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Regulation of the kRAS Promoter in Pancreatic Cancer by Proteins and Small Molecules

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REGULATION OF THE kRAS PROMOTER IN PANCREATIC CANCER BY

PROTEINS AND SMALL MOLECULES

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ABSTRACT

DNA-binding proteins play a pivotal role in cell biology. The major class of DNA-binding proteins are transcription factors (TFs). TFs are central to almost every fundamental cellular process such as cell development, differentiation, cell growth, and gene expression. They account for 10% of the genes in eukaryotes. In mammals, more than 700 TFs are identified to be DNA-binding TFs. They bind to the TF binding sites (TFBSs) in the genome and regulate the expression of their target genes. kRAS is a proto-oncogene with intrinsic GTPase activity, that contributes to cell proliferation, division, and apoptosis. kRAS mutations are observed in >95% of pancreatic adenocarcinoma and in 30 % of all human tumors. Pancreatic cancer is the fourth most deadly cancer, with 5 year survival rate of ~6%. When kRAS is mutated it leads to constitutive activity and uncontrolled proliferation, which results in increased tumorigenicity and poor prognosis. Other than mutation, kRAS gene amplification, overexpression, or increased upstream activation is also observed. Downregulating kRAS expression has shown to halt proliferation and lead to cellular death in pancreatic cancer models, but to date no small molecule capable of silencing expression has been described. Moreover, the kRAS promoter region is G-rich and is a hot spot for binding of TFs. TF binding and function in respect to kRAS transcription, is not yet mapped, leading to a gap in understanding of kRAS transcriptional regulation. In the current study, our purpose was to: a) identify and evaluate the function and binding interactions of TF's on the regulation of kRAS, with a particular focus on two putative G-quadruplex (G4)-forming regions (herein termed near

and mid) and the core region from 0 - +50, respective to the transcriptional start site and (b) to evaluate the effect of novel G4 stabilizing compounds on the kRAS expression. This study evaluated biological effects in both an isolated system with plasmids in HEK-293 cells by luciferase assay, and in complex in vitro milieus within pancreatic cancer cell lines by RT-qPCR. Protein changes were evaluated by western blotting. TF binding to the kRAS promoter was predicted based on consensus binding sites by online tools, and by direct binding was probed by Qiagen, and by us using a promoter binding array kit, and DNA pulldown followed by LC-MS/MS. EMSA was utilized for binding studies and effects on G4 formation and stability profile was probed by ECD. For the identification of kRAS-G4 interactive molecules we used FRET, ECD, luciferase assay and RT-qPCR. This mapping of TF binding to the kRAS promoter, and the demonstration of their function as their transcriptional silencers and activators and identification of G4-interactive molecules is important piece of the puzzle associated with the kRAS regulation.

DEDICATION

This dissertation is dedicated to my beloved parents, aunt, and friends. I could never have succeeded on this path without the guidance of my mom and dad. Thank you for instilling in me the passion for knowledge. I look to the examples that you have set for me as a blueprint for living a fulfilled life.

LIST OF ABBREVIATIONS AND SYMBOLS

5'UTR   5' Untranslated region

5-FAM   5-carboxyfluorescein

5-hmC   5-hydroxymethylcytosine

5-mC   5-methylcytosine

6-OHDA   6-hydroxydopamine

AP2   Adaptor protein 2

APS   Ammonium persulfate

Bcl-2   B-cell lymphoma 2

ChIP   Chromatin immunoprecipitation

CK2   Casein kinase 2

CNBP   Cellular nucleic acid-binding protein

DBD   DNA-binding domain

DHFR   Dihydrofolate reductase

DMEM	Dulbecco's Modified Eagle Medium
DMS	Dimethyl sulfate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
E2F-1	E2F transcription factor 1
ECD	Electronic circular dichroism
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epithelial growth factor receptor
EMSA	Electrophoretic mobility shift assay
EV	Empty vector plasmid
FBS	Fetal Bovine Serum
G4	G-quadruplex
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate

GEF	Guanine nucleotide exchange factor
GO	Gene Ontology
GTFs	General transcription factors
GTP	Guanosine-5'-triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNP	Heterogeneous nuclear ribonucleoprotein
HRAS	Harvey rat sarcoma viral oncogene homolog
hTERT	Telomerase reverse transcriptase
IC$_{50}$	Half maximal inhibitory concentration
kRAS	Kirsten rat sarcoma viral oncogene
MAPK	Mitogen-activated protein kinase
MMP7	Matrix metalloproteinase 7
MAZ	MYC associated zinc finger protein
MT	Mutant
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl) -2-(4-sulfophenyl)-2Htetrazolium
mut-kRAS	mutant kRAS

NHE	Nuclease hypersensitivity element
NRAS	Neuroblastoma rat sarcoma viral oncogene homolog
p53	Tumor protein 53
PAGE	Polyacrylamide gel electrophoresis
PARP-1	Poly[ADP-ribose] polymerase 1
PCR	Polymerase chain reaction
PI3K	Phosphatidyl-inositol-3-kinase
PIC	Preinitiation complex
PMS	Phenazine methosulfate
PMSF	Phenylmethylsulfonyl fluoride
PNMT	Phenylethanolamine N-methyltransferase
PPAR-γ	Peroxisome proliferator-activated receptor
pRB	Retinoblastoma protein
PSWM	Position-specific weight matrix
PWMs	Position weight matrices
RT-qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNA Pol II	RNA polymerase II

RRMs	RNA recognition motifs
siRNA	Small interfering RNA
Sp1	Specificity protein 1
ssDNA	Single-stranded DNA
SVD	Singular value decomposition
TBE	Tris/Borate/EDTA buffer
TEMED	Tetramethylethylenediamine
TFs	Transcription factors
TFBS	Transcription factor binding sites
T_M	Melting temperature
TRANSFAC	TRANScription FACtor database
TSS	Transcriptional start site
VEGF	Vascular endothelial growth factor
WT1	Wilm's tumor 1
YBX-1	Y-box 1
YBX-2	Y-box 2
YBX-3	Y-box 3

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Next, I would like to express thanks to my committee members, Dr. Asok Dasmahapatra, Dr. Robert Doerksen, and Dr. Kristine Willett. I appreciate all of their help and encouragement, and I am truly grateful for the time and effort they have given to be on my committee.

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CHAPTER 1. INTRODUCTION

1.1. RAS background and function

The enzymes of RAS superfamily are responsible for signal propagation. This family consists of over 100 GTP hydrolysis switch proteins (small GTPases) which include the RAS, Rho, Arf, Ran, and Rab proteins (Wennerberg et al. 2005). The name RAS is derived from RAt Sarcoma, the model system in which this class of enzymes was first described in 1962 (Harvey et al. 1964). RAS human homologs as genes important in human cancer were identified by Scolnick and colleagues (Chang et al. 1982). Structurally these small GTPases resemble the Gα subunit of the heterotrimeric G proteins (large GTPases), lacking the presence of the Gβ and Gγ subunits as additional regulators (Hurowitz et al. 2000). RAS protein exists in an inactive form when bound to GDP, and an active form when bound to GTP. In the active state, the RAS proteins are capable of engaging multiple effector proteins such as RAF, ERK, PI3K etc. Direct binding of effectors to RAS enzymes changes their conformation, creating an active state in which downstream signaling is affected, leading to immediate effects on cellular processes like metabolism and survival and can influence DNA transcription leading to global cellular outcomes such as altered motility, growth, and differentiation.

The six identified human RAS proteins (kRAS4A, kRAS4B, nRAS, hRAS, mRAS, and rRAS) share approximately 85% sequence homology. They differ mainly at the C-terminus in a string of residues termed the "hypervariable region" responsible for the appropriate localization of these enzymes (Colicelli et al. 2004). The two kRAS proteins are produced from the same gene as

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a result of alternative splicing and differ in this hypervariable region. Gamma-phosphate of GTP interacting at the two switch regions (usually at residues threonine 35 of switch 1 and glycine 60 of switch 2) within the RAS proteins leads to kRAS activation (Milburn et al. 1990). The RAS proteins undergo self-hydroxylation of their GTP molecule to GDP, inactivating the protein, but the process is slow and needs to be amplified by additional regulatory proteins such as p120 and NF2.

1.2. The RAS activation cycle

The RAS proteins are activated in response to the engagement of growth factor receptors. After the binding of growth factors/ligands to the extracellular domain of the tyrosine kinases (RTK) receptor, the intracellular region of the receptor undergoes a conformational change and is phosphorylated on tyrosine residues (Fortini et al. 1990). This phosphorylation promotes binding of proteins containing SH2 domains to the tyrosine sites. SH2 domain-containing proteins, also known as adaptor proteins, bind to an activated receptor and activate Guanine Exchange Factors (GEFs). GEF's interact with the kRAS proteins, the result of which is an exchange of the previously utilized GDP nucleotide for a GTP, also facilitated because GTP occurs at a ten-fold higher concentration in the cytosol (Vetter et al. 2001). The kRAS enzyme is thus activated and transduces intracellular signals through other GTPases and kinases, linking the presence of extracellular growth factors to intracellular signaling cascades.

SOS1, the most extensively characterized GEF, was first identified as an essential regulator of *Drosophila melanogaster* eye development (Simon et al. 1993) and later characterized as an intracellular messenger for the epithelial growth factor receptor (EGFR) in human cells that was essential for the neurodevelopmental pathway (Rogge et al. 1991). Later Grb2 was found to

facilitate the activation of Sos1 by growth factor receptors either by direct binding (Lowenstein et al. 1992) or through binding to other adaptor proteins which are directly bound to these receptors such as FRS for FGFR signaling (Kouhara et al. 1997) and IRS to elicit insulin receptor induced Sos1 activation and the RAS signaling cascade (Skolnik et al. 1993). Additionally, in yeast S. cerevisiae the RasGEF Cdc25 was found as a link between the adenylate cyclase pathway and RAS activation (Robinson et al. 1987), and later the mammalian homolog was established (Wei et al. 1992). The Cdc25 mediated activation of RAS signaling was found to be an important regulator of early development in murine embryonic cells (Chen et al. 1993).

The kRAS enzymes have an intrinsic rate of hydrolysis thus deactivating the enzyme; however, this rate is insufficient for appropriate biological control. Later, several RAS-GTPase activating proteins (RASGAPs), such as p120 and NF2, which bind the kRAS enzymes and accelerated the rate of hydrolysis of GTP, were discovered (Trahey et al. 1987; Trahey et al. 1988; Martin et al. 1990). These RasGAPs increase the hydrolysis of GTP bound Ras by binding to the catalytic site on the RAS and reorienting a water molecule using an arginine residue dubbed the "arginine finger." Upon RasGAP binding to RAS this water molecule becomes oriented in a position favoring a nucleophilic attack on the GTP nucleotide (Resat et al. 2001).

1.3. kRAS in development

Despite considerable homology, the RAS proteins still differ in their functions, most notably in signaling. kRAS found to be indispensable for normal development in mice (Plowman et al. 2003). The development of mouse embryos beyond the two-cell stage was halted when they were injected with a dominant negative kRAS N-17, which abolishes all kRAS signaling. Knocking out of kRAS4B leads to embryonic lethality, which can be rescued by the introduction

of alternative kRAS isoforms at the kRAS locus (Potenza et al. 2005). The hematopoietic defects caused by a dysfunctional microenvironment in the fetal liver led to failure of embryos in gestation between day 12 and day 14 post-fertilization in kRAS4B deficient mice (Yamauchi et al. 1994). RASopathies is collective term for growth deformities due to series of inheritable and spontaneous genetic modifications. These arises from mutations that result in the improper activation of the RAS pathway and thus may serve as a model for the role of RAS in normal development (Rauen et al. 2010). Noonan syndrome is the most common RASopathy inheritable disorder, occurring in 1/1000 to 1/2500 individuals in the United States. Activating mutations in SOS1, kRAS, or inactivating mutations in SHP2, a phosphatase that acts on growth factor regulated pathways, including RAS is responsible for Noonan syndrome. The sites of these activating mutations in kRAS are different than those seem in human tumors (Schubbert et al. 2006). An extensive set of developmental problems including facial dysmorphism, skeletal defects, blood defects, heart problems, neurological disorders, and dwarfism are observed in these patients due to deregulated kRAS signaling (Tartaglia et al. 2011).

1.4. kRAS signal transduction

kRAS activates seven downstream signaling proteins (Cully & Downward 2008). Detailed screening in Drosophila melanogaster led to the identification of the first one, RAF1 (Dickson et al. 1992). This enzyme important for growth and development and is characterized as an activator of Mek-Erk signal transduction (Kyriakis et al. 1992). Mammals process three RAF isoforms, ARAF, BRAF, and RAF1. kRAS is known to primarily utilize B-Raf to elicit the Mek-Erk response, while C-Raf and A-Raf may have evolved to serve both alternative functions including the inhibition of differentiation and promotion of endocytosis respectively (Kern et al. 2012;

Nekhoroshkova et al. 2009). The p110 catalytic subunit of PI3K was the next RAS effecter elucidated (Rodriguez-Viciana et al. 1994). Conversion of the membrane lipid PIP2 to PIP3 is catalyzed by enzyme PI3K. A specific subset of signaling kinases containing a pleckstrin homology (PH) domain are recruited by PIP3 to the membrane for activation. Akt is the best characterized of the PH domain containing proteins in this group which plays an important role in growth and metabolism. Four isoforms of PI3K exist: alpha, beta, delta and gamma of which alpha and gamma are confirmed RAS effecters (Ihle & Powis 2010). Mice created with a PI3Kα isoform which was genetically modified to be deficient in kRAS binding showed embryonic lethality due to deficient lymphogenesis, displaying a phenotype similar to mice with vascular endothelial growth factor receptor-3 (VEGFR3) deficiency (Gupta et al. 2007). These proteins allowed the elucidation of the common structural features of kRAS effectors, the kRAS binding domain or the RAS association domain (RA), and more effectors began to emerge as a result of massive screening efforts. The first of these was RALGDS, an activator the RALA and RALB GTPases, which contains an RA domain (Kikuchi et al. 1994). The Ral GTPases have been demonstrated to play an important role in endocytosis, the exocyst complex, and nutrient sensing. kRAS also binds PLCε (Lambert et al. 2002), a phospholipase C isoform with an RA domain responsible for RAS mediated production of the membrane lipid DAG which results in calcium release and activation of the PKC signaling cascade (Wing et al. 2003). Finally, kRAS binds Tiam1, an enzyme which is utilized in integrin signaling (O'Toole et al. 2011) and plays an extensive role in T cell trafficking through its control of the chemokine and S1P response making it necessary for the mounting of an appropriate immune response (Gerard et al. 2009).

While these effectors are associated with growth and survival another set of kRAS effecter RA domain containing proteins associated with apoptosis have been identified; namely the RASSF

family of enzymes (Vavvas et al. 1998). While the entire set of RASSF proteins have a RA domain, only a subset is confirmed as kRAS binders. While it is known that the Ras-RASSF complex engages the pro-apoptotic complex Mst1 many details of this pathway remain to be discovered (Avruch et al. 2009). The kRAS effectors are shown in (**Figure 1-1)**.
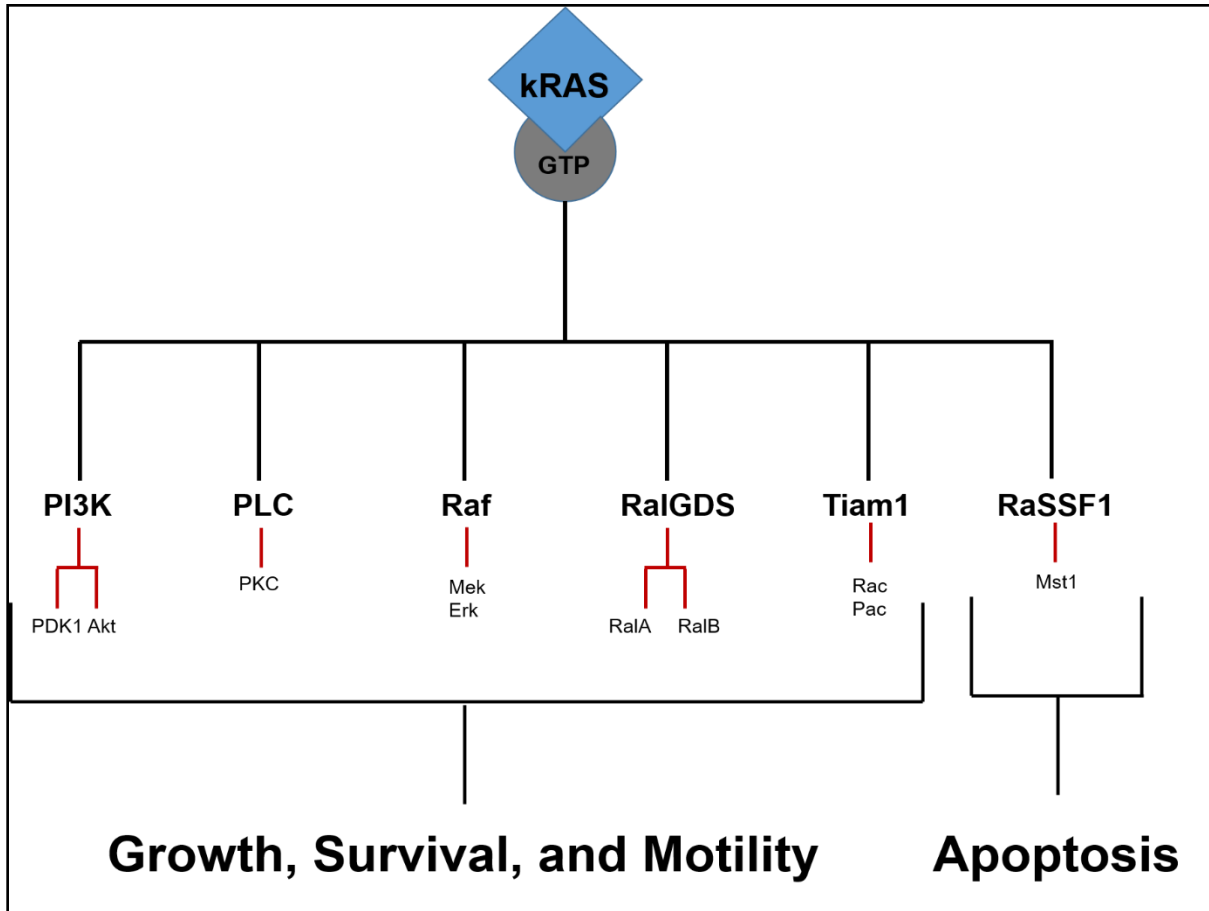
Figure 1-1. kRAS effector pathways. Validated effecters of Ras signaling and the pathways activated by each. A majority of Ras effecter pathways promote cellular functions such as growth, survival, and motility. Unique among these effecters is RASSF1 which is known to induce apoptosis.

1.5. kRAS in cancer

The kRAS has been linked with the study of cancers since their initial discovery as the Kirsten murine sarcoma virus in 1970 (Harvey et al. 1964; Kirsten et al. 1970). In 1982 it was discovered that transformation was due to the mutations present in kRAS (Chang et al. 1982). In the US it is estimated that 320,000 individuals will carry mutant kRAS (mut-kRAS) (Jemal et al. 2010). Of the kRAS isoforms, kRAS4B is the most frequently oncogenically activated through mutation in human cancers. kRAS mutations are observed in cancers that arise from the ectoderm, such as colorectal cancer (Bos et al. 1987), lung cancer (Rodenhuis et al. 1987) and pancreatic cancer (Almoguera et al. 1988). kRAS4A, hypothesized to be a tumor suppressor due to its mediation of apoptosis, is not activated in cancer (Luo et al. 2010).

The kRAS activation by amino acid substitution mutations occurring at codon 12 is most frequent in human tumors, followed by codons 13 and 61. These mutations result in the enzyme being "locked" in the GTP-bound state due to an insensitivity to RASGAPs (Trahey et al. 1987). The wild-type kRAS protein has a glycine, an amino acid lacking a side chain, at both 12 and 13 codons. The introduction of any side chain containing amino acid at the codon 12 or 13 positions, except proline, serves to increase the activity of kRAS enzyme (Seeburg et al. 1984). The formation of Van der Waals bonds between RASGAP and kRAS is inhibited by these amino acids at codon 12, further disrupting the proper orientation of the catalytic glutamine for the γ-phosphate of GTP found at codon 61 (Wittinghofer et al. 1993). Particular codon and amino acids relate to specific types of cancer (Prior et al. 2012). The most common mutation for kRAS occurs at residue 12 and is seen in 30% of all cancers (majority being solid malignancies) including lung, pancreatic, and colon (Lowy & Willumsen 1993; Ostrem & Shokat 2016). In melanoma and myeloid leukemia mutation of nRAS at position 61 is seen, in breast cancer, mutants of mRAS typically occur on

residues 22 or 71, and lastly, frequently expressed in bladder cancer, hRAS mutations happen at either residue 12 or 61 (Ward et al. 2004).

Early studies could not prove kRAS to be driver of tumorigenesis. Activated kRAS resulted in growth arrest in mouse embryonic fibroblasts only when cell cycle inhibitory checkpoint proteins were deleted (Serrano et al. 1997). It was later discovered that endogenous levels of active kRAS in these cells leads to overexpression of key components of the cell cycle and enhanced proliferation without the deletion of these checkpoints (Tuveson et al. 2004). kRAS has also been established in multiple systems to be integral in driving other hallmarks of cancer development and progression, such as the evasion of apoptosis and angiogenesis under hypoxic conditions (Zeng et al. 2010), cancer invasion into proximal tissues and the establishment of cancer metastases at distant sites (Campbell et al. 2004).

In human tumors, activating kRAS mutations occur frequently in lung adenocarcinomas, particularly non-small cell lung cancer (NSCLC), where around 30% of patients have kRAS mutations (Malumbres et al. 2003) and in colorectal cancer, where approximately 35–40% of patients have mutations (Bos et al. 1987). In pancreatic ductal adenocarcinomas alone, mutated kRAS is overexpressed in over 90% of patients (Bailey et al. 2016, cBioPortal for Cancer Genomics).

1.6. kRAS promoter

The kRAS promoter was first investigated in the 1980s (Jordano et al. 1986; Jordano et al. 1988; Yamamoto et al. 1988), but not much work has been done in the subsequent 40 years to describe its regulation. The kRAS promoter region responsible for transcriptional initiation and elongation lie between -510 bp to +50 bp relative to the transcription start site (TSS). This region

of the kRAS promoter is > 70% rich in guanines (G). The G-rich region of the kRAS promoter contains 21 bp SV40 repeats, which show resistance to DNAse I (Jonstra et al. 1984) and are a binding site for Sp1 (Gidoni et al. 1984). Like other housekeeping genes, kRAS contains several putative Sp1 binding motifs (CCCGCCC), lacks characteristic TATA and CCAAT boxes, and contains multiple transcriptional initiation sites (Jordano et al. 1986). These data are indicative of the importance of this region of the kRAS promoter in transcriptional regulation.

1.7. G-quadruplexes (G4's)

kRAS promoter G-rich regions are clustered around the TSS (Balasubramanian et al. 2011) and are expected to be the sites for the binding of transcription factors (TFs). Moreover, these G-rich regions are capable of forming G-quadruplexes or (G4s). The key component to a G4 structure is the G-quartet. A G-quartet is composed of four guanine bases that self-assemble to form a square planar cyclic array. Unlike the hydrogen-bonding of the classical Watson-Crick G-C base pairing in duplex DNA, the cyclic guanine array is instead held together by eight Hoogsteen hydrogen-bonds. By utilizing the N1 and N2 of one face and the O6 and N7 of another, each guanine base becomes a hydrogen bond donor on one face and a hydrogen bond acceptor on the other and when two or more G-quartets stack together, they construct the G4 scaffold that may be held together by loops which are a specific feature of unimolecular and bimolecular G4s. Thus, the definition of a G4 refers to any four-stranded DNA containing stacked guanine quartets (Parkinson 2006; Simonsson 2001) are held together by the sugar-phosphate backbone (Huppert 2009). All G4 structures possess a central cavity which is a unique characteristic of a G4 and due to the inwardly facing O6 guanine carbonyls of the tetrameric arrangement of the four guanine bases, there is an aggregation of negative charge resulting in electrostatic repulsion that can be stabilized by a locally

placed cation in the central cavity. For a sequence to form a stable G4, instead of a duplex, it should have a minimum of four stretches of two or more adjacent guanine nucleotides: $G_{2-5}X_nG_{2-5}X_nG_{2-5}X_nG_{2-5}X_n$, where X is any base and can be up to seven nucleotides (Bochman 2012) (**Figure 1-2**).
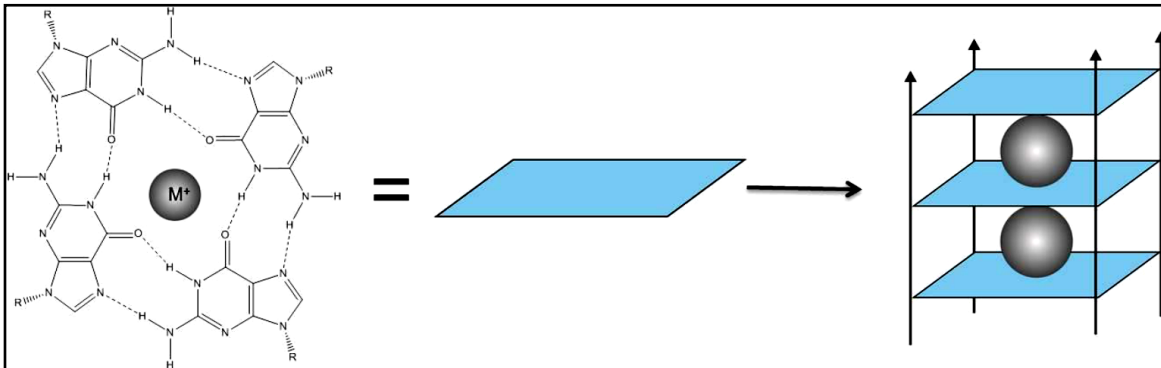
Figure 1-2. Assembly of a G-quadruplex (G4) structure. Cyclic arrangement of a G-quartet where each guanine base interacts with its neighbour in Hoogsteen hydrogen bonding. G-quartets stack on top of one another which are stabilised by a central cation. Schematic representation of three stacked G-quartets to form the parallel G4 structure (Adapted from Brooks & Hurley 2010).

1.8. G4s in promoter

G4-forming motifs are widely dispersed in eukaryotic genomes. They can be found in immunoglobulin switch regions (Sen & Gilbert 1988; Sen & Glibert 1990), in telomeres (Blackburn 1991), in the retinoblastoma susceptibility gene (Murchie & Lilley 1992), upstream of the insulin gene (Bell et al. 1982), and in the promoter regions of many oncogenes and tumor suppressor genes such as MYC, kRAS, hRAS, nRAS, c-KIT, Bcl-2, pRB, VEGF, HIF-1, hTERT, and more. In fact, there is at least one example of a G4 forming and acting upon critical genes responsible for each of the ten hallmarks of cancer (Brooks & Hurley 2010; Cogoi & Xodo 2006; Morgan & Brooks 2016; Morgan et al. 2016; Onyshchenko et al. 2009; Sun et al. 2014; Phan et al. 2004; Huang et al. 2012; Bonnal et al. 2003; Ambrus et al. 2006; Aggarwal et al. 2013; Williamson 1994). With focus on whole genome, putative G4 forming regions promoters have been shown to cluster 1 kb up-and down-stream of the TSS of approximately 40% of all known genes (Huppert & Balasubramanian 2007). The core kRAS promoter (-510 to +50) contains three putative G4-forming regions termed near (-157 to -129), mid (-231 to -179), and far (-278 to -243), based on their distance from the TSS (**Figure 1-3**). These G4 forming regions span the two nuclease hypersensitive regions of the promoter and harbor potential TF binding sites (Quante et al. 2012; Ishi et al. 1986). Regulatory proteins binding to G-rich DNA regions can have transcriptional silencing or enhancing effects by recruiting co-proteins or by altering the DNA topologies within the promoter region. It is notable that while there are three putative G4-forming regions in the kRAS promoter, only two form inducible structures, and only one has a biological function (Morgan et al. 2016). Thus, it is likely that while over 40% of promoters may contain potential G4-forming regions, not all will be stable, form in physiological conditions, or have a function related to transcription or translation.

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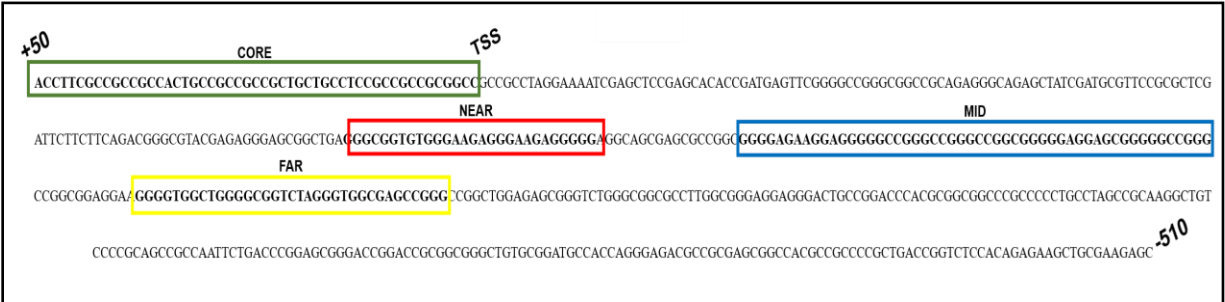
Figure 1-3. kRAS promoter architecture. kRAS promoter extends from (+50 to -510 bp). The 0 – +50 bp from the TSS is the core promoter region with a role in guiding and initiating transcription. The complex G/C-rich region of the kRAS promoter downstream to the TSS also contains three distinct G4 forming regions – termed near, mid, and far.

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1.9. Role of regulatory proteins in transcriptional regulation

Cells recognize and respond to signaling molecules from the extracellular environment. TFs can be activated due to extracellular ligand binding and subsequent signaling cascades. For successful transcription by RNA polymerase in eukaryotic cells, diverse arrays of proteins are crucial. These proteins include general TFs, cofactors, and histones and chromatin remodeling proteins. TFs are key cellular components that control gene expression, and their activities are responsible for determining how cells function and respond to their environment. TFs are critical for the regulation of gene expression. They represent the largest family of proteins in humans, at nearly 10% of all proteins (Babu et al. 2003).

Several classes of TFs exist including (a) General transcription factors (GTFs) that are involved in the formation of a preinitiation complex (which are associated with TATA regulatory elements near TSS's, such as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH; (b) upstream TFs which are proteins that stimulate or repress transcription by binding upstream of the TSS; and (c) inducible TFs that are similar to upstream TFs, but require activation or inhibition (Myers et al. 2000; Spiegelman et al. 2004).

Typical sequence-specific TFs are composed of a DNA-binding domain (DBD), a dimerization domain, and a regulatory domain also termed TRD. All TFs contain DBD's and proteins that cooperate with them but lack their own DBD's are termed as transcription cofactors. Sequence-specific DNA binding TFs are critical in the regulation of gene expression in eukaryotic cells. In general, they will either increase (enhancers) or decrease (repressors) the rate of transcription by stabilizing or destabilizing the formation of the preinitiation complex (PIC) respectively (Latchmann 1997). They impact the stability of the PIC both directly and indirectly (Semenza 1994). A typical eukaryotic promoter contains one or more copies of a particular cis-

regulatory element which is recognized by TFs. Generally, promoters contain cis-regulatory elements for multiple sequence-specific TFs (Wray et al. 2003). These cis-regulatory modules are involved in the integration of signals from several TFs, thus giving rise to a broad spectrum of expression patterns from a single promoter in a context-dependent manner. The activity of several TFs in various combinations is termed as combinatorial control of transcription. Moreover, cooperative functioning between multiple copies of the same TF, or between different TF's could lead to synergistic effects a phenomenon in which the regulatory effect of multiple factors working together is greater than the sum of the activities driven by each factor individually on transcription (Maston et al. 2006). Enhanceosome is a stable nucleoprotein complex formed by cooperating activators (Thanos & Maniatis 1995) and generates one output to the target promoter by integrating regulatory information from multiple signaling cascades. These activators seem to cooperate not in binding, but in activation. In the case of the interferon beta (IFNβ) promoter, the cofactor CBP/p300 simultaneously contact with multiple activators to elicit a response (Merika et al. 1998). Similar clusters can also interact to repress transcription (Gowri et al. 2003).

As mentioned above, TFs can bind to other regulator proteins (Co-regulators) to elicit a response adding yet another layer of regulation to the function of sequence-specific TFs. They are broadly defined as factors that do not possess DNA-binding properties themselves, but which are required for the function of sequence-specific TFs (Malik et al. 2000) and are recruited through protein-protein interactions. Co-factors increase the diversity of responses exhibited by a TF. The final activity of TF as activators or repressors is dependent on co-regulators (Thomas et al. 2006). Some examples include chromatin modifiers that work by altering chromatin structure, leaving the promoter more accessible to the general transcription machinery, and that by linking the TF directly to the RNA polymerase and the GTFs.

Sequence-specific TFs often have the ability to form oligomers. Oligomerization leads to changes in DNA-binding affinity or alterations of sequence specificity (Funnell et al. 2012). Furthermore, the formation of hetero-dimers represents a form of combinatorial control, as it allows different combinations of TFs to bind a cis-regulatory element (Funnell et al. 2012; Singh et al. 1998).

As described, DBD's recognize the cis-regulatory element within promoters and provide gene specificity. DNA-binding domains from different families of TFs have distinct but well-defined structures. The classification of TFs based on the structure of their DNA-binding domains is (Luscombe et al. 2000; Shrivastava et al. 2010):

1) Basic helix-loop-helix (e.g. Helix-loop-helix / leucine zipper factors (bHLH-ZIP), Helix-loop-helix factors (bHLH), and Leucine zipper factors (bZIP))

2) Zinc coordinated DNA binding domains (e.g. diverse Cys4 zinc fingers – GATA factors, Krüppel factors, Cys4 zinc finger of nuclear receptor type)

3) Helix-turn-helix (e.g. Heat Shock Factors, Ets domain – PU.1, Homeo domain, and Fork head/winged helix)

4) Beta-scaffold factors (minor groove contact) (e.g. p53, MAD, TATA, CCAAT, NF-kappaB, NFAT, STS).

In general, helix-turn-helix or a beta-scaffold factors insert into the major groove of double-stranded DNA to facilitate binding to DBD's of TF. Usually, the DNA-protein contact spans approximately 5 bp, with a relatively high affinity (Kd between 10^{-9} and 10^{-10} M) and sequence-specificity (Wray et al. 2003). Van der Waals contacts between protein and DNA base-pairs is the dominant force holding the two together, although contact is also made with the highly negatively charged sugar-phosphate backbone (Luscombe et al. 2001) (**Figure 1-4**).
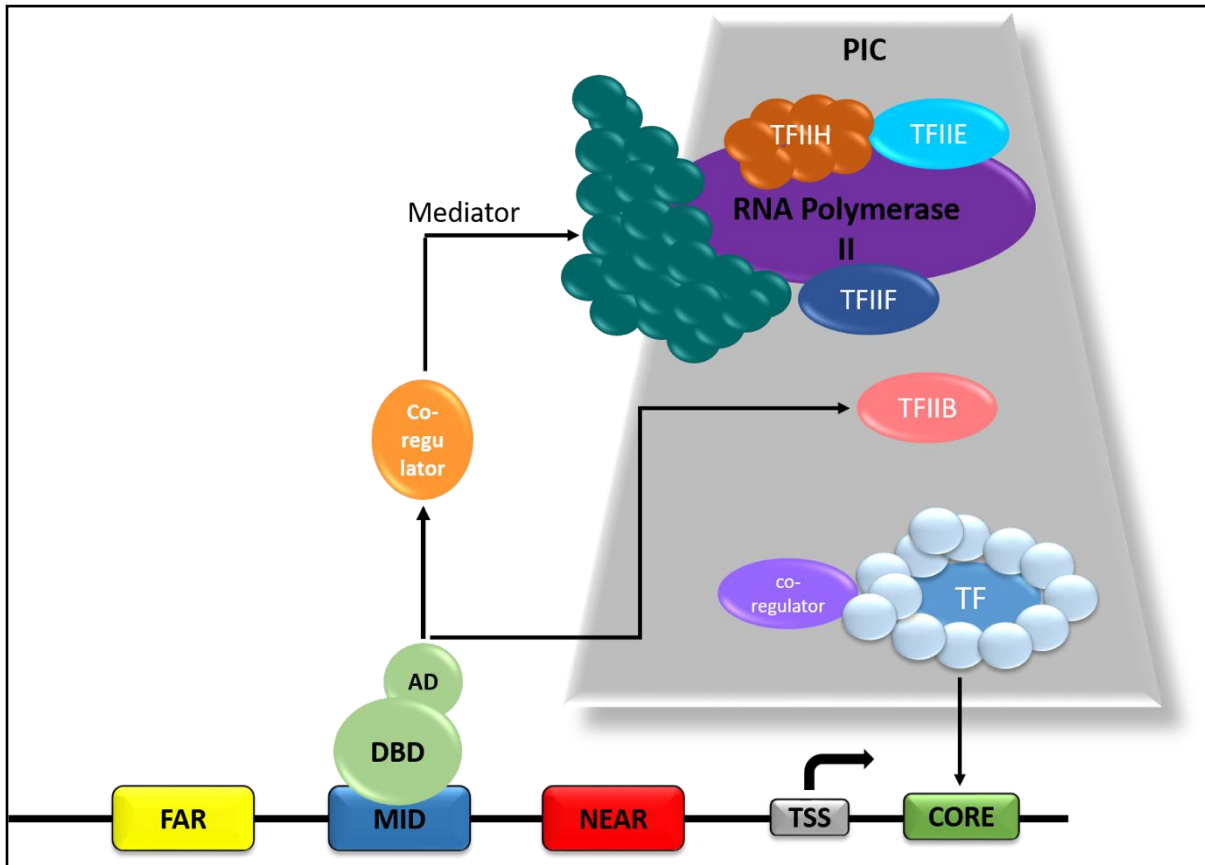
Figure 1-4. The eukaryotic transcriptional machinery. Transcription by RNA polymerase II involves the assembly of several factors including TFIIA, TEIIB, TFIID, TFIIE, TFIIF, TFIIH, co-activators, and activators (sequence-specific transcription factors (TFs)) to form a pre-initiation complex (PIC). Transcriptional activity is initiated when sequence-specific TFs bind upstream regulatory cis-elements on the promoter and stimulate PIC formation. Sequence-specific TFs consist of a DNA-binding domain (DBD) and an activation domain (AD) that is responsible for protein-protein interaction with components of the PIC, such as TFIIA or subunits in the mediator complex. The AD can also interact with co-activators or co-regulators, which in turn can interact with components of the PIC. Adapted from (Maston et al. 2006).

1.10. G4 binding proteins

Several G4 DNA binding proteins from different organisms have been reported. Based on their function they are classified into five major groups by function: (a) G4 DNA stability enhancers; (b) non-catalytic G4 DNA destabilizer (c) catalytic G4 DNA destabilizer in an ATP-dependent fashion; (d) proteins that promote the formation of G4 DNA formation promoters; (e) DNA at or adjacently to a G4 domain nucleases (Paramasivam et al. 2009). The mammalian MYC locus G4 structures are one of the well-studied ones. G-rich region of NHE III_1 in the c-MYC forms G4 structure which regulate transcription (Siddiqui-Jain et al. 2002). In 80% of human cancer cells, increased levels of MYC expression are observed, and this increase promotes tumorigenesis. MYC G4 was shown to be a transcriptional repressor. Further, it was shown that through the binding of nucleolin to G4 structures formed within the NHE III_1, act as signaling elements (Gonzales et al. 2009). Nucleolin is a nucleolar phosphoprotein that is highly expressed in proliferating cells, known mainly for its role in ribosome biogenesis (Ginisty et al. 1998); however, nucleolin also functions in chromatin remodeling (Angelov et al. 2006), transcription (Yang et al. 1994; Grinstein et al. 2007), G4 binding (Dempsey et al. 1999), and apoptosis. It has been shown that nucleolin binds with higher affinity to the MYC G4 structure over its consensus's-RNA substrate (González et al., 2009). Another hypothesis on nucleolin-G4 binding associated transcription regulation is that nucleolin-mediated G4 formation in NHE III 1 inhibits MYC transcription by masking binding sites for MYC transcriptional activators, such as the TF SP1 (Bochman et al. 2012) and cellular nucleic acid-binding protein (CNBP) (Borgognone et al. 2010).

Nucleophosmin a multifunctional protein plays an important role in the pathogenesis of several human malignancies by interacting with different protein partners including p53, p14arf,

etc. It specifically recognizes G4 through its intrinsically unfolded C-terminal region (Gallo et al. 2012) which contributes largely to the binding of MYC G4 motif (Scognamiglio et al. 2014).

A nuclear zinc-finger protein PARP-1 is present in approximately one in every 50 nucleosomes and becomes catalytically active upon binding to DNA breaks (Benjamin et al. 1980). PARP-1 activity is also linked to coordination of chromatin structure and gene expression (Soldatenkov et al. 2002). PARP-1 was shown to bind DNA hairpins and promoter region in superhelical DNA (Chasovskikh et al. 2005). PARP-1 binds to intramolecular DNA G4s in vitro with high affinity and with a stoichiometry of two proteins for one G4 (Soldatenkov et al. 2002). Using an enzymatic assay, it was shown that PARP-1 gets catalytically activated upon binding to G4s localized at the c-kit promoter (Soldatenkov et al. 2002).

The most extensively studied tumor suppressor gene is p53. The p53 mutations are observed in more than 50% of all human tumors, and these mutations inactivate this gene which plays a critical role in the induction of malignant transformation (Dey et al. 2008). As a TF, sequence-specific DNA target sites binding is associated with p53. Previously it was shown that p53 also binds to superhelical DNA and cruciform structures (Palecek et al. 1994; Brazda et al. 2000). Furthermore, p53 binding to bent DNA (Nagaich et al. 1997), DNA bulges, and three- and four-way junctions (Subramanian et al. 2005), mismatched duplexes, cruciform structures (Jagelska et al. 2008; Jagelska et al. 2010; Coufal et al. 2013), and structurally flexible chromatin DNA (Kim & Deppert 2003) has been demonstrated. Recently, the mutant p53 protein was reported to bind to G-C rich DNA sequences and stabilize G4 structure in vitro (Quante et al. 2012).

1.11.   kRAS binding proteins

Within the last ten years last decade, the Xodo et al group has focused on one G4-forming region of the kRAS promoter, G4$_{near}$ (denoted as 32R), in the nuclease hypersensitive element (NHE) of the kRAS promoter. They have explored the 32R element as an important region in formation of G4 structure and role in transcription (Cogoi et al. 2016). They utilized pull-down assays with Panc-1 nuclear extract to identify proteins showing an affinity for the kRAS G4$_{near}$ region. The proteins identified by LC-MS/MS include poly [ADP-ribose] polymerase 1 (PARP-1), ATP-dependent DNA helicase 2, subunit 1 (Ku70) and heterogeneous ribonucleoprotein A1 (hnRNP A1) (Cogoi et al. 2008). hnRNP A1 was selected for further study due to its role in altering G4 profiles in other promoters (Xodo et al. 2009; Zhang et al. 2006). hnRNP A1 is a member of the heterogeneous ribonucleoprotein family found in abundance in the nuclei of actively growing mammalian cells (Dreyfuss et al. 1993; McAfee et al. 1997). There are two highly conserved RNA recognition motifs at the N-terminus and a glycine-rich domain at the C-terminus (Cobianchi et al. 1986; Shamoo et al. 1994) of all the proteins members of the hnRNP family. It was previously reported that hnRNP A1 interacts with the kRAS promoter and destabilizes the 32R G4 structure formed by the GA-element (Cogoi et al. 2009).

The Xodo lab also focused on the MAZ protein and showed that the murine analog of 32R binds to MAZ (myc-associated zinc-finger), a zinc-finger factor that activates transcription whose consensus sequence is GGG(A/C)GG (Parks et al. 1996, Cogoi et al. 2010). Both MAZ and PARP-1 recognize parallel G4 conformation adopted by the G4-forming element and activate kRAS promoter, which may be a key in recruiting these proteins to the promoter (Soldatenkov et al. 2008; Cogoi et al. 2013). Both the murine and the human G4$_{near}$ sequences show a 70% homology, and the human sequence contains two MAZ-binding sites- GGGCGG and GGGAGG at the 5′- and 3′-

ends. In human pancreatic cancer cells they observed that when MAZ was overexpressed, kRAS transcription was upregulated 3.5-fold as compared to the controls (cells treated with a non-specific plasmid). When MAZ was downregulated (to 10% of control), kRAS transcription was downregulated by 60% (Cogoi et al. 2013). Notable work in our lab with the whole kRAS promoter emphasized that it is not the $G4_{near}$, but $G4_{mid}$ region, which might effects kRAS transcription (Morgan et al. 2016) and proteins regulating this region are not known.

In light of these different regions and their role in kRAS transcription, there is a need for extensive exploration of the kRAS transcriptional machinery. There is currently a knowledge gap about the TFs binding to the kRAS promoter, particularly to different DNA topologies, and their role in kRAS expression. The long-term goal of this study is to define the endogenous regulation of kRAS transcription. The first step to achieving that purpose is to examine the binding of TFs to various DNA regions and topologies within the kRAS promoter, as well as to elucidate the biological consequence of their binding on transcription. Our central hypothesis is that the TFs bind to the kRAS G-rich and core promoter regions to modulate expression. G-rich binding proteins mediate changes in the topology of the kRAS promoter DNA leading to altered expression. To address our central hypothesis, we will:

1. Identify and examine the TFs and their protein: DNA interactions within the kRAS promoter.

2. Determine the biological role of regulatory proteins binding to the kRAS promoter.

Overview of dissertation

This work describes the identification of TFs that bind to kRAS promoter and demonstrates the functional outcome of TF-kRAS promoter interactions. Chapter 1 gives an introduction about the thesis research and some relevant background. In Chapter 2, we discuss materials and methods employed for the studies mentioned in the thesis. After that in Chapter 3 we identify the TFs based on online putative binding by using PROMO tool, which were subjective to biological evaluation by dual luciferase assay and RT-qPCR. Chapter 4 is focused on global binding of TFs as determined by actual binding studies utilizing promoter-TF array kit and Oligo-nuclear extract pulldown followed by LC-MS/MS. In Chapter 5 we tried to identify kRAS G4mid interacting compounds which were synthesized by our collaborator. Finally, conclusion and future directions for this project are described in Chapter 6.

CHAPTER 2. MATERIALS & METHODS

2.1. Reagents

All oligonucleotides (**Table 1**) were purchased from Operon (Huntsville, AL). Acrylamide/bisacrylamide (29:1) solution and ammonium persulfate were purchased from Bio-Rad laboratories (Hercules, CA), and 4,5,6,7-Tetrabromobenzotriazole (TBB) was obtained from Fisher Scientific (Pittsburgh, PA). MAZ, Sp1, E2F-1, AP-2, PPAR-γ, nucleolin, and YBX-1 plasmids were purchased from Addgene (Cambridge, MA). pGL4.17 plasmid (Empty vector, EV) and dual luciferase assay kits were acquired from Promega (Madison, WI). All other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Putative Binding: Computational Studies

TRANSFAC is a repository that contains data on TFs, their experimentally proven binding sites, and regulated genes. The online tool PROMO predicts DNA:protein interaction using the TFBS predicted in TRANSFAC database to construct specific binding site weight matrices for TFBS prediction (Messeguer et al. 2002). The PROMO algorithm can be accessed at: http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promo.cgi?dirDB=TF_8.3&calledBy=alggen.

Table 2-1. List of all oligonucleotides sequences.

Name	5'-3' sequence
kRAS core	ATTTTCCTAGGCGGCGGCCGCGGCGGCGGAGGCAGCGG CGGCGGCAGTGGCG
Core complement	CGCCACTGCCGCCGCCGCTGCTGCCTCCGCCGCCGCGGC CGCCGCCTAGGAAAAT
Biotin-kRAS core	[Biotin~5]ATTTTCCTAGGCGGCGGCCGCGGCGGCGGAGG CAGCAGCGGCGGCGGCAGTGGG
kRAS G4$_{near}$	AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG
kRAS G4$_{near}$ complement	CCTCCCCCTCTTCCCTCTTCCCACACCGCCCTC
Biotin-kRAS G4$_{Near}$	[Biotin~5]GAGGGCGGTGTGGGAAGAGGGAAGAGGGGGA GTTTTT
kRAS G4$_{mid}$	CGGGGAGAAGGAGGGGGCCGGGCCGGGCCGGCGGGGG AGGAGCGGGGGCCGGGC
kRAS G4$_{mid}$ complement	GCCCGGCCCCCGCTCCTCCCCCGCCGGCCCGGCCCGGCC CCCTCCTTCTCCCCG
Biotin-kRAS G4$_{Mid}$	[Biotin~5]GCGGGGAGAAGGAGGGGGCCGGGCCGGGCCG GCGGGGGAGGAGCGGGGGCCGGGCCTTTTT
kRAS G4$_{far}$	AAGGGGTGGCTGGGGCGGTCTAGGGTGGCGAGCCGGGC

2.3. Cell Culture

The pancreatic cancer cell lines, MiaPaCa-2 (G12C mutant kRAS), Panc-1 (G12D mutant kRAS), and AsPc-1 (G12D mutant kRAS), and the non-cancerous HEK-293 cells, were obtained from ATCC (Manassas, VA). They were maintained in exponential growth in a 37 °C incubator with a humid environment maintained with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO) and 1% Penicillin-Streptomycin.

2.4. Transfection and Luciferase Assay

HEK-293 cells were plated at 4×10^4 in 24 well plates and allowed to attach overnight before transfection with MAZ or Sp1 or E2F-1 or AP-2 or PPAR-γ or nucleolin or YBX-1 expression plasmids (0, 250, 500, or 1000 ng), 62.5 ng of Renilla luciferase plasmid, and 125 ng of empty vector (promoter-less) or kRAS promoter (Morgan et al. 2016) luciferase. For MAZ and Sp1 combination studies, cells were transfected with 500 ng of both the plasmids alone or with the renilla and firefly plasmids listed above. After 48 hrs, cells were washed with PBS, lysed with passive lysis buffer, and luciferase expression extrapolated from the firefly and renilla luciferase activities using the dual luciferase assay kit (Promega, Madison, WI); light output was measured with a Lumat LB9507 luminometer. Relative luciferase units (RLU, firefly:renilla) were normalized to no protein expression plasmid, and again to correlative protein-induced-expression changes in EV, to assess fold changes in kRAS promoter activity. Experiments were run in triplicate with internal technical duplicates; one-way ANOVA with post-hoc Tukey analysis was used to determine statistical significance.

2.5. Quantitative Real-Time PCR (RT-qPCR)

The Panc-1, MiaPaca-2, and AsPc-1 cells were plated in 12-well plates at a concentration of 1×10^5 cells per well for MiaPaCa-2 and Panc-1, and at 2×10^5 cells per well for AsPc-1 in 1 mL of media, and were allowed to attach overnight. The following day, the cells were transfected with increasing concentration of MAZ, Sp1, E2F-1, AP-2, PPAR-γ, nucleolin, or YBX-1 plasmids or the combination of MAZ and Sp1. 48 hr later, RNA was harvested from the cells using the Thermo Scientific GeneJet RNA Purification kit (Fisher Scientific); the yield and quality was determined by NanoDrop 2000, and only samples with 260/230 values >2 were used for further analysis. We used 500 ng of RNA and the Bio-Rad iScript cDNA synthesis kit to synthesize cDNA; qPCR was run on a Bio-Rad CFX Connect real-time PCR detection system using TaqMan primers from ABI kRAS: Hs00364284_g1, GAPDH: Hs99999905_m1, MAZ: Hs00911157_g1, Sp1: Hs00916521_m1, E2F-1: Hs00153451_m1, AP-2: Hs01029413_m1, PPAR-γ: Hs00234592_m1, Nucleolin: Hs: 01066668_m1, and YBX-1: Hs00358903_g1). kRAS, MAZ, Sp1, E2F-1, AP-2, PPAR-γ, Nucleolin, and YBX-1 mRNA expression were normalized to GAPDH, and to untreated control by the ΔΔCq method. Experiments were run in triplicate with internal technical duplicates; one-way ANOVA with post-hoc Tukey analysis was used to determine statistical significance.

2.6. Protein Extraction and Western Blotting

Cells were plated in 12-well plates at a concentration of 1×10^5 cells per well for MiaPaCa-2 and Panc-1, and at 2×10^5 cells per well for AsPc-1 in 1 mL of media, and were allowed to attach overnight. The following day, the cells were transfected as described above. 48 hr later, cells were lysed in RIPA lysis buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) containing 100X Halt protease inhibitor (1 µl) and lysed by vortexing after

every 5 minutes for 30 minutes; proteins were quantified by the bicinchoninic acid (BCA) assay (Smith et al. 1985). The resultant proteins were solubilized in 2X Laemmli buffer (4% SDS, 20% glycerol and 0.125 M Tris–HCl, pH 6.8). Equal amounts (40 µg) of proteins were separated on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred at 25 V, 2.5 A for 7 minutes in Trans-Blot® Turbo™ Transfer System (Hercules, CA). The membranes were incubated with a primary anti-kRAS antibody (diluted 1:1,000, Cell Signaling, cat#3965), anti-MAZ antibody (diluted 1:500, Active Motif, cat#39936), anti-Sp1 antibody (diluted 1:1,000, Active Motif, cat#39058), anti-E2F-1 antibody (diluted 1:1,000, Cell Signaling, cat#3742) , and a secondary rabbit IgG peroxidase-conjugated antibody (1:10,000, Cell Signaling, cat#7074). The β-actin level in each sample was measured with anti–β-actin antibody (1:10,000, Cell Signaling, cat#3700) and a mouse IgM peroxidase-conjugated antibody (1:10,000, Cell Signaling, cat#7076). The antibodies were diluted in 10 mM Tris, pH 7.9, 150 mM NaCl, 0.05% Tween and 5% bovine serum albumin. The signal was developed by treating the membranes with enhanced chemiluminescence (ECL, Thermo Fisher Scientific, Rockford, IL) and detected on a ChemiDOC XRS using Quantity One 4.6.5 software (Bio-Rad Laboratories, CA, USA).

2.7. Nuclear Protein Extraction

HEK-293 cells ($1x10^6$) were plated in a 150 mm x 25 mm petridish supplied with 6 ml of complete growth medium (DMEM + 10% Fetal Bovine Serum), and allowed to attach overnight. When the dish was 70-80% confluent, cells were transfected with 20 µg of MAZ, Sp1, E2F-1, AP-2, PPAR-γ, Nucleolin or YBX-1 expression plasmids. After 48 hr, nuclear extraction was performed according to Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit (Waltham, MA). Briefly, cells were harvested with trypsin-EDTA and then centrifuged at $500 \times g$

for 5 minutes. After that, the cell pellet was suspended in PBS and was transferred to a 1.5 mL micro centrifuge tube before pelleting by centrifugation at $500 \times$ g for 2-3 minutes. Ice-cold 500 µl CER I was added to the pellets, the tube was vortex vigorously for 15 seconds and incubated on ice for 10 minutes. Next, ice-cold 27.5 µl CER II was added to the tube and tube was vortexed for 5 seconds on the highest setting before incubating on ice for 1 minute. The sample was centrifuged for 5 minutes at $16,000 \times$ g. The cytoplasmic extract was transferred to a new tube and 250 µl of NER was added to the cell pellet. The tube was vortexed at the highest setting for 15 seconds, every 10 minutes, for a total of 40 minutes. The tube was centrifuged at $16,000 \times$ g for 10 minutes. The lysate concentration was measured using the BCA assay kit (Pierce Biotechnology, USA), against a standard curve generated by serial dilutions of 10mg/ml bovine serum albumin (BSA). The nuclear fraction was aliquoted in a clean pre-chilled tube and was stored at -80°C until use.

2.8. Electrophoretic Mobility Shift Assay (EMSA)

Biotinylated oligonucleotides (5 µM) of the kRAS-G4 sequences of the near and mid G4-forming sequence were heated at 95 °C for 10 min followed by slow cooling to form G4s. The dsDNA were formed in the thermal cycler by incubating with the complementary strand at 95 ^{o}C for 5 minutes followed by a controlled cooling at -1 ^{o}C/ minute to a terminal temperature of 25 ^{o}C. MAZ or SP1 nuclear extract (20 µg) was incubated in 2× binding buffer (40 mM Tris–HCl, pH 7.5, 40 mM NaCl, 40 mM KC1, 10% (w/v) glycerol, 2 mM EDTA, and 2 mM DTT) for 30 minutes on ice (Sam & Club 2012). The biotin-labeled oligonucleotide of different DNA topologies (ss-, ds-, or G4-DNA) was added to the mixture and was incubated for 20 minutes at room temperature. Before loading the samples, 0.25 µl of Eva Green (20X) to visualize DNA and 0.5 µl 6X loading dye was added to each sample. Samples were loaded on a 0.5% Agarose gel prepared in $0.5 \times$ TBE

buffer (1.25 mM boric acid, 12.5 mM Tris, 0.25 mM EDTA, pH 8.0). Electrophoresis was in 0.5 × TBE buffer at 100 V for 45 min at room temperature. The gel was visualized under blue light LED using a FotoDyne Investigator FX Imager.

2.9. Electronic Circular Dichroism (ECD)

Unlabeled oligonucleotides (5 µM) of the kRAS-G4 sequences of the near and mid G4-forming sequences were heated at 95 °C for 10 min followed by slow cooling to form G4s. After 2 hr the samples were incubated with either 5% NE or 5% NE from cells transfected with MAZ or Sp1 protein. Background-corrected ECD spectra were recorded on an Olis DSM-20 Spectrophotometer fitted with a CD 250 Peltier cell holder (Bogart, GA) from 225 to 350 nm with scanning time as a function of high volts (generally 0.5 – 1 sec per nm). Thermal stability was determined by collecting full (225-350 nm) over a range of increasing temperatures (20-100 °C, spectra collected every 10 °C after a 1-minute hold at temperature). T_{MS} were determined by performing singular value decomposition (SVD) (DeSa & Matheson 2004). All spectra were baselined for signal contributions from the buffer.

2.10. MTS Cellular Viability Assay

For cellular viability assays, MiaPaCa-2, Panc-1, and AsPc-1 cells were plated at 7,500 cells/well in 90 □L in a 96-well plate and allowed to attach overnight. The following day, a 10x stock plate of TBB diluted from 10 mM over a 5-6 log range in 0.5 log steps was made, and 10 µL of this stock was added to the cell plate, in triplicate such that the highest dose tested was 1 mM; cells were incubated with compound; wells with no cells served as background after compound was added for this colorimetric assay. After 48 hr, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) + 5% phenazine methosulfate

(PMS) was added to each well and incubated for 2-4 h before the absorption at 490 nm was measured on a BioTek Synergy 2 plate reader (Winooski, VT) (Mossman 1983). Background absorptions were subtracted, and data were normalized to control cells. GraphPad Prism (San Diego, CA) was used to calculate the IC_{50} using a non-linear regression model.

2.11. Promoter-Binding Transcription-Factor Profiling Array Assay

To screen for TFs that bind to the kRAS promoter (-510 – +50), the activities of 48 TFs in Panc-1 cells were assayed using a Promoter-Binding TF Profiling Array (Signosis, CA). The assay was performed as recommended by the manufacturer's instructions. Briefly, nuclear extract was isolated from $1x10^6$ Panc-1 cells using a Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit (Waltham, MA). The reaction mixture was prepared using 15 μl of the TF binding buffer, 3 μl of the probe, 10 μg of nuclear extract and 5 μl of the kRAS promoter fragment (-510/+50) and was incubated at room temperature for 30 min to allow for the formation of the TF-DNA complex. Unbound probes were separated from the complex, while bound probes were eluted and then hybridized to the plate and incubated overnight at 42°C. Bound probe was detected using an HRP-streptavidin conjugate incubated with the chemiluminescent substrate. Luminescence is reported as relative light units (RLUs) on a SpectraMax® M5/M5e Multimode Plate Reader, (Sunnyvale, California).

2.12. DNA- Pulldown Assay

One mg of nuclear protein extract (0.25 mg/ml), prepared as described (Cogoi et al. 2008), was incubated for 1 hr at 37 °C with 4 μM biotinylated G4-DNA (annealed with 100 mM KCl) or biotinylated duplex (G4-biotin annealed with its complementary strand in 100 mM NaCl) in a solution containing 20 mM Tris-HCl, pH 8, 8% glycerol, 150 mM KCl, 25 ng/ml poly(dI-dC), 1

mM Na3VO4, 5 mM NaF, 1 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride. Then 250 µg of Streptavidin MagneSphere Paramagnetic Particles (Promega) pretreated for 30 min with 0.25 mg/ml BSA were added and incubated for 30 min at 37 °C. Particles were captured with a magnet, and the proteins were eluted with the buffer containing 0.5 and 1 M NaCl. The eluted proteins were used for further analysis.

2.13. Sample Preparation

In order to digest the proteins, the proteins were solubilized in Tris buffer, pH 8.0 with 1 mM $CaCl_2$. The proteins were denatured by heat at 95 °C for 20 minutes in the presence of 10 mM dithiothreitol (DTT). After the solution was cooled to room temperature, 20 mM iodoacetamide was added and samples were kept at room temperature in dark for 45 minutes. After incubation, 20 mM DTT was added to quench the iodoacetamide followed by sequencing grade trypsin in 1:20 of protease to protein ratio and incubated at 37 °C overnight with rotation.

2.14. Mass Spectrometry

The samples were acidified with 0.1% formic acid, spun briefly and the supernatant was transferred to sample vials. An Orbitrap Fusion tribid mass spectrometer coupled to an ultimate 3000 HPLC system was used for the nano-LC-MS/MS analysis. The samples were first loaded onto a trap cartridge containing C18 stationary phase to desalt the samples. After 4 minutes, samples were loaded onto nano PepMap column (75 µm inner diameter, 150 mm length, particle size 3 µm). The mobile phase (B) consisted of 100% acetonitrile and 0.1% formic acid. The peptide separation was achieved with a linear gradient from 0 to 40 % B for 25 min at 0.3 µL/min. The mass spectrometer was operated at a capillary temperature of 300 °C. The peptides were fragmented by collision-induced dissociation and the normalized collision energy was set to 35%.

The MS scan range was m/z 150-2000 and the top 15 peaks were selected in precursor scan for the data dependent CID fragmentation in each cycle.

2.15. Database Search

The resulting RAW files were searched by Byonic database search engine (version v2.10.5) against human proteome database (downloaded from Uniprot). Proteins were filtered by log probability and only proteins that have log probability >5 were considered. False discovery rate was set at 1%.

CHAPTER 3. TRANSCRIPTION MAPPING AND FUNCTIONAL ANALYSIS OF THE TFs

TO THE kRAS PROMOTER

3.1. Introduction

kRAS is a GTPase that, upon activation, initiates a cascade leading to enhanced transcription and cellular proliferation. kRAS proteins can act as signal transducers, converting upstream extracellular signals to downstream intracellular effects through three important pathways (RAF serine/threonine kinases, phosphatidyl-inositol-3-kinase (PI3K), and RAS-like G proteins RALA and RALB) (reviewed in Shapiro 2002; Wellbrock, Karasarides et al. 2004; reviewed in Vivanco and Sawyers 2002; reviewed in Katz and McCormick 1997; Ferro and Trabalzini 2010). The physiologic roles of the above kRAS pathways can be broadly categorized as mitogenic, anti-apoptotic, and pro-cell-survival. Thus, RAS sits at the apex of a signaling hub that, if activated to a pathophysiologic extent, could promote cancer. Pancreatic, as well as colorectal and lung cancers, frequently present with a mutated kRAS protein mediating enhanced intracellular signaling cascades, as compared to non-tumor tissues (Rall et al. 1996; Grunewald et al. 1989). In the absence of a mutation, some cancers demonstrate gene amplification and overexpression of kRAS at the transcriptional and translational level. For example, in esophageal adenocarcinomas, 40% of the tumors were found to have amplification of the kRAS gene (Galiana et al. 1995), and in low-grade ovarian serous carcinomas, overexpression of kRAS was responsible for 41% of active MAPK in tumors (Hsu et al. 2004). Amplifications of kRAS are also noted in

endometrial and uterine cancers, testicular cancers, and triple negative breast cancers (cbioportal.com).

kRAS plays a distinctive role in different stages of cancer. Interestingly, several genes involved in metabolism are identified to be regulated by kRAS (Ying et al. 2012). Additionally, it was reported that to aid in the maintenance of the tumor cell's redox state, kRAS plays a role in the regulation of glutamine metabolism (Son et al. 2013). In mice, oncogenic kRAS (G12D) is required for initiation and maintenance of pancreatic cancer. kRAS leads to extracellular matrix remodeling by upregulating Hedgehog signaling pathway, fibroblast activation, inflammatory cell infiltration, and production of enzymes (matrix metalloproteinase 7 (MMP7)). In early stage PanIN, inactivation of kRAS causes re-differentiation of PanIN cells into acinar cells which leads to remodeling of the stroma and complete tumor regression. At late stages of PanIN, this reversal of tumor development is not possible due to addiction of cells to continuous expression of oncogenic kRAS (Ying et al. 2012; Collins et al. 2012; Collins et al. 2012).

The kRAS promoter was first described in the 1980s (Jordano & Perucho 1986; Jordana & Perucho 1988; Yamamoto & Perucho 1988); however, little has been done since to describe its regulation. The kRAS promoter is > 70% rich in guanines (G). Such G-rich regions of DNA are clustered around the TSS (Balasubramanian et al. 2011) and are known to be the sites for the binding of TFs. The proteins identified to bind kRAS promoter were poly[ADP-ribose] polymerase I (PARP-1), ATP-dependent DNA helicase 2 subunit 1 (Ku70) and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1). In addition, Myc-associated zinc-finger protein (MAZ) on the basis of its consensus sequence was predicted to have binding sites on GA element of hRAS and kRAS promoter. This putative interactions was confirmed by EMSA and chromatin

immunoprecipitation experiments, and MAZ was established as transcriptional activator of kRAS (Cogoi et al. 2010; Cogoi et al. 2013; Cogoi et al. 2014).

TFs are critical for the regulation of gene expression (Thomas et al. 2006). They represent the largest family of proteins in humans at nearly 10% (Babu et al. 2004). TFs are mainly classified into two types: general and sequence-specific. The general TFs are involved in the transcription of a large fraction of genes by cooperatively acting with RNA polymerase II (Lee & Young 2000). The sequence-specific TFs bind to specific subsets of target genes and lead to distinct comprehensive patterns of gene expression (Kadoonga 2004). TF's function to recruit cofactors, RNA polymerase II, and binding enhancer elements to target genes (Lelli et al. 2012; Ong & Corces 2011; Spitz & Furlong 2012). Multiple TFs regulate transcription from the core promoters of nearby genes by binding to individual enhancers in cooperative fashion (Panne 2008). This physical interaction results in looping of the DNA between the core promoters and enhancers (Krivega & Dean 2012).

The easiest way to determine DNA-TF binding is through in silico methods. The computational method are used to determine the link of "TF to Transcription factor binding sites (TFBS)" by predicting/identifying TFBSs on the DNA sequence (Stormo & Zhao 2010). The links of the form "TFBS-gene" are normally found by looking at the proximity of predicted/identified TFBS to the gene location. Since TFs are considered to control the key aspects of transcription initiation and thus have a great impact on the regulation of gene expression, one of the significant goals in molecular biology has been to understand sequence-specific binding of TFs to DNA. Important issues that need to be considered when using these approaches for TFBSs prediction are to minimize false positive rate (over-prediction error) and to minimize false negative rate (under-prediction error) (Pickert et al. 1998). The most popular models of the TFBS families are those

based on position weight matrices (PWMs) of mononucleotides (Stormo 2000). PWM, also known as position-specific weight matrix (PSWM), is a simple statistical representation of the set of TFBS motifs. They are the most popular way of representing collections of aligned motifs as PWM, are relatively simple, and they result in much more accurate models of TFBS families (Stormo 2000). Models based on Markov chain (Zhou et al. 2005; Ellrott et al. 2002), Hidden Markov Models (Bailey et al. 1995) and models that use remote dependencies between the positions in TFBSs are more complex and more sophisticated models for the same purpose. Unfortunately, these models, while providing some improvements in accuracy over the PWM models, still appear not to be sufficiently accurate. Two major databases, TRANSFAC (Wingender 1996) and JASPAR (Sandelin 2004), store many of these experimentally determined TFBSs and their derived PWM models. The method was also employed to determine the putative binding of the MAZ to the kRAS promoter (Cogoi et al. 2013). With very few exceptions such as PARP-1, Ku70, and hnRNP A1 identified by pulldown assay (Cogoi et al. 2010) the TF's regulating the expression of kRAS remain a mystery.

In addition to serving as TF binding sites, G-rich regions are capable of forming G4's, which are higher order DNA structures in which guanines Hoogsteen-base pair with each other. The core kRAS promoter (+50 to -510) contains three putative G4-forming regions termed near (-129 to -157), mid (-179 to -231), and far (-243 to -278) based on their distance from the TSS. Within the kRAS promoter, the G-rich regions putatively serve both as sites for TF binding and G4 formation. G4 formation and the impact of TF binding and transcriptional regulation in the promoter region of important oncogenes like MYC, Bcl-2, c-KIT, and VEGF, has been studied before (Sun et al. 2008; Siddiqui-Jain et al. 2002; Dexheimer et al. 2006; Shirude et al. 2007; Morgan et al. 2016). Most often, although not always, G4's function to suppress transcription by

sequestering TF binding sites and are an interest of study for various oncogenic promoters. Several proteins such as nucleolin, nm23-H2, MAZ, UP1, and Sp1 have been noted to bind to these G-rich sequences and/or G4 structures and alter the stability profile of G4's (Brooks & Hurley 2009; Thakur et al. 2009; Paramasivam et al. 2009; Cogoi et al. 2010; Cogoi et al. 2013; Raiber et al. 2012; Piekna-Przybylska et al. 2014). Other than their role in transcription of TATA-less GC-rich promoters, MAZ (5'-GGGGGAGGGGGG-3') and Sp1 (5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3) are also known to share their consensus binding sequence (Kadonaga et al. 1986; Ashfield et al. 1994). Both of these proteins have been found to have a role in activating transcription and in binding to alternate DNA (G4) topologies (Her et al. 2003; Cogoi et al. 2013).

With such similarities in consensus binding sites, other regulatory mechanisms of MAZ and Sp1 have been studied. In particular, studies within the promoter of phenylethanolamine N-methyltransferase (PNMT) were undertaken to examine general affinity and regulation of these two TFs. MAZ demonstrated a higher affinity to the PNMT promoter as compared to Sp1 but demonstrated a lower activation level. For either protein to bind and activate this promoter, both proteins must be phosphorylated (Her et al. 2003); casein kinase 2 (CK2) mediates this phosphorylation. Casein kinase 1 and CK2 were the first protein kinases to be discovered (Burnett & Kennedy 1954). CK2 is an essential protein kinase found ubiquitously in a variety of eukaryotic tissues and organisms; it is highly conserved from protozoan level (Kikkawa et al. 1992; Ole-Moyoi et al. 1992; Ospina et al. 1992) and can phosphorylate more than 300 substrates, including both Sp1 and MAZ (Meggio & Pinna 2003). CK2-mediated phosphorylation of MAZ and Sp1 was shown to lead to alternative binding of these proteins to the MYC promoter (**Figure 3-1**). In MAZ, CK2 phosphorylated serine residue at position 480 in the carboxy-terminal region and resulted in an increase in the DNA-binding of MAZ to the pyrimidine-rich c-myc NHE (5'-

TCCTCCCCACCTTCCCCACCCTCCCCACCCTCCCC-3') which in turn enhanced the MYC gene transcription (Tsutsui et al. 1999). On the other hand, phosphorylation of the carboxy terminus of Sp1 by CK2 resulted in a decrease of the DNA-binding activity. (Armstrong et al. 1997).
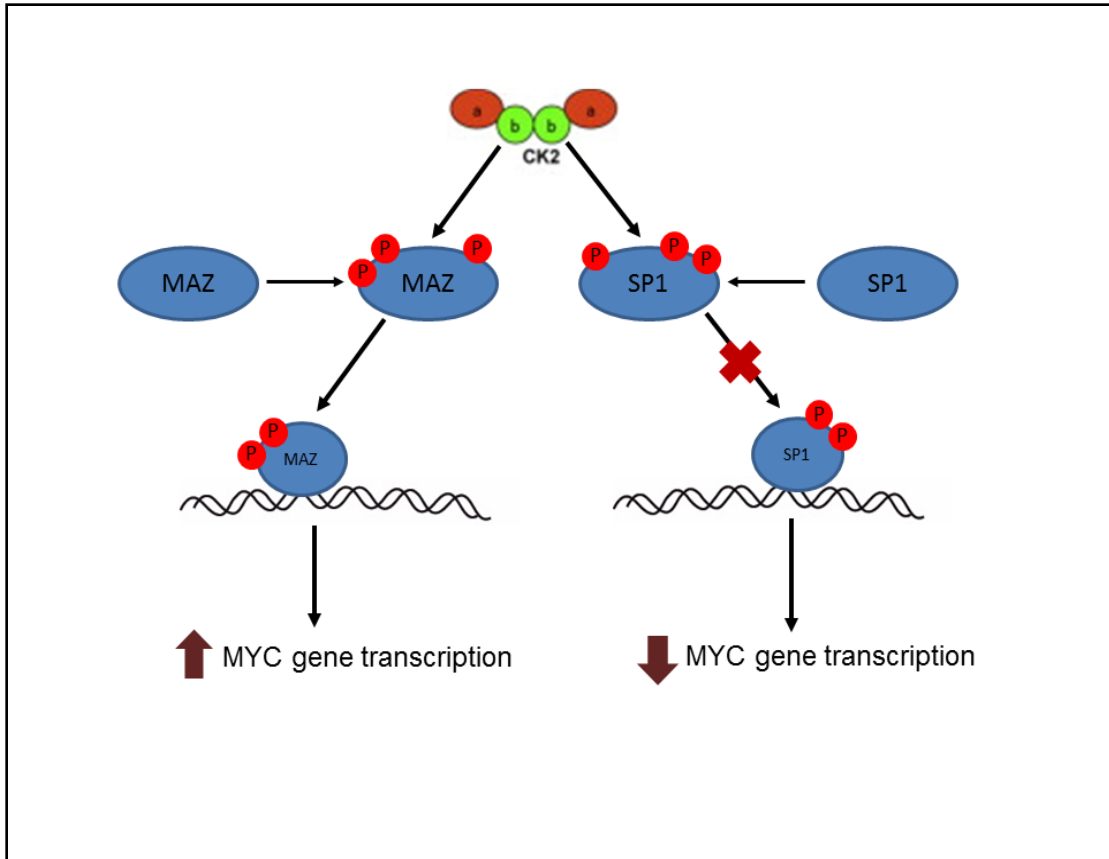
Figure 3-1. CK2 phosphorylation of MAZ and Sp1. CK2 phosphorylates MAZ and Sp1. This leads to altered binding profiles and opposite effects on MYC transcription.

Globally, the regulatory proteins for the kRAS promoter, and the modulation of various topologies (single strand (ssDNA), double strand (dsDNA), and G4 DNA), is an untold story, which needs to be explored further. We hypothesized that regulatory proteins bind to the kRAS promoter and elicit a response based on their different region binding and affinities. The hypothesis was investigated by examining binding of MAZ, Sp1, and E2F-1 to the kRAS promoter, including a consideration of the structural forms of DNA to which the proteins were binding, and the functional outcome of their binding on kRAS promoter activity. The knowledge of the kRAS regulation is important for the drug discovery efforts as well as to fill up the puzzle related to kRAS biology. We aim to gain such knowledge through following aims:

Specific Aim 1. Identify and examine the MAZ, Sp1 and E2F-1 and DNA interactions within the kRAS promoter.

Specific Aim 2. Determine the biological role of MAZ, Sp1 and E2F-1 binding to the kRAS promoter.

3.2. Sp1, MAZ, and E2F-1 Putative binding sequences on kRAS promoter

Select regions of the minimal promoter of kRAS from -510 to +50, in relation to the TSS (Jordano & Perucho 1986; Jordana & Perucho 1988; Yamamoto & Perucho 1988) were used to map TF binding sites. Specifically, PROMO online tool was used for identification of putative TFBS in the kRAS promoter region. In particular, we used the core region (0 – +50), the $G4_{near}$-forming region (-128 – -160), and the $G4_{mid}$-forming region (-174 – -226). A number of proteins mapped to these regions, including E2F-1, p53, WT1, MAZ, and Sp1. Of those, MAZ and Sp1 were of particular interest as they have overlapping consensus sequences that are highly prevalent in G4-forming regions, both have been shown to bind both dsDNA and G4-DNA, and MAZ has particularly been linked to the kRAS promoter previously (Cogoi et al. 2013). MAZ binding was mapped to the $G4_{near}$-region, in agreement with previous literature, and also had two potential binding sites within the $G4_{mid}$-region. For Sp1, we found several binding sites throughout the kRAS promoter region with approximately two potential binding sites per each of the three regions of interest. Lastly, for E2F-1 there were four binding sites in the core promoter region from 0 to +50 bp. (**Figure 3-2**).
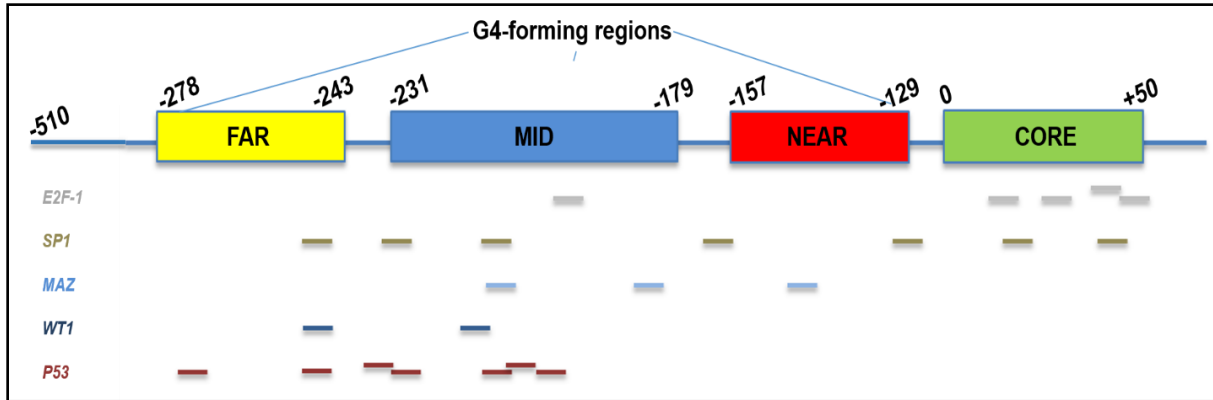
Figure 3-2. Transcription factors binding sites on the kRAS promoter. The complex G-rich region of the kRAS promoter contains three distinct G4 forming regions – termed near, mid, and far. Several transcription factor binding sites lie within this extensive region, including a number for the MAZ (three), Sp1 (seven), E2F-1(five), WT1 (two), and P53 (seven).

3.3. Biological role of MAZ, Sp1, and E2F-1 on kRAS promoter regulation

These putative binding techniques help identify the binding sites between TFs and target genes. However, many of the binding events are not functionally relevant as they do not result in a change in target gene expression. Thus, the effects of TF binding target gene expression needs to be functionally validated. DNA binding proteins can have varying functional outcome, dependent on context and binding partner (Fry & Farnham 1999). We sought to define the effect of MAZ and Sp1 on kRAS transcription with two studies. First we analyzed the effect of these proteins on isolated kRAS promoter in HEK-293 cells by dual luciferase assay, and second we tested the overexpression of MAZ, Sp1, and E2F-1 on endogenous expression by PCR. For dual luciferase assay, a promoter-less vector (EV) was included as a control. A luciferase assay was performed in HEK-293 cells transfected with a luciferase plasmid driven by the kRAS promoter Full Length (FL) or with EV, in the presence of increasing expression of the MAZ and Sp1 plasmid. In both cases, increasing MAZ and Sp1 expression led to a significant decrease in kRAS promoter activity, as measured by up to 50% (at 500 ng) (**Figure 3-3A**). Sp1 induced an even more marked change, as kRAS promoter activity was decreased by up to 80% at 500 and 1000 ng respectively (**Figure 3-3B**). As MAZ and Sp1 are known to share the binding sites, we transfected them together at 500 ng concentration with the FL kRAS promoter. MAZ and Sp1 combination abrogated their specific silencing effect and led to significant increase in the kRAS promoter activity as measured by luciferase expression. The increase was by 270% as compared to control (**Figure 3-3C**). Increasing E2F-1 expression led to significant increase in kRAS promoter activity as measured by up to 60% (at 500 ng) (**Figure 3-3D**) In MAZ, Sp1, and E2F-1 dose dependent studies $*p < 0.05$ (versus control), was determined by one-way ANOVA with Tukey post-hoc

testing. For the MAZ + Sp1 combination studies *p< 0.05 (versus control) was determined by two-tailed Student t-test.

The effects of MAZ, Sp1, and E2F-1 proteins, alone and in combination, on kRAS expression was also studied in pancreatic cancer cell lines. In particular three cell lines homozygous for mutant kRAS with varying levels of addiction to the oncogene, MiaPaCa-2 (moderate addiction), Panc-1 (low addiction), and AsPc-1 (high addiction) were transfected with increasing MAZ, Sp1, E2F-1, and MAZ + Sp1 expression plasmids and the expression of MAZ, Sp1, E2F-1, and kRAS were monitored at the transcript and protein levels. We noted the basal levels of MAZ, Sp1, and kRAS in different cell lines. The basal levels of all these transcripts varied with AsPc-1 showing maximum levels of kRAS, MAZ, and Sp1 followed by Panc-1, with MiaPaCa-2 showing the lowest level of kRAS (**Figure 3-4A**). The data from the cell lines with each TF individually was in accordance with the luciferase findings – kRAS transcription was significantly decreased by in the presence of MAZ or Sp1, respectively (**Figure 3-5A and B**, respectively). On the other hand, E2F-1 showed opposite effects. E2F-1:kRAS promoter interaction showed significant increase in kRAS mRNA expression (**Figure 3-5C**). All changes in mRNA were also examined at the protein level in MiaPaCa-2 cells transfected with 500 ng MAZ plasmid, 100 ng Sp1 plasmid and 100 ng E2F-1 plasmid. The results concurred with the RT-qPCR as kRAS decreased with increasing MAZ or Sp1 (**Figure 3-5D-E**). With E2F-1, increase in kRAS protein expression was observed as that by RT-qPCR (**Figure 3-5F).** The panel of cell lines were also transfected with 500 ng of MAZ and 100 ng of Sp1 plasmid (consistently mediating an increase in TF transcription by less than 15-fold). The combination of MAZ and Sp1 abrogated the silencing effect of either protein alone, as observed by either no change in kRAS mRNA, or an increase in expression (**Figure 3-6A**); again this finding was in agreement with the plasmid

findings. Increase in western blot with the MAZ and Sp1 combination was also observed (**Figure 3-6B**). Collectively these data support that the MAZ and Sp1 individually act as transcriptional silencer but in combination, the effects are abrogated or even reversed. E2F-1 was classified as a transcriptional activator of kRAS.
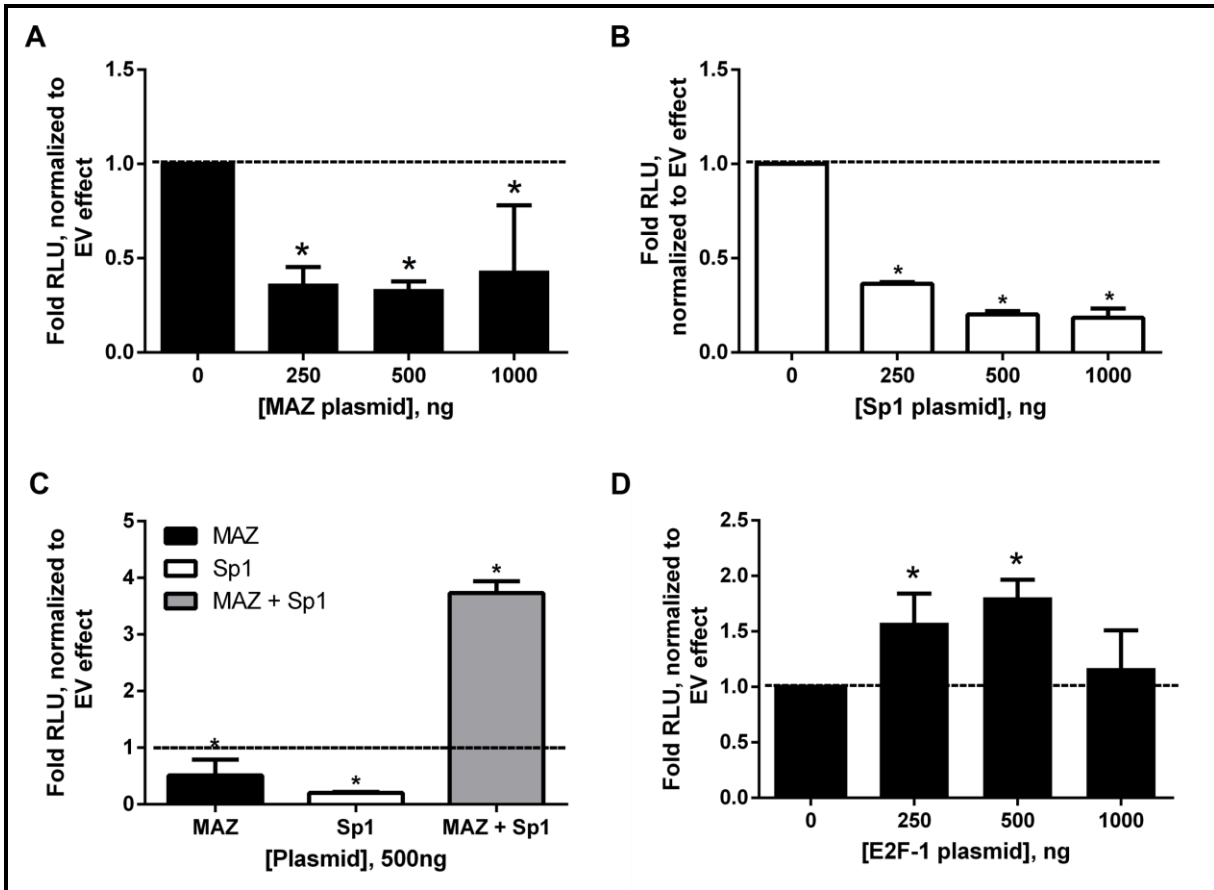
Figure 3-3. MAZ, Sp1, MAZ +SP1, and E2F-1 effect on kRAS promoter activity. A luciferase assay was performed in HEK-293 cells transfected with a luciferase plasmid driven by the kRAS promoter in the presence of increasing MAZ, Sp1, MAZ + Sp1, E2F-1 expression plasmid. Promoterless (EV) was included as a control. Increased MAZ & Sp1 expression leads to decreased kRAS promoter activity as measured by fold change in RLU, normalized to the EV effect. (A, B) The combination of 500 ng of MAZ + Sp1 abrogates the decreased kRAS promoter activity seen with either protein alone (C). Increased E2F-1 expression leads to increased kRAS promoter activity as measured by fold change in RLU, normalized to the EV effect (D). *$p < 0.05$ (versus control), as determined by one-way ANOVA with Tukey post-hoc testing. All experiments were performed in a minimum of triplicate.
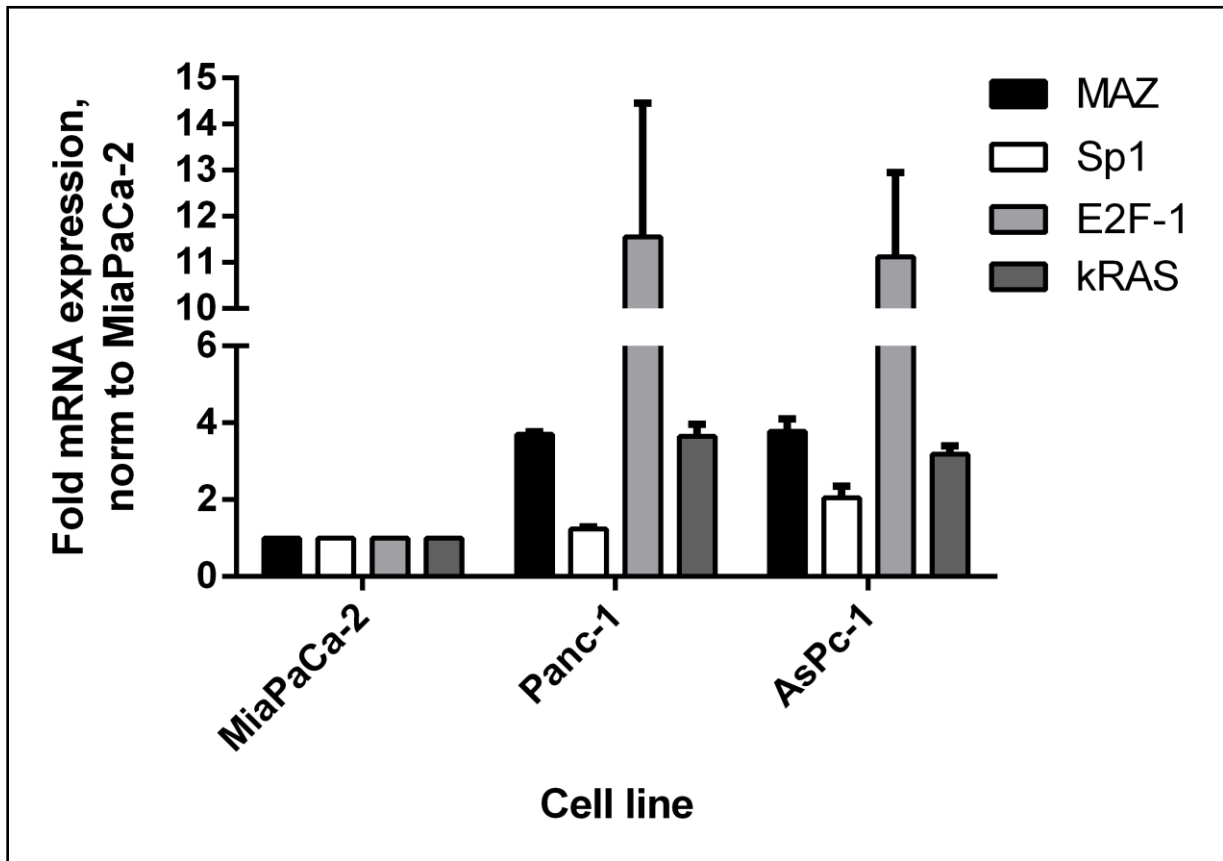
Figure 3-4. Basal levels of MAZ, Sp1, E2F-1, and kRAS in pancreatic cancer cell lines. At Basal level (0 ng) levels of all the proteins were normalized to the MiaPaCa-2 cells. AsPc-1 and Panc-1 nearly similar levels of all proteins.
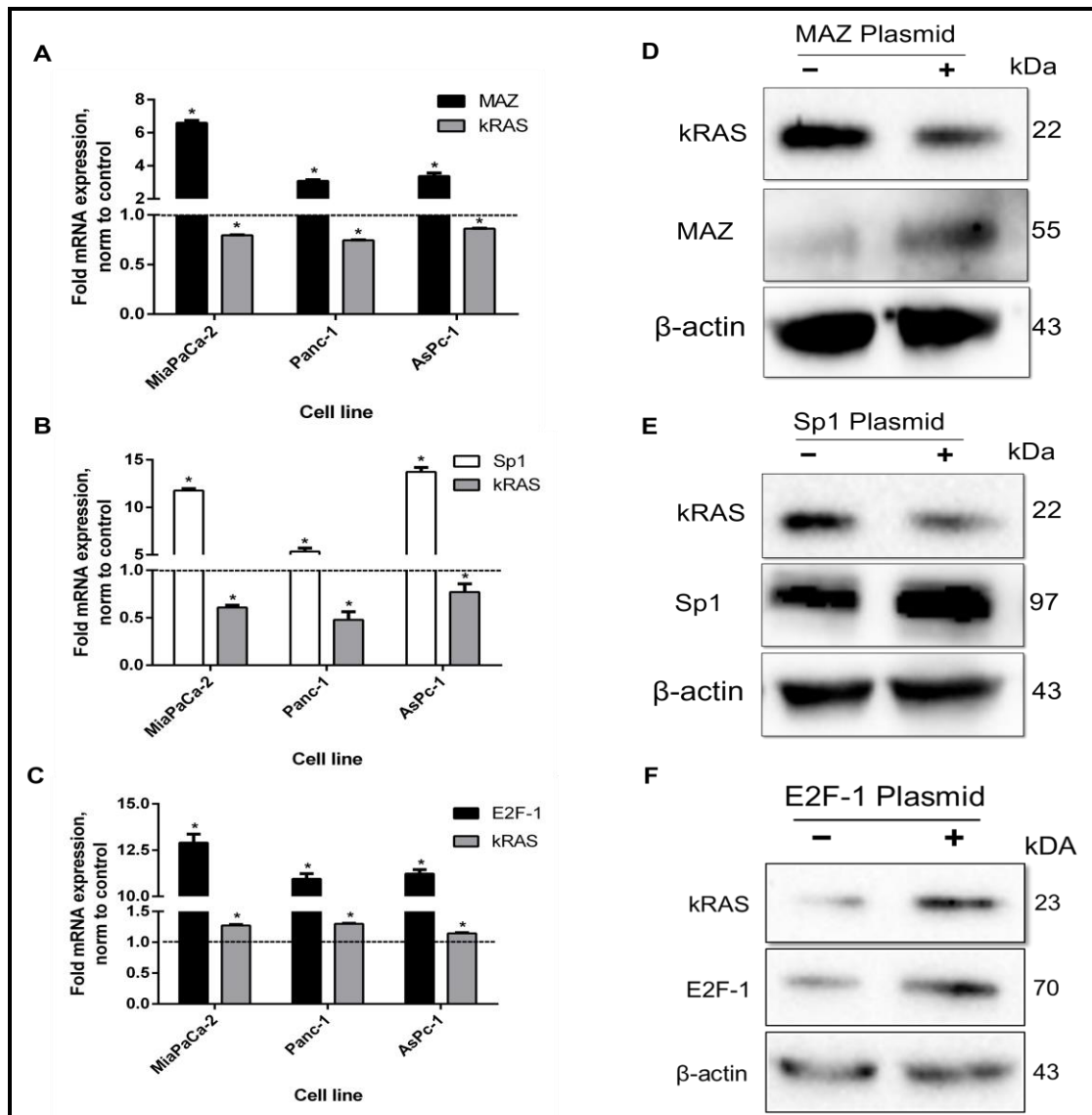
Figure 3-5. kRAS, MAZ, Sp1, and E2F-1 expression change on pancreatic cell lines. The pancreatic cancer cells MiaPaCa-2, Panc-1, and AsPc-1 were transfected with increasing MAZ, Sp1, and E2F-1 expression plasmid; the mRNA expression of MAZ, Sp1, E2F-1, and kRAS were monitored. (A) The MAZ:kRAS promoter interaction in the pancreatic cell lines showed similar results as luciferase assay. The decrease in mRNA kRAS expression with increasing MAZ expression. (B) The Sp1:kRAS promoter interaction in the pancreatic cell lines followed the same pattern as MAZ. (C) The E2F-1:kRAS promoter interaction the increase in mRNA kRAS expression with increasing E2F-1 expression was observed. The protein level by western blot at 500, 250, and 100 ng of MAZ, Sp1, and E2F-1 respectively also followed the RT-qPCR results. *$p < 0.05$ (versus control), as determined by Student's t-test. All experiments were performed in a minimum of triplicate.

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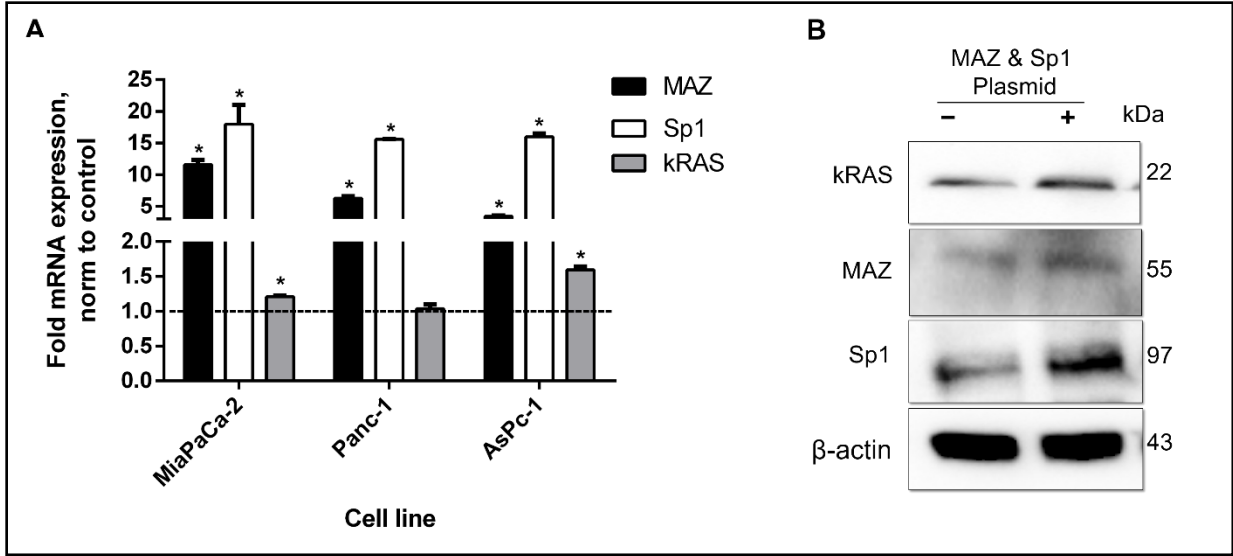
Figure 3-6. MAZ + Sp1 combination effect on pancreatic cancer cell lines. The pancreatic cancer cells MiaPaCa-2, Panc-1, and AsPc-1 were transfected with MAZ (500 ng) + Sp1 (100 ng) plasmid; the mRNA expression of MAZ, Sp1, and kRAS were monitored. (A) Combination of MAZ (500ng) and Sp1 (100ng) abrogated the decrease shown by MAZ and Sp1 individually and lead to significant increase in fold mRNA kRAS expression in AsPc-1 and MiaPaCa-2 cell lines. (B) The protein level by western blot showed the darkening of the band. *p< 0.05 (versus control), as determined by Student's t-test.

3.4. Pancreatic cancer cell line specific TBB effects on kRAS

        The protein CK2 is capable of phosphorylating MAZ and Sp1, and modulating their affinity for binding DNA. Thus, we studied the effect of the CK2 inhibitor, TBB, on kRAS promoter activity transcript and protein expression. The panel of pancreatic cancer cells were treated with a dose-range of TBB to determine its IC_{50} in all the cell lines. The 48 hr IC_{50s} for TBB was determined to be 28 ± 0.6, 27 ± 0.1, and 13 ± 0.3 µM for MiaPaCa-2, Panc-1 and AsPc-1 respectively (**Figure 3-7A**). Cells were treated with their respective IC_{50s} of TBB for 48 hr and the mRNA levels of kRAS, MAZ and Sp1 were all monitored. The results were varied across cell lines, with a significant decrease in kRAS measured in MiaPaCa-2 and Panc-1 cells, but significantly increased kRAS noted in AsPc-1 cells (**Figure 3-7B**). Unexpectedly, significant changes were also noted in MAZ transcription with a decrease in MiaPaCa-2 cells, and in Sp1 transcription with an increase in AsPc-1 cells. The mechanisms of such changes need to be determined. Furthermore for the protein level also followed the RT-qPCR results with increase kRAS protein levels in AsPc-1 and decrease in MiaPaCa-2 and Panc-1 cells (**Figure 3-7C**).
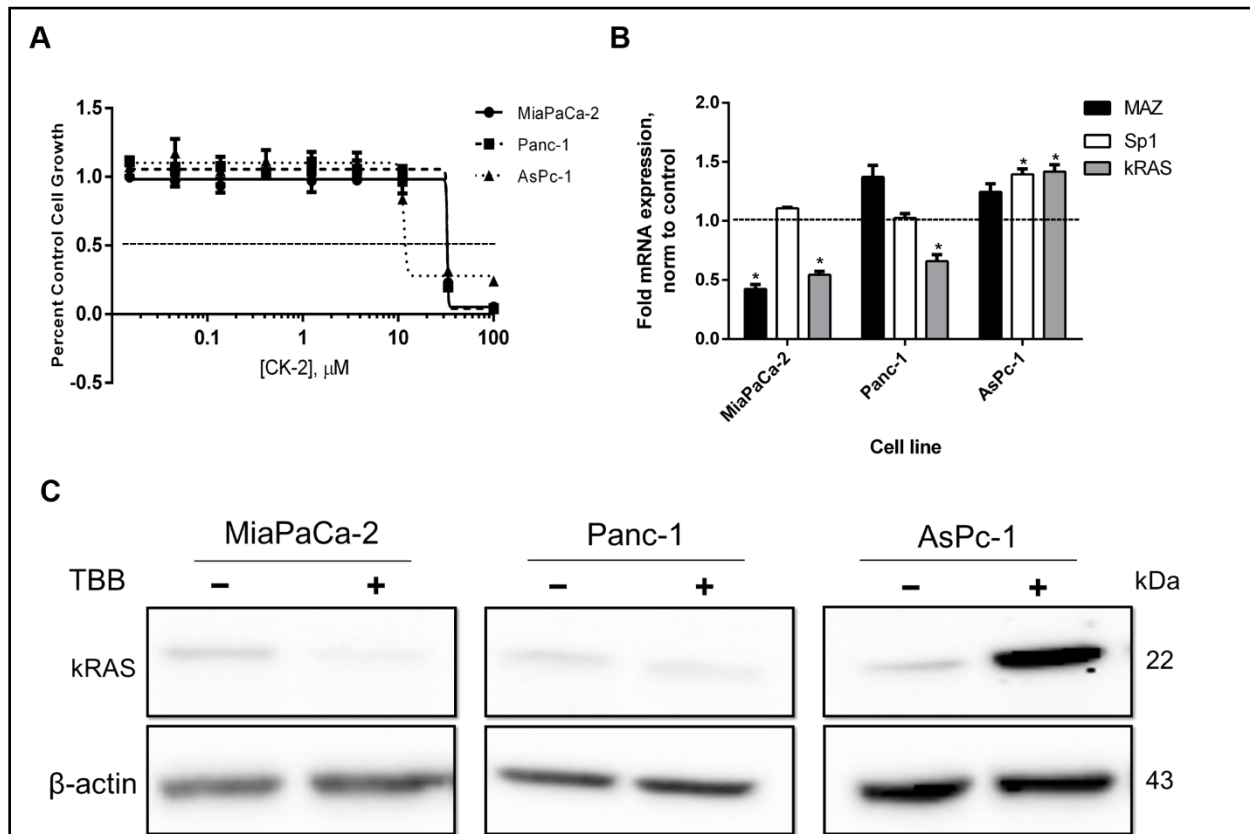
Figure 3-7. TBB effect on kRAS expression in pancreatic cancer cell lines. (A) IC_{50} values of pancreatic cancer cells treated with TBB after 48 hr. (B) MiaPaCa-2, Panc-1, and AsPc-1 were treated with CK2 at their determined IC_{50s}. There was an increase in fold change mRNA expression of kRAS in AsPc-1, whereas TBB lead to significant decrease in MiaPaCa-2. Changes in level of protein level on kRAS was also seen (C). *$p < 0.05$ (versus control), as determined by Student t-test. All experiments were performed in a minimum of triplicate.

*3.5. MAZ, Sp1, and E2F-1 preferential binding to kRAS promoter regions and topologies*

G4-forming regions are commonly associated with regulatory proteins such as Sp1, which most often binds to dsDNA (Kumar et al. 2011), but stabilize some G4 structures (Raiber et al. 2012; Piekna-Przybylska et al. 2014). MAZ binds to and unfolds the hRAS promoter G4, leading to the reactivation of gene expression (Cogoi et al. 2014). Recently in the same study where Sp1 bound H19 gene promoter G4 structure, E2F-1 also interacted with its G4 (Fukhura et al. 2017). With such variations in structure recognition and effects, the binding of MAZ, Sp1, and E2F-1 to varying topologies of the $G4_{near}$, $G4_{mid}$, and core kRAS promoter regions was examined using EMSA. MAZ bound near ssDNA, all regions in dsDNA with more preference to near and core region over mid region, and to both $G4_{near}$ and $G4_{mid}$ region of the kRAS promoter. Overall, MAZ showed preferable binding to near regions of all three topologies (**Figure 3-8A**). Sp1 bound mid-ssDNA, dsDNA, and G4-DNA respectively. Sp1 also showed binding to the near dsDNA (**Figure 3-8B**). Finally, E2F-1 ubiquitously bound all the regions and the topologies in the kRAS promoter (**Figure 3-8C**).

By ECD, further investigation of the effect of MAZ, Sp1, and E2F-1 to the kRAS promoter G4s stability was studied to find out any G4-interactive proteins. No significant effect on G4 stabilization was observed with MAZ or Sp1 (**Figure 3-9**). The E2F-1 pronouncedly destabilized $G4_{near}$ by decreasing melting temperature by 7.68 °C (**Figure 3-9**). MAZ and E2F-1 modestly stabilized $G4_{mid}$ by 2.56 and 2.11 ºC respectively. On the other hand, Sp1 led to moderate destabilization of G4mid by 2.57 ºC (**Table 3-1**). ΔT_M for MAZ and Sp1 compared to control to -2.56 ºC and -2.57 ºC change in mid G4 respectively (**Table 3-1**). No effect on the number of G4 isoforms present compared to control was observed with MAZ, Sp1, and E2F-1 can be denoted by less than 1% change of the Hill slope as compared to the control.
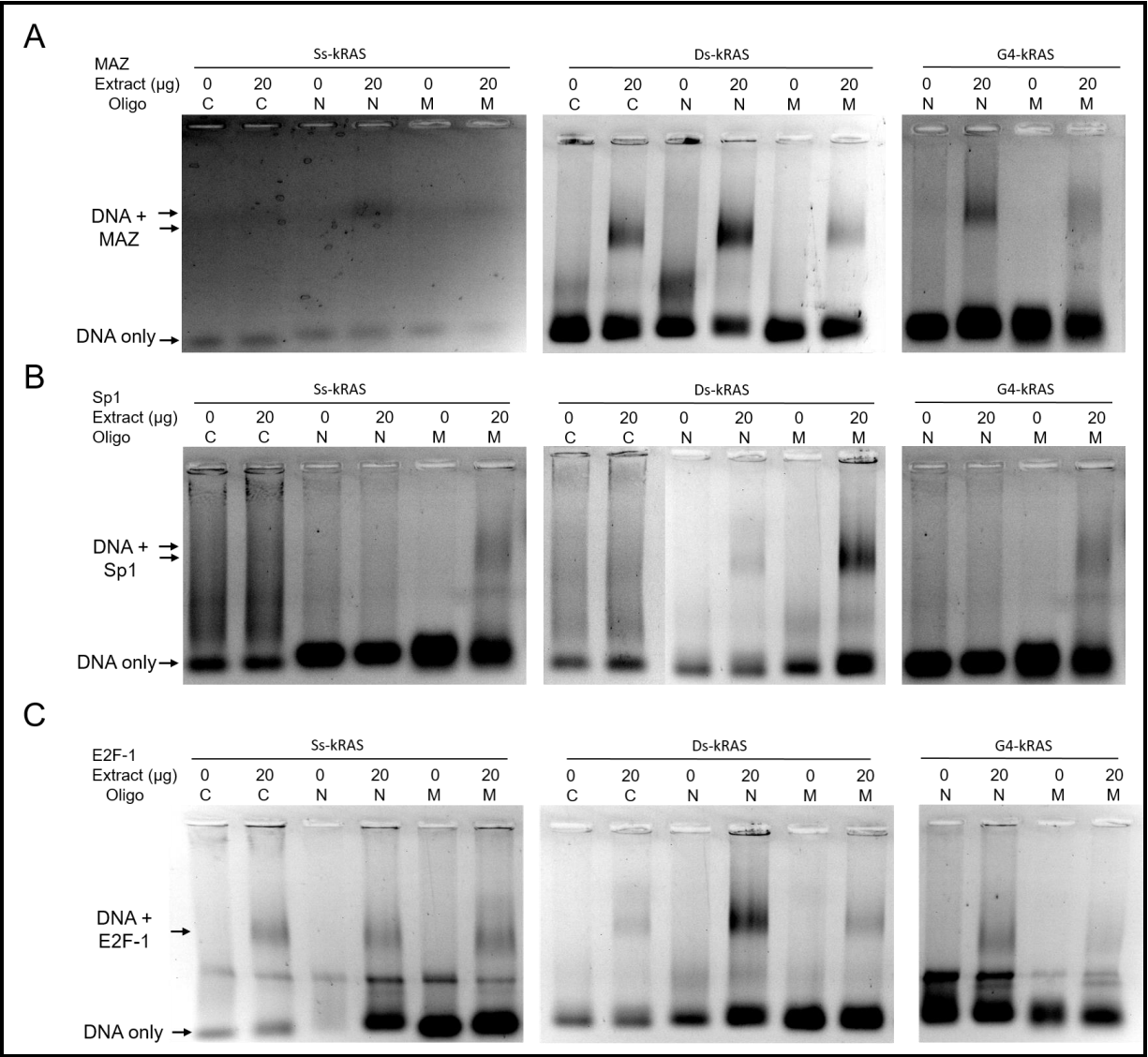
Figure 3-8. MAZ, Sp1, and E2F-1 binding to the kRAS promoter. EMSA was performed to show binding of MAZ and Sp1 to different topologies (ss-, ds, and G4-DNA) and regions (core, near, and mid). MAZ showed preferable binding to near (A), whereas Sp1 bind preferably to mid all topologies (B). E2F-1 showed binding to all the regions and topologies (C).
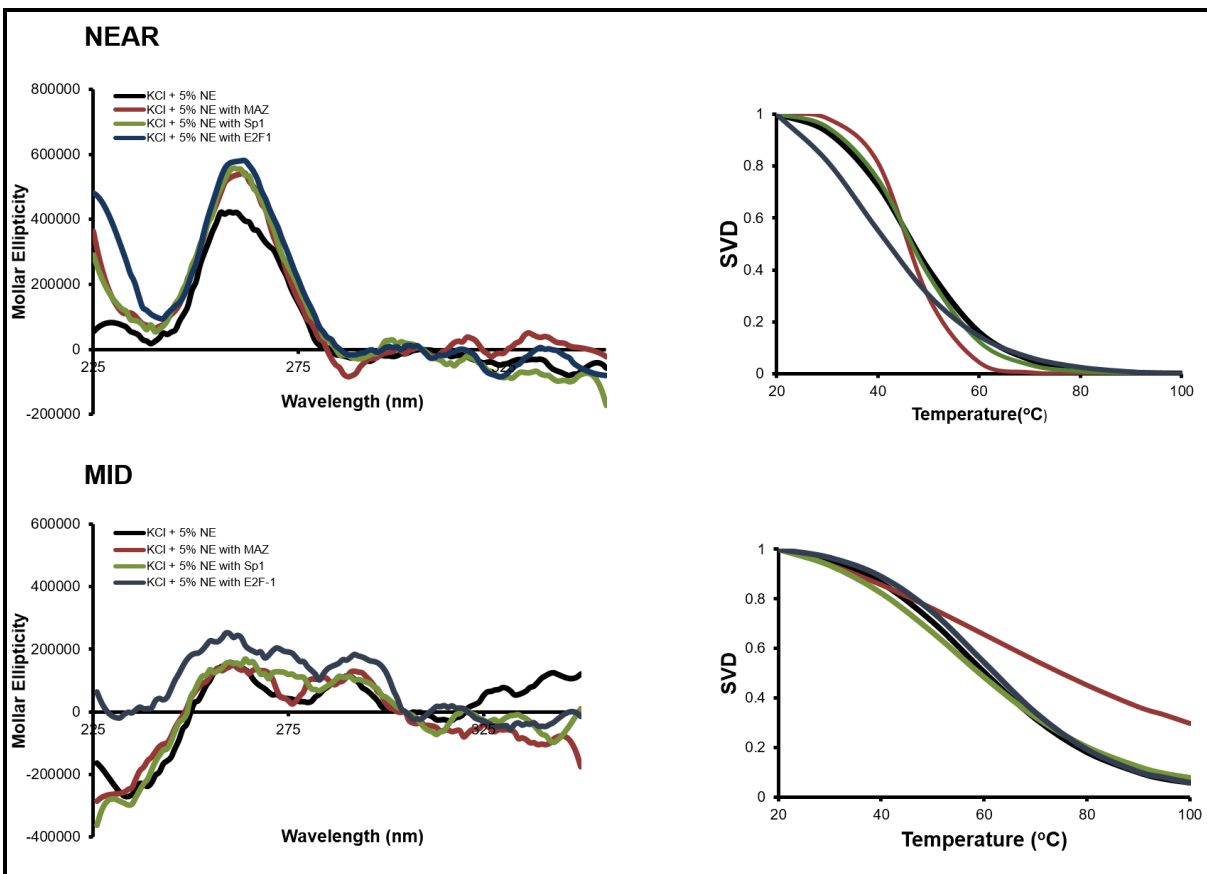
Figure 3-9. MAZ, Sp1, and E2F-1 effect on G4_near and G4_mid stability. By ECD, further investigation of the effect of MAZ, Sp1, and E2F-1 binding to G4-DNA was studied. No significant effect on G4 stabilization was observed with MAZ or Sp1. MAZ led to a slight right shift in the G4_near SVD graph, which is indicative of stabilization of G4 structure. E2F-1 leads to a left shift in the G4_near SVD graph, which is indicative of destabilization of G4 structure. Not much effect on number of G4 isoforms were seen.

Table 3-1. Effect of proteins on G4 stabilization. Denoted by ΔT_M and number of G4 isoforms shown by percent change of $G4_{near}$ and $G4_{mid}$ compare to control (KCl + 5% NE).

NEAR	KCl + 5% NE	KCl + 5% NE with MAZ	KCl + 5% NE with Sp1	KCl + 5% NE with E2F-1
TM	46.5	46.2	46.57	38.82
ΔTM	--	-0.3	-0.07	-7.68
Hill Slope	-0.05587	-0.1006	-0.06621	-0.04014
Percent Change	--	80.06	-1.03	-39.18

MID	KCl + 5% NE	KCl + 5% NE with MAZ	KCl + 5% NE with Sp1	KCl + 5% NE with E2F-1
TM	58.72	N/D	56.15	60.83
ΔTM	--	N/D	-2.57	2.11
Hill Slope	-0.03527	-0.01786	-0.0299	-0.03719
Percent Change	--	-49.36	-15.22	5.44

3.6. Discussion

The region of the kRAS promoter between +50 and -510 has been previously identified as being essential for its expression, but the TFs contributing to promoter region have not yet been fully described. In this study, we mapped particular regions of the kRAS promoter for TF binding sites, and further evaluated the binding and function of the TFs MAZ and Sp1 to the kRAS promoter region, with a focus on three regions of interest – two G4-forming regions (near and mid) and the core region from 0 to +50, relative to the TSS. MAZ and Sp1 individually act as transcriptional silencers for kRAS expression as demonstrated in plasmid studies and pancreatic cancer cell lines. Interestingly, the combination of MAZ and Sp1 leads to an increase in kRAS promoter activity in both plasmids and pancreatic cancer cell lines. The role of CK2, a protein kinase that can phosphorylate MAZ and Sp1 was also studied; changes in kRAS expression were not consistent across cell lines as it decreased in MiaPaCa-2 and Panc-1 cells but increased in AsPc-1 cells. It is worth noting that the TFs were not overexpressed in this experiment, and the interplay between expression and phosphorylation is an area still under investigation. We also demonstrated preferential affinity of the TFs for varying regions and DNA topologies within the kRAS promoter.

To comprehensively understand the regulation of transcription initiation, one has to know which TFs are controlling which gene. We used PROMO, which uses TRANSFAC database for TF mapping studies. Our putative binding search showed that there are several consensus sites for binding of several factors to the core promoter region: Sp1 (one), E2F-1 (four), WT1, GR-alpha, p53, STAT4, and NF-AT1/2. Furthermore, near- and mid- G4-forming regions each contain a number of putative transcriptional regulator binding sites as well, including two for MAZ (one at the end of the near region and one in the midst of the mid-region), three for Sp1 (one in the near

region and two within the mid-region), and several for p53, E2F-1, STAT4, WT1, NF-kB, and more (Morgan et al. 2016).

We found out that MAZ binds different regions and topologies in kRAS promoter and this interaction leads to decrease in kRAS promoter activity, transcripts and proteins level. Our finding here contradicts the work done by Cogoi group, in which they showed MAZ binding to near G4 and duplex kRAS and this interaction leads to 3.5 fold increase in kRAS transcriptional activity in Panc-1 cell lines (Cogoi et al. 2013). In murine kRAS promoter by luciferase assay, they proved that MAZ as a kRAS transcriptional activator (Cogoi et al. 2010). We accomplished our studies by a step-by-step approach, starting from the basic level to minimize the variables in our studies. We first focused on the effect of MAZ in an isolated system of non-cancerous cell lines (HEK-293), such as less noise or variation due to other factors as present in cancerous cells. Secondly, we took in account broader panel of pancreatic cancer cell lines based on their kRAS addiction pattern, and we also calculated the level of these proteins at basal levels in cell lines which are not accounted for in previous studies. Further, we confirmed RT-qPCR transcriptional downregulation by western blotting and found similar results showing reduce kRAS protein levels in these cell lines at 500 ng of MAZ plasmids. Lastly, cell passage number plays a critical role in studies. The studies performed by Xodo group doesn't report the passage number of cells, this variability in result might be due to older cell lines used. It has been shown previously with other cell lines that expression levels of genes vary with different passage number (Chang et al. 1997; Driscoll et al. 2006; Hughes et al. 2007). On the other hand, to overcome this variability we performed our studies with freshly acquired stock from ATCC.

Further work has been done to understand the transcriptional mystery behind kRAS. Recently one such mechanism was studied. kRAS-E2F1-ILK-hnRNPA1 regulatory loop has been

linked to oncogenic kRAS signaling promotion. This signaling involves destabilization of the G4 on the kRAS promoter by hnRNPA1, this activated kRAS that through E2F-1-mediated transcriptional activation lead to the increase expression of ILK (Chu et al. 2016). It was further shown that the destabilization by hnRNP A1 might be due to the unfolding of the kRAS G4 structure (Cogoi et al. 2017).

The TF MAZ is present in most, if not all, tissues (Bossone et al. 1992; Kennedy & Rutter 1992; Pyrc et al. 1992; Parks & Shenk 1996). MAZ was first identified as a GA-box–binding TF in the c-MYC promoter, where it controls transcription initiation and termination (Bossone et al. 1992). MAZ has a complex functional role as it activates some genes (Her et al. 2003; Parks & Shenk 1996; Song et al. 1998; Parke & Shenk 1997; Leroy et al. 2004), and represses others (Himeda et al. 2008; Palumbo et al. 2008). We identified a silencer function for MAZ alone in relation to the kRAS gene, although this finding disagrees with previous reports (Cogoi et al. 2010), as described above. However, in combination of its partner Sp1, this silencing effect was abrogated.

Sp1 (5′-(G/T)GGGCGG(G/A)(G/A)(C/T)-3) recognition sites highly overlap with the consensus binding sequences for MAZ (5'-GGGGGAGGGGGG-3') (Kadonaga et al. 1986; Ashfield et al. 1994), both sequences are often found in G4-forming regions. The Sp/XKLF (Specificity protein/Krüppel-like factor) family first member identified was Sp1, which has three contiguous Cys2His2 Zinc (Zn) finger DNA-binding domains, with Zn fingers 2 and 3 being essential for DNA binding activity (Song et al. 2001). Sp1 forms complexes with many factors associated with transcription (Li et al. 2004) and can act as transcriptional activator or repressor. Recent CHIP-on-CHIP studies suggested the overlap of Sp1 (87%) binding sites with G4 forming sequences. Which indicates Sp1 as an important G4 interactive protein. Since then several studies have been published related to binding of Sp1 to several genes G4. In the c-KIT gene, Sp1 TF was

shown to bind the G4 formed in the Sp1 binding sites (Raiber et al. 2012). Sp1 was also shown to bind the HIV-1 promoter G4 and alter the promoter activity through this binding (Piekna-Przybylska et al. 2014). Recently it was shown that Sp1 also binds to the G4 in H19 gene and this interaction suppresses the H19 transcription (Fukhura et al. 2017). Linking kRAS promoter to Sp1 has not been done before. Our studies demonstrated Sp1 acts as a transcriptional silencer for kRAS expression though no effect or minimal effect on kRAS G4 stabilization.

Interestingly, the combination of MAZ and Sp1 expression mediated either no change or an increase in kRAS expression in a cell line-specific manner. The binding of both proteins is also regulated at the phosphorylation level by the kinase CK2. We indirectly studied the role of CK2 with its inhibitor TBB, and the findings were even more confounding, with a decrease in kRAS noted in MiaPaCa-2 and Panc-1 cells, but an increase in AsPc-1 cells. The underlying mechanisms of these changes remain to be determined. The role of CK2 in phosphorylation of MAZ and Sp1 their effects on another G4-containing promoter, c-MYC, was previously studied. In the 5'-end promoter region of the c-MYC gene phosphorylation of MAZ by CK2 enhanced the binding affinity of MAZ to the G4-forming pyrimidine-rich region of the nuclease-hypersensitive element (NHE) (Tsutsui et al. 1999). On the other hand, CK2 phosphorylation of the C terminus of Sp1 resulted in a decrease in DNA binding activity; ultimately the differential binding of the proteins led to transcriptional downregulation (Armstrong et al. 1997). In our studies treatment with CK2 inhibitor, TBB had cell specific effect on pancreatic cancer cell lines which might be related to different basal levels of proteins, varied effects of binding of phosphorylated MAZ and Sp1, or another unknown mechanism.

The E2F-1 was our selected third hit. The E2F TFs are key regulators of cell cycle progression. They are known to control the transcription of gene products required for S phase.

Whereas the recent microarray and CHIP studies shown wide variety of functions of E2F proteins such as cell cycle transitions, differentiation, apoptosis, DNA repair, and checkpoint signaling (Ishida et al., 2001; Ma et al., 2002; Muller et al., 2001; Polager et al., 2002; Ren et al., 2002). Ten E2F family member genes, E2F1-E2F8 (He et al., 2000; Leone et al., 2000) exist. Among the E2F family members, E2F-1 is unique in its ability to regulate a number of key genes that participate in both cell cycle progression and apoptosis and behaves as both oncogene and a tumor suppressor gene (Yamasaki et al., 1996). Thus, E2F-1 is a multi-functional damage responsive protein that is involved in numerous aspects of the DNA damage response. Recently it was shown that E2F-1 binds to G4 structures in H19 gene (Fukhura et al. 2017). H19 is a gene found in humans which is related to long noncoding RNA. Physiologically H19 is required for cell proliferation and as a negative regulator (or limiting) of body weight (Gabory et al. 2009). H19 is highly expressed in various cancers such as breast, colorectal, bladder, endometrial, esophageal, and lung (Kondo et al. 1995; Elkin et al. 1995; Adriaenssens et al. 1998; Tanos et al. 2004; Ariel et al. 2000). H19 gene is responsible for tumor cell proliferation, colony formation, and tumor metastasis (Lottin et al. 2002; Berteaux et al. 2005; Matouk et al. 2014).The interaction of H19 gene G4 to E2F-1 leads to increase in endogenous H19 levels indicative of its role as transcriptional activator.

Overall, we have tried to understand the binding and functional role of MAZ, Sp1, and E2F-1 in relation to the kRAS promoter. MAZ and Sp1 act as a transcriptional silencers for kRAS and show binding to different DNA topologies, including the G4s. On the other hand E2F-1 acts as transcriptional activator and binds to all DNA topologies and leads to significant destabilization of G4$_{near}$. Identification of the major transcriptional silencers and their effect on the dynamic regulation of the kRAS promoter will provide an insight that is necessary for the development of novel drug therapies, as this information can be a tool for future drug discovery related to kRAS

transcriptional regulation. The strategies have been developed at downregulating kRAS by blocking the formation of DNA–protein complexes at NHE by triplex-forming oligonucleotides (Paramasivam et al. 2008) and use of decoy oligonucleotides against proteins binding to NHE of hRAS promoter (Cogoi et al 2009; Cogoi et al. 2013). The understanding of significant TFs regulating kRAS is necessary for the comprehension of a mechanism of action of exogenous (small molecule) regulators of the kRAS expression and to utilize proteins as a target for pancreatic cancer therapeutics in the future which has an abysmal 5-year survival rate (9%) and requires novel therapeutic advancement to overcome the peril of pancreatic cancer.

CHAPTER 4. GLOBAL BINDING OF TFs TO THE kRAS PROMOTER

4.1. Introduction

The largest group of human genes are enzymes, which account for 10% of all human genes; TFs are the second largest group of proteins, comprising 6% (Venter et al. 2001). There are 1510 TFs (http://dbd.mrc-lmb.cam.ac.uk/DBD/index.cgi?Home), according to the current human TF database, out of which less than 5% have been purified and characterized (Gadgil et al. 2001). In the previous chapter, we did mapping studies on kRAS promoter which were based on weight matrices and evolution. We found out that nearly 150-200 TFs bind to the kRAS promoter different regions. Physical mapping and understanding the biological activity of these proteins is of utmost importance in determining how genes are regulated. To understand these interactions, it is necessary to understand the major proteins which are involved in transcriptional machinery.

Promoter regions contain several parts that play a vital role in transcriptional regulation. The core promoter is regarded as the region of DNA, typically spanning from positions -50 to +50 relative to the transcription start site that is necessary to drive transcription of RNA polymerase II (reviewed in Juven-Gershon et al. 2009). RNA polymerase II along with a number of basal TFs are needed to drive transcription. Transcription Factor IIA (TFIIA), TFIIB, TFIID, TFIIE, TFIIF, and TFIIH are the essential factors for RNA polymerase II-dependent transcription (Thomas & Chiang 2006).The core promoter is comprised of TATA box, initiator element, Downstream Promoter Element (DPE), Motif Ten Element (MTE), TFIIB Recognition Element (BRE), and binding sites for RNA polymerase holoenzyme. TATA boxes, in eukaryotes, is frequently required

for initiation of transcription. Approximately 30 nucleotides upstream of the transcription start site (TSS) exist an A/T-rich sequence named as TATA box was the first core promoter element discovered (Goldberg 1979). Aside from the TATA box, PIC assembly also requires several other elements (Smale et al. 2003; Juven-Greshon et al. 2008; Butler et al. 2002). The first indication for this came from the observation that the TBP-Taf complex TFIID gives an extended DNase I footprint downstream from the transcription start site of certain promoters, suggesting that there are elements besides the TATA box that can interact with the transcription machinery in a sequence-specific manner (Sypes et al. 1994; Nakatani et al. 1990). Further investigation led to the discovery of the Inr (Initiator), DPE (Downstream Promoter Element), DCE (Downstream Core Element), and the MTE (Motif 10 element), all of which are conserved from drosophila to humans and directly interact with various TFIID subunits (Burke et al. 1996; Burke et al. 1997; Burke et al. 1998; Kadonaga 2002; Kutach et al. 2000; Lim et al. 2004). Additionally, the BRE elements, located upstream and downstream of the TATA box (independently or together) are recognized by the general transcription factor TFIIB (Deng et al. 2005; Deng et al. 2006; Deng et al. 2007).

TATA box, it is utilized by a limited number of promoters (approximately 20%), and other elements are equally important for PIC assembly (Basehoar et al. 2004; Yang et al. 2007). Extensive mapping of transcription start sites and promoter elements in humans and mice revealed that the majority of promoters of genes with constitutive levels of expression lack a well-defined transcription start site or a TATA box (Yang et al. 2007; Carninci et al. 2006). The class of promoters which initiate from a single well-defined nucleotide is much smaller and is enriched in highly expressed tissue-specific genes regulated by a TATA box (Carninci et al. 2006). The majority of genes fall into the TATA-less category and overlap extensively with the class of genes

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that are TFIID dependent and have steady levels of expression (Basehoar et al. 2004). kRAS is a TATA-less promoter whose transcription regulation is not yet fully understood. These various elements in the core promoter localize the binding of the RNA polymerase. After binding to the DNA, the polymerase converts from the closed complex to the open complex to be available to other co-regulators. To reveal the mechanism of gene expression of kRAS, the promoter regions and binding sites for TFs must be characterized. For TATA-less promoters, the other core promoter elements usually involved in transcriptional initiation include the binding of TFIID-associated TBP-associated proteins (TAF) (Smale & Kadonaga 2003; Maston et al. 2006).

The activation or inhibition of transcription involves DNA response elements (REs) in promoter regions, which are the binding sites for TFs. Other than common elements, GC-rich promoters like kRAS consist of the GC-box element, to which TF's like Sp1 bind (Yamamoto & Perucho 1988). Other than that there are developmentally specific (e.g., the B3 TF of Xenopus oocytes), tissue specific (e.g., MyoD), and hormone specific TF's (e.g., the estrogen receptor) also (Cooper 2000). A better understanding of transcriptional machinery requires promoter characterization. Promoter characterization requires identification of each DNA element and their protein binding profile. We showed in chapter 3 multiple putative binding sites for TFs in the kRAS promoter regions. Herein we explore proteins shown to bind within the proximal promoter region of the kRAS gene.

TFs can bind specifically to DNA cis-elements, modulating their binding in response to external stimuli generally via protein or metabolite allosteric interactions. In making this connection, they become the critical link in transferring information from the environment into functional consequences. The prototypical prokaryotic TFs are mainly of the helix-turn-helix

(HTH) fold family that selectively bind to the major groove of DNA using the second helix of the TF (Cooper 2000).

TF functional investigation in gene regulation, biochemical, and structural studies involves protein purification as a first step. Most complications in identification arise due to the TF purification step, believed to be related to their large numbers and low abundance in human cells (around <0.1%) (Woo et al. 2002), often leading to significant amounts of starting material required for investigation.

Several methodologies have been employed to study the binding of TF to promoters. From the early 1980's, EMSAs have been used to quantify DNA binding activity and protein binding (Matsuoka et al. 2003; Fried & Crothers 1981). The EMSA is a relatively simple procedure in which an oligonucleotide of usually 20–25 bp, containing a response element (RE), and nuclear extract or recombinant protein are incubated in binding buffer. When subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE), any TF-DNA complex migrates more slowly than the free DNA, which can be visualized radioactively, fluorescently, or with ultraviolet light. The combination of EMSA with proteomic techniques is frequently used for TF identification from complex intracellular milieus. Two-dimensional gel electrophoresis (2-DE), which is a combination of isoelectric focusing (IEF) and SDS-PAGE, is an example of TF-DNA binding technique (Woo et al. 2002). Efficient renaturing of protein from a denaturing (SDS-PAGE) gel for DNA binding activity analysis by EMSA is a prerequisite in this method, which can only detect and characterize monomeric or homodimeric proteins. The second dimension of SDS-PAGE is applied to the proteins bands and samples are identified by MS.

The binding affinity of TFs to REs is usually in the nM-pM range. In traditional affinity chromatography, DNA is covalently attached to columns at µM or higher concentrations, which

often leads to nonspecific protein binding and over-identification of proteins. Promoter trapping was developed to overcome this shortcoming. In promoter trapping, nM concentrations of TFs are incubated with duplex DNA (oligonucleotide or promoter) with a $(GT)_5$ single-stranded tail, enabling the formation of the specific TF-RE complex in solution. An $(AC)_5$–sepharose column traps the complex by annealing with the single-stranded $(GT)_5$ of the oligonucleotide or promoter (Gadgil & Jarrett 2002; Moxley & Jarrett 2005; Jiang et al. 2008). To further prevent the binding of non-specific proteins, the trapping mixture is supplemented with specific binding modifiers, such as heparin, T18, poly dI:dC and non-ionic detergent. Lastly, the trapped protein is eluted by a high salt to disrupt TF-RE interactions, or by low salt with moderate temperatures. It must be taken into consideration that the single stranded $(GT)_5$ tail at 3′-end of double-stranded oligonucleotide or promoter DNA leads to a nonspecific binding of hnRNP family members and several other abundant nuclear proteins.

DNA affinity chromatography is the oldest technique used which takes into consideration that TFs show higher affinity for binding to their consensus sites, as compared to for non-specific DNA. In this technique, TFs are purified by exploiting their inherent capacity to bind to DNA by utilizing chromatographic support (Vaquerizas et al. 2009). Currently, TF purification often involves four or five different chromatographic steps that may include gel filtration, ion exchange, and sequence-specific/nonspecific DNA affinity columns (Gadgil et al. 2001). Another modification of DNA affinity chromatography is stable isotope labeling by/with amino acids in cell culture (SILAC) (Mittler et al. 2009). It combines stable isotope labeling with an amino acid in cell culture and one-step DNA affinity chromatography with quantitative proteomics to identify specific TFs that bind to the methyl-CpG site in a promoter. It gives a better prediction and less background by preventing identification of non-specific proteins (Mittler et al. 2009). These

approaches are multiple-step, are not relevant to all research as they require homogenous tissue culture, and are laborious.

In our experiments, we utilized a streptavidin-agarose pulldown approach to analyze protein-DNA binding. This technique overcomes the shortcomings of EMSA, promoter trapping, and DNA-affinity chromatography. It is an easy assay with minimal steps, not requiring special columns or radiolabeled probes. The number of purification steps are fewer, as compared to DNA-affinity chromatography. The 5'-biotinylated double-stranded DNA probes and streptavidin-agarose beads are incubated with nuclear extract proteins. The proteins are eluted from the pulled down complex with 1M NaCl and analyzed by capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Wu 2006).

TF purified by the above-mentioned procedures exist in very minute amounts (femtomole, 10^{-18}) sample volumes. Only LC-MS/MS, or other sensitive proteomic approaches, can be used for the characterization and identification of such small quantities of proteins. This process requires several critical steps from sample preparation to analysis. The first step requires the removal of contaminants such as plastics, salts, detergents, buffer components and any others that may interfere with digestion. This is followed by pre-concentration, desalting, separation, and ionization (Grosveld et al. 2005; Sebastian et al. 2002). The most critical step involves the digestion of the TFs with sequence-specific exogenous protease(s); two types of digestions are possible: in-gel and on-blot digestion.

To understand the function of TFs on kRAS expression, we first must identify the proteins that bind to its promoter regions of interest. We hypothesized that several proteins bind to the kRAS promoter different topologies and the binding of these proteins are expected to have a different effect on kRAS transcription. Some might act as transcriptional activators, others as

inhibitors, while still other may have no relevant biological activity. To investigate the hypothesis, and overall tactic, a DNA pulldown-based purification approach, combined with proteomic techniques for TF identification was used. Binding studies to different topologies of kRAS (ssDNA, dsDNA, G4 DNA) of the regions of interest (core, G4$_{near}$, and G4$_{mid}$) within the kRAS promoter were performed with nuclear extract to characterize protein interactions with the kRAS core promoter region. Further, the biological role of these protein:DNA interactions to determine the functional outcomes of these binding was determined.

4.2. Binding and affinity of regulatory proteins and the kRAS promoter

To identify and characterize different regulatory proteins binding to the kRAS promoter we utilized three approaches. The first approach utilized a DECipherment Of DNA Elements (DECODE) database of proteins binding with the kRAS promoter. Several proteins were shown to bind to the kRAS promoter. AP-2 and PPAR-γ were selected from the list as they show binding in the core kRAS promoter region ± 50bp, which is involved in transcriptional initiation. The whole genome rVISTA search portal (Zombon et al. 2005) showed four binding sites for AP2 and one binding site for PPAR-γ (**Figure 4-1**).

The second approach utilized a Promoter Binding TF Profiling Plate Array kit (Signosis, Santa Clara, CA). To characterize the TFs that bind to the kRAS core promoter region (-510/+50) and potentially regulate the activation of the kRAS gene, 48 TFs were assessed for binding to the kRAS promoter that was amplified by PCR from Panc-1 cell DNA, using a competitive promoter-binding TF profiling array. The kRAS promoter is a TATA-less promoter (Jordano & Perucho 1988); the protein TFIID binds to the TATA box only, so we were able to use it as a control to normalize across duplicate data sets (Sawadogo & Sentenac 1990; Signosis protocol). A significant change in transcriptional activity was assumed by 1.5-fold up- or down-regulation. Statistical differences were determined by comparing the RLU of TFIID by use of a two-sided unpaired Student's t-test (* $p \leq 0.05$). Of the 48 total TFs, 14 showed an ability to compete for binding to the kRAS promoter at significant levels (**Figure 4-2B**), including GATA (subtype undetermined), Gr/PR, HIF, MYC-MAX, NF-1, NFAT, NF-E2, NF-κB, PPAR (subtype undetermined), Sp1, Stat1, Stat3, Stat4, and Stat5.

For the third approach, the proteins that bind various regions and topologies of the kRAS promoter were evaluated by us, in collaboration with the laboratory of Dr. Joshua Sharp, by DNA

pulldown-LC/MS/MS (Wu 2006), which involves two major steps. First, biotinylated oligonucleotides for different topologies (ss-, ds-, and G4-DNA) and regions (core, G4$_{near}$, and G4$_{mid}$) were incubated with nuclear extract from Panc-1 and AsPc-1 respectively to form the DNA-protein complex. This complex then underwent several steps of washing and cleaning before proteins were eluted with buffer containing 1M NaCl; excess detergent was removed using the Pierce™ Detergent Removal Spin Column. A BCA assay was performed on the eluted proteins to determine their concentrations, and proteins were identified and characterized by LC-MS/MS (**Figure 4-3**). Data showed a significant number of proteins from Panc-1 and AsPc-1 nuclear extract binding to different regions and topologies of the kRAS promoter (**Figure 4-4**). Proteins of greatest interest were filtered on the basis of different # of spectra identified, their rank, and role in transcription. # of spectra identified is a characteristic in MS identification and provides a maximum probability of finding these proteins in samples. We had 33 proteins after the last step (**Table-2**). We further selected 15 proteins including nucleophosmin, nucleolin, YBX-1, S100 A8/A9, hnRNPA1, TRIM28, PABPC-1, IGF2BP1, THO, 14-3-3, LYRIC, and BTF3 based on their binding to different regions and topologies. Specifically, nucleolin, YBX-1, S100 A8/A9, and 14-3-3 bind to all regions and topologies. Addgene database was checked for the availability of plasmids for future work. Out of them AP-2, PPAR-γ, YBX-1, and nucleolin were selected for subsequent biological investigation.

PPAR-γ

chr12:25,393,854   25,423,854

PPAR-gamma1 | | | |

Legend: ↵ Transcription Starting site of kRAS | Transcription factor binding site
Scale: ⌐ 670 bp

AP-2

Chrom	Start	End	Strand	Relative Start	Relative End
chr12	25403898	25403909	+	44	55
chr12	25403898	25403910	-	44	56
chr12	25403899	25403910	-	45	56
chr12	25403899	25403914	+	45	60
chr12	25403900	25403915	-	46	61
chr12	25403902	25403914	-	48	60
chr12	25403904	25403916	+	50	62
chr12	25403904	25403919	-	50	65
chr12	25403905	25403916	+	51	62
chr12	25403905	25403917	-	51	63
chr12	25403906	25403918	-	52	64
chr12	25403907	25403919	+	53	65
chr12	25403908	25403919	-	54	65
chr12	25403908	25403920	-	54	66
chr12	25403909	25403920	-	55	66
chr12	25403909	25403921	-	55	67
chr12	25403925	25403940	-	71	86

Figure 4-1. PPAR-γ & AP-2 binding sites. Qiagen Champion ChiP Transcription Factor search portal showed and one binding site for PPAR-γ in a region of core kRAS promoter. rVista online tool was used for identification of AP-2 binding sites to core kRAS core promoter. It combines database searches with comparative sequence analysis, reducing the number of false positive predictions by ~95% while maintaining a high sensitivity of the search. Four binding sites for AP-2 in a region of core kRAS promoter were predicted.
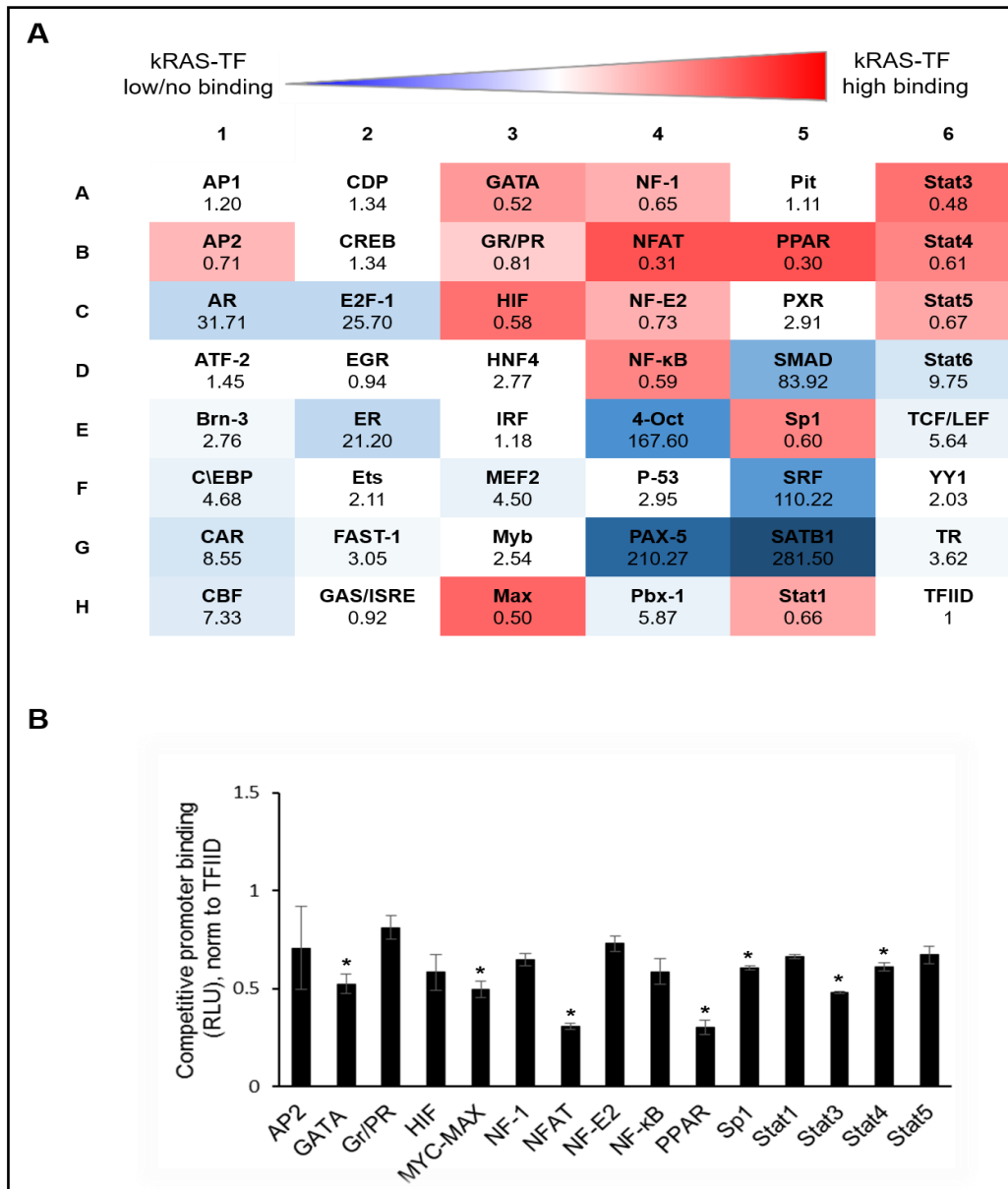
Figure 4-2. kRAS promoter-binding transcription factor profiling array. Binding of various transcription factors present in the nuclear extract isolated from Panc-1 cells to the kRAS promoter (-510 to +50) was assessed using the Promoter-Binding Transcription Factor Profiling Array I. (A) The binding of each transcription factor to kRAS was indicated by average reduction in chemiluminescence of transcription factor-specific oligonucleotide probe specific to each factor from duplicate samples. This 48-well plate shows the replicate of lead compounds suggesting approximately 15 hits based on decrease in RLU (red wells) indicative of high kRAS promoter-TF binding, and (blue wells) indicative
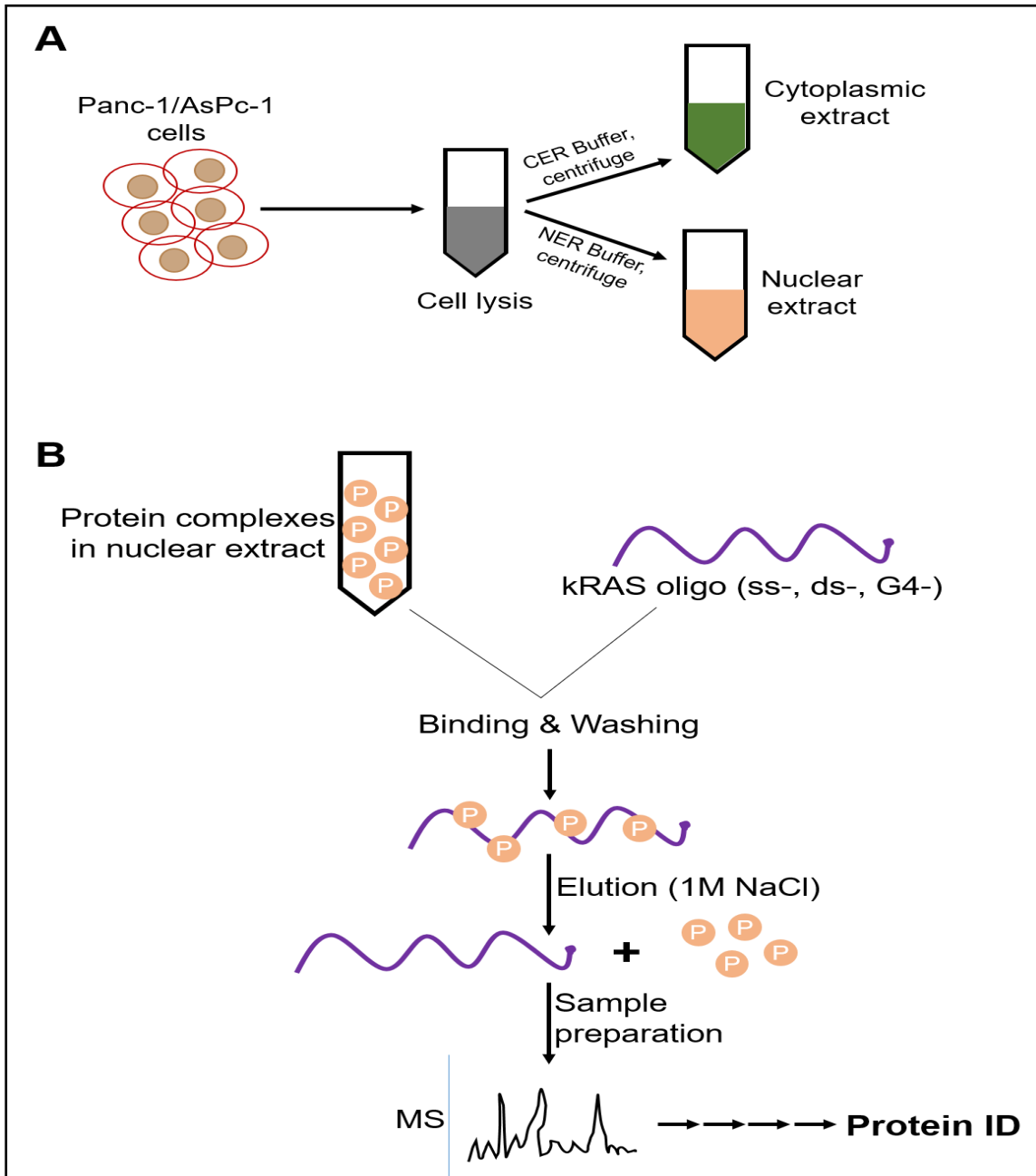
Figure 4-3. Schematic representation of DNA-pulldown method for identification of kRAS binding TFs. (A) Pancreatic cancer cells nuclear extraction. Nuclear extract incubation with kRAS oligos (ss-, ds-, and G4-DNA), followed by multiple washing steps, and protein identification by LC-MS/MS.
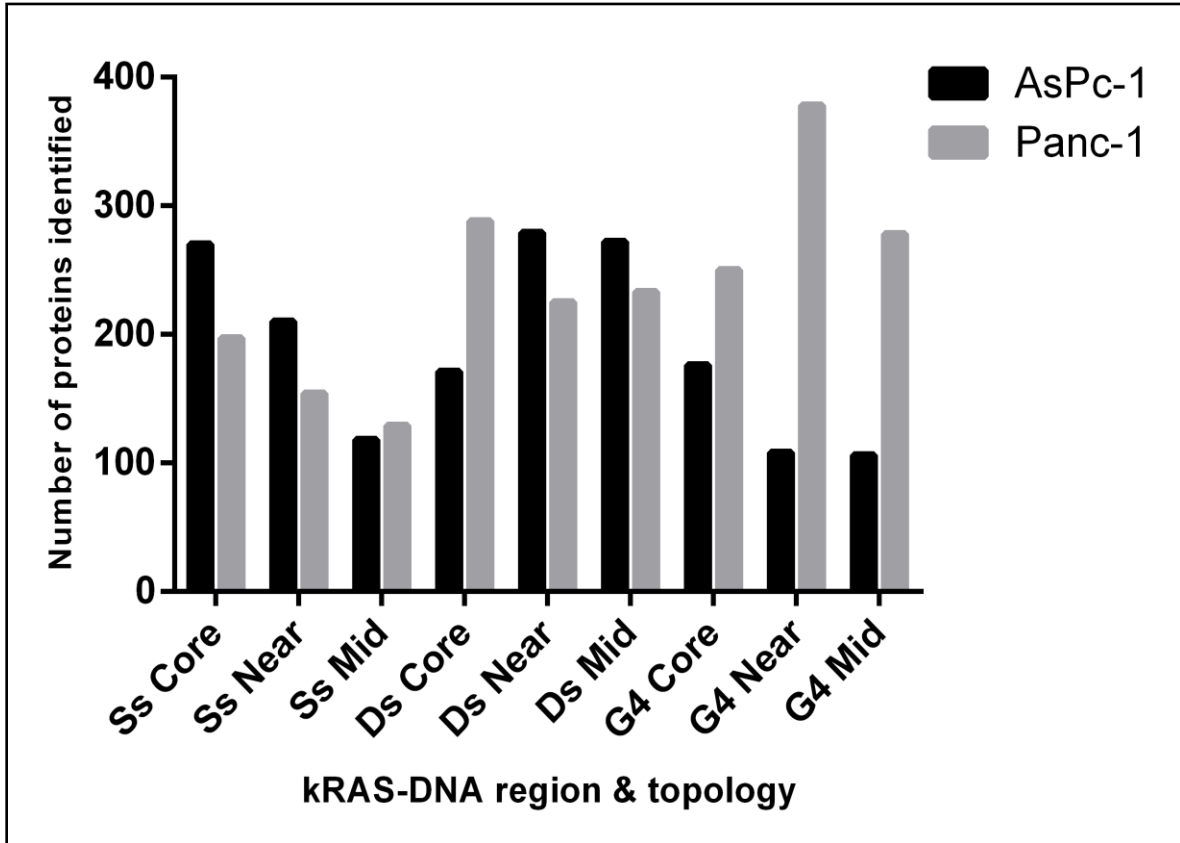
Figure 4-4. Number of proteins determined by LC-MS/MS. AsPc1 and Panc-1 nuclear extract, number of proteins identified for each region (core, near, mid) and topology (ss-, ds-, and, G4-DNA n=1).

Table 4-1. Proteins identified by LC-MS/MS. The table represent the name of protein its protein identification number, number of spectra identified, and ranking in parenthesis which was based on best score assigned by the software.

| Protein	PDB	Ss Core	Ss Near	Ss Mid	Ds Core	Ds Near	Ds Mid	G4 Core	G4 Near	G4 Mid
Nucleolin	P19338	17 (67)	9 (70)	15 (31)	18 (34)	45 (26)	36 (39)	17 (50)	9 (69)	7 (69)
YBX-1	P67809	12 (34)	20 (35)	6 (50)	16 (16)	41 (24)	52 (19)	27 (10)	22 (16)	19 (16)
YBX-3	P16989	7 (71)	0	0	0	22 (30)	46 (27)	17 (32)	8 (41)	8 (51)
Nucleophosmin	P06748	21 (74)	15 (127)	6 (136)	10 (96)	7 (163)	12(205)	0	0	4 (116)
S100 A8	P05109	8 (108)	7 (117)	7 (62)	10 (56)	6 (227)	3(264)	10 (68)	6 (101)	10 (26)
S100 A9	P06702	4 (129)	3 (146)	3 (159)	4 (101)	0	0	10 (64)	9 (60)	6 (61)
hnRNP R	O43390	0	0	0	0	7 (239)	0	0	0	0
hnRNP F	P52597	12 (145)	0	0	0	8 (87)	20 (52)	13 (102)	8 (99)	7 (113)
hnRNP A1	P09651	9 (167)	0	0	0	0	3 (328)	9 (72)	0	0
hnRNP U	Q00839	0	9 (220)	5 (56)	6 (107)	6 (176)	13 (59)	0	0	0
hnRNP M	P52272	0	0	2 (158)	0	7 (206)	0	3 (116)	4 (81)	6 (79)
hnRNP L	O14979	4 (128)	0	0	0	7 (194)	10 (89)	0	0	0
TRIM28	Q13263	4 (161)	0	0	4 (129)	14 (49)	10 (85)	4 (97)	8 (73)	11 (29)
PABPC1	P11940	13 (111)	11 (79)	6 (61)	8 (60)	27 (162)	28 (209)	8 (99)	10 (57)	4 (97)
IGF2BP1	Q9NZI8	10 (104)	4 (150)	3 (63)	10 (79)	0	13 (74)	0	0	0
IGF2BP2	Q9Y6M1	0	0	0	0	0	3 (254)	0	0	0
IGF2BP3	O00425	0	4 (143)	0	0	0	3 (313)	0	0	0
THO	Q96FV9	0	8 (58)	2 (60)	4 (103)	9 (60)	8 (80)	0	0	0
14-3-3, gamma	P61981	9 (59)	13 (49)	0	4 (53)	5 (203)	5 (200)	5 (128)	0	0
14-3-3, zeta/delta	P63104	16 (37)	7 (54)	10 (25)	20 (37)	9 (121)	10 (95)	17 (43)	4 (127)	10 (119)
14-3-3, epsilon	P62258	10 (49)	14 (61)	8 (38)	12 (52)	25 (36)	24 (37)	7 (29)	0	0
14-3-3, beta/alpha	P31946	4 (227)	4 (71)	3 (123)	0	2 (278)	6 (73)	0	0	5 (45)
14-3-3, eta	Q04917	0	0	0	0	2 (81)	0	0	0	0
14-3-3, theta	P27348	5 (75)	5 (83)	0	4 (68)	10 (66)	14 (48)	3 (76)	4 (90)	2 (99)
LYRIC	Q86UE4	0	0	0	0	0	3 (384)	0	0	0
BTF3	P20290	0	0	0	0	7 (249)	6 (287)	4 (151)	4 (105)	3 (130)
PA2G4	Q9UQ80	8 (108)	6 (152)	0	0	13 (85)	0	0	0	0
EWS	Q01844	5 (73)	4 (98)	0	5 (71)	7 (179)	6 (149)	6 (78)	6 (68)	4 (94)
FUBP2	Q92945	15(78)	5 (76)	5 (75)	2 (159)	10 (84)	9 (68)	4 (73)	0	4 (53)
IRS4	O14654	0	0	0	0	0	3 (340)	0	0	0
NME2	P22392	7 (134)	10 (178)	5 (87)	0	15 (147)	0	0	5 (102)	0

4.3. Transcriptional activator/silencing effect of proteins on kRAS promoter

As described in previous chapters, we first sought to examine the effects of the identified proteins effect on kRAS transcription and to further define the DNA regions and structures to which the proteins bind. A luciferase assay was performed in HEK-293 cells transfected with a luciferase plasmid driven by the kRAS promoter Full Length (FL) in the presence of increasing amounts of the AP-2, PPAR-γ, YBX-1, and nucleolin the expression plasmids; a promoter-less vector (EV) was included as a control. A correlative relationship was found between the proteins AP-2, PPAR-γ, and nucleolin with kRAS transcription as inferred from luciferase expression. As compared to EV, AP-2 increased promoter activity by 30% (1.300 ± 0.104), over 170% (2.767 ± 0.209), and by 300% (4.064 ± 0.295), at 250, 500, and 1000 ng of expression plasmid, respectively (**Figure 4-5A**). PPAR-γ showed an increase by 100% (2.094 ± 0.143), and almost 180% (2.782 ± 0.075), at 500 and 1000 ng of expression plasmid, respectively (**Figure 4-5B**). Nucleolin increased kRAS promoter activity by over 30% (1.355 ± 0.155), and 90% (1.920 ± 0.068), at 500 and 1000 ng, respectively (**Figure 4-5C**). Our fourth protein of interest, YBX-1, showed an inverse relationship with kRAS expression, with significant decreases in the promoter activity by almost 70% (0.336 ± 0.013), 80% (0.200 ± 0.015), and by 83% (0.170 ± 0.015), at 250, 500, and 1000 ng of expression plasmid, respectively (**Figure 4-5D**).

We extended these studies to examine the effect of AP-2, PPAR-γ, YBX-1, and nucleolin on kRAS transcription in more complex milieus with pancreatic cancer cell lines. MiaPaCa-2, Panc-1, and AsPc-1 were transfected with increasing protein expression plasmids and the effects on endogenous kRAS expression was monitored and normalized to the housekeeping gene, GAPDH. In these more complicated environments, with variations in basal levels of the proteins (**Figure 4-6**) studied, as well as in overall genetics, the findings are not as easy to interpret. In the

cells, AP-2 had no effect on kRAS transcription in the MiaPaCa-2 and AsPc-1 cells. In contrast to the plasmid findings, kRAS expression decreased in Panc-1 cells (**Figure 4-7A**). The PPAR-γ:kRAS promoter interaction was more consistent with the plasmid findings: while there was no change in kRAS expression in MiaPaCa-2 cells, it significantly increased in both Panc-1 and AsPc-1 cells by 2.5 fold and 1 fold respectively **(Figure 4-7B)**. The nucleolin:kRAS promoter interaction was confounding with an array of responses. At lower increased of nucleolin (1.5 – 4.5-fold), kRAS expression increased 0.50 in MiaPaCa-2 cells, did not change in Panc-1 cells, and significantly decreased in AsPc-1 cells (**Figure 4-7C**). Transfections with more plasmid and subsequent higher expression of nucleolin demonstrated a complex interaction with a significant decrease in kRAS expression in MiaPaCa-2 cells, a significant *increase* in Panc-1 cells, and no change in AsPc-1 cells (**Figure 4-7D**). More work is ongoing to decipher the precise interaction of this known G4-interactive protein with the kRAS promoter. YBX-1, seen to significantly decrease kRAS promoter activity in plasmids, mediated a significant increase in kRAS transcription in AsPc1 cells lines (**Figure 4-7E**).
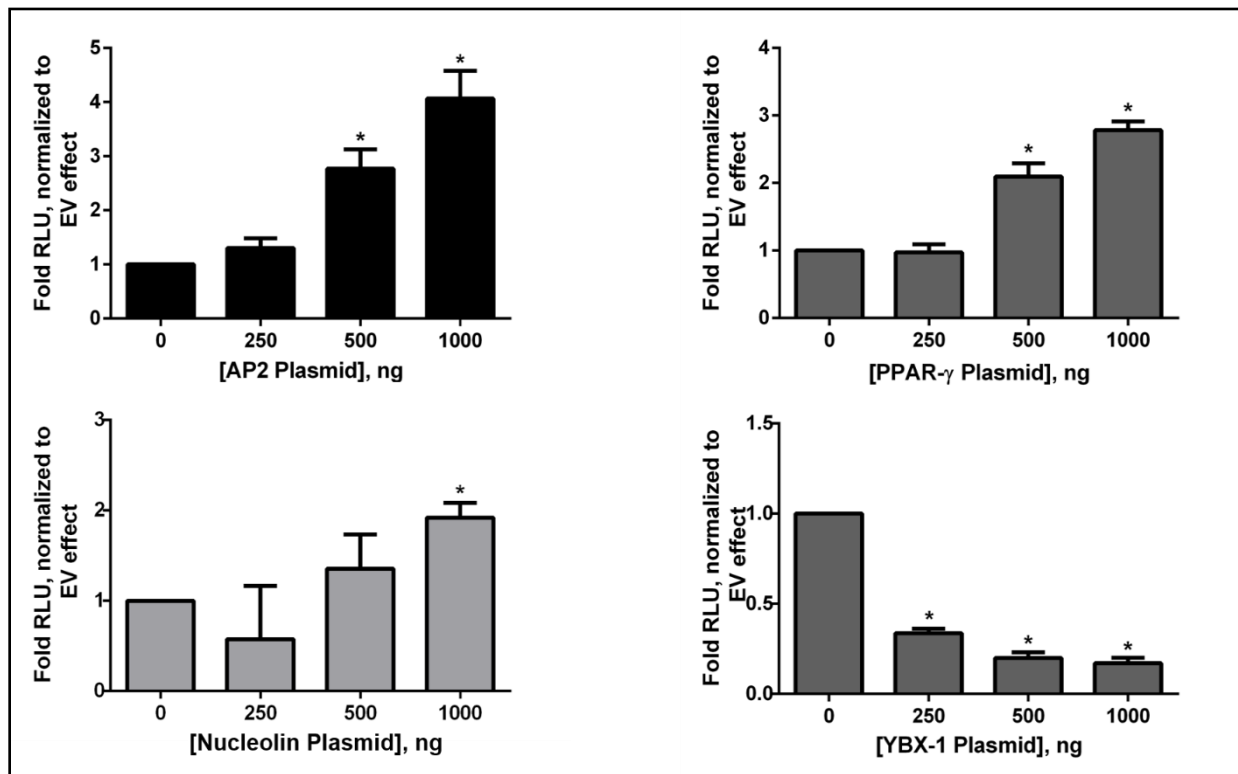
Figure 4-5. AP-2, PPAR-γ, nucleolin, and YBX-1 effect on kRAS promoter activity. A luciferase assay was performed in HEK-293 cells transfected with a luciferase plasmid driven by the kRAS promoter in the presence of increasing AP-2, PPAR-γ, nucleolin, and YBX-1 expression plasmid. Promoterless (EV) was included as a control. Increased AP-2, PPAR-γ, and nucleolin expression led to decreased kRAS promoter activity as measured by fold change in RLU, normalized to the EV effect. (A-C). YBX-1 led to a significant decrease in kRAS promoter activity (D). *$p < 0.05$ (versus control), as determined by one-way ANOVA with Tukey post-hoc testing. All experiments were performed in a minimum of triplicate.
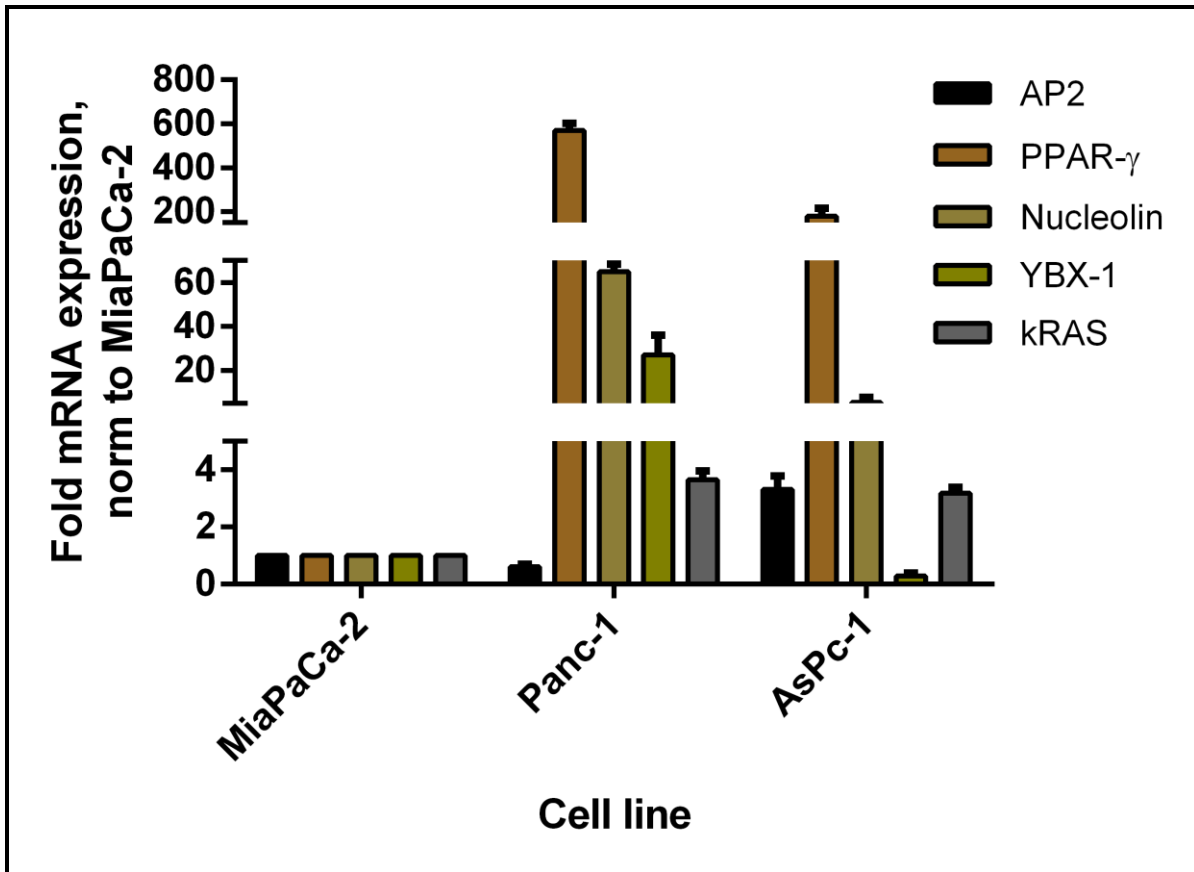
Figure 4-6. Basal levels of AP-2, PPAR-γ, nucleolin, YBX-1, and kRAS in pancreatic cancer cell lines. At Basal level (0 ng) the proteins expression are normalized to MiaPaca-2 cells. AsPc-1 showed highest level of kRAS and AP-2. On the other hand Panc-1 showed the highest level of PPAR-γ, nucleolin, and YBX-1.
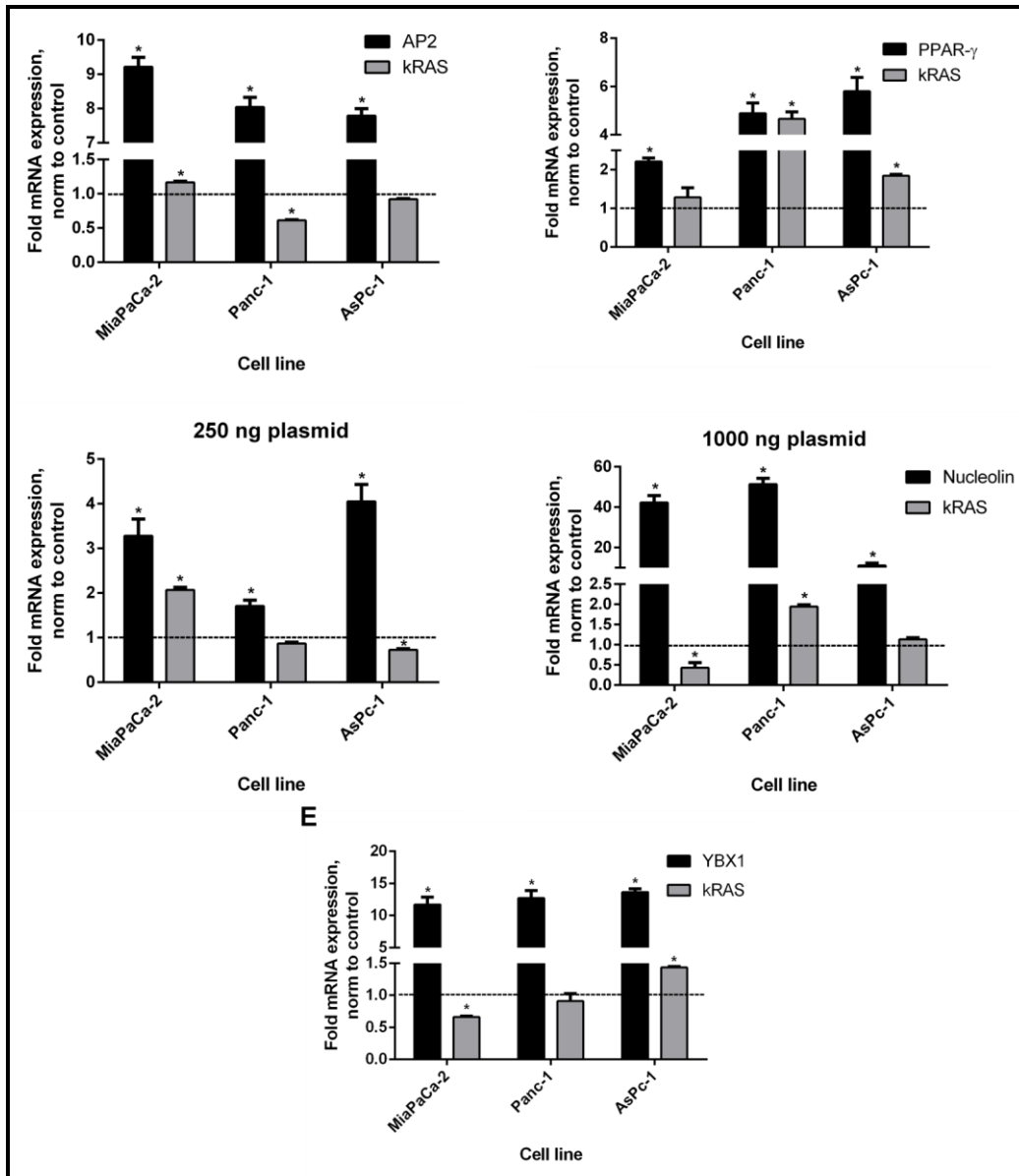
Figure 4-7. AP-2, PPAR-γ, nucleolin, and YBX-1 effect on pancreatic cancer cells. The pancreatic cancer cells MiaPaCa-2, Panc-1, and AsPc-1 were transfected with increasing AP-2, PPAR-γ, nucleolin, and YBX-1 plasmid; the mRNA expression of proteins and kRAS were monitored. (A) The AP-2:kRAS interaction led to significant decrease in Panc-1. (B) The PPAR:kRAS, promoter interaction led to significant increase in Panc-1 and AsPc-1 cell lines at 500 ng. (C) The effect of nucleolin:kRAS, promoter interaction varied on on the amount of plasmid transfected. (D) Finally, the effect of YBX-1:kRAS, promoter interaction showed a significant increase in kRAS mRNA expression in AsPc-1 cell lines only. *$p < 0.05$ (versus control), as determined by one-way ANOVA with Tukey post-hoc testing. All experiments were performed in a minimum of

4.4. The G4 kRAS (near and mid) stability profile of proteins

The binding affinity of AP-2, PPAR-γ, YBX-1, and nucleolin to various regions and topologies of the kRAS promoter was examined using EMSA. As detailed previously, biotin-labeled oligonucleotides (4 µM) of the near- and mid -G4 forming regions were incubated at 95 °C for 10 min followed by slow cooling to form G4s. The dsDNA were formed in the thermal cycler by incubating with the complementary strand at 95 °C for 5 minutes followed by 70 cycles of 95 °C (-1 °C/ cycle). The 1X agarose gel was run to confirm the formation of ssDNA and dsDNA (Data not shown). The ss-, ds-, and G4-DNAs were incubated with AP-2, PPAR-γ, YBX-1, and nucleolin nuclear extract (0-20 µg/µl) for 30 min at room temperature. We also did a dose-response to see the effect of incubation of different amount of nuclear extract on DNA binding (data not shown). AP-2 showed favorable binding to ss, ds-core and to G4-near regions (**Figure 4-8A**). PPAR-γ bound only to the ds mid region (**Figure 4-8B**). Nucleolin showed binding to all the regions and topologies (**Figure 4-8C**). YBX-1 showed binding to various regions and topologies as well. Shifts were notable with the ss and G4 forms of the mid-region, and with all regions in dsDNA form, although most markedly with the near region (**Figure 4-8D**).

Further investigation of these proteins binding to G4-DNA was done by ECD. Unlabeled oligonucleotides (5 µM) of the kRAS G4 sequences of the near, and mid G4-forming sequence after the formation of G4 with heating and cooling at 95 °C for 10 minutes with 100 mM KCl were incubated with either protein elution buffer, or the nuclear extract, with (to see effect on stabilization profile of a G4) and without (as control for comparison). No significant effect on G4$_{mid}$ stabilization was observed with AP-2, PPAR-γ, and YBX-1 (**Figure 4-9A**). The nucleolin pronouncedly destabilized G4$_{mid}$ (**Figure 4-9B**) by decreasing the melting temperature by 15 °C and showed no significant effect on G4$_{near}$ (**Table 4-3**). As nucleolin has been previously shown

to interact with and stabilize RNA and the MYC G4, this was an unexpected finding, although it is in agreement with an increase in transcription noted with plasmids and to a varying degree in cell lines, as described above.
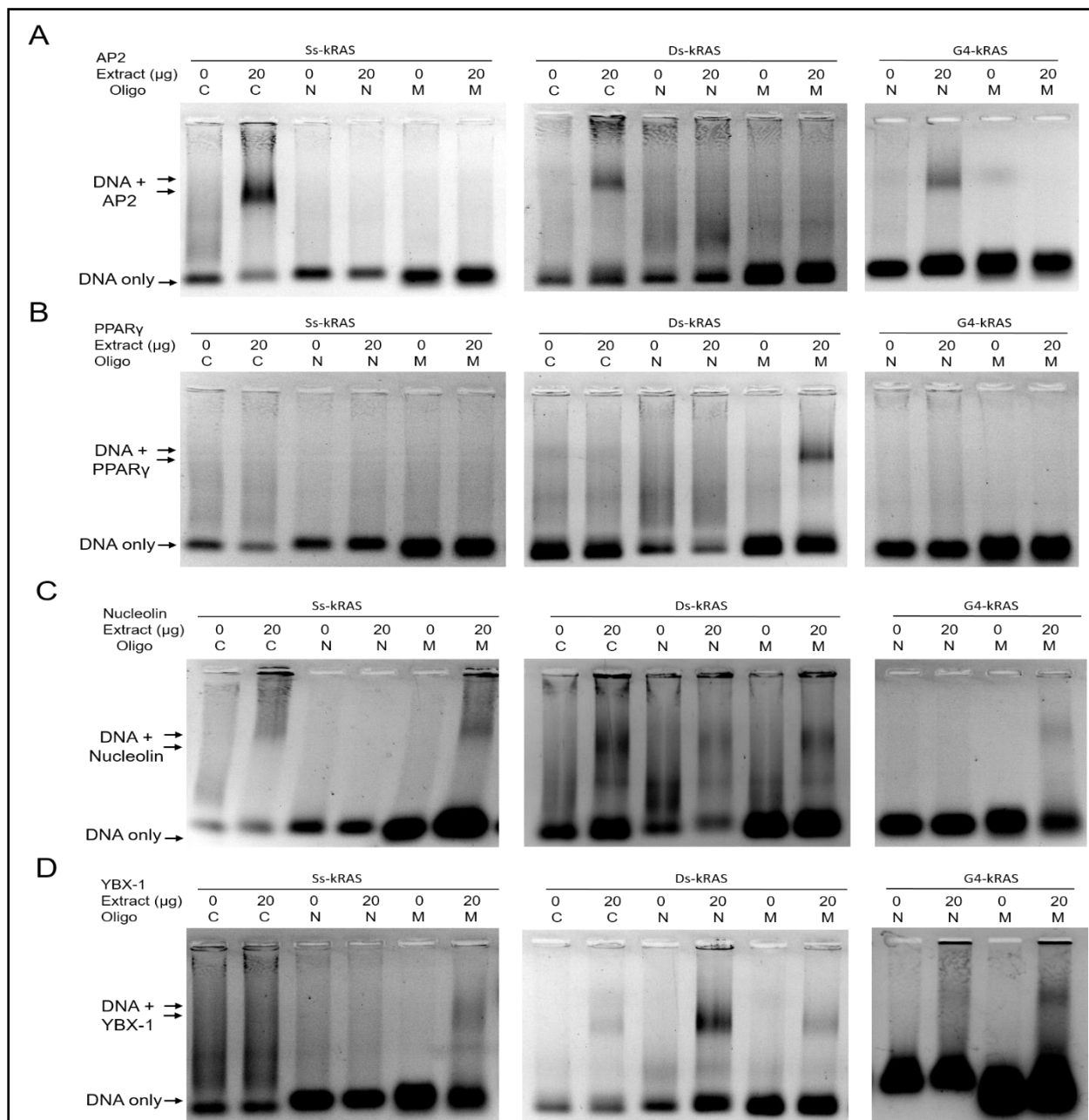
Figure 4-8. AP-2, PPAR-γ, nucleolin, and YBX-1 binding to the kRAS promoter. EMSA was performed to show binding of AP-2, PPAR-γ, nucleolin, and YBX-1 to different topologies (ss-, ds, and G4-DNA) and regions (core, near, and mid). (A) AP-2 showed favorable binding to all near and mid regions. (B) PPAR bind preferably to ds mid region. (C) Nucleolin showed binding to all the regions and topologies. (D) Finally, YBX-1, like nucleolin showed binding to all with more preference to ds-DNA.
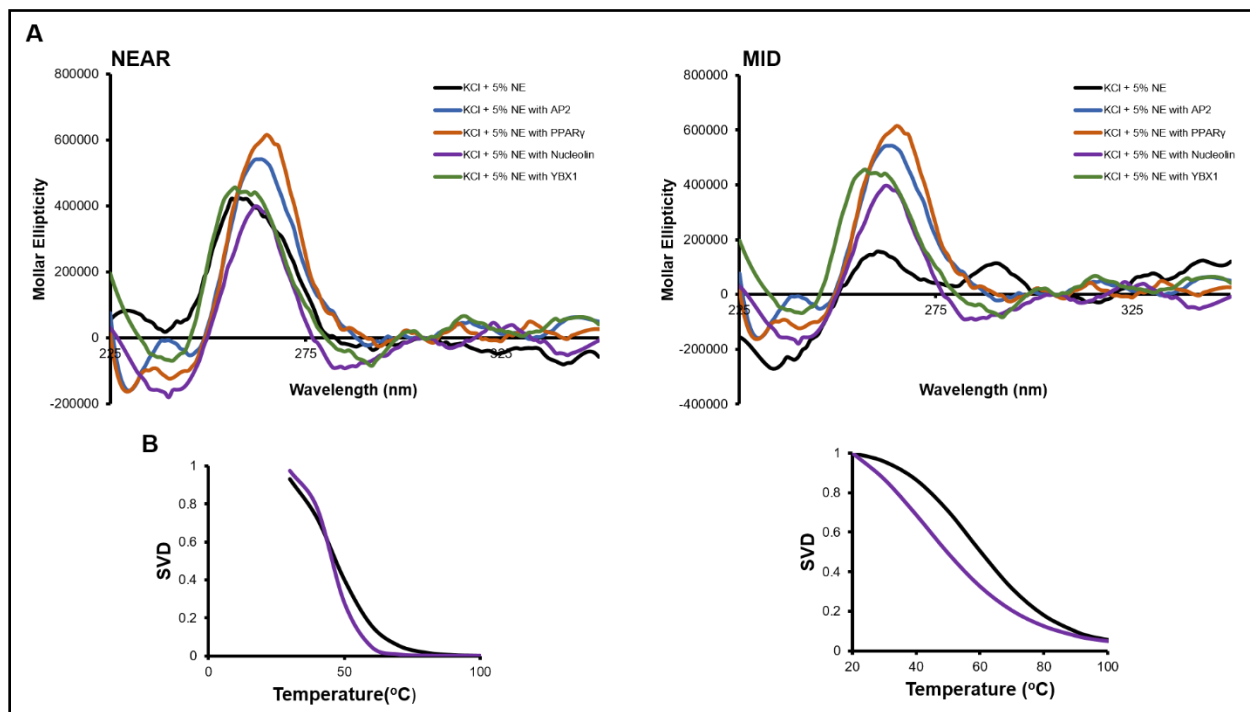
Figure 4-9. Effect on kRAS G4 stability profile. By ECD, further investigation of the effect of AP-2, PPAR-γ, nucleolin, and YBX-1 binding to G4-DNA was studied. No significant effect on G4 stabilization was observed with AP-2, PPAR-γ, and YBX-1. The nucleolin pronouncedly destabilized G4$_{mid}$ by decreasing melting temperature by 15 °C and showed no significant effect on G4$_{near}$.

Table 4-2. Effect of proteins on G4 stabilization as denoted by ΔT_M and number of G4 isoforms shown by percent change of G4$_{near}$ and G4$_{mid}$ compare to control (KCl + 5% NE).

NEAR	KCl + 5% NE	KCl + 5% NE with Nucleolin	KCl + 5% NE with YBX-1
TM	46.5	44.5	44.53
ΔTM	--	-2.0	-1.97
Hill Slope	-0.05587	-0.07054	-0.05385
Percent Change	--	-26	-3

MID	KCl + 5% NE	KCl + 5% NE with Nucleolin	KCl + 5% NE with YBX-1
TM	58.72	43.75	58.57
ΔTM	--	-14.97	0.015
Hill Slope	-0.03527	-0.02835	-0.0489
Percent Change	--	+19	+39

4.5. Discussion

Herein, we utilized empirical methods to identify proteins of interest binding to the kRAS promoter, including a database of ChIP data, binding studies to the whole promoter, and identification of proteins binding to distinct regions of interest. This was done to generate a more global picture of TF binding to the kRAS promoter that extends from 300-500 bases downstream from the transcriptional start site (TSS) to 50 bases upstream and contains two distinct putative G4-forming regions. From these studies, there were several hit proteins, including many whose function and involvement is yet to be determined, such as GATA, Gr/PR, HIF, MYC-MAX, NF-1, NFAT, NF-E2, NF-κB, PPAR, Sp1, Stat1, Stat3, Stat4, and Stat5. From the cumulative approaches, we focused on four proteins: AP2, PPAR-γ, YBX-1, and nucleolin.

AP2 had four binding sites at the core promoter region from 0 to +50 bp according to the Qiagen Champion ChiP Transcription Factor detection. It also was identified as a hit protein in our TF array kit. Homologous proteins of 52 kDa form the AP2 family of proteins, which include Tfap2a, Tfap2b, Tfap2c, Tfap2d, and Tfap2e (Cheng et al. 2002; Feng & Williams 2003; Hilger-Eversheim et al. 2000; Tummala et al. 2003; Williams et al. 1988; Zhao et al. 2001). In the nucleus, AP2 mediates both activating and repressing stimuli to their targets by binding to the GC-rich consensus sequences, fostering homo- or hetero-dimerization of the AP2 proteins via their dimerization motifs (Bosher et al. 1996; Hilger-Eversheim et al. 2000; Williams & Tjian 1991a; Williams & Tjian 1991b).

AP2 proteins are involved in several targets associated with a different level of cancer development, in a cell-specific manner. Reduced tfap2a is observed in tumor cells of malignant melanoma, during metastasis, inhibition of tumor growth by increasing cell death is observed by forced expression of Tfap2a in these cells (Bar-Eli 2001; Huang et al. 1998). On the other hand,

overexpression of Tfap2c promotes cell proliferation and tumor growth in a murine breast cancer model (Jager et al. 2005). AP2 proteins because of their structural conservation share similar transactivating properties (Bosher et al. 1996; McPherson & Weigel 1999). For example, both Tfap2a and Tfap2c are capable of inducing p21WAF1 expression and inhibiting proliferation (Li et al. 2006; Zeng et al. 1997). The expression of human epidermal growth factor receptor-2 (HER2) is activated by Tfap2a and Tfap2c, as well as Tfap2b, in breast cancer cell lines (Bosher et al. 1996; Bosher et al. 1995; Hollywood & Hurst, 1993; Li et al. 2002). Cell adhesion molecule MCAM/MUC18, protease-activated receptor 1 (PAR-1), estrogen receptor alpha (ER-α), and the tyrosine-kinase c-Kit are some other targets of AP2 (Bar-Eli 2001; Huang et al., 1998; Jean et al., 1998; McPherson et al., 1997; Tellez et al., 2003). The complexity of AP-2 proteins' regulatory control increases manifold with its transcriptional co-factors, such as Ying Yang 1 (YY1), p53, Sp1, Myc, Pax6, retinoblastoma protein (pRB), cAMP response element binding protein (CREB), CREB-binding protein (CBP)/p300, and CBP/p300-interacting transactivator with ED-rich tail 2 and 4 (CITED 2 and 4) (Batsche et al. 1998; Braganca et al. 2003; Braganca et al. 2002; McPherson et al. 2002; Pena et al. 1999; Sivak et al. 2004; Wu & Lee 1998; Wu & Lee 2001).

Moreover, several lines of evidence suggested that AP2α might behave as a tumor-suppressor gene (TSG) in several tissues. For example, loss of AP2α expression was reported in metastatic melanoma cells (Tellez et al. 2003), in prostate (Ruiz et al. 2001) and colon cancer cells (Schwartz et al. 2007). In breast cancer cells, contrasting results were reported in the literature. Earlier studies suggested that AP2 proteins promote the malignant potential of breast cancer cells by transactivating the promoters of ErbB-2 and ErbB-3 (Bosher et al. 1996). However, recent data using shRNA to knockdown AP2α expression in breast cancer cell lines showed that AP2α inhibits tumor growth both in vitro and in vivo (Orso et al. 2008). Colon and breast cancer cells sensitivity

towards chemotherapeutic drugs is controlled by AP2α (Wajapeyee et al. 2005). Despite its potential interest, the roles of AP-2α in pancreatic cancer is not yet studied extensively (Vernimmen et al. 2003). In human pancreas, AP2α was expressed by almost 66% of non-tumoral ductal cells and endocrine cells, whereas its expression was decreased to only 5.5% of pancreatic ductal adenocarcinoma (PDAC) (Fauquette et al. 2007). Moreover, AP2α expression was mutually exclusive with that of the human epithelial mucin, MUC4, which is a specific marker of PDAC and is expressed in 83% of the patients (Jonckheere 2009). The relationship of AP2 and kRAS transcription has not been explored before. Herein, we showed the binding of AP2 to the kRAS promoter, and that binding leads to increase kRAS promoter activity in HEK-293 cells and differential effects on pancreatic cancer cells, which might have a role in pancreatic cancer which has not been looked into before.

Peroxisome proliferator-activated receptors (PPARs), of which our second hit, PPAR-γ, is a member, are a group of ligand-activated TFs and nuclear hormone receptor superfamily members. There are three separate genes that give rise to the different isoforms of PPAR, termed PPAR-α, β/δ, γ. Due to differential splicing and alternate promoter usage, there are three isoforms of gamma - γ1, γ2, γ3 (Fajas et al. 1998; Desvergne & Wahli 1999; Roberts-Thomson 2000). Each of the PPAR isoforms has a different tissue distribution, function, and to some extent, different ligand specificity. PPARα is expressed primarily in the liver, heart, kidney, skeletal muscle and brown adipose tissue. PPARβ/δ expression is ubiquitous, while PPAR-γ is primarily found in white and brown adipose tissue, large intestine and spleen (Desvergne & Wahli, 1999). In normal physiology, PPAR-γ is involved in adipogenesis, insulin resistance, regulation of lipid homeostasis, and in the development of various organs. In addition to these metabolic processes, PPAR-γ is overexpressed in several types of human cancers, including breast, colon, bladder, and

prostate cancer (Sikka et al. 2012). The mechanism of binding of PPARs is a multistep process. Activated PPARs first form a heterodimer with the retinoid X receptor and this complex binds to peroxisome proliferator response element (PPRE), which is a specific sequence in the promoter region of target genes (Isseman et al. 1993; Roberts-Thomson 2000). Its activation leads to inhibition of cell growth and promotion of differentiation in many epithelial derived cancer cell lines (Sarraf et al. 1995; Tontonoz et al. 1997; Elstner et al. 1998; Mueller et al. 1998; Kubota et al. 1998; Mueller et al. 2000; Chang & Szabo 2000).

Activated PPAR-γ decreases pancreatic cancer cell growth and their migration and invasive capacity (Motomura et al. 2000; Toyota et al. 2002; Tsujie et al. 2003; Motomura et al. 2004; Adrian et al. 2008; Kumei et al. 2009). Using a pancreatic carcinoma xenograft model of nude mice, PPAR-γ activation inhibited pancreatic cancer growth and suppressed tumor angiogenesis (Dong et al. 2009). However, like in other types of cancers, the role of PPAR-γ in pancreatic cancer remains controversial (Eibl 2008). In contrast to the above studies, PPAR-γ expression in pancreatic cancer was correlated with shorter patient survival suggesting a role for PPAR-γ in tumor progression (Kristiansen et al. 2006). Due to contradictory results in mice versus human, further investigations are still needed to clarify the role of PPAR- γ in pancreatic cancer. So, having a profound effect on several types of cancers and it being identified as a hit in our studies resulted in moving with PPAR-γ as the second hit against kRAS regulation.

In 1970's Y-box binding proteins were first described and characterized in animals including duck, rabbit, and amphibians (Morel et al. 1973; Morel et al. 1971; van Venrooij et al. 1977). Y-box-1 protein was sequenced in 1988 and was found to interact with the Y-box motif in the promoter of the major histocompatibility complex class II genes (Didier et al. 1988). It binds to an inverted CCAAT box (5′-CTGATTG-GC/TC/TAA-3′) in the Y box of the promoter. There

are three families of YB proteins namely YBX-1, -2, and -3 respectively. In embryonic tissues, there is a high-level YBX-1 expression in heart, muscle, lung, adrenal gland and brain, and low amounts in thymus, kidney, bone marrow and spleen (Spitkovsky et al. 1992). YBX-1 has been demonstrated to be vital for normal embryonic development, particularly late embryonic development in mice (Lu et al. 2005). Mice that were YBX-1 (-/-) developed growth retardation after 13.5 days, exencephaly, craniofacial defects and progressive mortality. In prostate, breast, ovarian, brain, colorectal cancer YBX-1 overexpression is detected as an adverse prognostic factor. It has been implicated in several other cancers, including gastric cancer (Wu et al. 2012), bladder cancer (Shiota et al. 2011), oesophageal cancer (Li et al. 2011), osteosarcoma (Oda et al. 2003; Oda et al. 1998), lung cancer (Gessner et al. 2004; Shibahara et al. 2001), melanoma (Schittek et al. 2007) and liver cancer (Yasen et al. 2005). YBX-1 binds single-stranded DNA with high affinity (Izumi et al. 2001), although in our findings we demonstrated binding to multiple DNA topologies, including dsDNA.

The increased YBX-1 expression is observed in PDAC. Nuclear YBX-1 expression is associated with dedifferentiation, lymphatic/venous invasion, and unfavorable prognosis. On the other hand YBX-1 knockdown leads to inhibition of cell proliferation via cell cycle arrest by S-phase kinase-associated protein 2 downregulation, and decrease in the invasion due to downregulated membranous-type 2 MMP expression in PDAC cells. YBX-1 targeting antisense oligonucleotide significantly inhibited the growth of subcutaneous tumors. In conclusion, YBX-1 was shown to be involved in aggressive natures of PDAC and is a promising therapeutic target (Shinkai et al. 2016). For these reasons and due to its availability, we selected it as our third hit for biological evaluation. The results obtained were not conclusive and require further investigation to determine its role in pancreatic cancer.

The fourth protein of interest which was chosen for further study was nucleolin. In 1973, nucleolin was discovered in rat liver cells (Pinard 1973). It is present in the nucleolus of plants, yeast, and mammals (Genisty et al. 1999; Yang et al. 2008). Human nucleolin consists of 707 amino acids with a calculated mass of 77 kDa (Srivastava et al. 1989). Nucleolin helps to regulate RNA polymerase II transcription of genes by interacting with several TFs (Tajrishi et al. 2011; Masumi et al. 2006; Huddleson 2006). Nucleolin also functions in chromatin remodeling (Hans et al. 2006; Mongeland & Bouvet 2007), transcription (Yang et al. 1994; Grinstein et al. 2002; Grinstein et al. 2007; Brys & Maizels 1994; Dampsey et al. 1990), apoptosis (He et al. 1998), and G4 binding (Dampsey et al. 1990; Hanakahi et al. 1999; Bates et al. 1999). Nucleolin binds and stabilizes the c-MYC gene G4 formed in the promoter region. This lead to the downregulation of c-MYC transcription, acting as a repressor of c-MYC (Gonzalez et al. 2009; Gonzalez et al. 2010). It binds the G4 structure in the promoter region of human vascular endothelial growth factor (VEGF) (Sun et al. 2011) and in ribosomal RNA (Hanakahi et al. 1999).

Nucleolin inhibition reduces tumor growth in renal cell cancer and breast cancer (Bates et al. 2009; Pichiorri et al. 2013). In the recent study, a low level of nucleolin was linked with significantly increased survival in PDAC patients. The growth of primary tumors and liver metastasis in an orthotopic mouse model of PDAC (mPDAC) was impaired by the nucleolin antagonist N6L. In pancreatic cancer, targeting nucleolin by N6L is hailed as a new anti-cancer therapeutic strategy as it leads to blocking tumor progression, normalizing tumor vasculature, improving the delivery and efficacy of chemotherapeutic drugs (Gilles et al. 2016). One study described interactions between nucleolin and kRAS in the nucleolus (Birchenall-Roberts et al. 2006). Endogenous nucleolin t C-terminal to interacts simultaneously in vivo with endogenous RAS and ErbB1 (EGFR) in cancer cells (Farin et al. 2011). Most importantly, these three

oncogenes synergistically facilitate anchorage-independent cell growth in vitro and tumor growth in vivo (Farin et al. 2011). In our studies, nucleolin binds to the kRAS core and mid, but not much to near and only a little in the ds form and led to an increase in transcription. Previous studies showed that overexpression of nucleolin is associated with tumor growth. On the other hand, nucleolin was also showed to destabilize the kRAS G4 structure, which might contribute to its role in activating transcription. Further studies on folding profile and its role in cancer progression is warranted.

Through our studies, we identified four potential transcriptional regulators of kRAS. AP2, YBX-1, and PPAR-γ alter the kRAS transcription in a non-G4 interactive manner. In contrast, and in a similar manner to the MYC and VEGF structures, nucleolin also binds to the $G4_{mid}$ kRAS promoter. Interestingly our studies demonstrated a destabilization effect, whereas nucleolin has been seen to stabilize the MYC structure; nucleolin apparently mediated increase in kRAS expression overall, by destabilization of the kRAS $G4_{mid}$ structure. The laboratory will continue to investigate the relevance of the interaction of all TFs and kRAS promoter to understand the mechanism behind the regulation. The mechanism of regulation for the G4-binding proteins, such as nucleolin, might be through altering the stability profile of the G4 present in the kRAS promoter. Understanding the transcription machinery of kRAS is important to understand the biological role of these regulatory proteins behind tumor formation, maintenance, and metastasis. This information will be useful in the future for the development of novel cancer therapies by deliberately and specifically altering their binding to the kRAS promoter or by utilizing them to alter the G4 stability profile with the final aim of altering the expression of kRAS.

CHAPTER 5. G4-INTERACTIVE COMPOUNDS AS NOVEL SMALL MOLECULE
TRANSCRIPTION INHIBITORS OF PANCREATIC CANCER

5.1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive form of pancreatic cancer that accounts for 90% of total pancreatic cancer cases (Ryan et al. 2014). The treatment of PDAC has not improved in 30 years (Wolfgang et al. 2013) as the 5-year survival rate is dismal at 7% (SEER Cancer Statistics, 2015. PDAC progression also involves the loss or mutation of several other tumor suppressor genes (Vincent el al. 2011) such as cyclin-dependent kinase inhibitor 2A (CDKN2A) (Schutte et al. 1997), TP53, and the SMAD4 (Malkoski et al. 2012). Out of these, kRAS is the prime player in pancreatic cancer, and was identified as a key target for cancer research by a kRAS pancreatic cancer working group (NCI) in the establishment of the RAS Initiative Center in Frederick, MD (Abbruzzese 2013).

The RAS family of small GTPases includes three genes: HRAS, NRAS, and kRAS which encode: HRAS, NRAS, kRAS4A, and kRAS4B proteins. RAS mutations are observed in residues 12, 13 and 61, and effect the GTP hydrolysis activity (Vigil et al. 2010). RAS In bladder, ovary, thyroid, lung, colon and pancreatic cancer kRAS is mutated. kRAS codon 12 mutations occur most frequently in pancreatic cancer (Prior & Lewis 2010). kRAS protein is critical in cancer signaling due to their active role in cell differentiation, proliferation, migration, and apoptosis (Cox et al. 2010). After activation from upstream signals and mutations, RAS-GTP acts as a molecular switch

and activates different downstream effectors which regulate a myriad of cytoplasmic signaling networks.

After more than three decades of intensive effort, no anti-RAS therapy has been clinically employed (Stephen et al. 2014; Bryant et al. 2014; Cox et al. 2014). Six promising paths have been looked at to find an effective treatment for kRAS-mutant cancers, including kRAS direct inhibitors, membrane association inhibitors, synthetic lethal interactors, kRAS downstream effector signaling pathways inhibitors of kRAS, kRAS metabolic process regulators, and harnessing the immune response.

Direct targeting of kRAS has primarily focused on inhibiting protein-protein interactions necessary for activation. Targets of this approach include the RAS-GEF1 recognition pocket (Sun et al. 2012; Maurer et al. 2012) and downstream effector pathway interactions (Shima et al. 2013). Notably, an RNA interference (RNAi) strategy was used to suppress kRAS expression and has shown positive pre-clinical results in kRAS mutant-driven mouse models of lung and colorectal cancer (Yuan et al. 2014; Xue et al. 2014), but due to delivery issues and poor pharmacokinetic profile, it has not reached clinical phase. This last strategy is most applicable to our approach and its limitations are ones that G4-targeting can putatively overcome.

Interruption of kRAS plasma membrane localization by inhibiting the farnesyltransferase enzyme has also been pursued therapeutically (Queneau et al. 2001; Rowinsky et al. 1999; End 1999). Clinical trials of Farnesyl Transferase Inhibitors (FTIs) to treat PDAC were unsuccessful (Basso et al. 2006; Berndt et al. 2011), mainly due to an alternative localization pathway with the geranylgeranyl transferase enzyme, which overcomes the actions of FTI treatment. A third approach has been exploiting synthetic lethal interactions. Synthetic lethality interactors are the combination of genes whose loss of function in the presence of mutant kRAS would lead to cell

death. These include CDK1, TPX2, TFs such as GATA2, serine/threonine protein kinase 33 (STK33) and TBK1 (Sarthy et al. 2007; Morgan-Lappe et al. 2007; Stecket et al. 2012; Kumar et al. 2012; Barbie et al. 2009; Muvaffak et al. 2014). Inhibition of these has not shown any promise in inhibiting pancreatic cancer progression.

The most encouraging of all approaches has been targeting the downstream effector pathways. Six of the eleven RAS effector families identified to date, have validated roles in contributing to RAS-dependent cancer initiation and maintenance (Cox et al. 2015; Baines et al. 2011). MEK1/2 and ERK1/2 interaction with BRAF serine/threonine kinases are a major downstream effector pathways of kRAS-mediated oncogenesis. Rigosertib (2-[(2-Methoxy-5-{[(E)-2-(2,4,6-trimethoxyphenyl)ethenesulfonyl]methyl}phenyl)amino]acetic acid), a benzyl-styryl sulfone, acts as RAS-mimetic and interacts with the RAS binding domains (RBDs) of RAF kinases. This binding alters their ability to bind to RAS, disrupts RAF activation, and ultimately leads to inhibition of the RAS-RAF-MEK pathway. Rigosertib also binds to the RBDs of Ral-GDS and PI3Ks (Ostrem & Shokat 2016; Athuluri-Divakar et al. 2016; Cox et al. 2015).

Another important parallel pathway is that of PI3K-AKT-mTOR. There are four main pharmacologic approaches for inhibition of PI3K signaling: PI3K inhibitors, AKT inhibitors, and mTOR inhibitors, and dual PI3K-mTOR inhibitors (Cox et al. 2014; Fruman & Rommel 2014). Previously, in a lung cancer murine model harboring upregulated kRAS, PI3K inhibition led to partial regression of tumor growth (Ebi et al. 2011; Castellano et al. 2013; Gupta et al. 2007). On the other hand, evidence suggests that this pathway is not a significant effector for RAS signaling alone. However, combination of PI3K pathway inhibitors with RAF-MEK-ERK cascade inhibitors leads to potent synergistic activity (Engelman et al. 2008). Specifically, treatment with MEK (GDC-0973) or PI3K inhibitors (GDC-0941) alone in mice PDAC model showed slight tumor

growth inhibition with no significant effect on survival. On the other hand, the combination of the two treatments resulted in a survival advantages (Junttila et al. 2015). Many of these anticancer drugs have not passed clinical trials due to their lack of specificity and selectivity for mutant kRAS, as well as, causing the activation of parallel pathways resulting in tumor growth and disease progression (Adjei 2001). Another approach includes kRAS-regulated metabolic targets related to glycolysis and glutamine metabolism (Bryant et al. 2014). Cancer cells display altered levels of glycolysis along with increased dependency on glutamine. Strategies include inhibition of enzymes related to these pathways such as glutaminase (GLS) and aspartate transaminase (GOT1)-dependent mechanism (Son et al. 2013). As PDAC cells require GOT1 for redox homeostasis, it could be an enticing therapeutic target (Son et al. 2013). Additionally, two previous GLS inhibitors have shown growth suppressive effect on PDAC cells whose effects are potentiated in the presence of hydrogen peroxide treatment (Son et al. 2013). Redox homeostasis in PDAC is disrupted by the inhibition of GOT1 or GLS and maybe effective treatment in combination with chemotherapy and radiation that increase reactive oxygen species (Lyssiotis et al. 2013).

To gain specificity and selectivity, immunological approaches have been utilized to differentiate between wild-type and mutant kRAS. Because mutated kRAS can be classified as being tumor specific, a synthetic peptide technique was used to target cytotoxic T-cells to tumors expressing the altered protein where p21RAS already underwent membrane localization (Gjertsen & Gaudernack 1998). This posed some difficulties as T-cells require full activation via multiple signals, and the kRAS mutation was found to be poorly immunogenic which resulted in ineffective vaccinations (Fossum et al. 1995; Gjertsen et al. 2001).

One of the newer approaches includes the effect of transcriptional regulators TAZ/YAP. Elevated nuclear levels of the transcriptional regulators TAZ and YAP (TAZ/YAP) are associated with a broad range of aggressive cancers (Harvey et al. 2013). YAP1 allows kRAS-dependent pancreatic cancer cells to survive even when the oncogenic kRAS is inhibited (Kapoor et al. 2014; Shao et al. 2014). Moreover, activation of YAP1 and TAZ in acinar cells up-regulate JAK-STAT3 signaling to promote the development of PDAC in mice (Gruber et al. 2016). Mechanistically, active YAP1 and TAZ interact with the TF TEAD, which binds and up-regulates the expression of several genes in the JAK–STAT3 pathway. Future studies are warranted for combination therapy against oncogenic kRAS and YAP1. Recently, targeting mutant kRAS via the small molecule inhibitors of kRAS-SOS binding pocket has yielded promising results (Wilson & Tolias 2016). Fragment-based screening has been used to synthesize GDP-bound kRAS mutant G12D compounds (Sun et al. 2012). This interaction with kRAS binding pocket inhibits its downstream signaling cascade by causing conformation change in protein and preventing kRAS-SOS interactions. However, further optimization of these compounds is needed to gain the affinity required to move the compounds into clinical trials. Recently, a series of inhibitors irreversibly targeting kRAS-G12C by forming a covalent attachment to the mutant cysteine was developed (Ostrem et al. 2013). These compounds bind in an allosteric pocket beneath switch II, causing decreased cell viability and increased cell death in lung cancer cell lines harboring the mutation when compared to cells with wild-type kRAS. Unfortunately, the compounds have a limited potency to move from preclinical to clinical trials and are not expected to be effective in pancreatic cancer as 3% of total mutations are G12C in pancreatic cancer. Based on these approaches, suppressing kRAS expression has shown preclinical promise, but no viable molecular target has yet been established.

Our approach is to transcriptionally downregulate kRAS through the stabilization of a non-B-DNA G4 structure in the proximal promoter. The region of the proximal promoter in the kRAS gene from -324 to -39 bp, in relation to the transcriptional start site (TSS), contains a high density of guanine residues. The three distinct guanine runs have potential to form multiple G4 isoforms that can act as silencing elements (Lavrado et al. 2015). These regions are labeled based on their proximity to the TSS, the near (-129 to -161), mid (-174 to -226), and far (-238 to -273) (Morgan et al. 2016). Previously, several varying structures have been reported for G4$_{near}$ (Cogoi et al. 2008; Cogoi & Xodo 2006; Paramasivam et al. 2011; Paramasivam et al. 2009; Cogoi et al. 2009). The predominant structure was confirmed in Dr. Brooks' laboratory by Dr. Vanessa Gaerig (data not shown). Recently, our lab showed kRAS G4$_{mid}$ fold into a G4 structure and act as the transcriptional silencer (Morgan et al. 2016).

Several G4 stabilizing compounds have been discovered to date (**Figure 5-1**). Telomere G4 stabilizing compound telomestatin, BRACO19, and RHSP4 showed antitumor activity in various xenograft models (Rodriguez et al. 2008; Rahman et al. 2009). SYUIQ-5 inhibits MYC promoter activity in a cell-free system and two leukemia cell lines (Liu et al. 2007) by acting as a telomerase inhibitor and inducer of telomere damage (possibly via stabilization of telomeric G4 DNA (Zhou et al. 2009). The telomestatin derivative S2T1-6OTD showed selectivity to MYC G4 structure and was shown to reduce the expression of MYC and TERT in childhood medulloblastoma and atypical teratoid–rhabdoid tumor cells (Shalaby et al. 2010). Trisubstituted isoalloxazines that bind to and stabilize the G4s formed by KIT1 and KIT2 reduce KIT transcript levels in cells that express KIT (Bejugam et al. 2007). Previous studies have looked at compounds that stabilize kRAS G4$_{near}$, such as guanidine phthalocyanines (DIGP and Zn-DIGP) and 7-carboxylate indolo[3,2-b]quinoline tri-alkylamine derivatives (IQ3A) (Brito et al. 2015; Cogoi et

al. 2010). Recently, compound benzophenanthridine alkaloid stabilizes the three existing kRAS

G4s and led to downregulation of gene expression (Kaiser et al. 2017).
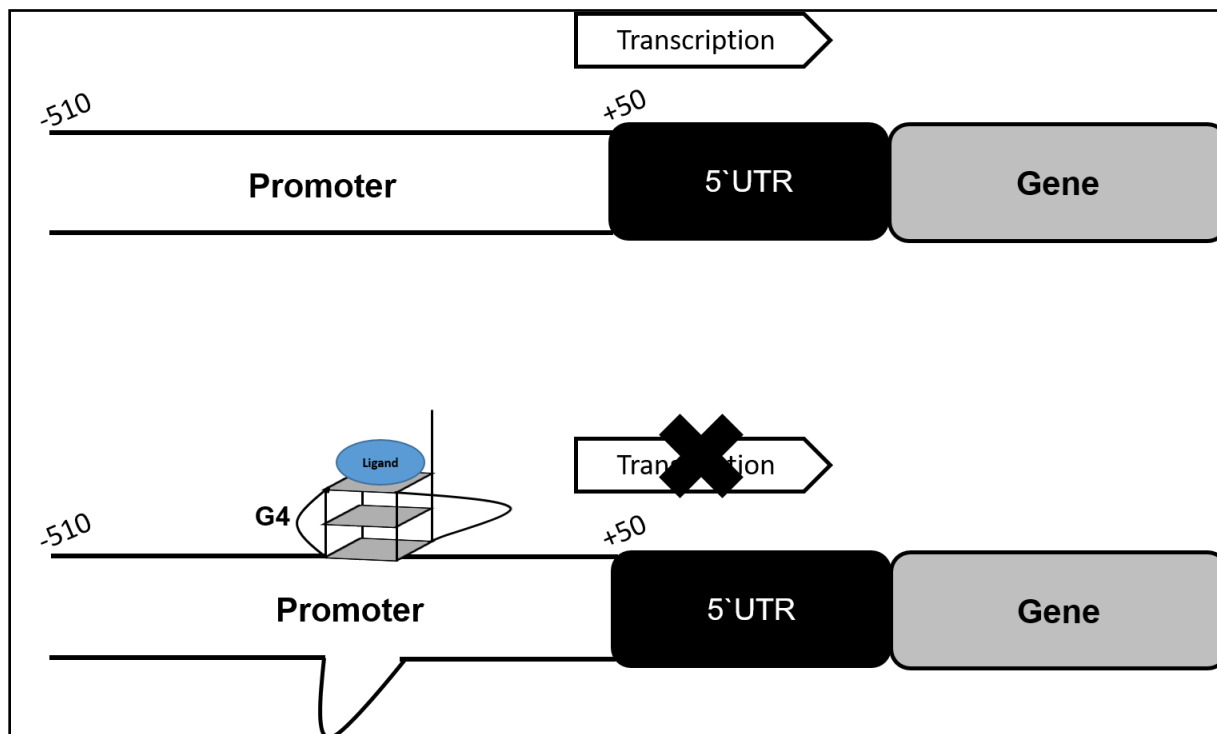
Figure 5-1. **Interaction and effect of G4-interacting compounds in promoter of genes.**

Pancreatic cancer is projected to become the third leading cause of cancer deaths surpassing breast cancer in the U.S. in 2016 (Siegel et al. 2016), and the second leading cause of cancer deaths in the U.S. by around 2020 (Rahib et al. 2014). Less transcription will lead to less translation of kRAS protein, and thus will affect the downstream signaling pathways associated with it which will finally cease the growth of the tumor. In the current work, we seek to identify and characterize compounds that bind and stabilize the G4 structure in the mid region of the kRAS, classifying them as $G4_{mid}$ and $G4_{near}$ stabilizers and to examine the mechanism of action of such agents in pancreatic cancer cells.

5.2. kRAS promoter G4 stabilization

We characterized compounds submitted by our collaborator, Dr. Khondaker Miraz Rahman from King's College in London. We were evaluating the compounds for selectivity to G4 structures within the kRAS promoter, specifically the mid region. Two different classes of compounds were analyzed. The BF series, (**Figure 5-2A**) a biphenylene and bipyridine connected bis-benzofuran, consisted of 12 compounds designed with the help of molecular modeling to facilitate interaction with G4 sequences. Tertiary amine side chains were incorporated to the C2-position of the benzofuran ring to improve the DNA binding affinity. The KN series is a tri-aryl benzofuran (**Figure 5-2B**) class of compounds, designed to interact with G4 sequences. As with the BF series, tertiary amines were incorporated at the C2-position of the benzofuran ring to improve DNA binding. 35 novel compounds were screened for their ability to stabilize G4 structures in the kRAS promoter, and to downregulate kRAS promoter activity. Thermodynamic stability and compound selectivity were examined for $G4_{mid}$ and $G4_{near}$ upon compound addition (**Table 1**). In the BF series, four compounds markedly increased $G4_{mid}$ stability over $G4_{near}$: BF-1.3, -2.2, -3.3, and -4.2 (**Figure 5-3A**). Within the KN series, six compounds - KN-119, -159, -212, -239, -257, and -267, showed stabilization of $G4_{mid}$ over $G4_{near}$, and seven compounds recognized and stabilized both G4s (KN-154, -217, -232, -237, -242, -247, and -272 (ECD performed by Dr. Rhianna M. Morgan, **Figure 5-3B**.) Further, the compounds were tested for their effect on kRAS promoter activity in the HEK-293 cells.
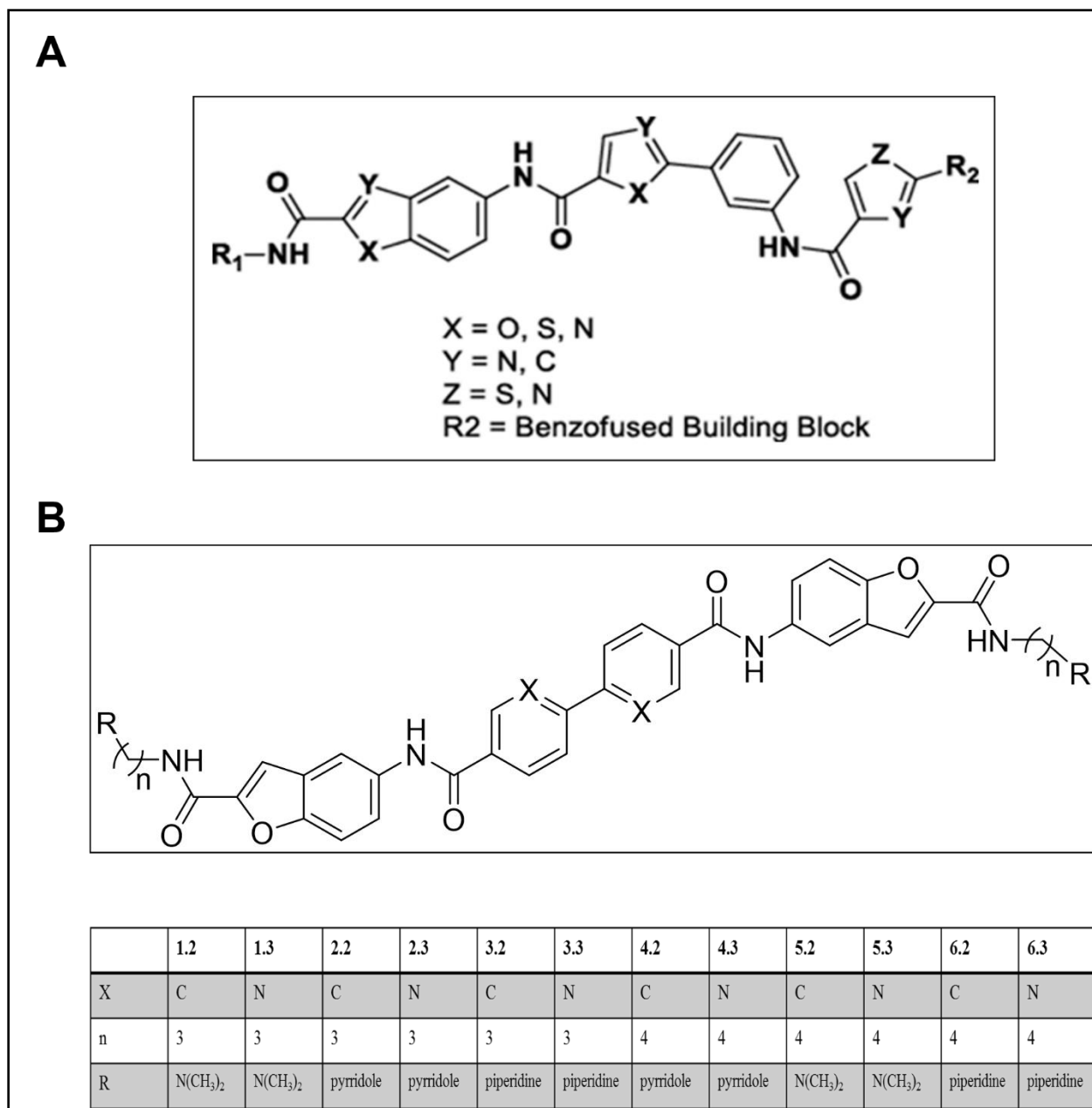
Figure 5-2. Basic structure of KN (A) and BF series compounds (B).

	1.2	1.3	2.2	2.3	3.2	3.3	4.2	4.3	5.2	5.3	6.2	6.3
X	C	N	C	N	C	N	C	N	C	N	C	N
n	3	3	3	3	3	3	4	4	4	4	4	4
R	$N(CH_3)_2$	$N(CH_3)_2$	pyrridole	pyrridole	piperidine	piperidine	pyrridole	pyrridole	$N(CH_3)_2$	$N(CH_3)_2$	piperidine	piperidine
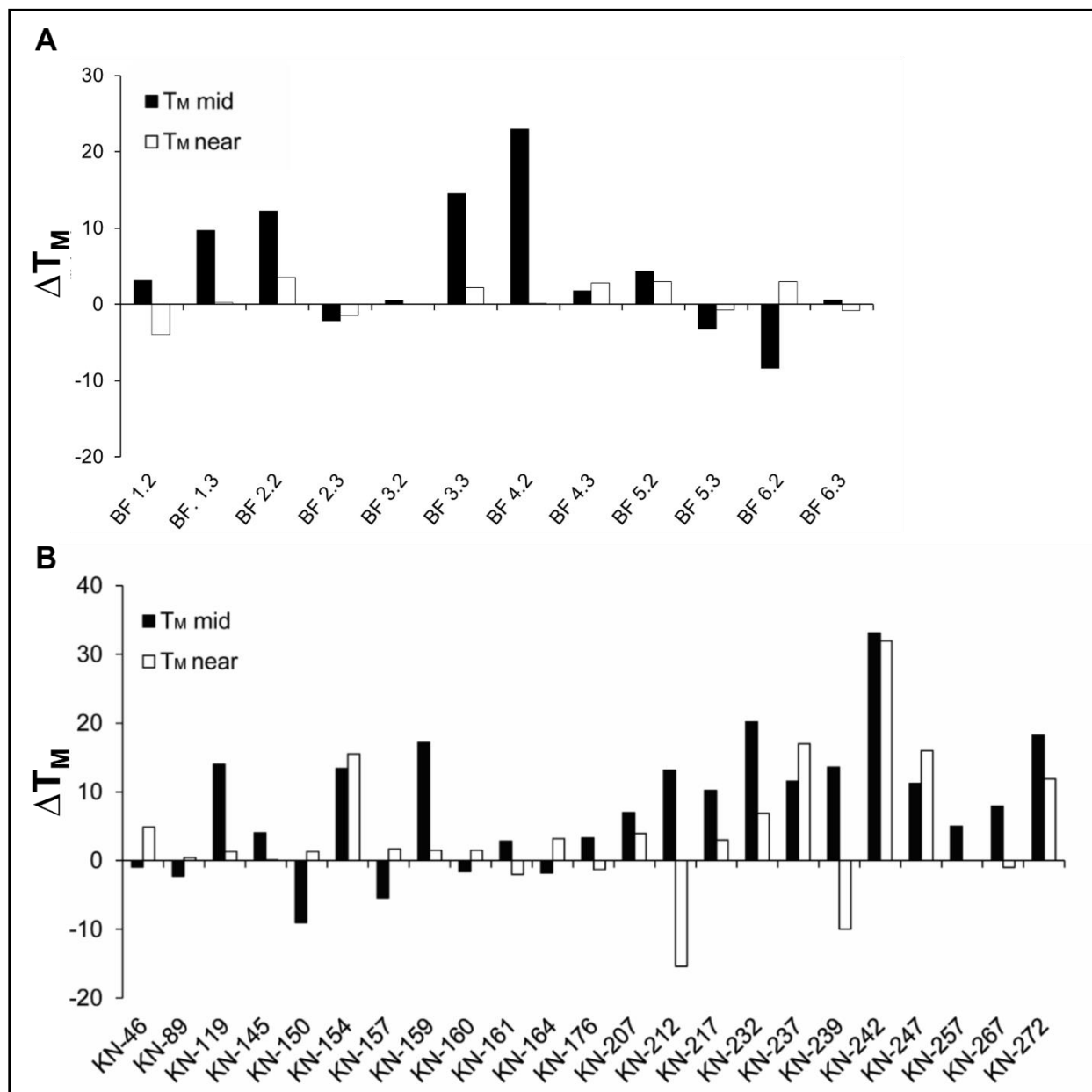
Figure 5-3. kRAS promoter G4 stabilization. 35 compounds of two different series (BF &KN) were screened by ECD to examine changes in thermal stability of $G4_{mid}$ and $G4_{near}$ (A). In BF series 4 compounds markedly increased $G4_{mid}$ stability over $G4_{near}$. On the other hand, for KN series 13 compounds markedly increased $G4_{mid}$ melting temperature compared to control while 7 increased that of G4near (B). Black box represent T_M mid and white boxes represent T_M near.

5.3. Changes in kRAS promoter activity

HEK-293 cells were transfected with a luciferase plasmid driven by the kRAS promoter (Morgan et al. 2016) and were treated with 1 μM of each BF and KN compound for 48 hr. BF-1.2, -2.2, -2.3, -4.2, -4.3, -5.3, and -6.3 significantly downregulated promoter activity in the kRAS plasmid (FL), as compared to EV negative control and DMSO-treated cells (**Figure 5-4A**). For the KN series of compounds, KN-89, -157, -159, -160, -161, -212, -237, -239, -242, and -272 significantly downregulated kRAS promoter activity (**Figure 5-4B**). Considering both the thermodynamic stability profile (for both promoter G4s) and the compounds' effects on promoter activity, BF-1.2, -2.2, -2.3, -4.2, -4.3, -5.3, and -6.3 the compounds of the BF series and 5 compounds of KN series KN-159, -212, -237, -239, -242, and -272 were selected for further studies in the pancreatic cancer cell line panel. BF 1.3, -3.3, and -6.2 were the negative control as they had no significant effect on promoter activity but affected the $G4_{mid}$ stability profile.
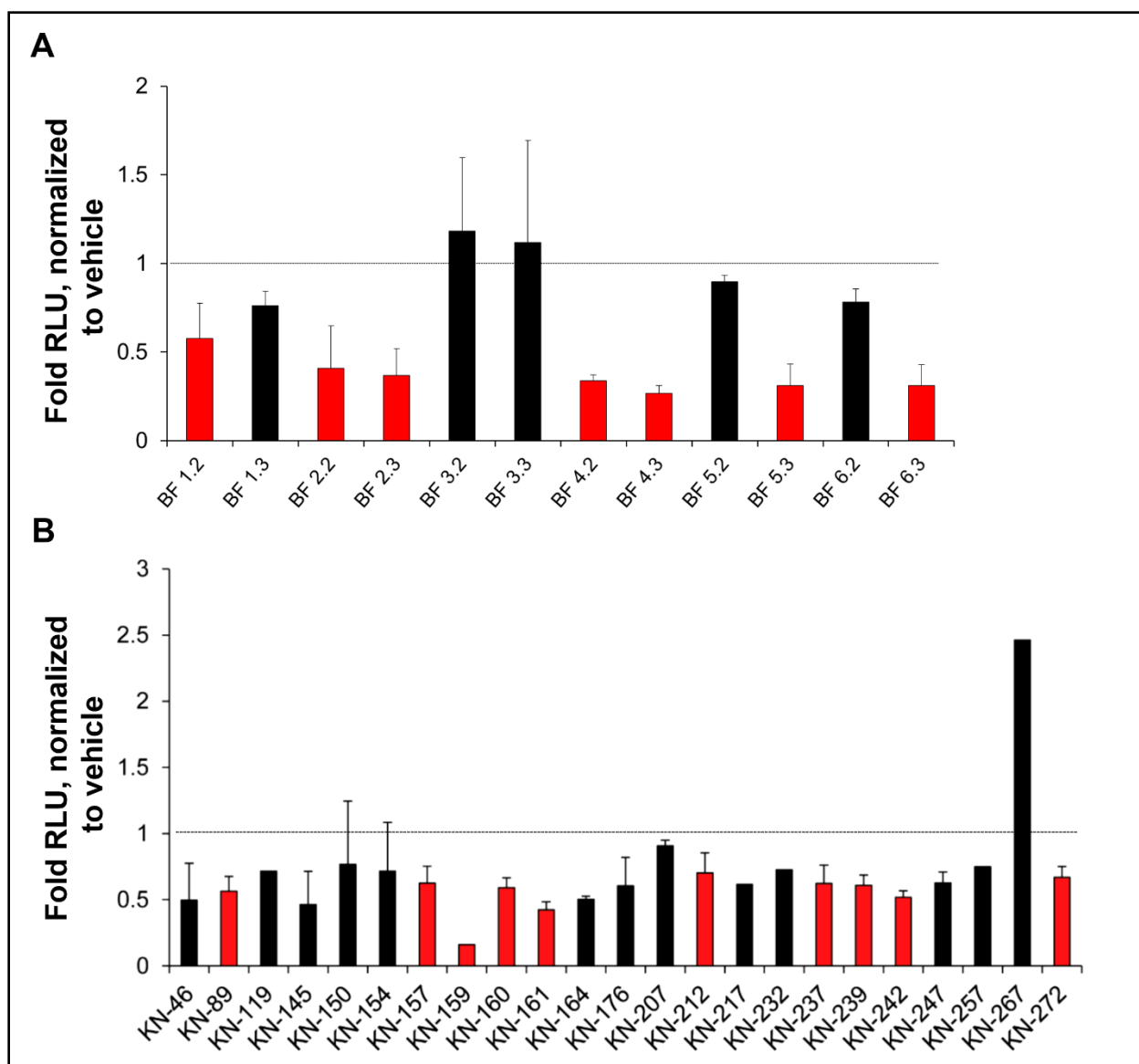
Figure 5-4. Changes in kRAS promoter activity. BF & KN compounds modulate kRAS promoter activity. HEK-293 cells were transfected with a luciferase plasmid driven by the kRAS promoter and were treated with 1 mM of each BF & KN compound for 48 hr. For BF series 7 compounds significantly downregulated kRAS promoter activity (A). Whereas for KN compounds 10 compounds led to this decrease (B). Experiments were run minimally in triplicate, with technical duplicates; Red bars, *p< 0.05 versus control).

5.4. Compound gene regulation and G4 stabilization

Two-thirds of the BF series compounds only showed inhibition of cell viability at 100 µM after 72 hr exposure. Only three compounds – BF-2.3, -4.3, and -5.3 were selected for further studies in pancreatic cancer cell lines at 30 and 100 µM based on their cytotoxicity profile. BF-6.3 was selected as a negative control. We wanted to take into account the effects of these compounds on cell lines with different levels of kRAS addiction, so the compounds were tested in both Panc-1 and MiaPaCa-2 cell lines. Panc-1 is less addicted to kRAS than MiaPaCa-2 cells (Aoki et al. 1997). We sought to correlate a decrease in cell viability with a decrease in kRAS transcription by determining each compounds' influence on kRAS mRNA expression. None of the BF series of compounds downregulated kRAS mRNA expression; rather, BF-5.3 and -6.3 lead to significant increase in kRAS mRNA levels in Panc-1 cell lines (**Figure 5-5A**) and BF-4.3 and -5.3 showed a significant increase in kRAS mRNA levels in MiaPaCa-2 cell line (**Figure 5-5B**). The mechanism behind such an increase is unknown.

Within the KN series of compounds, only KN-242 and -272 demonstrated a dose-dependent cytotoxic effect on pancreatic cancer cells. KN-242 had IC_{50} values of 2 and 11 µM in Panc-1 and MiaPaCa-2 cells, respectively (**Figure 5-6A**) and KN-272 had IC_{50} values of 60 and 6 µM in Panc-1 and MiaPaCa-2 pancreatic cancer cells, respectively (cell viability with KN-272 treatment performed by Dr. Rhianna Morgan, results not shown).

KN-272 had no marked impact on kRAS mRNA expression in MiaPaCa-2, compared to untreated cells (KN-272 RT-qPCR performed by Dr. Rhianna M. Morgan, results not shown). KN-242 significantly reduced kRAS expression Panc-1 and MiaPaCa-2 cells at a dose correlating with inhibition of cellular viability. In an effort to examine G4-selectivity, the transcriptional regulation of other genes (MYC, Bcl-2, and ADAM-15) with biologically active promoter G4s was measured

(Siddiqui-Jain et al. 2002; Dexheimer et al. 2006; Brown et al. 2013). KN-242 also affected the expression levels of MYC and Bcl-2 but had no effect on ADAM-15 in Panc-1 cells. On the other hand, in MiaPaCa-2 cells, KN-242 altered the expression of all three genes. (**Figure 5-6C**). Overall, after the characterization of 35 novel compounds, KN-242 was identified as a lead compound to be further characterized for its selectivity for kRAS, its ability to distinguish DNA structures (G4 vs dsDNA) and a determination of its mechanism of action.
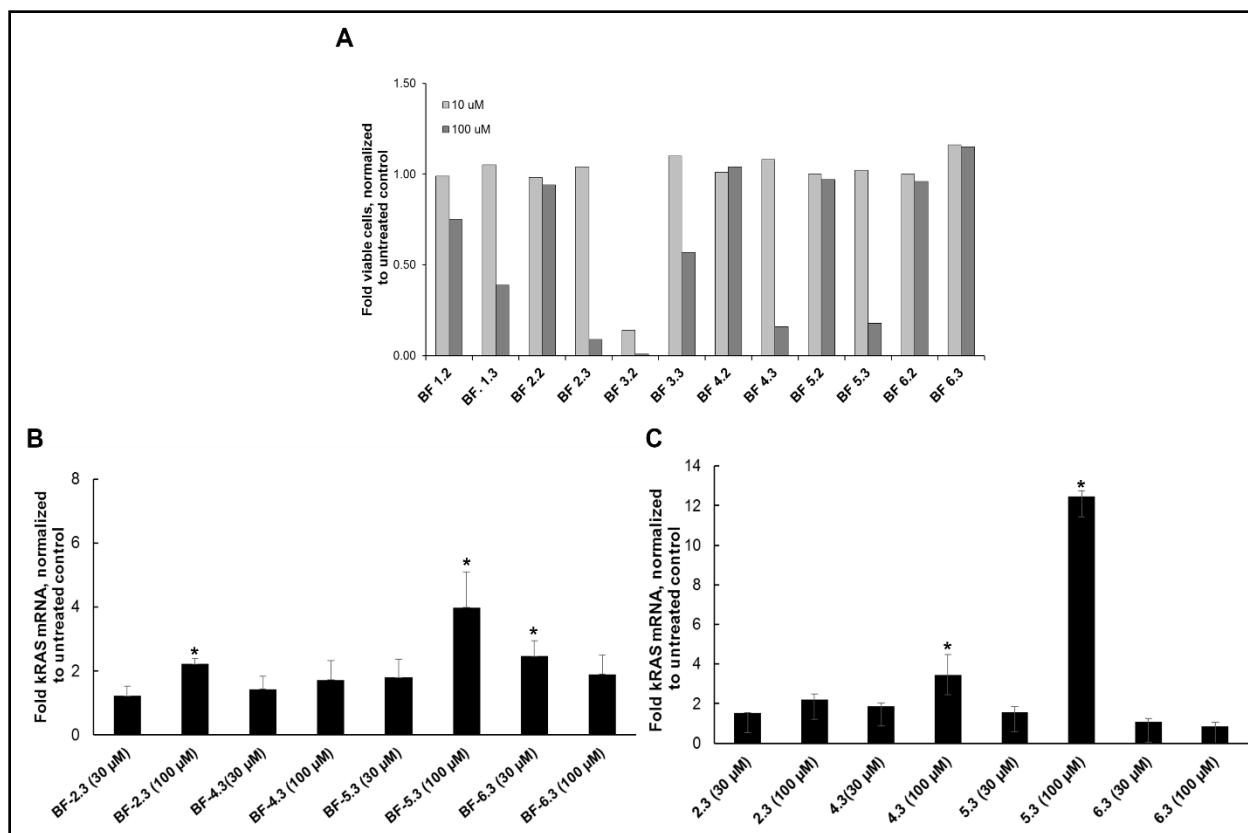
Figure 5-5. Changes in kRAS gene regulation. (A) Cytotoxicity of BF series of compounds. The dose-dependent effects of the pyridine series of BF compounds was examined following 72 hr of treatment in MiaPaCa-2 (B) and Panc-1 (C) cell lines. No significant decrease in mRNA was noted with any compound. Experiments were run minimally in triplicate with technical duplicates used for qPCR; $*p<0.05$ indicates a significant increase in expression, as compared to untreated controls. These findings indicate that there is a different mechanism of action of decreased cell viability in vitro than expected**.**
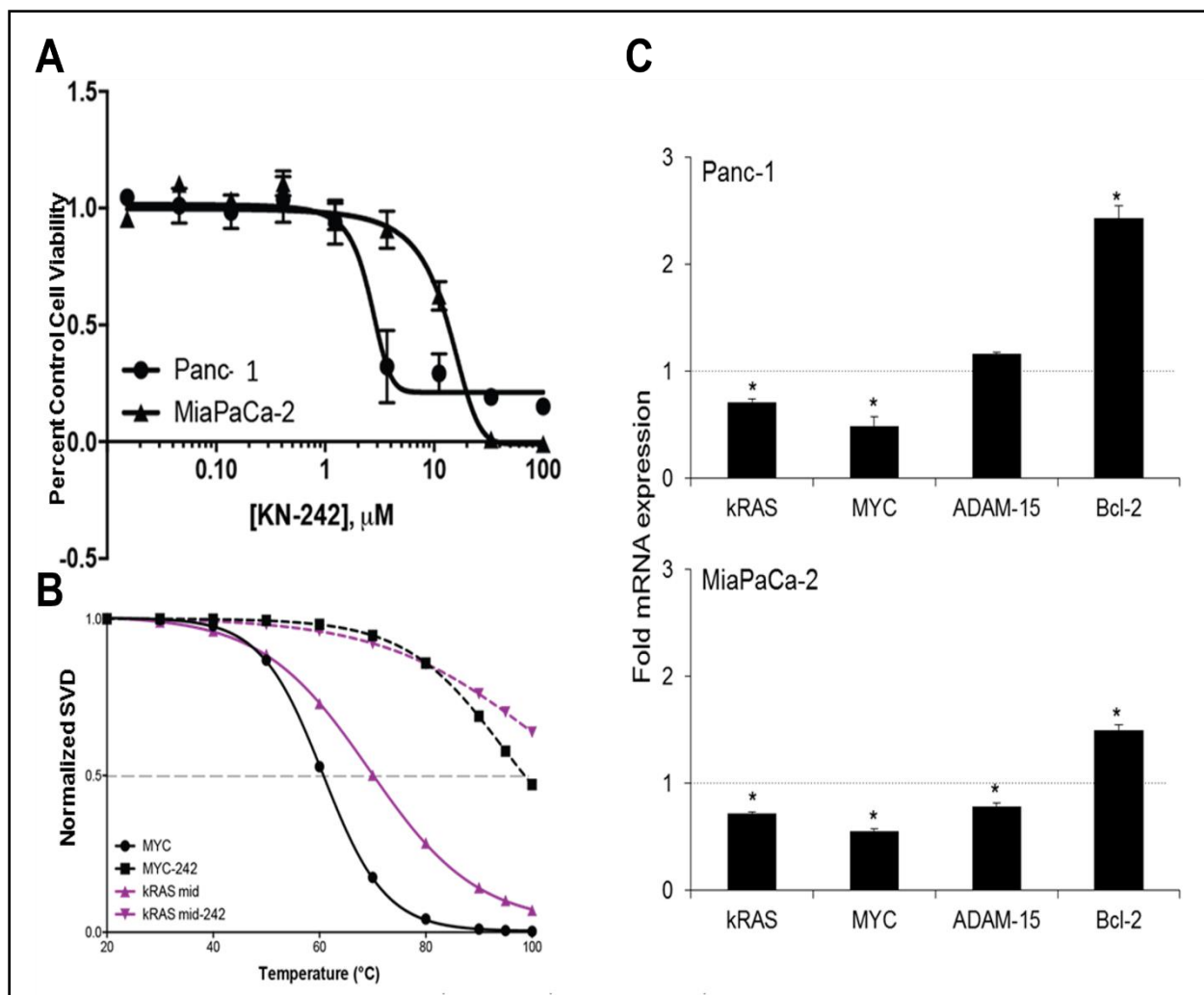
Figure 5-6. KN-242 cancer gene regulation & G4 stabilization. (A) Cytotoxicity of KN-242 on Panc-1 (IC$_{50}$ = 2 µM) and MiaPaCa-2 (IC$_{50}$= 11 µM) cells. (B) SVD for KN-242 in kRAS mid and MYC with KN-242, which stabilizes kRAS mid and MYC G4 by 33 and 40°C, respectively. (C) The effect of KN-242 on endogenous promoter activity was evaluated by qPCR. KN-242 decreased kRAS expression by nearly 30% in both MiaPaCa-2 and Panc-1 cells. MYC expression was also decreased by 40% in both cell lines. *$p<0.05$, 2-tailed student t-test versus untreated control.

5.5. Discussion

In the current work, we examined 35 compounds from two pharmacophore series developed based on molecular modeling. These compounds were screened against two G4s within the kRAS promoter, and after thermodynamic stability validation using ECD, nine compounds (four for BF series and five for the KN series) stabilized $G4_{mid}$ only, and seven compounds from the KN series stabilized both $G4_{mid}$ and $G4_{near}$. These compounds were further filtered based on the luciferase assay, cytotoxicity profiles, and qPCR results. We were able to identify a lead compound, KN-242, which showed a significant effect on kRAS expression, as well as several other oncogenes involved in tumorigenesis. These G4 stabilizing compounds typically lack selectivity with a particular G4 structure as they have tendency of ubiquitously binding to all G4 structures; future development efforts will involve increasing selectivity of this compound for the target promoter G4 structures through variations in side chains and halogen group replacements. Additionally, compounds already synthesized and future analogs will be screened against other biologically active G4s such as MYC, VEGF, Bcl-2, c-kit, hTERT, and ADAM-15 (Morgan & Brooks 2016).

Previously, the G4-forming region within the kRAS promoter has been examined for its effects on kRAS regulation. This has been performed through the use of protein transfection and G4-decoys that differ from G4s structurally based on locked or twisting base modifications. In each of these instances, treating cells with decoys of the $G4_{near}$ led to transcriptional silencing, putatively by sequestering G4-destabilizing proteins that normally unfolded chromosomal structures (Cogoi et al. 2008; Paramasivam et al. 2009; Cogoi et al. 2010). Previous studies have also looked at small molecules targeting kRAS $G4_{near}$ and $G4_{mid}$. Guanidine phthalocyanines such as DIGP and Zn-DIGP stabilize $G4_{near}$ and this interaction led to increase in kRAS transcription (Cogoi et al. 2010). On the other hand, in kRAS-dependent colon cancer cell lines, 7-carboxylate

indolo[3,2-b]quinoline tri-alkylamine derivatives (IQ3A) down-regulates kRAS expression, by stabilizing G4$_{near}$ (Brito et al. 2015). Compound benzophenanthridine alkaloid stabilizes the three existing kRAS G4s and led to downregulation of gene expression (Kaiser et al. 2017).

Recently it was described by our group that the silencing effect of G4s within the promoter of kRAS is seemingly contained in the mid region from -226 to -174, relative to the TSS, whereas G4$_{near}$ had no discernible effect on kRAS transcription (Morgan et al. 2016). These studies indicate that the G4$_{mid}$ is a more optimal target to modulate kRAS expression, a therapeutic approach which has been validated to have anti-cancer efficacy (Gray et al. 1993; Zhang et al. 1993; Aoki et al. 1997). Therapies targeting mutant RAS protein GTP binding, protein localization or downstream signaling pathways have failed to yield clinical agents related to mutant RAS activity (Ghobrial & Adjei 2002; Adjei 2001; Cho & Lee 2002; Queneau et al. 2001; Rowinsky et al. 1999; End 1999). Drugging the 'undruggable' kRAS requires a new approach, such as stabilization of unique DNA structures. Our approach of targeting G4 structures in the kRAS promoter for transcriptional silencing will halt all the downstream pathways associated with kRAS and ultimately lead to cancer cell death. Cumulatively, this information could positively impact the ongoing research related to the development of new chemotherapeutic agents for downregulating kRAS transcription against pancreatic cancer.

CHAPTER 6. CONCLUSION

This work looked at the regulation of kRAS promoter at the transcriptional level, and by small compound modulation. Our regulation focus was on TFs, their interactions with DNA regions and topologies, and their biological effects on kRAS transcription. Characterization of the promoter region and its regulatory proteins is important to understand the transcriptional regulation of any gene. We classified TFs as both transcriptional activators and silencers of kRAS, filling a knowledge gap in kRAS regulation. We identified seven proteins and evaluated their biological effects on kRAS transcription. We also identified two proteins (E2F-1 and nucleolin) which affect the stability of kRAS promoter G4 structures. Further, as described below, we looked at modulating these G4s with small molecules to regulate transcription.

We looked into the TFs that were previously reported in the literature, as well as others identified by their putative binding consensus sequence from the PROMO database. In addition, we identified TFs by direct binding studies. Overall, we explored the function of seven TFs. The kRAS basic promoter extends from -510bp to + 50bp (Jordano et al. 1986; Jordano et al. 1988; Yamamoto et al. 1988). Our lab demonstrated that transcriptional activity was contained from -324 to + 50bp (Morgan et al. 2016); therefore, for our future work we utilized the -324 to + 50 bp plasmid. Previously, it was shown that core promoter region from 0 ±50 bp plays a critical role in transcription initiation and guiding and that the upstream region is necessary for full expression. The upstream region contains three putative G4-forming regions, two of which form stable structures (G4near and G4mid) (Cogoi et al. 2013; Morgan et al. 2016). Cumulatively, we

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examined MAZ, Sp1, E2F-1 AP-2, PPAR-γ, YBX-1, and nucleolin for their effects and function, binding and G4-interactivity.

Of these, only TF examined previously was MAZ. By EMSA and filter binding assay MAZ was shown to bind to the G4$_{near}$ kRAS promoter and this binding led to MAZ sequestration and effects the kRAS transcription (Cogoi et al. 2010; Cogoi et al. 2013; Cogoi et al. 2014). Our finding here contradicts the work done by Cogoi group, in which they showed MAZ binding to near G4 and duplex kRAS and this interaction leads to 3.5 fold increase in kRAS transcriptional activity in Panc-1 cell lines (Cogoi et al. 2013). We accomplished our studies by a step-by-step approach, starting from the basic level to minimize the variables in our studies. We first focused on the effect of MAZ in an isolated system of non-cancerous cell lines (HEK-293), such as less noise or variation due to other factors as present in cancerous cells. Secondly, we took in account broader panel of pancreatic cancer cell lines based on their kRAS addiction pattern, and we also calculated the level of these proteins at basal levels in cell lines which are not accounted for in previous studies. Further, we confirmed RT-qPCR transcriptional downregulation by western blotting and found similar results showing reduce kRAS protein levels in these cell lines at 500 ng of MAZ plasmids. Sp1 interacts with the TATA-less promoter and is shown to interact with hRAS. Sp1 was also identified in our PROMO database search. E2F-1 was our third hit which showed four binding sites in core promoter region. After identifying these hits, our aim was to examine TF mediated regulation of the kRAS promoter and to understand their biological role.

We decided on a two-step approach. The first approach was to test these TFs in simplified promoter system using non-cancerous cell line. We performed the luciferase assay in HEK-293 cells with the plasmid containing the kRAS promoter from -324 to +50 bp relative to the TSS. We found out that MAZ and Sp1 downregulate kRAS promoter when used individually, but in

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combination, the effects were abrogated, leading to an increased kRAS promoter activity. On the other hand, E2F-1 increased kRAS promoter activity. The second approach was to overexpress these proteins in pancreatic cancer cells, i.e., Panc-1, MiaPaCa-2, and AsPc-1 and to study the effects in a multicellular mileu. We also examined an important protein kinase CK2 shown to phosphorylate these proteins and alter the binding of these proteins to DNA. The result was surprising as AsPc-1 cells showed a significant increase with CK2 inhibitor TBB treatment while Panc-1 and MiaPaCa-2 showed a significant decrease. Future studies are warranted to look into the mechanism leading to such variation.

Lastly, we examined the binding of these proteins on different regions and topologies in the kRAS promoter. We also tested all these proteins to determine their effect on G4 stability by ECD. We found that out of three proteins only E2F-1 significantly destabilized the kRAS G4$_{near}$.

We further wanted to evaluate the global binding and regulation of kRAS promoter. The three-way approach was utilized to identify and characterize different regulatory proteins binding to the kRAS promoter. These included rVISTA genome tool (Zombon et al. 2005), Promoter Binding TF Profiling Plate Array kit (Signosis, Santa Clara, CA) and DNA pulldown assay followed by LC-MS/MS for protein identification (Wu 2006). After filtering out, the data were selected for subsequent biological investigation.

These selected proteins underwent the same biological evaluation as mentioned in chapter 3. Over time, the increased AP-2, PPAR-γ, and nucleolin expression led to an increase in kRAS promoter activity as measured by luciferase expression. Our fourth hit YBX-1 however, showed a significant decrease in the promoter activity. Ultimately from luciferase data, we classified the identified proteins in two categories as promoter activators (AP-2, PPAR-γ, and nucleolin) and promoter silencers (YBX-1). Whereas after comparing the result obtained from endogenous

regulation through RT-qPCR, PPAR-γ and nucleolin (500ng) were classified as the transcriptional activators as they caused an increase in kRAS promoter activity and increased % mRNA expression. On the other hand, AP-2 and YBX-1 showed opposite effect in luciferase assay and RT-qPCR, so further investigation is required for their classification. Finally, these proteins were tested for their binding profiles using EMSA and were further analyzed for their effects to G4 stabilization by ECD. Nucleolin was the only hit obtained with a significant destabilization of the kRAS G4$_{mid}$ structure. Previously it was shown that nucleolin stabilizes MYC G4 structures and by doing so, it led to decrease in MYC transcription. However, for kRAS promoter results obtained were opposite. Future work is required to understand the role of two G4 interacting proteins identified in our studies i.e., E2F-1 and nucleolin. With CHIP-seq, binding to the specific DNA sequence will be evaluated. E2F-1 and nucleolin will be tested for their role in folding or unfolding of structures, and by DMS foot printing, the guanines involved in destabilizing the structure will be studied. As these two proteins lead to an increase in kRAS transcription, they can also be used as a drug target by small molecules to prevent their binding to the kRAS promoter or by using the G4 stabilizing compounds to prevent the disruption of G4 stability by these proteins. Moreover, the other TFs which were detected in our methods such as NME1, YBX-3, Nucleophosmin, BTF3, LYRIC, hnRNPs, 14-3-3s, IGFs, STATs, NFAT, and GATA need to be evaluated to complete the puzzle of kRAS transcriptional regulation.

In addition to understanding the molecular basis of kRAS regulation, we also focused on finding new drug targets against this oncogene. kRAS is one of the highly deregulated oncogenes that promotes tumorigenesis. In 30% of all cancers and 90% of PDAC, kRAS is mutated (Ryan et al. 2014). Several approaches have been utilized to target kRAS with little to no success. These six promising paths include- direct inhibitors of kRAS, disturbing its membrane association,

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synthetic lethal interactors, the kRAS downstream inhibitors, targeting kRAS metabolic regulatory processes, and harnessing the immune response (Ghobrial & Adjei 2002; Adjei 2001; Cho & Lee 2002; Queneau et al. 2001; Rowinsky et al. 1999; End 1999). Targeting kRAS transcriptionally through G4 stabilization with small molecule has not been used before. We screened 35 novel compounds for their ability to stabilize G4 structures in the kRAS promoter and downregulate kRAS promoter activity. Of all those compounds, KN-242 was a hit compound. KN-242 significantly reduced kRAS expression, as well as the expression of other important genes involved in tumorigeneses, such as MYC, ADAM-15, and Bcl-2. As KN-242 is not specific as a target, future work is warranted to develop more specific and selective analogs of $G4_{mid}$ and to further screen libraries to find compounds against other oncogenes in which G4 structure has been established. Ultimately, G4-stabilization-mediated downregulation of kRAS has a high potential for anti-cancer efficacy in pancreatic cancers, wherein there is a dire need for novel therapeutic development.

There is currently a gap in the knowledge regarding the TFs binding to the kRAS promoter, particularly to varying DNA topologies, and their role in kRAS expression. We focused on several TFs and classified them as activators and silencers; some of these worked through G4 while others had no effect on G4 stability profile. Understanding the mechanism of action and combinatorial effects of TFs is an important part of the larger puzzle leading to a targeted drug discovery program focused on G4-regulation and utilizing TFs as drug targets themselves. In conclusion, this information will positively impact the ongoing research related to the development of new chemotherapeutic agents for downregulating kRAS transcription by unveiling the mechanism of transcription control and to confirm the mechanism of action of developed compounds.

LIST OF REFERENCES

REFERENCES

Abbruzzese, J.L. Pancreatic cancer: Scanning the horizon for focused interventions. Available online: http://deainfo.nci.nih.gov/advisory/ctac/archive/0313/PCwgReport.pdf 1-22.

Aboul-Ela, F., Murchie, A.I. & Lilley, D.M., 1992. NMR study of parallel-stranded tetraplex formation by the hexadeoxynucleotide d (TG4T). *Nature, 360(6401), pp.280-282.*

Adjei, A., 2001. Blocking oncogenic Ras signaling for cancer therapy. *J Natl Cancer Inst, 93(14), pp.1062–1074.*

Adriaenssens, E., Dumont, L., Lottin, S., Bolle, D., Leprêtre, A., Delobelle, A. & Curgy, J.J., 1998. H19 Overexpression in Breast Adenocarcinoma Stromal Cells Is Associated with Tumor Values and Steroid Receptor Status but Independent of p53 and Ki-67 Expression. *The American Journal of Pathology, 153(5), pp.1597–1607.*

Adrian, T.E., Hennig, R., Friess, H. & Ding, X., 2009. The role of PPARgamma receptors and leukotriene B(4) receptors in mediating the effects of LY293111 in pancreatic cancer. *PPAR Res, 2008, pp.827096.*

Aguirre, A.J., Bardeesy, N., Sinha, M., Lopez, L., Tuveson, D.A., Horner, J. & DePinho, R.A., 2003. Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. *Genes Dev, 17(24), pp.3112- 3126.*

Almoguera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N. & Perucho, M., 1988. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell 53(4), pp.549-554.*

Angelov, D., Bondarenko, V.A., Almagro, S., Menoni, H., Mongelard, F., Hans, F., Mietton, F., Studitsky, V.M., Hamiche, A., Dimitrov, S. & Bouvet, P., 2006. HY5 stability and activity in arabidopsis is regulated by phosphorylation in its COP1 binding domain. *EMBO J, 19(18), pp.4997-5006.*

Ariel, I., de Groot, N. & Hochberg, A., 2000. Imprinted H19 gene expression in embryogenesis and human cancer: the oncofetal connection. *Am J Med Genet, 91(1), pp.46-50.*

Armstrong, S.A., Barry, D.A., Leggett, R.W. & Mueller, C.R., 1997. Casein Kinase II-mediated Phosphorylation of the C Terminus of Sp1 Decreases Its DNA Binding Activity. *The Journal of Biological Chemistry, 272(21), pp.13489-13495.*

Ashfield, R. Patel, A.J., Bossone, S.A., Brown, H., Campbell, R.D., Marcu, K.B. & Proudfoot, N.J., 1994. MAZ-dependent termination between closely spaced human complement genes. *The EMBO Journal, 13(23), pp.5656–5667.*

Athuluri-Divakar, S.K., Vasquez-Del Carpio, R., Dutta, K., Baker, S.J., Cosenza, S.C., Basu, I. & Vogt, P.K., 2016. A Small Molecule RAS-Mimetic Disrupts RAS Association with Effector Proteins to Block Signaling. *Cell, 165(3), pp.643–655.*

Avruch, J., Xavier, R., Bardeesy, N., Zhang, X.F., Praskova, M., Zhou, D. & Xia, F., 2009. Rassf family of tumor suppressor polypeptides. *Journal of Biological Chemistry, 284(17), pp.11001-11005.*

Babu, M.M., Luscombe, N.M., Aravind, L., Gerstein, M. & Teichmann, S.A., 2004. Structure and evolution of transcriptional regulatory networks. *Curr Opin Struct Biol, 14(3), pp.283–291.*

Bailey, T.L. & Elkan, C., 1995, July. The value of prior knowledge in discovering motifs with MEME. *In Ismb (Vol. 3, pp. 21-29).*

Bailey, T.L. & Elkan, C., 1995. Unsupervised learning of multiple motifs in biopolymers using expectation maximization. *Machine learning, 21(1), pp.51-80.*

Bain, G., Maandag, E.C.R., Izon, D.J., Amsen, D., Kruisbeek, A.M., Weintraub, B.C. & van der Valk, M., 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell, 79(5), pp.885-892.*

Baines, A.T., Xu, D. & Der, C.J., 2011. Inhibition of Ras for cancer treatment: The search continues. *Future Med. Chem, 3(14), pp.1787–1808.*

Balasubramanian, S., Hurley, L.H. & Neidle, S., 2011. Targeting G-quadruplexes in gene promoters: a novel anticancer strategy? *Nat Rev Drug Discov, 10(4), pp.261-275.*

Barbie, D.A., Tamayo, P., Boehm, J.S., Kim, S.Y., Moody, S.E., Dunn, I.F. & Fröhling, S., 2009. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature, 462(7269), pp.108–112.*

Bar-Eli, M., 2001. Gene regulation in melanoma progression by the AP-2 transcription factor. *Pigment Cell Res, 14(2), pp.78-85.*

Bart Vanhaesebroeck, I.G. & Fry, M.J., 1986. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Acta B0, 317, p.328.*

Basehoar, A.D., Zanton, S.J. & Pugh, B.F., 2004. Identification and distinct regulation of yeast TATA box-containing genes. *Cell, 116(5), pp.699-709.*

Basso, A.D., Kirschmeier, P. & Bishop, W.R., 2006. Lipid posttranslational modifications. Farnesyl transferase inhibitors. *J. Lipid Res, 47(1), pp.15–31.*

Bates, P.J., Kahlon, J.B., Thomas, S.D., Trent, J.O. & Miller, D.M., 1999. Antiproliferative activity of G-rich oligonucleotides correlates with protein binding. *J. Biol. Chem, 274(37), 26369–26377.*

Bates, P.J., Laber, D.A., Miller, D.M., Thomas, S.D. & Trent, J., 2009. Discovery and development of the G-rich oligonucleotide AS1411 as a novel treatment for cancer. *Exp Mol Pathol, 86(3), pp.151–164.*

Batsché, E., Muchardt, C., Behrens, J., Hurst, H.C. & Crémisi, C., 1998. RB and c-Myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2. *Mol Cell Biol, 18(7), pp.3647-3658.*

Bejugam, M., Sewitz, S., Shirude, P.S., Rodriguez, R., Shahid, R. & Balasubramanian, S., 2007. Trisubstituted isoalloxazines as a new class of G-quadruplex binding ligands: small molecule regulation of c-kit oncogene expression. *J. Am. Chem. Soc, 129 (43), pp.12926–12927.*

Bell, G.I., Selby, M.J. & Rutter, W.J., 1982. The highly polymorphic region near the human insulin gene is composed of simple tandemly repeating sequences. *Nature, 295(5844), pp.31-35.*

Benjamin, R.C. & Gill, D.M., 1980. Poly (ADP-ribose) synthesis in vitro programmed by damaged DNA. A comparison of DNA molecules containing different types of strand breaks. *Journal of Biological Chemistry, 255(21), pp.10502-10508.*

Benoist, C. & Chambon, P., 1981. In vivo sequence requirements of the SV40 early promotor region. *Nature, 290(5804), pp.304-310.*

Berndt, N., Hamilton, A.D. & Sebti, S.M., 2011. Targeting protein prenylation for cancer therapy. *Nat. Rev. Cancer, 11(11), pp.775–791.*

Berteaux, N., Lottin, S., Monté, D., Pinte, S., Quatannens, B., Coll, J. & Adriaenssens, E., 2005. H19 mRNA-like noncoding RNA promotes breast cancer cell proliferation through positive control by E2F1. *J Biol Chem, 280(33), pp.29625-29636.*

Biankin, A.V., Waddell, N., Kassahn, K.S., Gingras, M.C., Muthuswamy, L.B., Johns, A.L. & Chang, D.K., 2012. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature, 491(7424), pp.399-405.*

Birchenall-Roberts, M.C., Fu, T., Kim, S.G., Huang, Y.K., Dambach, M., Resau, J.H. & Ruscetti, F.W., 2006. K-Ras4B proteins are expressed in the nucleolus: interaction with nucleolin. *Biochem Biophys Res Commun, 348(2), pp.540–549.*

Blackburn, E.H., 1991. Structure and function of telomeres. *Nature, 350(6319), pp.569-573.*

Bochman, M.P., Paeschke, K. & Zakian, V.A., 2012. DNA secondary structures: stability and function of G quadruplex structures. *Nat Rev Genet, 13(11), pp.770-780.*

Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan–de Vries, M., van Boom, J.H., van der Eb, A.J. & Vogelstein, B., 1987. Prevalence of ras gene mutations in human colorectal cancers. *Nature, 327(6120), pp.293-297.*

Bosher, J.M., Totty, N.F., Hsuan, J.J., Williams, T. & Hurst, H.C., 1996. A family of AP-2 proteins regulates c-erbB-2 expression in mammary carcinoma. *Oncogene, 13(8), pp.1701-1707.*

Bosher, J.M., Williams, T. & Hurst, H.C., 1995. The developmentally regulated transcription factor AP-2 is involved in c-erbB-2 overexpression in human mammary carcinoma. Proc Natl Acad Sci U S A, 92(3), pp.744-747.

Bossone, S.A., Asselin, C., Patel, A.J. & Marcu, K.B., 1992. MAZ, a zinc finger protein, binds to c-MYC and C2 gene sequences regulating transcriptional initiation and termination. *PNAS, 89(16), pp.7452-7456.*

Bragança, J., Swingler, T., Marques, F.I., Jones, T., Eloranta, J.J., Hurst, H.C., Shioda, T. & Bhattacharya, S., 2002. Human CREB-binding protein/p300-interacting transactivator with ED-rich tail (CITED) 4, a new member of the CITED family, functions as a co-activator for transcription factor AP-2. *J Biol Chem, 277(10), pp.8559-8565.*

Bragança, J., Swingler, T., Marques, F.I., Jones, T., Eloranta, J.J., Hurst, H.C. & Bhattacharya, S., 2003. Physical and functional interactions among AP-2 transcription factors, p300/CREB-binding protein, and CITED2. *J Biol Chem, 278(18), pp.16021-16029.*

Brázda, V., Paleĉek, J., Pospîšilová, Š., Vojtêšek, B. & Paleĉek, E., 2000. Specific modulation of p53 binding to consensus sequence within supercoiled DNA by monoclonal antibodies. *Biochemical and biophysical research communications, 267(3), pp.934-939.*

Brito, H., Martins, A.C., Lavrado, J., Mendes, E., Francisco, A.P., Santos, S.A. & Neidle, S., 2015. Targeting KRAS Oncogene in Colon Cancer Cells with 7-Carboxylate Indolo[3,2-b]quinoline Tri-Alkylamine Derivatives. *PLoS ONE, 10(5), e0126891.*

Britten, C.D., 2013. PI3K and MEK inhibitor combinations: Examining the evidence in selected tumor types. *Cancer Chemotherapy and Pharmacology, 71(6), pp.1395–1409.*

Brooks, T.A. & Hurley, L.H., 2009. The role of supercoiling in transcriptional control of MYC and its importance in molecular therapeutics. *Nat Rev Cancer, 9(12), pp.849-861.*

Brooks, T.A. & Hurley, L.H., 2010. Targeting MYC Expression through G-Quadruplexes. *Genes Cancer, 1(6), pp.641-649.*

Brown, R.V., Gaerig, V.C., Simmons, T. & Brooks, T.A., 2013. Helping Eve overcome ADAM: G-quadruplexes in the ADAM-15 promoter as new molecular targets for breast cancer therapeutics. *Molecules, 18(12), pp.15019-15034.*

Bryant, K.L., Mancias, J.D., Kimmelman, A.C. & Der, C.J., 2014. KRAS: Feeding pancreatic cancer proliferation. *Trends Biochem. Sci, 39(2), pp.91–100.*

Brys, A. & Maizels, N., 1994. LR1 regulates c-myc transcription in B-cell lymphomas. *Proc. Natl. Acad. Sci. U.S.A. 91(11), pp.4915–4919.*

Burke, T.W. & Kadonaga, J.T., 1996. Drosophila TFIID binds to a conserved downstream basal promoter element that is present in many TATA-box-deficient promoters. *Genes Dev, 10(6), pp.711-724.*

Burke, T.W. & Kadonaga, J.T., 1997. The downstream core promoter element, DPE, is conserved from Drosophila to humans and is recognized by TAFII60 of Drosophila. *Genes Dev, 11(22), pp.3020-3031.*

Burke, T.W., Willy, P.J., Kutach, A.K., Butler, J.E.F. & Kadonaga, J.T., 1998. The DPE, a conserved downstream core promoter element that is functionally analogous to the TATA box. *Cold Spring Harb Symp Quant Biol, 63, pp.75-82.*

Burnett, G. & Kennedy, E.P., 1954. The enzymatic phosphorylation of proteins. *J Biol Chem, 211, pp. 969-980.*

Butler, J.E. & Kadonaga, J.T., 2002. The RNA polymerase II core promoter: a key component in the regulation of gene expression. *Genes Dev, 16(20), pp.2583-2592.*

Campbell, P.M. & Der, C.J., 2004. Oncogenic Ras and its role in tumor cell invasion and metastasis. *Semin Cancer Biol, 14(2), pp.105-114.*

Carey, M., 1998. The enhanceosome and transcriptional synergy. *Cell, 92(1), pp.5-8.*

Carninci, P., Sandelin, A., Lenhard, B., Katayama, S., Shimokawa, K., Ponjavic, J. & Forrest, A.R., 2006. Genome-wide analysis of mammalian promoter architecture and evolution. *Nat Genet, 38(6), pp.626-635.*

Castellano, E., Sheridan, C., Thin, M.Z., Nye, E., Spencer-Dene, B., Diefenbacher, M.E. & Lassailly, F., 2013. Requirement for Interaction of PI3-Kinase p110?? with RAS in Lung Tumor Maintenance. *Cancer Cell, 24(5), pp.617–630.*

Chang, E.H., Gonda, M.A., Ellis, R.W., Scolnick, E.M. & Lowy, D.R., 1982. Human genome contains four genes homologous to transforming genes of Harvey and Kirsten murine sarcoma viruses. *Proc Natl Acad Sci U S A, 79 (16), pp.4848-485.*

Chang, T.H. & Szabo, E., 2000. Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor γ in non-small cell lung cancer. *Cancer Res, 60(4), pp.1129-1138.*

Chang-Liu, C.M. & Woloschak, G.E., 1997. Effect of passage number on cellular response to DNA-damaging agents: cell survival and gene expression. *Cancer Letters, 26(113), pp.77-86.*

Chasovskikh, S., Dimtchev, A., Smulson, M. & Dritschilo, A., 2005. DNA transitions induced by binding of PARP-1 to cruciform structures in supercoiled plasmids. *Cytometry Part A, 68(1), pp.21-27.*

Chen, L., Zhang, L.J., Greer, P., Tung, P.S. & Moran, M.F., 1993. A murine CDC25/ras-GRF-related protein implicated in ras regulation. *Genesis, 14(5), pp.339-346.*

Cheng, C., Ying, K., Xu, M., Zhao, W., Zhou, Z., Huang, Y., Wang, W., Xu, J., Zeng, L., Xie, Y. & Mao, Y., 2002. Cloning and characterization of a novel human transcription factor AP-2 beta like gene (TFAP2BL1). *Int J Biochem Cell Biol, 34(1), 78-86.*

Cho, K. & Lee, K., 2002. Chemistry and biology of Ras farnesyltransferase. *Arch Pharm Res, 25(6), pp.759–769.*

Chu, P.C., Yang, M.C., Kulp, S.K., Salunke, S.B., Himmel, L.E., Fang, C.S., Jadhav, A.M., Shan, Y.S., Lee, C.T., Lai, M.D. & Shirley, L.A., 2016. Regulation of oncogenic KRAS signaling

via a novel KRAS-integrin-linked kinase-hnRNPA1 regulatory loop in human pancreatic cancer cells. *Oncogene, 35(30), pp.3897-3908.*

Chu, P.C., Yang, M.C., Kulp, S.K., Salunke, S.B., Himmel, L.E., Fang, C.S., Jadhav, A.M., Shan, Y.S., Lee, C.T., Lai, M.D. & Shirley, L.A., 1986. Structure of rodent helix-destabilizing protein revealed by cDNA cloning. *J. Biol. Chem, 261(8), pp.3536–3543.*

Cogoi, S. & Xodo, L.E., 2006. G-quadruplex formation within the promoter of the KRAS proto-oncogene and its effect on transcription. *Nucleic Acids Research, 34(9), pp.2536–2549.*

Cogoi, S., Paramasivam, M., Filichev, V., Géci, I., Pedersen, E.B. & Xodo, L.E., 2008. Identification of a new G-quadruplex motif in the KRAS promoter and design of pyrene-modified G4-decoys with antiproliferative activity in pancreatic cancer cells. *Journal of medicinal chemistry, 52(2), pp.564–568.*

Cogoi, S., Paramasivam, M., Membrino, A., Yokoyama, K.K. & Xodo, L.E., 2010. The KRAS promoter responds to Myc-associated zinc finger and poly(ADP-ribose) polymerase 1 proteins, which recognize a critical quadruplex forming GA-element. *Journal of Biological Chemistry, 285(29), pp.22003–22016.*

Cogoi, S., Paramasivam, M., Spolaore, B. & Xodo, L.E., 2008. Structural polymorphism within a regulatory element of the human KRAS promoter: formation of G4-DNA recognized by nuclear proteins. *Nucleic Acids Res, 36(11), pp.3765–3780.*

Cogoi, S., Rapozzi, V., Cauci, S. & Xodo, L.E., 2016. Critical role of hnRNP A1 in activating KRAS transcription in pancreatic cancer cells: A molecular mechanism involving G4 DNA. *Biochimica Et Biophysica Acta (BBA), 1861(5 Pt B), pp.1389-1398.*

Cogoi, S., Shchekotikhin, A.E. & Xodo, L.E., 2014. HRAS is silenced by two neighboring G-quadruplexes and activated by MAZ, a zinc-finger transcription factor with DNA unfolding property. *Nucleic Acids Res, 42(13), pp.8379-8388.*

Cogoi, S., Zorzet, S., Rapozzi, V., Géci, I., Pedersen, E.B. & Xodo, L.E., 2013. MAZ-binding G4-decoy with locked nucleic acid and twisted intercalating nucleic acid modifications suppresses KRAS in pancreatic cancer cells and delays tumor growth in mice. *Nucleic Acids Research, 41(7), pp.4049-4064.*

Colicelli, J., 2004. Human RAS superfamily proteins and related GTPases. *Sci STKE, (250), RE13.*

Collins, M.A., Bednar, F., Zhang, Y., Brisset, J.C., Galbán, S., Galbán, C.J., Rakshit, S., Flannagan, K.S., Adsay, N.V. & di Magliano, M.P., 2012. Oncogenic Kras is required for

both the initiation and maintenance of pancreatic cancer in mice. *J Clin Invest, 122(2), pp.639-653.*

Collins, M.A., Brisset, J.C., Zhang, Y., Bednar, F., Pierre, J., Heist, K.A., Galbán, C.J., Galbán, S. & di Magliano, M.P., 2012. Metastatic Pancreatic Cancer Is Dependent on Oncogenic Kras in Mice. *PLoS One, 7(12), e49707.*

Cooper, G.M., 2000. Regulation of Transcription in Eukaryotes. Sunderland (MA): Sinauer Associates; 2000. Available from: https://www.ncbi.nlm.nih.gov/books/NBK9904.

Coufal, J., Jagelská, E.B., Liao, J.C. & Brázda, V., 2013. Preferential binding of p53 tumor suppressor to p21 promoter sites that contain inverted repeats capable of forming cruciform structure. *Biochemical and biophysical research communications, 441(1), pp.83-88.*

Cox, A.D. & Der, C.J., 2010. Ras history: The saga continues. *Small GTPases, 1(1), pp.2–27.*

Cox, A.D., Der, C.J. & Philips, M.R., 2015. Targeting RAS Membrane Association: Back to the Future for Anti-RAS Drug Discovery? *Clin. Cancer Res, 21(8), pp.1819–1827.*

Cox, A.D., Fesik, S.W., Kimmelman, A.C., Luo, J. & Der, C.J., 2014. Drugging the undruggable RAS: Mission possible? *Nat. Rev. Drug Discov, 13(11), pp.828–851.*

Czerwinska, I., Sato, S., Juskowiak, B. & Takenaka, S., 2014. Interactions of cyclic and non-cyclic naphthalene diimide derivatives with different nucleic acids. *Bioorg. Med. Chem, 22(9), 2593–2601.*

Davis, J.T. 2004. G-Quartets 40 Years Later: From 5′-GMP to Molecular Biology and Supramolecular Chemistry. *Angew. Chem. Int. Ed. Engl, 43(6), pp.668–698.*

Dempsey, L.A., Sun, H., Hanakahi, L.A. and Maizels, N., 1999. G4 DNA binding by LR1 and its subunits, nucleolin and hnRNP D, A role for G-G pairing in immunoglobulin switch recombination. *J. Biol. Chem, 274(2), pp.1066–1071.*

Deng, W. & Roberts, S.G., 2005. A core promoter element downstream of the TATA box that is recognized by TFIIB. *Genes Dev, 19(20), pp.2418-2423.*

Deng, W. & Roberts, S.G., 2006. Core promoter elements recognized by transcription factor IIB. Biochem Soc Trans, 34(pt 6), pp.1051-1053. Deng, W. & Roberts, S.G., 2007. TFIIB and the regulation of transcription by RNA polymerase II. *Chromosoma, 116(5), pp.417-429.*

DeSa, R.J. & Matheson, I.B., 2004. A practical approach to interpretation of singular value decomposition results. *Methods Enzymol, 384, pp.1-8.*

Desvernge, B. & Wahli, W., 1999. Peroxisome proliferator-activated receptors, nuclear control of metabolism. *Endocrine Reviews, 20(5), pp.649-688.*

Dexheimer, T.S., Sun, D. & Hurley, L.H., 2006. Deconvoluting the structural and drug-recognition complexity of the G-quadruplex-forming region upstream of the bcl-2 P1 promoter. *J Am Chem Soc, 128(16), pp.5404-5415.*

Dey, A., Verma, C.S. & Lane, D.P., 2008. Updates on p53: modulation of p53 degradation as a therapeutic approach. *British journal of cancer, 98(1), pp.4.*

Dickson, B., Sprenger, F., Morrison, D. & Hafen, E., 1992. Raf functions downstream of Rasl in the Sevenless signal transduction pathway. *Nature, 360(6404), pp.600-603.*

Didier, D.K., Schiffenbauer, J., Woulfe, S.L., Zacheis, M. & Schwartz, B.D., 1988. Characterization of the cDNA encoding a protein binding to the major histocompatibility complex class II Y box. *Proc Natl Acad Sci U S A, 85(19), pp.7322-7326.*

Ding, J., Hayashi, M.K., Zhang, Y., Manche, L., Krainer, A.R. & Xu, R.M., 1999. Crystal structure of the two-RRM domain of vhnRHP A1 (Up1) complexed with single-stranded telomeric DNA. *Genes Dev, 13(9), pp.1102–1115.*

Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kurtev, V., Brosch, G., Wintersberger, E. & Seiser, C., 1999. Histone deacetylase 1 can repress transcription by binding to Sp1. *Mol Cell Biol, 19(8), pp.5504-5511.*

Dong, Y.W., Wang, X.P. & Wu, K., 2009. Suppression of pancreatic carcinoma growth by activating peroxisome proliferator-activated receptor gamma involves angiogenesis inhibition. *World J Gastroenterol, 15(4), pp.441–448.*

Downward, J., 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature, 370 (6490), pp.527-532.*

Dreyfuss, G., Matunis, M.J., Pinol-Roma, S. & Burd, C.G., 1993. hnRNP proteins and the biogenesis of mRNA. *Annu. Rev. Biochim, 62, pp.289–321.*

Dynan, W.S., Sazer, S., Tjian, R. & Schimke, R.T., 1986. Transcription factor Sp1 recognizes a DNA sequence in the mouse dihydrofolate reductase promoter. *Nature, 319(6050), pp.246-248.*

Dynlacht, B.D., 1997. Regulation of transcription by proteins that control the cell cycle. *Nature, 389(6647), pp.149-152.*

Ebi, H., Corcoran, R.B., Singh, A., Chen, Z., Song, Y., Lifshits, E., Ryan, D.P., Meyerhardt, J.A., Benes, C., Settleman, J. & Wong, K.K., 2011. Receptor tyrosine kinases exert dominant control over PI3K signaling in human KRAS mutant colorectal cancers. *The Journal of Clinical Investigation, 121(11), pp.4311–4321.*

Eferl, R. & Wagner, E.F., 2003. AP-1: a double-edged sword in tumorigenesis. *Nat Rev Cancer, 3(11), pp.859-868.*

Eibl, G., 2008. The role of PPAR-gamma and its interaction with COX-2 in pancreatic cancer. *PPAR Res 2008, pp.326915.*

Elkin, M., Shevelev, A., Schulze, E., Tykocinsky, M., Cooper, M., Ariel, I., Pode, D., Kopf, E., de Groot, N. & Hochberg, A., 1995. The expression of the imprinted H19 and IGF-2 genes in human bladder carcinoma. *FEBS Lett, 374(1), pp.57-61.*

Ellrott, K., Yang, C., Sladek, F.M. & Jiang, T., 2002. Identifying transcription factor binding sites through Markov chain optimization. *Bioinformatics, 18(suppl_2), pp.S100-S109.*

Elstner, E., Müller, C., Koshizuka, K., Williamson, E.A., Park, D., Asou, H., Shintaku, P., Said, J.W., Heber, D. & Koeffler, H.P., 1998. Ligands for perosixome proliferator-activated receptor γ and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. *PNAS, 95(15), pp.8806-8811.*

End, D., 1999. Farnesyl protein transferase inhibitors and other therapies targeting the Ras signal transduction pathway. *Invest New Drugs, 17(3), pp.241–258.*

Engelman, J.A., Chen, L., Tan, X., Crosby, K., Guimaraes, A.R., Upadhyay, R., Maira, M., McNamara, K., Perera, S.A., Song, Y. & Chirieac, L.R., 2008. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nature medicine, 14(12), pp.1351–1356.*

Estrem, S.T., Gaal, T., Ross, W. & Gourse, R.L., 1998. Identification of an UP element consensus sequence for bacterial promoters. *Proc. Natl. Acad. Sci. U.S.A, 95(17), pp.9761-9766.*

Fajas, L., Auboeuf, D., Raspé, E., Schoonjans, K., Lefebvre, A.M., Saladin, R., Najib, J., Laville, M., Fruchart, J.C., Deeb, S. & Vidal-Puig, A., 1997. The organization, promoter analysis, and expression of the human PPARγ gene. *J Biol Chem, 272(30), pp.18779-18789.*

Farin, K., Schokoroy, S., Haklai, R., Cohen-Or, I., Elad-Sfadia, G., Reyes-Reyes, M.E., Bates, P.J., Cox, A.D., Kloog, Y. & Pinkas-Kramarski, R., 2011. Oncogenic synergism between ErbB1, nucleolin, and mutant Ras. *Cancer Res, 71(6), pp.2140-2151.*

Fauquette, V., Aubert, S., Groux-Degroote, S., Hemon, B., Porchet, N., Van Seuningen, I. & Pigny, P., 2007. Transcription factor AP-2a represses both the mucin MUC4 expression and pancreatic cancer cell proliferation. *Carcinogenesis, 28(11), pp.2305–2312.*

Feng, W. & Williams, T., 2003. Cloning and characterization of the mouse AP-2 epsilon gene: a novel family member expressed in the developing olfactory bulb. *Mol Cell Neurosci, 24(2), pp.460-475.*

Ferro, E. & Trabalzini, L., 2010. RalGDS family members couple Ras to Ral signalling and that's not all. *Cell Signal, 22(12), pp.1804-1810.*

Fortini, M.E., Simon, M.A. & Rubin, G.M., 1992. Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation. *Nature 355(6360), pp.559-561.*

Fossum, B., Olsen, A.C., Thorsby, E. & Gaudernack, G., 1995. CD8+ T cells from a patient with colon carcinoma, specific for a mutant p21-Ras-derived peptide (Gly13-->Asp), are cytotoxic towards a carcinoma cell line harbouring the same mutation. *Cancer Immunol Immunother, 40(3), pp.165–172.*

Friday, B.B. & Adjei, A.A., 2005. K-ras as a target for cancer therapy. *Biochim. Biophys. Acta, 1756(2), pp.127-144.*

Fried, M. & Crothers, D.M., 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res, 1981, 9(23), pp.6505-6525.*

Fruman, D.A. & Rommel, C., 2014. PI3K and cancer: Lessons, challenges and opportunities. *Nat. Rev. Drug Discov, 13(2), pp.140–156.*

Fry, C.J. & Farnham, P.J., 1999. Context-dependent Transcriptional Regulation. *The Journal of Biological Chemistry, 274(42), pp.29583-29586.*

Fukuda, H., Katahira, M., Tsuchiya, N., Enokizono, Y., Sugimura, T., Nagao, M. & Nakagama, H., 2002. Unfolding of quadruplex structure in the G-rich strand of the minisatellite repeat by the binding protein UP1. *Proceedings of the National Academy of Sciences of the United States of America, 99(20), pp.12685–12690.*

Fukuhara, M., Ma, Y., Nagasawa, K. & Toyoshima, F., 2017. A G-quadruplex structure at the 5′ end of the *H19* coding region regulates *H19* transcription. *Scientific Reports, 7, pp.45815.*

Funnell, A.P. & Crossley, M., 2012. Homo- and heterodimerization in transcriptional regulation. *Adv Exp Med Biol, 747, pp.105-121.*

Gabory, A., Ripoche, M.A., Le Digarcher, A., Watrin, F., Ziyyat, A., Forné, T., Jammes, H., Ainscough, J.F., Surani, M.A., Journot, L. & Dandolo, L., 2009. H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development, 136(20), pp.3413-3421.*

Gadgil, H. & Jarrett, H.W., 2002. Oligonucleotide trapping method for purification of transcription factors. *J. Chromatogr A, 966(1-2), pp.99-110.*

Gadgil, H., Jurado. L.A., Jarrett, H.W., 2001. DNA affinity chromatography of transcription factors. *Analytical Biochemistry. 290(2), pp.147–178.*

Galiana, C., Lozano, J.C., Bancel, B., Nakazawa, H. & Yamasaki, H., 1995. High frequency of Ki-ras amplification and p53 gene mutations in adenocarcinomas of the human esophagus. *Mol. Carcinog, 14(4), pp.286-293.*

Gallo, A., Sterzo, C.L., Mori, M., Di Matteo, A., Bertini, I., Banci, L., Brunori, M. & Federici, L., 2012. Structure of nucleophosmin DNA-binding domain and analysis of its complex with a G-quadruplex sequence from the c-MYC promoter. *Journal of Biological Chemistry, 287(32), pp.26539-26548.*

Gérard, A., van der Kammen, R.A., Janssen, H., Ellenbroek, S.I. & Collard, J.G., 2009. The Rac activator Tiam1 controls efficient T-cell trafficking and route of transendothelial migration. *Blood, 113(24), pp.6138-6147.*

Gessner, C., Woischwill, C., Schumacher, A., Liebers, U., Kuhn, H., Stiehl, P., Jürchott, K., Royer, H.D., Witt, C. & Wolff, G., 2004. Nuclear YB-1 expression as a negative prognostic marker in nonsmall cell lung cancer. *Eur Respir J, 23(1), pp.14-19.*

Ghobrial, I. & Adjei, A., 2002. Inhibitors of the ras oncogene as therapeutic targets. *Hematol Oncol Clin North Am, 16(5), pp.1065–1088.*

Gidoni, D., Dynan, W.S. & Tjian, R., 1984. Multiple specific contacts between a mammalian transcription factor and its cognate promoters. *Nature, 312(5993), pp.669-680.*

Gill, G., Pascal, E., Tseng, Z.H. & Tjian, R., 1994. A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the Drosophila TFIID complex and mediates transcriptional activation. *PNAS, 91(1), pp.192-196.*

Gilles, M.E., 2016. Nucleolin Targeting Impairs the Progression of Pancreatic Cancer and Promotes the Normalization of Tumor Vasculature. *Cancer Res, 76(24), pp.7181-7193.*

Ginisty, H., Sicard, H., Roger, B. & Bouvet, P., 1999. Structure and Functions of Nucleolin. *J Cell Sci, 112, 761–772.*

Gjertsen, M.K. & Gaudernack, G., 1998. Mutated Ras peptides as vaccines in immunotherapy of cancer. *Vox sanguinis, 74(2), pp.489–95.*

Gjertsen, M.K., Buanes, T., Rosseland, A.R., Bakka, A., Gladhaug, I., Søreide, O., Eriksen, J.A., Møller, M., Baksaas, I., Lothe, R.A. & Sæterdal, I., 2001. Intradermal ras peptide vaccination with granulocyte-macrophage colony- stimulating factor as adjuvant: Clinical and immunological responses in patients with pancreatic adenocarcinoma. *International journal of cancer. Journal international du cancer, 92(3), pp.441–50.*

Goldberg M. L. 1979. Ph. D. Thesis, Stanford University, Stanford, CA, USA.

González, V. & Hurley, L.H., 2010. The C-Terminus of Nucleolin Promotes the Formation of the C- MYCG-Quadruplex and Inhibits C- MYC Promoter Activity. *Biochemistry, 49(45), pp.9706–971.*

González, V., Guo, K., Hurley, L. & Sun, D., 2009. Identification and Characterization of Nucleolin as a C-Myc G-Quadruplex-Binding Protein. *J. Biol. Chem, 284(35), pp.23622–23635.*

Gowri, P.M., Yu, J.H., Shaufl, A., Sperling, M.A. & Menon, R.K., 2003. Recruitment of a repressosome complex at the growth hormone receptor promoter and its potential role in diabetic nephropathy. *Molecular and cellular biology, 23(3), pp.815-825.*

Grinstein, E., Du, Y., Santourlidis, S., Christ, J., Uhrberg, M. & Wernet, P., 2007. Nucleolin regulates gene expression in CD34-positive hematopoietic cells. *J. Biol. Chem, 282(17), pp.12439–12449.*

Grinstein, E., Wernet, P., Snijders, P.J., Rösl, F., Weinert, I., Jia, W., Kraft, R., Schewe, C., Schwabe, M., Hauptmann, S. & Dietel, M., 2002. Nucleolin as activator of human papillomavirus type 18 oncogene transcription in cervical cancer. *J. Exp. Med, 196(8), pp.1067–1078.*

Grosveld, F., Rodriguez, P., Meier, N., Krpic, S., Pourfarzad, F., Papadopoulos, P., Kolodziej, K., Patrinos, G.P., Hostert, A. & Strouboulis, J., 2005. Isolation and characterization of hematopoietic transcription factor complexes by in vivo biotinylation tagging and mass spectrometry. *J. Ann N Y Acad Sci, 1054, pp.55-67.*

Gruber, R., Panayiotou, R., Nye, E., Spencer-Dene, B., Stamp, G. & Behrens, A., 2016. YAP1 and TAZ Control Pancreatic Cancer Initiation in Mice by Direct Up-regulation of JAK–STAT3 Signaling. *Gastroenterology, 151(3), pp.526–539.*

Grünewald, K., Lyons, J., Fröhlich, A., Feichtinger, H., Weger, R.A., Schwab, G., Janssen, J.W. & Bartram, C.R., 1989. High frequency of Ki-ras codon 12 mutations in pancreatic adenocarcinomas. *Int J Cancer, 43(6), pp.1037-1041.*

Gupta, S., Ramjaun, A.R., Haiko, P., Wang, Y., Warne, P.H., Nicke, B., Nye, E., Stamp, G., Alitalo, K. & Downward, J., 2007. Binding of Ras to Phosphoinositide 3-Kinase p110?? Is Required for Ras- Driven Tumorigenesis in Mice. *Cell, 129(5), pp.957–968.*

Haider, S.M. & Neidle, S., 2011. Parkinson, G.N. A structural analysis of G-quadruplex/ligand interactions. *Biochimie, 93(8), pp.1239–1251.*

Haigis, K.M., Kendall, K.R., Wang, Y., Cheung, A., Haigis, M.C., Glickman, J.N., Niwa-Kawakita, M., Sweet-Cordero, A., Sebolt-Leopold, J., Shannon, K.M. & Settleman, J., 2008. Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. *Nat Genet, 40(5), pp.600- 608.*

Hanakahi, L.A., Sun, H. & Maizels, N., 1999. High affinity interactions of nucleolin with G-G-paired rDNA. *J. Biol. Chem, 274(22), pp.15908–15912.*

Angelov, D., Bondarenko, V.A., Almagro, S., Menoni, H., Mongelard, F., Hans, F., Mietton, F., Studitsky, V.M., Hamiche, A., Dimitrov, S. & Bouvet, P., 2006. Nucleolin is a histone chaperone with FACT-like activity and assists remodeling of nucleosomes. *EMBO J. 25(8), pp.1669–1679.*

Harvey, J.J., 1964. An unidentified virus which causes the rapid production of tumours in mice. *Nature 204, pp.1104-1105.*

Harvey, K.F., Zhang, X. & Thomas, D.M., 2013. The Hippo pathway and human cancer. Nat. Rev. *Cancer, 13(4), pp.246–257.*

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., Da Costa, L.T., Morin, P.J., Vogelstein, B. & Kinzler, K.W., 1998. Identification of c-MYC as a target of the APC pathway. *Science, 281(5382), pp.1509–1512.*

Her, S., Bell, R.A., Bloom, A.K., Siddall, B.J. & Wong, D.L., 1999. Phenylethanolamine N-methyltransferase gene expression. Sp1 and MAZ potential for tissue-specific expression. *Journal of Biological Chemistry 274(13), pp.8698-8707.*

Her, S., Claycomb, R., Tai, T.C. & Wong, D.L., 2003. Wong Regulation of the Rat Phenylethanolamine N-Methyltransferase Gene by Transcription Factors Sp1 and MAZ. *Molecular Pharmacology, 64(5), pp.1180-1188.*

Hilger-Eversheim, K., Moser, M., Schorle, H. & Buettner, R., 2000. Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell-cycle control. *Gene, 260(1-2), pp.1-12.*

Himeda, C.L., Ranish, J.A. & Hauschka, S.D. 2008. Quantitative proteomic identification of MAZ as a transcriptional regulator of muscle-specific genes in skeletal and cardiac myocytes. *Mol. Cell Biol, 28(20), pp.6521-3655.*

Hollywood, D.P. & Hurst, H.C., 1993. A novel transcription factor, OB2-1, is required for overexpression of the proto-oncogene c-erbB-2 in mammary tumour lines. *Embo J, 12(6), pp.2369-2675.*

Hong, S.M., Vincent, A., Kanda, M., Leclerc, J., Omura, N., Borges, M., Klein, A.P., Canto, M.I., Hruban, R.H. & Goggins, M., 2012. Genome-wide somatic copy number alterations in low-grade PanINs and IPMNs from individuals with a family history of pancreatic cancer. *Clin. Cancer Res, 18(16), pp.4303–4312.*

Hsu, C.Y., Bristow, R., Cha, M.S., Wang, B.G., Ho, C.L., Kurman, R.J., Wang, T.L. & Shih, I.M., 2004. Characterization of active mitogen activated protein kinase in ovarian serous carcinomas. *Clin Cancer Res, 10(19), pp.6432-6436.*

Huang, S., Jean, D., Luca, M., Tainsky, M.A. & Bar-Eli, M., 1998. Loss of AP-2 results in downregulation of c-KIT and enhancement of melanoma tumorigenicity and metastasis. *Embo J, 17(15), pp.4358-69.*

Huddleson, J.P., 2006. Regulation of the KLF2 Transcription Factor Gene in Endothelial Cells by Fluid Shear Stress.

Hughes, P., Marshall, D., Reid, Y., Parkes, H. & Gelber, C., 2007. The costs of using unauthenticated, over-passaged cell lines: how much more data do we need? *Biotechniques, 43(5), pp.575-578.*

Huppert, J. L., 2010. Structure, location and interactions of G-quadruplexes. *FEBS Journal 277(17), pp.3452–3458.*

Huppert, J., 2008. Four-stranded nucleic acids: structure, function and targeting of G-quadruplexes. *Chem Soc Rev, 37(7), 1375–1384.*

Huppert, J.L. & Balasubramanian S., 2007. G-quadruplexes in promoters throughout the human genome. *Nucleic acids research, 35(2), pp.406-413.*

Hurowitz, E.H., Melnyk, J.M., Chen, Y.J., Kouros-Mehr, H., Simon, M.I. & Shizuya, H., 2000. Genomic characterization of the human heterotrimeric G protein alpha, beta, and gamma subunit genes. *DNA Res, 7 (2), pp.111-120.*

Ihle, N.T. & Powis, G., 2010. The biological effects of isoform-specific PI3-kinase inhibition. *Current opinion in drug discovery & development, 13(1), pp.41-49.*

Ikezoe, T., Miller, C.W., Kawano, S., Heaney, A., Williamson, E.A., Hisatake, J., Green, E., Hofmann, W., Taguchi, H. & Koeffle, H.P., 2001. Mutational analysis of the peroxisome proliferator-activated receptor γ gene in human malignancies. *Cancer Research, 61(13), pp.5307-5310.*

Ishii, S., Kadonaga, J.T., Tjian, R., Brady, J.N., Merlino, G.T. & Pastan, I., 1986. Binding of the Sp1 transcription factor by the human Harvey ras1 proto-oncogene promoter. *Science, 232(4756), pp.1410-413.*

Issemann, I., Prince, R.A., Tugwood, J.D. & Green, S., 1993. The retinoid X receptor enhances the function of the peroxisome proliferator-activated receptor. *Biochime, 75(3-4), pp.251-256.*

Izumi, H., Imamura, T., Nagatani, G., Ise, T., Murakami, T., Uramoto, H., Torigoe, T., Ishiguchi, H., Yoshida, Y., Nomoto, M. & Okamoto, T., 2001. Y box-binding protein-1 binds preferentially to single-stranded nucleic acids and exhibits 3'-- >5' exonuclease activity. *Nucleic Acids Res, 29(5), pp.1200-1207.*

Jagelská, E.B., Brázda, V., Pečinka, P., Paleček, E. & Fojta, M., 2008. DNA topology influences p53 sequence-specific DNA binding through structural transitions within the target sites. *Biochemical Journal, 412(1), pp.57-63.*

Jagelská, E.B., Pivoňková, H., Fojta, M. & Brázda, V., 2010. The potential of the cruciform structure formation as an important factor influencing p53 sequence-specific binding to natural DNA targets. *Biochemical and biophysical research communications, 391(3), pp.1409-1414.*

Jäger, R., Friedrichs, N., Heim, I., Büttner, R. & Schorle, H., 2005. Dual role of AP-2gamma in ErbB-2-induced mammary tumorigenesis. *Breast Cancer Res Treat, 90(3), pp.273-280.*

Jean, D., Gershenwald, J.E., Huang, S., Luca, M., Hudson, M.J., Tainsky, M.A. & Bar-Eli, M., 1998. Loss of AP-2 results in up-regulation of MCAM/MUC18 and an increase in tumor growth and metastasis of human melanoma cells. *J Biol Chem, 273(26), pp.16501-16508.*

Jemal, A., Siegel, R., Xu, J. & Ward, E., 2010. Cancer statistics, 2010. *CA Cancer J Clin, 60(5), pp.277-300.*

Jiang, D., Jia, Y., Zhou, Y. & Jarrett, H.W., 2009. Two-dimensional southwestern blotting and characterization of transcription factors on-blot. *J Proteome Res. 8(7), pp.3693-3701.*

Jiang, D., Moxley, R.A. & Jarrett, H.W., 2006. Promoter trapping of c-jun promoter-binding transcription factors. *J Chromatogr A. 1133(1-2), pp.83-94.*

Jiang, D., Zhou, Y., Moxley, R.A. & Jarrett, H.W., 2008. Purification and identification of positive regulators binding to a novel element in the c-Jun promoter. *Biochemistry, 47(35), pp.9318-9334.*

Jonckheere, N., Fauquette, V., Stechly, L., Saint-Laurent, N., Aubert, S., Susini, C., Huet, G., Porchet, N., Van Seuningen, I. & Pigny, P., 2009. Tumour growth and resistance to gemcitabine of pancreatic cancer cells are decreased by AP-2α overexpression. *British Journal of Cancer, 101(4), 637–644.*

Jones, S., Zhang, X., Parsons, D.W., Lin, J.C.H., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Kamiyama, H., Jimeno, A. & Hong, S.M., 2008. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science, 321(5897), pp.1801-1806.*

Jordano, J. & Perucho, M., 1986. Chromatin structure of the promoter region of the human c-K-ras gene. *Nucleic Acids Res, 14(18), pp.7361–7378.*

Jordano, J. & Perucho, M., 1988. Initial characterization of a potential transcriptional enhancer for the human c-K-ras gene. *Oncogene, 2(4), pp.359–366.*

Junttila, M.R., Devasthali, V., Cheng, J.H., Castillo, J., Metcalfe, C., Clermont, A.C., Den Otter, D., Chan, E., Bou-Reslan, H., Cao, T. & Forrest, W., 2015. Modeling targeted inhibition of MEK and PI3 kinase in human pancreatic cancer. *Mol. Cancer Ther, 14(1), pp.40–47.*

Juven-Gershon, T., Hsu, J. & Kadonaga, J.T., 2008. Caudal, a key developmental regulator, is a DPE-specific transcriptional factor. *Genes and Development, 22(20), pp.2823-2830.*

Kadonaga, J.T., 2002. The DPE, a core promoter element for transcription by RNA polymerase II. *Exp Mol Med, 34(4), pp.259-264.*

Kadonaga, J.T., 2004. Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. *Cell, 116(2), pp.247-257.*

Kadonaga, J.T., Jones, K.A. & Tjian, R., 1986. Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem. Sci, 11(1), pp.20–23.*

Kaiser, C., Van Ert, N., Agrawal, P., Chawla, R., Yang, D. & Hurley, L.H., 2017. Insight into the Complexity of the i-Motif and G-Quadruplex DNA Structures Formed in the KRAS Promoter and Subsequent Drug-Induced Gene Repression. *J Am Chem Soc. 2017 Jun 15. doi: 10.1021/jacs.7b02046.*

Kanda, M., Matthaei, H., Wu, J., Hong, S.M., Yu, J., Borges, M., Hruban, R.H., Maitra, A., Kinzler, K., Vogelstein, B. & Goggins, M., 2012.. Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. G*astroenterology, 142(4), pp.730–733.*

Kapoor, A., Yao, W., Ying, H., Hua, S., Liewen, A., Wang, Q., Zhong, Y., Wu, C.J., Sadanandam, A., Hu, B. & Chang, Q., 2014. Yap1 activation enables bypass of oncogenic Kras addiction in pancreatic cancer. *Cell, 158(1), pp.185–197.*

Katz, M.E. & McCormick, F., 1997. Signal transduction from multiple Ras effectors. *Curr Opin Genet Dev, 7(1), pp.75-79.*

Kennedy, G.C. & Rutter, W.J., 1992. Pur-1, a zinc-finger protein that binds to purine-rich sequences, transactivates an insulin promoter in heterologous cells. *PNAS, 89(23), pp.11498-11502.*

Kern, F., Doma, E., Rupp, C., Niault, T. & Baccarini, M., 2013. Essential, non-redundant roles of B-Raf and Raf-1 in Ras-driven skin tumorigenesis. *Oncogene, 32(19), p.2483.*

Kikuchi, A., Demo, S.D., Ye, Z.H., Chen, Y.W. & Williams, L.T., 1994. ralGDS family members interact with the effector loop of ras p21. *Molecular and Cellular Biology, 14(11), pp.7483-7491.*

Kim, E. & Deppert, W., 2003. The complex interactions of p53 with target DNA: we learn as we go. *Biochemistry and cell biology, 81(3), pp.141-150.*

Kim, T.K. & Maniatis, T., 1997. The mechanism of transcriptional synergy of an in vitro assembled interferon-b enhanceosome. *Mol Cell, 1(1), pp.119-129.*

Kirsten, W.H., Schauf, V. & McCoy, J., 1970. Properties of a murine sarcoma virus. *Bibl Haematol, 36, pp.246-249.*

Klimczak, L.J., Collinge, M.A., Farini, D., Giuliano, G., Walker, J.C. & Cashmore, A.R., 1995. Reconstitution of Arabidopsis casein kinase II from recombinant subunits and phosphorylation of transcription factor GBF1. *Plant Cell, 7(1), pp.105-115.*

Kondo, M., Suzuki, H., Ueda, R., Osada, H., Takagi, K. & Takahashi, T., 1995. Frequent loss of imprinting of the H19 gene is often associated with its overexpression in human lung cancers. *Oncogene, 10(6), pp.1193-1198.*

Kouhara, H., Hadari, Y.R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I. & Schlessinger, J., 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell, 89(5), pp.693-702.*

Kristiansen, G., Jacob, J., Buckendahl, A.C., Grützmann, R., Alldinger, I., Sipos, B., Klöppel, G., Bahra, M., Langrehr, J.M., Neuhaus, P. & Dietel, M., 2006. Peroxisome proliferator-activated receptor gamma is highly expressed in pancreatic cancer and is associated with shorter overall survival times. *Clin Cancer Res, 12(21), pp.6444–6451.*

Krivega, I. & Dean, A., 2012. Enhancer and promoter interactions-long distance calls. *Curr. Opin. Genet. Dev, 22(2), pp.79-85.*

Kubota, T., Koshizuka, K., Williamson, E.A., Asou, H., Said, J.W., Holden, S., Miyoshi, I. & Koeffler, H.P., 1998. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both in vitro and in vivo. *Cancer Res, 58(15), pp.3344-3352.*

Kumar, M.S., Hancock, D.C., Molina-Arcas, M., Steckel, M., East, P., Diefenbacher, M., Armenteros-Monterroso, E., Lassailly, F., Matthews, N., Nye, E. & Stamp, G., 2012. The GATA2 transcriptional network is requisite for RAS oncogene-driven non-small cell lung cancer. *Cell, 149(3), 642–646.*

Kumar, M.S., Hancock, D.C., Molina-Arcas, M., Steckel, M., East, P., Diefenbacher, M., Armenteros-Monterroso, E., Lassailly, F., Matthews, N., Nye, E. & Stamp, G., 2012. Zinc-finger transcription factors are associated with guanine quadruplex motifs in human, chimpanzee, mouse and rat promoters genome-wide. *Nucleic Acids Res, 39(18):8005-8516.*

Kumei, S., Motomura, W., Yoshizaki, T., Takakusaki, K. & Okumura, T., 2009. Troglitazone increases expression of E-cadherin and claudin 4 in human pancreatic cancer cells. *Biochem Biophys Res Commun 380(3): 414–419.*

Kutach, A.K. & Kadonaga, J.T., 2000. The downstream promoter element DPE appears to be as widely used as the TATA box in Drosophila core promoters. *Mol Cell Biol, 20(13), pp.4754-4764.*

Kyriakis, J.M., App, H., Zhang, X.F., Banerjee, P., Brautigan, D.L., Rapp, U.R. & Avruch, J., 1992. Raf-1 activates MAP kinase-kinase. *Nature, 358(6385), pp.417-421.*

Lambert, J.M., Lambert, Q.T., Reuther, G.W., Malliri, A., Siderovski, D.P., Sondek, J., Collard, J.G. & Der, C.J., 2002. Tiam1 mediates Ras activation of Rac by a PI (3) K-independent mechanism. *Nature cell biology, 4(8), p.621.*

Latchman, D.S., 1997. Transcription factors: an overview. *Int J Biochem Cell Biol, 29(12), pp.1305-1312.*

Lee, J., Jang, K.T., Ki, C.S., Lim, T., Park, Y.S., Lim, H.Y. & Park, J.O., 2007. Impact of epidermal growth factor receptor (EGFR) kinase mutations, EGFR gene amplifications, and KRAS mutations on survival of pancreatic adenocarcinoma. *Cancer, 109(8), pp.1561-1569.*

Lee, T.I. & Young, R.A., 2000. Transcription of eukaryotic protein-coding genes. *Annu Rev Genet, 34, pp.77-137.*

Lelli, K.M., Slattery, M. & Mann, R.S., 2012. Disentangling the many layers of eukaryotic transcriptional regulation. *Annu. Rev. Genet, 46, pp.43-68.*

Leroy, C., Manen, D., Rizzoli, R., Lombes, M. & Silve, C., 2004. Functional importance of Myc-associated zinc finger protein for the human parathyroid hormone (PTH)/PTH-related peptide receptor-1 P2 promoter constitutive activity. *J. Mol. Endocrinol, 32(1), pp.99-113.*

Li, H., Goswami, P.C. & Domann, F.E., 2006. AP-2gamma induces p21 expression, arrests cell cycle, and inhibits the tumor growth of human carcinoma cells. *Neoplasia, 8(7), pp.568-77.*

Li, L., He, S., Sun, J.M. & Davie, J.R., 2004. Gene regulation by Sp1 and Sp3. *Biochem Cell Biol, 82(4), pp.460-471.*

Li, M., Wang, Y., Yu, Y., Nishizawa, M., Nakajima, T., Ito, S. & Kannan, P., 2002. The human transcription factor activation protein-2 gamma (AP-2gamma): gene structure, promoter, and expression in mammary carcinoma cell lines. *Gene, 301(1-2), pp.43-51.*

Li, Y., Wen, Z. S., Yang, H. X., Luo, R.Z., Zhang, Y., Zhang, M.F. & Jia, W.H., 2011. High Expression of Y-Box-Binding Protein-1 is Associated with Poor Survival in Resectable Esophageal Squamous Cell Carcinoma. *Ann Surg Oncol, 18(12), pp.13370-13376.*

Lim, C.Y., 2004. The MTE, a new core promoter element for transcription by RNA polymerase II. *Genes Dev, 18(13), pp.1606-1617.*

Lim, S.M., Westover, K.D., Ficarro, S.B., Harrison, R.A., Choi, H.G., Pacold, M.E. & Sim, T., 2014. Therapeutic targeting of oncogenic K-RAS by a covalent catalytic site inhibitor. *Angew. Chem. Int. Ed. Engl, 53(1), pp.199–204.*

Liu, J.N., Deng, R., Guo, J.F., Zhou, J.M., Feng, G.K., Huang, Z.S. & Zhu, X. F., 2007. Inhibition of myc promoter and telomerase activity and induction of delayed apoptosis by SYUIQ-5, a novel G-quadruplex interactive agent in leukemia cells. *Leukemia, 21(6), pp.1300–1302.*

Lonskaya, I., Potaman, V.N., Shlyakhtenko, L.S., Oussatcheva, E.A., Lyubchenko, Y.L. &Soldatenkov, V.A., 2005. Regulation of poly (ADP-ribose) polymerase-1 by DNA structure-specific binding. *Journal of Biological Chemistry, 280(17), pp.17076-17083.*

Lottin, S., Adriaenssens, E., Dupressoir, T., Berteaux, N., Montpellier, C., Coll, J. & Curgy, J.J., 2002. Overexpression of an ectopic H19 gene enhances the tumorigenic properties of breast cancer cells. *Carcinogenesis, 23(11), pp.1885-1895.*

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R. & Schlessinger, J., 1992. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell, 70(3), pp.431-442.*

Luo, F., Ye, H., Hamoudi, R., Dong, G., Zhang, W., Patek, C.E. & Arends, M.J., 2010. K-ras exon 4A has a tumour suppressor effect on carcinogen-induced. murine colonic adenoma formation. *J Pathol. 220(5), pp.542-550.*

Luscombe, N.M., Austin, S.E., Berman, H.M. & Thornton, J.M., 2000. An overview of the structures of protein-DNA complexes. *Genome Biol, 1(1), REVIEWS001.*

Luscombe, N.M., Laskowski, R.A. & Thornton, J.M., 2001. Amino acid-base interactions: a three-dimensional analysis of protein-DNA interactions at an atomic level. *Nucleic Acids Res, 29(13), pp.2860-2874.*

Lyssiotis, C.A., Son, J., Cantley, L.C. & Kimmelman, A.C., 2013. Pancreatic cancers rely on a novel glutamine metabolism pathway to maintain redox balance. *Cell Cycle, 12(13), pp.1987–1988.*

Malik, S. & Roeder, R.G., 2000. Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. *Trends Biochem Sci, 25(6), pp.277-283.*

Malkoski, S.P. & Wang, X.J., 2012. Two sides of the story? Smad4 loss in pancreatic cancer versus head-and-neck cancer. *FEBS Lett, 586(14), pp.1984–1992.*

Malumbres, M. & Barbacid, M., 2003. RAS oncogenes: the first 30 years. *Nat Rev Cancer, 3(6), pp.459-465.*

Martin, G.A., Viskoohil, D., Bollag, G., McCabe, P.C., Crosier, W. J., Haubruck, H. & Innis, M.A., 1990. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. Cell, 63(4), 843-849.1990. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell, 63(4), pp.843-849.*

Maston, G.A., Evans, S.K. & Green, M.R., 2006. Transcriptional regulatory elements in the human genome. *Annu Rev Genomics Hum Genet, 7, pp.29-59.*

Masumi, A., Fukazawa, H., Shimazu, T., Yoshida, M., Ozato, K., Komuro, K. & Yamaguchi, K., 2006. Nucleolin Is Involved in Interferon Regulatory Factor-2-Dependent Transcriptional Activation. *Oncogene, 25(37), pp.5113-5124.*

Matouk, I.J., Raveh, E., Abu-lail, R., Mezan, S., Gilon, M., Gershtain, E. & Richler, C., 2014. Oncofetal H19 RNA promotes tumor metastasis. *Biochim Biophys Acta, 1843(7), pp.1414-1426.*

Matsuoka, T.A., Zhao, L., Artner, I., Jarrett, H.W., Friedman, D., Means, A. & Stein, R., 2003. Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells. *Mol Cell Biol, 23(17), pp.6049-6062.*

Maurer, T., Garrenton, L. S., Oh, A., Pitts, K., Anderson, D.J., Skelton, N.J. & Ludlam, M.J., (2012). Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange activity. Proceedings of the National Academy of Sciences, 109(14), 5299-5304.2012. Small-molecule ligands bind to a distinct pocket in Ras and inhibit SOS-mediated nucleotide exchange activity. *Proc. Natl. Acad. Sci. USA, 109(14), pp.5299–5304.*

McPherson, L.A. & Weigel, R.J., 1999. AP2alpha and AP2gamma: a comparison of binding site specificity and trans-activation of the estrogen receptor promoter and single site promoter constructs. *Nucleic Acids Res, 27(20), pp.4040-4049.*

McPherson, L.A., Baichwal, V.R. & Weigel, R.J., 1997. Identification of ERF-1 as a member of the AP2 transcription factor family. *Proc Natl Acad Sci U S A, 94(9), pp.4342-4347.*

Meggio, F. & Pinna, L.A., 2003. One-thousand-and-one substrates of protein kinase CK2? *FASEB J, 17(3), pp.349-368.*

Membrino, A., Cogoi, S., Pedersen, E. B. & Xodo, L. E., 2011. G4-DNA formation in the HRAS promoter and rational design of decoy oligonucleotides for cancer therapy. *PLoS One, 6(9), e24421.*

Menkens, A.E., Schindler, U. & Cashmore, A.R., 1995. The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. *Trends Biochem. Sci, 20(12), pp.506-551.*

Merika, M., Williams, A.J., Chen, G., Collins, T. & Thanos, D., 1998. Recruitment of CBP/p300 by the IFNβ enhanceosome is required for synergistic activation of transcription. *Molecular cell, 1(2), pp.277-287.*

Milburn, M.V., 1990. Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. *Science, 247(4945), pp.939-945.*

Mittler, G., Butter, F. & Mann, M., 2009. A SILAC-based DNA protein interaction screen that identifies candidate binding proteins to functional DNA elements. *Genome Research, 19(2), pp.284–293.*

Monchaud, D. & Teulade-Fichou, M.P., 2008. A hitchhiker's guide to G-quadruplex ligands. *Org. Biomol. Chem, 6(4), pp.627–636.*

Mongelard, F. & Bouvet, P., 2007. Nucleolin: a multiFACeTed protein. *Trends Cell Biol. 17(2), pp.80–86.*

Morel, C., Gander, E.S., Herzberg, M., Dubochet, J. & Scherrer, K., 1973. The duck-globin messenger-ribonucleoprotein complex. Resistance to high ionic strength, particle gel electrophoresis, composition and visualisation by dark-field electron microscopy. *Eur J Biochem, 36(2), pp.455-464.*

Morel, C., Kayibanda, B. & Scherrer, K., 1971. Proteins associated with globin messenger RNA in avian erythroblasts: Isolation and comparison with the proteins bound to nuclear messengerlikie RNA. *FEBS Lett, 18(1), pp.84-88.*

Morgan, R.K., Batra, H., Gaerig, V. C., Hockings, J. & Brooks, T. A., 2016. Identification and characterization of a new G-quadruplex forming region within the kRAS promoter as a transcriptional regulator. *Biochim Biophys Acta, 1859(2), pp.235-245.*

Morgan-Lappe, S.E., Tucker, L.A., Huang, X., Zhang, Q., Sarthy, A.V., Zakula, D. & Fesik, S.W.2007. Identification of Ras-related nuclear protein, targeting protein for xenopus kinesin-like protein 2, and stearoyl-CoA desaturase 1 as promising cancer targets from an RNAi-based screen. *Cancer Res, 67(9), 4390–4398.*

Mossman, B.T., 1983. In vitro approaches for determining mechanisms of toxicity and carcinogenicity by asbestos in the gastrointestinal and respiratory tracts. *Environ. Health Perspect, 53, pp.155-161.*

Motomura, W., Nagamine, M., Tanno, S., Sawamukai, M., Takahashi, N., Kohgo, Y. & Okumura, T., 2004. Inhibition of cell invasion and morphological change by troglitazone in human pancreatic cancer cells. *J Gastroenterol, 39(5), pp.461–468.*

Motomura, W., Okumura, T., Takahashi, N., Obara, T. & Kohgo, Y., 2000. Activation of peroxisome proliferator-activated receptor gamma by troglitazone inhibits cell growth through the increase of p27KiP1 in human. Pancreatic carcinoma cells. *Cancer Res, 60(19), pp.5558–5564.*

Moxley, R.A. & Jarrett, H.W., 2005. Oligonucleotide trapping method for transcription factor purification systematic optimization using electrophoretic mobility shift assay. *J Chromatogr A, 1070(1-2), pp.23-34.*

Mueller, E., Sarraf, P., Tontonoz, P., Evans, R.M., Martin, K.J., Zhang, M. & Spiegelman, B.M., 1998. Terminal differentiation of human breast cancer through PPARγ. *Molecular Cell, 1(3), pp.465-470.*

Mueller, E., Smith, M., Sarraf, P., Kroll, T., Aiyer, A., Kaufman, D. S. & Eng, C. 2000. Effects of ligand activation of peroxisome proliferator-activated receptor γ in human prostate cancer. *PNAS, 97(20), pp.10990-10995.*

Muvaffak, A., Pan, Q., Yan, H., Fernandez, R., Lim, J., Dolinski, B. & Zhang, W., 2014. Evaluating TBK1 as a therapeutic target in cancers with activated IRF3. *Mol. Cancer Res, 12(7), pp.1055–1066.*

Myers, J.C., Moore, S.A. & Shamoo, Y., 2003. Structure-based incorporation of 6-methyl-8-(2-deoxy-beta-ribofuranosyl) isoxanthopteridine into the human telomeric repeat DNA as a probe for UP1 binding and destabilization of G-tetrad structures. *J. Biol. Chem, 278, pp.42300–42306.*

Myers, L.C. & Kornberg, R.D., 2000. Mediator of transcriptional regulation. *Annu Rev Biochem, 69, pp.729-749.*

Nagaich, A.K., Appella, E. & Harrington, R.E., 1997. DNA bending is essential for the site-specific recognition of DNA response elements by the DNA binding domain of the tumor suppressor protein p53. *Journal of Biological Chemistry, 272(23), pp.14842-14849.*

Nakatani, Y., Horikoshi, M., Brenner, M., Besnard, F. & Freese, E., 1990. A downstream initiation element required for efficient TATA box binding and in vitro function of TFIID. *Nature, 348(6296), pp.86-88.*

Nekhoroshkova, E., Albert, S., Becker, M. & Rapp, U.R., 2009. A-RAF kinase functions in ARF6 regulated endocytic membrane traffic. *PLoS One, 4(2), p.e4647.*

Nielsen, M.C. & Ulven, T., 2010. Macrocyclic G-Quadruplex Ligands. *Curr. Med. Chem, 17(29), pp.3438–3448.*

O'Driscoll, L., Gammell, P., McKiernan, E., Ryan, E., Jeppesen, P.B., Rani, S. & Clynes, M., 2006. Phenotypic and global gene expression changes in low and high passage MIN6 cells. *J of Endocrinology, 191, pp.665-676.*

Oda, Y., Ohishi, Y., Saito, T., Hinoshita, E., Uchiumi, T., Kinukawa, N., Iwamoto, Y., Kohno, K., Kuwano, M. & Tsuneyoshi, M., 2003. Nuclear expression of Y-box-binding protein-1 correlates with P-glycoprotein and topoisomerase II alpha expression, and with poor prognosis in synovial sarcoma. *J Pathol, 199(2), pp.251-258.*

Oda, Y., Sakamoto, A., Shinohara, N., Ohga, T., Uchiumi, T., Kohno, K., Tsuneyoshi, M., Kuwano, M. & Iwamoto, Y., 1998. Nuclear expression of YB-1 protein correlates with P-glycoprotein expression in human osteosarcoma. *Clin Cancer Res, 4(9), pp.2273-2277.*

Ohnmacht, S.A., Marchetti, C., Gunaratnam, M., Besser, R.J., Haider, S.M., Di Vita, G., Lowe, H.L., Mellinas-Gomez, M., Diocou, S., Robson, M. & Šponer, J., 2015. A G-quadruplex-binding compound showing anti-tumour activity in an in vivo model for pancreatic cancer. *Scientific Reports, 5, pp.11385.*

Ong, C.T. & Corces, V.G., 2011. Enhancer function: new insights into the regulation of tissue-specific gene expression. *Nat. Rev. Genet, 12(4), pp.283-293.*

Orso, F., Penna, E., Cimino, D., Astanina, E., Maione, F., Valdembri, D., Giraudo, E., Serini, G., Sismondi, P., De Bortoli, M. & Taverna, D., 2008. AP-2a and AP-2g regulate tumor progression via specific programs. *FASEB J, 22, pp.2704–2714.*

Ostrem, J. & Shokat, K., 2016. Direct small-molecule inhibitors of K-Ras: from structural insights to mechanism-based design. *Nature Publishing Group, 15(11), pp.771–785.*

Ostrem, J.M., Peters, U., Sos, M.L., Wells, J.A. & Shokat, K.M., 2013. K-RAS (G12C) inhibitors allosterically control GTP affinity and effector interactions. *Nature, 503(7477), pp.548–551.*

O'Toole, T.E., Bialkowska, K., Li, X. & Fox, J.E., 2011. Tiam1 is recruited to β1-integrin complexes by 14-3-3ζ where it mediates integrin-induced Rac1 activation and motility. *Journal of cellular physiology, 226(11), pp.2965-2978.*

Ou, T.M., Lu, Y.J., Tan, J.H., Huang, Z.S., Wong, K.Y. & Gu, L.Q., 2008. G-Quadruplexes: Targets in Anticancer Drug Design. Chem. *Med. Chem, 3(5), pp.690–713.*

Palecek, E., Vlk, D., Stankova, V., Brazda, V., Vojtesek, B., Hupp, T.R., Schaper, A. & Jovin, T.M., 1997. Tumor suppressor protein p53 binds preferentially to supercoiled DNA. *Oncogene, 15(18), pp.2201-2210.*

Palumbo, S.L., Memmott, R.M., Uribe, D.J., Krotova-Khan, Y., Hurley, L.H. & Ebbinghaus, S.W., 2008. A novel G-quadruplex-forming GGA repeat region in the c-myb promoter is a critical regulator of promoter activity. *Nucleic Acids Res, 36(6), pp.1755-1769.*

Panne, D., 2008. The enhanceosome. *Curr. Opin. Struct. Biol, 18(2), pp.236-242.*

Paramasivam, M., Cogoi, S., Filichev, V.V., Bomholt, N., Pedersen, E.B. & Xodo, L.E., 2008. Purine twisted-intercalating nucleic acids: a new class of anti-gene molecules resistant to potassium-induced aggregation. Nucleic acids research, 36(10), pp.3494-3507.2008. Purine twisted-intercalating nucleic acids: a new class of anti-gene molecules resistant to potassium-induced aggregation. *Nucleic Acids Research, 36(10), pp.3494–3507.*

Paramasivam, M., Membrino, A., Cogoi, S., Fukuda, H., Nakagama, H. & Xodo, L.E., 2009. Protein hnRNP A1 and its derivative Up1 unfold quadruplex DNA in the human KRAS promoter: implications for transcription. *Nucleic Acids Res, 37(9), pp.2841-2853.*

Parks, C.L. & Shenk, T., 1996. The serotonin 1a receptor gene contains a TATA-less promoter that responds to MAZ and Sp1. *J. Biol. Chem, 23(8), pp.4417–4430.*

Parks, C.L. & Shenk, T., 1997. Activation of the adenovirus major late promoter by transcription factors MAZ and Sp1. *J. Virol, 71(12), pp.9600-9607.*

Pena, P., Reutens, A.T., Albanese, C., D'amico, M., Watanabe, G., Donner, A., Shu, I.W., Williams, T. & Pestell, R.G.,1999. Activator protein-2 mediates transcriptional activation of the CYP11A1 gene by interaction with Sp1 rather than binding to DNA. *Mol Endocrinol, 13(8), pp.1402-1416.*

Philipsen, S. & Suske, G., 1999. A tale of three fingers: the family of mammalian Sp/ XKLF transcription factors. *Nucleic Acids Res, 27(15), pp.2991-3000.*

Pickert, L., Reuter, I., Klawonn, F. & Wingender, E., 1998. Transcription regulatory region analysis using signal detection and fuzzy clustering. *Bioinformatics, 14(3), pp.244-251.*

Piekna-Przybylska, D., Sullivan, M.A., Sharma, G. & Bambara, R.A., 2014. U3 Region in the HIV-1 Genome Adopts a G-Quadruplex Structure in Its RNA and DNA Sequence. *Biochemistry, 53(16), pp.2581–2593.*

Pinard, J. & de Lamirande, G., 1973. Isolation of Arginine Acceptor-Proteins From Normal Rat-Liver and Novikoff Hepatoma Supernatant. *British Journal of Cancer, 27(1), pp.18–26.*

Plowman, S.J., Williamson, D.J., O'Sullivan, M.J., Doig, J., Ritchie, A.M., Harrison, D.J., Melton, D.W., Arends, M.J., Hooper, M.L. & Patek, C.E., 2003. While K-ras is essential for mouse development, expression of the K-ras 4A splice variant is dispensable. *Mol Cell Biol, 23(24), pp.9245-9250.*

Podsypanina, K., Politi, K., Beverly, L.J. & Varmus, H.E., 2008. Oncogene cooperation in tumor maintenance and tumor recurrence in mouse mammary tumors induced by Myc and mutant Kras. *Proc. Natl. Acad. Sci. U. S. A., 105(13), pp.5242-5247.*

Potenza, N., Vecchione, C., Notte, A., De Rienzo, A., Rosica, A., Bauer, L., Affuso, A., De Felice, M., Russo, T., Poulet, R. & Cifelli, G., 2005. Replacement of K-Ras with H-Ras supports normal embryonic development despite inducing cardiovascular pathology in adult mice. *EMBO Rep, 6(5), pp.432-437.*

Prior, I.A., Lewis, P.D. & Mattos, C., 2012. A comprehensive survey of Ras mutations in cancer. *Cancer Res, 72(10), pp.2457-2467.*

Pyrc, J.J., Moberg, K.H. & Hall, D.J., 1992. Isolation of a novel cDNA encoding a zinc-finger protein that binds to two sites within the c-myc promoter. *Biochemistry, 31(16), pp. 4102-4110.*

Quante, T., Otto, B., Brázdová, M., Kejnovská, I., Deppert, W. & Tolstonog, G.V., 2012. Mutant p53 is a transcriptional co-factor that binds to G-rich regulatory regions of active genes and generates transcriptional plasticity. *Cell cycle, 11(17), pp.3290-3303.*

Rahib, L., Smith, B.D., Aizenberg, R., Rosenzweig, A.B., Fleshman, J.M. & Matrisian, L.M., 2014. Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res, 74(11), 2913–2921.*

Rahman, K.M., Reszka, A.P., Gunaratnam, M., Haider, S.M., Howard, P.W., Fox, K.R., Neidle, S. & Thurston, D.E., 2009. Biaryl polyamides as a new class of DNA quadruplex-binding ligands. *Chem. Commun, 27, pp.4097–4099.*

Raiber, E.A., Kranaster, R., Lam, E., Nikan, M. & Balasubramanian, S., 2012. A non-canonical DNA structure is a binding motif for the transcription factor SP1 in vitro. *Nucleic Acids Research, 40(4), pp.1499–1508.*

Rall, C.J., Yan, Y.X., Graeme-Cook, F., Beauchamp, R., Yandell, D.W., Povoski, S.P. & Rustgi, A.K., 1996. Ki-ras and p53 mutations in pancreatic ductal adenocarcinoma. *Pancreas, 12(1), pp.10-17.*

Rauen, K.A., Schoyer, L., McCormick, F., Lin, A.E., Allanson, J.E., Stevenson, D.A., Gripp, K.W., Neri, G., Carey, J.C., Legius, E. & Tartaglia, M., 2010. Proceedings from the 2009 genetic syndromes of the Ras/MAPK pathway: From bedside to bench and back. *American Journal of Medical Genetics Part A, 152(1), pp.4-24.*

Resat, H., Straatsma, T.P., Dixon, D.A. & Miller, J.H., 2001. The arginine finger of RasGAP helps Gln-61 align the nucleophilic water in GAP-stimulated hydrolysis of GTP, *Proc Natl Acad Sci U S A, 98(11), pp.6033-6038.*

Roberts-Thomson, S.J., 2000. Peroxisome proliferator-activated receptors in tumorigenesis: Targets of tumor promotion and treatment. *Immuni Cell Biol, 78(4), pp.436-441.*

Robinson, L.C., Gibbs, J.B., Marshall, M.S., Sigal, I.S. & Tatchell, K., 1987. CDC25: a component of the RAS-adenylate cyclase pathway in Saccharomyces cerevisiae. *Science, 235(4793), pp.1218-1221.*

Rodenhuis, S., van de Wetering, M.L., Mooi, W.J., Evers, S.G., van Zandwijk, N. & Bos, J.L., 1987. Mutational activation of the K-ras oncogene. A possible pathogenetic factor in adenocarcinoma of the lung. *N Engl J Med, 317(15), pp.929-935.*

Rodriguez, R., Müller, S., Yeoman, J.A., Trentesaux, C., Riou, J.F. & Balasubramanian, S., 2008. A novel small molecule that alters shelterin integrity and triggers a DNA-damage response at telomeres. *J. Am. Chem. Soc, 130(147), pp.15758–15759.*

Rogge, R.D., Karlovich, C.A. & Banerjee U., 1991. Genetic dissection of a neurodevelopmental pathway: Son of sevenless functions downstream of the sevenless and EGF receptor tyrosine kinases. *Cell, 64(1), pp.39-48.*

Rowinsky, E.K., Windle, J.J. & Von Hoff, D.D., 1999. Ras protein farnesyltransferase: A strategic target for anticancer therapeutic development. *Journal of Clinical Oncology, 17(11), pp.3631–3652.*

Ruiz, M., Troncoso, P., Bruns, C. & Bar-Eli, M., 2001. AP-2a transcription factor expression is associated with luminal differentiation and is lost in prostate cancer. *Clin Cancer Res, 7(12), pp.4086– 4095.*

Ryan, D.P., Hong, T.S. & Bardeesy, N., 2014. Pancreatic adenocarcinoma. *N. Engl. J. Med, 371(22), pp.2140–2141.*

Sam, M D. & Clubb, R.T., 2012. Preparation and Optimization of Protein–DNA Complexes Suitable for Detailed NMR Studies. *Methods in Molecular Biology (Clifton, N.J.), 831, 219–232.*

Sandelin, A., Alkema, W., Engström, P., Wasserman, W.W. & Lenhard, B., 2004. JASPAR: an open-access database for eukaryotic transcription factor binding profiles. *Nucleic Acids Res, 32(Database issue), pp.D91-94.*

Sarraf, P., Mueller, E., Jones, D., King, F.J., DeAngelo, D.J., Partridge, J.B., Holden, S.A., Chen, L.B., Singer, S., Fletcher, C. & Spiegelman, B.M., 1998. Differentiation and reversal of malignant changes in colon cancer through PPARγ. *Nature Medicine, 4(9), pp.1046-1052*

Sarraf, P., Mueller, E., Smith, W.M., Wright, H.M., Kum, J.B., Aaltonen, L.A., de la Chapelle, A., Spiegelman, B.M. & Eng, C., 1999. Loss-of-function mutations in PPARγ associated with human colon cancer. *Mol Cell, 3(6), pp.799-804.*

Sarthy, A.V., Morgan-Lappe, S.E., Zakula, D., Vernetti, L., Schurdak, M., Packer, J.C., Anderson, M.G., Shirasawa, S., Sasazuki, T. & Fesik, S.W., 2007. Survivin depletion preferentially reduces the survival of activated K-Ras-transformed cells. *Mol. Cancer Ther, 6(1), pp.269– 276.*

Satoh, T., Endo, M., Nakafuku, M., Nakamura, S. and Kaziro, Y., 1990. Platelet-derived growth factor stimulates formation of active p21ras.GTP complex in Swiss mouse 3T3 cells. *Proc Natl Acad Sci U S A, 87(15), pp.5993-5997.*

Sawadogo, M. & Sentenac, A., 1990. RNA Polymerase B (II) and General Transcription Factors. *Annu. Rev. Biochem, 59, pp.711-75.*

Schittek, B., Psenner, K., Sauer, B., Meier, F., Iftner, T. & Garbe, C., 2007. The increased expression of Y box-binding protein 1 in melanoma stimulates proliferation and tumor invasion, antagonizes apoptosis and enhances chemoresistance. *Int J Cancer, 120(10), pp.2110-2118.*

Schubbert, S., Zenker, M., Rowe, S.L., Böll, S., Klein, C., Bollag, G., van der Burgt, I., Musante, L., Kalscheuer, V., Wehner, L.E. & Nguyen, H., 2006. Germline KRAS mutations cause Noonan syndrome. *Nat Genet, 38(3), pp.331-336.*

Schutte, M., Hruban, R.H., Geradts, J., Maynard, R., Hilgers, W., Rabindran, S.K., Moskaluk, C.A., Hahn, S.A., Schwarte-Waldhoff, I., Schmiegel, W. & Baylin, S.B., 1997. Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. *Cancer Res, 57(15), pp.3126–3130.*

Schwartz, B., Melnikova, V.O., Tellez, C., Mourad-Zeidan, A., Blehm, K., Zhao, Y.J., McCarty, M., Adam, L. & Bar-Eli, M., 2007. Loss of AP-2a results in deregulation of E-cadherin and MMP-9 and an increase of tumorigenicity of colon cancer cells in vivo. *Oncogene, 26(28), pp.4049–4058.*

Scognamiglio, P.L., Di Natale, C., Leone, M., Poletto, M., Vitagliano, L., Tell, G. & Marasco, D., 2014. G-quadruplex DNA recognition by nucleophosmin: new insights from protein dissection. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1840(6), pp.2050-2059.*

Seeburg, P.H., Colby, W.W., Capon, D.J., Goeddel, D.V. & Levinson, A.D., 1984. Biological properties of human c-Ha-ras1 genes mutated at codon 12. *Nature, 312(5989), pp.71-75.*

Semenza, G.L., 1994. Transcriptional regulation of gene expression: mechanisms and pathophysiology. *Hum Mutat, 3(3), pp. 180-199.*

Sen, D. & Gilbert, W., 1988. Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis. *Nature, 334(6180), pp.364-366.*

Sen, D. & Gilbert, W., 1990. A sodium-potassium switch in the formation of four-stranded G4-DNA. *Nature, 344(6265), pp.410-414.*

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D. and Lowe, S.W., 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell, 88(5), pp.593-602.*

Shalaby, T., von Bueren, A.O., Hürlimann, M.L., Fiaschetti, G., Castelletti, D., Masayuki, T., Nagasawa, K., Arcaro, A., Jelesarov, I., Shin-ya, K. and Grotzer, M., 2010. Disabling c-Myc in childhood medulloblastoma and atypical teratoid/rhabdoid tumor cells by the potent G-quadruplex interactive agent S2T1-6OTD. *Mol. Cancer Ther, 9(1), pp.167–179.*

Shamoo, Y., Abdul-Manan, N., Patten, A.M., Crawford, J.K., Pellegrini, M.C. & Williams, K.R., 1994. Both RNA-binding domains in heterogenous nuclear ribonucleoprotein A1 contribute toward single stranded-RNA binding. *Biochemistry, 33(27), pp.8272–8281.*

Shao, D.D., Xue, W., Krall, E.B., Bhutkar, A., Piccioni, F., Wang, X., Schinzel, A.C., Sood, S., Rosenbluh, J., Kim, J.W. & Zwang, Y., 2014. KRAS and YAP1 converge to regulate EMT and tumor survival. *Cell, 158(1), pp.171–184.*

Shapiro, P., 2002. Ras-MAP kinase signaling pathways and control of cell proliferation: relevance to cancer therapy. *Crit Rev Clin Lab Sci, 39(4-5), pp.285-330.*

Shibahara, K., Sugio, K., Osaki, T., Uchiumi, T., Maehara, Y., Kohno, K., Yasumoto, K., Sugimachi, K. & Kuwano, M., 2001. Nuclear expression of the Y-box binding protein, YB-1, as a novel marker of disease progression in non-small cell lung cancer. *Clin Cancer Res, 7(10), pp.3151-3155.*

Shima, F., Yoshikawa, Y., Ye, M., Araki, M., Matsumoto, S., Liao, J., Hu, L., Sugimoto, T., Ijiri, Y., Takeda, A. & Nishiyama, Y., 2013. In silico discovery of small-molecule Ras inhibitors that display antitumor activity by blocking the Ras-effector interaction. *Proc. Natl. Acad. Sci. USA, 110(20), pp.8182–8187.*

Shinkai, K., Nakano, K., Cui, L., Mizuuchi, Y., Onishi, H., Oda, Y., Obika, S., Tanaka, M. & Katano, M., 2016. Nuclear expression of Y-box binding protein-1 is associated with poor prognosis in patients with pancreatic cancer and its knockdown inhibits tumor growth and metastasis in mice tumor models. *Int J Cancer, 139(2), pp.433-445*

Shiota, M., Yokomizo, A., Itsumi, M., Uchiumi, T., Tada, Y., Song, Y., Kashiwagi, E., Masubuchi, D. & Naito, S., 2011. Twist1 and Y-box-binding protein-1 promote malignant potential in bladder cancer cells. *BJU Int, 108, pp.142-149.*

Shirude, P.S., Okumus, B., Ying, L., Ha, T. & Balasubramanian, S., 2007. Single-molecule conformational analysis of G-quadruplex formation in the promoter DNA duplex of the proto-oncogene c-kit. *J Am Chem Soc, 129(24), pp.7484-7485.*

Shrivastava, T. & Tahirov, T.H., 2010. Three-dimensional structures of DNA-bound transcriptional regulators. *Methods Mol Biol, 674, pp.43-55.*

Siddiqui-Jain, A., Grandm, C.L. & Bearss, D.J., 2002. Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. *Proc Natl Acad Sci USA, 99(18), pp.11593-11598.*

Siegel, R.L., Miller, K.D. & Jemal, A., 2016. Cancer statistics. *CA Cancer J. Clin, 66(1), pp.7–30.*

Sikka, S., Chen, L., Sethi, G. & Kumar, A.P., 2012. Targeting PPARγ Signaling Cascade for the Prevention and Treatment of Prostate Cancer. *PPAR Research, 968040*. http://doi.org/10.1155/2012/968040.

Simon, M.A., Dodson, G.S. & Rubin G.M., 1993. An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to sevenless and Sos proteins in vitro. *Cell, 73(1), pp.169-177.*

Simonsson, T., Kubista, M. & Pecinka, P., 1998. DNA tetraplex formation in the control region of c-myc. *Nucleic acids research, 26(5), pp.1167-1172.*

Singh, K.B., 1998. Transcriptional regulation in plants: the importance of combinatorial control. *Plant Physiol, 118(4), pp.1111-1120.*

Sivak, J.M., West-Mays, J.A., Yee, A., Williams, T. & Fini, M.E., 2004. Transcription factors Pax6 and AP-2alpha interact to coordinate corneal epithelial repair by controlling expression of matrix metalloproteinase Gelatinase B. *Mol Cell Biol, 24(1), pp.245-257.*

Skolnik, E.Y., Lee, C.H., Batzer, A., Vicentini, L.M., Zhou, M., Daly, R., Myers Jr, M.J., Backer, J.M., Ullrich, A. & White, M.F., 1993. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signaling. *EMBO J, 12(5), pp.1929-1936.*

Smale, S.T. & Baltimore, D., 1989. The "initiator" as a transcriptional control element. *Cell 57: 103-113.*

Smale, S.T. & Kadonaga, J.T., 2003. The RNA Polymerase II Core Promoter. *Annual Review of Biochemistry, 72(1), pp.449-479.*

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. *Analytical biochemistry, 150(1), pp.76-85.*

Soldatenkov, V.A., Chasovskikh, S., Potaman, V.N., Trofimova, I., Smulson, M.E. & Dritschilo, A., 2002. Transcriptional repression by binding of poly (ADP-ribose) polymerase to promoter sequences. *Journal of Biological Chemistry, 277(1), pp.665-670.*

Soldatenkov, V.A., Vetcher, A.A., Duka, T. & Ladame, S., 2008. First evidence of a functional interaction between DNA quadruplexes and poly (ADP-ribose) polymerase-1. *ACS chemical biology, 3(4), pp.214-219.*

Son, J., Lyssiotis, C.A., Ying, H., Wang, X., Hua, S., Ligorio, M., Perera, R.M., Ferrone, C.R., Mullarky, E., Shyh-Chang, N. & Kang, Y.A., 2013. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature, 496(7443), pp.101-105.*

Song, J., Murakami, H., Tsutsui, H., Tang, X., Matsumura, M., Itakura, K., Kanazawa, I., Sun, K. & Yokoyama, K.K., 1998. Genomic organization and expression of a human gene for Myc-associated zinc finger protein (MAZ). *J. Biol. Chem, 273(32), pp.20603-20614.*

Song, J., Ugai, H., Ogawa, K., Wang, Y., Sarai, A., Obata, Y., Kanazawa, I., Sun, K., Itakura, K. & Yokoyama, K.K., 2001. Two consecutive zinc fingers in Sp1 and in MAZ are essential for interactions with cis-elements. *J Biol Chem, 276(32), pp.30429-30434.*

Spiegelman, B.M. & Heinrich, R., 2004. Biological control through regulated transcriptional coactivators. *Cell, 119(2), pp.157-167.*

Spitz, F. & Furlong, E.E., 2012. Transcription factors: from enhancer binding to developmental control. *Nat. Rev. Genet, 13(9), pp.613-626.*

Srivastava, M., Fleming, P.J., Pollard, H.B. & Burns, A.L., 1989. Cloning and Sequencing of the Human Nucleolin Cdna. *Febs Letters, 250(1), pp.99–105.*

Stark, A. & Hines, O.J., 2015. Endoscopic and operative palliation strategies for pancreatic ductal adenocarcinoma. *Semin. Oncol, 42(1), pp.163–176.*

Stead, J.A. & McDowall K.J., 2007. Two-dimensional gel electrophoresis for identifying proteins that bind DNA or RNA. *Nat Protoc, 2(8), pp.1839-1848.*

Steckel, M., Molina-Arcas, M., Weigelt, B., Marani, M., Warne, P.H., Kuznetsov, H., Kelly, G., Saunders, B., Howell, M., Downward, J. & Hancock, D.C., 2012. Determination of synthetic lethal interactions in KRAS oncogene-dependent cancer cells reveals novel therapeutic targeting strategies. *Cell Res, 22(8), pp.1227–1245.*

Stephen, A.G., Esposito, D., Bagni, R.K. & McCormick, F., 2014. Dragging RAS back in the ring. *Cancer Cell, 25(3), pp.272–281.*

Stormo, G.D. & Zhao, Y., 2010. Determining the specificity of protein-DNA interactions. *Nat Rev Genet, 11(11), pp.751-760.*

Stormo, G.D., 2000. DNA binding sites: representation and discovery. *Bioinformatics, 16(1), pp.16-23.*

Štros, M., Muselíková-Polanská, E., Pospíšilová, Š. & Strauss, F., 2004. High-affinity binding of tumor-suppressor protein p53 and HMGB1 to hemicatenated DNA loops. *Biochemistry, 43(22), pp.7215-7225.*

Subramanian, D. & Griffith, J.D., 2005. Modulation of p53 binding to Holliday junctions and 3-cytosine bulges by phosphorylation events. *Biochemistry, 44(7), pp.2536-2544.*

Sun, D., Guo, K. & Shin, Y.J., 2011. Evidence of the Formation of G-Quadruplex Structures in the Promoter Region of the Human Vascular Endothelial Growth Factor Gene. *Nucleic Acids Research, 39(4), pp.1256–1265.*

Sun, D., Liu, W.J., Guo, K., Rusche, J.J., Ebbinghaus, S., Gokhale, V. & Hurley, L.H., 2008. The proximal promoter region of the human vascular endothelial growth factor gene has a G-quadruplex structure that can be targeted by G-quadruplex-interactive agents. *Mol Cancer Ther, 7(4), pp.880-889.*

Sun, D., Thompson, B., Cathers, B.E., Salazar, M., Kerwin, S.M., Trent, J.O., Jenkins, T.C., Neidle, S. & Hurley, L.H., 1997. Inhibition of human telomerase by a G-quadruplex-interactive compound. *J Med Chem, 40(14), pp.2113-2116.*

Sun, Q., Burke, J.P., Phan, J., Burns, M.C., Olejniczak, E.T., Waterson, A.G., Lee, T., Rossanese, O.W. & Fesik, S.W., 2012. Discovery of small molecules that bind to K-Ras and inhibit Sos-mediated activation. *Angew. Chem. Int. Ed. Engl, 51(25), pp.6140–6143.*

Sypes, M.A. & Gilmour, D.S., 1994. Protein/DNA crosslinking of a TFIID complex reveals novel interactions downstream of the transcription start. *Nucleic Acids Res, 22(5), pp.807-814.*

Tajrishi, M.M., Tuteja, R. & Tuteja, N., 2011. Nucleolin: the Most Abundant Multifunctional Phosphoprotein of Nucleolus. *Commun Integr Biol, 4(3), pp.267–275.*

Tanos, V., Ariel, I., Prus, D., De-Groot, N. & Hochberg, A., 2004. H19 and IGF2 gene expression in human normal, hyperplastic, and malignant endometrium. *Int J Gynecol Cancer, 14(3), pp.521-525.*

Tartaglia, M., Gelb, B.D. & Zenker, M., 2011. Noonan syndrome and clinically related disorders. *Best practice & research Clinical endocrinology & metabolism, 25(1), pp.161-179.*

Tellez, C., McCarty, M., Ruiz, M. & Bar-Eli, M., 2003. Loss of activator protein-2alpha results in overexpression of protease-activated receptor-1 and correlates with the malignant phenotype of human melanoma. *J Biol Chem, 278(47), pp.46632-46642.*

Thakur, R.K., Kumar, P., Halder, K., Verma, A., Kar, A., Parent, J.L., Basundra, R., Kumar, A. & Chowdhury, S., 2009. Metastases suppressor NM23-H2 interaction with G-quadruplex DNA within *c-MYC* promoter nuclease hypersensitive element induces *c-MYC* expression. *Nucleic Acids Research*, *37*(1), pp.172–183.

Thanos, D. & Maniatis, T., 1995. Virus induction of human IFNβ gene expression requires the assembly of an enhanceosome. *Cell, 83(7), pp.1091-1100.*

Thomas, M.C. & Chiang, C.M., 2006. The general transcription machinery and general cofactors. *Crit Rev Biochem Mol Biol, 41(3), pp.105-178.*

Trahey, M. & McCormick, F., 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science, 238(4826), pp.542-545.*

Trahey, M., Wong, G., Halenbeck, R., Rubinfeld, B., Martin, G.A., Ladner, M., Long, C.M., Crosier, W.J., Watt, K., Koths, K. & McCormick, F., 1988. Molecular cloning of two types of GAP complementary DNA from human placenta. *Science, 242(4886), pp.1697-1700.*

Tsai, F.D., Lopes, M.S., Zhou, M., Ponce, O., Fiordalisi, J.J., Gierut, J.J., Cox, A.D., Haigis, K.M. & Philips, M.R., 2015. K-Ras4A splice variant is widely expressed in cancer and uses a hybrid membrane-targeting. Motif. Proc. *Natl. Acad. Sci. USA, 112(3), pp.779–784.*

Tsujie, M., Nakamori, S., Okami, J., Hayashi, N., Hiraoka, N., Nagano, H., Dono, K., Umeshita, K., Sakon, M. & Monden, M., 2003. Thiazolidinediones inhibit growth of gastrointestinal, biliary, and pancreatic adenocarcinoma cells through activation of the peroxisome proliferator-activated receptor gamma/retinoid X receptor alpha pathway. *Exp Cell Res, 289(1), pp.143–151.*

Tsutsui, H., Geltinger, C., Murata, T., Itakura, K., Wada, T., Handa, H. & Yokoyama, K.K., 1999. The DNA-binding and transcriptional activities of MAZ, a myc-associated zinc finger protein, are regulated by casein kinase II. *Biochem Biophys Res Commun, 262(1), pp.198-205.*

Tummala, R., Romano, R.A., Fuchs, E. & Sinha, S., 2003. Molecular cloning and characterization of AP-2 epsilon, a fifth member of the AP-2 family. *Gene, 321, pp.93-102.*

Tuveson, D.A., Shaw, A.T., Willis, N.A., Silver, D.P., Jackson, E.L., Chang, S., Mercer, K.L., Grochow, R., Hock, H., Crowley, D. & Hingorani, S.R., 2004. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell, 5(4), pp.375-387.*

Van Venrooij, W.J., Van Eekelen, C.A.G., Jansen, R.T.P. & Princen, J.M.G., 1977. Specific poly-A-binding protein of 76,000 molecular weight in polyribosomes is not present on poly A of free cytoplasmic mRNP. *Nature, 270(5633), pp.189-191.*

Vaquerizas, J.M., Kummerfeld, S.K., Teichmann, S.A. & Luscombe, N.M., 2009. A census of human transcription factors: function, expression and evolution. *Nat Rev Genet, 10(4), pp.252-263.*

Vavvas, D., Li, X., Avruch, J.& Zhang, X.F., 1998. Identification of Nore1 as a potential Ras effector. *Journal of Biological Chemistry, 273(10), pp.5439-5442.*

Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A. & Gocayne, J.D., 2001. Science. The sequence of the human genome. *Science, 291(5507), pp.1304-1351.*

Vernimmen, D., Guéders, M., Pisvin, S., Delvenne, P. & Winkler, R., 2003. Different mechanisms are implicated in ERBB2 gene overexpression in breast and other cancers. *Br J Cancer, 89(5), pp.899–906.*

Vetter I.R. & Wittinghofer A., 2001. The guanine nucleotide-binding switch in three dimensions. *Science, 294(5545), pp.1299-1304.*

Vigil, D., Cherfils, J., Rossman, K.L. & Der, C.J., 2010. RAS superfamily GEFs and GAPs: Validated and tractable targets for cancer therapy? *Nat. Rev. Cancer, 10(12), pp.842–857.*

Vivanco, I. & Sawyers, C.L., 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer, 2(7), pp.489-501.*

Wajapeyee, N., Ganpat, R.C. & Somasundaram, K., 2005. AP-2a status determines the chemosensitivity of cancer cells: implications in cancer chemotherapy. *Cancer Res, 65(19), pp.8628–8634.*

Wei, W., Mosteller, R.D., Sanyal, P., Gonzales, E., McKinney, D., Dasgupta, C., Li, P., Liu, B.X. & Broek, D., 1992. Identification of a mammalian gene structurally and functionally related to the CDC25 gene of Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences, 89(15), pp.7100-7104.*

Wellbrock, C., Karasarides, M. & Marais, R., 2004. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol, 5(11), pp.875-885.*

Wennerberg, K., Rossman, K.L. & Der, C.J., 2005. The Ras superfamily at a glance. *J Cell Sci, 118 (5), pp.843-846.*

Williams, T. & Tjian, R., 1991a. Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev, 5(4), pp.670-682.*

Williams, T. & Tjian, R., 1991b. Characterization of a dimerization motif in AP-2 and its function in heterologous DNA-binding proteins. *Science, 251(4997), pp.1067-1071.*

Williams, T., Admon, A., Lüscher, B. & Tjian, R., 1988. Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes Dev, 2(12A), pp.1557-1569.*

Wilson, D., Charoensawan, V., Kummerfeld, S.K. & Teichmann, S.A., 2008. DBD--taxonomically broad transcription factor predictions: new content and functionality. *Nucleic Acids Res, 36, pp.88-92.*

Wing, M.R., Bourdon, D.M. & Harden, T.K., 2003. PLC-ε: a shared effector protein in Ras-, Rho-, and Gαβγ-mediated signaling. *Molecular Interventions, 3(5), p.273.*

Wingender, E., Dietze, P., Karas, H. & Knüppel, R., 1996. TRANSFAC: A Database on Transcription Factors and Their DNA Binding Sites. *Nucleic Acids Research, 24(1), pp.238-241.*

Winkler, G.S., Lacomis, L., Philip, J., Erdjument-Bromage, H., Svejstrup, J.Q. & Tempst, P., 2002. Isolation and mass spectrometry of transcription factor complexes. *Methods, 26(3), pp.260-269.*

Wittinghofer, A., Franken, S.M., Scheidig, A.J., Rensland, H., Lautwein, A., Pai, E.F. & Goody, R.S., 1993. Three-dimensional structure and properties of wild-type and mutant Hras-encoded p21. *Ciba Found Symp, 176, pp.6-21.*

Wolfgang, C.L., Herman, J.M., Laheru, D.A., Klein, A.P., Erdek, M.A., Fishman, E.K. & Hruban, R.H., 2013. Recent progress in pancreatic cancer. *CA Cancer J. Clin, 63(5), pp.318–348.*

Woo, A.J., Dods, J.S., Susanto, E.etUlgiati, D. & Abraham, L.J., 2002. A proteomics approach for the identification of DNA binding activities observed in the electrophoretic mobility shift assay. *Mol Cell Proteomics, 1(6), pp.472-478.*

Wray, G.A., Hahn, M.W., Abouheif, E., Balhoff, J.P., Pizer, M., Rockman, M.V. & Romano, L.A., 2003. The evolution of transcriptional regulation in eukaryotes. *Mol Biol Evol, 20(9), pp.1377-1419.*

Wu, F. & Lee, A.S., 1998. Identification of AP-2 as an interactive target of Rb and a regulator of the G1/S control element of the hamster histone H3.2 promoter. *Nucleic Acids Res, 26(21), pp.4837-4845.*

Wu, F. & Lee, A.S., 2001. YY1 as a regulator of replication-dependent hamster histone H3.2 promoter and an interactive partner of AP-2. *J Biol Chem, 276(1), pp.28-34.*

Wu, K.K., 2006. Analysis of protein-DNA binding by streptavidin-agarose pulldown. *Methods Mol Biol, 338, pp.281-290.*

Wu, Y., Yamada, S., Izumi, H., Li, Z., Shimajiri, S., Wang, K.Y., Liu, Y.P., Kohno, K. & Sasaguri, Y., 2012. Strong YB-1 expression is associated with liver metastasis progression and predicts shorter disease-free survival in advanced gastric cancer. *J Surg Oncol, 105(7), pp.724-730.*

Xue, W., Dahlman, J.E., Tammela, T., Khan, O.F., Sood, S., Dave, A., Cai, W., Chirino, L.M., Yang, G.R., Bronson, R. & Crowley, D.G., 2014. Small RNA combination therapy for lung cancer. *Proc. Natl. Acad. Sci. USA, 111(34), 3553–3661.*

Yamamoto, F. & Perucho, M., 1988. Characterization of the human c-K-ras gene promoter. *Oncogene Res, 3(2), pp.125–130.*

Yamauchi, N., Kiessling, A.A. & Cooper, G.M., 1994. The Ras/Raf signaling pathway is required for progression of mouse embryos through the two-cell stage. *Mol Cell Biol, 14(10), pp.6655-6662.*

Yang, C., Bolotin, E., Jiang, T., Sladek, F.M. & Martinez, E., 2007. Prevalence of the initiator over the TATA box in human and yeast genes and identification of DNA motifs enriched in human TATA-less core promoters. *Gene, 389(1), pp.52-65.*

Yang, L., Reece, J.M., Cho, J., Bortner, C.D. & Shears, S.B., 2008. The Nucleolus Exhibits an Osmotically Regulated Gatekeeping Activity That Controls the Spatial Dynamics and Functions of Nucleolin. *J. Biol. Chem. 283(17), pp.11823–11831.*

Yang, T.H., Tsai, W.H., Lee, Y.M., Lei, H.Y., Lai, M.Y., Chen, D.S., Yeh, N.H. & Lee, S.C., 1994. Purification and characterization of nucleolin and its identification as a transcription repressor. *Mol. Cell. Biol, 14(9), pp.6068–6074.*

Yasen, M., Kajino, K., Kano, S., Tobita, H., Yamamoto, J., Uchiumi, T., Kon, S., Maeda, M., Obulhasim, G., Arii, S. & Hino, O., 2005. The up-regulation of Y-box binding proteins (DNA binding protein A and Y-box binding protein-1) as prognostic markers of hepatocellular carcinoma. *Clin Cancer Res, 11(20), pp.7354-7361.*

Ying, H., Kimmelman, A.C., Lyssiotis, C.A., Hua, S., Chu, G.C., Fletcher-Sananikone, E., Locasale, J.W., Son, J., Zhang, H., Coloff, J.L. & Yan, H., 2012. Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell, 149(3), pp.656-670.*

Yuan, T.L., Fellmann, C., Lee, C.S., Ritchie, C.D., Thapar, V., Lee, L.C., Hsu, D.J., Grace, D., Carver, J.O., Zuber, J. & Luo, J., 2014. Development of siRNA payloads to target KRAS-mutant cancer. *Cancer Discov, 4(10), pp.1182–1197.*

Zambon, A.C., Zhang, L., Minovitsky, S., Kanter, J.R., Prabhakar, S., Salomonis, N., Vranizan, K., Dubchak, I., Conklin, B.R. & Insel, P.A., 2005. Gene expression patterns define key transcriptional events in cell-cycle regulation by cAMP and protein kinase A. *Proc Natl Acad Sci USA, 102(24), pp.8561-8566.*

Zeng, M., Kikuchi, H., Pino, M.S. & Chung, D.C., 2010. Hypoxia activates the K-ras protooncogene to stimulate angiogenesis and inhibit apoptosis in colon cancer cells. *PLoS One, 5(6), e10966.*

Zeng, Y.X., Somasundaram, K. & El-Deiry, W.S., 1997. AP2 inhibits cancer cell growth and activates p21WAF1/CIP1 expression. *Nat Genet, 15(1), pp.78-82.*

Zhao, F., Satoda, M., Licht, J.D., Hayashizaki, Y. & Gelb, B.D., Cloning and characterization of a novel mouse AP-2 transcription factor, AP-2 delta, with unique DNA binding and transactivation properties. *J Biol Chem, 276(44), pp.40755-40760.*

Zhao, X., Huang, H & Speed, T.P., 2005. Finding short DNA motifs using permuted Markov models. *Journal of Computational Biology, 12(6), pp.894-906.*

Zhou, W.J., Deng, R., Zhang, X.Y., Feng, G.K., Gu, L.Q. & Zhu, X.F., 2009. G-quadruplex ligand SYUIQ-5 induces autophagy by telomere damage and TRF2 delocalization in cancer cells. Mol. Cancer Ther, 8(12), pp.3203–3213.

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