INVESTIGATION OF RAS-DEPENDENT GENE EXPRESSION AND CELL SURVIVAL SIGNALING IN COLORECTAL CANCER

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2016

Declaration

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

The thesis has also not been submitted for any degree in any university previously.

Pek Mi Xue Michelle

7January 2016

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To God be the glory, the best is yet to be.

Summary

KRAS mutation occurs in up to 60% of colorectal cancers (CRC) and development of effective targeted therapies still remains a formidable challenge. Studies have highlighted the issue of KRAS dependency and independency, implying that KRAS mutation status is inadequate as a biomarker of treatment response. There is a need to identify better biomarkers to predict response to proposed treatment strategies. Intratumoral heterogeneity in CRC tumors, however, hindered the establishment of a clinically derived KRAS dependency gene signature. Here, through integration of quantitative KRAS mutation pyrosequencing data and gene expression profiling in CRC tumors, we are able to define a KRAS dependency gene signature which exhibits enrichment in cell cycle and mitotic processes, together with elevated FOXM1 expression. We proposed a therapeutic strategy using CDK4/6 and MEK inhibitors that targets KRAS-driven molecular signature and reduces viability of KRAS-dependent and BRAF-mutant CRC via the synergistic depletion of FOXM1 and reduction of other mitotic transcription factors E2F1 and CMYC, both in vitro and in vivo. Moreover, depletion of FOXM1 is able to subvert growth of KRAS-dependent CRC. No toxicity was observed in vivo and, intriguingly, addition of CDK4/6 inhibitor reduces sensitivity of normal colon epithelial lines to MEK inhibitor, suggesting that this combination therapy could mitigate effects of MEK inhibition on normal noncancerous cells. Our study thus establishes KRAS dependency gene signature as a potential biomarker of response and the combinatorial inhibition of CDK4/6 and MEK as a promising treatment strategy against KRAS/MAPK-dependent CRC.

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List of Abbreviations

APC	Adenomatous polyposis coli
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
°C	Degrees centigrade
CCNB1	Cyclin B1
CCND1/2/3	Cyclin D1/2/3
CDK4/6	Cyclin-Dependent Kinase 4/6
cDNA	Complementary Deoxyribonucleic acid
Cip	CDK interacting protein
CIMP	CpG island methylator phenotype
CRC	Colorectal cancer
CIN	Chromosomal instability
dsDNA	Double stranded Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
FACs	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FOLFIRI	Folinic acid, fluorouracil, irinotecan
FOLFOX	Folinic acid, fluorouracil, oxaliplatin
FOXM1	Forkhead box M1
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GRB2	Growth factor receptor- bound protein 2
GTP	Guanosine triphosphate
HER2	Human epidermal growth factor receptor 2
HDAC	Histone deacetylase
IGF-2R	Insulin-like growth factor 2 receptor
INK4	Inhibitor of kinase 4
IPA	Ingenuity pathway analysis
IRS	Insulin receptor substrate
kDa	Kilodalton
KSR	Kinase repressor of KRAS
Kip	Kinase inhibitor protein
KRAS	Kirsten rat sarcoma viral oncogene
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
$MgCl_2$	Magnesium chloride
MLH1	MutL homolog 1
mRNA	Messenger RNA
MSI	Microsatellite instability
mTORC2	Mechanistic target of rapamycin complex 2
NaCl	Sodium chloride
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDEδ	prenyl-binding protein

PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIP2	phosphatidylinositol-3,4-diphosphate
PIP3	phosphatidylinositol 3, 4, 5 triphosphate
RAF	MAP kinase kinase kinase
RB	Retinoblastoma protein
RBD	Ras binding domain
RNA	Ribonucleic acid
rpm	Revolutions per minute
RTK	Receptor tyrosine kinase
SAH	Stabilized alpha helices
ssDNA	Single stranded DNA
siRNA	Small interfering RNA
SDS	Sodium dodecyl sulphate
SOS	Son of Sevenless
SH2	Src-homology domain 2
SHC	Src homology and collagen domain
TGF	Transforming growth factor
WT	Wild type
VEGF	Vascular endothelial growth factor

List of Publications

1. Conference paper: Michelle Mi Xue Pek & Qiang Yu. Investigation of RAS-dependent gene expression and cell survival signaling in colorectal cancer. Gordon Research Conference: Cell Growth and Proliferation. West Dover, VT. June 23-28, 2013
2. Conference paper: Michelle Mi Xue Pek & Qiang Yu. Investigation of RAS-dependent gene expression and cell survival signaling in colorectal cancer for therapeutic perturbation. Cell Symposia, Hallmarks of Cancer: Asia. Beijing, China. November 9-11,2014
3. Conference paper: Michelle Mi Xue Pek & Qiang Yu. Investigation of RAS-dependent gene expression and cell survival signaling in colorectal cancer for therapeutic perturbation. European Association for Cancer Conference Series 2015: 2nd Special Conference: Cancer Genomics 2015. Cambridge, UK. June 28- July 1, 2015.

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CHAPTER 1: INTRODUCTION

1.1 Colorectal Cancer

Colorectal cancer arises from the large intestine which comprises of the ascending colon, transverse colon, descending colon, sigmoid colon and the rectum (NCI).The large intestine functions to absorb water and nutrients from food and to store and control excretion of fecal materials. Similar to most of the gastrointestinal tract, the large intestine consists of 4 layers (Figure 1.1) namely: the mucosa which is a columnar epithelium with numerous mucus-secreting goblet cells; the submucosa which contains blood vessels and submucous plexus; the muscularis propria where the circular and longitudinal muscles and myenteric nerve plexus are found; and lastly the serosa which consists of the visceral peritoneum. Tumor growth initiates from the mucosa layer and invades outwards to the sercosa. Adenocarcinomas originating from the epithelium of the mucosa make up more than 90% of all colorectal carcinoma (Bosman FT 2010), with neuroendocrine, spindle cell, adenosquamous, squamous and undifferentiated carcinomas making up the rest of the cases.

Upon invasion of tumor cells beyond the mucosa layer as the tumor grows larger in size, there is an increase likelihood of colorectal cancer cells invading the blood or lymphatic vessels to metastasize to other sites in the body. Metastatic colorectal cancers are frequently presented in the liver due to the drainage of the venous blood flow from the large intestine into the hepatic portal vein leading into the liver. The next most common sites for metastases are peritoneum and lungs.

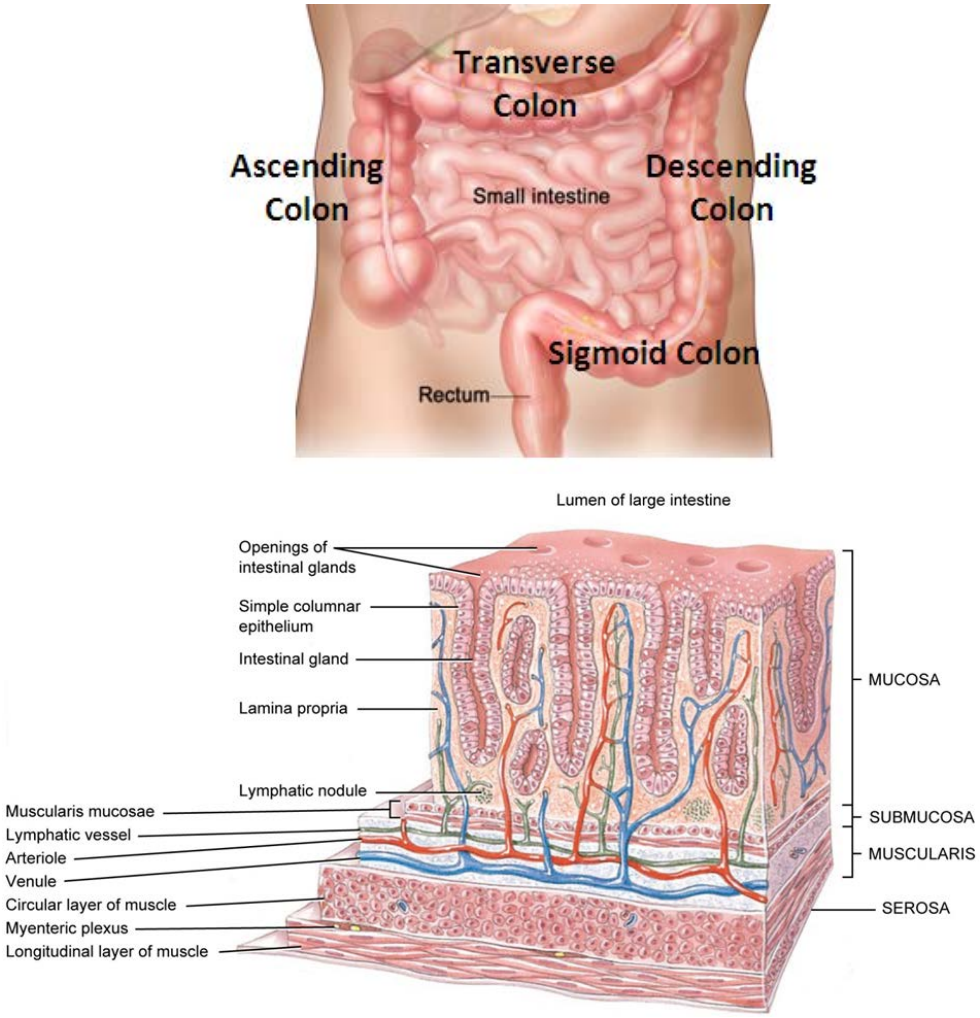


Figure 1.1 Anatomy of the colon and rectum

Anatomical structure of the colon and the rectum (Figure adapted from https://www.nlm.nih.gov/medlineplus/magazine/issues/spring09/articles/spring09pg7-8.html and http://www.kln.ac.lk/science/depts/zoology/images/stories/zoo/Kumudu/histology%20of%20large%20intestine%20%20fig%202.jpg

1.1.1 Epidemiology of Colorectal Cancer

Colorectal cancer (CRC) is the third leading cause of death in all cancers in both male and female with an estimated 50,000 deaths and more than 130,000 new cases of CRC were expected to surface in the United States in 2015, making CRC the cancer with the third highest incidence rate (Siegel, Miller et al. 2015). Incidence of colorectal cancer increases with age, with the median age of diagnosis at about 70 years in developed nations (Siegel, DeSantis et al. 2012).

With improved treatment methods and screening, mortality has decreased in developed countries. Screening through colonoscopy allows for the removal of any premalignant adenomatous polyps at the same time, preventing the development of cancer. 5-year relative survival rate is 90% when CRC is detected at early stages (localized spread) but survival rate decreased drastically to 13% when the cancer has metastasized to distant organs (DeSantis, Lin et al. 2014).

1.1.2 Treatment of Colorectal Cancer

The treatment of colorectal cancer depends on the stage of the cancer at the time of diagnosis. Colorectal cancer patients are stratified into the four stages based on the TNM staging criteria where T refers to the size of the tumor and the extent at which the tumor had spread through the walls of the colon; N indicates if the cancer cells has infiltrated into the nearby lymph nodes and the higher the number of lymph nodes involved, the higher the possibility of the systemic spread of the cancer; and M indicates the presence or absence of metastases at a distant organ or lymph nodes (Shia, Klimstra et al. 2012, 2014). Another factor to determine the appropriate treatment is the grade of the cancer (Derwinger, Kodeda et al. 2010). Low grade cancer means the cancer cells are highly differentiated and look similar to normal colorectal tissue and they are likely to grow slower and be less invasive. High grade cancer, however, displays poorly differentiated or undifferentiated morphology and indicates a poorer prognosis as these cancer cells tend to grow faster and are more

invasive. Thus, more aggressive treatment in terms of adjuvant chemotherapy may be given.

Stage I and II CRC, where the tumor is confined within the colon, the cancer is curable through colostomy (Skibber 2001) by removing the section of the colon or rectum containing the tumor. When the cancerous cells have spread to the lymph nodes or beyond the walls of the colon (stage III), adjuvant chemotherapy and/or radiation may be given after surgical resection of the colon to improve survival by reducing recurrence (Sargent, Sobrero et al. 2009). Stage IV CRC, where the cancer had further metastasized to other organs or tissues, chemotherapy is mainly used in attempt to systemically remove metastases in the body and surgery and radiation may also be given to relieve blockage of colon caused by the primary tumor (Skibber 2001, 2014).

1.1.2.1 Treatment of metastatic colorectal cancer using conventional chemotherapy

For metastatic CRC, till 2009, the first line treatment in CRC uses a combination of 5-fluorouracil with leucovorin or capecitabine with either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) which is able to target and kill all rapidly growing and dividing cells, both normal and cancerous (Meyerhardt and Mayer 2005). 5-fluorouracil is the cornerstone of CRC treatment, inhibiting thymidylate synthase which is the rate-limiting enzyme in pyrimidine nucleotides synthesis (Longley, Harkin et al. 2003). Combination with leucovorin enhances the ability of 5-fluorouracil to bind to thymidylate synthase. Oxaliplatin is a platinum-based drug which generates inter- and intra-strand cross linkages in DNA, preventing DNA replication and transcription (Graham, Mushin et al. 2004). Irinotecan is a topoisomerase I inhibitor and prevents type 1 topoisomerase from relieving supercoils formed during DNA replication and transcription (Wang 2002). There are many advances over the years in the development of chemotherapeutic drugs for advanced CRC, resulting in the increase

of the median overall survival time from 10 months with the use of fluorouracil in the 1980s and 1990s to about 20 months using the current standard chemotherapy drugs in various combinations (Goldberg, Sargent et al. 2004). However, even with better chemotherapeutic drugs, the median survival time stagnated at around 20 months. Furthermore, these cytotoxic drugs have very narrow therapeutic index, implying that the optimal effective dose is very close to the lethal dose. The flexibility to change the drug dosage to suit the patients' response to the treatment is limited, especially for patients who do not respond. Thus, a new approach to CRC treatment is needed.

1.1.2.2 Use of targeted therapy for metastatic colorectal cancer

The past decades have seen unprecedented advances in the understanding of the molecular biology of cancer and this knowledge has aided identification of pathways and targets that are vital for the survival of the cancer cells. Development of specific small molecule inhibitors against kinases soon followed with hopes of abrogating the cancer cells and sparing the normal cells to improve treatment response and reduce adverse side effects which are almost certainly observed in the use of chemotherapy. Specifically in colorectal cancer, the use of monoclonal antibodies against vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) together with chemotherapy are approved for use.

The use of anti-VEGF therapy started due to the role of VEGF in mediating pro-survival pathways in endothelial cells (Gerber, McMurtrey et al. 1998, Fujio and Walsh 1999) and endothelial cells are the building blocks of the formation of new vessels. By blocking the VEGF-activated pathways, tumor angiogenesis which is the sprouting of new vessels from existing vessels, together with the loss of adhesion between pericyte and endothelial cells, vasodilation, increased permeability of vessels and the integration of bone marrow-derived endothelial progenitor cells (Jain 2005), can be inhibited and tumor growth and metastasis can be hindered. Bevacizumab, the monoclonal antibody used in the treatment of metastatic CRC, binds to VEGF and

prevent it from interacting with its receptor on the endothelial cells. Phase III clinical trials using a chemotherapy regimen of irinotecan, fluorouracil and leucovorin with bevacizumab to treat metastatic CRC improved the median overall survival by 4.7 months and progression free survival by 4.4 months as compared to the patients receiving only chemotherapy (Hurwitz, Fehrenbacher et al. 2004). This combination is currently used as one of the first-line treatments for metastatic CRC.

Treatment with monoclonal antibody against EGFR prevents ligand binding of EGF to the extracellular domain of EGFR and this binding leads to the internalization of EGFR, preventing the activation of survival pathways in the cancers cells which present EGFR on their cell membranes. The binding of the antibody to EGFR could also trigger an immune response via the antibody dependent cellular cytotoxicity via the binding of its humanized immunoglobulin G1 heavy chain to the Fc receptors on phagocytic cells (Kurai, Chikumi et al. 2007). In the 2009 CRYSTAL phase III clinical trial, epidermal growth factor receptor EGFR monoclonal antibody, cetuximab was shown to be effective when used in combination with FOLFIRI as a First-Line therapy in metastatic CRC cases with KRAS (Kirsten Rat Sarcoma) wild type status and is therefore approved for use by FDA (Van Cutsem, Kohne et al. 2009).

However, based on phase III clinical trials, cetuximab is only effective in extending the overall survival time of patients with KRAS wild type status CRC. In KRAS mutant CRC, the use of cetuximab is ineffective due to the mutant KRAS being downstream of EGFR. With KRAS mutated, KRAS is constitutively activated and phosphorylates its downstream targets to activate the MEK/ERK and the PI3K/AKT pathway to promote cell cycle progression, transcription and cell survival. Its independence of upstream regulations and response to growth factors render inhibition of EGFR useless in the treatment of KRAS mutant CRC. Currently, KRAS mutation is used as a negative biomarker for the use of cetuximab and all patients

have to undergo KRAS mutation testing before cetuximab treatment can be administered (De Roock, Claes et al. 2010, Dienstmann, Vilar et al. 2011).

This group of mutant KRAS CRC patients, that are ineligible for cetuximab targeted therapy, makes up about 40% of all CRC and more studies are emerging, showing that other common mutations such as PIK3CA and BRAF may cause cetuximab to be ineffective as well (Therkildsen, Bergmann et al. 2014). In chemotherapy-refractory CRC, it has also been shown that CRC with KRAS, BRAF, PIK3CA and PTEN loss are resistant to monoclonal anti-EGFR therapy (Luo and Xu 2014). Thus, it is necessary to find specific therapies for these groups of CRC.

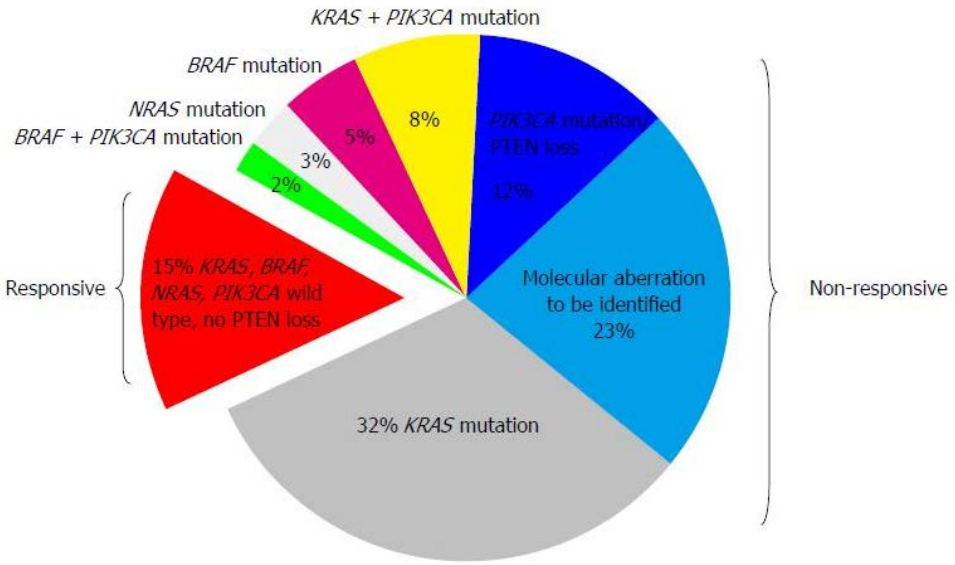


Figure 1.2 Large percentage of colorectal cancer not responsive to EGFR monoclonal antibody treatment

Only 15% of chemorefractory CRC are responsive to EGFR monoclonal antibody treatment and the rest are not responsive due to mutation in KRAS, BRAF, PIK3CA, loss of PTEN function and other molecular aberration that is yet to be identified. Figure is taken from (Luo and Xu 2014).

1.1.3 Common molecular aberration in the progression of Colorectal Cancer

1.1.3.1 Common mutations/alterations in CRC

Some of the common alterations found in sporadic CRC include loss of adenomatous polyposis coli (APC) tumor suppressor, activating mutations in proto-oncogenes KRAS and BRAF and these are acquired early in the onset of tumorigenesis. Other commonly mutated genes included PIK3CA and p53. Loss of APC leads to constitutively activated WNT signaling as the role of APC is to promote beta-catenin degradation and prevent its nuclear localization where it can bind to T-cell factor-lymphocytes factors to activate transcription of its target genes mainly involved in proliferation, survival and self-renewal in stem cells (Korinek, Barker et al. 1997, Anastas and Moon 2013). In recent study by The Cancer Genome Atlas Network, it was shown that about 93% of all CRC tumors showed altered WNT signaling with APC inactivated in 77% of all CRC tumors (2012). In the same study, alteration in receptor tyrosine kinase-RAS signaling, PI3K signaling and p53 signaling were 62%, 50% and 61% respectively in CRC tumors (2012), showing that these 4 pathways including WNT signaling are most frequently altered pathways and perhaps the most important pathways driving the development of CRC.

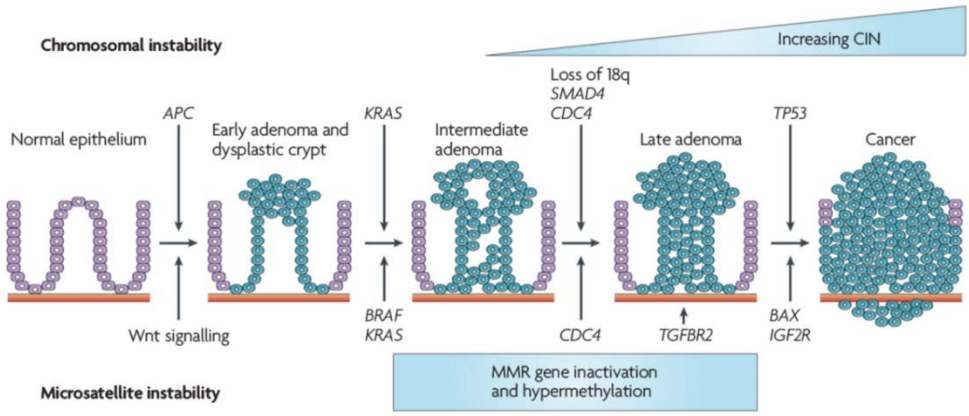


Figure 1.3 Progression of colorectal cancer with common molecular alterations

Diagram is extracted from (Walther, Johnstone et al. 2009). This simplified diagram shows the progression from an adenoma to carcinoma. Increasing chromosomal instability often takes place via the acquisition of KRAS mutation, followed by the loss of chromosome 18q containing SMAD4 and loss of p53 functions. CRC driven by microsatellite instability often exhibit dysregulated WNT signaling (Grady and Carethers 2008), BRAF mutation (Rajagopalan, Bardelli et al. 2002) and aberration in the DNA mismatch repair genes, leading to positive selection for mutated TGF-beta receptor 2 (Parsons, Myeroff et al. 1995) and further mutation in BAX (Rampino, Yamamoto et al. 1997) and insulin-like growth factor 2 receptor (Souza, Appel et al. 1996) leading to carcinoma formation.

Loss of genomic stability is the driver for CRC development and in about 80% of all sporadic CRC, chromosomal instability is the major cause of genomic instability (Lengauer, Kinzler et al. 1997). The chromosomal instability pathway (CIN) manifests through changes in chromosomal number and structures, leading to the loss of heterozygosity at multiple loci (Lengauer, Kinzler et al. 1997). These changes often led to physical loss of a functional copy of a tumor suppressor gene such APC, SMAD4 and p53, which roles are to prevent uncontrolled proliferation in cells by regulating the beta catenin pathway, mediating the TGF-beta signaling pathway in suppressing growth and arresting cell cycle and repairing any damage to the genomic material respectively (Campo, de la Calle-Martin et al. 1991, Goss and Groden 2000, Kinzler KW 2002, Miyaki and Kuroki 2003).

In other sporadic CRC tumors, genomic instability arose differently through DNA mismatch-repair defects where there is methylation on the promoter of DNA mismatch-repair gene MLH1, resulting in microsatellite instability (MSI) (Deng, Peng et al. 2002). Microsatellites are nucleotide repeat sequences scattered along the genome and DNA polymerases are error-prone when copying short repeat sequences and require mismatch-repair genes to repair the mistakes. Loss of function of MLH1 on both alleles leads to inability to repair strand slippage and this changes the length of microsatellite, leading to the microsatellite instability phenotype (Boland and Goel 2010).

Another mechanism of genome instability results from the aberrant methylation of CpG islands in the genome (Weisenberger, Siegmund et al. 2006) and this phenomenon is termed as CpG island methylator phenotype (CIMP). CpG islands are cytosine and guanine rich regions often found at the gene promoters and are mostly unmethylated in a normal cell (Carninci, Sandelin et al. 2006). When the CpG islands at the promoter region are hypermethylated, transcription of the respective genes are repressed. Often in CRC, the genes that are silenced via CpG methylation are mostly involved in mismatch-repair such as MLH1, MINT1, MINT2 and MINT3 (Toyota, Ahuja et al. 1999, Issa 2004, Barault, Charon-Barra et al. 2008), thus these CRC tumors also display microsatellite instability.

1.1.3.2 Adenoma to carcinoma progression in Colorectal Cancer

Tumorigenesis in colorectal cancer usually follows the adenoma-carcinoma development where it begins with a benign adenomatous polyp from colon lumen epithelium (Kinzler KW 2002) (Figure 1.3). Upon acquisition of more mutations, the cells in the polyp proliferate rapidly to form larger adenoma. Defects in the DNA repair machinery permit the accumulation of mutations as the cells continue to divide. Activating mutations in KRAS, a small GTPase (guanosine triphosphatase) and PIK3CA, a catalytic subunit of phosphoinositide-3-kinase (PI3K) are common as they

drive the growth and survival signaling pathways in the cells (Baba, Nosho et al. 2011). Chromosomal instability often increases upon the acquisition of KRAS mutation and followed by the loss of chromosome 18q containing SMAD4 and loss of p53 function due to mutation and loss of chromosome 17. CRC driven by microsatellite instability often exhibit dysregulated WNT signaling (Grady and Carethers 2008), BRAF mutation (Rajagopalan, Bardelli et al. 2002) and the aberration in the DNA mismatch repair genes, leading to positive selection for mutated TGF-beta receptor 2 (Parsons, Myeroff et al. 1995) and further mutation in pro-apoptotic BAX (Rampino, Yamamoto et al. 1997) and insulin-like growth factor 2 receptor (Souza, Appel et al. 1996) leading to p53-independent progression to carcinoma.

If left untreated, the adenoma will develop into an early carcinoma as the cancerous cells spread to the surrounding tissue (Baker, Fearon et al. 1989, Baker, Markowitz et al. 1990). As the tumor grows, it develops a network of blood supply, a process termed angiogenesis to obtain sufficient nutrients and oxygen to maintain its excessive growth rate (Wyckoff, Jones et al. 2000). The cancerous cells then can enter the blood circulation via these or other blood vessels or they could indirectly enter the blood stream via the lymphatic system (Chambers, Groom et al. 2002). This is why the presence of cancerous cells in the draining lymph nodes is an indicator of the progression of the cancer beyond its primary site.

To successfully metastasize, the cancer cells in the blood stream have to arrest at a distant organ and extravasate into the tissue surrounding the vessel. The common sites of CRC metastasis are liver, peritoneum and lungs. Once they are lodged in their new site, they have to establish their own network of blood supply in order to grow (Chambers, Groom et al. 2002).

1.2 RAS superfamily of GTPases

The RAS superfamily comprises 154 members which are small GTPases including the founding members HRAS, NRAS and KRAS. They are segregated into 5 main families according to their sequence and functions namely: Ras, Rho Rab, Arf and Ran (Colicelli 2004).

Ras family members are mainly involved in the regulation of extracellular signal transduction to the intracellular signaling networks that controls cell proliferation and survival. Rho family members are also involved in signal transduction but they signal to pathways that regulate actin cytoskeleton organization and this affects cell movement, polarity and shape. Rab and Arf family members regulate membrane trafficking and intracellular transport and Ran regulates the transport of macromolecules between the nucleus and cytoplasm and the spindle organization during mitosis.

1.2.1 KRAS

Kirsten rat sarcoma viral oncogene homolog, KRAS, belongs to the RAS superfamily of small GTPases proteins which bind and hydrolyze guanosine triphosphates (GTP) to guanosine diphosphates (GDP), leading to their activation and subsequently the phosphorylation and activation of their downstream targets. It has 2 protein isoforms, KRAS4A and KRAS4B due to alternative splicing at the KRAS locus (Malumbres and Barbacid 2003). Together with the other RAS family members HRAS and NRAS, they share over 90% similarity in sequences in the first 168 amino acids but differ in the hypervariable regions at the C-terminal which affect their subcellular membrane localization as well as plasma membrane targeting (Bourne, Sanders et al. 1991, Hancock 2003). The mammalian homologues of KRAS together with Harvey-RAS was first discovered in the rat genome through the studies of Kirsten murine sarcoma virus and the mutated KRAS allele were soon found in many of the cancer cell lines.

KRAS, is a proto-oncogene that is frequently mutated in many cancers; it is estimated to occur at about 90% in pancreatic adenocarcinoma (Almoguera, Shibata et al. 1988, Capella, Cronauer-Mitra et al. 1991), 40% in colorectal cancer (Vaughn, Zobell et al. 2011) and 30% in lung cancer (Capella, Cronauer-Mitra et al. 1991).

1.2.2 Regulation of KRAS activation

In normal, non-transformed cells, activation of KRAS require the presence of growth factors that bind and activation the receptor tyrosine kinases (RTK) found on the cellular membrane. Epidermal growth factor receptors upon binding to their ligands, mitogenic signals, lead to phosphorylation of tyrosine residues in their intracellular domains (Figure 1.4). Activated RTKs then recruit adaptor proteins such as Growth factor receptor- bound protein 2 (GRB2) through recognition and binding to the Src-homology domain (SH2 in the adaptor proteins) (Buday and Downward 1993, Okutani, Okabayashi et al. 1994). GRB2 also binds indirectly to RTK via another adaptor protein, src homology and collagen domain protein (SHC) (Schlessinger and Bar-Sagi 1994). Son of the sevenless (SOS) bound to Grb2 through the SH3 domain is then brought to close to the plasma membrane where inactive KRAS is found (Chardin, Camonis et al. 1993). SOS, a guanine nucleotide exchange factor, acts to catalyze the exchange of GDP to GTP, promoting the formation of active RAS-GTP complexes (Bar-Sagi 1994).KRAS has a weak intrinsic GTPase activity and the binding of a GTPase-activating protein, GAP, such as p120 RASGAP or neurofibromin increases the rate of catalysis of GTP to GDP, inactivating KRAS and stopping the downstream signaling cascade (Scheffzek, Ahmadian et al. 1998, Cichowski and Jacks 2001, Donovan, Shannon et al. 2002).

Activation of RAS also requires that a series of post-translational modifications at the C-terminal which is important to direct it to the various cellular membranes and for RAS to be activated by its upstream RTK, it needs to be first localized at the plasma membrane (Ahearn, Haigis et al. 2012).

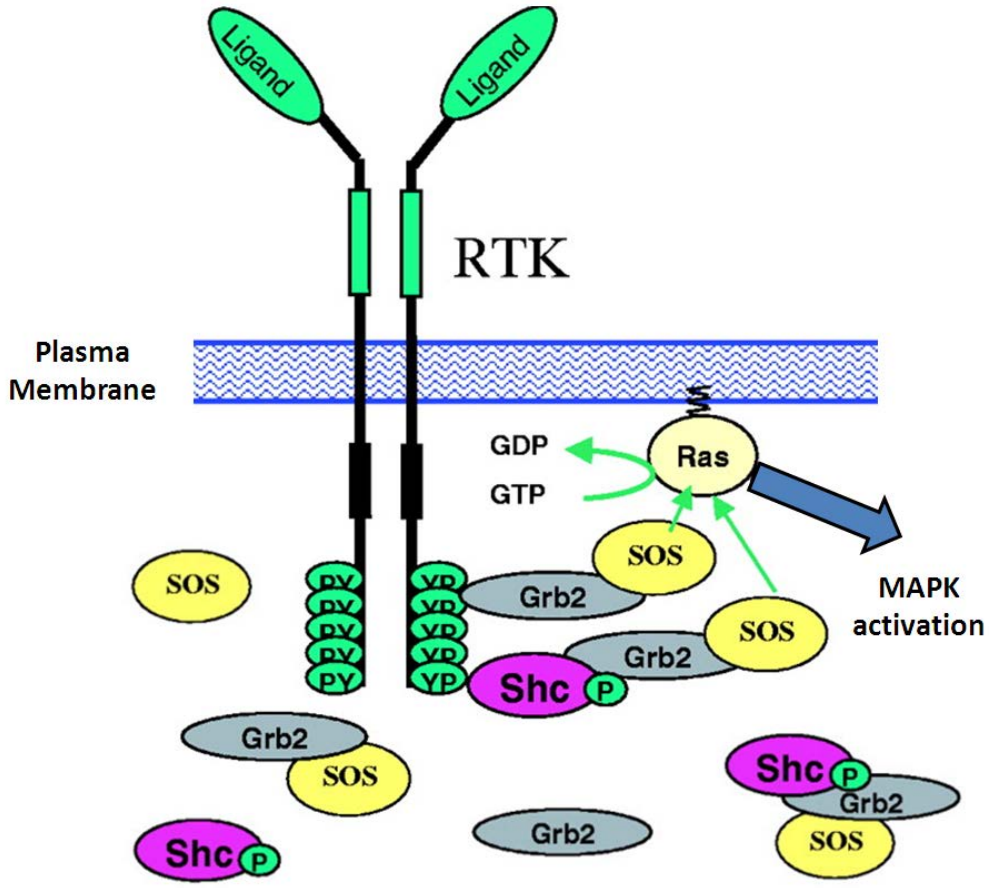


Figure 1.4 RAS activation via ligand binding to receptor tyrosine kinase

Diagram is adapted from (Kholodenko 2003). Upon ligand/mitogens binding to the extracellular domain of receptor tyrosine kinase (RTK) such as epidermal growth factor receptor, tyrosine residues in their intracellular domains are phosphorylated (YP), leading to recruitment of adaptor proteins growth factor receptor-bound protein 2 (GRB2) directly or indirectly through src homology and collagen domain protein (SHC). Son of sevenless (SOS), a guanine nucleotide exchange factor, bound to GRB2 is then brought into close proximity to inactive RAS at the plasma membrane. SOS then catalyzes the exchange of GDP to GTP, activating RAS leading to the subsequent downstream activation of MAPK signaling.

1.2.3 Regulation of RAS signaling

1.2.3.1 Regulation of downstream MAPK signaling

RAF family consists of 3 serine/threonine kinase members A-RAF, B-RAF and C-RAF, with B-RAF being the member most frequently mutated in cancer. Upon formation of active RAS-GTP complex, RAF is recruited and bound to the effector loop of the RAS isoform via its RAS-binding domain (RBD) in the N-terminus (Vojtek, Hollenberg et al. 1993) and this disrupts the inhibitory interaction between the adjacent cysteine rich domain and the catalytic domain in RAF (Cutler, Stephens et al. 1998). The binding of RAS also interrupts the interaction between 14-3-3 that stabilizes the inactive state of RAF (Light, Paterson et al. 2002), leading to the dephosphorylation of serine 259 by protein phosphatase 2A (Ory, Zhou et al. 2003) (Figure 1.5). This results in a conformational change in RAF that primes it for subsequent activating phosphorylation at serine 338 (S338) and threonine 341 (Y341) with the latter likely due to Src-family kinases or casein kinase 2 (Marais, Light et al. 1997). These 2 sites of phosphorylation enable interaction with MEK1 and MEK2 and lead to phosphorylation on serine 218/222 and serine 222/226 respectively (Zheng and Guan 1994, Shaul and Seger 2007), and subsequently, activated MEK1/2 then phosphorylates and activates downstream ERK1 on tyrosine 202 and threonine Y204 (Ferrell and Bhatt 1997) and ERK2 on tyrosine 185 and threonine 187 (Haystead, Dent et al. 1992, Burack and Sturgill 1997).

This signaling cascade is aided by the recruitment of Kinase repressor of KRAS (KSR) to the RAS-GTP complex, where it functions as a scaffold to bring MEK and ERK proteins into close proximity to the RAF proteins, facilitating the activation of the MAPK pathway (Roy, Laberge et al. 2002). Dimerization between different members of the RAF kinases can result in allosteric activation by the B-RAF kinase on its other family members, driving downstream phosphorylation and activation (Hu, Stites et al. 2013). The interaction and dimerization between the RAF family and

other kinases is complex, not surprisingly as this is the start of the signaling cascade which needs to be tightly regulated and not all about RAF regulation is known at this moment.

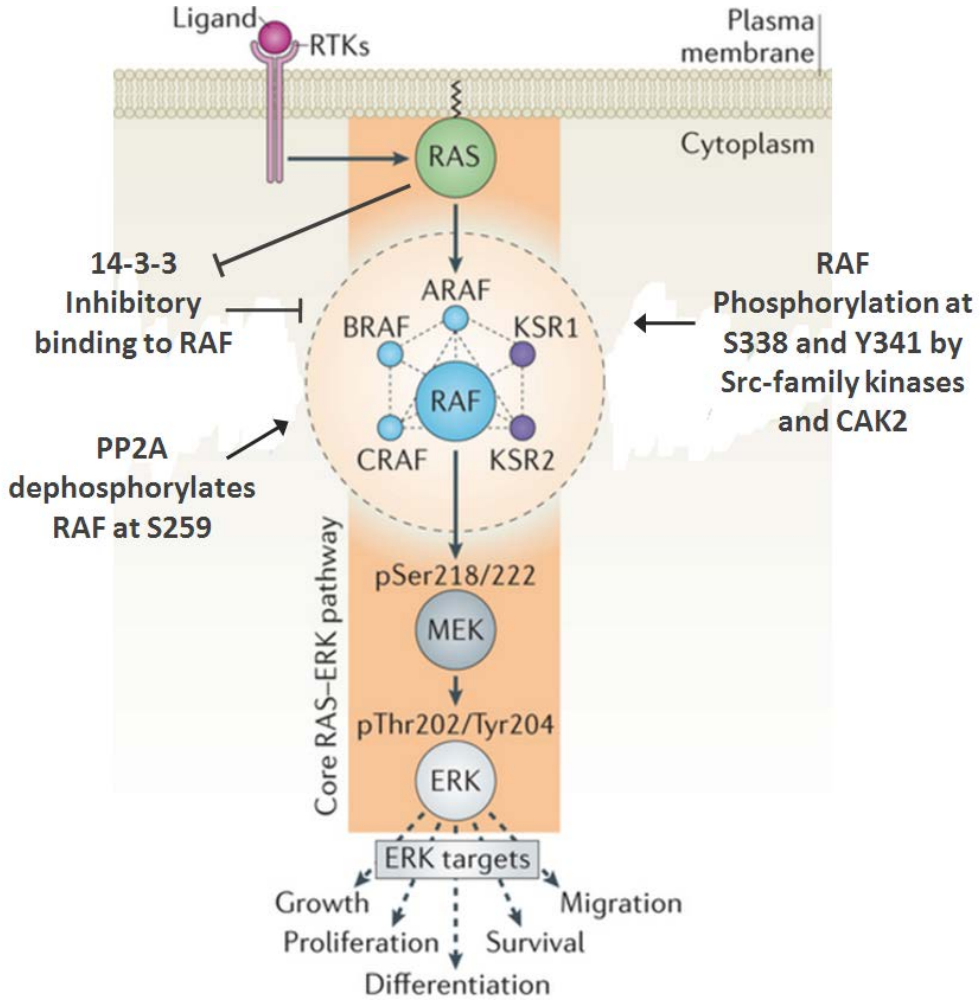


Figure 1.5 Core RAS-activated MAPK signaling

Diagram is adapted from (Lavoie and Therrien 2015). Simplified diagram shows the activation of MAPK signaling after RAS activation. RAF binds to activated RAS and this disrupts inhibitory interaction between 14-3-3 and RAF. Dephosphorylation of S259 on RAF leads to conformation change in RAF. Src-family kinases and casein kinase 2 (CAK2) subsequently phosphorylated RAF on S338 and Y341. RAF then phosphorylates MEK1 and MEK2 on S218/222 and S222/226 respectively. Activated MEK1/2 further phosphorylates ERK1 and ERK2 on Y202/204 and Y185/187 respectively. KSR1/2 functions as a scaffold to bring the components of the MAPK signaling into close proximity of each other to facilitate signaling. More details in text.

Phosphorylated ERK1/2 further catalyze the phosphorylation of its cytoplasmic substrates such as 90kDa ribosomal S6 kinases (p90RSK) family, consisting RSK1-4 (Roux and Blenis 2004) as well as multiple nuclear transcription factors driving processes such as proliferation, survival, differentiation, angiogenesis, migration and chromatin remodeling (Dunn, Espino et al. 2005, Yoon and Seger 2006). The temporal differences in the strength of activation and cellular localization of ERK determines the activation of the processes (Murphy and Blenis 2006). Early gene products such as MYC, JUN, FOS and EGR-1 induced by ERK signaling may function as sensors detecting ERK signaling dynamics through their expression level and phosphorylation and duration of phosphorylation (Murphy, Smith et al. 2002, Murphy, MacKeigan et al. 2004). Only sustained ERK signaling promotes the phosphorylation and stabilization of genes such as cyclin D1, thus promoting entry to cell cycle.

Activated ERK also regulates the MAPK signaling via a negative feedback loop where it inhibits RAF via phosphorylation, leading to RAS binding inhibition and disruption of BRAF-CRAF complexes (Dougherty, Muller et al. 2005, Ritt, Monson et al. 2010). Activated ERK also phosphorylates SOS1, inhibiting its and RAS activity (Corbalan-Garcia, Yang et al. 1996). It also induces the expression of dual-specificity phosphatases (DUSPs) and Sprouty, which dephosphorylate ERK and impair RAS activation by tethering GRB2 away from SOS respectively (Hanafusa, Torii et al. 2002, Amit, Citri et al. 2007), contributing to the negative regulation of MAPK signaling.

1.2.3.2 Other downstream effectors of RAS signaling

RAS-GTP can also bind directly to phosphatidylinositol-3-kinase (PI3K) and allosterically activate it via the activation of its p110 catalytic subunit (Ong, Hadari et al. 2001) (Figure 1.6). PI3K then catalyzed the generation of phosphatidylinositol-3, 4, 5-triphosphate (PIP3), which recruits the protein kinase AKT to the membrane where it is activated by phosphorylation at threonine 308 and serine 473 by 3-phosphoinositide-dependent kinase 1 (PDK1) and mTORC2 complex (Alessi, James et al. 1997, Sarbassov, Guertin et al. 2005) respectively. Activated AKT signals downstream to drive protein synthesis through mTORC1 (Wang and Proud 2006), survival through its inhibition on pro-apoptotic proteins, cell cycle through cyclin D1 (Liang and Slingerland 2003), p21 and p27 (Testa and Bellacosa 2001), metabolism and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway (Romashkova and Makarov 1999). AKT pathway thus has been found to be dysregulated in cancer and its downstream targets shown to be important in tumorigenesis (Vivanco and Sawyers 2002).

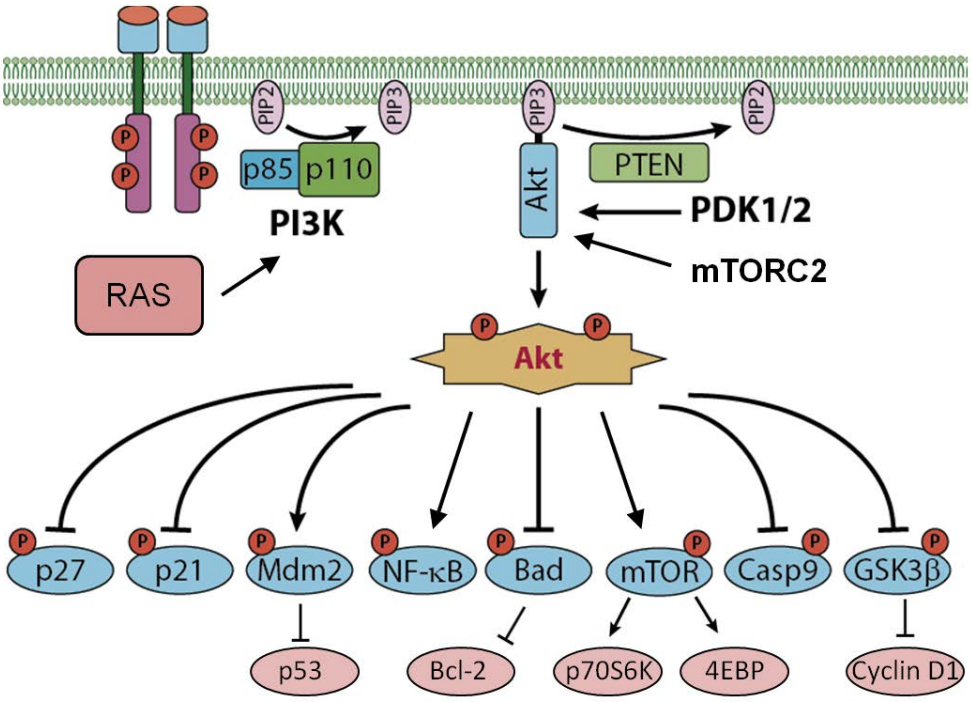


Figure 1.6 Activation of PI3K/AKT pathway and its downstream effectors.

Diagram is adapted from (http://www.sfb773.de/html/projectC5.html). Activation of receptor tyrosine kinase activate PI3K (p85 and p110 subunits) and PI3K then catalyzed the generation of phosphatidylinositol 3, 4, 5 triphosphate (PIP3) from phosphatidylinositol-3,4-diphosphate (PIP2), which recruits the protein kinase AKT to the membrane where it is activated by phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1) and mTORC2 complex. Activated AKT signals downstream to drive protein synthesis through mTORC1 (Wang and Proud 2006), survival through its inhibition on pro-apoptotic proteins (Bad and Casp9), cell cycle through cyclin D1, p21, p27 and MDM2 and the nuclear factor kappa-light-chain-enhancer of activated B cells to prevent cell death. Activated RAS can activate p110, the catalytic subunit of PI3K. PTEN inhibit PI3K/AKT signaling by converting PIP3 back to PIP2. More details in text

1.2.4 Role of KRAS in cancer

KRAS mutation is found in multiple cancer types and its role in driving tumorigenesis has been well established (Lawrence, Stojanov et al. 2014). In CRC, the most frequent KRAS mutation in is on codon 12 and 13, followed by codon 61. Mutations on G12 and G13 account for more than 99% of all mutations, with G12D, G12V and G13D contributing 35%, 20% and 19% respectively (Prior, Lewis et al. 2012). Substitution in codon 12 and 13 results in steric hindrance which prevents van der Waals bonds formation between RAS and GAP and this disturbs the orientation of catalytic glutamine in codon 61, attenuating GTP hydrolysis (Scheffzek, Ahmadian et al. 1997). Similarly, a direct substitution in glutamine 61 other than glutamic acid will also block GTP hydrolysis (Der, Finkel et al. 1986). With impaired GTP hydrolysis, KRAS is trapped in a GTP-bound activated state, constitutively driving its downstream effector pathway signaling.

1.2.5 Challenges in KRAS mutant cancer treatment

In the past, mutant KRAS was considered to be not targetable via pharmacological inhibition presumably due to its nucleotide-binding pocket of KRAS protein having very high affinity for GTP (Young, Lyons et al. 2009, Baines, Xu et al. 2011). Alternative strategy to block RAS activation indirectly was to inhibit farnesyltransferase, which is required for the farnesylation of RAS. This post-translational lipid modification of RAS allows the binding of RAS to the membrane and its subsequent activation but in the presence of farnesyltransferase inhibitors, KRAS and NRAS are still activated due to the geranylgeranyltransferase providing sufficient lipid modification (James, Goldstein et al. 1996, Whyte, Kirschmeier et al. 1997). Thus, there is switch of focus to target KRAS mutant cancer by inhibiting its downstream signaling, specifically the MAPK pathway.

1.2.6 RAF inhibition predominantly ineffective in KRAS mutant cancer

The BRAF inhibitors used in the clinical such as vemurafenib and dabrafenib, suppress RAF activity effectively in cancer cells harboring BRAF(V600E) mutation. In BRAF wild type cancer cells, in the presence of oncogenic RAS, non-saturating BRAF inhibition can lead to activation of CRAF through RAS-dependent BRAF binding to CRAF, ultimately activating MEK-ERK signaling (Heidorn, Milagre et al. 2010). This transactivation of RAF via dimerization reveals the importance of inhibition of CRAF as well as BRAF even though BRAF has a higher basal activity than CRAF (Mason, Springer et al. 1999) and is mutated at a higher frequency. The use of sorafenib, a multi-kinase inhibitor, which is developed as a wild type CRAF and BRAF inhibitor, has showed promising efficacy in combination with chemotherapy in phase I/II clinical trials in KRAS mutant cancers (Dingemans, Mellema et al. 2013, Samalin, Bouche et al. 2014), suggesting that this may be a treatment strategy for KRAS mutant cancers. However, toxicity to normal cells may limit the dosage used since sorafenib or similar inhibitors target wild type CRAF and BRAF which are also important in normal cells.

1.2.7 Use of MEK inhibitors in clinical trials for CRC

Since MEK1 and MEK2 are positioned directly downstream of RAS and RAF, multiple highly selective MEK inhibitors have been designed and tested in the clinical trials. Currently the only clinically approved MEK inhibitor is trametinib for the treatment of metastatic melanoma with BRAF(V600E/K) mutation which improved overall survival and progression-free survival (Flaherty, Robert et al. 2012). However, such efficacy is not observed with single MEK inhibitor treatment in KRAS mutant cancer. This is likely due to the toxicity in normal cells limiting the dose used and lack of enrichment of KRAS mutant patient cohorts (Rinehart, Adjei et al. 2004, Haura, Ricart et al. 2010). Moreover, RAS signaling to multiple downstream

effectors other than the MAPK pathway could be the reason as to why single MEK inhibition is ineffective due to activated compensatory pathways still present.

Most of the MEK inhibitors are reversible, allosteric inhibitors that bind to a site adjacent to the ATP-binding site on MEK1 and MEK2 (Samatar and Poulikakos 2014). PD0325901, a second generation MEK1/2 inhibitor, inhibits MAPK signaling by binding to MEK1/2 and inhibit their kinase activity, thus preventing the phosphorylation of downstream ERK1/2. Trametinib, the only approved MEK inhibitor for clinical use, inhibits both MEK1/2 kinase activity as well as RAF-dependent phosphorylation of MEK1 on serine 217, preventing dual phosphorylation and complete activation of MEK1 and MEK2 (Gilmartin, Bleam et al. 2011).

1.2.7.1 Compensatory pathways leading to MEK inhibitor inefficacy

1.2.7.1.1 PI3K/AKT pathway activation/dependence upon MEK inhibition

Ebi et al showed that upon MEK inhibition in KRAS mutant cancer, there is an increase in AKT phosphorylation via the increased association of the PI3K and IRS proteins (insulin receptor substrate is an adaptor protein which recruits PI3K to RTK via association with p85 subunit) as well as the increased phosphorylation in insulin like growth factor 1 receptor (IGF-1R) (Ebi, Corcoran et al. 2011). MEK inhibition resulted in decrease in TORC1 activation downstream, leading to the loss of feedback inhibition of TORC1 on IRS. Recently, phosphorylated ERK has been shown to transcriptionally activate the negative regulators of mTORC1 such as TSC1, TSC2, Deptor and REDD1 (Komatsu, Fujita et al. 2015).

She et al showed that in tumors with both PIK3CA and KRAS mutation, single inhibition is insufficient in blocking cap-dependent translation (She, Halilovic et al. 2010). Only when both MEK and AKT pathways are inhibited, there is dephosphorylation of 4EBP1and the binding of unphosphorylated 4EBP1 to eIF4E inhibit cap-dependent translation, decreasing cell viability.

Mutation in PIK3CA in KRAS mutant cancers reestablishes cyclin D1 expression and G1-S phase progression in cell cycle via constitutive activation of AKT signaling, rendering cyclin D1 expression and therefore cell cycle progression, independent of KRAS/MEK/ERK signaling (Halilovic, She et al. 2010).

Sun et al showed once again AKT pathway involvement in MEK inhibition resistance in KRAS mutant CRC and lung cancer via a MYC-dependent transcription upregulation of ERBB3, the kinase-defective member of the ERBB receptor tyrosine kinase family (Sun, Hobor et al. 2014). Increase in phosphorylated AKT and phosphorylated ERBB3 were induced upon MEK inhibition, promoting cell survival. Only MEK inhibitor treatment together with afatinib, a dual EGFR/ERBB2 inhibitor which can block the formation of kinase-active heterodimer between ERBB3 and its binding partners EGFR and ERBB2, and prevent the downstream activation of AKT pathway and induce apoptosis.

1.2.7.1.2 Re-activation of RAF

Upon the MEK inhibition, phosphorylation of ERK decreases together with its inhibitory phosphorylation on CRAF (Dougherty, Muller et al. 2005) and after time, this leads to the induction of RAF-MEK complexes and CRAF-dependent reactivation of ERK (Lito, Saborowski et al. 2014), preventing the durable inhibition of ERK. Through this finding, there is increased interest in newly improved MEK inhibitor that inhibits MEK activity as well as prevent CRAF reactivation as this may improve MEK inhibitor efficacy in KRAS mutant cancer.

1.2.7.1.3 MEK inhibition alone insufficient in eliciting death signals

Suppression of ERK phosphorylation via MEK inhibition induces expression of pro-apoptotic BIM but with no reduction in the expression of anti-apoptotic protein BCL-xL, BCL-2 and MCL-1. This negates the increase in BIM levels as the anti-apoptotic proteins formed inhibitory complexes with BIM, preventing apoptosis. Only upon treatment with BCL-xL and MEK inhibitors, formation of BCL-XL and BIM

complexes is inhibited and the increased level of BIM then induces apoptosis in KRAS mutant cancer (Corcoran, Cheng et al. 2013).

The identification of compensatory pathway activation or dependence in KRAS mutant cancer upon MEK inhibition leads to the idea of combinatorial treatment strategies and various treatment strategies were proposed and are currently being tested out in clinical trials.

1.2.8 Development of ERK inhibitors

Upon the discovery of negative feedback loops in the RAF/MEK/ERK pathway as well as RAF and MEK inhibitors resistance that result in the reactivation/recovery of ERK (Lito, Saborowski et al. 2014), development of specific ERK inhibitors started. Currently, SCH900353, a specific inhibitor able to inhibit both ERK1 and ERK2 intrinsic kinase activity and prevent their phosphorylation by MEK, is in phase I clinical trials (Chaikuad, Tacconi et al. 2014, Deng, Shipps et al. 2014). Other ERK inhibitors are either in very early phases of clinical testing or still in preclinical studies and are unlikely to be available for clinical use in the near future.

1.2.9 KRAS direct inhibitors

In 2013, Ostrem et al developed small molecule inhibitors that bind specifically to KRAS with G12C mutations, disrupting switch I and II and causing the mutant KRAS G12C to favor binding to GDP instead of GTP (Ostrem, Peters et al. 2013). This inactivates mutant KRAS and also prevent it from binding to RAF without affecting the activity of wild type RAS.

Attempts to interfere with the subcellular localization and activation of RAS have been successful in preclinical studies using deltarasin which binds to the farnesyl-binding pocket of prenyl-binding protein PDEδ. Deltarasin inhibits the interaction between KRAS and PDEδ interaction, and relocalized mutant KRAS proteins to the

endomembranes, thus suppressing mutant KRAS signaling and inhibiting tumor growth (Zimmermann, Papke et al. 2013).

Stabilized alpha helices of son of sevenless 1 (SAH-SOS1) peptides also were recently developed and shown to be able to disrupt KRAS and SOS1 interaction and prevent nucleotides from binding to wild type and mutant KRAS (Leshchiner, Parkhitko et al. 2015) and therefore inhibiting downstream KRAS signaling *in vitro*.

These KRAS direct inhibitors mentioned above are still in preliminary preclinical testing stages and much more characterization and validation *in vivo* models need to be done. Another potential challenge in targeting KRAS directly is the phenomenon of KRAS independence which a few studies have shown that in KRAS mutant cancer lines, some are resistant to the knockdown of KRAS (Scholl, Frohling et al. 2009, Singh, Greninger et al. 2009, Singh, Sweeney et al. 2012). This strategy may not be effective in cancers with KRAS mutation but are not dependent or addicted to the oncogenic KRAS.

1.3 CDK4/6 pathway

Cyclin-dependent kinase 4 and 6 (CDK4/6) belong to the family of cyclin-dependent kinases which have important functions in cell cycle and transcription and their activity are dependent on their binding to specific cyclin subunits. There are 11 classical CDK ranging from CDK1 to CDK11. CDK4/6 specific binding cyclin subunits are cyclin D1, D2 and D3 and they share over 50% similarity in their amino acid sequences (Xiong, Menninger et al. 1992).

1.3.1 Functions of CDK4/6

1.3.1.1 CDK4/6 regulates G1 to S phase transition in cell cycle

In the classical model of cell cycle in mammalian cells, the specific formation of cyclins-CDKs complexes drive the cell cycle progression in an orderly and sequential manner. In G1 phase, the accumulation of cyclin D leads to the formation of active CDK4/6-cyclin D complexes, which partially inactivate retinoblastoma (RB), a tumor suppressor, to promote the expression of cyclin E. Cyclin E then binds to CDK2, forming cyclin E-CDK2 complexes that further inactivate RB and to drive expression of genes required for S phase (Lundberg and Weinberg 1998, Harbour, Luo et al. 1999). During the late stage of DNA replication in S phase, CDK2 is then activated by cyclin A2 to drive transition from S phase to G2 phase(Yam, Fung et al. 2002). Cyclin A2 is thought to then binds to and activate CDK1 to mediate the onset of mitosis (Malumbres and Barbacid 2009). Upon the disintegration of the nuclear envelope, cyclin A is degraded, enabling the formation of cyclin B-CDK1 complexes to drive mitosis (den Elzen and Pines 2001, Jackman, Lindon et al. 2003, Malumbres and Barbacid 2005).

In normal cells, activated cyclin D-CDK4/6 and cyclin E-CDK2 complexes, through their combined phosphorylation of RB, drive cells pass the restriction point, committing the cell to cell cycle. When CDK4 and CDK6 were genetically ablated in mice models, development of most organs and tissues were unaffected and isolated

mouse embryonic fibroblasts from these mice were still able to undergo proliferation (Malumbres, Sotillo et al. 2004). CDK2 knockout mice are shown to be viable and it is essential only in meiosis but not mitosis (Berthet, Aleem et al. 2003, Ortega, Prieto et al. 2003), suggesting that there is redundancy in the functions of CDK2 and CDK4/6 in cell cycle in normal mammalian cells. In colon cancer cells, however, inhibition of CDK4 was sufficient to cause G1 arrest but inhibition of CDK2 did not prevent cell cycle progression, suggesting that CDK4/6 has a greater role in driving cell cycle entry in cancer as compared to CDK2 (Tetsu and McCormick 2003).

Loss of CDK4/6 activity can lead to senescence through the loss of phosphorylation on RB and FOXM1, leading to the inhibition of G1-S transcriptional program driven largely by E2F and FOXM1 transcription factors (Anders, Ke et al. 2011).

1.3.1.2 Role of CDK4/6 in development

Studies in mice have shown CDK4 to be important during postnatal development for the proliferation of pancreatic β-cells and pituitary lactrotrophs (Rane, Dubus et al. 1999, Tsutsui, Hesabi et al. 1999)and loss of CDK6 to only results in minor defects erythroid lineage cells (Malumbres, Sotillo et al. 2004).

1.3.2 Regulation of CDK4/6

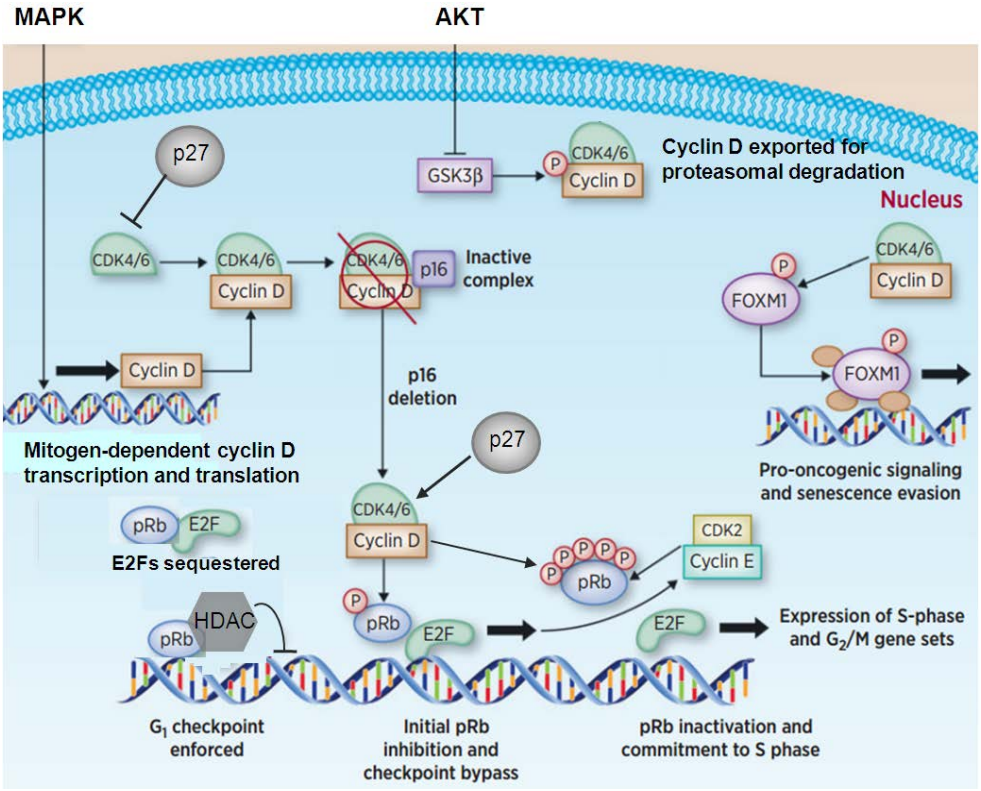


Figure 1.7 Regulation of CDK4/6-cyclin D complex and its role in cell cycle

Diagram is adapted from (VanArsdale, Boshoff et al. 2015). In early G1 phase, RB is hypophosphorylated, sequestering E2Fs and forming repressive transcriptional complexes with histone deacetylases, preventing the transcription of S-phase and G2/M phase genes driven by E2Fs. CDK4/6 monomer activity is inhibited by p27. Upon mitogen stimulation and activation of upstream MAPK and PI3K/AKT pathway, cyclin D accumulates via increased transcription and translation and decreased degradation, forming active CDK4/6-cyclin D complexes facilitated by phosphorylated p27. These complexes together with CDK2-cyclin E complexes then phosphorylate and inactivate RB, releasing E2Fs, allowing for transcription of genes regulated by E2Fs and transition from G1 phase to S phase. This FOXM1 is also phosphorylated and activated by active CDK4/6-cyclin D complexes, leading to transcription of FOXM1 target genes, driving pro-oncogenic signaling and preventing cellular senescence

1.3.2.1 Cyclin D availability

Activation of CDK4/6 requires the binding of its catalytic partners, cyclin D1, D2 or D3. Cyclin D expressions change throughout the different phases of cell cycle according to the presence of external mitogenic stimuli (Sherr and Roberts 1999). Increase in cyclin D protein level could be due to increase in transcription of cyclin D genes by transcription factors activated by mitogenic signals such as CMYC, FOS and JUN (Brown, Nigh et al. 1998, Bouchard, Thieke et al. 1999, Klein and Assoian 2008), increased cyclin D protein translation via PI3K/AKT/mTOR/S6 kinase 1 signaling (Muise-Helmericks, Grimes et al. 1998), or GSK3β inhibition preventing the phosphorylation of cyclin D at its C-terminus, leading to its stabilization and nuclear localization (Diehl, Cheng et al. 1998, Alt, Cleveland et al. 2000).

1.3.2.2 Regulation by Cip/Kip proteins

Another layer of regulation is contributed by the Cip/Kip proteins namely, p21, p27 and p57. In the presence of antiproliferative signals, unphosphorylated p21 and p27 directly inhibit CDK4/6 by blocking its kinase site (Harper, Elledge et al. 1995, Ray, James et al. 2009). However, upon phosphorylation of p21 and p27 at tyrosine 76 and tyrosine 88 and 89 respectively, Cip/Kip proteins release their inhibitory blockage of CDK4/6 kinase site, allowing for phosphorylation of CDK4/6 to take place (James, Ray et al. 2008, Hukkelhoven, Liu et al. 2012). Moreover, Cip/Kip proteins have been observed to be bound to active cyclin D-CDK4/6 complexes (Kaldis, Ojala et al. 2001) and since they harbor bipartite nuclear localization sequences absent in CDK4/6, they could possibly mediate the nuclear localization of CDK4/6, granting them access to their substrates (LaBaer, Garrett et al. 1997, Cheng, Olivier et al. 1999).

Likely after the formation of the ternary complex of cyclin D-CDK4/6 and Cip/Kip proteins, phosphorylation of the activation loop or T-loop at threonine 172 in CDK4 and threonine 177 in CDK6 take place to enable the full activation of the kinases, with the T-loop then facilitating the binding of cyclin and substrates (Kato, Matsuoka

et al. 1994, Bockstaele, Kooken et al. 2006, Day, Cleasby et al. 2009). CDK7, together with cyclin H and Mat1, is suggested to be responsible for the T-loop phosphorylation, although there are other evidences suggesting that there are other proline-directed kinases activating CDK4 (Bockstaele, Bisteau et al. 2009).

1.3.2.3 Negative regulation by INK4 proteins

The third layer of regulation is carried out by the INK4 proteins also known as CDK inhibitory proteins, comprising of p15, p16, p18 and p19. INK4 proteins bind to the ternary complex containing CDK4/6, cyclin D and Cip/Kip and distort the ATP-binding site of CDK4/6 and change its conformation, leading to reduction in its binding interface to cyclin D and interaction with Cip/Kip (Russo, Tong et al. 1998, Jeffrey, Tong et al. 2000). p16 expression can be induced by increasing cellular age, presence of oncogenic RAS and presence of BRAF V600E mutation, leading to cell senescence or cell cycle arrest (Serrano, Lin et al. 1997, Michaloglou, Vredeveld et al. 2005, Ressler, Bartkova et al. 2006). TGF-beta signaling can also trigger p15 expression, leading to cell cycle arrest (Hannon and Beach 1994).

1.3.3 Downstream substrates of CDK4/6

1.3.3.1 Retinoblastoma protein and E2F transcription factors

Retinoblastoma proteins (RB1, RBL1 and RBL2) function as transcriptional repressor, blocking S phase entry and cell cycle when it is hypophosphorylated (Weinberg 1995). By directly binding to the transactivation domains of E2F transcription factor family, which activates transcription of genes required for S phase entry, the RB/E2F complex then binds to the promoters of these genes, repressing transcription and further inducing chromatin remodeling via recruitment of histone deacetylase 1 (HDAC1) (Luo, Postigo et al. 1998, Talluri and Dick 2012). Active CDK4/6 phosphorylates RB proteins at their N and C terminus and this possibly destabilizes their binding to E2F and HDAC1 and results in the disintegration of the transcriptional repressor complexes, freeing E2Fs to function as transcription

activators (Harbour, Luo et al. 1999, Burke, Deshong et al. 2010). The transcription activators of the E2F family are E2F1-3 and the genes transcribed by them are involved in DNA replication, DNA repair and DNA damage checkpoints, processes that are crucial in S phase (Ren, Cam et al. 2002, Bracken, Ciro et al. 2004).

1.3.3.2 FOXM1 transcription factor

Another major substrate of CDK4/6 is forkhead box M1 (FOXM1) which is a transcription activator important for G1 to S phase transition as well as G2 to M phase transition. Activate CDK4/6 phosphorylates FOXM1 at its N and C terminus, stabilizing it and preventing it from proteasomal degradation. Multiple phosphorylations on its C-terminal also induce FOXM1 transactivation, with more phosphorylation resulting in higher level of transactivation (Anders, Ke et al. 2011). FOXM1 phosphorylation is further regulated by other proteins such checkpoint kinase 2 in response to DNA damage leading to increased FOXM1 stability (Tan, Raychaudhuri et al. 2007) as well as phosphorylated ERK which promotes the nuclear translocation and transactivation of FOXM1 (Ma, Tong et al. 2005).

FOXM1 is overexpressed in many cancers, such as ovarian cancer where high FOXM1 expression is associated with poor patient outcome and paclitaxel (microtubule-stabilizing chemodrug) resistance (Zhao, Siu et al. 2014). Specifically, in CRC, the 5 year survival rate for patients with high FOXM1 expression is significantly lower than those with low FOXM1 expression and is also correlated to lymph node and liver metastasis and advanced TNM stage (Chu, Zhu et al. 2012). In addition, in the comprehensive molecular profiling of 276 colon and rectal cancer tumors carried out by The Cancer Genome Altas, integrated analysis from exome sequencing, promoter methylation, DNA copy number alterations and mRNA and microRNA expression have shown elevated FOXM1 as well as MYC and E2F expression in almost all CRC tumors (2012). FOXM1 transcription factor is also

implicated in cell migration, invasion and angiogenesis, prompting the development of FOXM1 inhibitors (Koo, Muir et al. 2012, Halasi and Gartel 2013).

1.3.3.3 Others CDK4/6 downstream substrates

Other well-known substrates of CDK4/6 include SMAD2 and SMAD3 which have mainly roles in G1 to S phase transition (Matsuura, Denissova et al. 2004). Phosphorylation of SMAD2/3 by CDK4/6 prevents the SMAD proteins from repressing the transcription of *MYC* and this results in the blocking of the cytostatic effect of TGF-beta signaling on the cells (Chen, Kang et al. 2002, Matsuura, Denissova et al. 2004).

1.3.4 Dysregulation of CDK4/6 pathway in cancer

Alterations in CDK4/6 is rarely observed in cancer, however, mutations and changes in expression of other members of the CDK4/6 pathway are frequently seen. In colorectal cancer, *CCND1* overexpression and *CCND1* amplification are present in about 55% and 2.5% of all cases respectively (McKay, Douglas et al. 2000, Toncheva, Petrova et al. 2004). Aberrant methylation on *CDKN2A* locus resulting in loss of p16 protein expression is detected in 25-42% of CRC, more frequently in advanced CRC and may indicate poorer prognosis (Yi, Wang et al. 2001, Goto, Mizukami et al. 2009, Veganzones-de-Castro, Rafael-Fernandez et al. 2012).

Amplification of HER2/ERBB2 receptors in about 20% of breast cancer and KRAS mutation can drive the overexpression of cyclin D1 through the RAS/MEK/ERK and PI3K/AKT pathways (Lenferink, Busse et al. 2001, Klein and Assoian 2008, 2012). In CRC, loss of APC deregulates WNT/APC/beta-catenin signaling, stimulating the constitutive transcription of cyclin D1 and D2 through T-cell factor and its target gene, *CMYC* respectively (Bouchard, Thieke et al. 1999, Shtutman, Zhurinsky et al. 1999). In T-cell lymphoblastic leukemia, NOTCH1 activating mutation, which is present in over 50% of all cases, also drives cyclin D3 overexpression (Ferrando

2009, Joshi, Minter et al. 2009). With higher level of cyclin D and the loss of expression of negative regulator p16, CDK4/6 activity is elevated, leading to unregulated hyperphosphorylation of RB and FOXM1 and deregulated cell cycle progression.

Mutations in RB is rarely observed in CRC but in other cancers, RB inactivation is commonly observed especially in small cell lung cancer where more than 90% of all cases exhibit RB inactivation (Burkhart and Sage 2008). This further emphasizes the role of CDK4/6 in promoting tumorigenesis through its role in regulating the activity of important tumor suppressor RB.

1.3.5 Clinical Application of CDK4/6 in cancer

Due to CDKs crucial role in cell cycle, there have been many inhibitors developed in the past decades and the first generation inhibitors are largely non-specific as they inhibit multiple CDKs. Flavopiridol, the most investigated CDK inhibitor, has been shown to inhibit CDK1/2/4/6/7/9 (Sedlacek, Czech et al. 1996, Shapiro 2006) in vitro but it was discontinued due to its lower than expected efficacy in clinical trials. Reasons behind the failure of these CDK inhibitors with low specificity could be the targeting of multiple important CDKs in the normal cells such as CDK1, leading to toxicities which prevent the drug from being used at a higher and more efficacious dose (Asghar, Witkiewicz et al. 2015). The lack of specificity in the earlier generation of CDK inhibitors prevented their use in combination therapies and there was a need to design more specific CDK inhibitors.

Currently Palbociclib, a CDK4/6 small molecule kinase inhibitor, has already been approved by the U.S. FDA for the treatment of estrogen receptor-positive and human epidermal growth factor receptor-negative breast cancer together with letrozole. It shows high specificity for CDK4 and CDK6 with IC_{50} values for CDK4 and CDK6 at

11nM and 16nM respectively (Toogood, Harvey et al. 2005). Palbociclib competes with ATP for binding for the ATP-binding pocket of CDK4/6.

Palbociclib treatment leads to sustained repression of RB phosphorylation and tumor regression in colorectal cancer xenografts, and also G1 arrest in breast, lung and colon cancer cell lines (Fry, Harvey et al. 2004). In oncogenic KRAS-driven murine non-small cell lung cancer model, Palbociclib was able to drastically slow down the tumor growth through inducing senescence response in the tumors (Puyol, Martin et al. 2010). LEE011, another specific CDK4/6 inhibitor, has also shown to elicit cell cycle arrest and senescence response in neuroblastoma (Rader, Russell et al. 2013), showing that CDK4/6 inhibition is indeed effective in a wide range of cancer types.

Putative positive biomarkers of response to CDK4/6 inhibition include *CCND1* amplification/overexpression and loss of p16 (Konecny, Winterhoff et al. 2011). However, studies have shown that tumors still responded to CDK4/6 inhibitors even without the presence of positive biomarkers. More importantly is the presence of functional RB as absence of functioning RB predicts resistance to CDK4/6 (Fry, Harvey et al. 2004, Dean, Thangavel et al. 2010). CDK4/6 inhibitors are currently in numerous clinical trials for melanoma, breast cancer and lung cancer.

1.4 Aims and objectives of study

In the past decades, intensive effects have been made to find effective targeted therapies for KRAS mutant cancer including CRC. However, up to this point, there is still no treatment other the standard chemotherapy available in the clinics. Much of the combinatorial treatments suggested in the recent years are based on cell lines models where high throughput small interference RNA screenings were done to identify synthetic lethal genes specifically in existing KRAS mutant cell lines or where differential gene expression signature were obtained by comparing KRAS mutant and wild type cancer cell lines to identify targetable KRAS dependent pathways (Sarthy, Morgan-Lappe et al. 2007, Scholl, Frohling et al. 2009, Cox, Fesik et al. 2014). Cancer cell lines models may not accurately represent the deregulated expression profile in patients' tumors and this might be one of the reasons for the lack of efficacy of combination treatment in the clinical trials. Other synthetic lethal genes such as STK33 and TBK1 identified in KRAS mutant cancer cells either do not have their specific inhibitors or their inhibitors are still in preclinical studies (Barbie, Tamayo et al. 2009). Moreover, studies have also revealed the presence of KRAS dependency and independency in KRAS mutant cancer and found KRAS mutation to be an inadequate biomarker for MEK/ERK pathway activation, further increasing the complexity in finding a suitable treatment strategy for KRAS mutant cancer (Singh, Greninger et al. 2009, Yeh, Routh et al. 2009, Singh, Sweeney et al. 2012).

Our approach is to first identify a clinically relevant KRAS dependency gene signature from patients-derived CRC tumors. By obtaining gene expression profile driven by KRAS, we can proceed to identify pathways other than the MEK/ERK pathway that oncogenic KRAS is dependent on. We hypothesize that by identifying these pathways, we can then use pharmacological tools to inhibit the pathway that KRAS mutant CRC are co-dependent on together with MEK/ERK pathway inhibition. And specifically, we hypothesized that by inhibiting the CDK4/6 and

MEK, the growth of KRAS dependent CRC will be impeded. In addition, this gene signature could be potentially used together with existing method of KRAS mutation detection to better predict CRC tumors that will respond to our proposed treatment strategy.

Clearly, our aims are:

1. To develop an effective treatment strategy against KRAS mutant CRC.
2. To identify a clinically relevant KRAS mutation associated gene signature.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines and Drug treatment

DLD1, HCT116, HCT15, RKO, HT29, FHs74 Int and CCD841 CoN cell lines were obtained from American Type Culture Collection. KRAS isogenic DLD1 lines were obtained from Horizons Discovery. DLD1, HCT116, HCT15, RKO and HT29 were grown in Dulbecco's modified Eagles's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 5000U/ml penicillin/streptomycin (Gibco). Growth media for FHs 74 Int and CCD841 CoN were cultured in DMEM with additional supplement of 30ng/ml EGF, non-essential amino acid (Gibco), 10ug/ml insulin, 1mM oxaloacetate and 0.5mM sodium pyruvate (Gibco). KRAS isogenic DLD1 lines were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum and 5000U/ml penicillin/streptomycin. All cells were maintained in $75cm^2$ flasks in a humidified incubator at 37°C with 5% carbon dioxide. Patient tumor derived spheres were grown in DMEM/Ham's F12 (Nacalai Tesque) and supplemented with 1X B27 (Gibco), 0.5ug/mL Hydrocortisone, Heparin, 20ug/mL EGF and 20ug/mL FGF. Patient tumor derived adherent line 14S was grown in DMEM supplemented with 10% fetal bovine serum, 5000U/ml penicillin/streptomycin, 4ug/ml insulin and non-essential amino acid.

Palbociclib and PD0325901 were obtained from Axon Medchem (Groningen, Netherlands) and drug treatments for all experiments were given one day after seeding. 1.5×10^5cells were seeded for 48 hour drug treatment for protein extraction as well as RNA extraction.

2.2 Cell viability assay, Fluorescence Activated Cell Sorting

Optimal cell seeding density was determined empirically for all cell lines by examining the growth of a range of seeding densities in a 96-well format. For the cell viabilitly assay, 1000 cells were then seeded per well 24 h before drug treatment in at least triplicate. Media were carefully removed before 100µl of fresh media with inhibitor were added to each well. To measure cell viability, 50µl of CellTiter-Glo®

substrate were added to each well to lyse the cells and the 96-well plate was then incubated at room temperature for 15 minutes inclusive of 5 minutes of shaking. Cell viability was then measured via chemiluminescent signals using GloMax Explorer (Promega). CellTiter-Glo values obtained were normalized to either the day of seeding (day 0) or the day of drug addition (day 1).

For fluorescence activated cell sorting assay, 5000 cells were seeded per well in 6-well plate with at least 3 wells per treatment condition. Media were removed with the addition of fresh media with inhibitors the next day and cells were treated for 7 days with a top-up of media and drug on day 5. Cells were harvested by trypsinization, washed with cold PBS and fixed with 70% ethanol for at least 1 hour. Cells were washed with PBS again before treating with RNase for 5 minutes. Cells were then stained with propidium iodide (50ug/ml) for 30 minutes, analyzed with FACScalibur (BD Biosciences) and quantified by CellQuest software (BD Biosciences).

2.3 RNA extraction

1ml Trizol added directly to lyse monolayer cells, followed by the addition of 200µL of chloroform and centrifugation at 12,000g to separate RNA from DNA and protein. Top liquid phase containing RNA was transferred to a fresh eppendorf tube with 70% ethanol added. RNAeasy mini kit (Qiagen) was used for RNA purification. Briefly, samples were transferred to columns to allow RNA binding and then subjected to centrifugation at 10,000rpm for 30 seconds. Columns were then washed once with 750µL RW1 buffer and twice with 500µL RPE buffer and eluted with 35-50µL of nuclease free water. RNA concentration and purity were then assessed using Nanodrop machine

2.4 cDNA conversion and Real time quantitative PCR

Using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), RNA samples were reverse transcribed and converted into complementary DNA

(cDNA). In short, 750ng of RNA were diluted in 25µL of nuclease free water and a reaction mixture containing 5µL of reverse transcriptase buffer, 5µL of random primers, 2µL of dNTP mix and 2.5µL of MultiScribe™ reverse transcriptase and 10.5µL of nuclease-free water was added. Reaction mixture was subjected to PCR using thermo cycler and the protocol of 10 minutes at 25°C followed by 2 hours at 37°C. Quantitative PCR was performed using 0.44µL of cDNA samples together with 0.4µL of 10µM gene specific primer mix and 5µL of KAPA SYBR FAST qPCR Kits (Kapa Biosystems). Reaction mixture was topped up to 10µL with nuclease-free water, amplified and quantified with PRISM 7900 Sequence Detection System (Applied Biosystems). Sequences for the real time quantitative PCR primers are as followed:

Gene	Forward Primers 5'-3'	Reverse Primers 5'-3'
ACTB	GCACAGAGCCTCGCCTT	GTTGTCGACGACGAGCG
FOXM1	AACCGCTACTTGACATTGGC	GCAGTGGCTTCATCTTCC
BUB1	ATCTCCCTGGGTAGCTTCGT	CCATCAAGCCCAAGACTGAA
PBK	CAGCTGCCGGGCGTATGTGT	CTCAGTCCAGAGTCTCACCGCCT
CDCA7	GGCTTTTCAGAAAGTGAGGTGC	AACTTCATCGCCACCCTGAG
FOXM1B	AGGTGTTTAAGCAGCAGAAACG	GCTAGCAGCACCTTGGGGGCAA
CDK2	ATCCGCCTGGACACTGAGAC	TTGCAGCCCAGGAGGATTTC
KIF11	CTGCCAGCAAGCTGCTTAAC	CCTGGGAATGGGTCTGCTTT
TIMELESS	ATGACAGGTCTTCCAGTCGC	TGGATGATCTGCTTGCGTGT
BUB1B	GCAAAGGGAAAAAGACAGCA	TGCATCTGTTGAGGAAATGG

2.5 Microarray Gene Expression Profiling

Microarray hybridization was carried out using the Illumina Gene Expression SentrixBeadChip HumanRef-8_V2 according to the manufacturer's protocol. In brief, 500ng of RNA samples were reverse transcribed into cDNA and processed into

double-stranded cDNA. Biotinylated cRNA was then generated from purified cDNA, purified and hybridized onto BeadChip. After washing, the cRNA was then bound with streptavidin. BeadChip was then scanned using Illumina BeadArray Reader and the images were processed using Illumina GenomeStudio™. The generated data was imported into GeneSpringGX™ (Aglient Technologies) for analysis. Signals were normalized to median expression and fold change were analyzed using pairwise comparisons to the controls (untreated samples). Median-normalized data were represented in $\log_2$ transformed values and were processed by Cluster and Treeview software to get heatmap. Illumina gene expression data of human CRC and matched normal controls can be found in GEOarchive under accession number GSE10972 and GSE74604. Gene expression for DLD1 treated with Palbociclib and PD0325901 for 24, 48 and 72 hours can be found under accession number GSE74604.

2.6 Synergy scoring

To identify synergistically upregulated and downregulated genes after treatment with Palbociclib and PD0325901, formula previously used in (McMurray, Sampson et al. 2008) were applied. Let a be the expression value of a given gene after Palbociclib treatment, b represents the expression value for the same gene after PD0325901 treatment and d represents the expression value for this gene after Palbociclib and PD0325901 combination treatment. The formula defines synergistic genes as $\frac{a+b}{d} \leq 0.9$ for upregulated genes and $\frac{d}{a} + \frac{d}{b} \leq 0.9$ for downregulated genes. This formula was implemented after fold change 2 cut-off was applied gene expression data from DLD1 to obtain differentially regulated genes after treatment.

2.7 Gene ontology analysis

For analysis of the effect of the treatment of Palbociclib and PD0325901 on cellular signaling, Ingenuity Pathway Analyses (IPA) software was used. Genes that were differentially expressed with a fold change of 2 in any of the treatment groups as compared to controls were imported into IPA. Activation Z-score for predicted

upstream transcription factors were obtained. Enriched signaling pathways and diseases/biological functions were also obtained. The same analysis was repeated for synergistic genes using expression values of 72 hour timepoint. Results were exported and plotted on GraphPad.

2.8 siRNA transfection

SiRNA transfection was done with Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's protocol. Both non-targeting control, siNC, and target-specific siRNA were purchased from Integrated DNA Technologies. The sequences for the specific siRNA are as follow: KRAS siRNA: 5'-GACGATACAGCTAATTCAGAA-3'; FOXM1 siRNA: 5'-GGACCACUUUCCCUACUUU-3'. For siRNA transfection, 1.5µL of siRNA (20uM) and 2µL of Lipofectamine RNAiMAX were added separately to 100µL of OPTI-MEM (Invitrogen). After incubation at room temperature for 5 minutes, the two diluents were mixed and further incubated at room temperature for 20 minutes. The transfection reaction was then added onto cells with 800µL of complete media. After 24 hours, transfected cells were then reseeded for cell viability assay or for RNA and proteins.

2.9 Anchorage Independent colony formation assay

3% agar mixture was prepared by heating dissolving bactoagar in PBS. Agar mixture was then further diluted to 0.6% using complete media and coated onto 6 wells plates. 5000 cells in 0.3% agar mixture and seeded onto the pre-coated agar plate. 1ml of media or media containing drug treatment were added to each well the following day. After 12 days, the colonies were stained overnight with iodonitrotetrozolium chloride (Sigma, St Louis), scanned and counted using GelCount. Average of three replicates was determined.

2.10 Pyrosequencing

Genomic material extracted from tumor samples were amplified along the KRAS codon 12 and 13 region with KRAS PCR forward primer, 5'-AGGCCTGCTGAA AATGACTG-3', and a biotinlyated KRAS PCR reverse primer, 5'-[Biotin]CAAGATTTACCTCTATTG-3'. Using the Qiagen PyroMark PCR kit, two 40 cycles of PCR were done get sufficient templates. Briefly, genomic material was added to reaction mixture containing 5µL of PyroMark master mix, 0.4µL of KRAS forward and reverse primers (5uM) and nuclease free water added to a final volume of 10µL. The reaction mix is subjected to PCR in a thermo cycler with the following program: Initialization for 15min at 95°C to activate DNA polymerase; Denaturation for 30sec at 94°C; Annealing of primers to single strand template for 30sec at 56°C; Elongation for 30sec at 72°C: repeat denaturation to elongation step for 39 cycles; Final elongation step for 5min at 72°C; Hold at 4°C. Agarose gel was run to semi-quantitatively check the amount of PCR products obtained for both PCR reactions.

To prepare for the sequencing reaction, PCR product is incubated for 30min at room temperature in a reaction mixture containing 40µL of binding buffer, 2µL of Streptavidin Sepharose beads and top up with nuclease free water to 80µL to allow the binding of the biotinlyated PCR products to the beads. The KRAS sequencing primer (5'-TTGTGGTAGTTGGAGC-3') is diluted using annealing buffer from 100uM to 0.3uM and 25µL was added to the each well on the Qiagen PyroMark Q24 plate. The PCR products bound to the beads were denatured, leaving only the biotinlyated single strand temple and released into the plate containing the sequencing primer. The mixture is then incubated at 80°C for 2min and cooled to room template to allow the sequencing primer to hybridize to the template. The reaction mixture is then placed in the machine where DNA polymerase, ATP sulfurylase, luciferase, and apyrase as well as their substrates adenosine 5' phosphosulfate (APS) and luciferin are added. The different deoxyribonucleotide triphosphates (dNTPs) were added

sequentially. Incorporation of a complementary nucleotide to the template released pyrophosphate which is a substrate for the production of ATP from ATP sulfurylase reaction and the ATP produced led to the production of visible light from luciferase reaction. Apyrase degrades unused dNTP and ATP from the addition of the next dNTP. The light produced generates a peak and the height of the peak is proportional to the number of nucleotide incorporated, allowing for the quantification of KRAS mutation found on codon 12 and 23 in each tumor samples. Reaction mixtures were ran on Qiagen PyroMark Q24 using PyroMark Q24 Gold Q24 Reagents Kits and data was analyzed using the PyroMark Q24 software.

2.11 Antibodies and Immunoblotting

Antibodies against the following proteins were obtained from Cell Signaling Technology (Danvers,MA, USA) and used at the indicated dilution: RB (4H1) #9309 (1:2000), P-RB Ser780 #9307 (1:1000), P-p44/22 MAPK (ERK1/2) Thr202/Tyr204 #9101 (1:1000), Total p44/22 MAPK (ERK1/2) #9102 (1:2000), c-Myc #9402 (1:2000) and pFOXO3A Ser253 #9466 (1:1000). Antibodies to the following proteins were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA): FOXM1 (A11) #sc-271746 (1:500), Cyclin B1 (GNS1) #sc-245 (1:500), E2F1(C-20) #sc-193 (1:1000), E2F4 (A-20) #sc-1082 (1:1000) and Cyclin D1 (HD11) #sc-246 (1:1000). Beta actin antibody was obtained from Sigma-Aldrich and used at the dilution of 1:200,000.

For immunoblotting, cells were trypsinized and washed in PBS before being subjected to lysis using radioimmunoprecipitation assay lysis buffer (50mM Tris-HCl pH7.4, 1mM EDTA, 150mM NaCl, 1% Igepal CA630, 0.5% sodium deoxycholate, 1mM Na_2VO_4, 20mM NaF, 1mM PMSF and complete protease inhibitor (Roche)). Samples were incubated on ice for 30 minutes and subjected to sonication before centrifugation at 13200rpm for 15 minutes at 4°C. Supernatant was transferred into fresh tubes and protein concentration was estimated using the BioRad Bradford dye

with known BSA concentration as standards and measured using Tecan Xfluor™ software.

To obtain protein lysates from xenograft studies, tumors were lysed in the same buffer with the use of the Tissuelyser II (Qiagen) at the frequency of 30 rounds/second for 45 seconds for 3 cycles. The lysates were then subjected to the same downstream process as monolayer cultured cells.

Protein samples (30µg) were separated by 8% or 12% SDS-PAGE gel and subsequently transferred onto PVDF membrane (Millipore) using Trans-Blot SD Semi-Dry transfer cell (Bio-Rad). Membranes with immobilized proteins were blocked with 5% BSA (Sigma) or 5% non-fat milk (Bio-Rad) for at least 2 hour followed by primary antibodies for 1 hour to overnight incubation depending on the specific antibodies and HRP-conjugated secondary antibodies for 1 hour. Membrances were incubated with chemiluminescent Supersignal® West Femto maximum sensitivity substrate (Thermo Scientific) and signals were detected using ChemiDoc™ MP imaging systems (Biorad) with Imagelab software.

2.12 *In Vivo* Treatment Studies

DLD1 and RKO xenografts were generated via the injection of 3 x 10^6 cells with Matrigel in a ratio of 1:1 into the flank of 4-8 week old female NOD/SCID mice. Mice were randomized to 4 treatment groups once the tumors reached the average size of 100mm^3. Palbociclib were administered daily at 35mg/kg for DLD1 xenografts and at 50mg/kg for RKO xenografts via oral gavage. PD0325901 were administered daily for 5 days at 20mg/kg via oral gavage. Vehicle for Palbociclib was PBS and for PD0325901 was 0.5% hydropropylmethylcellulose, 0.2% Tween-80 and 5% DMSO. Tumor volume was measured by electronic caliper twice a week and calculated with the following formula: Length x (Width^2) x 0.5. Palbociclib (CT-PD2991) and PD0325901 (CT-PD03) for in vivo use were obtained from ChemieTek

(Indianapolis, IN). Mice were obtained from Invivos (Singapore) and housed in Biological Resource Centre.

2.13 Study Approval

All animal studies were performed in compliance with protocols approved by Biopolis Institutional Animal Care and Use Committee of Singapore. Human CRC tissue DNA and RNA samples were originally obtained from Singapore Tissue Network and National University of Singapore (NUS) using protocols approved by Institutional Review Board of NUS. Informed consent was obtained from each individual who provided the tissues.

2.14 Statistical analyses

In-vitro experiments were repeated at least three times and data are reported as mean+s.e.m. Statistical significances were assessed by two-tailed Student's using Student's *t*-test, one-way or two-way analysis of variance for multiple group comparisons using GraphPad Prism 6 software. $P \leq 0.05$ was considered significant.

2.15 Computational Modeling

This portion of the work was done by Fan Zhang and Dr Zheng Jie from Nanyang Technological University.

Network construction

More genes were observed to be down-regulated synergistically as compared to the up-regulated ones, we chose to focus on the down-regulated genes and synergistically downregulated genes were selected (23 genes). Next, from the UCSC_TFBS [1] database, transcription factors that are able to transcriptionally regulate these 23 genes were identified. Next, signaling pathways containing the drug targets (i.e., CDK4/6 and MEK) and related neighbors were extracted from GeneGoMetaCore [2]. The transcription factors that are not downstream of the extracted signaling pathways were excluded because their activities are unlikely to be perturbed by the drugs.

Altogether, the network constructed is composed of 11 signaling proteins, 23 transcription factors and 23 synergistically downregulated genes. In total, there are 122 edges in the network: 14 edges among signaling proteins with the interaction type of phosphorylation; 35 edges leading from signaling proteins to transcription factors with interaction type also phosphorylation; and 73 edges representing transcription factors transcriptionally regulating the targeted genes.

Network Component Analysis

To reversely derive the activities of the transcription factors from the gene expression profile, Network Component Analysis (NCA) [3, 4] was employed. NCA considers the biochemical reactions of the network to deduce the activity profiles of transcription factors using temporal gene expression data and relationships between transcription factors and their respective targeted genes. The gene regulatory network is represented as a "bipartite network model", which requires time-series gene expression data and interaction relationships between transcription factors and target genes (in the form of a connectivity matrix) as the input data. Given the time-series gene expression data $[E]_{g\times t}$ and the connectivity matrix $[C]_{g\times f}$, where g, t and f indicate the numbers of genes, time points and transcription factors, respectively. NCA is able to obtain the activities of transcription factors over time $[A]_{f\times t}$, by decomposing the matrix [E] in the following equation into two matrices [C] and [A].

$$[E]_{g\times t} = [C]_{g\times f}[A]_{f\times t}$$

Here matrix [E] contains the expression profiles of the 23 synergistically down-regulated genes at 3 time points (i.e., 24h, 48h and 72h), and [C] is the prior knowledge of the regulatory relationships between the transcription factors and genes obtained from UCSC_TFBS and GeneGoMetaCore (i.e., the 73 edges, which represent relations of transcriptional regulation, in the aforementioned network).

According to [4], the elements in the connectivity matrix [C] for all the transcription factors that have no connection with respective genes were set to zero; the rest of the elements were then assigned to an arbitrary value. Next, the columns of [C] were normalized such that the mean absolute value of the non-zero elements in the matrix for each column would be equal to the number of controlled genes. For example, if the *i*-th transcription factor regulates *j* genes, the mean absolute value of the non-zero elements in the *i*-th column of [C] should be equal to *j*.

CHAPTER 3: RESULTS

3.1 Identification of a KRAS mutation associated gene signature showed enrichment for cell cycle and mitosis in colorectal tumors and predict an dependency for CDK4/6 activity

In search of an effective treatment strategy for KRAS mutant colorectal cancer, many groups have intensely studied the molecular signaling in KRAS mutant colorectal cancer and came up with combinatorial treatment strategies by inhibiting both MEK together the function of AKT (She, Halilovic et al. 2010), IGFR1 (Ebi, Corcoran et al. 2011), HER3 (Human Epidermal Growth Factor receptor 3) (Turke, Song et al. 2012), BCL-2/BCL-xL/MCL1 (B-cell lymphoma 2/xL/Myeloid cell leukemia 1)(Corcoran, Cheng et al. 2013) or RAF (Lamba, Russo et al. 2014). These treatment strategies were proposed through the use of KRAS isogenic cell lines or different colorectal cancer cell line models with different KRAS mutation status to identify pathways that are important for the survival of KRAS mutant cells especially in the presence of MEK inhibition or through the differential gene expression between KRAS mutant and wild type cancer cell. These models were based on *in vitro* cell lines model as the heterogeneity in patient CRC tumors, due to presence of sub-clones driven by different oncogenic mutations, had hindered the identification of gene expression driven by aberrant KRAS signaling.

Here in our studies, to identify a KRAS dependent colorectal cancer gene expression, we performed gene expression profiling on 55 colorectal tumors using Illumina 24K human BeadArray-V3 as well as pyrosequencing of KRAS at codon 12 and 13 to quantify the level of KRAS mutation in the tumors in order to overcome the issue of tumor intraheterogeneity. We stratified the tumors into KRAS wildtype (<10% of KRAS mutation), low KRAS mutation (10-40%) and high KRAS mutation group (>40%) (Figure 3.1A) and proposed that the high KRAS mutation tumors contain mainly KRAS mutant cells whereas KRAS wildtype or low KRAS mutation tumors consists of mainly KRAS wild type cells. Supervised gene clustering analysis of these

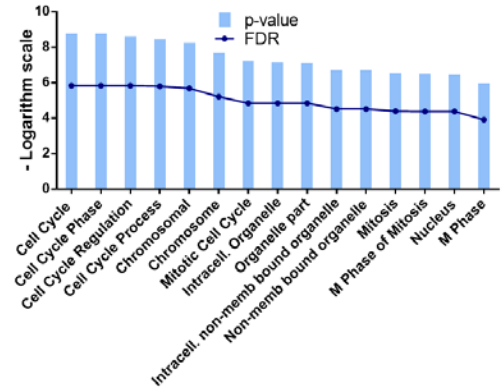
tumors revealed 97 genes were significantly correlated with KRAS mutation ($P<0.05$) with56 genes meeting the cutoff of Pearson correlation of <-0.3 or >0.3 (Figure 3.1B). 34and 22 genes were differentially upregulated and downregulated respectively in the high KRAS mutation group. In a recent study, polycomb protein EZH2, a methyltransferase, has been positively associated with KRAS mutation in lung cancer and inhibiting EZH2 can sensitize KRAS mutant cells to chemotherapy (Riquelme, Behrens et al. 2015). Among the downregulated genes associated with KRAS mutation, FBXO32, an EZH2-repressed apoptotic target, when repressed by EZH2, has been associated with chemoresistance, suggesting that EZH2 functions similarly in KRAS mutant CRC (Tan, Yang et al. 2007, Wu, Lee et al. 2011). We chose to focus on the upregulated genes which we termed as KRAS dependency gene signature.

Figure 3.1 Identification of a KRAS associated Gene Signature in Colorectal Cancer

A. Stratification of colorectal tumors in groups according to the level of KRAS mutation (in codon G12 and G13) detected via pyrosequencing. KRAS Wildtype consists of tumors with less than 10% KRAS mutation; Low KRAS mutation for those between 10-40% mutation; High KRAS mutation for tumors with more than 40% mutation detected.
B. Heatmap showing the 56 genes which expression correlates with level of KRAS mutation. Pearson correlation $\leq$-0.3 or $\geq$0.3 with a p-value $\leq$0.05 were used as a cut-off. The 34 upregulated genes (in red brackets) were then termed as the KRAS dependency gene signature. Colored scale bar represents log2 transformed values.
C. Heatmap showing expression of genes which are markers for various cell types found in the tumor microenvironment that could possibly contribute to the tumor gene expression profile and none of the genes were found to be significantly differentially expressed in any of the group using one way ANOVA test. *CDH1*, *EPCAM* and *KRT20* were used as markers for epithelial cells; *CD2*, *CD19* and *PTPRC* for leukocytes; *CDH5*, *ENG* and *VWR* for endothelial cells; *DCN*, *PDPN* and *FAP* for fibroblasts. Colored scale bar represents log2 transformed values.


A
KRAS Wildtype
<10%
Low KRAS mutation
10-40%
High KRAS mutation
>40%
100
80
60
40
20
0
Percentage of KRAS mutations
B
PTN
FBXO32
RPIB9
SMPD1
SERTAD4
FNBP1
CPEB3
APOD
ILK
RELN
GCNT2
C1ORF71
SLC36A1
TRPM6
TCF21
ITGA8
CST1
ABI3BP
FLJ21986
IFITM3
GLDN
LRRN2
PTPLAD1
CDCA7
TRIP13
PBK
MCM3
RPL6
POP5
TROAP
RPS5
ECT2
FOXM1
C7ORF24
LOC653505
NUSAP1
SLC12A2
KNTC1
SNRPD2
BUB1B
NUP37
RFC5
ILF3
ACN9
GMNN
CDK2
KIF11
ZNF185
SHMT2
TMPO
KRT6B
LIPG
CIT
TSFM
TIMELESS
RPLP0
Min
Max
C
CDH1
EPCAM
KRT20
CD2
CD19
PTPRC
CDH5
ENG
VWF
DCN
PDPN
FAP
Min
Max

A



B

Top upstream regulators	P-value of overlap
CDKN1A	4.18×10^{-7}
SMARCB1	6.56×10^{-7}
RB	1.26×10^{-5}
Methylselenic acid	1.82×10^{-5}
1-alpha, 25-dihydroxy vitamin D3	2.18×10^{-5}

Figure 3.2 KRAS dependency gene signature highly associated with cell cycle and mitosis processes

A. Top 15 enriched gene ontology biological processes from Molecular Signature Database which significant overlaps were observed with the 34 genes from the KRAS dependency gene signature. Majority of the top enriched gene ontology are related to cell cycle and mitotic processes.
B. The top upstream regulators of the 97 genes which expression were significantly correlated with KRAS mutation ($P<0.05$). CDKN1A, SMARCB1 and RB all have roles in cell cycle and mitosis.

To counter the possibility that the differential gene expression seen in the three group was due to different level of stroma content in the tumor sampled, we checked the expression of markers that were previously used to identify the various cell types (Calon, Lonardo et al. 2015): *CDH1*, *EPCAM* and *KRT20* for epithelial cells; *CD12*, *CD19* and *PTPRC* for leukocytes; *CDH5*, *ENG* and *VWF* for endothelial cells; *DCN*, *PDPN* and *FAP* for fibroblasts. Solid tumors do contain cells of other origins as mentioned above (Egeblad, Nakasone et al. 2010)and these cells types though found in much less quantity than the tumor cells, may skew the gene expression of the tumors, especially the tumors in the low KRAS mutation group. Thus, it was important to show that the differentially lower expression of KRAS dependency gene signature in the low KRAS mutation group was not due to the increased presence of other non-tumor cells 'diluting' the expression from the tumor cells. None of the markers were found to be significantly different in any group, strengthening our

findings that the upregulation of the gene signature in the high KRAS mutation group is due to KRAS mutation (Figure 3.1C).

To gain further insights, we used the Molecular Signature Database and found significant overlaps of the upregulated KRAS associated genes with gene sets involved in cell cycle and mitosis processes (Figure 3.2A). We also performed Ingenuity Pathway Analysis (IPA) on the 97 genes significantly correlated with KRAS mutation level and $p21^{CIP/WAF}$ or CDKN1A and Retinoblastoma protein, RB, were predicted to be top upstream regulators (Figure 3.2B). It is interesting to note that both p21 and RB regulate the cell cycle checkpoint from G1 to S phase; p21 mainly inhibits CDK2 activity which is required for the phosphorylation of RB for the subsequent release and activation of E2F-regulated gene transcription program and other than CDK2, CDK4/6-cyclin D complex is the other important regulator of RB activity. Another top upstream regulator, SMARCB1 (Figure 3.2B), though not directly involved in G1 phase of cell cycle, is a core component of the SWI/SNF nucleosome remodeling complex which modulates the transcription of lineage-specific genes as well as represses genes involved proliferation such as MYC, CCNB1 and CDK1(Wilson and Roberts 2011), which are required for the mitotic phase. In addition, FOXM1, a transcription factor regulating cell cycle and mitotic genes is also upregulated in the high KRAS mutant tumors and it is activated by CDK4/6 (Anders, Ke et al. 2011) and phosphorylated ERK (Ma, Tong et al. 2005). Thus these findings suggest that the KRAS dependency gene signature identified in high KRAS mutation tumors showed elevated cell cycle and mitosis processes, likely through the abrogation of the G1 checkpoint.

In KRAS mutant cells, it is known that the RAS/MEK/ERK pathway is constitutively activated, leading to increased proliferation and cell survival. However, single MEK inhibitor has shown no clinical efficacy in KRAS mutant cancers. Here, we hypothesize by looking at the transcriptional output of the high KRAS mutation

group that the KRAS mutation not only upregulates the MEK/ERK signaling but also dysregulates G1 checkpoint to drive tumorigenesis and we proposes that by co-inhibiting the CDK4/6 pathway and MEK/ERK pathway, we can better target KRAS mutant colorectal cancer cells.

3.2 CDK4/6 inhibition sensitizes specifically KRAS-dependent colorectal cancer cells to MEK inhibition

Previous studies on various CDK4/6 inhibitors, including Palbociclib, have shown that a wild type retinoblastoma status and a low expression of p16 predicts the best response to CDK4/6 inhibition in ovarian cancers (Konecny, Winterhoff et al. 2011), breast cancers (Dean, Thangavel et al. 2010) and glioma (Wiedemeyer, Dunn et al. 2010). Using datasets on cBioPortal (Cerami, Gao et al. 2012, Gao, Aksoy et al. 2013), we observed that the alteration frequency of RB1 in colorectal cancer is one of the lowest (Table 3.1, Figure 3.3A), suggesting that in colorectal cancer RB is mostly wild type and functional. To evaluate the activity of CDK4/6 activity in colorectal cancer, we downloaded the Kaiser Colon (Kaiser, Park et al. 2007) and TCGA Colorectal (2012) dataset from Oncomine and checked the expression of cyclin D1 (*CCND1*) and *CDK4* and found both proteins significantly upregulated in colorectal tumors as compared to normal colon tissue (Figure 3.3B-C). These evidence, combined with prior reports on 40-50% of CRC exhibiting aberrant methylation on p16 (Guan, Fu et al. 1999, Goto, Mizukami et al. 2009), showed that the CDK4/6 pathway is frequently activated in colorectal cancer and the presence of positive biomarkers of response suggests that CDK4/6 inhibitors will be effective in colorectal cancers.

Table 3.1 Datasets from cBioPortal for alteration frequency of *RB* in various cancers.

STUDY ABBREVIATION	STUDY NAME	PERCENT OF CASES ALTERED
Lung (small cell)	Small Cell Lung Cancer (CLCGP, Nature Genetics 2012)	65.50%
Prostate	Prostate Adenocarcinoma, Metastatic (Michigan, Nature 2012)	27.90%
Bladder	Bladder Cancer (MSKCC, Eur Urol 2014)	22.90%
Uterine	Uterine Carcinosarcoma (TCGA, Provisional)	21.40%
Sarcoma	Sarcoma (TCGA, Provisional)	16%
Ovarian	Ovarian Serous Cystadenocarcinoma (TCGA, Provisional)	12.90%
Liver	Liver Hepatocellular Carcinoma (TCGA, Provisional)	11.40%
Glioblastoma	Glioblastoma (TCGA, Nature 2008)	11%
Lung (squamous)	Lung Squamous Cell Carcinoma (TCGA, Provisional)	10.70%
Esophagus	Esophageal Squamous Cell Carcinoma (ICGC, Nature 2014)	8%
Cervical	Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma (TCGA, Provisional)	7.90%
Stomach	Stomach Adenocarcinoma (TCGA, Nature 2014)	7.70%
Breast	Breast Invasive Carcinoma (TCGA, Provisional)	6.50%
Lung (adenocarcinoma)	Lung Adenocarcinoma (TCGA, Nature 2014)	6.50%
Melanoma	Skin Cutaneous Melanoma (Broad, Cell 2012)	5%
Colorectal	Colorectal Adenocarcinoma (TCGA, Nature 2012)	3.30%
Myeloma	Multiple Myeloma (Broad, Cancer Cell 2014)	2%
Kidney	Kidney Renal Clear Cell Carcinoma (TCGA, Provisional)	1%

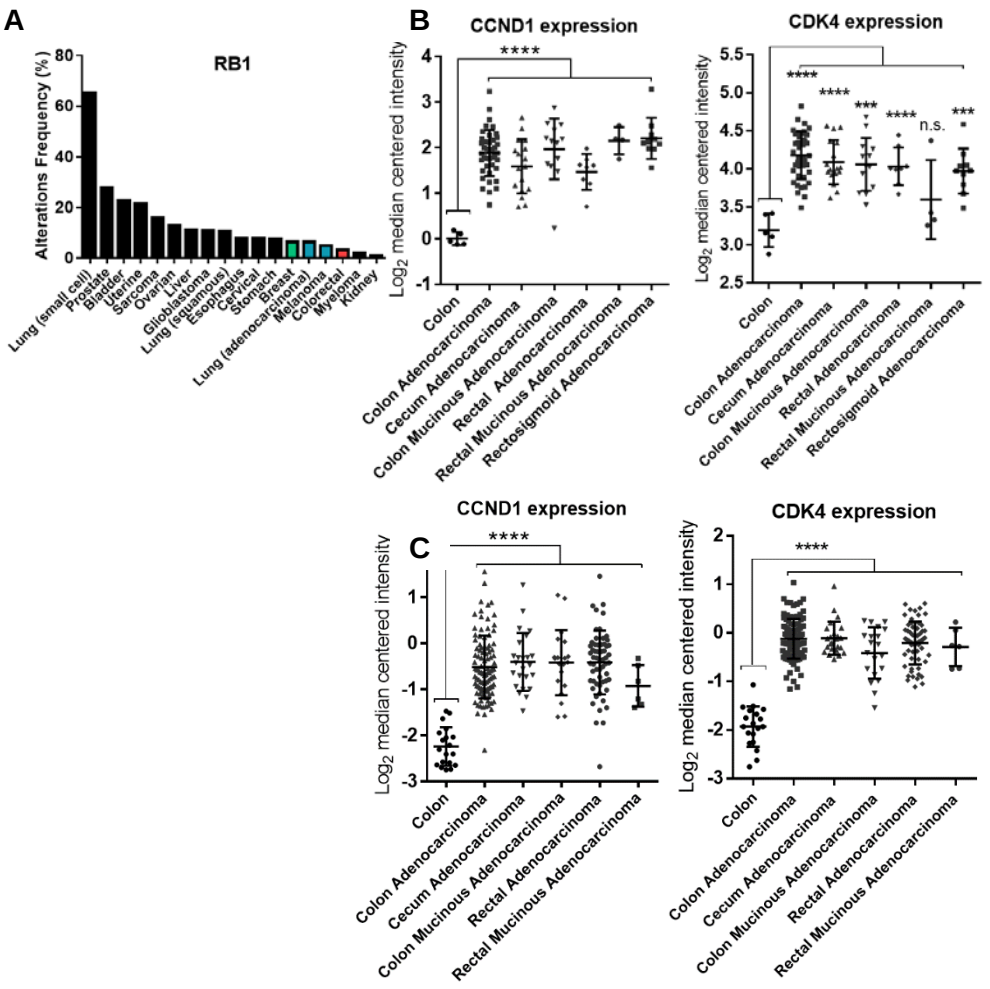


Figure 3.3 Positive indicators of response to CDK4/6 inhibition present in colorectal cancer

A. Low alteration frequency of RB1 observed in colorectal cancer highlighted in red. Breast cancer, lung cancer and melanoma also showed very low level of RB alteration and CDK4/6 inhibitors are either in clinical use or in clinical trials for these cancer types. Dataset were obtained and analyzed in cBioPortal.
B. Expression of *CCND1* and *CDK4* observed to be significantly higher in the colorectal cancer as compared to normal colon tissue in the Kaiser Colon dataset
C. Expression of *CCND1* and *CDK4* observed to be significantly higher in the colorectal cancer as compared to normal colon tissue in the TCGA colorectal dataset.

To evaluate if inhibiting CDK4/6 pathway is able to sensitize KRAS mutant colorectal cancer to MEK inhibition, we first characterize the EC_{50} value of Palbociclib, a first-in-class CDK4/6 inhibitor, in 3 KRAS mutant CRC lines, DLD1, HCT116 and HCT15, as well as RKO, a BRAF V600E mutant line and found their EC_{50} value of Palbociclib in the micro molar scale. We then compared the dose response of MEK inhibitor, PD0325901, in the presence or absence of a fixed concentration of 1μM CDK4/6 inhibitor and found that in DLD1 and HCT116, the addition of CDK4/6 inhibitor led to an significant increase in MEK inhibitor sensitivity (7.69-fold and 11.9-fold reduction in EC_{50} of PD0325901 in DLD1 and HCT116 respectively) (Figure 3.4A). In HCT15, however, CDK4/6 inhibition did not significantly reduce its EC_{50} of PD0325901 (1.95-fold reduction) (Figure 3.4C). In the recent years, there have been findings that identified the phenomenon of KRAS independence, specifically cancer cells with KRAS mutations but upon KRAS knockdown or knockout, no significance decrease in cell viability were observed in these cells. These KRAS independent cells may have KRAS mutation but are no longer dependent on KRAS for growth and survival. KRAS mutation may be one of the key oncogenic drivers in many cancers but as cancer progresses, more mutations are acquired and KRAS mutation may be rendered dispensable. Not surprisingly, HCT15 has been found to be KRAS-independent (Scholl, Frohling et al. 2009), despite having a KRAS mutation, and a validation was done by knocking down KRAS, showing that colony formation ability of HCT15 was not affected in anchorage independent growth conditions (Figure 3.5). However, for KRAS dependent DLD1 and HCT116, the number of colony formed in soft agar condition were greatly reduced (Figure 3.5). This suggests that the effect of CDK4/6 inhibition on sensitizing cells to MEK inhibition required the cells to be dependent on KRAS.

To further investigate if this effect of CDK4/6 inhibition sensitizing cells to MEK inhibition is unique to KRAS dependent cells, we also tested on BRAF mutant cells.

BRAF mutant cancer cells have shown to have elevated ERK activation and are highly dependent on the MAPK signaling, by doing this experiment, we can determine if this sensitization effect is also applicable on cells that are dependent MAPK signaling but do not have KRAS mutation. RKO, a BRAF V600E mutant CRC line, also showed similar increased sensitivity to MEK inhibitor after CDK4/6 inhibition (5.48 fold reduction) (Figure 3.4B), suggesting that a dependence on the RAS-MAPK signaling pathway is required for MEK inhibition sensitization via CDK4/6 inhibition.

To study the response of the normal colon epithelial cells to the addition of CDK4/6 inhibitor, we repeated the same experiment on FHs 74 Int, a small intestinal epithelial cell line and CCD 841CoN, a normal colon cell line and observed a significant decrease in sensitivity to MEK inhibition in the presence of CDK4/6 inhibitor (Figure 3.4D). This seems to suggest that CDK4/6 inhibition could reduce the toxic effect MEK inhibitor has on normal epithelial cells.

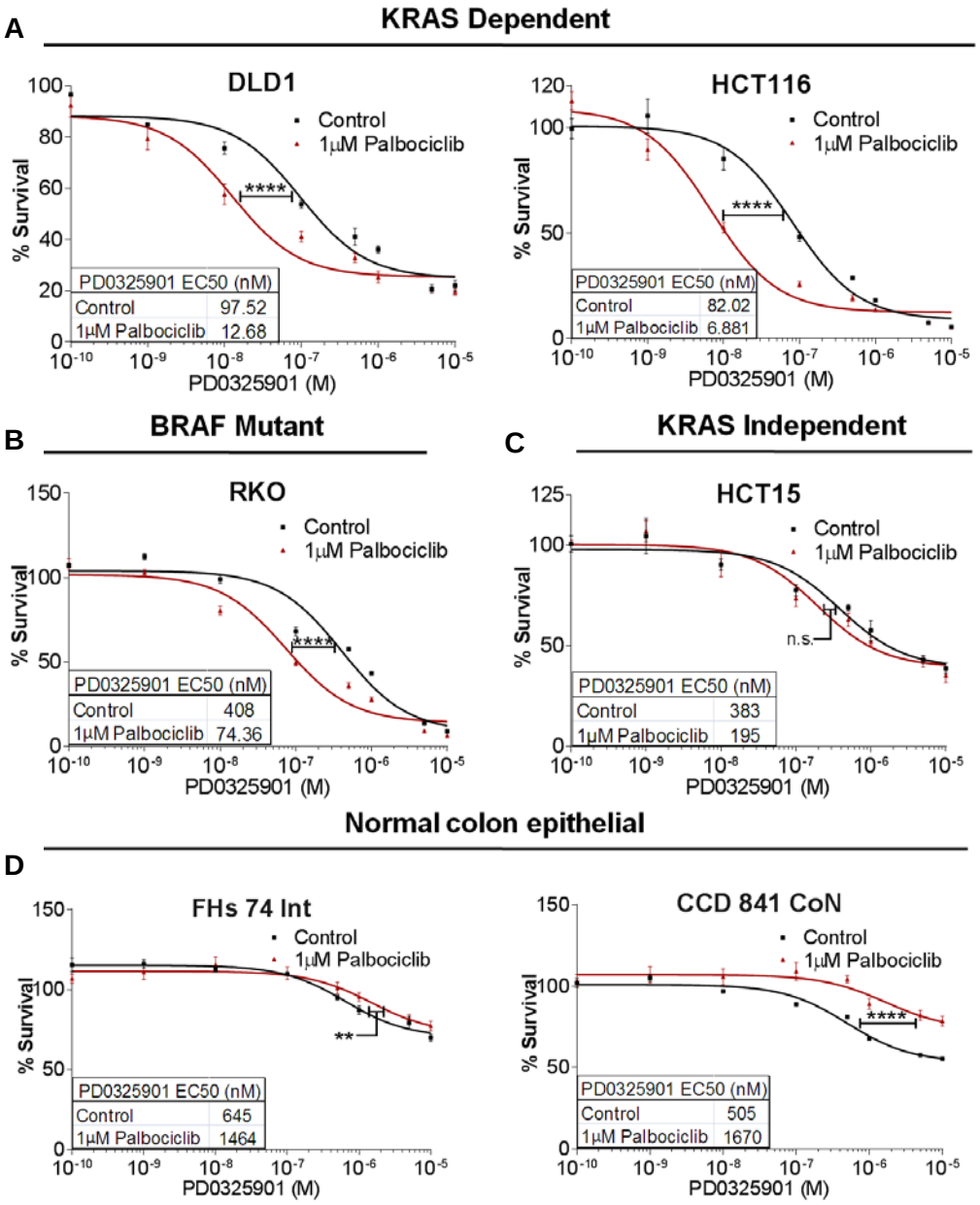


Figure 3.4 CDK4/6 inhibition specifically increases KRAS dependent/BRAF mutant CRC sensitivity to MEK inhibitor

A. Addition of 1μM Palbociclib increased sensitivity to PD0325901 in KRAS-dependent CRC, DLD1 and HCT116 as seen through reduction in PD0325901 EC_{50}. Assay was measured using CellTiter-Glo.
B. Addition of 1μM Palbociclib also increased sensitivity to PD0325901 in BRAF mutant CRC, RKO.
C. In KRAS-independent HCT15, addition of 1μM Palbociclib only showed insignificant increase in sensitivity to PD0325901.
D. In normal colon epithelial cells, FHs 74 Int and CCD 841 CoN, addition of 1μM Palbociclib reduced sensitivity to PD0325901 as seen in the increase in PD0325901 EC_{50}.
Two way ANOVA used for statistical analysis of interaction between EC50 curves. **p<0.01,****P<0.0001, ns, not significant

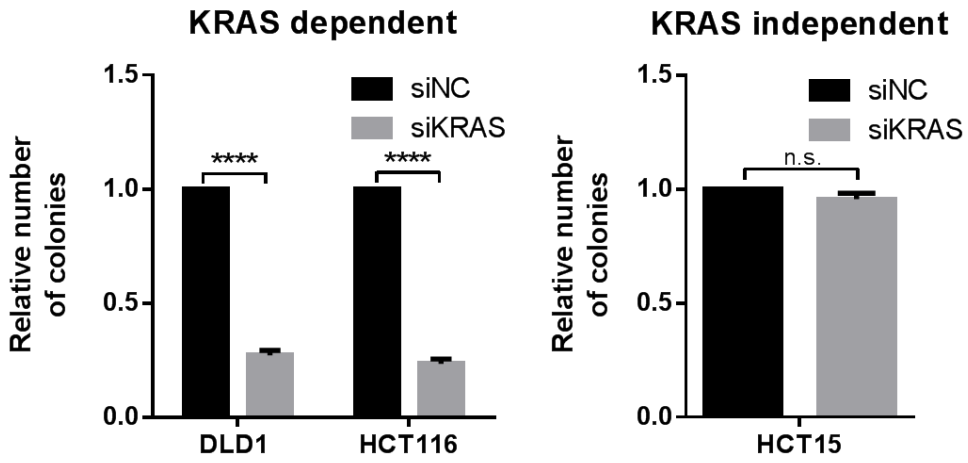


Figure 3.5 KRAS knockdown reveals KRAS dependency in DLD1 and HCT116 and KRAS independency in HCT15

(Left) Knockdown of KRAS carried out using short interfering RNA specific to KRAS led to reduction of colony formation in anchorage independent soft agar conditions for DLD1 and HCT116 (grey bars) as compared to the knockdown using non-targeting siNC (black bars). (Right) No significant reduction in colony formation was observed in HCT15. Unpaired Student T-test was used. ****P-value<0.0001. n.s., not significant

3.3 Pharmacological inhibition of CDK4/6 and MEK reduces cell viability in KRAS dependent and BRAF mutant colorectal cancer cells

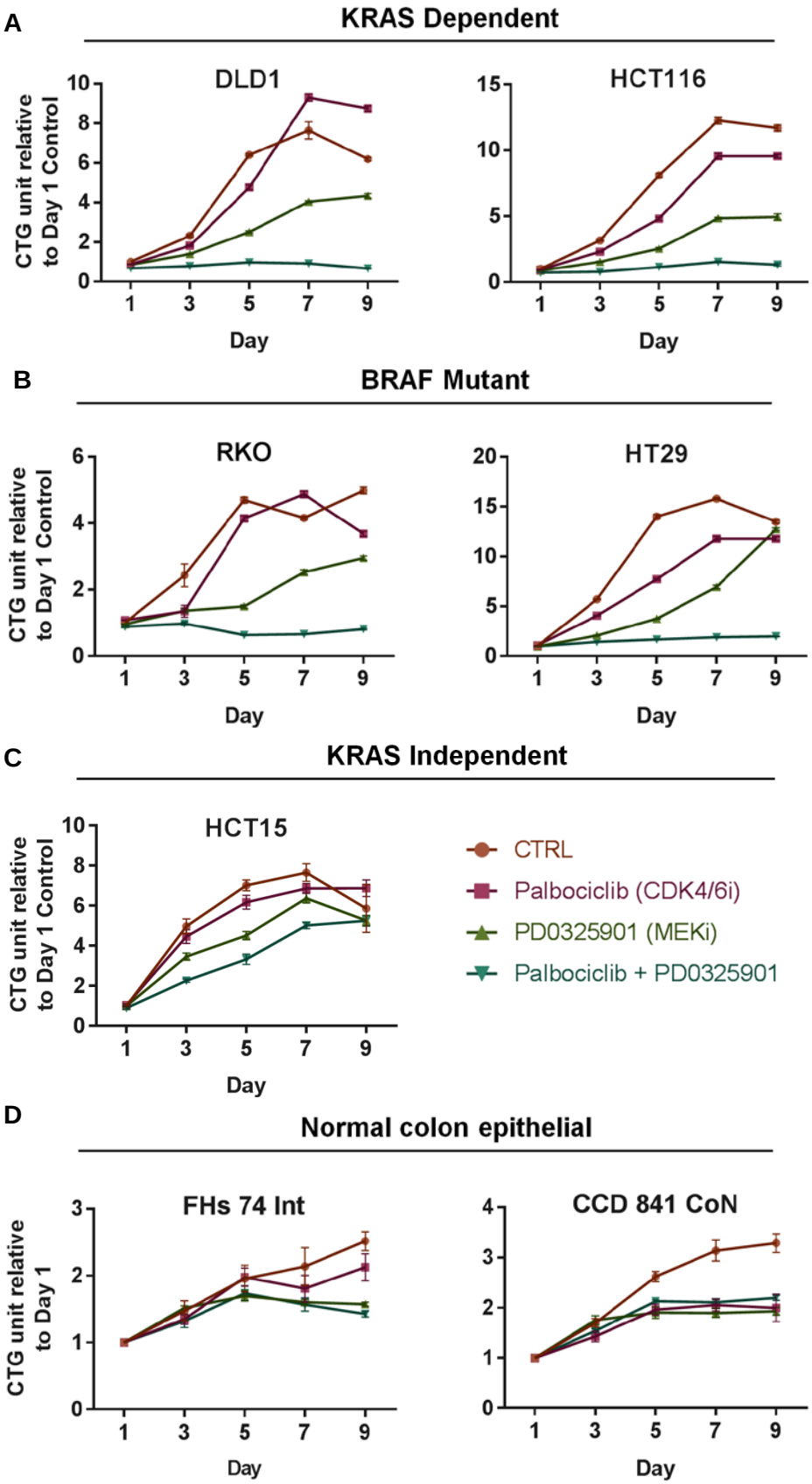
Based on our findings that the addition of CDK4/6 inhibitor is able to sensitize the KRAS dependent and BRAF mutant CRC lines to MEK inhibition, we proceeded to investigate the effect of combining the two inhibitors on cellular viability. Treatment of the cells was carried out using fixed concentration of Palbociclib and PD0325901, either alone or in combination over the course of 9 days and we monitored their viability via the level of ATP detected. The combination of CDK4/6 and MEK inhibitors led to a drastic decrease in cell proliferation when compared to single treatment of either inhibitor only in KRAS-dependent (DLD1 and HCT116) (Figure 3.6A) and BRAF mutant (RKO and HT29) (Figure 3.6B) but this was not observed in KRAS-independent HCT15 (Figure 3.6C). Similarly in the normal colon epithelial cells, no synergistic or additive growth inhibitory effect were seen when the cells were treated with the combination treatment (Figure 3.6D).

Figure 3.6 Increased reduction in cell viability in Palbociclib and PD0325901 combination treatment in KRAS-dependent and BRAF mutant CRC

(A-D) Growth inhibition response to treatment of Palbociclib and PD0325901 either alone or in combination. Top up of fresh media and drug on day 5. Relative viability measured by CellTiter-Glo and compared to Day 1 untreated control. Concentrations used are as follow:

A. KRAS Dependent:DLD1 (1μM Palb, 2μM PD); HCT116 (1μM Palb, 0.1μM PD)
B. BRAF Mutant: RKO (1μM Palb, 1μM PD); HT-29 (1μM Palb, 0.05μM PD);
C. KRAS Independent: HCT15 (1μM Palb, 1μM PD)
D. Normal colon epithelial: FHs 74 Int and CCD 841 CoN (2μM Palb, 2μM PD)

All data in the graphs represent mean±SEM, n=3.


A
KRAS Dependent
DLD1
HCT116
CTG unit relative to Day 1 Control
Day
B
BRAF Mutant
RKO
HT29
C
KRAS Independent
HCT15
CTRL
Palbociclib (CDK4/6i)
PD0325901 (MEKi)
Palbociclib + PD0325901
D
Normal colon epithelial
FHs 74 Int
CCD 841 CoN
CTG unit relative to Day 1

We also evaluated the colony formation ability of the different cell lines after treatment in 2-dimensional (2D) monolayer and 3-dimensional (3D) anchorage-independent soft agar conditions. After exposure to CDK4/6, MEK or the combination of both inhibitors for 12 days, the cells were stained with crystal violet for the 2D culture for visualization and for the soft agar culture, cells were stained with iodonitrotetrazolium chloride for quantification. Only in the KRAS-dependent or BRAF mutant cells, the combination treatment completely eradicated colony formation in monolayer and anchorage-independent growth conditions (Figure 3.7A-B). No substantial inhibitory effect was observed for KRAS-independent HCT15 (Figure 3.7C). These finding are consistent with earlier findings in Figure 3.4 where CDK4/6 inhibitor sensitized only the KRAS-dependent and BRAF-mutant cancer cells to MEK inhibition.

Furthermore, we also showed that treatment with the two inhibitors led to significant increase in apoptosis when compared to single inhibitor treatment as indicated by the Sub-G1 population in HCT116 and RKO but not in HCT15 as well as the normal colon epithelial lines (Figure 3.8). In DLD1, combination treatment did not significantly increased apoptosis as compared to Palbociclib-treated cells but increase was significant when compared to PD0325901-treated cells. Moreover in the normal epithelial lines, we observed that there was no significant difference in the level of apoptosis between untreated cells and cells treated with the combination treatment, once again suggesting that the combined inhibition of CDK4/6 and MAPK pathway does not cause toxicity in normal non-cancerous cells. All in all, these data put together demonstrated that pharmacological inhibition of CDK4/6 and MEK in combination greatly reduced cell viability as compared to single inhibitor treatment specifically in KRAS-dependent and BRAF mutant CRC.

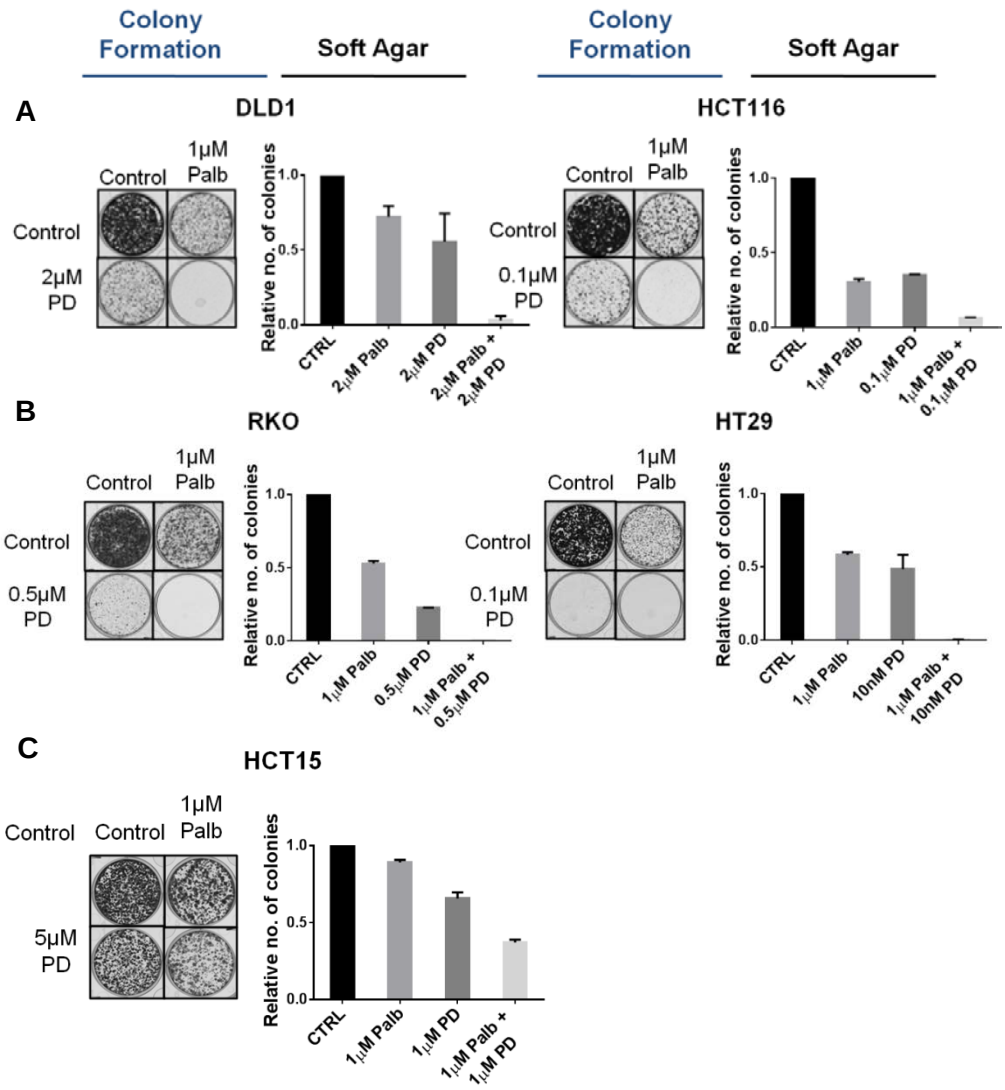


Figure 3.7 Colony formation after combination treatment with Palbociclib and PD0325901 in 2D monolayer culture and anchorage-independent soft agar inhibited in KRAS-dependent and BRAF Mutant CRC only.

A. Combination treatment with CDK4/6 and MEK inhibitors for 12 days in KRAS dependent DLD1 and HCT116 led to complete inhibition of colony formation in 2D monolayer condition as shown in the images. Combination treatment also led to an almost complete reduction in number of colonies formed in soft agar.
B. Similar to (A), combination treatment in BRAF Mutant RKO and HT29 led to reduction in colony formation in both 2D monolayer and soft agar conditions.
C. Combination treatment in KRAS independent HCT15 did not lead to as marked reduction in colony formation as seen in the KRAS dependent and BRAF mutant in both 2D monolayer and soft agar conditions.
All data in the graphs represent mean±SEM, n=3.

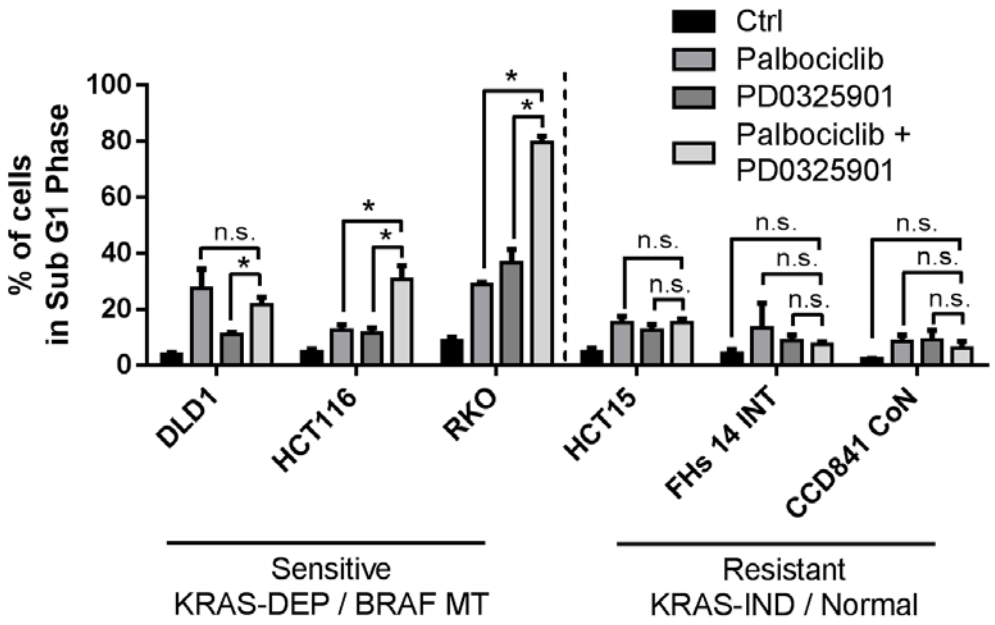


Figure 3.8 Significant induction of apoptosis in KRAS Dependent and BRAF Mutant CRC with combination treatment of Palbociclib and PD0325901.

Significant increase of cell death was observed in the combination treated group over single inhibitor treated group in KRAS dependent and BRAF mutant CRC only. Percentage of apoptotic cells as indicated by sub G1 phase after 7 days treatment with Palbociclib, PD0325901 or both inhibitors as quantified by fluorescence-activated cell sorting (FACS) analysis. Concentrations used are as followed: DLD1 (2µM Palb, 2µM PD); HCT116 (2µM Palb, 0.1µM PD); RKO (1µM Palb, 1µM PD); HCT15 (1µM Palb, 1µM PD); FHs 74 Int and CCD 841 CoN (2µM Palb, 2µM PD). Statistical analysis was done using paired Student's T-test. * indicates p value <0.05.

All data in the graphs represent mean±SEM, n=3.

3.4 KRAS mutant isogenic colorectal cancer line are more sensitive to pharmacological inhibition of CDK4/6 and MEK

To specifically study the effect of the combination treatment on KRAS isogenic cell lines (cell lines with genetically identical background other than their KRAS mutation status), we tested the combination treatment versus single treatment in DLD1 KRAS isogenic cell line. Parental DLD1 is a heterozygous KRAS mutant, with one wild type KRAS allele and one mutated KRAS allele at codon 13, G13D. Using Horizon GENESIS^{TM} gene editing technology which enables precision genome editing down to single base pair resolution, knockout of either the KRAS wild type allele or the KRAS mutant G13D allele was done in parental DLD1 cells, generating DLD1 KRAS wildtype only cells, (KRAS WT) and DLD1 KRAS mutant G13D only cells (KRAS G13D) respectively (Figure 3.9A). The knockout was done using an adenoviral associated vector by homologous recombination resulting in isogenic cell lines, each containing one allele of KRAS.

We first characterized these commercially obtained cell lines and found that DLD1 KRAS WT cells grew slower and were unable to grow in anchorage independent condition (Figure 3.9B) which was not surprising as mutated KRAS is known to transform normal cells, making them more tumorigenic. Functionally, DLD1 KRAS wild type cells also expressed lower levels of phosphorylated ERK in low serum (0.1%) and high serum (10%) conditions (Figure 3.9C), suggesting that after KRAS mutant knockout, the activity of the RAS/MEK/ERK pathway is decreased and no longer constitutively active. We then tested the combined treatment of CDK4/6 and MEK inhibitors on parental, KRAS WT and KRAS G13D DLD1 and found short term treatment up to 5 days had similar effect on all three lines (Figure 3.10A). After 5 days, however, we observed a differential effect of the combination treatment on DLD-1 KRAS WT. DLD-1 KRAS WT under the combination treatment grew at a faster rate as compared to control and the single treatment whereas, the combination

treated Parental and KRAS G13D continued to display slower growth as compared the single treated cells (Figure 3.10B). Notably, DLD1 KRAS G13D under combination treatment showed negative growth at day 7 and 11 compared to day 5, suggesting that there might be cell death occurring. From this data, we showed that colorectal cancer cells with KRAS mutation are more sensitive to the combined inhibition of CDK4/6 and MEK as compared to its isogenic KRAS wild type counterpart.

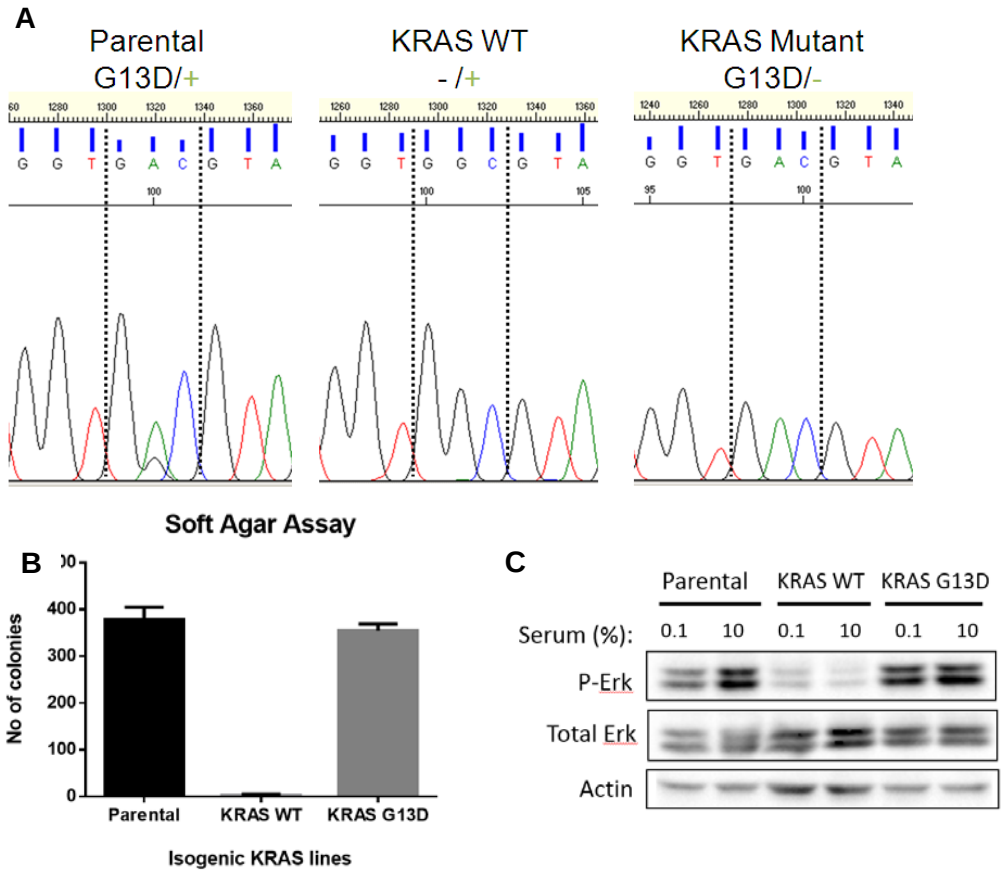


Figure 3.9 Presence of KRAS mutation in DLD1 KRAS isogenic cell lines determines cells' ability to grow in anchorage independent conditions.

A. Sequencing results of the three DLD1 KRAS isogenic cell lines, showing in DLD1 parental cells, there are both KRAS wild type and KRAS mutation at codon 13, DLD1 KRAS WT where there is only KRAS wild type and DLD1 KRAS G13D where only KRAS mutation is present.
B. DLD1 KRAS wild type cells were unable to form colonies in anchorage independent soft agar condition unlike DLD1 Parental and KRAS G13D which have the mutant KRAS allele and therefore still able to form colonies.
C. DLD1 KRAS wild type cells expressed lower levels of phosphorylated ERK as compared to isogenic DLD1 lines with KRAS mutant in both low (0.1%) and high (10%) serum culture condition.

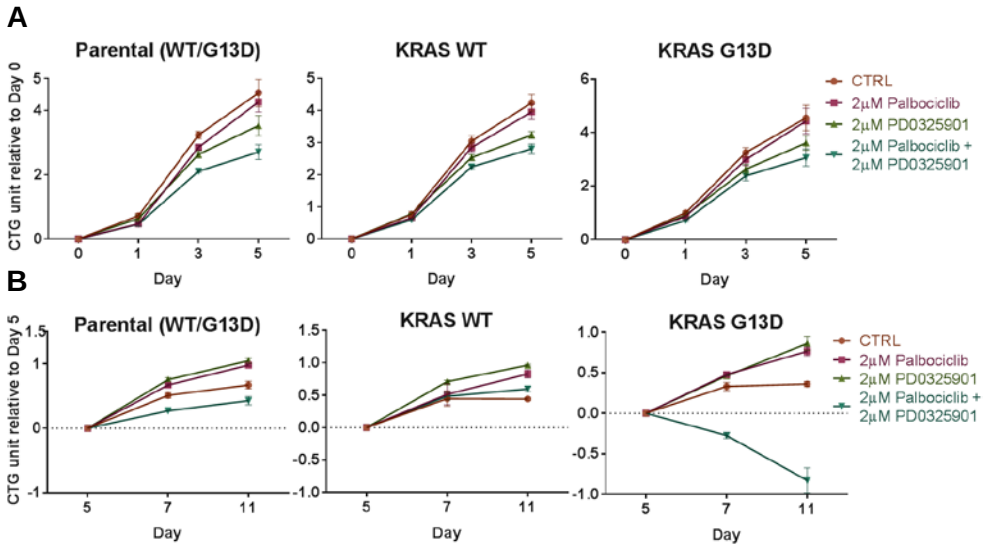


Figure 3.10 Greatest inhibition in proliferation seen in DLD1 KRAS G13D mutant treated with Palbociclib and PD0325901 with longer duration of treatment.

A. No differential growth rate was observed in the three DLD1 KRAS isogenic cell lines treated with Palbociclib, PD0325901 or both. Readings from day 0 to 5 were measured using CellTiter-Glo and were plotted relative to day 0 and then $\log_2$ transformed.
B. Greatest reduction in proliferation observed in KRAS G13D mutant from Day 5 treatment onwards. Readings from day 5 to 11 were measured using CellTiter-Glo and were plotted relative to day 5 and then $\log_2$ transformed.
All data in the graphs represent mean±SEM, n=3.

3.5 Combination treatment of CDK4/6 and MEK inhibitors converge to downregulate KRAS associated gene signature

To better understand the reason behind the effectiveness of the combined inhibition of CDK4/6 and MEK, we aimed to identify the possible mediators in the combination treatment. To this end, we performed a microarray analysis on DLD1 treated with Palbociclib or PD0325901 alone or in combination for 24h, 48h and 72h with Illumina 24K human BeadArray-V3 and we analyzed the data output as shown in Figure 3.11.

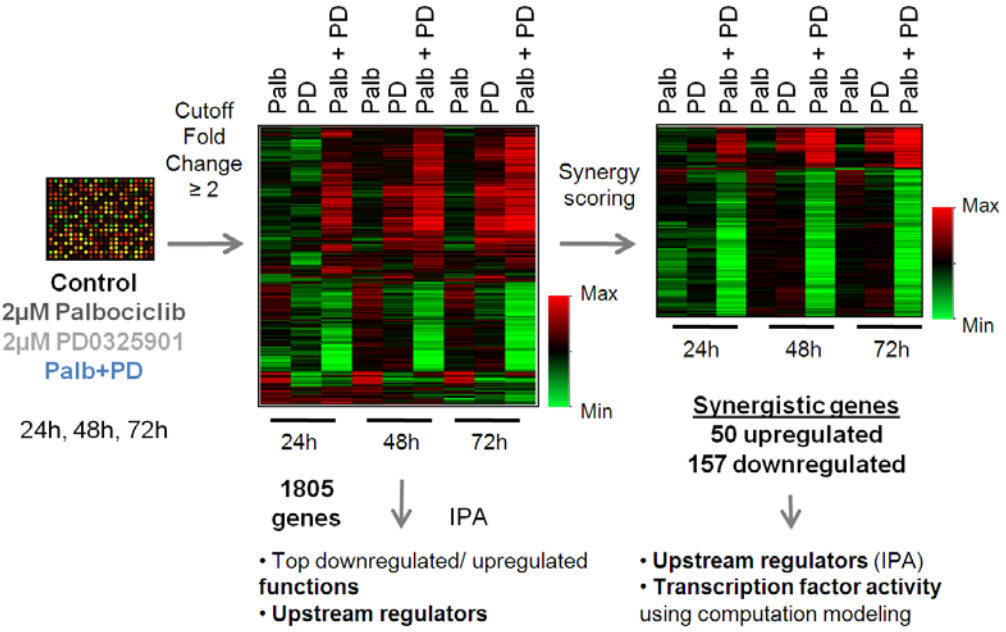


Figure 3.11 Diagram illustrating the analysis of microarray data to identify mediators of the combined inhibition of CDK4/6 and MEK.

Microarray analysis was performed on DLD1-treated with Palbociclib, PD0325901 or combination of both at time points of 24, 48 and 72 hour. IPA analysis was performed with 1805 genes (cutoff of fold change ≥2) and top regulated functions and upstream regulators were predicted. 207 genes synergistically regulated by the combination treatment (in at least 1 time point) were further identified and IPA and computation modeling were used to predict upstream regulators and activity of predicted transcription factor. Through these prediction methods, possible mediators behind the effectiveness of the combined inhibition of CDK4/6 and MAPK pathways can then be identified. Colored scale bars in heatmaps represent $\log_2$ transformed values.

Using Genespring, 1805 genes were found to have a fold change of at least 2 in their expression in one or more treatment groups (Figure 3.11 centre). We observed the expression of some genes were obviously very much more elevated or reduced upon combination treatment and proceeded to use a synergy scoring method previously used by McMurray et al. (McMurray, Sampson et al. 2008) to identify these genes. Since these genes were synergistically regulated by the combination treatment, it is likely that these genes are under regulation of both the CDK4/6 and MEK pathways, rather than just one pathway and the changes in the expression of these synergistic genes is possibly crucial to the effectiveness of the combination treatment. 50 synergistically upregulated and 157 synergistically downregulated genes were found (Figure 3.11 right).

The 2 gene set were then input into Ingenuity Pathway Analysis to predict potential upstream regulator of these genes. In the first gene set of 1805 differentially regulated genes of at least fold change 2, at the 48h and 72h treatment time points, FOXM1, MYC and E2F1 were observed to have incremental decrease in their activation z-score in the combination treated samples as compared to the single treatment samples (Figure 3.12A). Activation z-score is the prediction of the activity of the transcriptional regulators after considering the number of target genes and their expression directional changes in the data set input that correspond to the curated target genes in the IPA database. The second dataset using the expression of the 207 synergistic genes after 72h combination treatment also revealed the E2F1 and FOXM1 as the transcription factors with the most downregulated activation z-score (Figure 3.12B). TBX2 was not considered to be as important as it did not show incremental decrease in predicted activity after combination treatment so it is probable that the changes in its target genes were similar in both single and combination treatment (Figure 3.12A). There was also an increase in activation z-score in tumor suppressors CDKN2A, SMARCB1 and RB1, suggesting that their

target genes might be repressed by the combination treatment (Figure 3.12A). In addition, RB1 and SMARCB1 were also the predicted upstream regulators of genes correlated to KRAS mutation shown in Figure 3.2B, an indication that the combination treatment may be specifically targeting the KRAS dependency gene signature identified. Functions related to proliferation of cancer cells, DNA repair and mitosis were also predicted to be more downregulated in combination treatment and functions relating to apoptosis of cancer cell lines were predicted to be more gulated by the combination treatment (Figure 3.13).

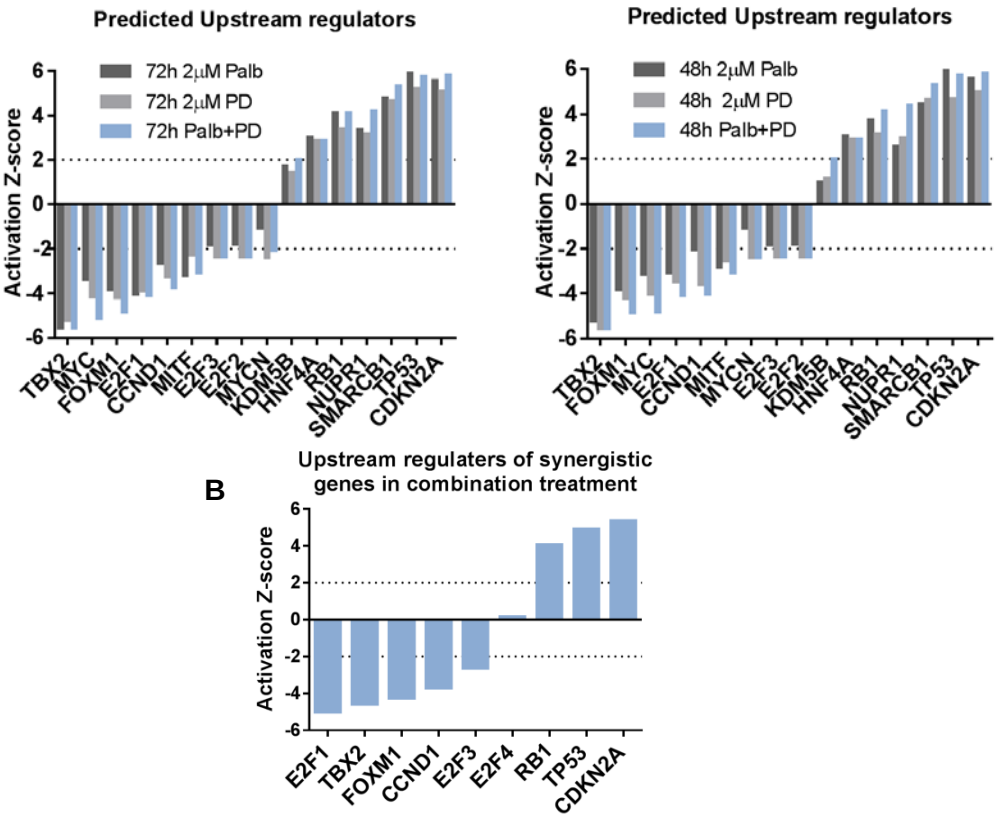


Figure 3.12 Top predicted upstream regulators, including FOXM1, E2F1 and MYC, related to cell cycle and mitosis

A. FOXM1, E2F1 and MYC were observed in top predicted upstream regulators showing greater decrease in activation z-score in combination treatment at 48 and 72h time point in IPA analysis using 1805 differentially regulated genes.
B. Using 207 genes synergistically regulated by combination treatment and their expression at 72h time point under combination treatment, E2F1 and FOXM1 activity were predicted to be greatly decreased.

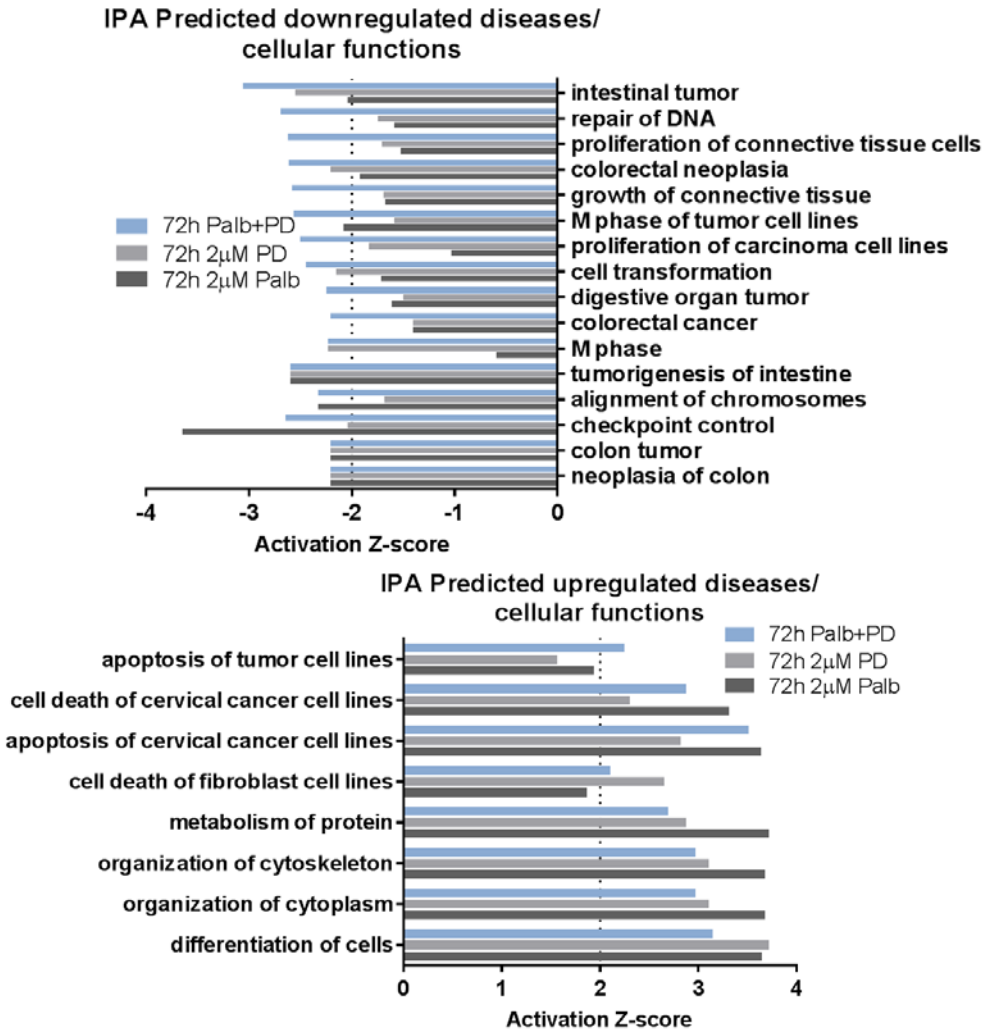


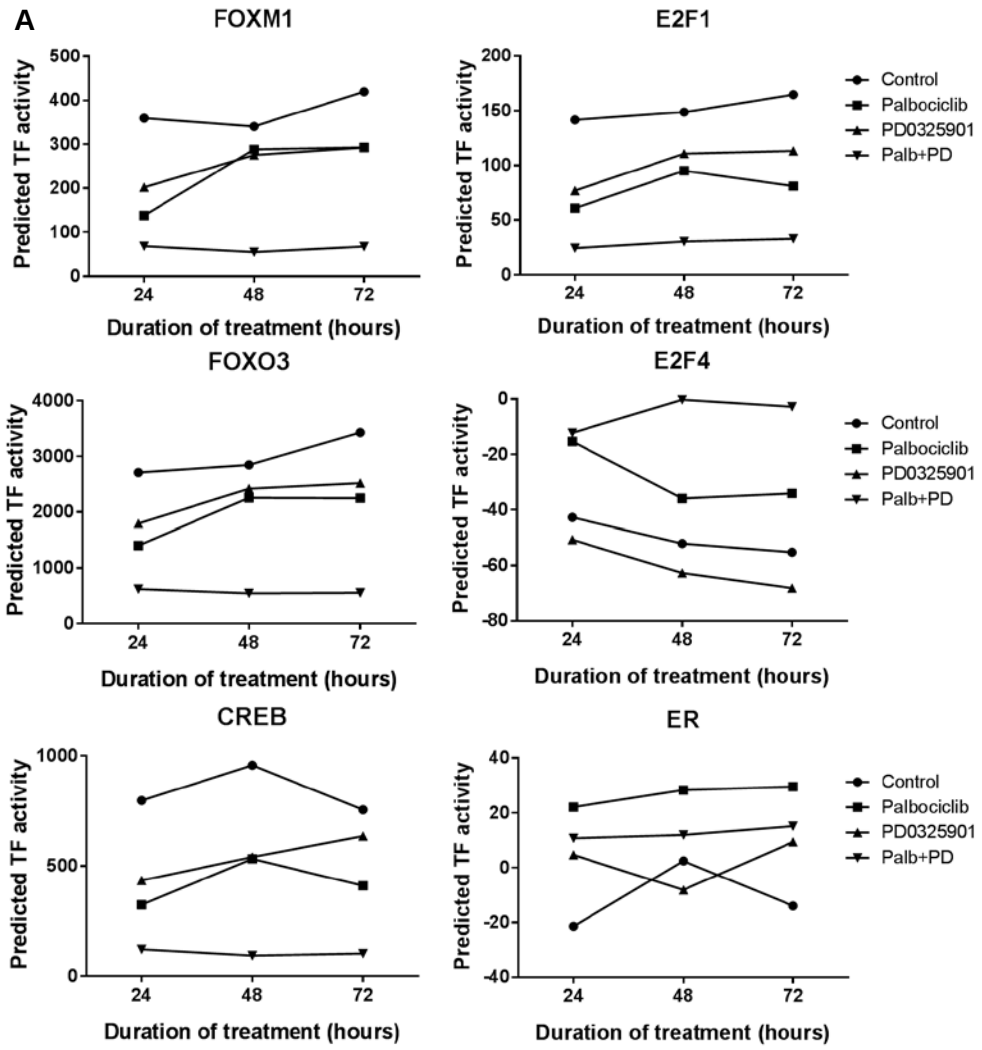
Figure 3.13 Proliferation and cell cycle processes predicted to be downregulated and apoptosis to be upregulated after combined treatment of Palbociclib and PD0325901.

Cellular functions related to proliferation, cell cycle, mitosis and DNA repair were predicted to be more downregulated in the combination treatment of Palbociclib and PD0325901 after 72 hour treatment. Apoptosis of tumor cell lines was predicted to be upregulated in the same treatment conditions in IPA analysis.

Computational modeling was also carried out to simulate the inhibition of CDK4/6 and MEK pathway and using the expression of the 157 synergistically downregulated genes, the mathematical algorithm included the known transcription factors in existing literature and predicted their activity at each time point for each of the treatment group. A simplified network of the prediction modeling is shown in Figure 3.14B where some of the known downstream transcription factors of CDK4/6 and MEK/ERK pathway are shown. Figure 3.14A showed FOXM1 activity was also predicted to be synergistically reduced after combination treatment among other transcription factors as well. All the bioinformatics and computational data together strongly suggest the possible involvement of FOXM1 and E2F1 in the combination treatment.

Figure 3.14 Computational simulation predicts involvement of potential downstream mediators including FOXM1 and E2F1 in combined CDK4/6 and MEK inhibition

A. Activity of transcription factors as predicted by the computational simulation of CDK4/6 and MEK inhibition. FOXM1, E2F1, FOXO3 and CREB showed synergistic reduction of activity under combination inhibition as compared to single inhibition. E2F4 showed synergistic increase in activity under combination inhibition but no synergistic increase or decrease was observed in ER activity. Expression of 157 synergistically downregulated genes together with known downstream transcription factors of CDK4/6 and MEK pathways were used in the prediction.
B. Simplified network of known signaling proteins (pink nodes) in the CDK4/6 and MEK/ERK pathways with some of the predicted transcription factors (blue nodes) and their target genes (yellow nodes) from the 157 synergistically regulated genes. The green and red arrows denote activation and inhibition respectively.



The combined inhibition of CDK4/6 and MEK pathway has shown to be efficacious in KRAS dependent CRC lines on the phenotypic level in terms of inhibiting

proliferation, colony formation and inducing apoptosis. Since this combination treatment strategy was proposed through the identification of the KRAS associated gene signature, we wanted to find out if the genes in the gene signature were downregulated. From the previous microarray performed, 27 out of the 37 genes in the signature were downregulated after the combination treatment (Figure 3.15A) with 12 genes synergistically downregulated (Figure 3.15B). This showed that by inhibiting CDK4/6 and MEK pathway, the upregulated KRAS dependency gene signature, likely crucial for the survival of KRAS-dependent cancer cells, can be abrogated. Another observation is that MEK inhibition alone was able to downregulate these genes to a certain extent and since single inhibition of MEK has proved to be ineffective in clinical settings, it is likely that the residual expression of these genes after MEK inhibition was sufficient to drive the survival of the cancer cells. Only after combination with CDK4/6 inhibition, the expression of these genes was then probably abrogated to a degree that is insufficient for the cancer cells to survive. Validation of the microarray data was performed using qPCR in KRAS dependent DLD1 and HCT116 showing a large decrease in representative KRAS dependency gene signature genes *FOXM1*, *BUB1*, *PBK* and *CDCA7* under combination treatment (Figure 3.15C). Combination treatment in KRAS-independent HCT15, however, did not result in a further decrease in the expression of these genes as compared to single MEK inhibition (Figure 3.15D), mirroring the phenotypic lack of response seen in the earlier figures. These results suggest that the inhibition of both CDK4/6 and MEK pathway is able to downregulate the KRAS dependency gene signature and the change in the gene expression is consistent with the cell line responsiveness to the combination treatment.

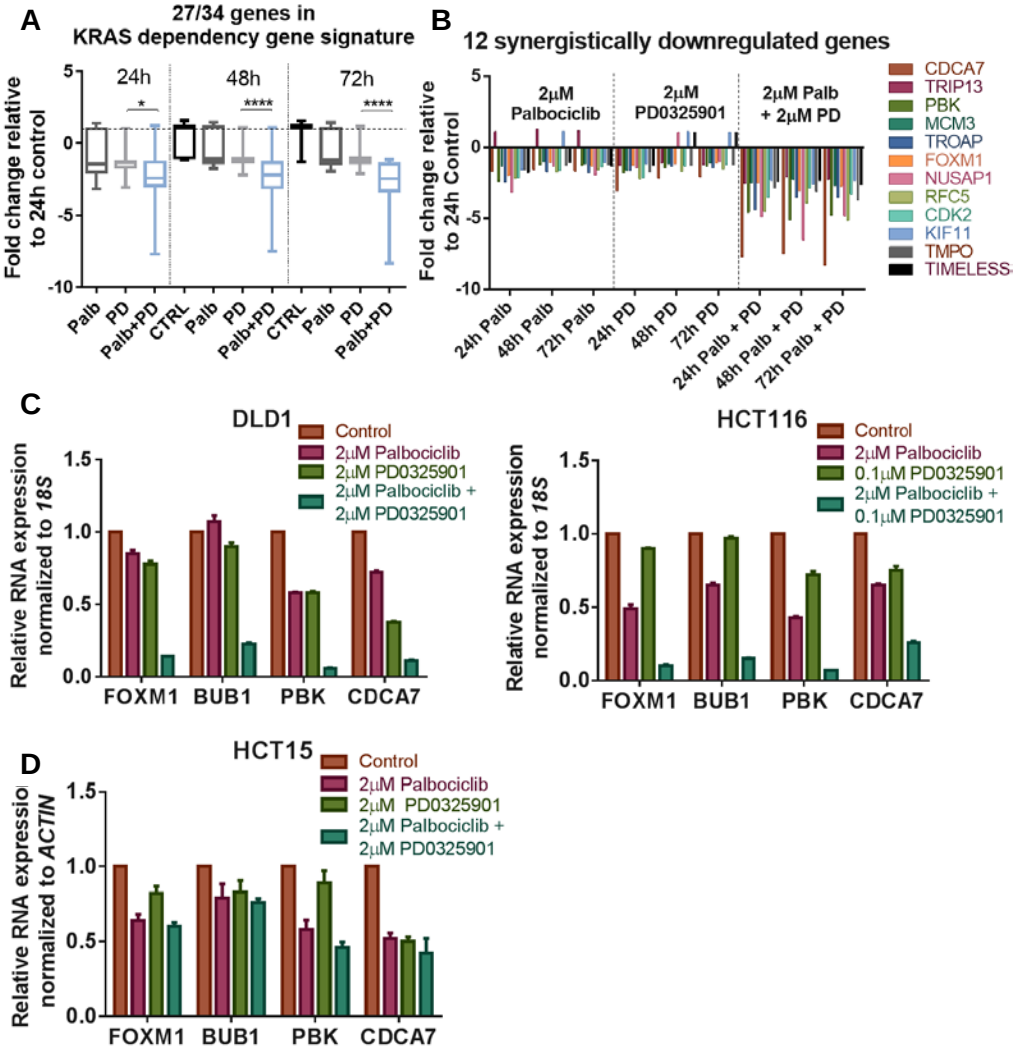


Figure 3.15 KRAS Dependency Gene Signature is effectively downregulated by the inhibition of CDK4/6 and MEK in KRAS dependent CRC.

A. Expression of KRAS dependent genes relative to 24h control in after treatment in DLD1 showed in a box and whiskers plot with the 25th and 75th percentile indicated by the edges of the box with the median shown. Statistical significance was analyzed by paired Student's T test. *P-value <0.05 and ****P-value <0.0001.
B. 12 of the KRAS dependent gene signature synergistically downregulated by combination treatment in at least 1 of the time point. Expression fold change relative to 24h control in DLD1 from microarray data was plotted.
C. qPCR validation of microarray data in KRAS dependent DLD1 and HCT116 on 4 representative genes, showing a robust downregulation by the combination treatment as compared to single treatment.
D. RNA expression of 4 representative genes in KRAS independent HCT15, not showing much difference in expression in combination treatment as compared to single MEK treatment.
All data in the C, D and E represent mean±SEM, n=3.

3.6 Transcription factors crucial for cell cycle and mitosis, including FOXM1, are synergistically downregulated by CDK4/6 and MEK inhibition in KRAS-dependent colorectal cancers

Through the microarray data analysis comparing the combination treatment of CDK4/6 and MEK inhibitors to single treatment with either of the inhibitors, several transcription factors were identified to be possibly involved downstream of these two inhibited pathways, based on the genes that were differentially regulated in at least one of the treated groups as well as the synergistically downregulated genes in the combination treatment group. FOXM1, E2F1 and MYC were predicted through IPA and E2F4, FOXO3 together with FOXM1 and E2F1 were predicted through the computational simulation.

FOXM1 has already been known to be a target of CDK4/6 and phosphorylated ERK, where these kinases phosphorylate FOXM1 at different sites, leading to its activation and nuclear localization where FOXM1 carries out its transcriptional activity (Ma, Tong et al. 2005, Anders, Ke et al. 2011). As for E2F1, it is known to be indirect target of CDK4/6 where CDK4/6 phosphorylates RB and hyperphosphorylated RB releases E2F1 from its inhibitory binding pockets for E2F1 to allow it to activate transcription of its target genes (Choi and Anders 2014). C-MYC, an oncogene frequently amplified in cancer, is known to be phosphorylated and stabilized by p-ERK (Sears, Nuckolls et al. 2000) amidst other regulation from pathways such as PI3K/AKT (Sears, Nuckolls et al. 2000), WNT and TGF-beta (Dang 2012).

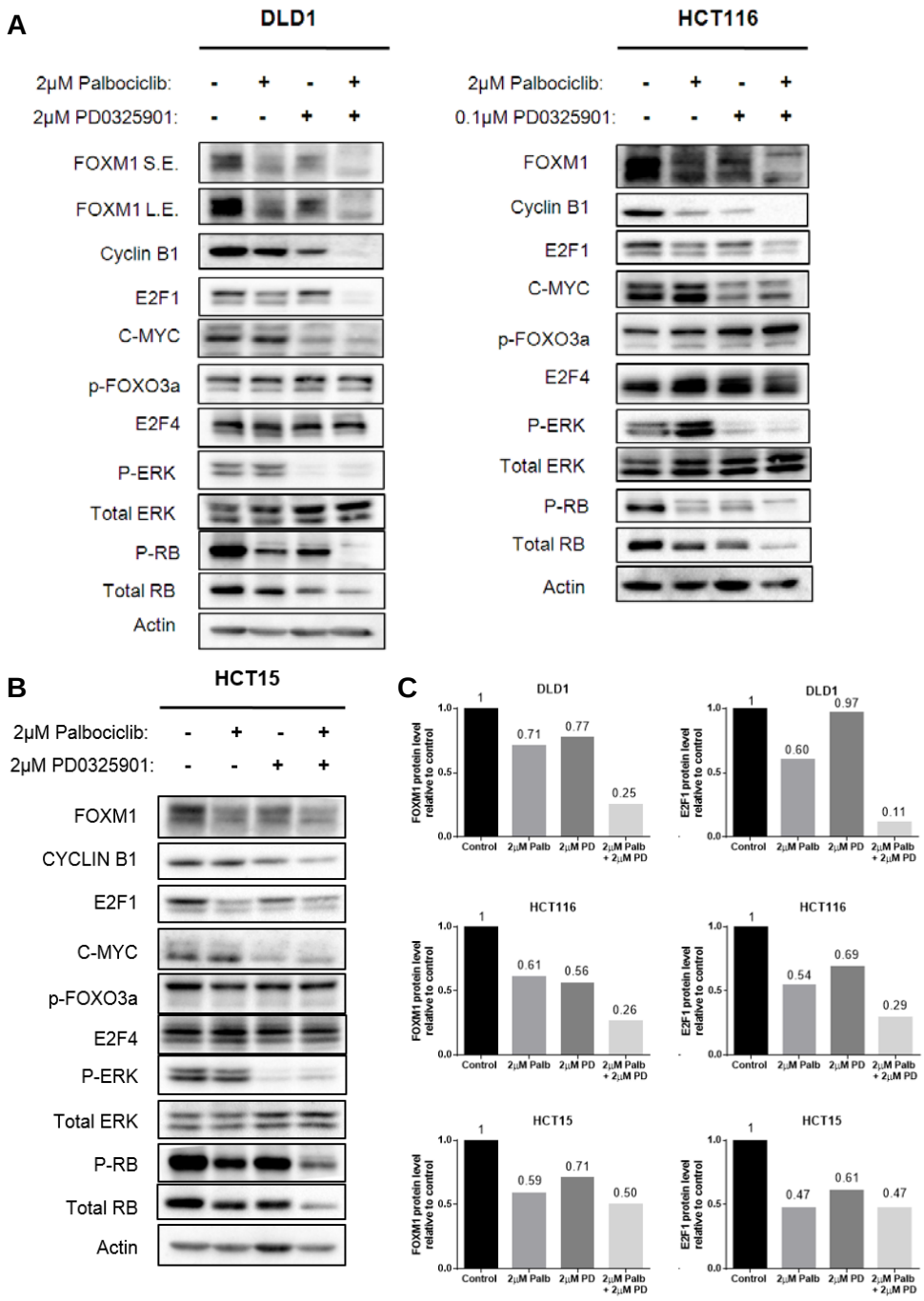


Figure 3.16 CDK4/6 and MEK inhibition synergistically downregulate FOXM1 and E2F1 in KRAS dependent CRC, but not in KRAS independent CRC

A. Western blotting validation of inhibition of CDK4/6 and MEK pathway and also the complete downregulation of FOXM1, E2F1 and C-MYC on the protein level in combination-treated DLD1 and HCT116. (S.E. and L.E. indicate short exposure and long exposure respectively.
B. Western blotting validation of inhibition of CDK4/6 and MEK pathway in KRAS independent HCT15 but without the complete downregulation of FOXM1 and E2F1 as seen in DLD1 and HCT116.
C. Quantification of FOXM1 and E2F1 protein expression relative to each cell line control.

We validated the predicted involvement of these transcription factors by observing their protein expression after treatment with Palbociclib, PD0325901 or with both inhibitors. We treated KRAS dependent DLD1 and HCT116 for 48 hours, harvested the cells and lysed them for protein extraction. The effects of the inhibitors on CDK4/6 and RAS/MAPK pathway were first characterized (Figure 3.16A). Single Palbociclib treatment did not have much effect on the RAS-MAPK pathway as no significant changes were observed in phosphorylated ERK1/2 and C-MYC, unlike single MEK inhibition which was adequate to downregulate ERK1/2 phosphorylation and C-MYC expression. This suggests that C-MYC downregulation is not a critical determinant of the synergistic effect elicited by the combination treatment. Palbociclib or PD0325901 treatment alone led to only modest reduction in phosphorylated RB and E2F1, suggesting that CDK4/6 pathway was only partly inhibited. This may suggest the reason behind ineffective single MEK inhibition may be due to remnant CDK4/6 pathway activity and notably, by treating with both CDK4/6 and MEK inhibitors, phosphorylated RB and E2F1 expression were fully abrogated.

Remarkably, FOMX1 and E2F1 which were predicted to be downregulated, showed synergistic downregulation in the combination treatment as compared to single treatment (Figure 3.16A, C) and FOXM1 downstream target, cyclin B1 also showed the same downregulation trend in both DLD1 and HCT116. For the other transcription factors predicted, E2F4 and phosphorylated FOXO3, their expression did not show any significant changes after combination treatment.

Next, we repeated the same treatment for KRAS independent HCT15, which has been shown to be not responsive to the combined inhibition of CDK4/6 and MEK. If CDK4/6 pathway and FOXM1 downregulation is critical for the effective of the combination treatment, in the less responsive HCT15, the pathway and FOXM1 should still be present after combination treatment. Indeed, the expression of

phosphorylated RB, FOXM1 and FOXM1 target cyclin B1 remained after combination treatment (Figure 3.16B, C). Single MEK inhibition in HCT15 led to only a very slight reduction in the expression of the components of the CDK4/6 pathway (phosphorylated RB and cyclin B1) as compared to DLD1 and HCT116. This suggests that in the KRAS dependent CRC, the RAS-MAPK pathway may have a greater role in activating the CDK4/6 pathway whereas in the KRAS independent CRC, CDK4/6 pathway may be more strongly driven by another mitogenic pathway. This lent greater weight of the role of CDK4/6 pathway in KRAS dependent CRC and the rationale behind using CDK4/6 and MEK inhibitors in combination

Since BRAF-mutant CRC also display activated MAPK pathway and are considered to be dependent on the MAPK pathway, we also treated two BRAF mutant CRC lines RKO and HT-29 with single or combined treatment of Palbociclib and PD0325901 for 48 hours. A similar abrogation of FOXM1 and CDK4/6 downstream targets were also observed when compared to the KRAS dependent CRC lines (Figure 3.17). From all these data, it strongly suggests that the complete downregulation of CDK4/6 pathway and its downstream signaling is crucial to inhibit proliferation in KRAS dependent and BRAF mutant CRC.

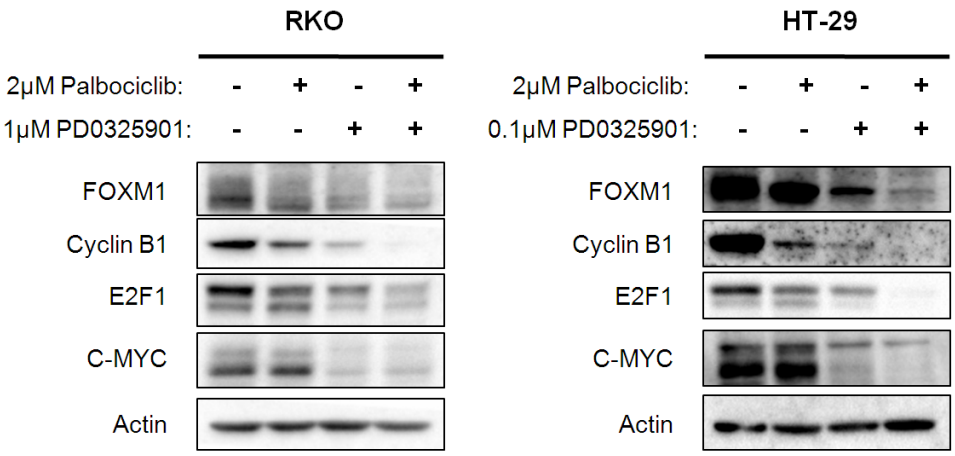


Figure 3.17 Inhibition of CDK4/6 and MEK in BRAF mutant CRC downregulates FOXM1, Cyclin B1, E2F1 and C-MYC.

Western blotting showing the complete downregulation of FOXM1 and its target gene, CyclinB1 and also the downregulation of E2F1 and C-MYC in BRAF mutant RKO and HT-29.

3.7 Combination treatment of CDK4/6 and MEK pathways leads to longer-lasting repression of CDK4/6 pathway and FOXM1 expression as compared to single inhibitor treatment

To explore the effect of Palbociclib and PD0325901 on CDK4/6 and MAPK pathways, we treated DLD1 and HCT116 with the two inhibitors individually and in combination and harvested the cells at 2, 4, 8, 24 and 48 hours for DLD1 and 4, 8, 24 and 48 hours for HCT116. An observation that we first made was that single treatment of Palbociclib was able to downregulate the phosphorylation of RB, E2F1 and FOXM1 over time up to 24 hours but at the 48 hour, there was a re-expression of phosphorylated RB, E2F1 and FOXM1 in DLD1 (Figure 3.18A). In HCT116, single treatment with CDK4/6 inhibitor was insufficient to completely downregulate phosphorylated RB and E2F1 at all time points (Figure 3.18B). For the MEK inhibitor treated cells, expression of phosphorylated RB and E2F1 was reduced but not completely abrogated in both DLD1 and HCT116. In the combination treated cells, however, phosphorylated RB, E2F1, FOXM1 and Cyclin B1, a target of FOXM1, remained downregulated after 48 hours. In HCT116, though FOXM1 seemed to be completely abrogated with single CDK4/6 inhibition, it was only in the combination treatment that Cyclin B1 expression was completely eradicated. This suggests that the combined treatment of Palbociclib and PD0325901 is effective in repressing or even shutting down the CDK4/6 and MAPK pathways for a longer duration as compared to single inhibitor treatment and this can prevent the remnant signals that may be sufficient to continue to drive survival and growth in KRAS dependent CRC.

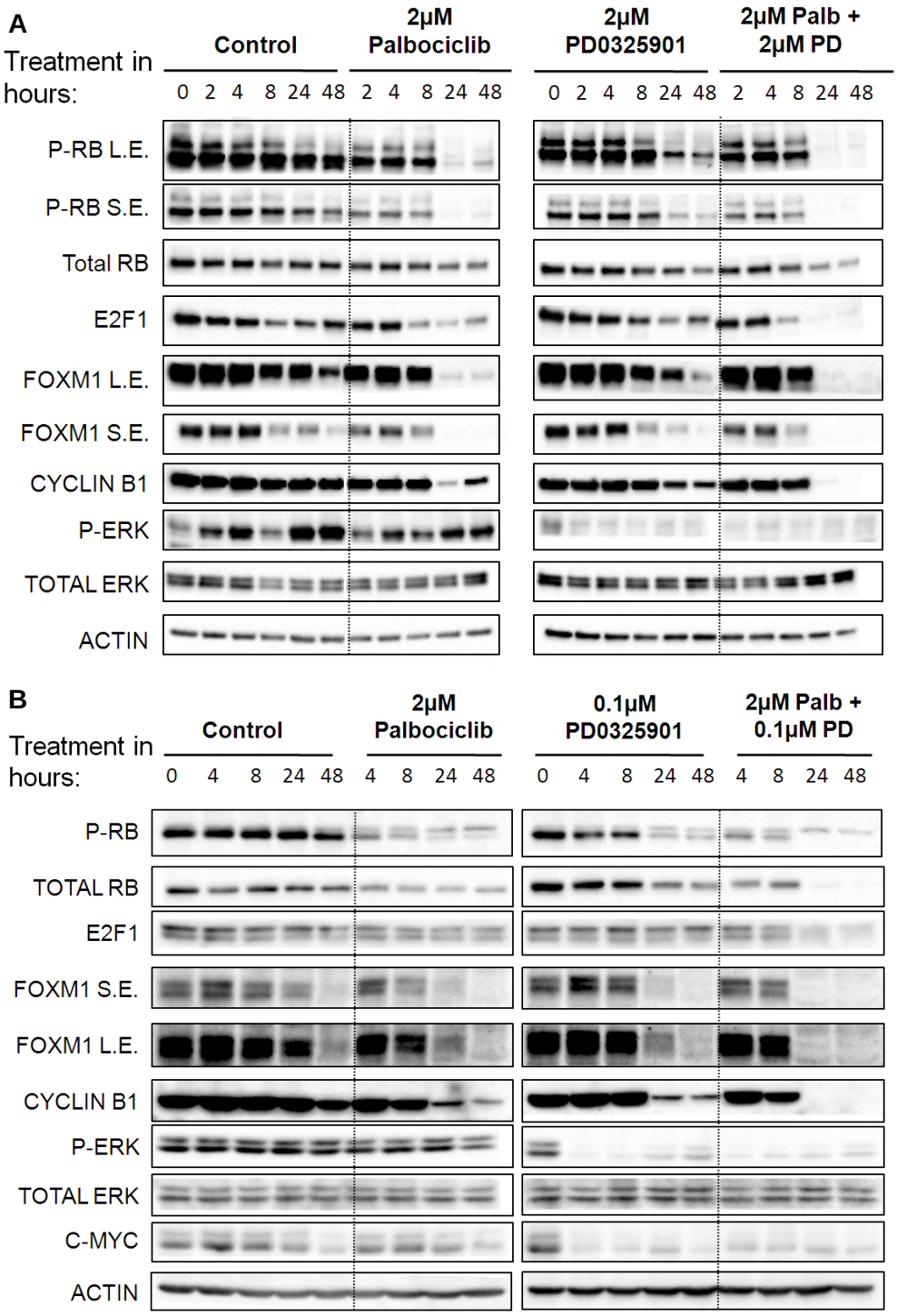


Figure 3.18 Combined inhibitions of CDK4/6 and MEK repressed the activity of CDK4/6 pathway and FOXM1 for a longer duration in KRAS dependent CRC

A. Western blotting showed longer suppression of the CDK4/6 pathway in the combination treated DLD1 as compared to single inhibitor treatment.
B. Western blotting in HCT116 showed similar longer suppression of CDK4/6 pathway and FOXM1 in combination treatment

3.8 KRAS dependent and independent colorectal cell lines showed differential sensitivity towards FOXM1 depletion

Earlier, we showed that upon inhibition of both CDK4/6 and MEK, FOXM1 and E2F1 were observed to be synergistically downregulated as compared to the single treatment in both KRAS-dependent DLD1 and HCT116 but in KRAS independent HCT15, and that the responsiveness of the cell lines to the combination treatment correlate with KRAS dependency. Thus we wanted to investigate if FOXM1 or E2F1 were important to the cell viability. Short interfering RNA was used to knockdown FOXM1 and E2F1. For E2F1 knockdown, no effect was observed in cell viability (Figure 3.19A). This could be attributed the functional redundancy in the E2F family. Triple knockdown of E2F1, E2F2 and E2F3 were therefore carried out as they function as transcriptional activators but cell viability were still not affected (Figure 3.19A). This could implies that the reduction of E2F1 seen in the combination treated cells was either an effect of the general reduction in growth or that there are other transcription factors that are able to replace the function of E2Fs when E2F1, E2F2 and E2F3 are knocked down. E2F family regulates many genes that are involved in cell growth, mitosis and survival but often these genes are tightly regulated by many other transcription factors and so it is likely upon only E2F knockdown, the cell viability was not affected. However, the role of E2Fs in the combined inhibition of CDK4/6 and MEK should not be rule out because there are a few crucial transcription factors that are downregulated upon the combination treatment and so it is still possible the functions of E2Fs are crucial in the absence of the other transcription factors.

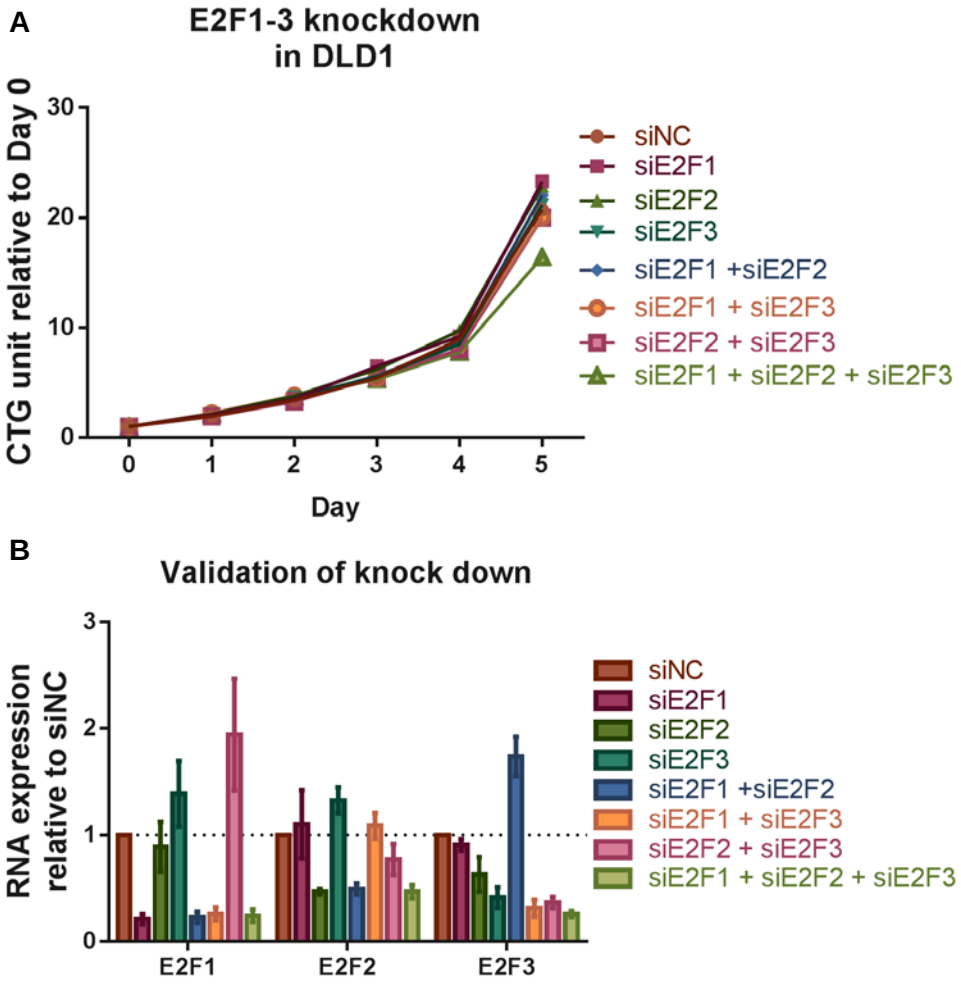


Figure 3.19 Knockdown of E2F1-3 did not affect cell viability

A. Short interfering RNA sequences (30nM) were used to specifically knockdown E2F1, E2F2 and E2F3 in DLD1. Cell viability was measured with CellTiter-Glo.
B. RT-qPCR validation of knockdown efficiency
All data in the graphs represent mean±SEM, n=3.

For FOXM1 knockdown, reduction in cell proliferation (Figure 3.20B) and colony formation in soft agar (Figure 3.20C) were observed in both KRAS dependent DLD1 and HCT116 but not in KRAS independent HCT15 (Figure 3.20A). It is also interesting to note that sensitivity to FOXM1 correlate with KRAS dependence (Figure 3.20C), suggesting that FOXM1 could be specifically important for the viability of KRAS dependent cancer cells.

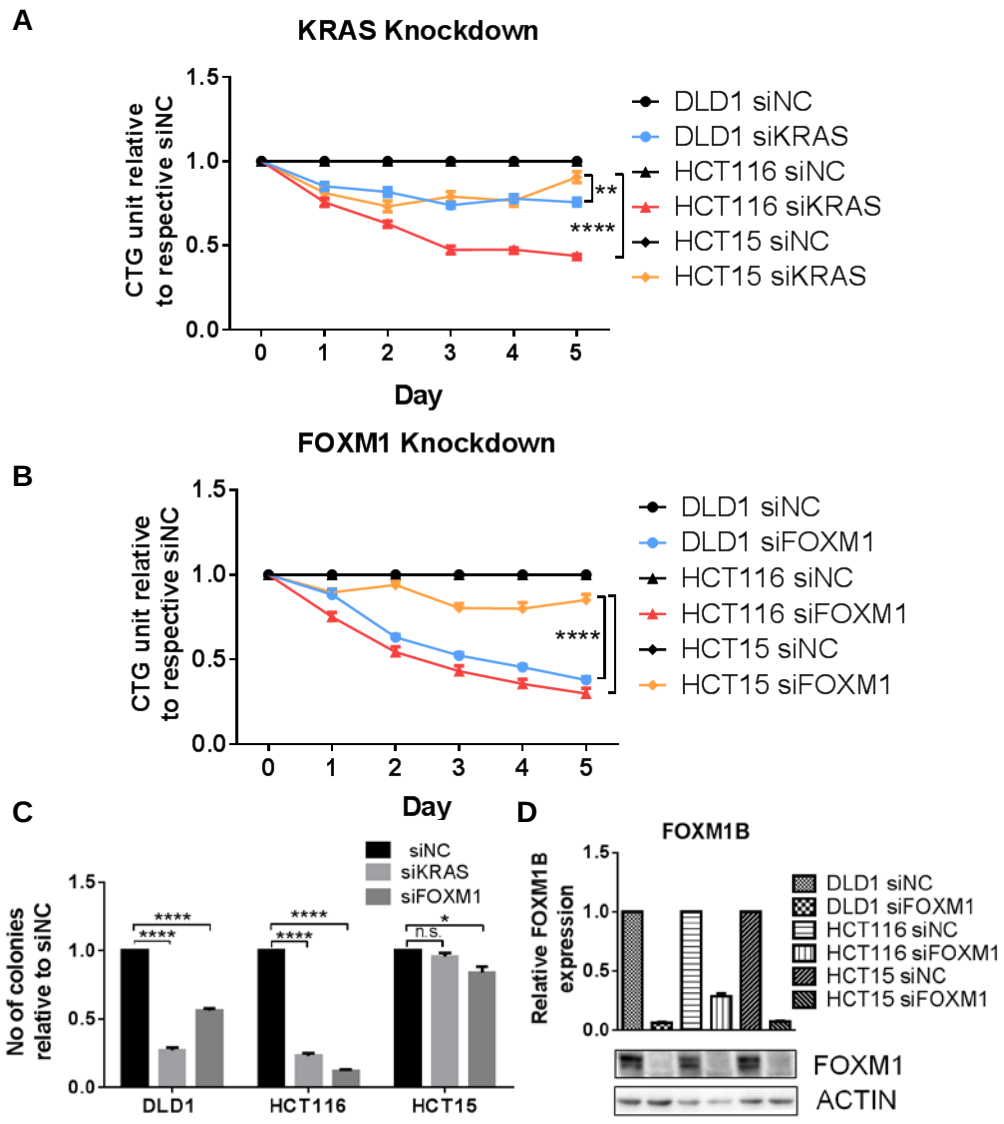


Figure 3.20 KRAS dependent CRC displayed sensitivity to FOXM1 knockdown

A. Knockdown of KRAS in KRAS dependent DLD1 and HCT116 led to a more significant reduction in cell viability as compared to KRAS knockdown in KRAS independent HCT15.
B. Knockdown of FOXM1 in KRAS dependent DLD1 and HCT116 led to a more significant reduction in cell viability as compared to FOXM1 knockdown in KRAS independent HCT15.
C. Knockdown of either KRAS or FOXM1 significantly reduced colony formation in anchorage independent conditions in KRAS dependent CRC DLD1 and HCT116 but not in KRAS independent HCT15. Unpaired Student's T-test was used for statistical analysis. *P-value<0.05; **P-value<0.01; ****P-value<0.0001.
D. Validation of FOXM1 knockdown at RNA and protein expression level.
All data in A-D graphs represent mean±SEM, n=3.

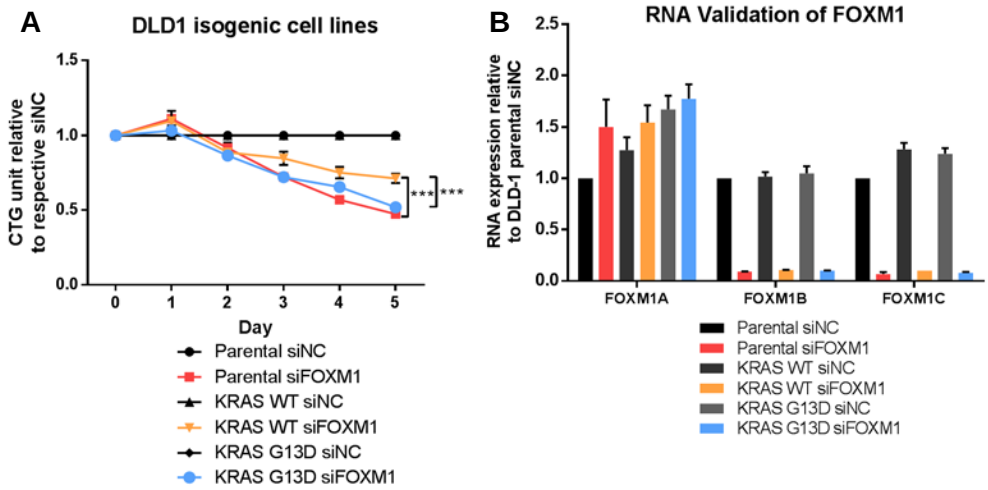


Figure 3.21 KRAS mutant DLD1 displayed greater sensitivity to FOXM1 knockdown as compared to KRAS wildtype DLD1

A. Growth of DLD1 KRAS isogenic cell lines after knockdown of FOXM1 with siFOXM1 (30nM) relative to siNC knockdown. Unpaired Student's T test was used. ***P-values<0.001.
B. qPCR validation of FOXM1 knockdown.
All data in the graphs represent mean±SEM, n=3.

We also performed FOXM1 knockdown in DLD1 KRAS isogenic cell lines, measured their proliferation and compared their growth to their respective negative control knockdown. We observed that the DLD1 parental and KRAS mutant lines displayed a significantly greater reduction in proliferation as compared to DLD1 KRAS wild type line upon FOXM1 knockdown (Figure 3.21A). Here, we once again showed that CRC lines with KRAS mutation are more sensitive to FOXM1 knockdown, suggesting FOXM1 is more important for the viability of KRAS mutant CRC than KRAS wild type CRC.

3.9 FOXM1 depletion cooperates with MEK inhibition to reduce cell viability and KRAS dependency gene signature

Since FOXM1 was downregulated in the Palbociclib and PD0325901 treated KRAS dependent cells and its expression important for the cell viability specifically in the KRAS dependent cells, we wanted to determine if knockdown of FOXM1 was able to cooperate with MEK inhibition. We first knocked down FOXM1 using short interfering RNA against FOXM1 and then after reseeding the cells, treated them with PD0325901. We found the combination of FOXM1 knockdown together with MEK inhibition led to a further decrease in proliferation and number of colonies formed in the anchorage independent soft agar specifically for KRAS dependent DLD1 and HCT116 (Figure 3.22 A, B). In KRAS independent HCT15, in 2D monolayer growth condition, the combination of FOXM1 knockdown and MEK inhibitor did lead to a significant reduction in growth when compared to FOXM1 knocked down cells (Figure 3.23A). However, in anchorage independent soft agar growth condition which is more representative of the *in vivo* tumor growth conditions, knockdown of FOXM1 with MEK inhibition did not further reduce the number of colonies formed when compared to MEK inhibitor treatment only (Figure 3.23B). The concentration of PD0325901 used in these assay were the EC50 values of their respective cell lines, 518nM for DLD1 and 126nM for HCT116. Lower concentrations of PD0325901 were used for soft agar assays as cells were generally more sensitive when growing in this condition. However, for HCT15, a higher concentration of 1μM was used to ensure that the difference observed between KRAS dependent and independent lines was not due to the ineffective inhibition of MEK. Here, we showed that specifically in KRAS dependent CRC, MEK inhibition with FOXM1 depletion led to greater reduction in growth and cell viability.

We also checked the representative genes from KRAS dependency gene signature upon the knockdown of FOXM1 and MEK inhibition and found that only in the

KRAS dependent cell lines, a cooperative decrease of these genes were observed (Figure 3.22C). In KRAS independent HCT15, even an increase of the RNA expression of *CDCA7*, *BUB1* and *PBK* was seen when comparing the FOXM1 knocked down and MEK inhibitor-treated sample to MEK inhibitor-treated only sample (Figure 3.23C). Even at the gene expression level, there was a distinct difference between KRAS dependent and independent CRC upon FOXM1 knockdown and MEK inhibition and this corresponds to the susceptibility of these lines to the combination treatment. This further suggests the potential role that FOXM1 has in the combined inhibition CDK4/6 and MAPK pathways.

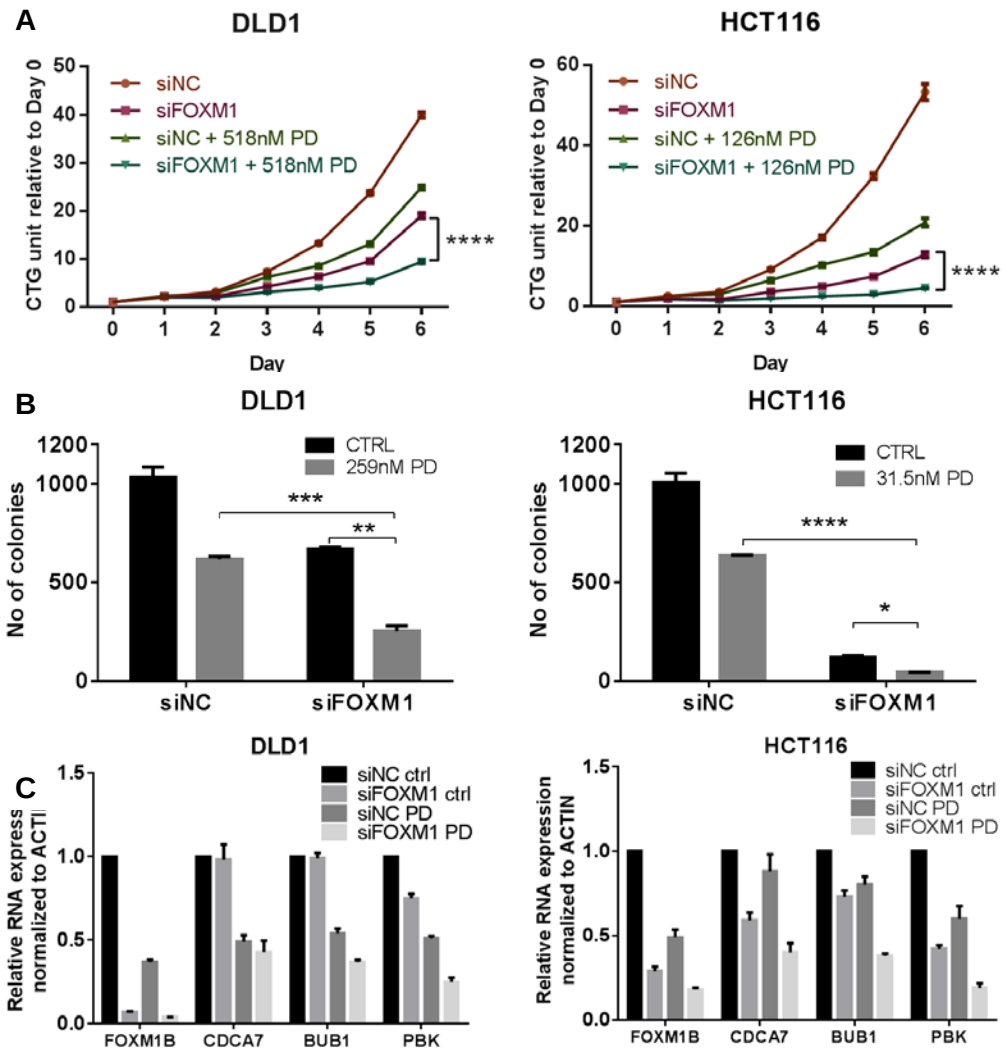


Figure 3.22 FOXM1 knockdown with MEK inhibition led to greater reduction in cell viability and expression of genes in the KRAS dependent gene signature in KRAS dependent CRC

A. An increase reduction in cell viability in MEK inhibitor treated FOXM1 knocked down cells in both KRAS dependent DLD1 and HCT116. EC_{50} of PD0325901 were determined for each of the cell lines and used for this assay.
B. Significant reduction in the number of colony formed in anchorage independent soft agar after FOXM1 knockdown and MEK inhibition treatment. Concentration of PD0325901 used was reduced to better observe the combined effect of FOXM1 knockdown and MEK inhibition. Student's T test was used. *, **,*** and ****P-values<0.05, 0.01,0.001 and 0.0001 respectively.
C. Total RNA were extracted after knockdown of FOXM1 for 72h and concurrent MEK inhibitor treatment in the last 24h. Representative genes from the KRAS dependency gene signature showed greatest downregulation after FOXM1 knockdown and MEK inhibition. Concentration of PD0325901 used was 512nM and 126nM for DLD1 and HCT116 respectively

All data in the graphs represent mean±SEM, n=3.

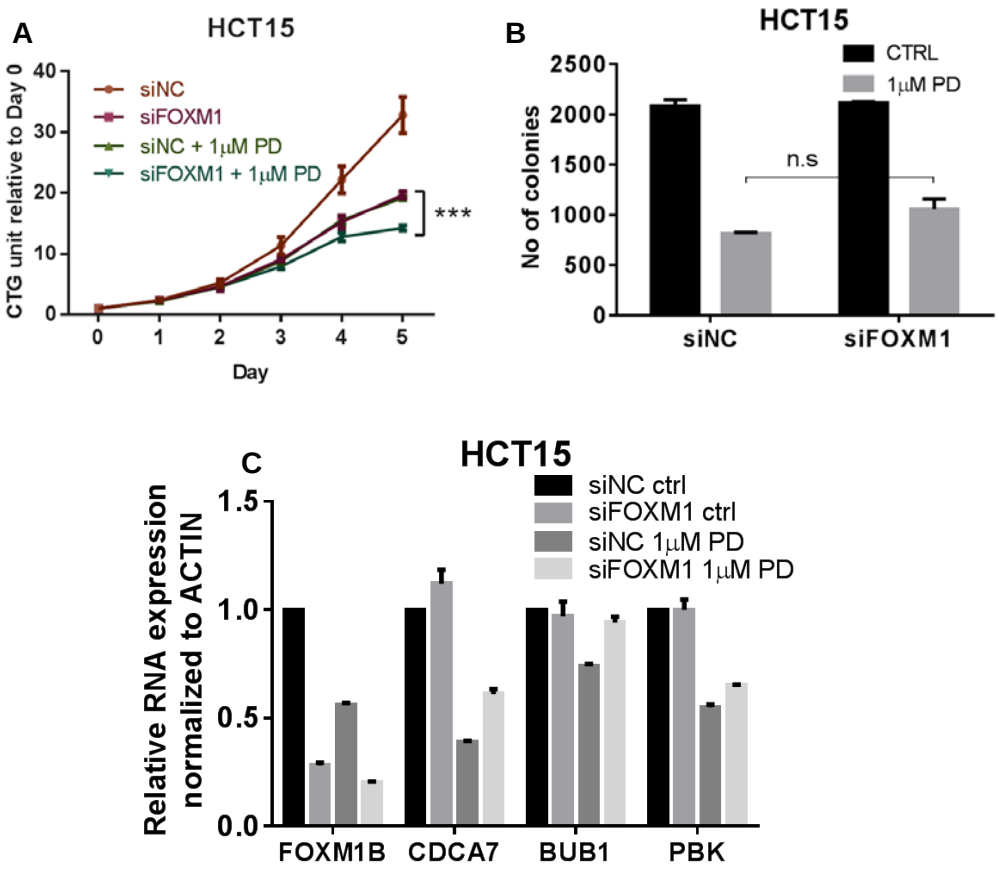


Figure 3.23 No significant reduction in colony formation and expression of KRAS dependent gene signature upon FOXM1 knockdown with MEK inhibition in KRAS independent CRC

A. An increase reduction in cell viability in MEK inhibitor treated FOXM1 knocked down HCT15 (KRAS independent) in 2D monolayer growth condition. 1μM PD0325901 was used. Paired Student's T-test was used. ***P-value<0.001.
B. No significant cooperative reduction in colony formation seen in FOXM1 knocked down and MEK inhibitor treated HCT15 in anchorage independent soft agar. Unpaired Student's T-test was used. N.s., not significant
C. Total RNA were extracted after knockdown of FOXM1 for 72h and concurrent MEK inhibitor treatment in the last 24h. Representative genes from the KRAS dependency gene signature showed no reduction in FOXM1 knocked down cells with MEK inhibition when compared to MEK inhibitor treated cells.
All data in the graphs represent mean±SEM, n=3.

3.10 Combined treatment of CDK4/6 and MEK inhibitors in patients derived colorectal cancer lines

So far we have been testing the combination treatment of Palbociclib and PD0325901 in cell line models and these lines have been propagated in culture for many decades and may not be the best model to represent patients' colorectal cancer cells. To this purpose, we obtained fresh primary colon tumors from patients after surgery, mechanically and enzymatically broke down the tissue to obtain the cells from the bulk tumor. These cells were then grown in serum-free growth medium supporting the growth of tumor spheres when grown in a low attachment conditions. This was done in order to eliminate the other cell types such as fibroblasts which are also found in the bulk tumor. If grown on plastic and supplemented with growth medium containing serum, the culture will be a mixture of fibroblasts and cancer cells and since cancer cells have the capability to grow in an anchorage-independent and serum-free condition, they can be selected for by using this culturing method. This method also enriched for cancer stem cells thus we can use this system to observe the effect of combination treatment on cancer stem cells.

We successfully established 3 patient derived sphere lines namely 14S, 29S and 47S and they can be passaged up to at least 25 times. We further characterized their KRAS mutation at G12 and G13 codon and BRAF mutation at V600 using pyrosequencing and found 14S harboring a heterozygous KRAS G13D mutation, 29S having a homozygous KRAS G12 and 47S having no KRAS mutation (Table 3.2). BRAF-V600 mutation was not detected in any of the lines. As with all sequencing techniques, there is a possibility of false positive detection so we have previously tested on a range of CRC cell lines with known mutation status to obtain a false positive rate of 5-10% for this method of mutation detection. For 14S, it is not immediately conclusive that it is a KRAS heterozygous mutant as mutation detection from 40-60% can either mean 50% of the population having a wildtype status and the

other 50% having a homozygous KRAS mutation. To be sure, we needed to derive a few separate lines from single cell and check their mutation status. However, sphere cells when grown in single cells or under low seeding density did not grow well to form spheres. To overcome this problem, we derived adherent lines from the patient derived spheres lines 14S and 47S, and named them 14SA and 47SA respectively. For 14SA, we further made subclones where we seeded the parental 14SA line in very low density, such that single cells were separated and clones arising from single cells were clearly separated. Clones were carefully trypsinized to avoid cross contamination between clones and removed and placed into a 24-well plate for expansion. Upon obtaining sufficient cells, pyrosequencing was carried out and we found all the clones, including the parental 14SA, harboring about 50% of KRAS mutation. From this result, we can safely concluded that 14S is a heterozygous KRAS mutant line.

	Codon G12 (GGT)				Codon G13 (GGC)				
Patient tumor-derived lines	**Wildtype** (GGT)	**G12D** (GAT)	**G12A** (GCT)	**G12V** (GTT)	**Wildtype** (GGC)	**G13D** (GAC)	**G13A** (GCC)	**G13V** (GTC)	**KRAS mutation status**
14S	**64**	**32**	1	3	**81**	**15**	0	4	**Heterozygous G12 mutant**
29S	6	1	**93**	0	**97**	1	1	2	**G12 mutant**
47S	**93**	2	1	5	**93**	1	0	6	**Wildtype**
14SA	**63**	**35**	1	2	**86**	3	0	**11**	**Heterozygous G12 mutant**

Table 3.2 Summary of KRAS mutation status of patient tumor derived lines determined via pyrosequencing.

Homozygous KRAS mutant and wild type were determined solely through pyrosequencing. Single clones from 14SA were expanded and sequenced together with parental 14SA for the determination of parental 14SA heterozygous G12 mutant status.

We went on to further characterize their RNA expression prolife where we examined the expression of the genes from the KRAS dependency gene signature such as *FOXM1B*, *FOXM1C*, *CDCA7*, *BUB1* and *PBK* and also *CDC20* and *CCNB2* which are known FOXM1 target genes and found 47S to have the highest levels of FOXM1 (Figure 3.24). It might seem contradictory to our prior finding that KRAS mutant cancer cells were likely to express higher levels of the KRAS dependency gene signature but upon closer inspection of the gene expression profile of all the three groups of tumors in Figure 3.1B, there were some KRAS wildtype tumors which possess similar elevated levels of the KRAS dependency gene signature. 47S also exhibited relatively higher level of phosphorylated ERK as compared to 14S and 29S (Figure 3.24), indicating that the RAS/MEK/ERK pathway was highly activated in the cells despite having wild type KRAS. Higher MAPK activity suggests greater dependence on this pathway. Thus, this suggests the KRAS dependency gene signature can better predict the activity of RAS/MEK/ERK pathway, and hence may more accurately predicts KRAS dependency in cancers.

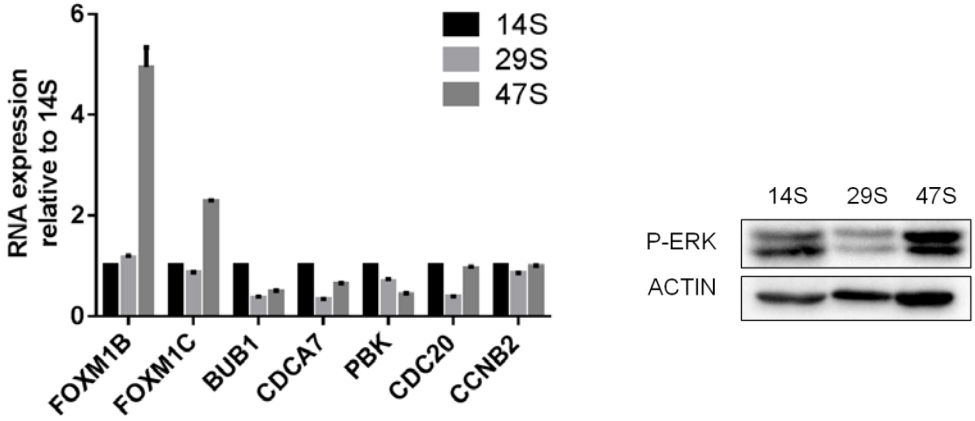


Figure 3.24 Expression of genes from KRAS dependency gene signature in patient tumor-derived sphere lines.

(Left) Expression of representative genes from KRAS dependency gene signature as well as FOXM1 target genes *CDC20* and *CCNB2*. Normalization was done with 18S and expression from 29S and 47S was plotted relative to 14S. (Right) Protein expression of phosphorylated ERK in the patient tumor derived sphere lines.

All data in bar graph represent mean±SEM, n=3.

We then proceeded to test and treat 3 patient tumor-derived sphere lines with Palbociclib, PD0325901 and the combination of both and measured their cell viability at day 7 and 14 after drug treatment. Concentration of inhibitors used was optimized such that single inhibitor treatment only led to about 50% reduction in viability. The growth of all three lines was significantly more inhibited by the combination treatment as compared to the single treatment (Figure 3.25A). If the status of KRAS mutation was used to predict the response to the combination treatment, we would expect 47S to not respond to the treatment. However, we observed that 47S, together with 14S and 29S showed significant further reduction upon treatment with both CDK4/6 and MEK inhibitors as compared to single treatment. This observation in 47S better correlated to its level of KRAS dependency gene signature, especially the level of FOXM1 in 47S than to its KRAS wild type status.

We also further treated of 29S and 47S with the same drug concentration used in 14S. In figure 3.25A, the growth of the spheres were not completely abrogated but with higher concentration of MEK inhibitor used, the growth of the sphere lines could be further reduced (Figure 3.25B). Notably, the concentrations of the inhibitors used here are in nanomolar range, and so highlights the efficacy of inhibition of the CDK4/6 and MEK in treating patient CRC–derived spheres, especially those with high level of KRAS dependency gene signature expressed such as 47S.

We did not have more lines to validate our findings but our preliminary findings supported our earlier stand that KRAS mutation might not be the best biomarker to predict the response to combination treatment. Instead, the KRAS dependency gene signature may be a more accurate method to determine the level of KRAS dependency of the tumor and thus be able to better predict the tumor response to the combination treatment. More lines need to be generated and tested in order to obtain a more conclusive and significant result on the KRAS dependency gene signature being a better biomarker.

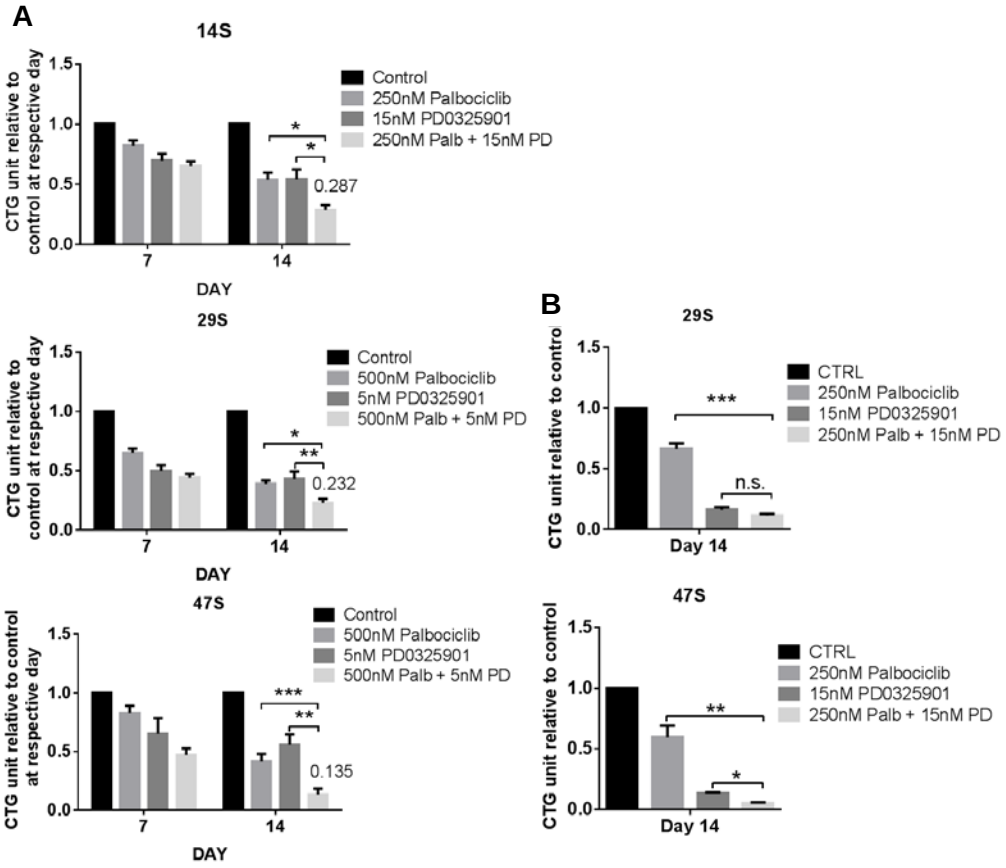


Figure 3.25 Combined inhibition of CDK4/6 and MEK significantly reduces viability in patient tumor-derived spheres.

A. Cell viability of patient CRC tumor derived sphere lines 14S, 29S and 47S were measured after 7 and 14 days of treatment with top up of fresh media and drugs at day 7.
B. Cell viability of patient CRC tumor derived sphere lines 29S and 47S were measured after 14 days of treatment with top up of fresh media and drugs at day 7 with similar concentration of drugs used for 14S. Statistical analysis was done with paired Student's T test. *p<0.05, **p<0.01, ***p<0.001. n.s.= not significant.

All data in the graphs represent mean±SEM, n=3.

Next, using the 14SA line, we treated the cells with Palbociclib and PD0325901 alone or in combination for 48 hours and harvested the cells for RNA to examine the expression of the respective genes of the KRAS dependency gene signature. We found that 14SA responds similarly to the KRAS dependent DLD1 and HCT116 where the combination treatment was able to significantly reduce the expression of the respective genes to a greater degree as compared to single treatment (Figure 3.26).

Once again, using a model derived from patient tumor, we showed the combined inhibition of CDK4/6 and MAPK pathway was able to target and downregulate the KRAS dependency genes.

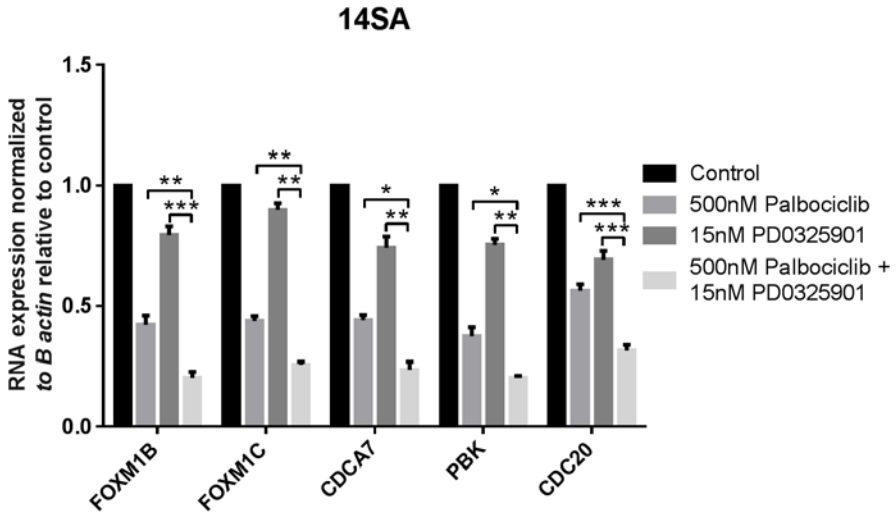


Figure 3.26 Combination treatment of CDK4/6 and MEK inhibitors in patient derived CRC line downregulates expression of genes in the KRAS dependency gene signature

14SA (adherent line derived from 14S) was treated for 48 hours with CDK4/6 and MEK inhibitors at the indicated concentrations and total RNA was extracted. RT-qPCR was then carried out on selected genes from KRAS dependency gene signature. Statistical analysis was performed with paired Student's T test. *P<0.05, **P<0.01, ***P<0.001. All data in the graph represent mean±SEM, n=3.

3.11 Therapeutic effect of combined CDK4/6 and MEK inhibitors treatment *in vivo*

Up to this point, all the experiments involving the testing of the efficacy of the combined inhibition of CDK4/6 and MAPK pathways had been done in vitro. For the better understanding on how the tumor would respond to the combination treatment of Palbociclib and PD0325901, we used xenograft models of KRAS dependent DLD1 and BRAF mutant RKO where the cells were injected into the flanks of mice. Upon tumor formation, the mice were treated with either vehicle, Palbociclib, PD0325901 or a combination of both for 14 or 17 days for DLD1 and RKO respectively. Combination treatment led to a greater reduction in tumor growth in both xenografts as compared to single treatment (Figure 3.27A, B). In addition, the same downregulation in the protein expression of phosphorylated RB, E2F1, FOXM1 and FOXM1 target cyclin B1 were observed to a greater extent in the combination treatment (Figure 3.28A, B). Importantly, there was no overt toxicity seen in the combination treated mice, as there was no more than 10% loss in weight when compared to the initial weight of the mice at the start of the experiment (Figure 3.27C). All in all, through the *in vivo* animal study, we showed the combined inhibition of CDK4/6 and MAPK pathway using Palbociclib and PD0325901 to be effective *in vivo* and yet does not cause overt toxicity to the mice. This, together with the *in vitro* data, supports the use of Palbociclib and PD0325901 in KRAS dependent or BRAF mutant CRC.

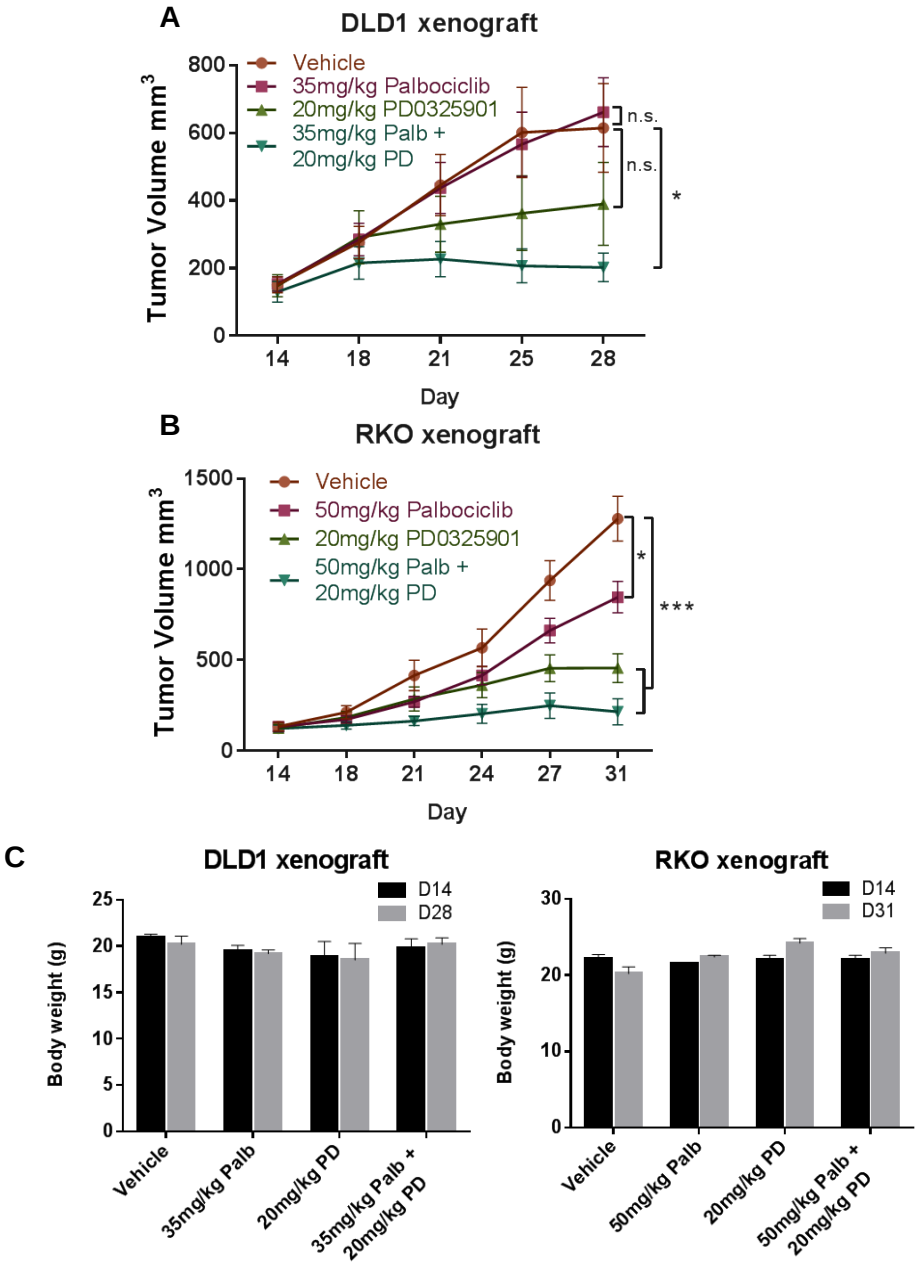


Figure 3.27 CDK4/6 and MEK inhibitors in combination effectively reduce tumor growth in KRAS dependent and BRAF mutant CRC xenograft models.

A. DLD1 xenograft tumor growth in NOD/SCID mice treated with vehicle (n=9), 35mg/kg Palbociclib (n=10), 20mg/kg PD0325901 (n=5) or combination (n=6). Mean tumor volume ± s.e.m. was shown. *P<0.05.

B. RKO xenograft tumor growth in NOD/SCID mice treated with vehicle (n=9), 50mg/kg Palbociclib (n=10), 20mg/kg PD0325901 (n=8) or combination (n=7). Mean tumor volume ± s.e.m. was shown. *P<0.05, ***p<0.001

C. Body weight change (± s.e.m.) in mice with DLD1 and RKO xenograft comparing Day14 (start of treatment) and Day 28/31 (end of treatment).

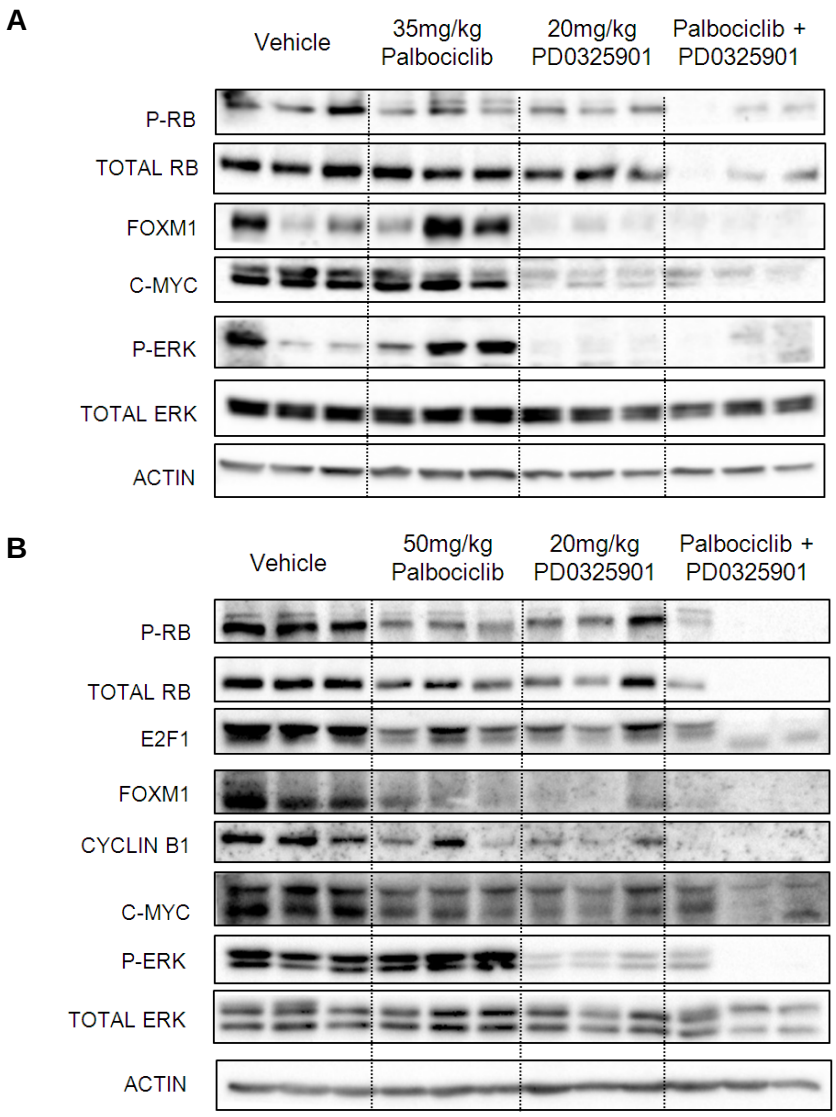


Figure 3.28 CDK4/6 and MEK inhibitors inhibit expression of FOXM1 together with signaling members of CDK4/6 and MEK pathway *in vivo*.

A. Western blot analysis of indicated proteins from resected DLD1 xenograft tumors.
B. Western blot analysis of indicated proteins from resected RKO xenograft tumors.

CHAPTER 4: DISCUSSION

4.1 Role of CDK4/6-FOXM1 axis in KRAS driven colorectal cancer

In our study, we have, through the derivation of the KRAS dependency gene signature from colorectal cancer tumors, hypothesized that the activity of CDK4/6 pathway is important in KRAS dependent/KRAS driven cancer. The gene signature being enriched for cell cycle and mitotic processes as well as the presence of FOXM1 and its target genes, PBK, MCM3, BUB1B, CDK2, TRIP13 and KIF11 (Buchner, Park et al. 2015), has suggested the involvement of CDK4/6, as it is a major player in the G1/S checkpoint and it phosphorylates and activates FOXM1.

Initial studies on RAS-transformed cells showed that the loss of function of tumor suppressor RB, which activity is regulated by CDK4/6, is necessary for the prevention of RAS oncogene-induced senescence, suggesting that these two pathways cooperate to drive robust cell growth (Collins, Napoli et al. 2012). Although loss of RB is rarely observed in colorectal cancer, CDK4/6 pathway is frequently activated as seen by the occurrence of *CCND1* amplification and aberrant methylation of the *CDKN2A* locus, resulting in the increase in CDK4/6 activation and hyperphosphorylation and inactivation of RB as mentioned in chapter 1.3.4. This suggests that in CRC, through the activation of CDK4/6 pathway instead of RB loss of function might be involved in the prevention of RAS oncogene-induced senescence.

Downstream effectors of KRAS have also been known to regulate components of the CDK4/6 pathway. Together with the AKT pathway, phosphorylated ERK phosphorylates and activates AP-1 , promoting the binding of AP-1 proteins to the *CCND1* promoter and increasing cyclin D1 transcription and expression(Shaulian and Karin 2001). eIF4E downstream of AKT too promotes cyclin D1 post-translationally via increasing nuclear export of CCND1 transcripts together with a sub-set of growth-related mRNA (Rousseau, Kaspar et al. 1996, Culjkovic, Topisirovic et al. 2005). CDK4 has also been shown to regulate and activate FOXM1 together with

phosphorylated ERK, another indication that the KRAS/MEK/ERK and CDK4/6 pathway cooperate to drive cell cycle and mitosis, since FOXM1 is vital for the transcription of various genes important for mitotic phase.

Tumors with RAS mutation frequently displayed significantly higher levels of mitotic figures (Liu, Jakubowski et al. 2011) and in melanoma cell lines, a knockdown of NRAS led to reduction in many cell cycle genes (Kwong, Costello et al. 2012). Here, our study also reveals that cancer cells that are dependent on KRAS are dependent on FOXM1 as well and this is not surprising as FOXM1 is required for transcription of genes that are important in mitosis. Activation of KRAS increases cell proliferation and this suggests that KRAS may drive proliferation partly through FOXM1. All in all, prior research and our studies suggest that the CDK4/6 pathway and KRAS driven pathways interact at many levels and that for KRAS mutation to exert its oncogenic effect in the cells, it requires the dysregulation and subsequent activation of the CDK4/6 pathway.

4.2 Targeting CDK4/6 and MAPK pathway as a potential therapeutic strategy in KRAS dependent CRC

Previous studies used Palbociclib as a single agent at higher dosage up to 150mg/kg (Fry, Harvey et al. 2004) in order to achieve a substantial reduction in tumor growth. In our studies, an addition of a suboptimal dosage of 30mg/kg of Palbociclib together with PD0325901 was sufficient to achieve a reduction in tumor growth, suggesting the inhibition of the CDK4/6 pathway synergizes with the inhibition of MEK/ERK pathway to elicit anti-tumor growth in KRAS dependent and BRAF mutant CRC. And this combination treatment was shown to be well-tolerated in the animal studies.

This effective halting of KRAS-dependent tumor growth could be due to the abrogation of expression of important transcription factors FOXM1, E2F1 and CMYC. In the single MEK inhibitor-treated DLD1 and HCT116, only the expression of CMYC was almost completely downregulated with FOXM1 and E2F1 expression slightly reduced. The inhibition of MEK prevents the activation of effectors downstream such as RSK, ELK-1 and ETS-2 which would decrease translation and transcription machinery. However, with the presence of E2F1 and FOXM1, which together regulates genes involved in all phases of cell cycle (Bracken, Ciro et al. 2004, Costa 2005), the cancer cells may still have sufficient activating signaling to continue with cell cycle progression.

Cell cycle progression is tightly regulated by cyclin-dependent kinases and their binding cyclins which upon activation or repression regulate a complex network of transcription factors and many of the transcription factors shared the same target genes, leading to redundancy. Through microarray gene expression analysis after combination, we identified three important transcription factors that are downregulated together with their target genes and analysis using IPA also revealed that cell cycle and mitosis pathways are significantly downregulated. All these suggested that it is likely that the inhibition of CDK4/6 and MEK could have

inhibited the activity of pro-survival and pro-proliferation transcription factors beyond a certain threshold to inhibit growth and even trigger apoptosis.

Malumbres et al had shown that the loss of CDK4/6 did not affect mouse embryo development and that mouse embryonic fibroblasts lacking CDK4/6 were still able to proliferate (Malumbres, Sotillo et al. 2004). In parallel, Kozar et al knocked out D-type cyclins in mice and found that in their absence, there are other alternative mechanisms to enable the cell cycle progression (Kozar, Ciemerych et al. 2004), once again demonstrating that CDK4/6 is not vital for normal cell survival and proliferation. The discovery of CDK1 as the likely predominant cyclin-dependent kinase in normal mammalian cells(Bashir and Pagano 2005), may explain why the use of CDK4/6 inhibitors is likely to be less toxic to normal cells.

MEK inhibitor, though was shown largely to be ineffective in clinic other than BRAF-mutant and NRAS mutant melanoma when administered alone, is still considered to be potentially effective against KRAS or BRAF driven cancer due to its strategic location in the RAS/RAF/MEK/ERK pathway. The reasons limiting MEK inhibitor efficacy, specifically for PD0325901 for non-small cell lung cancer, in the clinical trials are toxicity and lack of primary objective response in patients (Haura, Ricart et al. 2010). However, with the discovery of compensatory pathways such as PI3K/AKT pathway being activated in the KRAS mutant cancer cells upon MEK inhibition, it is not surprising that even at high dose of MEK inhibitor which elicited toxicity, no objective response was observed.

In our studies, we observed with single MEK inhibitor treatment, there was remnant of CDK4/6 activity as indicated by E2F1 and phosphorylated RB, which suggests that the insufficient inhibition of CDK4/6 by MEK inhibitor could be adequate from the survival of these cancer cells. Indeed, the combination treatment of both inhibitors completely shut down the CDK4/6 signaling and further potentiates the effects of

MEK inhibition on the cancer cells both in vitro and in vivo, suggesting that there is possibility of achieving objective response in clinical trials.

Combination with MEK inhibitor with a dual PI3K and mTOR inhibitor, PF-04691502, in phase 1 study failed to study the combined effects of both the inhibitors at their effective dose due to toxicity (Zhao and Adjei 2014) and these may be a potential problem in our study. However, we have shown that there is no significant increase in apoptosis or growth retardation in the normal colon epithelial cells in the combination treated group as compared to the single inhibitor treated group. This should be due to the CDK4/6 being much downstream in the signaling cascade and its main function in regulating cell cycle entry. For PI3K or mTOR, they regulate a variety of cellular processes ranging from protein synthesis and translation to metabolism and proliferation. Thus, upon inhibition of PI3K or mTOR, these pathways which are also vital to normal cells will be affected whereas in CDK4/6 inhibition, only cell cycle entry is inhibited.

Moreover, there is an interesting observation that the normal epithelial cells in the presence of CDK4/6 inhibitor becomes more resistant to the effect of MEK inhibitor, suggesting that the combination treatment may increase the narrow therapeutic window of MEK inhibitor, resulting in less toxic effect as compared to single MEK inhibition treatment. This scenario where combination therapy results in fewer off-targets adverse effects as compared to single agent therapy had been previously observed when BRAF and MEK inhibitors were used together (Flaherty, Infante et al. 2012). Definitely, more in depth studies using in vivo models need to be carried out to verify if there is indeed a widening of therapeutic window, which if proven true, will translate into more effective treatment of KRAS-dependent CRC and yet reduce adverse side-effects.

4.3 Conundrum of KRAS dependency and independency in KRAS mutant cancer and its therapeutic implications

In our studies, we demonstrated the efficacy of the combination treatment of CDK4/6 and MEK inhibitors in KRAS-dependent CRC cell lines. In KRAS independent CRC HCT15, the combination treatment was very much less effective and this could be due to the RAS/MEK/ERK pathway being less vital for survival and thus the combination treatment was not targeting the pathway that was crucial for viability.

Our findings are in line with recent reports on the existence of KRAS mutant cancer cells whose viability are not impaired upon the knockdown of oncogenic KRAS (Scholl, Frohling et al. 2009, Singh, Greninger et al. 2009, Singh, Sweeney et al. 2012). It is clear that we are underestimating the complexity of KRAS mutant cancers. Studies have shown that in pancreatic ductal adenocarcinoma, YAP amplification drives KRAS-independent tumor relapse in mice with TEAD and E2F1 to promote cell cycle (Kapoor, Yao et al. 2014) and in mutant KRAS non-small cell lung cancer, expression of MUC1-C confers KRAS independence (Kharbanda, Rajabi et al. 2014). Thus it is time to develop more specific biomarkers that can predicts response to proposed therapeutic strategies in the future rather than to continue to use the status of KRAS mutation as the mode of stratification.

Surprisingly, the KRAS wild type CRC cell line derived from patients tumor, 47S, which expressed relatively high amount of FOXM1 was also sensitive to CDK4/6 and MEK inhibition, suggesting that this combination treatment could be an effective treatment for KRAS wild type CRC as well. This also suggests that this cell line is dependent on KRAS and that the gene expression of the KRAS dependency gene signature is more accurate in predicting response to the combination treatment as compared to KRAS mutation status. This is not surprisingly as the activity of RAS/MEK/ ERK pathway is regulated by many components and it may be more

useful to examine the downstream output of the pathway to predict the response to the combination treatment.

In light of the development of resistance towards anti- EGFR therapy in KRAS wildtype CRC where the emergence of KRAS mutant clone was detected in some patients, it may not be wise to continue to just treat KRAS wild type CRC with cetuximab and chemotherapy. Chemotherapy with cetuximab, monoclonal antibody against EGFR, could only increase the median progression-free survival from 8.7 to 9.9 months (hazard ratio of 0.68) (Van Cutsem, Kohne et al. 2009) and studies are showing the presence of KRAS mutation clone in previously KRAS wild type classified tumor (Baldus, Schaefer et al. 2010, Richman, Chambers et al. 2011), suggesting that resistance is inevitable in these tumors. Morover, clinical trials have also shown KRAS mutant CRC to fare worse if cetuximab was given in addition to chemotherapy. Thus, it may be more beneficial to determine whether these KRAS wild type tumors are KRAS dependent and if so, the combination treatment be able to target the KRAS wild type and any possible KRAS mutant subclone within the tumor.

4.4 FOXM1 dependency in KRAS Dependent CRC

In our findings, FOXM1 is observed to be important for the viability of KRAS dependent CRC as shown when FOXM1 was knocked down, proliferation and colony formation in anchorage independent condition was reduced. Likewise, the isogenic KRAS mutant CRC line is more dependent on the FOXM1 expression as compared to its KRAS wild type counterpart. Under MEK inhibition condition, addition knock down of FOXM1 further suppressed growth in monolayer and anchorage-independent conditions, as well as expression of representative genes from the KRAS dependency gene signature.

This firstly showed that MEK inhibition is unable to completely deplete FOXM1 expression and the remnant of FOXM1 expression may contribute to the survival of KRAS dependent CRC and the ineffectiveness of single MEK inhibitor treatment. Definitely, FOXM1 is not the only transcription factor vital for the survival of KRAS-dependent CRC as FOXM1 depletion is unable to completely recapitulate the phenotype elicited by the combined inhibition of CDK4/6 and MEK. However, it is intriguing as to how FOXM1 depletion only has an effect on KRAS dependent but not on KRAS independent CRC. One major characteristic of KRAS driven cancer is that pathways associated with cell cycle and mitosis are highly enriched in these cancers which is not surprising since KRAS-transformed cells often displayed faster rate of proliferation (Tuveson, Shaw et al. 2004, Liu, Jakubowski et al. 2011). FOXM1 is known to transcriptionally activate genes essential to cell cycle, DNA replication, mitosis and DNA damage repair (Koo, Muir et al. 2012, Zona, Bella et al. 2014).

KRAS mutant cancer cells are often under replicative stress and are highly dependent on ATR/checkpoint kinase 1 (CHK1) for DNA damage repair (Gilad, Nabet et al. 2010, Grabocka, Pylayeva-Gupta et al. 2014). Upon inhibition of CHK1, there is increased DNA damage and abrogation of G2/M checkpoint. Interestingly, FOXM1

is also reported to activate the transcription of CHK1 proteins together with MYC and E2F1 (Tan, Chen et al. 2010, Hoglund, Nilsson et al. 2011). In addition during DNA damage, checkpoint 2 (CHK2) also mediates stabilization of FOXM1 to activate expression of DNA repair genes (Tan, Raychaudhuri et al. 2007). Thus it could be possible that by depleting FOXM1 in KRAS-dependent CRC, DNA damage response could be inhibited, leading to cell death from replicative stress and cumulative of lethal mutations.

CHAPTER 5: CONCLUSIONS

5.1 Conclusion

In our study, we have identified a gene signature that is associated with KRAS mutation from patient colorectal tumors and through pathway analysis carried out on the correlated genes that were upregulated in tumors with high KRAS mutation, pathways related to cell cycle and mitosis were observed to be enriched. Together with the observation that FOXM1 transcription factor was also upregulated in CRC tumors with high KRAS mutation, we hypothesized that the CDK4/6 pathway is also involved as a co-driver with the KRAS/MEK/ERK pathway in promoting the growth and proliferation of KRAS mutant CRC.

Inhibition of CDK4/6 sensitizes KRAS dependent and BRAF mutant CRC to MEK inhibition, but not in KRAS independent CRC. Combination treatment with CDK4/6 and MEK inhibitors reduces cell viability, induces apoptosis and downregulate the KRAS dependency gene signature in only KRAS dependent CRC. Importantly, treatment with Palbociclib and PD0325901 led to decreased tumor growth *in vivo* xenograft models with no overt toxicity,

We have also demonstrated that the combination treatment downregulates mitotic transcription factor FOXM1 in a synergistic manner together other cell cycle transcription factors such as E2F1 and CMYC and this may potentially contribute to the synergistic action of CDK4/6 and MEK inhibition. In addition, our study showed that KRAS dependent CRC is also dependent on FOXM1, suggesting FOXM1 plays a important role in the viability of KRAS dependent cancers.

In conclusion, our study identified a KRAS dependent gene signature and established a promising therapeutic strategy against KRAS dependent CRC via the inhibition of CDK4/6 and MAPK pathways.

5.2 Significance

In our study, we are the first to use the combination of pyrosequencing technology and gene expression analysis to identify a KRAS dependency gene signature in patients CRC tumor samples. Through the use of quantitative KRAS mutation detection, we overcame the issue of intratumoral heterogeneity that previously hindered the identification of a KRAS associated expression prolife from tumor samples.

We have also proposed and validated a treatment strategy for KRAS dependent and BRAF mutant CRC using a combination of CDK4/6 and MEK inhibitors, demonstrating *in vivo* efficacy with a satisfactory safety profile in the animal model used. Moreover, Palbociclib and PD0325901 have been well-studied in clinical trials, with the former already approved for use in clinic, thus allowing easier translation of this treatment strategy into future clinical trials. This will be of great significance as currently there is no targeted therapy available for KRAS mutant CRC.

We have also shown normal colon epithelial cells to be less sensitive to the effects of the combination treatment as compared to single MEK inhibition. MEK inhibitors previously failed in clinical trials due to toxicity and lack of efficacy when given as a single treatment. This could imply that combination treatment may have a wider therapeutic window than MEK inhibitor alone. At best, additional CDK4/6 inhibition may even reduce adverse toxicity induced by PD0325901 and yet more effectively target the cancer cells.

We have also shown FOXM1 to be important in CRC lines that are dependent on KRAS. FOXM1 together with the rest of the KRAS dependency gene signature can be further developed as a biomarker for the positive response to this proposed treatment strategy. Upregulation of the RAS/MEK/ERK pathway is not solely determined by KRAS or BRAF mutation status but is also determined by changes in

other activators, repressors and negative feedback mechanism. Therefore, by utilizing this gene signature, we can more effectively identify not only tumors with KRAS mutation but also tumors with elevated RAS/MEK/ERK signaling which we predict to be responsive to the combination treatment of CDK4/6 and MEK inhibitors. Since the gene signature is the summation of transcriptional output from different upstream signaling and signaling generated by a myriad of genetic and epigenetic changes, it will definitely be more informative than just KRAS mutation status, considering the fact that tumors have different combination of alterations and are highly heterogeneous.

5.3 Future Prospects

We have identified a KRAS dependency gene signature specifically from CRC tumors samples that will be useful in the prediction of KRAS dependency and responsive to CDK4/6 and MEK inhibitor treatment. However, more validation needs to be done on a larger cohort of patient tumors to obtain their level of expression of the gene signature and simultaneously, culture the tumor cells in vitro to test their response to CDK4/6 and MEK inhibitors treatment. This will be the best way to prove the reliability and the clinical utility of the KRAS-dependency gene signature. Further work can also be done to refine the gene signature by selecting genes which exhibit better association with response to CDK4/6 and MEK inhibition.

Based on our data, we have shown KRAS dependent CRC to be dependent on FOXM1 as well and this could be due to KRAS driven cancers having high levels of replicative stress, resulting in a dependency on DNA damage response pathway in which FOXM1 has also a critical role. Reports have shown the FOXM1 regulates expression of CHK1 and FOXM1 is a downstream effector of CHK2, a hint that KRAS dependent CRC may be dependent on FOXM1 due to its role in DNA damage response. Furthermore since MYC and E2F1 are also reported to regulate expression of CHK1, it will be interesting to investigate if the combined inhibition of CDK4/6 and MEK abrogate the activity of CHK1 and CHK2 since the combination treatment has been shown to abrogate FOXM1, CMYC and E2F1.

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