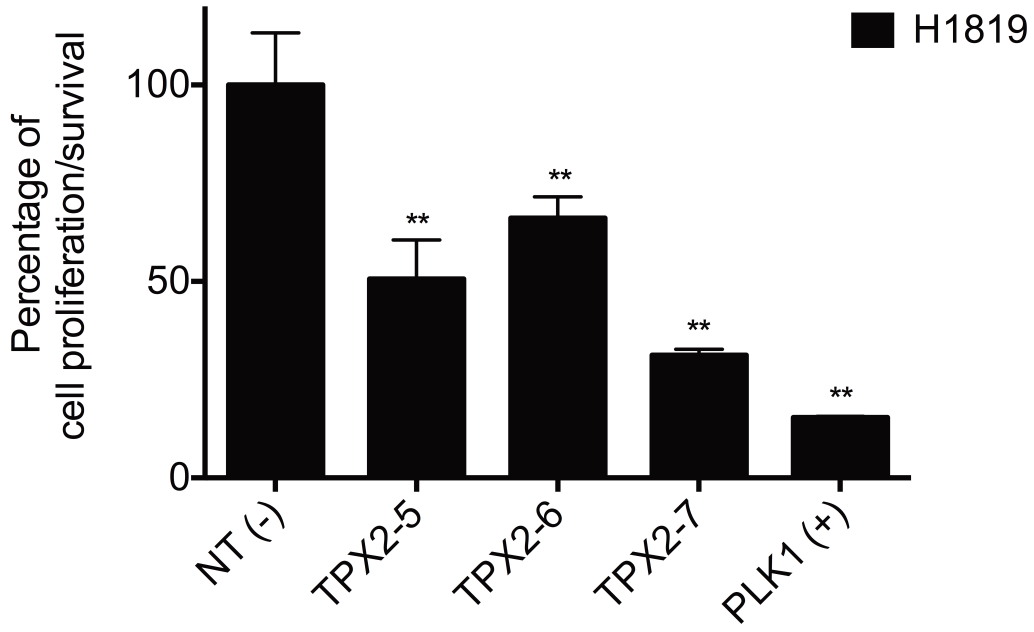
2-7


Nucleus
Cytoplasm
VX-680
TPX2
TPX2
RAN
GTP
AURKA
P

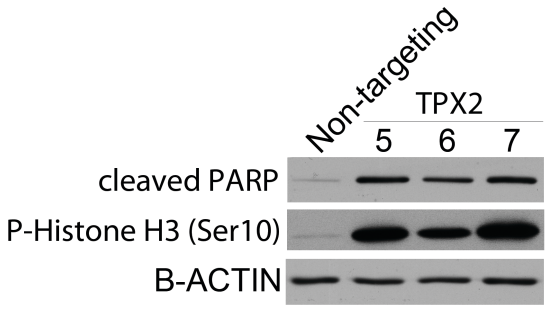
2-8


Non-targeting
TPX2
5
6
7
8
pool
TPX2
B-ACTIN

2-9

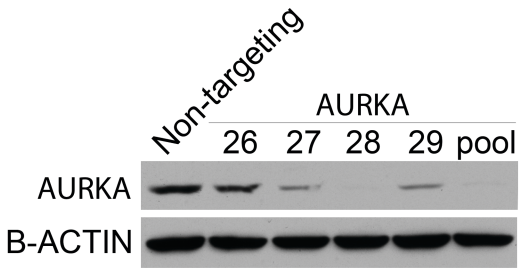

H1819
Percentage of
cell proliferation/survival
100
50
0
**
**
**
**
NT (-)
TPX2-5
TPX2-6
TPX2-7
PLK1 (+)

2-10

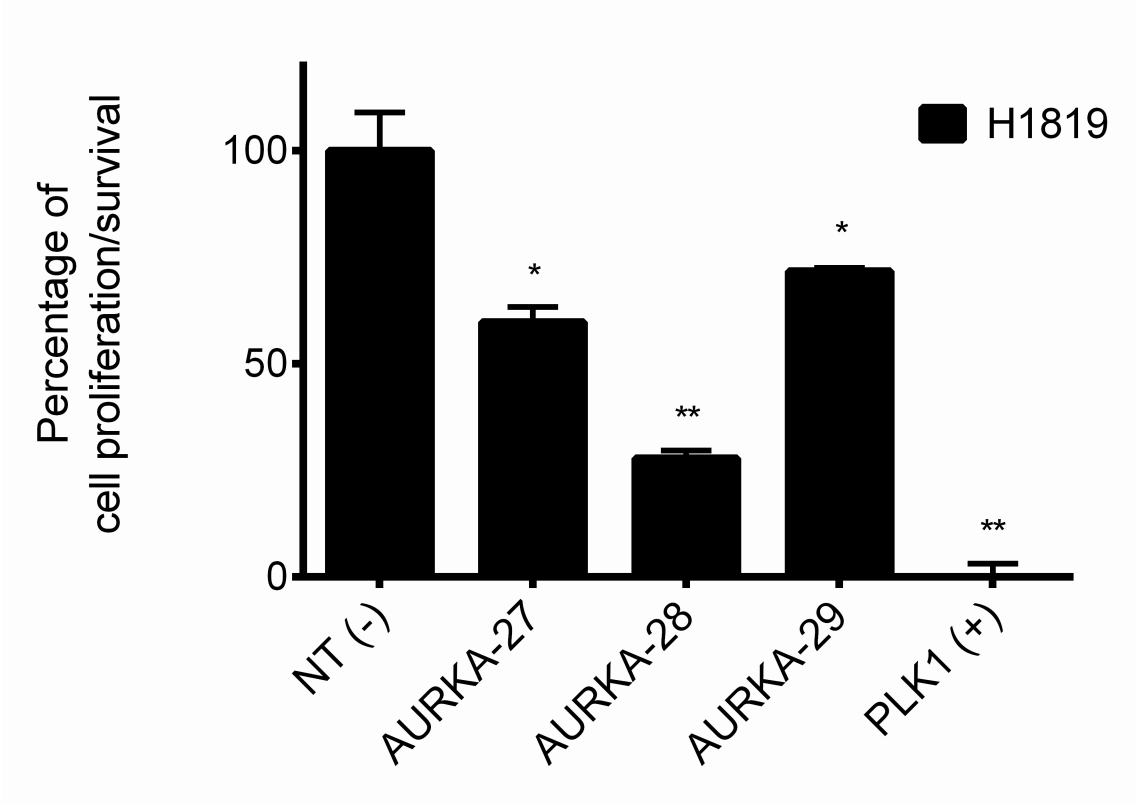


Figures 2-7/10: Depletion of TPX2, a binding partner and activator of Aurora kinase A, is detrimental in H1819 cells. **(2-7)** TPX2, one of the top hits, is a critical component of RAN-dependent mitotic spindle formation machinery. 4 individual siRNAs against different regions of the TPX2 gene product were obtained and whether it is a true hit and regulator of mitosis were investigated. **(2-8)** H1819 cell lysates were collected 3 days after transfecting the cells with either non- or TPX2-targeting siRNAs and cell lysates were subjected to western blotting to monitor TPX2 protein levels. **(2-9)** 5 days after transfecting H1819 cells with non-targeting siRNAs or individual siRNAs targeting TPX2, cell viability was measured with cell titer-glo assay. **(2-10)** Western blotting analysis with monoclonal cleaved PARP, phospho-Histone H3 and B-ACTIN antibodies. Cleaved PARP levels as a marker of apoptosis and phospho-Histone H3 levels as a marker of mitotic arrest were monitored.

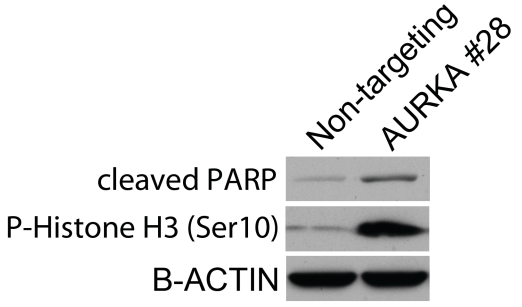
2-11


Non-targeting
AURKA
26
27
28
29
pool
AURKA
B-ACTIN

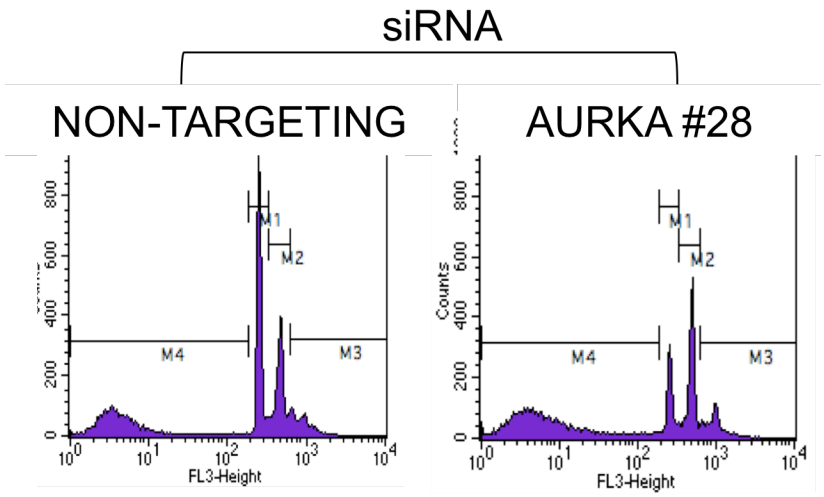
2-12


Percentage of cell proliferation/survival
100
50
0
H1819
*
**
*
**
NT (-)
AURKA-27
AURKA-28
AURKA-29
PLK1 (+)

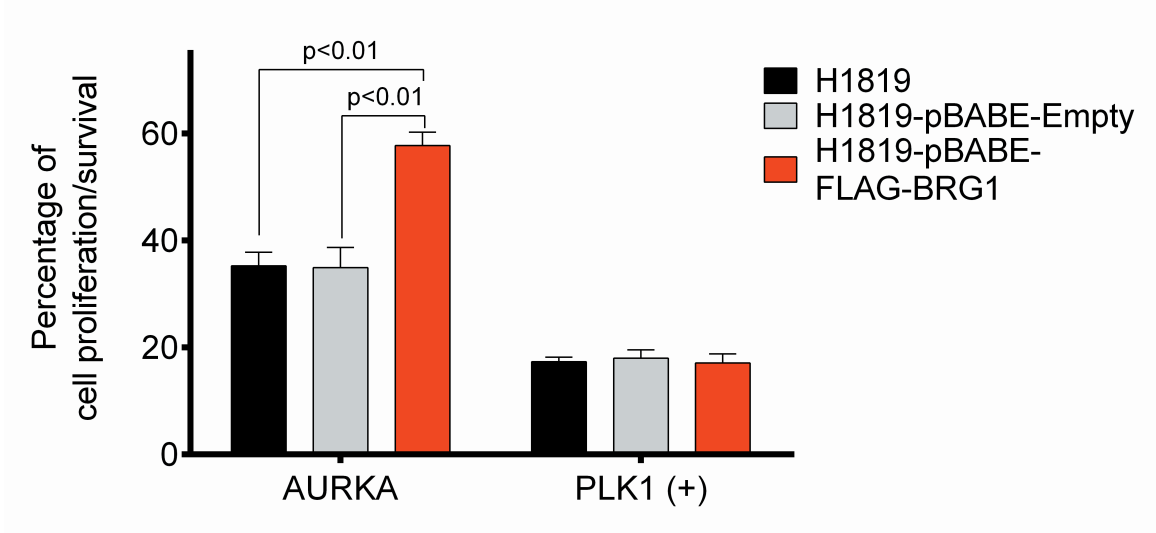
2-13


Non-targeting
AURKA #28
cleaved PARP
P-Histone H3 (Ser10)
B-ACTIN

2-14

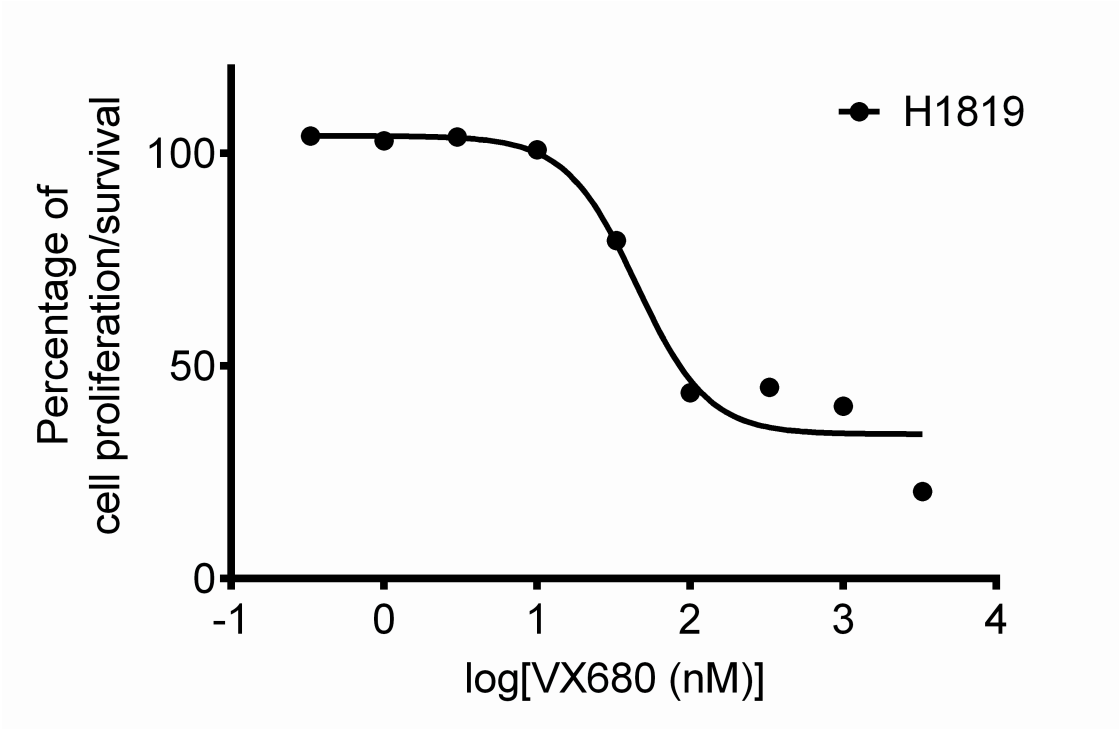


2-15

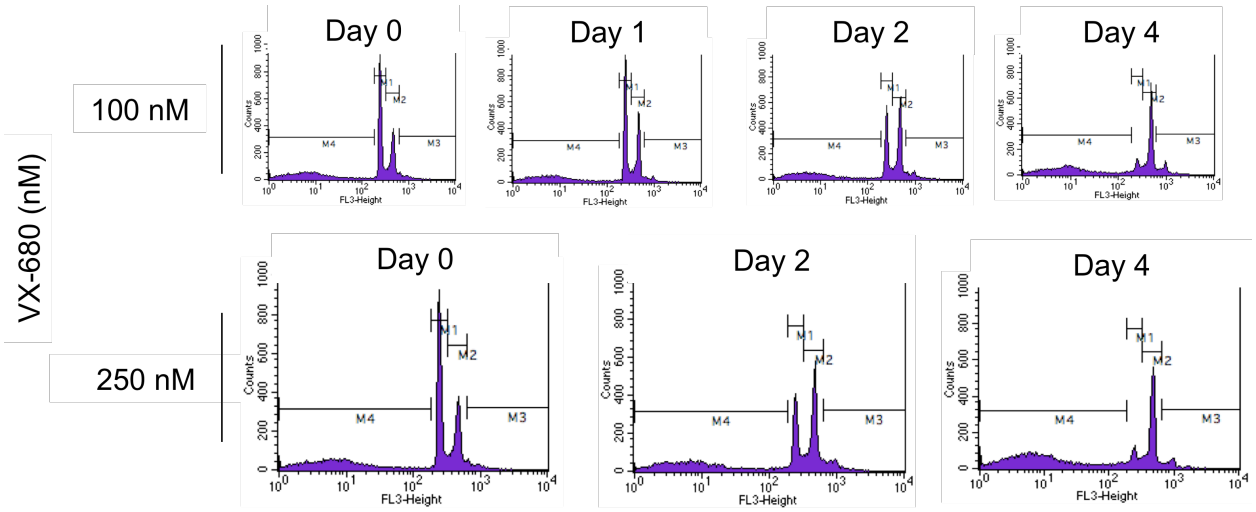


Figures 2-11/15: Depletion of Aurora kinase A (AURKA) with specific siRNAs causes toxicity in H1819 cells. **(2-11)** Western blotting analysis with monoclonal AURKA antibodies. AURKA was depleted with four individual siRNAs or their pool. Cell lysates were collected and subjected to immunoblotting to monitor AURKA protein levels **(2-12)** 5 days after transfecting H1819 cells with individual siRNAs, cell viability was measured with cell-titer glo assay. **(2-13)** Western blotting analysis with monoclonal cleaved PARP and phospho-Histone H3 antibodies. Increase in phospho-histone 3 as a marker of mitotic arrest and cleaved PARP as a marker of apoptosis was monitored. **(2-14)** DNA content was measured via flow cytometry after transfecting cells with non-targeting or AURKA-targeting siRNAs. **(2-15)** 5 days after depleting AURKA in H1819, H1819-pBABE and H1819-pBABE-FLAG-BRG1 cells, cell viability was measured with cell-titer glo assay.

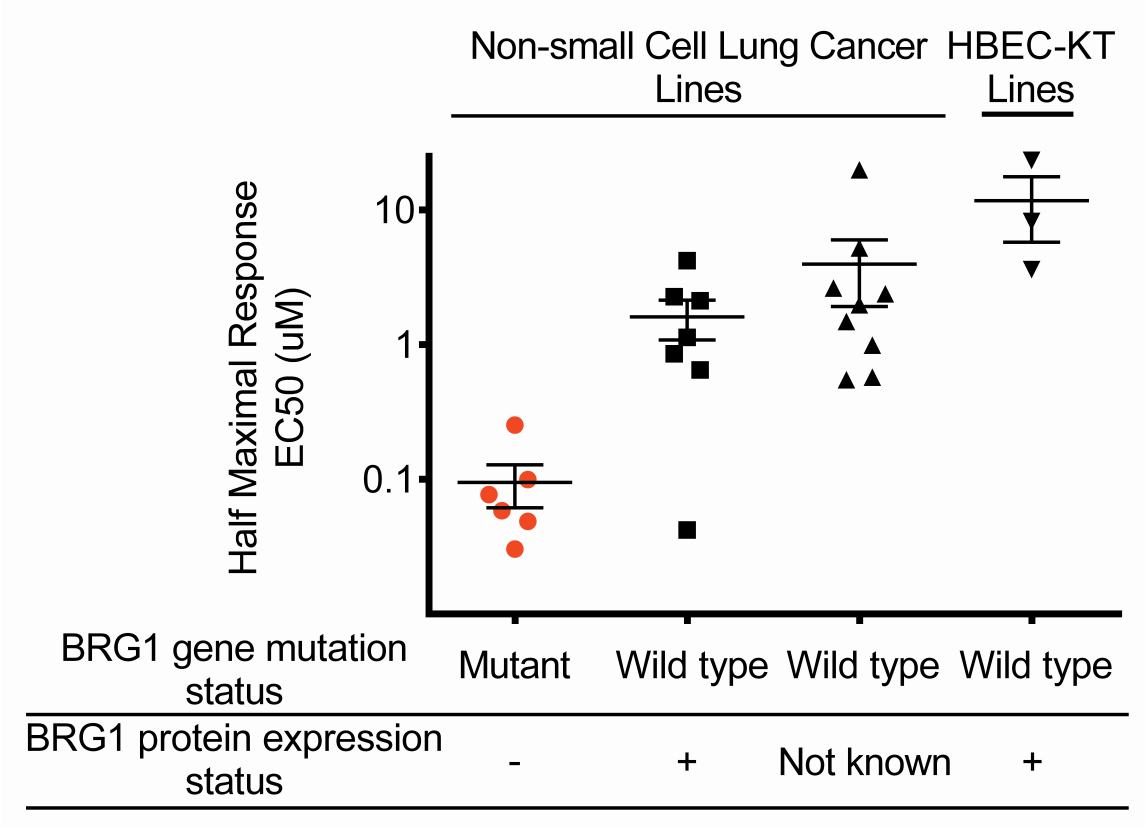
2-16


H1819
Percentage of cell proliferation/survival
100
50
0
-1
0
1
2
3
4
log[VX680 (nM)]

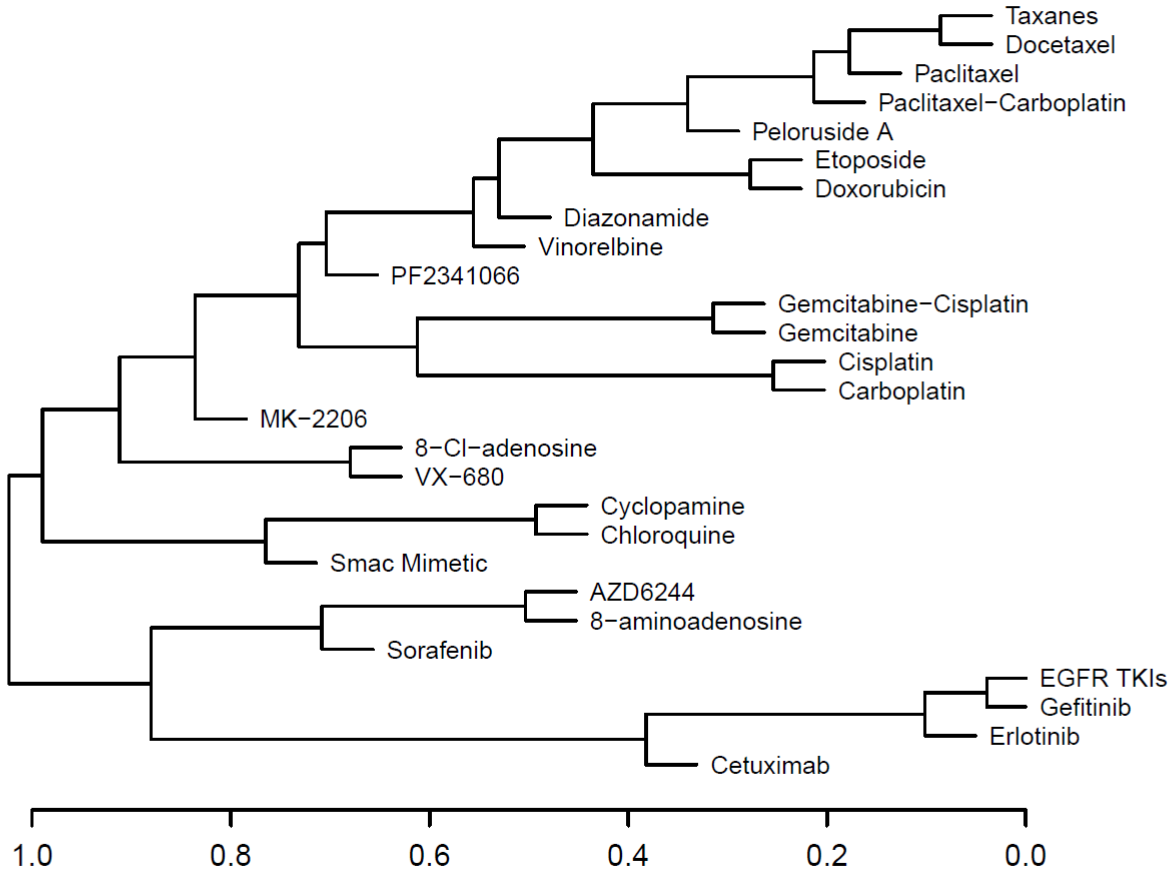
2-17


VX-680 (nM)
100 nM
250 nM
Day 0
Day 1
Day 2
Day 4
Day 0
Day 2
Day 4
M1
M2
M3
M4
Counts
FL3-Height

2-18


Non-small Cell Lung Cancer Lines
HBEC-KT Lines
Half Maximal Response EC50 (uM)
10
1
0.1
BRG1 gene mutation status
Mutant
Wild type
Wild type
Wild type
BRG1 protein expression status
-
+
Not known
+

2-19



Figures 2-16/19: Survey of human NSCLCs reveals preferential cytotoxicity of BRG1-mutant cells after Aurora kinase inhibitor, VX-680 treatment. **(2-16)** H1819 cell line was treated with different concentrations of VX-680. After 5 days of treatment, cell viability was measured with cell-titer glo assay. **(2-17)** The content of DNA was measured by propidium iodide staining in response to VX-680. **(2-18)** A panel of NSCLC and HBEC lines were individually treated with VX-680 and EC50 of their response was calculated with 5-parameter logistic (5-PL) equation. **(2-19)** Unsupervised clustering of VX-680 response with the response against other anti-cancer agents. Response pattern of NSCLCs to VX-680 was compared with the sensitivity profiles of NSCLC lines to other anti-cancer therapeutic agents.

2-20

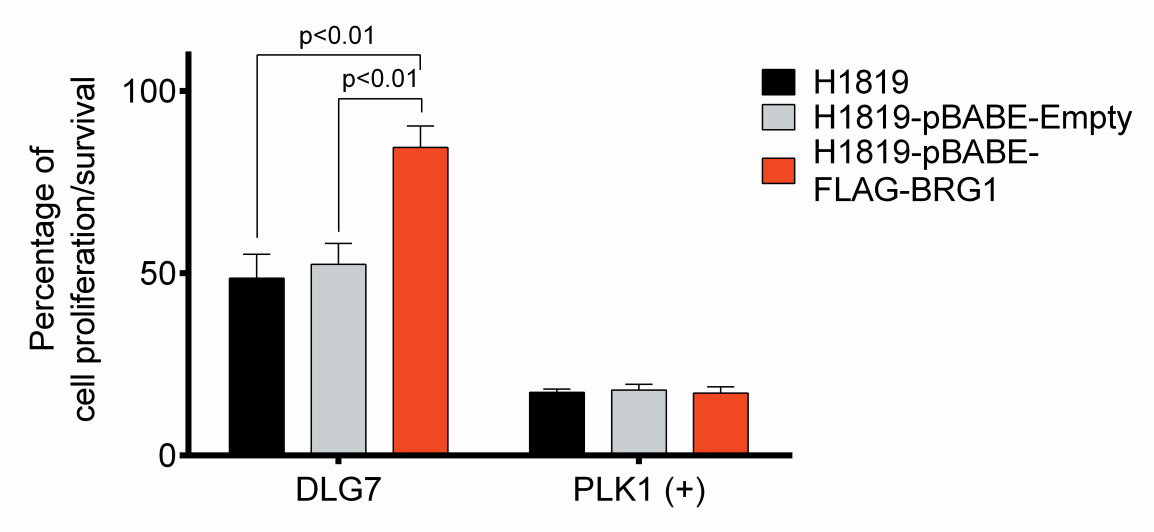


Figure 2-20. Restoring wild-type BRG1 in H1819 cells rescues the cytotoxicity induced by the depletion of DLG7, a specific component of centrosome-independent mitotic spindle assembly machinery. DLG7 was depleted in H1819, H1819-pBABE and H1819-pBABE-FLAG-BRG1 cells and viability was measured after 5 days with cell-titer glo assay.

CHAPTER III

PACLITAXEL TREATMENT POTENTIATES NSCLCs FOR AURORA KINASE A-TARGETED THERAPIES

Introduction

Limitless reproductive potential is a hallmark of cancer (Hanahan & Weinberg, 2011). Therefore, the conventional cancer therapies aim to block the growth of these rapidly dividing cells in tumors and utilize inhibitors of DNA replication or mitotic spindle assembly. Targeting mitosis, among all cell cycle phases, is important because any prolonged inhibitory effect of mitosis mostly causes the induction of cell death since cells are more vulnerable when there is minimal metabolism and minimum protection of cellular DNA. In comparison to M phase, other cell cycle phases can tolerate exogenous interventions and may show more cytostatic phenotypes when blocked (Sudakin & Yen, 2007).

Today, cytotoxic chemotherapies are the basis of cancer medicine since they are capable of blocking the cell division and thus, provide some survival benefit to the patients (Haber, Gray, & Baselga, 2011). However, their effect in healthy tissues due to their nature of inhibiting all dividing cells, causes severe side effects as well. For that reason, targeting the

molecular regulators of mitosis offers a more appealing and safer approach to eliminate many undesired side effects of chemotherapy (Hanahan, 2014).

Aurora kinase A is an important protein for mitosis, more specifically mitotic spindle assembly machinery (Bischoff et al., 1998; Hochegger, Hegarat, & Pereira-Leal, 2013; Kimura et al., 1997). Many reports have already shown the role of AURKA in a variety of cancers and there are several Aurora kinase A inhibitors in clinical trials. They are promising candidates to serve as an alternative to mitotic spindle inhibitors (Carmena, Ruchaud, & Earnshaw, 2009). However, due to the nature of kinase inhibitors, the specific concentration range against the kinase of interest is unfortunately often very narrow and this is also a challenge to overcome for the current inhibitors of Aurora kinase A(Anastassiadis, Deacon, Devarajan, Ma, & Peterson, 2011; Davis et al., 2011). Since cell cycle is regulated with a large collection of kinases and those kinases are mostly members of the protein families with a high degree of the homology, most of the off-target effects occur due to the co-inhibition of a subset of those. Subsequent to nonspecific inhibition of a group of cell cycle kinases, mostly cell cycle arrest occurs at an earlier phase than mitosis and this potentially prevents cell death that could happen with Aurora kinase A-specific inhibition. For that reason, it is critical to utilize

Aurora kinase A-specific concentrations of Aurora kinase inhibitors to have more cytotoxic effects in tumors and to decrease the probability of acquired drug resistance. Severe side effects may also be prevented due to the inhibition of only one particular target.

Results

Aurora kinase A plays a key role in mitotic spindle assembly. Inhibitors of Aurora kinase A have shown significant anti-cancer activity, but led to confusion about the appropriate setting of their utilization (Gorgun et al., 2010; Harrington et al., 2004). Here, we used a new generation Aurora kinase A inhibitor, MLN8237 and an Aurora kinase A-dependent NSCLC line, NCI-H1693 to study the inhibitory pattern of MLN8237 on Aurora kinase A and other targets as well as the phenotypic effect at different concentrations.

We first checked AURKA dependency of H1693 cells by depleting AURKA protein with RNAi approach. siRNA #28 successfully reduced AURKA protein(Figure 3-1) and induced significant toxic effects over the non-targeting siRNA-treated cells (Figure 3-2). We also verified the induction of apoptosis in response to AURKA depletion with assays of caspase 3/7 activity and by immunostaining for cleaved PARP (Figure 3-3, 3-1). Finally, we checked the effects of AURKA depletion on colony formation

by H1693 cells and showed that no colonies were able to form when AURKA was depleted (Figure 3-4). To investigate the effect of AURKA knockdown on mitosis, we checked the phosphorylation levels of Histone H3, which is phosphorylated only during mitosis. Cells treated with siRNA #28 targeting AURKA showed a dramatic increase in histone H3 phosphorylation. This suggested that lack of AURKA resulted in cell cycle arrest in mitosis (Figure 3-1). Altogether, these results confirmed that Aurora kinase A has a critical role in the survival and/or proliferation of H1693 cells.

Next, we checked the activity profile of an Aurora kinase A inhibitor, MLN8237 in H1693 cells at different concentrations and monitored toxicity, similar to the RNAi experiments, with four separate assays based on either ATP levels as a surrogate marker of living cells, caspase 3/7 activity as a marker of apoptosis, immunoblotting against markers of cell cycle arrest and activated apoptosis, and colony formation in the presence of serial concentrations of MLN8237. Primarily, we treated the cells with MLN8237 within a wide range of concentrations and measured the induced cell toxicity with ATP-based cell viability assay, Cell-titer Glo reagent. We obtained an unusual tri-phasic dose response curve unlike sigmoidal dose response patterns of most drugs (Figure 3-3). This dose response pattern suggested that MLN8237 has different target sets at

different concentrations and probably, the manifestation of the cytostatic or cytotoxic effects is dependent on the concentration of MLN8237. To test this hypothesis, we treated H1693 cells with different concentration of MLN8237 identical to the prior experiment and measured the caspase 3/7 activity as a surrogate marker for apoptotic cell death. Clarifying our previous observations, we showed that MLN8237 caused cytotoxic effects at concentrations ranging between 50 nM to 150 nM whereas treatments with concentrations higher than 150 nM progressively diminished the induction of apoptosis, becoming cytostatic and protective from cell death (Figure 3-4, 3-7).To better understand this unusual behavior of MLN8237, we tested whether this response pattern matches with the specificity pattern of MLN8237 to AURKA. RNAi against AURKA, in the earlier experiments, demonstrated that depleting AURKA caused a dramatic increase in the phosphorylation of Histone H3, arresting cells in mitosis. Assessing MLN8237 activity at the critical concentrations ranging from 16 nM to 1.2 uM by immunoblotting against phospho-histone H3, we showed that MLN8237 increases phospho-Histone H3 only at the concentrations where it also induces apoptosis, at 50-150 nM (Figure 3-5). This switch at the concentration of 150 nM most likely modulates the cytotoxic phenotype by inhibiting secondary targets that are normally active at an

earlier and safer cell cycle stage and inhibition of them does not allow the cells to reach M phase.

Regarding the higher clinical value of cytotoxic effects of any given anti-cancer drug, we proposed to prime the cells with Paclitaxel at minimal concentrations and co-treat the cells with MLN8237 to create synthetic lethality and expand the potency of MLN8237. First, we determined the dose response pattern of H1693 against Paclitaxel and calculated its half maximal inhibitory concentration (IC50) as 3.38 nM (Figure 3-7). To check whether Paclitaxel sensitizes H1693 cells to Aurora kinase A inhibition, we treated the cells with MLN8237 in the presence of absence of Paclitaxel at its IC10 concentration, 1.25 nM. With this combinational strategy, we observed significant increase in both the magnitude of the cytotoxic effects of MLN8237 and the potency by lowering its cytotoxic effects from 46 nM to 1.7 nM of concentrations (Figure 3-8).

Taken together, our findings revealed that currently MLN8237, an Aurora kinase A inhibitor, has key challenges in terms of specificity and this phenomenon may require new strategies to use it at the most potent and cytotoxic setting. In this regard, our data suggest that the microtubule-targeting, FDA-approved chemotherapy agent, Paclitaxel, at minimally effective concentrations is able to prime the cells to expand the specificity

range and magnitude of toxicity of MLN8237 for improved results in cancer therapeutics.

To assess the ability of MLN8237 to block tumor growth in vivo with minimal side effects, we plan a trial with mice xenografted with NCI-H1693 cells. In this experiment, first, we would like to extensively characterize the effects of MLN8237 for tumor growth and general toxicology by treating the mice with a wide dose range of MLN8237. Next, we are going to test the combinational effect of Paclitaxel and MLN8237.

Discussion

Current efforts to develop cancer therapeutics have had a major focus on targeting kinases that harbor high homology at their targetable ATP-binding sites (Anastassiadis et al., 2011; Davis et al., 2011). This phenomenon not only challenges drug candidates to compete with one of the most abundant molecules in the cells, but also creates a difficult task for them to bear significant specificity to the kinase of interest over homologous kinases. When the kinase of interest is a mitosis regulator like Aurora kinase A inhibitors, the efficacy of treatment may be affected due to the inhibition of kinases at an earlier cell cycle stage, like S or G2, that arrests the cells at a safer cell cycle phase than mitosis where the cells are more vulnerable with non-enveloped DNA and minimal

metabolism (Lapenna & Giordano, 2009; Sudakin & Yen, 2007). Thus, the potential problem with specificity may affect the fate of a promising drug candidate not because of its potency, but due to inability to control the dosage to the range where specificity is observed.

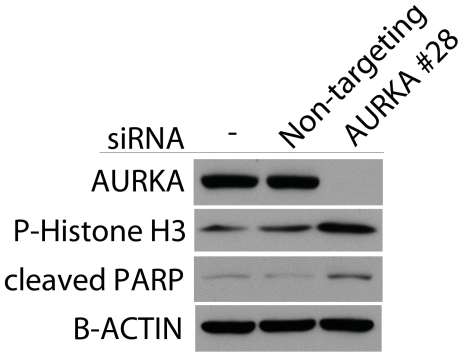
One way to improve the anticancer efficacy of an inhibitor is to combine it with another anticancer agent. Targeted therapies against the regulators of mitosis may rationally synergize with cytotoxic chemotherapies that target the same cellular machinery. In this regard, microtubule poisons at at concentrations having no significant effect alone, may create a better therapeutic window for mitosis-targeted therapies. Our studies showed that Aurora kinase A inhibitor, MLN8237, shows such combinatorial effect with Paclitaxel in NSCLCs.

In addition to providing a rationale for a clinical trial for combining paclitaxel with MLN8237, our data suggest that studies to test the combinational effects of inhibitors of related cellular mechanisms may have utility to minimize chemotherapy-induced side effects and maximize the utility of novel targeted therapies. Pairing nucleotide analogs or DNA-damaging agents with inhibitors of DNA damage repair (DDR) or anti-metabolites with metabolism-targeted therapies are two possible combinations for potential success with existing drugs.

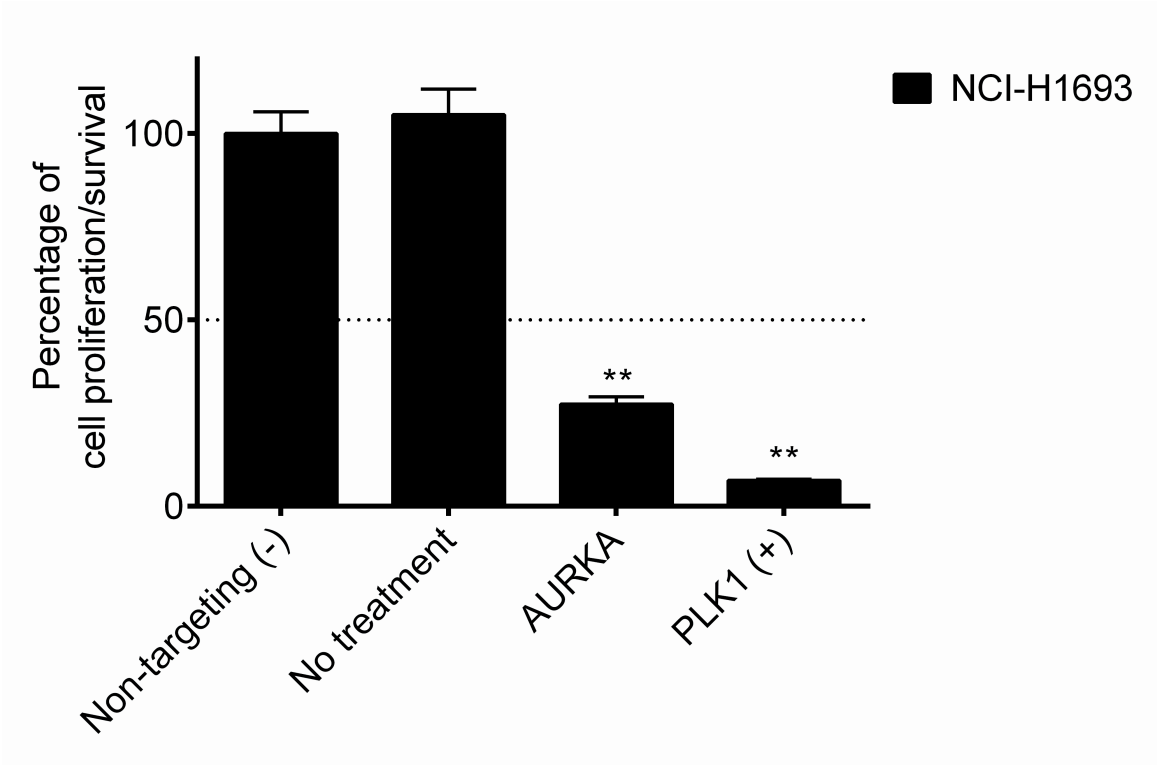
FIGURES OF CHAPTER III

PACLITAXEL TREATMENT POTENTIATES NON-SMALL CELL LUNG CANCERS TO AURORA KINASE A-TARGETED THERAPY

3-1



3-2



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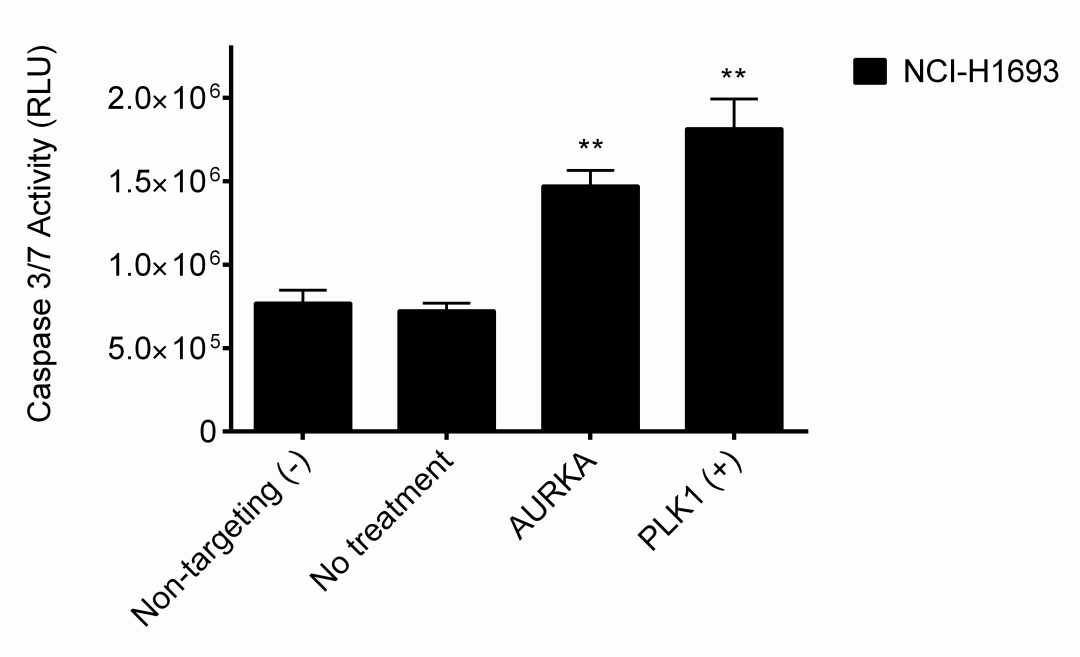
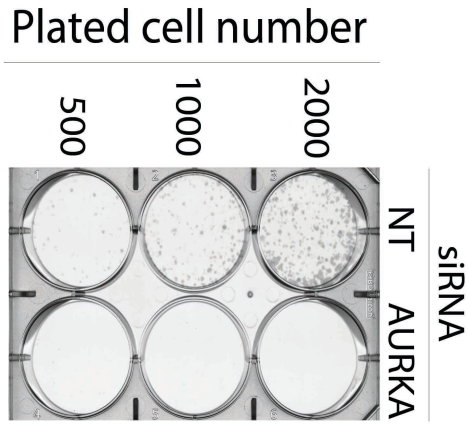
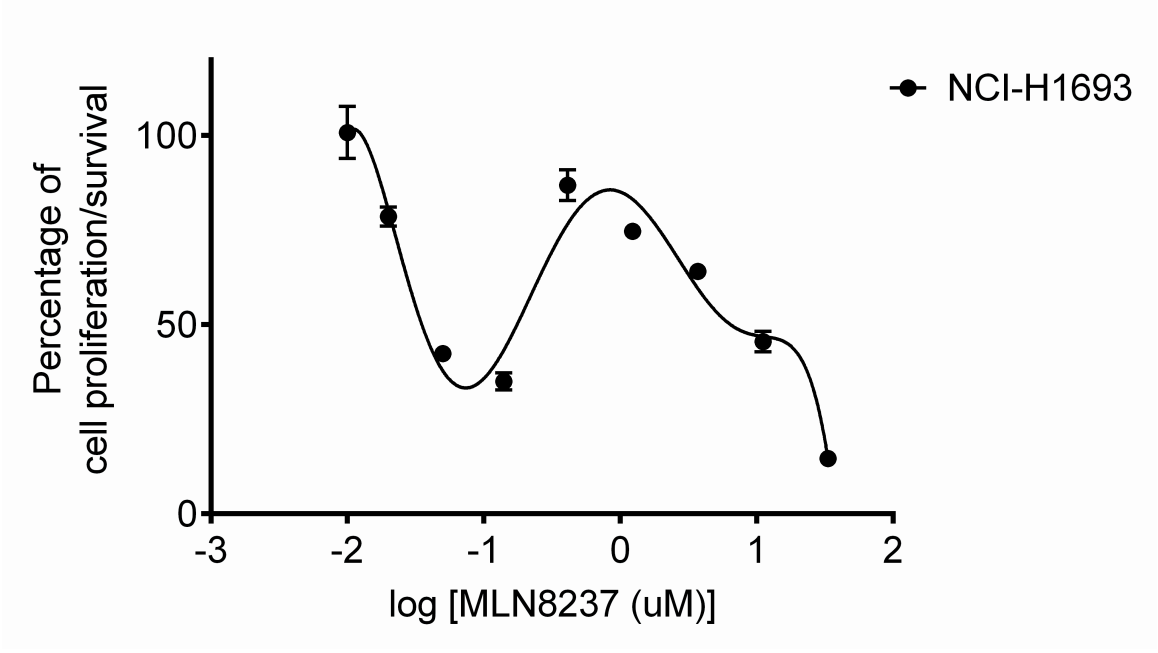


3-4

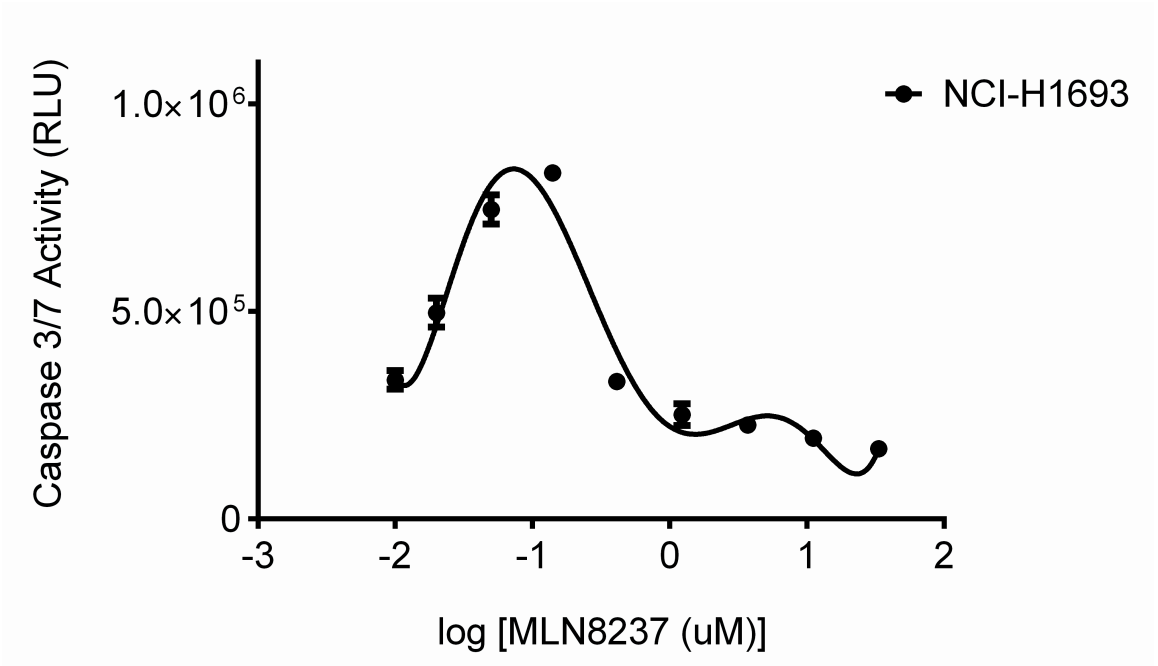


Figures 3-1/4: Viability of NCI-H1693 cells are dependent to the activity of Aurora kinase A. Aurora Kinase A (AURKA) was depleted with siRNA #28 and in different experiments, **(3-1)** cell lysates were collected and subjected to immunoblotting with specific AURKA, phospho-Histone H3 and cleaved PARP antibodies, **(3-2)** cell viability was measured with cell-titer glo assay, **(3-3)** increase in apoptosis was measured with Caspase-Glo 3/7 assay, **(3-4)** colony formation abilities of the cells were assessed with crystal violet staining.

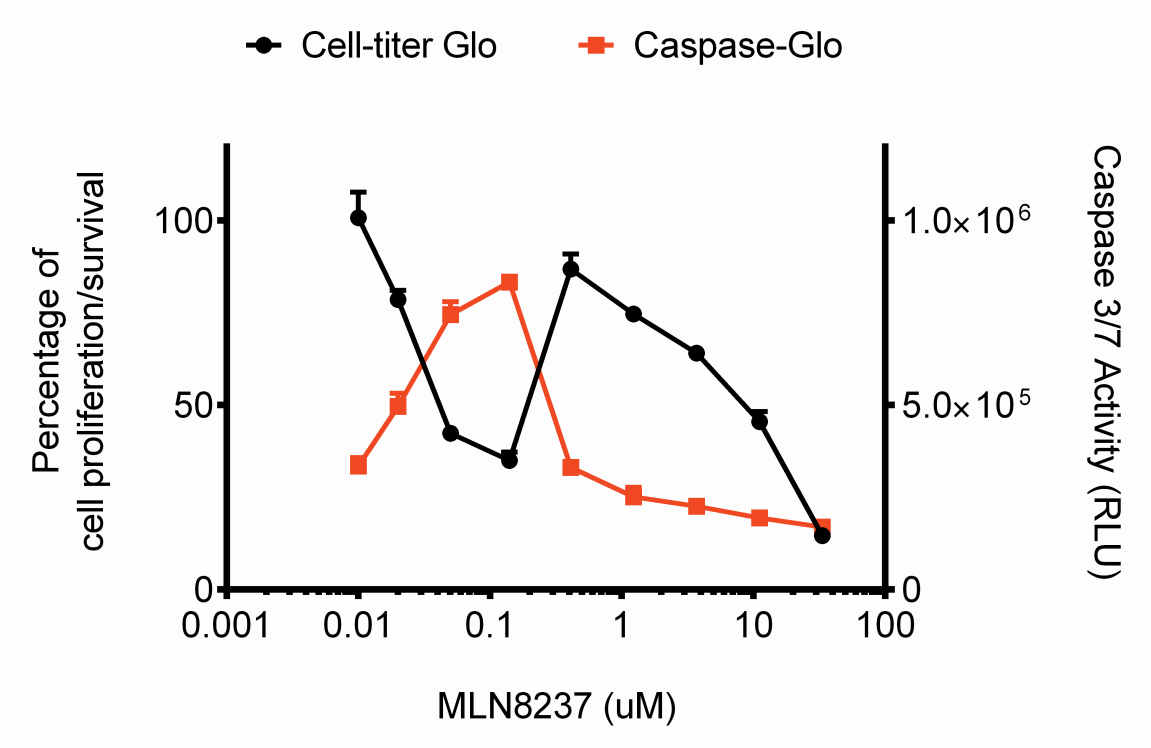
3-5


NCI-H1693
Percentage of cell proliferation/survival
100
50
0
-3
-2
-1
0
1
2
log [MLN8237 (uM)]

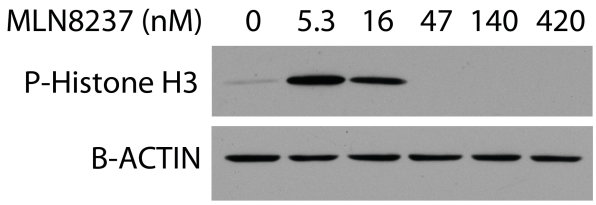
3-6


NCI-H1693
Caspase 3/7 Activity (RLU)
1.0×10^6
5.0×10^5
0
-3
-2
-1
0
1
2
log [MLN8237 (uM)]

3-7

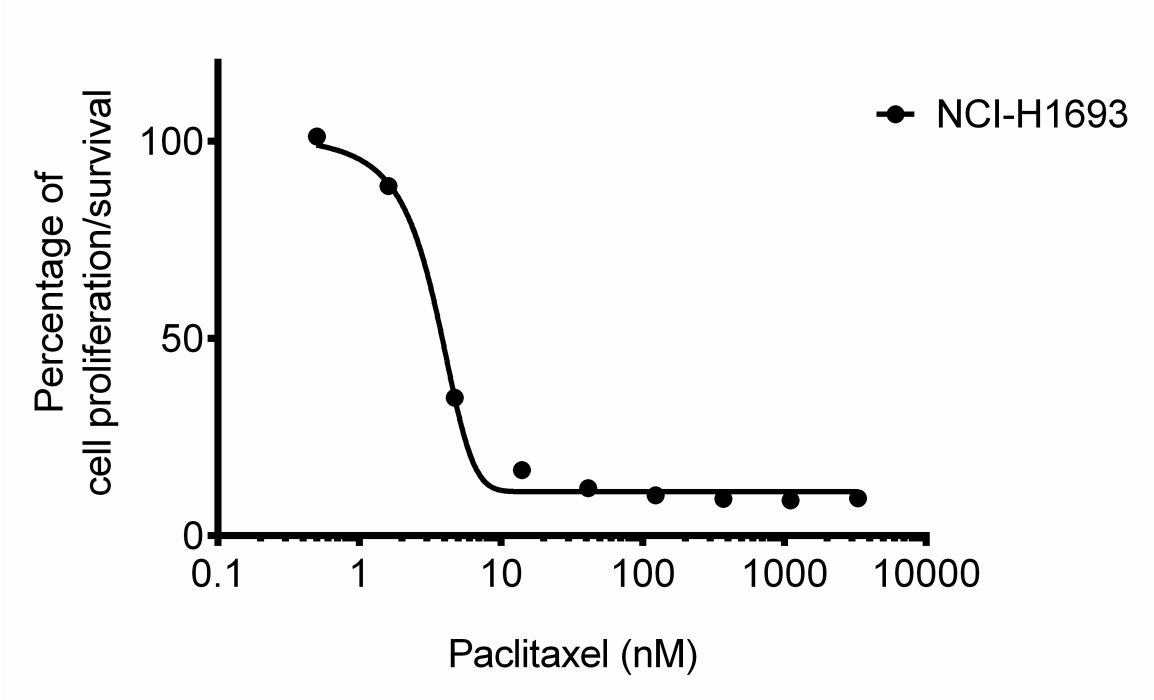


3-8

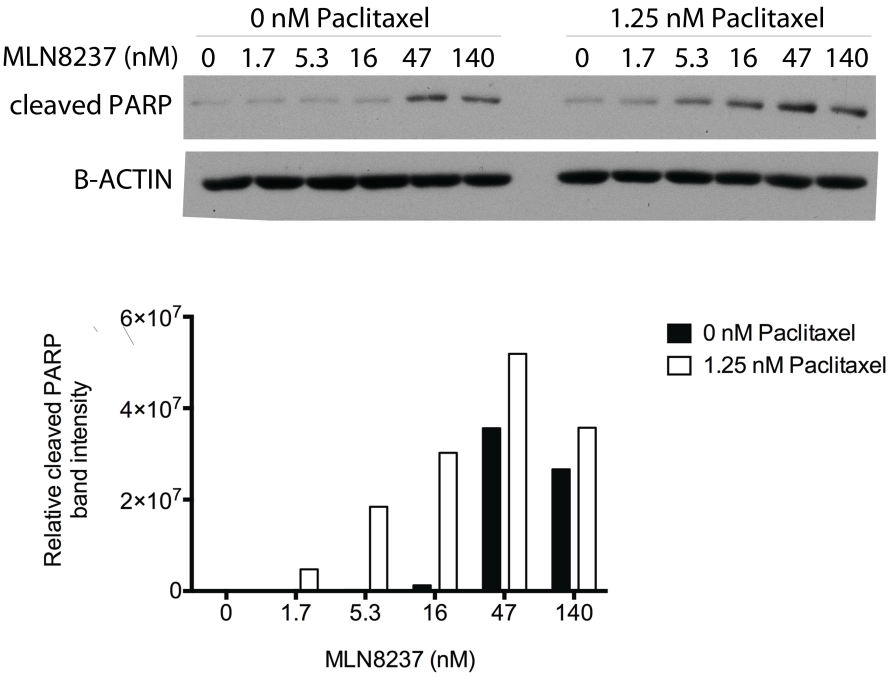


Figures 3-5/8: H1693 cells respond to MLN8237 treatment with an unusual triphasic drug response pattern. NCI-H1693 cells were treated with different concentrations of MLN8237. After 4 days or 3 days of treatment, cell viability was measured by **(3-5)** Cell-titer Glo assay or **(3-6)** Caspase-Glo Assay, respectively. **(3-7)** Overlay of Cell-titer Glo assay and Caspase-Glo assay. **(3-8)** H1693 cells were treated with different concentrations of MLN8237 and cell lysates were collected. Cell lysates were subjected to immunoblotting with monoclonal phospho-Histone H3 antibody. B-ACTIN levels were checked as an internal control.

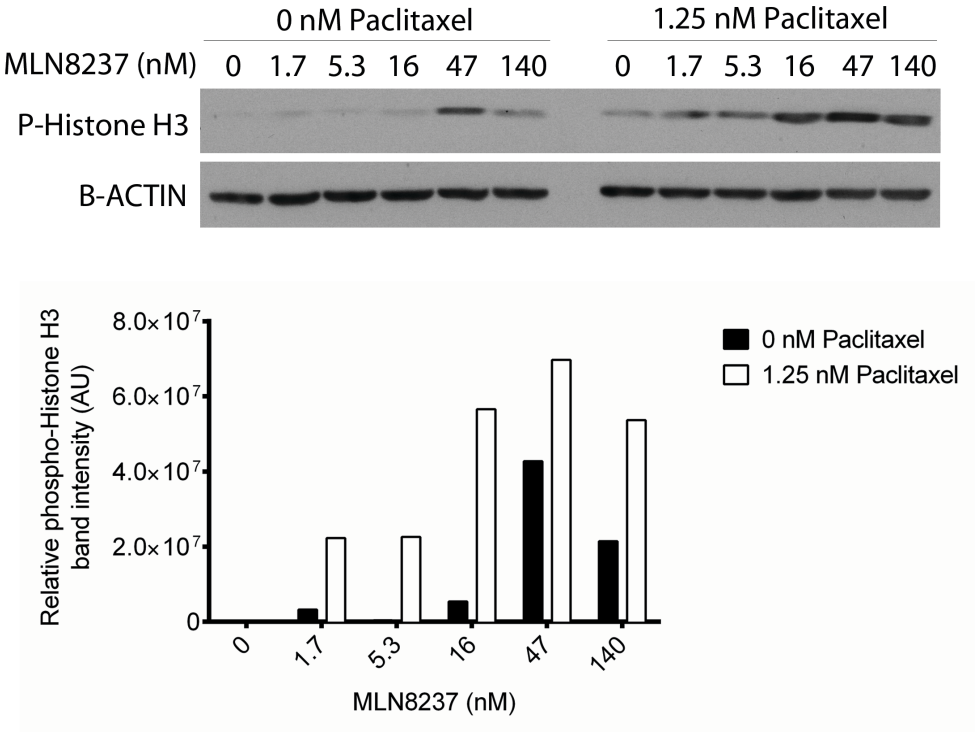
3-9


Percentage of cell proliferation/survival
100
50
0
0.1
1
10
100
1000
10000
Paclitaxel (nM)
NCI-H1693

3-10


0 nM Paclitaxel
1.25 nM Paclitaxel
MLN8237 (nM) 0 1.7 5.3 16 47 140 0 1.7 5.3 16 47 140
cleaved PARP
B-ACTIN
Relative cleaved PARP band intensity
6×10^7
4×10^7
2×10^7
0
0 1.7 5.3 16 47 140
MLN8237 (nM)
0 nM Paclitaxel
1.25 nM Paclitaxel

3-11



Figures 3-9/11: Paclitaxel synergistically increases the toxicity induced by MLN8237 treatment. **(3-9)** NCI-H1693 cells were treated with different concentrations of Paclitaxel. After 4 days of treatment, cell viability was measured with Cell-titer Glo assay. **(3-10)** H1693 cells were treated with six different concentration of MLN8237 in the presence or absence of 1.25 nM Paclitaxel. Cell lysates were collected and subjected to immunoblotting with monoclonal cleaved PARP antibody or **(3-11)** phospho-Histone H3 antibody. B-ACTIN levels were checked as an internal control. Band intensities of cleaved PARP or phospho-Histone H3 were measured with ImageQuant Software and normalized to the control band in each set of Paclitaxel treatment.

CHAPTER IV

MATERIALS AND METHODS

Materials and Methods Used for Experiments in Chapter II

Cell Lines

NSCLC and HBEC lines were generously provided by the laboratories of John Minna, MD and Adi Gazdar, MD (UT Southwestern Medical Center at Dallas, TX). NSCLC lines were cultured in RPMI-1640 medium (GIBCO, 11875) supplemented with 10% fetal bovine serum (v/v) (FBS) (Atlanta biological, S11550). HBEC lines were grown in Keratinocyte-SFM medium (GIBCO, 17005) with supplemented with EGF and keratinocyte extract (provided by the manufacturer). H1819, A549, H1299, H157, H23, H661 HCC15 and H1355 lines harbor SMARCA4/BRG1-inactivating mutations. H1792, H1975, H3255, H358, H820, HCC4006 and HCC827 express wild-type SMARCA4/BRG1. Calu-1, Calu-3, H1648, H2073, H2882, H3122, HCC1171, HCC193 and HCC78 are NSCLC lines harboring wild-type SMARCA4 gene with unknown BRG1 expression status. HBEC3-KT, HBEC30-KT AND HBEC34-KT are human bronchial epithelial cells immortalized with stable expression of CDK4 and hTERT.

Compounds, Antibodies and Plasmids

VX-680 (Chemietek, CT-VX680), MLN8237 (Selleck, S1133) and MLN8054 (Selleck, S1100) were purchased in powder form and stored at 20 mM, 10 mM or 10 mM stock concentrations in DMSO, respectively. TPX2 (Biolegend, mouse mAb, 628001), AURKA (Cell signaling, rabbit mAb, 4718), Phospho-Histone H3 (Cell signaling, rabbit mAb, 33770), cleaved PARP (Cell signaling, rabbit mAb, 9541), FLAG (Cell signaling, rabbit mAb, 2368), BRG1 (Novus Biologicals, rabbit mAb, NBP1-40785), PCM1 (Cell signaling, rabbit mAb, 5213), B-TUBULIN (Santa Cruz, mouse mAb, 55529), RAN (BD transduction laboratories, mouse mAb, 610341), DLG7/HURP (Bethyl laboratory, A300-852A), B-ACTIN (MP Biomedicals, mouse mAb, 69100) antibodies were used for immunoblotting or immunoflorescence assays. Propidium iodide was purchased from Sigma (P4170). pBABE-FLAG-BRG1 plasmid was obtained from Addgene (Gene ID: 1959). pBABE-Empty plasmid was a generous gift from Ugur Eskiocak, PhD in Jerry Shay laboratory.

High-Throughput Genomewide siRNA Library Screen

The siRNA library was purchased from Dharmacon. The siRNA library is designed to have 21124 pools of 4 individual siRNAs targeting approximately 85% of all identified genes in the human genome.

Transfection conditions were optimized with H1819 cell line with non-targeting siRNAs and siRNAs targeting PLK1 to achieve minimal unspecific toxicity with non-targeting siRNAs and maximal outcome with PLK1 siRNAs. For each gene target, siRNA pools were arrayed in 96-well plates. A reverse transfection protocol was utilized to maximize the transfection efficacy and siRNA pools at a final concentration of 50 nM were mixed with RNAiMax transfection reagent (Invitrogen, I3778-150) in OptiMEM medium (GIBCO, 31985) according to the optimized conditions. 3000 cells were dispensed into wells with an automated liquid dispenser. 96 hours after transfection, the culture medium was removed by centrifuging the plates upside down at 30g for 1 minute in a liquid collecting container and cell viability was measured with Cell-titer Glo (Promega, G7573) assay. Cell-titer Glo reagent was diluted in 1% Triton-X in PBS at 1:2 ratio.

Secondary Low-Throughput Confirmation Screen

A custom siRNA library with pools of 4 individual siRNAs for each of 38 target human genes was obtained from Dharmacon. Transfection conditions and methods same as the high-throughput screening were used. 96-hours after transfections in triplicates, cell viability was assessed with Cell-Titer Glo assay (Promega). Hits were primarily defined on the

basis of statistical and biological significance. The statistical assessments were made with student's T-test. Hits demonstrating a p-value of less than 0.01 were considered statistically significant. Biological significance was arbitrarily defined as a decrease in the Cell-titer Glo assay of more than 50% when compared to the Cell-titer Glo assay of non-targeting siRNA transfected cells. Hits identified as statistically and biologically significant were categorized as high-priority hits and statistically significant hits with failed biological significance were grouped as low-priority hits.

Microplate Apoptosis Assay

Transfections of the siRNA pools in the high-priority hits category were performed in the same manner with the high-throughput and low-throughput library screens in 96-well plate format. 24, 48 and 72 hours after transfections, culture medium was centrifuged upside down in liquid collecting containers at 3000g for 1 minute. Caspase 3/7 activity was assessed with Caspase-Glo 3/7 assay purchased from Promega (G8090). Caspase-Glo 3/7 reagent was diluted in 1% Triton-X in PBS at 1:1 ratio. Hits were identified on the basis of statistical significance. The statistical assessments were made with student's T-test. Hits demonstrating a p-value of less than 0.001 were considered statistically significant when compared to non-targeting siRNA transfected cells.

siRNA Confirmations with Cell Viability Assays

Four individual siRNAs used for knockdowns of TPX2, AURKA and DLG7 were purchased from Dharmacon. They were dissolved in DNAse/RNase free water overnight to make a final concentration of 5 uM. Reverse transfection protocol was utilized to maximize the transfection efficacy and siRNA pools at a final concentration of 50 nM were mixed with RNAiMax transfection reagent in OptiMEM medium 1000-2000 cells were dispensed into wells. 96 hours after transfection, the culture medium was removed by centrifuging the plates upside down at 30xG for 1 minute in a liquid collecting container and cell viability was measured with Cell-titer Glo assay. Cell-titer Glo was diluted in 1% Triton-X in PBS at 1:2 ratio.

Generating BRG1-expressing cell lines with retroviral infection

pBABE-Empty or pBABE-FLAG-BRG1 plasmids were packed into retroviruses in Jerry Shay's laboratory (UT Southwestern Med. Center). Cells were infected and BRG1-expressing H1819 cells were selected with puromycin. The cells were expanded as heterogeneous populations.

Western Blotting

$2x10^6$ cells were transfected with individual siRNAs at the concentration of 50 nM in 6-well format. 72 hours after transfection, the culture medium

was aspirated and cells were washed with PBS. Cells were lysed in RIPA lysis buffer (50 mM Tris buffer pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium, 0.1 % SDS, 0.5% Sodium deoxycholate) supplemented with protease and phosphatase inhibitors. Protein concentrations of cell lysates were measured with Protein DC assay (BioRad, 500-0111). Endogenous TPX2 or AURKA levels, phosphorylation of Histone H3 and B-ACTIN expression as internal control were monitored by immunoblotting with monoclonal antibodies.

Immunofluorescence

$5x10^6$ cells were cultured on a glass cover slip in 6-well plate format. 24 hours after seeding, culture media were aspirated and cells were washed with PBS. Cells grown attached to the cover glass were fixed with 3.7% formaldehyde (Sigma-Aldrich, 252549) in PBS (v/v) for 15 minutes. Excess formaldehyde was washed with PBS twice and cells were permeabilized with 0.25% Triton-X in PBS for 10 min. After washing with PBS twice, the samples were blocked with 10% BSA. After 1 hour blocking, the samples were stained with monoclonal antibodies against PCM1, B-TUBULIN or FLAG overnight at 4ºC. Alexaflour 488 or 568 secondary antibodies were used for detection of protein of interests. After secondary Alexaflour 488 or 568 antibody treatment and washing, cover

slips were mounted on microscope slides with the ProLong gold antifade mounting reagent.

Microplate Drug Sensitivity Assays

3000 cells were seeded in 96-well plates. 24 hours after, treatments with DMSO or 3X serial dilutions of VX-680 in 9-point concentration range were performed. 96 hours after treatments, media were removed and cell viability was measured with Cell-titer Glo assay.

A screen of a panel of NSCLC and HBEC cell lines with VX-680 was performed in 384-well plate format. 24 hours after plating cells at a density of 1000-1500 cells, VX-680 treatments at 12 half-log concentrations ranging from 50 pM to 50 uM were performed in triplicates. 96 hours after treatments, cell viability was measured with Cell-Titer Glo assay. Dose responses of each cell line were assessed and EC50 values were calculated with 5-parameter logistic (5PL) equation.

Mouse Xenograft and *In Vivo* Drug Studies

Cells derived from the human NSCLC line HCC827, were trypsinized and collected in RPMI 1640 media supplemented with 10% FBS (v/v). The cells were centrifuged at 1000g and the media were aspirated. After washing twice with sterilized PBS, cells were resuspended and diluted in

PBS to have 2.5 x 10^6 cells/100 µl concentration. 100 µl of the single cell suspensions were injected into the right flanks of NOD/SCID mice. Mice xenografted with HCC827 cells were maintained in hyper-sterile NG Animal Research Center (ARC). Tumor volumes were measured twice a week (on Mondays and Thursdays) with caliper measurements and calculated by the formula: tumor volume = (length x width^2)/2. VX-680 treatment was initiated when tumors reached a volume of 200 mm^3. Mice were assigned to treatment groups in serpentine style after sorted according to the tumor volume. VX-680 was formulated with 50% PEG-300 (Sigma, 90878) in PBS. Mice were injected intraperitoneally with 0, 5, 10, 20, 30 and 50 mg/kg VX-680 B.I.D. The experiment was terminated when the largest tumors reached 2000 mm^3.

Materials and Methods Used for Experiments in Chapter III

Cell Lines

The H1693 NSCLC line was generously provided by Adi Gazdar, MD (UT Southwestern Medical Center at Dallas, TX). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS).

Compounds and Antibodies

MLN8237 (S1133) was purchased from Selleck Chemicals in powder form and stored at 10 mM stock concentrations in DMSO. AURKA (Cell signaling, rabbit mAb, 4718), Phospho-Histone H3 (Cell signaling, rabbit mAb, 33770), cleaved PARP (Cell signaling, rabbit mAb, 9541) and B-ACTIN (MP Biomedicals, mouse mAb, 69100) antibodies were used for immunoblotting or immunofluorescence assays.

siRNA Transfections and Cell Viability Assays

Individual siRNA #28 used for knockdown of AURKA was purchased from Dharmacon. It was dissolved in DNAse/RNase free water overnight to make a final concentration of 5 uM. A reverse transfection protocol was utilized to maximize the transfection efficacy and siRNA pools at a final concentration of 50 nM were mixed with RNAiMax transfection reagent in OptiMEM medium according to the optimized conditions. 1000-2000 cells were dispensed into wells. 96 hours after transfection, the culture medium was removed by centrifuging the plates upside down at 30g for 1 minute in a liquid collecting container and cell viability was measured with Cell-titer Glo (Promega, G7573) assay. Cell-titer Glo was diluted in 1% Triton-X in PBS at 1:2 ratio.

Western Blotting

2x10^6 cells were transfected with individual siRNA, #28, at the concentration of 50 nM in 6-well format. A reverse transfection method was used. 72 hours after transfection, the culture medium was aspirated and cells were washed with PBS. Cells were lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentrations of cell lysates were measured with Protein DC assay. Endogenous AURKA levels, phosphorylation of Histone H3, cleaved PARP and B-ACTIN expression as internal control were immunoblotted with monoclonal antibodies.

Microplate Apoptosis Assay

Transfections of the siRNAs against AURKA and PLK1, and non-targeting siRNAs were performed in 96-well plate format. 24, 48 and 72 hours after transfections, culture medium was centrifuged upside down in liquid collecting containers at 3000g for 1 minute and caspase 3/7 activity was assessed with Caspase-Glo assay purchased from Promega. Caspase-Glo reagent was diluted in 1% Triton-X in PBS at 1:1 ratio. Hits were identified on the basis of statistical significance. The statistical assessments were made with student's T-test. Hits demonstrating a p-

value of less than 0.001 were considered statistically significant when compared to non-targeting siRNA transfected cells.

Colony Formation Assays

Cells were plated in triplicates in 6-well plates. Each well of the triplicates were seeded with either 500, 1000 or 2000 cells. Forward transfection for RNAi tests was preferred to decrease method-dependent toxicity. siRNAs at a final concentration of 10 nM and different concentrations of MLN8237 as described in the particular experiment in the results section were used. 10 days after transfections or treatments, cells were washed with cold PBS and stained with a staining buffer composed of 0.5% crystal violet (Sigma, C-3886) and 6% gluteraldehyde (Sigma, 340855) in MilliQ water for 4 hours at room temperature. After staining, wells were washed with running deionized water.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Since late 1990s, prior to our study, evidence has accumulated about the inactivating mutations in genes encoding the components of the SWI/SNF complex and their association with cancer development. Initially, pediatric rhabdoid tumors were linked with SNF5 mutations and then, BRG1 lesions were found in various adult tumors (Medina et al., 2004; Versteege et al., 1998). Detection of the mutant forms of SNF5 and BRG1 genes in cancers led the research for, and discoveries of, inactivating mutations in the other subunits of the SWI/SNF complex. Thus far, 9 known components of the SWI/SNF complex are found mutated in many cancer types including lung, breast, prostate, pancreatic, ovarian, colorectal, kidney, bladder, endometrium, liver, brain and blood cancers(Romero & Sanchez-Cespedes, 2013). Very recently, three simultaneous reports revealed that homozygous BRG1-inactivating mutations were observed in almost all of the Small Cell Carcinoma of The Ovary (SCCO) tumors (Jelinic et al., 2014; Ramos et al., 2014; Witkowski et al., 2014). Common cancer phenotypes associated with mutations in these genes point out a new hallmark of cancer as “deregulation of chromatin remodeling and epigenetics”.

The mutations in the genes encoding the SWI/SNF complex occur either somatically or in the germ line. Adult cancers happen after the accumulation of sufficient genomic changes following a process of random mutagenesis due to weakened DNA damage control. Therefore, today, it is hard to predict whether a genetic lesion found in a cancer is actively contributing to the development of cancer since many of them are passenger mutations. However, unlike adult cancers, childhood cancers possess significantly lower numbers of genetic mutations. Lawrence et. al recently showed that rhabdoid tumors are able to develop with 5 mutations on average and there are cases where even with a single mutation in SNF5 is sufficient to trigger tumor formation (Lawrence et al., 2013). Additionally, studies with SCCO yielded striking results showing that those tumors harbor no significant mutation other than SMARCA4/BRG1 (Jelinic et al., 2014; Ramos et al., 2014; Witkowski et al., 2014). This phenomenon highly suggests that the mutations in the genes encoding the SWI/SNF complex cause a dramatic shift in the gene expression profile, leading to an enormous change in the cellular protein network that is capable of transforming the cells without needing to acquire additional oncogenic lesions. Supporting this idea, large-scale genomic profiling of these tumors revealed that the majority of them have no mutations in the known, frequently mutated oncogenes found in many cancer types (Ding

et al., 2008; Oike et al., 2013). Consistent with the notion that deficiency of the SWI/SNF complex is able to reprogram cells to drive cancers in a self-sufficient way, BRG1 mutations in adult non-small cell lung cancers do not overlap with the mutations of many known oncogenes, including EGFR, ALK, MYC and so on (Oike et al., 2013; Romero & Sanchez-Cespedes, 2013).

As described in this dissertation, NSCLC lines harboring BRG1-inactivating mutations are hypersensitive to the Aurora kinase A inhibitor, VX-680. This is probably because Aurora kinase A has a central role in the cancer cells re-programmed by BRG1 loss. Although the biological connection between BRG1 activity and cancer has not been characterized yet, previous studies showed a link between the regulation of mitosis and BRG1 by observing mitotic catastrophe immediately after knocking-out BRG1 (Bourgo et al., 2009). This observation may explain the weakness in the mitotic spindle machinery in NSCLCs with inactivated BRG1. The cells that managed to survive after the loss of BRG1 may have a novel adaptation for assembling mitotic spindles successfully, but now are not tolerant to any interference of mitotic spindle assembly. Such possible change to adapt the absence of centrosomes would explain the link between increased toxicity of VX-680 in NSCLCs and their BRG1 status. This hypothesis is supported by my observation that centrosomal integrity

was lost in the BRG1-mutant NSCLC line H1819. In the future, more comprehensive studies should compare the kinetics of microtubule nucleation and mitotic spindle integrity in cells with and without BRG1 mutations to better understand the possible role of BRG1 for this process.

The absence of centrosomes in successfully dividing H1819 cells led us to consider centrosome-independent mitotic spindle assembly mechanisms more closely. Previous studies demonstrated that mammalian cells were able to control mitotic spindle assembly with at least two separate mechanisms (Barr & Gergely, 2007; Wadsworth & Khodjakov, 2004). One is centrosome-dependent and the other is chromosome-dependent. Loss of centrosomes, for that reason, may not create a complete loss of mitotic spindle formation ability of the somatic cells if the alternative acentrosomal pathway is properly functional. To test this hypothesis, with RNAi approach, we depleted DLG7 proteins as a key component of centrosome-independent machinery and performed cell viability assays with or without ectopic BRG1 expression in H1819 cells. Our results showed that DLG7 depletion was detrimental to the parental H1819 cells although DLG7 activity was identified as dispensible in mammalian somatic cells. Moreover, re-introduction of wild-type BRG1 expression rescued the toxicity induced by DLG7 depletion. The results suggest that BRG1 loss makes cells addicted to DLG7 activity for forming functional

mitotic spindles in centrosome-independent manner. Further studies are needed to explain how DLG7 functions in cancer cells and interacts with AURKA.

Why cancer cells lose centrosomes is one other important question arising if the relation between BRG1 loss and centrosome loss is correct. Since centrosome-dependent mitotic spindle assembly has evolved as a major mechanism in the somatic cells of animals, it would be expected that centrosomes would be fundamental units for safely accomplishing cell division to support the rapid growth of cancers. However, it is known that sustained growth signals are a hallmark of cancer and for uncontrolled tumor growth, cells first need to maintain a continuous state of cell proliferation signaling (Hanahan & Weinberg, 2011). Therefore, the primary need of cancer formation is to create the signaling machinery to trigger signals for fast growth. Regarding this fundamental need, centrosomes, when abnormally amplified, build cilia-like formations and become important platforms for cellular signaling pathways such as Wnt and hedgehog(Egeberg et al., 2012; Hassounah, Bunch, & McDermott, 2012; Koefoed, Veland, Pedersen, Larsen, & Christensen, 2013; Liu, Chen, Cheng, Jing, & Helms, 2014). From this point of view, sacrifice of centrosomes, when tolerated with the chromosome-dependent mitotic spindle assembly mechanism, may create an advantage for sustaining cell

proliferation signals and accomplish an important event for the progression of the disease. It is important to note that AURKA is the central component of acentrosomal mitotic spindle assembly, but a regulator of centrosome-dependent machinery. More than 200 centrosome associated proteins were pulled down in large scale proteomic studies whereas there are only 6 identified components of chromosome-dependent mitotic spindle machinery (Andersen et al., 2003). Thus, when centrosomes lose their proper functions, the redundancy in the microtubule organizing centers may also be lost and create this vulnerability and opportunity for cancer therapeutics.

Thus far, BRG1 is identified as a bona fide tumor suppressor with little information about its role in cancer progression. There is a growing need to understand the path to cancer starting with BRG1 mutations. To study the role of BRG1 mutations in cancer, new experimental platforms are required. However, the technological advances in gene editing provide a promising approach to study the contribution of gene losses to the diseases. The most direct way to characterize the role of BRG1 in cancer is to inactivate it in wild-type BRG1-expressing normal cells via RNAi or CRISPR methods (Koike-Yusa, Li, Tan, Velasco-Herrera, & Yusa, 2013). Since many interactions of BRG1 are identified, the sequence of those events may be understood with such an experimental setting.

The inevitable fate of targeted-therapies is acquired resistance. Besides studying the role of BRG1 in cancer progression, these VX-680 sensitive cell lines are important tools to find resistance mechanisms. Selection of resistant clones and investigation of alteration in those particular cell populations in respect to the parental cells may provide information about possible ways to escape from VX-680 interventions. These might inform about the way that BRG1 induces cancers and the new mutant gene products might be discovered to target for second-line therapy approaches.

Lastly, in my dissertation work, I found 37 other genes that are significantly critical for H1819 cells. Dependent or independent of BRG1, these genes are differentially toxic when compared with HBEC30-KT cells, indicating a potential for new anti-cancer drug target investigations. They cluster in splicing, protein degradation and RNA expression mechanisms.

By all means, knowing the genetic composition of each individual cancer provides a more deliberate platform to select the best drug in the clinic and, with the advent of genomic technologies, identification of each genetic aberration in individual tumors becomes increasingly achievable and promising to become a routine during the drug decision making process. Functional genomics approaches will continue providing new

candidate gene products as drug targets and developing drugs against them will gradually enrich the repertoire of anti-cancer agents to offer more therapeutic options for various genomic aberrations.

CHAPTER VI

APPENDICES

Appendix 1. List of FDA-Approved Targeted-Therapy and Chemotherapy Agents

	Targeted Therapy Agent	*Chemotherapy Agents*
1	Alemtuzumab	Actinomycin
2	Alitretinoin	All-trans retinoic acid
3	Anastrozole	Azacitidine
4	Bevacizumab	Azathioprine
5	Bexarotene	Bleomycin
6	Bortezomib	Bortezomib
7	Bosutinib	Carboplatin
8	Brentuximab vedotin	Capecitabine
9	Cabozantinib	Cisplatin
10	Carfilzomib	Chlorambucil
11	Cetuximab	Cyclophosphamide
12	Crizotinib	Cytarabine
13	Dasatinib	Daunorubicin
14	Denileukin diftitox	Docetaxel
15	Erlotinib hydrochloride	Doxifluridine
16	Everolimus	Doxorubicin
17	Exemestane	Epirubicin
18	Fulvestrant	Epothilone
19	Gefitinib	Etoposide
20	Ibritumomab tiuxetan	Fluorouracil
21	Imatinib mesylate	Gemcitabine
22	Ipilimumab	Hydroxyurea
23	Lapatinib ditosylate	Idarubicin
24	Letrozole	Imatinib
25	Nilotinib	Irinotecan
26	Ofatumumab	Mechlorethamine
27	Panitumumab	Mercaptopurine
28	Pazopanib hydrochloride	Methotrexate

29	Pertuzumab	Mitoxantrone
30	Pralatrexate	Oxaliplatin
31	Regorafenib	Paclitaxel
32	Rituximab	Pemetrexed
33	Romidepsin	Teniposide
34	Sorafenib tosylate	Tioguanine
35	Sunitinib malate	Topotecan
36	Tamoxifen	Valrubicin
37	Temsirolimus	Vinblastine
38	Toremifene	Vincristine
39	Tositumomab and 131I-tositumomab	Vindesine
40	Trastuzumab	Vinorelbine
41	Tretinoin	
42	Vandetanib	
43	Vemurafenib	
44	Vorinostat	
45	Ziv-aflibercept	

Appendix 2. Primary screen gene hits with mean viability equal to or less than 50%. (Overlapping hits from the screen performed with BRG1-expressing HBEC30-KT line are colored in Red and excluded in the follow-up experiments)

Gene ID	*Mean Viability*
UBB	0.030
UBC	0.030
NXF1	0.171
TPX2	0.225
POLR2A	0.294
CLEC1B	0.317
KIF9	0.339
RAN	0.343
BCAR4	0.344
PSMD6	0.350
NUP98	0.360
LOC400957	0.368
ROBLD3	0.379
XAB2	0.381
HES7	0.381
PLOD3	0.402
C16orf63	0.404
XKR6	0.412
NCLN	0.418
HDAC4	0.421
PSMD8	0.433
PLK1	0.436
FADS3	0.448
LOC283432	0.449
PTPRQ	0.458
PART1	0.462
SON	0.462
POTEH	0.465
ASB16	0.468
CNTFR	0.470

DNAJC6	0.473
MORF4L1	0.480
XIAP	0.480
LCE2A	0.483
C14orf115	0.486
PPARA	0.487
NAPA	0.489
MBNL3	0.491
SF3B1	0.493
NSMCE2	0.493
EIF4A3	0.496
CRYBB1	0.497
C20orf95	0.499
PEX11G	0.500
TENC1	0.500
PSG6	0.500

CHAPTER VII

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