Regulation of the Tumor Suppressor p53 and Survivin by Ras and Ral GTPases: Implications for Malignant Transformation

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Regulation of the Tumor Suppressor p53 and Survivin by Ras and Ral GTPases:
Implications for Malignant Transformation

by

Awet G. Tecleab

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Molecular Medicine College of Medicine University of South Florida

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Dedication

This thesis is dedicated to the loved ones: my mother and my siblings. Without their loving support and encouragement none of this would be possible.
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List of Abbreviations

ATP: Adenosine triphosphate
BCL-2: B-cell lymphoma 2
EGF: Epidermal growth factor
FTI: Farnesyl transferase inhibitor
FDA: Food and drug administration
CDK1: Cycline dependent kinase 1
GAP: GTPase activating protein
GDP: Guanosine diphosphate
GEF: Guanine nucleotide exchange factor
GGTI: Geranylgeranyltransferase inhibitor
GLUT-4: Glucose transporter type 4
GTP: Guanosine triphosphate
HEK: Human embryonic kidney cells
hTERT: human telomerase catalytic subunit
IAP: Inhibitors of apoptosis
NK: Natural killer cells
Pi3K: Phosphotydil inositol 3,4,5-triphosphate kinase
PKB: Protein kinase B
PLC: Phospholipase C
PLD-1: Phospholipase D-1
PP2A: Protein phosphotase-2A
RalA: Ras-like A
RalB: Ras-like B
RalBP1: Ral binding protein-1
RalGDS: Ral-guanine nucleotide dissociation stimulator
RalGEF: Ral-guanine nucleotide exchange factor
RasGAP: Ras GTPase activating protein
RBD: Ras binding domain
siRNA: Small interfering RNA
SV40T: Simian virus-40 large T antigen
S40t : Simian virus-40 small t antigen
TGF-β: Tumor growth factor- β
VEGF-A: Vascular endothelial growth factor-A
XIAP: X-linked inhibitor of apoptosis
Abstract

Although the critical role of the small GTPases Ras and Ral in oncogenesis has been well documented, much remains to be investigated about the molecular mechanism by which these GTPases regulate malignant transformation. The work under this thesis made two major contributions to this field. The first is the discovery that K-Ras, RalA and/or RalB are required for the maintenance of the high levels of the anti-apoptotic protein survivin in some human cancer cells, and the second is the demonstration that down regulation of K-Ras, RalA and/or RalB, but not Raf-1 or Akt1/2, stabilizes the tumor suppressor p53 and reactivates it to inhibit malignant transformation in human cancer cells with mutant K-Ras and wild type p53. Here we found that depletion of K-Ras leads to decreased survivin levels in human cancer cells that harbor mutant K-Ras but not those with wild type Ras. The mechanism by which this occurs involves ubiquitination and subsequent proteasome-mediated degradation of survivin. The presence of mutant K-Ras alone was not sufficient to predict the effects of RalA/B depletion on survivin levels. Indeed, depletion of RalA and/or RalB reduces survivin levels in human cancer cells with wild type p53 and mutant K-Ras, but not in those with mutant p53 and/or wild type K-Ras. The functional relevance of these findings to malignant transformation was further supported by the demonstration that compromising the expression of survivin by siRNA leads to reduction of mutant K-Ras-driven invasion and anchorage-independent growth. Furthermore, in this thesis, we have discovered that down regulation of K-Ras, RalA and/or RalB using siRNA leads to increased levels of
functional p53 that is capable of regulating its target genes. The mechanism by which depleting K-Ras, RalA and RalB increases the levels of p53 involves an increase in the half-life of the p53 protein concurrent with an increase in the phosphorylation of serine-15 of p53, a marker of p53 stability. Finally, we demonstrated that depletion of K-Ras, RalA and/or RalB interferes with cell cycle progression, anchorage-independent growth and invasion in a p53-dependent manner. In summary, the studies suggest that mutant K-Ras contributes to the maintenance of the aberrantly-high survivin levels by regulating its stability, and that the ability of mutant K-Ras to induce malignant transformation is, at least in part, dependent of these high levels of survivin. The work of this thesis also suggests that the expression of K-Ras, RalA and/or RalB proteins is critical to maintain low levels of p53, and that down regulation of these GTPases reactivates p53 by significantly enhancing its stability, and this contributes to suppression of malignant transformation.
Chapter 1

Introduction

Cancer versus normal cells

The transformation of normal cells to cancer cells is thought to arise from gradual accumulation of multiple genetic alterations such as mutations, chromosomal translocations and amplifications. These genetic alterations can lead to activation of cancer-causing genes (oncogenes) such as Ras GTPases and/or inactivation of tumor suppressor genes such as p53. Pioneering work over the last 3 decades documented that a single genetic alteration is not sufficient to elicit full-blown transformed phenotype. For example, Fearon and Vogelstein’s elegant work led to the discovery that colon cancer develops as a result of successive genetic alterations that are involved in driving malignant transformation or inhibiting tumor suppression\(^1\). These genetic alterations lead to deregulated signal transduction pathways that in turn result in several hallmarks of cancers such as apoptosis evasion, invasion, metastasis, resistance to growth suppressors, sustained proliferative signals, immortality, angiogenesis, evasion of immune destruction and deregulated cellular energetic\(^2\).

Many of the altered genes in cancer cells are intimately involved in signal transduction pathways that mediate these hallmarks of cancer. For example, mutations in the Ras gene persistently activate Raf/Mek/Erk, Pi3K/Akt and RalGDS/Ral signaling pathways that mediate many of the hallmarks of cancer including sustained proliferation,
survival, angiogenesis and metastasis\textsuperscript{2}. Up-regulation of the inhibitors of apoptosis (IAPs) such as survivin and the anti-apoptotic BCL-2 family proteins such as Bcl-xL and Mcl-1 contribute to apoptosis evasion. Amplification of telomerase enables immortality whereas mutations and/or deletions of the tumor suppressors p53 and RB lead to deregulation of the cell cycle and enable cells to proliferate continuously\textsuperscript{2}. Furthermore, up regulation of VEGF-A enables the formation of new tumor blood vessels, loss of E-cadherin promotes invasion and metastasis, up-regulation of GLUT-4 increase glucose import into the cytoplasm and reprogramming of energy metabolism and up-regulation of TGF-β enables cancer cells to evade immune system by disabling natural killer (NK) cells\textsuperscript{2}. While the above mentioned mechanism are cited separately, it needs to be emphasized that the complex crosstalk among the various aberrant signal transduction pathways is often required for normal cells to trespass the normal control mechanism, acquire certain hallmarks of cancer and eventually become fully transformed.

\textit{Ras, Ral and p53}

\textit{Ras}

Ras is a small GTPase that cycle between GTP and GDP binding states. Active Ras is GTP bound and capable of transducing signals from surface receptors to the nucleus to regulate a wide range of cellular processes including differentiation, survival and motility\textsuperscript{3}. Mutations of Ras are among the most frequently observed mutations in human cancer. The mutant version of Ras is defective of its intrinsic GTPase activity and becomes GTP-locked Ras which enables it to stay engaged to its downstream effectors persistently, and drive malignant transformation\textsuperscript{3}. This prompted many over the last several decades to target Ras directly and indirectly to discover novel anti-cancer drugs.
For example, attempts to identify small molecule that can bind Ras either at the GTP binding site, effector site or allosteric sites and induce a conformational change to reverse the GTP-lock to the GDP inactive form of Ras were unsuccessful; although renewed efforts in this area have yielded encouraging results recently\(^4\)\(^5\).

An indirect approach to inhibit Ras function that has been investigated extensively is to prevent Ras from associating with the plasma membrane\(^6\). Ras proteins undergo prenylation, a type of posttranslational modification, to be able to localize in the membrane. This localization of Ras on the membrane is required for its activation as well as for Ras to find and bind its downstream effectors. Previous studies have shown that this post-translational modification is required for the cancer-causing activity of Ras. Prenylation is defined as the attachment of a geranylgeranyl or a farnesyl group to the cysteine of the CAXX box at the C-terminus of Ras by either geranylgeranyl transferase-1 (GGT-1) or farnesyl transferase (FT). Since these enzymes transfer the prenyl group to Ras, it was reasonable to inhibit these enzymes to prevent Ras from localizing to the membrane. Subsequently, several potent and selective geranylgeranyl transferase inhibitors (GGTIs) and farnesyl transferase inhibitors (FTIs) have been developed and shown to inhibit Ras membrane association, Ras-driven oncogenic signaling and malignant transformation in vitro and tumor growth in mouse models\(^6\). Several FTIs have been tested clinically and one GGTI has recently entered clinical trials\(^6\). Unfortunately, the outstanding preclinical results with FTIs have not translated into efficacy in human clinical trials, and so far there is no FDA approved FTI that is used in the clinic. The reasons for this are not well understood. However, the fact that FT has several hundreds of potential substrates (the human genome encodes about 500 proteins
that harbor a CAXX box at their C-termini) may contribute to this\textsuperscript{6}. Indeed, at present it is not known yet which farnesylated proteins are some tumors addicted to. Another reason may be related to alternative prenylation. For example, among the isoforms of Ras that are implicated in tumorigenesis, H-Ras is known to undergo farnesylation exclusively, however, N-Ras and K-Ras have been shown to undergo geranylgeranylation when human cancer cells are challenged with FTIs\textsuperscript{7}.

Another indirect approach to inhibit the function of Ras is to target downstream effectors that have been implicated in cancer. For example, Raf-1 was the first identified effector of Ras known to mediate Ras driven malignant transformation. Subsequently, several Raf-1 kinase inhibitors were developed and many of them showed anti-tumor activity, however in some tumors, despite complete inhibition of Raf-1 kinase activity, the tumors still progressed, suggesting existence of other important downstream effectors that transduce Ras driven transformation\textsuperscript{8, 9}. Indeed subsequent studies identified the Pi3K/Akt, and RalGDS/Ral and other downstream signaling pathways as critical effectors that mediate Ras-driven malignant transformation. To date, there are several inhibitors of these pathways that are in clinical trials\textsuperscript{6}. Ras is known to activate more than 20 downstream effectors and these have been shown to be involved in different physiological functions of the cell, so the challenge at this moment is to identify the critical effectors of Ras that human tumors are addicted to. Recent studies have tried to address this issue using large scale RNAi screen. Using different cell lines that harbor mutant or wt-K-Ras genes, several target genes were identified, the depletion of which resulted in selective killing of cancer cells that harbor mutant but not wt K-Ras\textsuperscript{10-12}. Nonetheless, these studies identified different genes in different cell lines and there was
no consensus gene the depletion of which induces selective killing of cancer cell with K-Ras mutation. To date, studies that aim at identifying the critical downstream effectors of mutant K-Ras continue to be under intense investigation.

**Ral**

Ral (Ras like) proteins are small GTPases that belong to the Ras family of G proteins\(^\text{13}\). As the name indicated, Ral GTPases were first identified when searching for Ras like genes using oligonucleotide probes that correspond to a sequence of seven amino acid that are conserved in Ras\(^\text{14}\). The cDNA library screen from immortalized simian B-lymphocyte screen identified RalA as a Ras related gene. Using the simian RalA sequence human RalA and RalB were identified from a phechromocytoma library. Both RalA and RalB are exclusively geranygeranylated by GGT-1 and this geranylgeranylation is required for their localization and function\(^\text{15}\).

Initially, Ral GTPases didn’t get wide attention in cancer because they were found to have only minor role in Ras driven tumor formation in rodent cells. Later, however, the Ral guanine exchange factor RalGDS, which activates RalA and RalB, was shown to directly bind Ras and to play a critical role in Ras-driven tumorigenesis in human cancer cells of epithelial as well as of fibroblast origin\(^\text{16-20}\).

**Activation of Ral**

Like all small G proteins Ral GTPases are GTP/GDP binding proteins that are active when bound to GTP and inactive when bound to GDP\(^\text{21}\). Ral GTPases can be activated by two major mechanisms: Ras-dependent\(^\text{22-24}\) and Ras-independent activation\(^\text{25-27}\).
Ras-dependent activation of Ral

Activated Ras can bind directly to several proteins and subsequently induce activation of numerous downstream effector pathways that modulate a variety of biological functions. Activation of the Raf/Mek/Erk, Pi3K/Akt and RalGDS/Ral pathways through direct binding of their up-stream activators has been previously described\(^3\) (see figure 1 below).

**Figure 1. Activation of RalGTPases and their downstream effectors.** Upon activation, RalGDS activates RalA and RalB which in turn modulate downstream effectors that are involved in the regulation of different cellular processes.

Guanine nucleotide exchange factors (GEFs) such as Rgl, Rlf and RalGDS\(^{28, 29}\) can activate Ral proteins. Ras activate Ral proteins by binding RalGDS. Structurally RalGDS has a Ras binding domain (RBD) at its C terminus\(^{30, 31}\) which enables it to interact with Ras. When activated by Ras, RalGDS can then exchange GDP to GTP on Ral. RBD of RalGDS is critical for Ras-induced activation of RalGDS but activation of
RalGDS in the absence of RBD domain of RalGDS has also been reported. Linnemann et al show that, RalGDS that lack RBD but artificially targeted to the membrane, can still be activated by Ras.

*Ras-independent activation of Ral*

Activation of Ral independent of Ras has also been described. Hofer et al show that treating Rat-2 cells with Ca\(^{2+}\) activated Ral independently of Ras. The result show that inhibition of phospholipase C (PLC) blocks Ca\(^{2+}\) induced activation of Ral, implying that PLC was required for Ca\(^{2+}\) induced activation of Ral proteins. As suggested by Hofer et al, the mechanism by which Ca\(^{2+}\) activates Ral could be through phosphorylation of RalGDS by Calcuim-Calmodulin dependent kinase II or through direct interaction of Ca\(^{2+}\) with Ral. RalA has calmodulin binding site at its C terminus, and the binding of Ca\(^{2+}\) facilitate GTP binding to the RalA protein. For Ca\(^{2+}\) dependent activation to occur geranylgeranylation of Ral and calmodulin interaction are required. Different studies have also shown that RalGEF undergo posttranslational modification that enables it to localize in the membrane where it can be activated. RalGEF can also be stimulated upon association with PI3-K-dependent kinase 1 (PDK1). The activation of Ral GEF activity by PDK1 doesn’t involve its kinase activity, but rather depends on the Epidermal Growth Factor (EGF)-induced complex formation between PDK-1 and the N terminus of RalGDS which relieves the auto inhibitory effect on the catalytic domain of RalGDS.

*Functions of activated Ral*

RalA and RalB are two isoforms that share \(~85\%\) overall amino acid sequence identity and \(~100\%\) sequence identity in sequences important for effectors binding.
Once activated by RalGEF, they bind to different or similar molecules that play important roles in cell cycle and transformation\textsuperscript{35}.

Since RalA and RalB share identical effector binding domain, the two Ral proteins would be expected to bind to similar molecules and elicit similar functions but studies so far show that the two Ral proteins do not necessarily bind to same molecules or elicit same biological outcomes. In fact there are situation where the two proteins are shown to bind to different effectors and to elicit antagonistic functions.

![Figure 2. Role of RalA and RalB in cancer.](image)

The small GTPases RalA and RalB display distinct as well as overlapping functions in cancer.

**Significance of Ral protein in human tumorigenesis**

Evidence for the importance of Ral in human tumorigenesis started to emerge when RalGEF was identified as direct interacting protein of Ras and when Ral GDS was shown to be required for Ras-driven malignant transformation\textsuperscript{36}. Furthermore, other studies showed that activated Ral was found in human pancreatic cancer cells\textsuperscript{35} where the
frequency of K-Ras mutation is also high. Moreover, depletion of Ral protein reduced Ras ability to transform cells. Similarly, in vivo studies demonstrated that mice deficient of RALGDS, which is one of Ral GEFs, were developmentally normal but showed reduced skin tumor growth driven by oncogenic H-Ras\textsuperscript{16}. A very recent study showed that deletion of both RalA and RalB genes inhibit tumor formation\textsuperscript{37}. Other studies also showed RalA to be the target of known the tumor suppressor phosphatase, protein phosphates 2A (PP2A)\textsuperscript{38, 39}. Lim et al. also showed that stable knockdown of RalA in pancreatic cancer cells that harbor mutant K-Ras reduces anchorage independent growth in soft agar, which further support the role of Ral in transformation\textsuperscript{35}. All in all, these findings suggest deregulation and significant role of Ral in cancer.

**Role of Ral in cell survival**

Loss of function studies have shown that depletion of RalB but not RalA initiates programmed cell death in cancer cell line but there was no effect in normal cell line or immortalized cell lines. Interestingly, when RalA and RalB are depleted together, this reverses the death phenotype seen when only RalB is depleted in cancer cells and immortalized cells that harbor oncogenic H-Ras. This suggested that the two proteins function antagonistically to regulate cell survival\textsuperscript{40}.

Akt/ protein kinase B (PKB) is one of the survival protein downstream of Ras. Depletion of RalGDS by RNAi blocks insulin- as well as EGF-induced phosphorylation of Akt at T-308 and S-473. It is speculated that RalGDS activates Akt by acting as a scaffold to bring Akt closer to its activator PDK1. In fact shRNA depletion of RalGDS sensitizes cells to drug-induced apoptosis\textsuperscript{41}. These studies suggest a role of RalGDS in survival through its ability to modulate Akt activation.
Role of Ral in cell migration, invasion and metastasis

Another hallmark of cancer is invasion and metastasis. The ability of cancer cells to invade adjacent tissue and migrate to distant site constitute one of the worst phenotype of cancer pathogenesis. Therefore, the ability of cells to move helps them to metastasize to distant site. While RalA but not RalB is critical for Ras driven transformation, RalB plays critical a role in cell migration. While Rosse et al.\textsuperscript{42} and Oxford et al.\textsuperscript{43} show that depletion of RalB but not RalA by siRNA in UMUC-3 (bladder cancer cell line) and DU145 (prostate cancer cell line) reduces migration, Lim et al.\textsuperscript{35} show that depletion of RalA also reduce migration in some pancreatic cancer cells, suggesting the role of Ral in cell migration could be cell line specific. Nonetheless RalB seem to show dominant effect in migration. In addition, NIH-3T3 cells that were transformed by Ras effector mutant that activate Ral (Ras12V37G) promotes invasion and this was antagonized by over expressing dominant negative RalB\textsuperscript{44}. High level of activated RalA was also found more in metastatic cell than their counterpart non metastatic cells\textsuperscript{33} suggesting the role of activated RalA in this cancer hallmark.

In-vivo study show that HEK cells transformed by mutant H-Ras form fewer number of lung metastatic nodules when stably transfected with shRNA to RalA. Furthermore, even though RalB was dispensable for tumor establishment, cells where RalB was knockdown by shRNA had significantly impaired metastasis.\textsuperscript{35} Although RalA depletion reduces oncogenic Ras driven metastasis, dominant negative RalA had minimum effect on metastasis in Ras12V37G\textsuperscript{45}. This suggests that RalGTPases require additional proteins and/or molecules to promote metastasis.
Role of Ral in cell cycle

The ability of the body to replace old and dead cells largely depends on cell cycle. This activity is highly regulated in order to avoid uncontrolled proliferation. Cancer cells usually become insensitive or evade normal cell cycle control. Cell cycle checkpoints are important to ensure the completion of previously initiated cell cycle activity before proceeding with the subsequent steps. Like other signaling protein, Ral proteins have also been shown to play roles in cell cycle progression.

The two Ral proteins seem to work in collaboration for this activity. RalA mobilizes the exocyst, to the cytokinetic furrow during the early stage of cytokinesis and RalB pulls the complex toward the midbody of the cell and drives abscission to complete cytokinesis\textsuperscript{46}. RalA and RalB share Exo84 and PLD1 as effectors of cytokinesis but RalA additionally uses Sec5 for early recruitment of exocyst component.

CDK1 is the major mitotic kinase and is regulated by different inhibitory as well as activating kinases and also phosphatases. The Ras/Ral signaling pathway has been shown to decrease the inhibitory phosphorylation of cdc2 at Y15 after DNA damage induced by chemicals. This suggests that Ras/Ral signaling contributes to the activation of CDK1 and plays a role in mitosis.

Activation of the Ras/RasGDS/Ral pathway inhibits G2 arrest following DNA damage\textsuperscript{47}. This was demonstrated by showing that dominant negative RalA (N28) rescues from the loss of G2 arrest that was seen with cells that have activated Ras and that were treated with the DNA damage agent doxorubicin\textsuperscript{47}.
**p53**

p53 is a transcription factor that is known to regulate the expression of different genes that are involved in cell cycle as well as apoptosis. It is one of the most frequently mutated genes in cancer with ~50% prevalence in human tumor. It was first identified as a co-precipitant of SV40-Tag in SV40-infected cells and since the co-eluate of the protein runs at ~53 kilo Dalton in poly-acrylamide gel, the protein was named as p53. Since the expression of p53 goes up with SV40 levels in the cell, p53 was considered as an oncogene. Subsequently the cloned p53 from transformed cells has been shown to cooperate with known oncogenes such as H-Ras to transform cells. Moreover the cloned p53 by itself was able to immortalize cells. However, subsequent studies clearly demonstrated the tumor suppressive activity of p53 rather than the earlier reported oncogenic effect.

Functionally, p53 has been shown to induce cell cycle arrest at G1 and G2/M phases of the cell cycle as well as apoptosis. It is now widely accepted that the molecular mechanism by which p53 accomplish its tumor suppressive activity is through transactivation or repression of different genes that are involved in apoptosis as well as cell cycle.

Even though multiple genetical alterations are required for transformation, few genes seem to show potent effect at driving or preventing malignant phenotypes, such as sustained proliferation, resistance to antigrowth and apoptotic signals. Indeed previous studies have shown that, disabling of p53 function cooperate with Ras to transform cells. Moreover, using affymetrix microarrays, McMurray et al found a synergistic effect on the expression of 95 genes when mutant p53 and Ras are expressed together.
The study suggests that p53 and some genes that are regulated by p53 play critical role in Ras driven transformation.

The following are literature gaps filled by the work of this thesis concerning the mechanism by which Ral GTPases mediate malignant transformation and the role of the tumor suppressor p53 in this process.

Although Ral has been shown to have significant role in transformation as well as in regulating various malignant phenotype, the mechanism by which it regulates these phenotypes is not known. Furthermore, the ability of mt K-Ras to cause cancer depends on Ral GTPases, yet the mechanism by which Ral GTPases mediate K-Ras effects is not known. Recently, mutant K-Ras was shown to down regulate p53 but whether Ral GTPases down regulate p53 is not known. In this thesis we determined if the expression of RalA and/or RalB is required for maintaining low levels of wild type p53 in human cancer cells, and whether the expression of RalA/B is required for persistent degradation of p53. This thesis also investigated if Ral GTPases regulate the stability of p53 and if the ability of Ral GTPases to contribute to malignant transformation depends on suppression of p53.

**Survivin and Apoptosis**

**Cell death**

Cells have evolved to respond to proliferative as well as to death signals. Upon existence of irreparable abnormality, normal cells are doomed to death. Dying cells are characterized by loss of membrane potential, caspase activation, exposure of phosphatidylserine residues as well as permeabilization of mitochondrial outer membrane. However, since these signals can also be detected in the absence of non-lethal
processes, existence of one signal alone doesn’t represent cell death. So, when is a cell considered dead? According to the nomenclature committee on cell death (NCCD) cellular death is defined as the presence of the loss of membrane integrity as defined by incorporation of vital dyes in vitro, the cell undergo complete fragmentation into discreet bodies and engulfment of the corpse by adjacent cell in vivo\textsuperscript{62,63}.

Different types of cell death have been described based on the morphology of the dying cells and the biochemical events that trigger them. Accordingly, four major types of cell death exist and these are apoptosis, autophagy, necrosis and cornification. Morphologically apoptotic cells are presented as rounded cells, fragmented nucleus, retracted pseudopods, blebbing of plasma membrane and also engulfment of apoptotic cells by resident phagocytes, in-vivo. Autophagic cells are characterized by massive cytoplasmic vacuolation as well as accumulation of double-membraned autophagic vacuoles. Necrotic cells show swelling of the cytoplasm and its organelles as well as rupture of plasma membrane. Cornification is usually seen in skin cells (dermatocytes) and it is morphologically characterized by elimination of cytosolic organelles, accumulation of lipids in granules, as well as extrusion of lipids in the extracellular space.

In this thesis, we focus on apoptosis and hence this is further discussed below.

**Apoptosis**

The term apoptosis was first coined by Kerr et al to describe the specific morphological change of a dying cell as described above\textsuperscript{64}. Apoptosis occurs during development, aging as well as normal homeostatic mechanism to maintain the population of cells. Furthermore, a cell can undergo apoptosis following irreparable damage or abnormality in the cell.
The mechanism by which apoptosis is initiated in a cell vary. Some cells express death receptors, such as TNF-α or Fas in which binding of ligand to its receptor triggers apoptosis. On the other hand, some cells undergo apoptosis by default but it is prevented from dying by the binding of growth factor or hormones. In any case, the mechanism by which apoptosis occurred is highly complex and sophisticated. To date, two main apoptotic pathways have been identified: Extrinsic and intrinsic apoptotic pathways. The extrinsic death pathway is initiated when a death ligand bind to its receptor on cell membrane, in contrast, the intrinsic pathway is initiated upon cytochrome C release from the mitochondria. Although this two pathways were identified as two separate pathway based on how the apoptosis is initiated, subsequent studies show existence of cross-talk between the two pathways65 (see figure 3).

Biochemical features of apoptosis

Caspases are the major enzymes that are involved in apoptosis. Caspases are proteolytic enzymes that are capable of degrading proteins by cleaving at aspartic acid residues. They are widely expressed in a cell in their inactive form. Upon activation, the activated caspases activate other caspases thereby amplifying the apoptotic cascade and bringing rapid cell death. To date, 10 major caspases have been identified. They are majorly classified as initiator caspases (caspase 2, 8, 9 and 10) because they are known to ignite the caspase activation cascade. The other class of caspases are called effecter caspases (caspase 3,6, and 7) because they implement the execution phase of the cell killing. Finally there are other caspase (caspase 1,4, and 5) referred to as inflammatory caspases61.

Other biochemical features of apoptosis is protein cross linking, which is achieved
**Figure 3. Activation of intrinsic and extrinsic apoptosis pathways.** Intrinsic apoptotic pathways can be triggered by many events including DNA damage. This in turn results in activation of p53 and subsequent upregulation of pro-apoptotic Bcl-2 family proteins, which form pores in the mitochondrial membrane through which cytochrome-c is released. The released cytochrome-c leads to the activation of caspases-9 and -3 and induction of apoptosis. Alternatively, apoptosis can be triggered through the extrinsic pathway. In this case, a death ligand binds to its receptor activates caspases-8 and -3 and induces apoptosis. IAPs can inhibit activation of caspases and this effect could be antagonized by SMAC, which is also released from the mitochondria.

through activation of tissue transglutaminase\textsuperscript{66} as well as phagocytosis as a result of expression of cell surface signals, such as phosphatidylserine and annexin that send signals to phagocytic cells\textsuperscript{67} also exist.
The extrinsic pathway

The extrinsic pathway is initiated through activation of a receptor with transmembrane receptor binding to its ligand. One of the best characterized receptors is the Fas ligand receptor. This receptor has a death domain “DD” on its cytoplasmic portion that transmits the death signal from the cell surface to inside the cell. Upon binding of a death ligand (FasL) to its receptor (Fas R), the cytoplasmic domain of the receptor recruits adaptor proteins (FADD). This adaptor protein then recruits procaspase 8 and subsequently forms the DISC (death inducing signaling complex) which results in auto activation of procaspase-8. Activation of caspase-8 marks the beginning of cell death. Activated caspase-8 can then activate caspase-3, which is the executioner of cell death.

The intrinsic pathway

Unlike the extrinsic pathway, the intrinsic pathway is triggered by events that are not dependent on death receptors such as the Fas ligand receptor. The intrinsic pathway stimuli include the absence of growth factors or cytokines that normally inhibit cell death. Other stimuli are exposure to toxins, radiation or viral infection and other agents that stimulate cell death. The intrinsic pathway is also called the mitochondrial apoptotic pathway, because the apoptosis executed trough this pathway hinges on mitochondrial events. The death stimuli induce changes in the mitochondrial membrane which result in pores that allow the release of mitochondrial proteins such as cytochrome-C and Smac-DIABLO. The cytochrome-C released from the mitochondria binds Apaf-1 and procaspase-9 to form the apoptosome, ultimately culminating in the activation of caspase-9. Also released from the mitochondrial pores is Smac/Diablo, a protein that
augments apoptosis by inhibiting a family of proteins called Inhibitors of Apoptosis (IAPs)\textsuperscript{69}.

**Execution phase**

The execution phase is the final step in apoptosis. In this phase caspases 3, 6, and 7 get activated and stimulate different enzymes such as cytoplasmic endonucleases that degrades nuclear materials and proteases that degrade different nuclear and cytoskeletal proteins\textsuperscript{61}. Among the three executioner caspases, caspase-3 is considered as the most important executioner of apoptosis. It gets activated by caspase-9, which is the initiator of the intrinsic apoptosis pathway as well as by caspase 8, which is the initiator of the extrinsic apoptotic pathway. Once caspase-3 gets activated, it frees CAD from its inhibitor (i.e. ICAD). Activated CAD then degrades chromosomal DNA within the nuclei and cause degradation of chromosomal DNA, chromatic condensation and disintegration of the cell into apoptotic bodies\textsuperscript{70}.

**The BCL-2 family proteins**

The Bcl-2 (B-cell lymphoma-2) family members consist of approximately 20 proteins that are known to regulate apoptosis. While some of these members inhibit apoptosis and hence are termed anti-apoptotic, others promote it and hence are termed pro-apoptotic proteins. This family of proteins was first considered as proto-oncogene however since they lack the ability to drive cell proliferation, they are now considered as proteins that give support to oncogene-driven transformation. Indeed, over expressing Myc oncogene along with Bcl-2 has been shown to promote the development of lymphoma as well as other types of cancers\textsuperscript{71}.
The Bcl-2 family proteins have been classified into three groups based on their ability to protect or promote apoptosis and the domain they contain in their structure (see table below). Structurally, the Bcl-2 family of proteins contain up to four Bcl-2 homologue (BH) domains that are designated as BH1, BH2, BH3 and BH4\textsuperscript{72-74}. Many of the anti-apoptotic Bcl-2 family proteins contain all four BH domains while many of the pro-apoptotic Bcl-2 family members with multi-BH domain contain the first three BH domains (BH1-BH3), the remaining pro-apoptotic proteins harbor only the BH3 domain. (see table below)

Table 1. Classification of Bcl-2 family proteins

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<thead>
<tr>
<th>Bcl-2 family proteins with multiple BH domains</th>
<th>BH3 only proteins</th>
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<tr>
<td>Anti-apoptotic</td>
<td>Pro-apoptotic</td>
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<td>Bcl-2</td>
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**Mechanism of apoptosis regulation by Bcl-2 family proteins**

Apoptosis can be triggered by an insult to a cell. Upon such stimuli, the cell can be programmed to die. Bcl-2 family proteins have been shown to promote or inhibit apoptosis. Multitude of studies have shown that the Bcl-2 family proteins regulate
apoptosis through their ability to regulate the intrinsic apoptotic pathway, also called mitochondrial apoptotic pathway, by regulating membrane permeabilization of the mitochondria\textsuperscript{75}.

The anti-apoptotic bcl-2 family proteins are found majorly bound to mitochondria, endoplasmic reticulum as well as nuclear membranes\textsuperscript{75-78}. The pro-apoptotic proteins on the other hand are found primarily in the cytoplasm or bound to the cytoskeleton\textsuperscript{61}. Following death signal, pro-apoptotic proteins such as Bax and Bak not only translocate to the mitochondria but they also undergo conformational changes that enable them to be inserted into the mitochondrial membrane\textsuperscript{79, 80}. For example, Bax has been shown to insert itself into the outer mitochondrial membrane using its c-terminal membrane anchor\textsuperscript{81}. This step forms pores on the mitochondria that result in release of apoptogenic molecules such as cytochrome c as well as SMAC eventually resulting caspase activation as described above\textsuperscript{82}. The anti-apoptotic proteins on the other hand protect permeabilization of the mitochondrial membrane by binding and antagonizing the pro-apoptotic proteins.

The BH3 only proteins also induce apoptosis through their interaction with Bcl-2 family proteins. Furthermore, studies have shown that some BH3 only proteins, such as Bim or Bid, can interact with Bax and synergize at increasing cell-free permeabilization of membrane, which suggest that some BH3 induce apoptosis not only by interacting with anti-apoptotic proteins but also by interacting with pro-apoptotic proteins.
**IAP family proteins**

IAPs are another family of protein that is known to inhibit apoptosis. IAPs were first discovered by Lois Miller and her colleagues in baculovirus \(^{83,84}\). They were shown to inhibit cell death following viral infection.

**Expression of human IAPs**

Eight human IAPs have been identified in human cells and these are NAIAP, c-IAP1, c-IAP2, XIAP, survivin, Livin, bruce and ILP\(^{85-89}\). The expression of many IAPs but that of survivin has been detected in many normal adult tissue\(^6\). Overexpression of IAP family proteins (XIAP, c-IAP1 and c-IAP2 or survivin) has been shown to inhibit apoptosis induced by a variety of stimuli that include withdrawal of growth factors, apoptosis-inducing ligand such as Fas as well as drugs such as staurosporin and etoposide\(^{86,87,90,91}\).

**Mechanism of apoptosis inhibition by IAP family proteins**

Several studies investigated the mechanism by which IAPs inhibit apoptosis. Many studies show that XIAP, c-IAP1 and c-IAP2 and survivin inhibit directly\(^{92-94}\) the activities of caspases 3, 7 and 9 but not 1,6,8, or 10. While IAPs are capable of binding to pro-caspase 9 in vitro and inhibits activation of caspase 9 by cytochrome c\(^{94}\) they were shown to bind only to active caspase 3 and 7 \(^{92,93,95}\). Co-expression of c-IAP1 and c-IAP2 or survivin with caspase 3, 7, 9 were shown to reduce the ability of caspases to induce apoptosis.

IAPs contain BIR (baculoviral IAP repeat) domains, and depending on the IAP protein, they may contain additional domains such as RING or caspase recruiting domain (CARD) IAPs contains one to three copies of BIR domain, which is a ~70 residue zinc
binding domain. The BIR domain is considered the defining structural characteristics of IAP and all members of IAPs contain at least one BIR domain\textsuperscript{96-99}. The BIR domain is essential for the anti-apoptotic function of IAP proteins\textsuperscript{92, 93, 100, 101}.

Some IAPs (XIAP, ILP-2, c-IAP1 and c-IAP2 as well as livin) also contain a RING domain, which is another zinc binding motif. Studies have shown that the RING domain of IAPs functions as a ubiquitin ligase that leads the auto-degradation as well as the degradation of other target proteins.

**Figure 4. Structure of IAPs.** IAPs contain the BIR domain, which is the defining structure for this family of proteins. Survivin, which is the smallest IAP family protein and BRUCE contain only one BIR domain, where as pIAP contain 2 BIR domains and cIAP1, cIAP2, XIAP and NIAP contain three BIR domains. cIAP1 and cIAP2 contain caspase recruiting domain (CARD) and RING domain in their structure. BRUCE also contains the ubiquitin conjugating domain.

BIRs function to inhibit apoptosis majorly by mediating protein-protein interactions. In IAPs that contain more than one BIR, the second and third BIR domains have been shown to bind caspases and regulators of apoptosis\textsuperscript{61} but BIR-1 has been shown to interact with signaling intermediates. Overexpressed XIAP BIR1 interacts with TGF-B activating kinase 1 (TAK-1) associating subunit 1(TAB1) which then activate
MAPK signaling\textsuperscript{102}. Although survivin harbors only one BIR domain, it has been shown to interact with Aurora B through its BIR domain\textsuperscript{103}. In addition, BIR1 domain has been shown to interact with other IAP, Eg. XIAP BRI1 interacts with survivin upon apoptosis stimuli\textsuperscript{61}.

### IAPs and apoptosis

All IAPs were initially believed to inhibit apoptosis. This observation came from studies that overexpression of IAPs, i.e survivin, c-IAP1 and c-IAP2 as well as XIAP inhibits caspase 3, 7 and 9. Later, however, it was shown that only XIAP was capable of inhibiting caspases 3, 7, and 9\textsuperscript{61}. XIAP was shown to bind and inhibit the activation of caspases\textsuperscript{61}. The BIR3 domain of XIAP binds to caspase 9 and the BIR-2 domain binds to caspase 3 and 7\textsuperscript{104}.

### Survivin

Survivin is a member of the IAP family of proteins with dual function in apoptosis and cell proliferation\textsuperscript{105}. What makes survivin unique is its over-expression in most tumors with very little expression in normal cells\textsuperscript{90, 106}. Indeed studies have found high levels of survivin protein in fetal tissue but almost undetectable in most adult tissue\textsuperscript{90}.

The survivin gene is located on the 17q25 chromosome in humans\textsuperscript{91}. It undergoes alternative splicing and it gives rise to five transcripts. The wild-type survivin is derived from exons 1-4 and has 142 amino acids. The other isoform of survivin, 2B, harbors an additional segment in its exon 2. The third isoform, survivin ΔEX\textsubscript{3}, has 137 amino acids because of removal of exon 3\textsuperscript{107}. Survivin 2 alpha is another transcript that contains exon 1 and exon 2 and some regions of intron-2 which resulted in acquisition of new a stop
codon and a shorter survivin that has only 74 amino acid\textsuperscript{108}. Also another transcript which is termed as survivin 3B is encoded by exons one to three and a new DNA sequence from intron 3 of survivin resulting in a protein with 113 amino acids\textsuperscript{109}. Although different studies investigated the localization and function of the different isoforms of survivin, the wild-type survivin is the most studied isoform of survivin.

\textit{Structure of survivin}

Survivin is the smallest of all human IAPs and contains only one BIR domain and an extended C-terminal alpha helix\textsuperscript{61}. X-ray crystallography of survivin show that it forms a stable homodimer in solution\textsuperscript{110, 111}.

\textit{Expression of survivin in cancer and regulation of its expression}

Survivin is differentially expressed in cancer\textsuperscript{112}, and except for few normal cells, i.e thymocytes, CD34\textsuperscript{+} stem cells as well as basal colonic epithelial cells, it is undetectable in most terminally differentiated adult tissue\textsuperscript{61}. This differential expression has also been confirmed by the result from a genome wide search that show survivin as the fourth most expressed transcriptom in cancer\textsuperscript{106}. High expression of survivin in cancer has been reported in tumors of colon, breast, lung, pancreas, esophagus, stomach, liver, ovary, melanoma, neuroblastoma and other types of cancer\textsuperscript{61}.

How the expression of survivin is upregulated in cancer is not clearly understood but some studies shed light about the molecular mechanism. For example, while survivin exon1 is highly methylated in normal ovarian epithelium, in most ovarian cancer, it is found unmethylated and transcriptionalaly active\textsuperscript{61}. Also gain of 17q25 in neuroblastoma\textsuperscript{61}, negative regulation of survivin\textsuperscript{61} and loss of wild-type p53 in many human tumors suggest possible mechanism by which survivin could be upregulated in cancer.
Several studies show the correlation between increased tumor survivin expression and unfavorable patient prognosis or decreased overall survival. High levels of survivin have been correlated with decreased patient survival in lung, gastric, colorectal, breast cancers and neuroblastoma. Also, increased survivin expression is associated with increased risk of recurrence and metastasis in laryngeal carcinoma as well as osteosarcoma patients. In addition to this, survivin could be a predictive marker to chemotherapy and radiotherapy resistance.

**Regulation of survivin expression**

Survivin is expressed constitutively in cancer with peak expression at the G2/M phase of the cell cycle. The survivin promoter harbors cell cycle dependent elements (CDE) and cell cycle gene homologue region (CHR) and is believed to play role in the cell cycle regulated expression of survivin.

The transcription of survivin in cancer is believed to be regulated by several transcription factors. For example, studies have shown that the signal transducer and activator of transcription (STAT-3) and E2F positively regulate survivin expression. Over expression of dominant negative STAT-3 or pharmacological inhibition of STAT-3 has been shown to reduce the transcription of survivin. Similarly, the tumor suppressers p53 and APC negatively regulate survivin expression.

Beside the transcriptional regulation of survivin, post-translational modifications of survivin have been shown to regulate the expression of survivin. Survivin has been shown to undergo phosphorylation at Thr-34, which is believed to inhibit survivin degradation by the proteasome. The only known kinase to phosphorylate Thr-34 is cyclin-dependent kinase 1 (CDK1). This kinase is active at the G2/M phase of the cell
cycle, therefore it is possible to speculate that beside the CDE/CHR role, this phosphorylation could be responsible for the higher survivin expression levels at G2/M. Indeed, co-immunoprecipitation of survivin with CDK1 from cells that were synchronized at G2/M has been demonstrated and over expression of kinase dead CDK1 resulted in lower phosphorylation of survivin at this amino acid residue.

**Role of survivin in cell cycle and apoptosis**

The role of survivin in the regulation of cell cycle and apoptosis has been under intense scrutiny. With regards to cell cycle regulation, survivin as a member of the chromosomal passenger complex (CPC) is involved in the regulation of cell division through its association with INCENP and Aurora B on the centromere\(^6\). RNAi mediated depletion of survivin leads to impaired localization of the CPC at the centromere and mitotic spindle which resulted in interference with mitotic progression. Also, other studies have shown that siRNA mediated depletion of survivin resulted in the absence of aurora B and INCENP from centromere, misaligned chromosomes and in multi-nucleated cells\(^6\). In addition, in vitro studies showed that the G2M kinase Aurora B phosphorylates survivin at Thr-117.

**Role of survivin in apoptosis**

The evidence for the ability of survivin to inhibit apoptosis comes from different studies that were done both in-vitro as well as in-vivo. Several studies have shown that survivin is able to protect cells from extrinsic and intrinsic mediators of apoptosis including withdrawal of interleukins, stimulation with Fas ligand, over expression of pro-apoptotic proteins such as p53, Bax, caspase 3 and 8\(^8\), \(^9\), \(^10\), \(^11\). Additional evidence for survivin’s role in apoptosis comes from the observation that using survivin dominant
negative mutants, ribozymes, anti-sense as well as siRNA induces spontaneous apoptosis in vivo\textsuperscript{117-124}. Also, transgenic mice that express survivin at the basal layer of the epidermis using cytokeratin-14 promoter block apoptosis induced by UV irradiation\textsuperscript{125}.

Although the above studies convincingly demonstrate the anti-apoptotic role of survivin, the exact molecular mechanism by which survivin interferes in apoptosis is not known. Different studies have shown association of survivin with initiator and effector caspases\textsuperscript{94, 126, 127} however whether survivin inhibit caspase activation is uncertain\textsuperscript{128-130}. For example, while Tamm et al found that cellular survivin inhibits caspases, chemically synthesized survivin fails to do so. However, from x-ray crystallography structural studies, it is now clear that except for XIAP, all other IAPs lack the structure features to inhibit caspases\textsuperscript{61}.

The following are literature gaps filled by the work of this thesis concerning the mechanism by which cancer cells maintain high levels of the survivin protein and the contribution of the Ras and Ral GTPases in this process.

The high levels of survivin in cancer cells and the lack of expression in most normal tissues strongly suggest that some of the genetic alterations of cancer cells result in up regulation of survivin. While activation of STAT3, NFkB, Notch and Wnt as well as inactivation of the tumor suppressors p53 and Rb all have been shown to increase survivin expression, little is known about the regulation of survivin by the GTPases Ras and Ral. In this thesis, we investigated whether the expression of mutant K-Ras is required for the maintenance of the high levels of survivin in human tumors. We also determine whether K-Ras depletion attenuates both basal and drug-induced survivin levels. We also determined the mechanism by which K-Ras depletion decreases survivin
levels by investigating the phosphorylation, ubiquitination and proteasomal degradation of survivin. Furthermore, we investigated whether depletion of RalA, RalB, Raf-1, Akt1 or Akt2 decreases survivin levels. Finally, we evaluated whether the ability of mutant K-Ras to induce anchorage-independent growth, invasion and survival is compromised by depletion of survivin.

References


Chapter 2

K-Ras requirement for survivin stability and survivin contribution to K-Ras driven malignant transformation

Note to Readers

Portion of these results have been previously published (Awet Tecleab and Said Sebti, Cell Cycle 12:3, 522–532; February 1, 2013) and are utilized with permission of the publisher.

Abstract

Mutant K-Ras and high levels of survivin contribute to oncogenesis but little is known about K-Ras requirement for survivin maintenance and survivin contribution to K-Ras-driven malignant transformation. Here we demonstrate that K-Ras depletion significantly decreases survivin levels in human cancer cells that harbor mutant but not wild K-Ras. K-Ras depletion also suppresses the ability of drugs such as paclitaxel and VP16 to induce survivin levels. The mechanism by which K-Ras depletion decreases survivin levels is through ubiquitination and proteasomal degradation of survivin, and is independent of survivin-Thr-34 phosphorylation. Furthermore, the ability of mutant K-Ras to induce anchorage-independent growth, invasion and survival is compromised by depletion of survivin. These studies suggest that mutant K-Ras contributes to the
maintenance of the high survivin levels by regulating its stability, and that the ability of mutant K-Ras to induce malignant transformation is at least in part dependent on survivin.

Keywords: Apoptosis / Cancer / K-Ras / Protein degradation / Survivin

Introduction

One of the critical requirements for normal cells to become cancerous is to acquire the ability to evade programmed cell death (apoptosis) even when challenged with unfavorable conditions such as cytotoxic agent or radiation exposure \(^1\). The Bcl-2 family as well as and the inhibitors of apoptosis (IAP) family of proteins play essential roles in the regulation of apoptosis. While the Bcl-2 family proteins monitor cell death by controlling the release of cytochrome c from mitochondria, the IAP family proteins prevent cell death by inhibiting the activation of caspases\(^2\), \(^3\). Survivin is one of the members of the IAP family known to play critical roles in promoting cell cycle progression \(^4\) and in preventing apoptosis\(^5\). During cell cycle progression, survivin mediates proper loading of the chromosomal passenger complex, chromosomal segregation, spindle formation and microtubule stabilization\(^6\), \(^7\). The role of survivin as an anti-apoptotic protein has also been investigated thoroughly, and depending on the cell type, survivin inhibits either spontaneous apoptosis or drug-induced apoptosis\(^8\). Despite its prominent role in apoptosis, the mechanism by which survivin blocks apoptosis remains elusive. Some studies suggested that survivin protects against apoptosis through direct binding to caspases\(^5\) whereas other studies demonstrated that XIAP (another IAP member) but not survivin directly binds caspases\(^9\). More recent studies demonstrated that survivin binds and cooperates with XIAP to efficiently block caspase activation\(^10\).
Unlike other IAPs, little to no survivin is expressed in normal cells. In contrast, virtually all cancer cells maintain very high levels of survivin protein\textsuperscript{11}. The fact that survivin protein levels are much higher in cancer cells as compared to normal cells indicates that some oncogene may be responsible for the maintenance of these high levels. In deed, the induction of survivin expression in cancer cells has been attributed to some mutated or deregulated oncogenes/ proto-oncogenes in cancer. For example, aberrant activation of STAT3, NFkB, Notch and Wnt as well as inactivation of the tumor suppressors p53 and Rb all have been shown to increase survivin expression by regulating its transcription\textsuperscript{12}.

Mutations in the small GTPase Ras and high levels of the survivin protein are prevalent in human tumors but the role of mutant Ras in regulating survivin protein levels has not been thoroughly studied, and the very few studies reported, investigated mainly the role of ectopically expressed H-Ras but not K-Ras, the most frequently mutated isoform of Ras in human cancer. Previous studies showed that, ectopic over expression of c-H-Ras in Rat cells as well as in human keratinocytes induces survivin expression\textsuperscript{13, 14}. Furthermore, Fukuad et al. showed that exogenous H-Ras is required for interleukin induced survivin in Baf-3 cells\textsuperscript{15}. However, other studies showed that mutant H-Ras is not able to increase survivin\textsuperscript{16}. Furthermore, the requirements of endogenous Ras for the maintenance of the high levels of survivin in cancer cells have not been investigated. Finally, several studies have documented both in vitro and in vivo the importance of survivin to malignant transformation\textsuperscript{17, 18} but little is known about the contributions of survivin to mutant K-Ras-driven transformation.
In this thesis, we demonstrated that depletion of K-Ras decreases survivin levels in human cancer cells that harbor mutant K-Ras but not wild type Ras, and that this decrease in the survivin protein levels is due to proteasome degradation, suggesting that mutant K-Ras regulates survivin stability. Furthermore, we also demonstrated that depletion of survivin significantly reduces mutant K-Ras-driven anchorage-independent growth and invasion suggesting that survivin contributes to K-Ras driven malignant transformation.

**Materials and Methods**

**Cell culture**

The human tumor cell lines were grown in their respective media: A549 in Kaighn's modification medium (F12K) (Gibco Laboratories, Grand Island, NY), Calu-1 and SKOV-3 in McCoy’s 5a medium modified (Sigma-Aldrich, St. Louis, MO), H460, AsPC-1, Ovcar-5 and BxPC-3 in RPMI, (Invitrogen, Carlsbad, CA), MiaPaCa-2, Panc-1, SW1990, and HOP-92 in Dulbecco’s modified minima essential medium (DMEM) (Invitrogen). All cells were grown in their respective media containing 10% FBS and 1% penicillin streptomycin at 37°C in a humidified incubator at 5% CO2. Human Pancreatic Nestin Expressing (HPNE) cells were the kind gift of Dr Channing Der and Dr Paul Campbell (University of North Carolina) and were maintained in media containing 3:1 mixture of DMEM and M3F base media (INCELL, San Antonio, TX). NIH-3T3 cells that stably express mutant K-Ras and C7 cells that stably express mutant K-Ras were grown as described by us 19,20 respectively.
**siRNAs and antibodies**

Non-targeting (NT) and K-Ras siRNA were purchased from Dharmacon, Lafayette, CO and Ambion, Grand Island, NY, respectively. Survivin antibodies were purchased from Novus biologicals, Littleton, CO (used for immunoprecipitation), and Abcam, Cambridge, MA (used for western blotting). Bax, MCL-1, Bad and ubiquitin antibodies were purchased from SantaCruz Biotechnology Santa Cruz, CA. Cleaved PARP antibody was purchased from Cell Signaling, (Danvers, MA). β-actin, vinculin, α-tubulin and GAPDH antibodies were purchased from Sigma. K-Ras (OP-24), Bcl-2 and Bcl-X\_L antibodies were purchased from Calbiochem, Billerica, MA. XIAP antibody was purchased from BD Biosciences, San Jose, CA. Peroxidase conjugated Goat anti-mouse IgG, Rabbit anti-Goat IgG, Mouse anti-Rabbit IgG antibodies were purchased from Jackson immunoResearch Laboratories, West Grove, PA. TrueBlot mouse secondary was purchased from e-Bioscience San Diego, CA.

**DNA constructs and site directed mutagenesis**

Survivin T34 mutants were generated using QuickChange II XL site directed mutagenesis kit (Agilent Biotechnologies, Santa Clara CA). Survivin ORF plasmid was purchased from Origene and used as a template to do site directed mutagenesis according to the manufacturer’s recommendation. Briefly, primers were designed using an online primer designing tool from Agilient Biotechnology, www.agilent.com/genomics.

The following primers were purchased from Integrated DNA Technology (IDT).

Survivin T34D:  
Sense primer: 5’-GGGCTGCGCCTGCGACCCGGAGCGGATG-3’;  
Antisense primer: 5’-CATCCGCTCCGGTGTCGGCGAGCGGATG-3’.

Survivin
T34A: Sense primer: 5’-GCTGCGCCTGCGCCCCGGAGCGG-3’; Antisense primer: 5’-CCGCTCCGCGGCAGCGAGCGA-3’.

PCR conditions: The following components were added into a PCR tube-reaction buffer to 1X, 200ng DNA, 125ng of each sense and antisense primers, 1ul of dNTP mix, 3ul of quicksolution and distilled water to a final volume of 50ul and DNA polymerase (all reagents from Agilent Biotechnologies). PCR cycle conditions: 95°C-2min, 95°C-1min, 60°C-50 sec, 68°C 14min, 68°C 7min, repeat 18 times then end. Following temperature cycling, the reaction tubes were placed on ice for 2min, followed by the addition of 2 μL DPN1 restriction enzyme to each sample. This reaction was allowed to go for at least 4 hr at 37°C. After generation of the plasmid, bacteria (XL10-Gold Ultra competent Cells) were transformed per the manufacturer’s instructions. After overnight incubation, 10 colonies were picked for each sample. DNA was purified to determine mutation status.

Cloning of myc-tagged CA-K-Ras12V

We used our previously described K-Ras-Q12V pBABE expression construct as template to clone into myc-tagged pCMV-Tag3B vector. The following primers were used to shuttle the construct:

Forward primer: 5’-CGC GGA TCC AAG CTT ATG ACT GAA TAT AA CTT GTG GTA-3’,
Reverse primer: 5’-TGC TCT AGA CTC GAG TTA CAT AAT TAC ACA CTT TGT CTT-3’
**siRNA transfection**

Cells were transfected with siRNA using Lipofectamin RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were plated overnight to reach ~40-50% confluence at the time of transfection. Lipofectamine RNAiMAX and Opti-UM were mixed in one tube and a mixture of siRNA and Opti-UM was prepared in another tube, and the content of the two tubes were mixed. The mixture was incubated for 20 min at room temperature, after which the siRNA-RNAiMax mixture was added to cells drop-wise. After 24 hrs of transfection, appropriate media that contain 10% serum only was added until the desired time of transfection.

**DNA transfection and generation of stable cell lines**

Cells were plated overnight to reach 70-80% confluence and were then transfected with DNA using Lipofectamine 2,000 transfection reagent according to the manufacturer’s instructions. Briefly, Lipofectamine 2,000 reagent (1.5μl/μg of DNA) was diluted in OPTI-MEM medium (Invitrogen) and allowed to equilibrate for 5 min at room temperature. 2μg of DNA (vector or Myc-DDK-survivin construct), (Origene, Rockville, MD) was diluted in OPTI-MEM in a separate tube. The contents of the two tubes were mixed and allowed to complex for 20 min at room temperature, and added drop wise to cells that contain 10% FBS but no 1% PS. The cells were transfected for 6 hrs before changing the medium to medium containing 10% FBS only and incubated for an additional 18 hrs. The medium was then replaced with complete growth medium containing 1.2mg/mL of G418 (Fisher Scientific, Pittsburgh, PA). The cells were kept in selection medium for 2 weeks after which the resistant cells were pooled and grown for further experiments.
**Western blotting**

Cells were rinsed with ice cold PBS twice and lysed on the plate in lysis buffer containing 20mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1ug/mL leupeptin, and 1 mM PMSF. The lysate was collected by scraping and centrifuging at 13000g for 13 min to remove the debris. The protein content was measured using Bradford protein assay. Samples were resolved using 12.5% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5% milk in Tris-Buffered Saline and Tween 20 (TBST) and then probed with the desired antibodies. The enhanced chemiluminescence blotting system (PerkinElmer Inc, Waltham, MA) was used for antibody reaction.

**Immunoprecipitation**

Cells were rinsed with ice cold PBS twice and lysed on the plate in NP-40 lysis buffer containing 20 mM Tris pH 7.5, 0.05% Igepal, 250mM NaCl, 3mM EGTA, 3mM EDTA, 2mM NaVO4, 2mM PMSF and 7.4mg/ml PNPP, 0.5uM bortezomib, Ub-aldehyde 4μM, 20mM n-ethylmaleimide. After collecting the lysate, the lysate was sonicated (4 pulses), using Branson Sonifier, VWR Scientific, at output level 3, duty cycle (%) 30. After clearing the debris by centrifuging at 13,000g for 13 minutes, the protein amount was measured. 600-1000μg of protein was incubated with 30μl of 50/50 mix of protein A/ protein G agarose beads (Millipore) and 2μg/ml of survivin antibody (Novus) at 4°C overnight on an orbital shaker. After overnight incubation, the sample was centrifuged for 5 second to collect the beads. The supernatant was discarded and the beads were washed two times with 500μl lysis buffer. The agarose beads were re-
suspended in 30ul of sample buffer. The agarose beads were boiled for 5 minutes to dissociate the immunocomplex from the beads. The beads were collected by centrifuging and the proteins in the supernatant were run on a 12.5% SDS-PAGE.

**In-vivo ubiquitination assay**

Panc-1 cells were first transfected with siRNA for 48 hr. The cells were then treated with 0.5 uM of bortezomib (Velcade) for 30 min. The cells were then lysed with NP-40 lysis buffer. Survivin was immunoprecipitated as described above and ubiquitinated survivin was detected using ubiquitin antibody from SantaCruz Biotechnology and TrueBlot mouse secondary (eBioscience).

**Caspase activation assay**

Caspase-3 activity was measured in cell lysates using a fluorogenic substrate. The assay measures the cleavage of AMC groups from a Caspase-3 specific substrate (Ac-DEVD-AMC) (Biomol Research Labs, Inc., Plymouth Meeting, PA). Liberated AMC groups were quantified on fluorometer at excitation max.: 365–380 nm and emission max.: 430–460 nm. Briefly, cells were lysed in a protease inhibitor free lysis buffer (50 mM Tris, pH 8, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40). The lysate was collected by scraping. After clearing the lysate by centrifugation, protein was measured. 20 μg of protein and 20 μM caspase-3 substrate were added into 50 mM Tris, pH 7.5 reaction buffer in triplicate in a 96-well plate. The reactions were incubated for 1 h at 37°C and the plate was read by a fluorometer, WALLAC Victor 1420 Multilabel counter with 355 nm excitation and 460 nm emission filters (Perkin Elmer Life Sciences, Turku, Finland).

Assay for the chymotrypsin-like activity (CT-L) of the proteasome
The (CT-L of the proteasome was measured using fluorogenic peptide substrate as described elsewhere. Briefly, 20μg of cell lysate protein was incubated with 20μM of Suc-Leu-Leu-Val-AMC substrate for CT-L activity in 100μL of assay buffer (50mM Tris-HCl, pH 7.6) for 1hr at 37°C. After incubation, production of 7-hydrolyzed 7-amido-4-methyl-coumarin (AMC) groups were measured using WALLAC Victor 1420 Multilabel counter with 355nm excitation and 460nm emission filters (Perkin Elmer Life Sciences, Turku, Finland).

**Soft agar colony formation assay**

After 48 hrs of siRNA treatment, the cells were trypsinized, counted and seeded at a cell density of 2,000/well in triplicate in 12-well culture plates in 0.3% agar over a 0.6% bottom agar layer as previously described. Cultures were incubated until colonies formed (approximately 2-3 weeks). Plates were scanned after overnight incubation with 1 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Calbiochem), in cell growth medium. The colony numbers were visually determined and quantified.

**Cell invasion assay**

Analysis of cell invasion was performed using artificial extracellular matrix (Matrigel, 8μM pore size, BD Biosciences, Bedford, MA) according to the manufacturer’s instructions. Briefly, the inserts were rehydrated by adding warm serum-free medium for 2 hrs at 37°C, 5% CO₂ atmosphere. The medium was then removed from the inserts, and 750ul of medium containing FBS was added to each well of the plate and the inserts were placed on top of the medium. The transfected A549, Panc-1, and H460 (40,000 cells in 500ul of serum free-media) and HPNE cells (20,000) were added into the
inserts. The cells were incubated for 48 hrs at 37°C. Cell invasion was determined by staining the bottom parts of the insert with crystal violet (0.5% crystal violet in 25% methanol). The porous membrane was removed and placed on the slide. The number of cells that invaded were counted in three high-power microscope (40X) fields and averaged as the mean number of invaded cells per field for each membrane.

**Cell lines**

The human cancer cell lines Calu-1, Panc-1, Hop-92 and Tov-21G, A549, SKOV-3, ASPC-1, BxPC-3, and SW1990, H460 and Ovcar-5 were all obtained from ATCC but none of the cell lines have been authenticated.

**Results**

*Depletion of K-Ras decreases survivin levels in human cancer cells that harbor mutant, but not wild type K-Ras.*

Both mutant K-Ras and high levels of survivin contribute to malignant transformation and are associated with poor cancer patient prognosis. However, whether mutant K-Ras is required for the maintenance of high survivin levels is not known. To address this important question we first depleted K-Ras from several human cancer cell lines that either harbor mutant K-Ras or wild type Ras, and determined the effect of this depletion on the levels of survivin. Human cancer cell lines from various origins including pancreatic (Panc-1, MiaPaca-2, SW1990, ASPC-1, BXPC-3), lung (A-549, Calu-1, H460, HOP-92) and Ovarian (OVCAR5, SKOV-3), were transfected with non-targeting (NT) or K-Ras siRNA and processed for western blotting as described under Materials and Methods. Figure 5A show that siRNA to K-Ras depleted K-Ras and that this resulted in a significant decrease in the protein levels of survivin in cell lines that
harbor mutant K-Ras (Panc-1, Calu-1, H460, AsPC-1, MiaPaca-2, SW1990, A-549 and OVCAR5) but not in those cancer cell lines that express only wild type Ras (BXPC3, SKOV-3 and HOP-92).
Next we used another K-Ras siRNA that target a different region of K-Ras mRNA to confirm the results. Figures 5A and 5B show that depletion of K-Ras by two distinct K-Ras siRNAs reduces survivin levels. Whether depleting K-Ras also reduces the levels of ectopically expressed survivin was next investigated. To this end, cells were stably transfected with Myc-DDK-tagged survivin and survivin protein levels were determined following siRNA to NT or K-Ras transfection as described under Materials and Methods. Figure 5C shows that depletion of K-Ras reduces the levels of endogenous as well as exogenous survivin. The fact that depletion of K-Ras reduces the levels of exogenous survivin, the expression of which was driven by a foreign promoter, suggests that K-Ras may affect survivin levels by regulating its stability (see more below).

**Depletion of K-Ras reduces the ability of VP16 to increase survivin levels.**

Previous studies have shown that treatment with chemotherapeutic drugs such as paclitaxel and VP16 results in survivin induction, and that this is an intrinsic mechanism by which tumor cells develop resistance to chemotherapy. Since we found depletion of K-Ras to decrease basal survivin protein levels, we reasoned that depletion of K-Ras may also prevent induction of survivin by anticancer drugs in cancer cells that harbor mutant K-Ras. Therefore, we next investigated whether the VP16- and paclitaxel mediated increase in survivin levels requires mutant K-Ras. To this end, Panc-1 cells were transfected with siRNA to K-Ras or NT siRNA and treated with vehicle, VP16 or paclitaxel and processed for western blotting as described under Materials and Methods. Figures 5D and 5E show that in the absence of siRNA to K-Ras VP16 and paclitaxel treatment of Panc-1 cells resulted in a robust increase in survivin levels. In contrast, in Panc-1 cells where K-Ras was depleted, the ability of VP16 and paclitaxel to induce
survivin was significantly compromised (Figures 5D and 5E). These results suggest that K-Ras expression is required for the ability of VP16 and paclitaxel to increase survivin protein levels, further confirming the role of mutant K-Ras in maintaining high levels of survivin.

**Ectopic expression of mutant K-Ras is not sufficient to induce survivin**

Figure 5 demonstrated that depletion of K-Ras resulted in decreased levels of endogenous and exogenous survivin as well as drug-induced survivin. Next we determined if ectopic expression of mutant, constitutive active K-Ras (CA-K-Ras), increases survivin levels.

![Figure 6. Ectopic expression of CA-K-Ras is not sufficient to induce survivin.](image)

Human embryonic kidney HEK293 cells that ectopically express CA-K-Ras and their parental counterpart that were transfected with an empty vector construct were used. Figure 6A shows that forced expression of CA-K-Ras did not affect the protein levels of survivin. Similar results were also obtained with NIH-3T3 cells as well as two primary pancreatic ductal cell lines C7 and HPNE that stably express either CA-K-Ras or empty
vector (Figures 6B, 6C and 6D), suggesting that K-Ras is not sufficient to drive the expression of survivin. Taken together, the results from Figures 5 and 6 suggest that mutant K-Ras is required but not sufficient for the maintenance of the high survivin levels in human cancer cells.

**Depletion of K-Ras decreases the stability of survivin by promoting its ubiquitination and proteasome degradation.**

Previous studies have shown that survivin protein undergoes proteasomal degradation. To determine if depletion of K-Ras leads to decreased survivin protein levels by inducing its proteasomal degradation, we used the proteasome inhibitor bortezomib. First, we demonstrated that treatment of Panc-1 cells with bortezomib resulted in the accumulation of survivin in a time-dependent manner starting as early as 1 hr and reaching a maximum at 4 hrs (Figure 7A). Next, whether bortezomib can rescue from the K-Ras depletion-mediated decrease of survivin levels was investigated. To this end, Panc-1 cells that stably express Myc-DDKtagged survivin were transfected with either NT or K-Ras siRNA for 48 hrs prior to incubation with bortezomib for 1 hr and processed the cells for western blotting as described under Materials and Methods. Figure 7B shows that vector cells express only endogenous survivin whereas Myc-survivin transfected cells express both endogenous and exogenous survivin (compare lanes 1 and 2). In the absence of bortezomib, depleting K-Ras resulted in a significant decrease of both endogenous and exogenous survivin levels (compare lanes 3 and 5). In contrast, treatment of cells with bortezomib inhibited the ability of K-Ras siRNA to decrease the levels of both endogenous and ectopically expressed survivin (compare lanes 4 and 6 to lanes 3 and 5). Figure 7B also shows that bortezomib inhibited the CT-L
activity of the proteasome by 83% and 88% in Panc-1 cells transfected with NT siRNA and K-Ras siRNA, respectively.

We next determined whether K-Ras siRNA induces the degradation of survivin by increasing its ubiquitination. To this end, survivin was immunoprecipitated from cells that were transfected with siRNA to NT or K-Ras for 48 hrs followed by bortezomib treatment as described under Materials and Methods. Figure 7C shows more ubiquitinated survivin in the siRNA to K-Ras transfected cells. Thus, the results from Figures 7B and 7C suggest that K-Ras depletion leads to ubiquitination and proteasome-mediated degradation of survivin.

Phosphorylation of survivin at T34 has been shown to increase the stability of survivin\textsuperscript{22, 23}. One possible mechanism by which depletion of K-Ras could decrease the stability of survivin is by inhibiting the phosphorylation of survivin at T34. We therefore reasoned that the phospho mimic T34D survivin mutant should be resistant to K-Ras depletion. To this end, we generated the T34D survivin mutant as well as the non-phosphorylatable T34A mutant. Panc-1 cells that stably express either wild type (WT), T34D mutant or T34A mutant were transfected with siRNA to NT or K-Ras for 48 hrs and the expression of survivin was followed by western blotting as described under Materials and Methods.

Figure 7D shows that depletion of K-Ras reduces the expression of WT survivin as well as T34A and T34D survivin mutants suggesting that the ability of K-Ras siRNA to decrease survivin protein levels is not dependent on the phosphorylation status of survivin at T34.
Figure 7. Depletion of K-Ras decreases survivin protein levels in a proteasome dependent manner and a survivin-T34 phosphorylation independent manner

A. Panc-1 cells were treated with the indicated dose of bortezomib for 1 hr. The media was then replaced with drug-free media and the cells were allowed to grow for the indicated length of time and processed for western blotting as described in Materials and Methods. B. Panc-1 cells stably expressing Myc-DDK survivin were treated with siRNA to NT or K-Ras, followed by vehicle or bortezomib treatment. Cells were then harvested for western blot analysis or CT-L proteasome activity assay as described in the Materials and Methods. C. A549 cells were transfected with NT or K-Ras (K) siRNA for 48 hrs followed by bortezomib treatment for 30 min. Survivin was immunoprecipitated and processed for western blot analysis for either ubiquitin or survivin. D. Panc-1 cells that stably express vector, wt, T34A mutant or T34D mutant survivin were transfected with NT or K-Ras siRNA and the cells processed for western blotting as described in Materials and Methods. Data are representative of at least two independent experiments.
Depletion of K-Ras decreases selectively survivin over the anti-apoptotic proteins Bcl-2, Bcl-XL and Mcl-1, and does not increase the levels of the pro-apoptotic proteins Bax and Bad.

The ability of cancer cells to survive depends not only on IAP family members but also on a balance between anti-apoptotic and pro-apoptotic Bcl-2 family members. We therefore determined whether K-Ras depletion selectively affect survivin over other proteins that regulate cancer cell survival. To this end, in addition to survivin, we evaluated the effects of K-Ras depletion on anti-apoptotic (Bcl-2, Bcl-XL and Mcl-1) and pro-apoptotic (Bad and Bax) proteins. Figure 8 (in the following page) shows that siRNA to K-Ras decreased the levels of survivin but doesn’t affect the levels of the Bcl-2 family proteins in Panc-1 and MiaPaca-2 cells.

Survivin is required for mutant K-Ras-driven malignant transformation

Both survivin and mutant K-Ras contribute to malignant transformation, but whether survivin is required for K-Ras-driven malignant transformation is not known. In an attempt to address this question, we next determined if depletion of survivin induces apoptosis in HNPE cells where malignant transformation was induced by mutant K-Ras. Figure 9 shows that depletion of survivin induces apoptosis in HPNE-K-Ras cells as measured by caspase 3 activation and PARP cleavage, suggesting that survivin is a critical protein that mediates at least in part the anti-apoptotic function of K-Ras. To further confirm this result, we used different cell lines that harbor mutant K-Ras and determined if depletion of survivin induces apoptosis. Our result shows that depletion of survivin induces caspase 3 activation and PARP cleavage in A549 and H460 but not in panc-1 cells.
In addition to the well studied function of survivin in apoptosis, recent study have also demonstrated that survivin contributes to invasion and metastasis and this role is independent of its anti-apoptotic activity. The ability of cancer cells to grow in an anchorage-independent manner is a prerequisite to metastasis. We therefore determined if mutant K-Ras-induced colony formation in soft agar of HPNE pancreatic cells depends on survivin. Figure 10 shows that HPNE cells transfected with vector grew 62 colonies in soft agar. Transfection with mutant K-Ras enhanced the ability of HPNE cells to grow soft agar colonies by 6.6-fold from 62 (vector cells) to 412 (K-Ras cells) colonies. Figure

\[ \text{Figure 8. Depletion of K-Ras reduces survivin levels but does not affect the levels of Bcl-2 family proteins. } \]

Panc-1 and MiaPaCa-2 cells were transfected with siRNA to K-Ras or NT for 48 hrs and the expression of different members of BCL-2 family proteins as well as expression of survivin and XIAP were detected by western blot analysis. Data are representative of two (MiaPaCa-2) or three (Panc-1) independent experiments.
shows that depletion of survivin potently inhibited this K-Ras-dependent increase in colony formation in soft agar. This suggests that the ability of K-Ras to promote anchorage-independent growth partly depends on survivin. Furthermore, the ability of H-460 and Panc-1 cells, both of which harbor mutant K-Ras, to grow soft agar colonies was also inhibited by depletion of survivin.

Figure 9. Depletion of survivin induce apoptosis in some cells. HPNE-K-Ras, A549, Panc-1 and H460 cells were transfected with either control (NT) or survivin siRNA for 96 hrs. Top panel: western blot analysis showing knockdown of survivin and cleaved PARP and, bottom panel: caspase-3 activity following depletion of survivin. Experiment was done in triplicates and error bars show SD deviation between the triplicate wells. P value was calculated using the 3 values from the triplicates and SD. Data are representative of two independent experiments.
While anchorage-independent growth is a pre-requisite for metastasis, another close hallmark of metastasis is invasion. We therefore determined if mutant K-Ras-driven invasion requires survivin. Figure 11 shows that mutant K-Ras increased the ability of HPNE cells to invade by ~3.7-fold from 18 in vector cells to 67 in K-Ras transformed cells. Figure 11 also shows that depletion of survivin inhibited the ability of K-Ras to induce invasion. Similarly, in A-549, H-460 and Panc-1 cells, all of which harbor mutant K-Ras, depletion of survivin significantly compromised their ability to invade (Figure 11).
Discussion

The contributions of mutant K-Ras to several hallmarks of cancer including deregulation of anchorage-dependent and –independent cell growth, invasion, metastasis and apoptosis evasion have been well documented. In contrast, until recently, survivin has only been well documented in preventing apoptosis and promoting mitosis. Recent studies have demonstrated, as is the case for K-Ras, that survivin is also involved in other
hallmarks of cancer such as invasion and metastasis. However, little is known about the regulation of survivin by mutant K-Ras and the contribution of survivin to mutant K-Ras-mediated malignant transformation.

In this manuscript, we used siRNA to knock down K-Ras expression in several human cancer cell lines and demonstrated that depletion of K-Ras reduces survivin protein levels in cancer cells that harbor mutant K-Ras but not wild type Ras. In addition to decreasing the basal levels of survivin, depletion of K-Ras also inhibited the ability of paclitaxel and VP-16 to induce survivin, further confirming the importance of K-Ras in the maintenance of survivin. Furthermore, the K-Ras depletion-mediated reduction in survivin levels was demonstrated in cancer cell lines from different tissue of origin. Therefore, our results suggest that the requirement of K-Ras expression for the maintenance of high levels of survivin in human cancer cells depends on the mutation status of K-Ras but is not tissue specific.

Although our studies indicated that mutant K-Ras is required for the maintenance of survivin, they also clearly demonstrated that mutant constitutively active (CA) K-Ras, on its own, is not sufficient to induce survivin in several cell models including NIH-3T3 murine fibroblasts and human kidney embryonic HEK293 cells as well as 2 human pancreatic cell lines C7 and HPNE. Whether ectopic expression of CA-H-Ras can increase survivin levels is not clear with some studies showing that it can and other that it can not. Consistent with our results, expression of CA-K-Ras in mouse kidneys in the Wilms’ tumour mouse model was not able to induce survivin. Interestingly, in this model, expression of both CA-K-Ras and β-catenine was able to induce survivin. This study, coupled with our K-Ras depletion results, suggests that while several genetic
aberrations are required for increasing the levels of survivin, depletion of only K-Ras is able to decrease survivin. Therefore, K-Ras is necessary for the maintenance of survivin but not sufficient to increase survivin levels.

In addition to depleting the endogenous levels of survivin, our studies also demonstrated that depletion of K-Ras was able to decrease the levels of exogenous survivin whose ectopic expression was under the control of a foreign promoter. This suggested that depletion of K-Ras may affect the stability of the survivin protein, and this was further confirmed by the demonstration that depletion of K-Ras increased survivin ubiquitination, and that treatment with the proteasome inhibitor Bortezomib compromised the ability of K-Ras siRNA to decrease the levels of the survivin protein. Therefore, our studies indicate that depletion of K-Ras leads to decreased survivin levels through a proteasome degradation mechanism. Survivin has a fairly short half-life of 30 min \(26\), and its stability has been suggested to depend on phosphorylation of survivin at T34 by CDK1 \(22, 23\). We have made the phosphomimic T34D as well as the non-phosphorylatable T34A survivin mutants and have demonstrated that depletion of K-Ras was as effective at decreasing the levels of these mutants as it was at decreasing the levels of wild type survivin, suggesting that the ability of K-Ras depletion to promote the proteasome degradation of survivin is independent of T34 phosphorylation. While these results excludes the phosphorylation of survivin at T34 as the sole determinant of K-Ras regulated survivin stability, it doesn’t rule out possible involvement of this phosphorylation site in combination with other plausible K-Ras regulated mechanisms that are involved in the stability of survivin.
Depletion of K-Ras induces apoptosis in many human cancer cell lines that depend on mutant K-Ras for survival\textsuperscript{27, 28}. However, whether this depends on the K-Ras depletion-mediated decrease in survivin levels is not known. Using isogenic HCT-116 colon cell lines, Sarthy et al\textsuperscript{29} have found that survivin depletion induces apoptosis preferentially in cancer cells that harbor mutant K-Ras. However, several studies using RNAi screens have not all identified that depletion of survivin induces cell death in cancer cells with K-Ras mutation\textsuperscript{27, 28}. Consistent with this, our results also show that mutation of K-Ras alone may not predict sensitivity to survivin depletion. For example, depleting survivin in H460 and A549 but not in Panc-1 and MiaPaca 2 cells induces apoptosis, yet all 4 cell lines harbor mutant K-Ras. Depletion of K-Ras may induce apoptosis through other mechanisms, and this may include the decrease in the levels of another IAP family member, XIAP, that we have observed in both Panc-1 and MiaPaca 2 cells following K-Ras depletion. However, apoptosis following depletion of K-Ras in these 2 cell lines does not involve anti-apoptotic (Bcl-xL, Bcl-2 and Mcl-1) or pro-apoptotic (Bax and Bad) Bcl2 family members (Figure 8). Depletion of K-Ras may cause global reduction in the levels of IAP family of proteins that may induce apoptosis. Future studies that use a large number of cell lines that harbor mutant and wild type K-Ras are warranted to identify the effect of survivin depletion in the context of K-Ras mutation.

Survivin has been associated with malignant transformation as its expression increases at early stages of tumor development and its targeted expression in skin predispose mice to UV induced tumors\textsuperscript{30}. Our studies show that, mutant K-Ras also depends on survivin for its ability to transform cells as well as to induce invasion. Using a genetically-defined pancreatic cancer cell model, we have shown that the ability of
mutant K-Ras to induce anchorage-independent cell growth on soft agar was inhibited by depletion of survivin. Depletion of survivin also inhibited the anchorage-independent growth of human cancer cells that harbor mutant K-Ras such as Panc-1 and H-460 cells. Furthermore, the ability of mutant K-Ras to induce invasion in the genetically-defined HPNE pancreatic cell model was also inhibited by depletion of survivin. The ability of A-549, H-460 and Panc-1 cancer cells, all of which harbor mutant K-Ras, to invade was also inhibited when survivin expression was knocked down. These results suggest that survivin may be critical to the ability of mutant K-Ras to induce metastasis. This is consistent with recent studies that implicated survivin in invasion and metastasis\textsuperscript{24}. Furthermore, the demonstration that depletion of survivin induce apoptosis in H460 and A549 but not in panc-1 cells, and that its depletion antagonizes soft agar growth and invasion in all three cell lines, suggests that the ability of survivin to regulate invasion or soft agar growth is independent of its ability to regulate apoptosis.

In summary, this study identified an important biochemical link between the protein product of one of the most frequently mutated gene in human cancer, K-Ras, and the stability of a protein that is almost exclusively expressed in cancer, survivin; and that this mutant K-Ras-survivin biochemical link contributes to K-Ras-driven malignant transformation.

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Chapter 3

Requirement of Ral GTPases for survivin expression is mutation- but not tissue-specific

Abstract

Oncogenic K-Ras is known to induce malignant transformation by regulating several proteins that are important for abnormal proliferation and survival. Although previous studies have demonstrated the importance of survivin in Ras-mediated malignant transformation, the downstream effectors critical for this regulation are less studied. Here we show that, the small GTPase Ral, but not Akt or c-Raf, is critical for survivin expression in cancer cells that harbor mutant K-Ras. Furthermore, we show that Ral involvement in the regulation of survivin is mutation- but not -tissue specific. Indeed, Ral regulation of survivin requires mutant K-Ras and wild type p53, and the ability of Ral to regulate survivin depends on p53 that was induced following depletion of Ral. In summary we have identified Ral as a novel regulator of survivin and that this regulation is mutation- but not tissue-specific.

Keywords: K-Ras, Ral, survivin, p53, cancer,

Introduction

Ras is a member of the Ras super family of small GTPases that plays a critical role in signal transduction\(^1\). There are three Ras genes that encode four 21KDa Ras proteins, H-Ras, N-Ras, K\(_A\)-Ras and K\(_B\)-Ras. These signal transducers act as switches
that are inactive when bound to GDP and active when bound to GTP. For example, in normal cells when growth factors such as EGF bind their receptor, the receptors recruit complexes that contain guanine nucleotide exchange factors (GEF) such as mSOS-1 which converts Ras-GDP (inactive) to Ras-GTP (active)\(^2\). In its GTP-bound active form Ras can bind over 20 known effector proteins and trigger a multitude of signal transduction pathways that are intimately involved in the regulation of cell division, proliferation, differentiation and survival\(^3\).

Ras is mutated in about 30% of all human cancers. Mutation in amino acids 12, 13 or 61\(^4\) are frequently observed and this results in a constitutively active (GTP-locked) Ras that remains persistently activate and hence activates several down stream effectors (see above).

This aberrant activation of Ras mediates many of the hallmarks of cancer including uncontrolled cell division, sustained angiogenesis, metastasis and evasion of apoptosis. The mechanisms by which mutant Ras contributes to these cancer hallmarks has been extensively studied but much remains to be investigated.

Although Ras is known to engage over 20 effector proteins to regulate different malignant phenotypes, the Raf/MAPK, Pi3K/Akt, RalGDS/Ral pathways have been extensively studied: Ras binds and activates the kinase Raf which in turn activates Mek to activate Erk\(^5\). This so-called MAP kinase cascade has been implicated in uncontrolled proliferation of cancer cells. Ras also binds and activates phosphatidylinositol kinase (Pi3K) which activates protein kinase B (Aka, Akt) which in turn triggers many pathways involved in tumor survival, angiogenesis and metastasis\(^6\). Ras also bind the Ral GEF, RalGDS, which in turn activates the small GTPase, RalA and RalB\(^7\). Although early on the
Raf/Mek/Erk and Pi3K/Akt pathways were implicated, more recently the Ral GDS/RalA/B pathway has been shown to be just as important, and in some cases the only mediator, of Ras driven malignant transformation. 

One of the major means by which mutant Ras causes malignant transformation of human cancer is by inhibiting apoptosis and promoting tumor survival. The mechanism by which Ras inhibits apoptosis and hence maintains tumor survival is not well understood. Several mediators have been proposed such as Bcl2, cyclin D1, NFκb and others. More recently, the small IAP survivin has been shown to be induced by mutant Ras and has been suggested as a mediator of Ras anti-apoptotic activity. Moreover, recently, we have found that depletion of K-Ras reduce survivin expression selectively in cells that harbor mutant K-Ras. However the mechanism by which mutant Ras increases survivin levels is less known.

H-Ras has been shown to increase survivin levels in a dexamethasone-inducible mutant CA-H-Ras system. In this rat cell system, survivin expression was modulated by the level of H-Ras expression. The induction of survivin expression by H-Ras was cell cycle- and proliferation-independent. Furthermore, in Baf-3 cells, CA-H-Ras increased survivin levels in the absence of IL-3, whereas DN Ras suppressed the IL-3-stimulated survivin increase. Similarly, in Hela cells CA-H-Ras induced sustained survivin expression during all phases of the cell cycle, and protected against drug-induced apoptosis.

While the studies above suggested that H-Ras increased the levels of survivin and that this increase contributes to the ability of Ras to protect cancer cells from apoptosis, little is known about the mechanism by which Ras increases survivin levels. In
particular, the signal transduction pathways downstream of Ras that mediate the up regulation of survivin are ill-understood. The few studies that attempted to address this important question used only pharmacological inhibitors of Ras downstream effectors (see Figure 12).

![Figure 12. Inhibitors of Ras effector pathways and survivin expression.](image)

The PI3K inhibitor (LY294002) and the MEK inhibitor (U0126) were shown to reduce H-Ras driven survivin expression. The geranylgeranyl transferase inhibitor (GGTI-2417) was shown to reduce survivin in a mutant K-Ras harboring cells.

For example, one study showed that inhibitors of Mek, PI3K and mTOR but not Akt inhibited the ability of CA-H-Ras to increase the levels of survivin in BaF-3 cells\textsuperscript{11}. Similarly, in rat cells Mek and PI3K inhibitors also inhibited CA-H-Ras-mediated survivin increase and apoptosis\textsuperscript{10}. However, in one study PI3K inhibitors were unable to inhibit survivin levels. Finally, we have shown that geranylgeranyl transferase I inhibitors decreased the survivin levels in Mia-Pa-Ca-2 human pancreatic cancer cells, suggesting
geranylgeranylated proteins such as RalA and RalB may be involved in the regulation of survivin\textsuperscript{13, 14}. The drawback of these studies is that they used pharmacological inhibitors that may reduce survivin by off target effects. Moreover, the studies done so far investigated the downstream effectors of ectopically expressed H-Ras not endogenous K-Ras.

In summary, only a few studies investigated the role of these canonical downstream effectors in survivin expression that was induced by ectopic expression of H-Ras but the role of these effectors in cancer cells in the context of mutant K-Ras has not been studied. Much investigation is needed to determine the mechanism by which Ras maintains survivin levels.

Using different cancer cells from different tissue lineages and different genetical backgrounds, we have found that RalA and/or RalB is involved in the regulation of survivin expression only in cells with mutant K-Ras and wild-type p53 and the ability of Ral to regulate survivin in mutant K-Ras harboring cells depends on p53.

\textit{Material and methods}

\textbf{Cell culture}

Human tumor cell lines were grown in their respective media: A549 in Kaighn's modification medium (F12K) (Gibco Laboratories, Grand Island, NY), Calu-1 and SKOV-3 in McCoy’s 5a medium modified (Sigma-Aldrich, St. Louis, MO), H460, AsPC-1, Ovcar-5 and BxPC-3 in RPMI, (Invitrogen, Carlsbad, CA), Panc-1, SW1990, and HOP-92 in Dulbecco’s modified minimal essential medium (DMEM) (Invitrogen). All cells were grown in their respective media containing 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified incubator at 5% CO\textsubscript{2}. 
**siRNAs and antibodies**

Non-targeting (NT), K-Ras, Raf-1, RalA and RalB siRNA were purchased from Dharmaco, Lafayette, CO. Akt1/2 siRNA was obtained from Cell Signaling (Danvers, MA), respectively. Survivin antibodies were purchased from Abcam, Cambridge, MA (used for western blotting). Akt1/2 and Raf-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP antibody was purchased from Cell Signaling (Danvers, MA). β-actin, vinculin and α-tubulin antibodies were purchased from Sigma. K-Ras (OP-24), antibody was purchased from Calbiochem (Billerica, MA). Peroxidase conjugated goat anti-mouse IgG, rabbit anti-goat IgG, mouse anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**siRNA transfection**

Cells were transfected with siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were plated overnight to reach ~40-50% confluence at the time of transfection. Lipofectamine RNAiMAX and siRNAs were incubated with OptiMEM in separate tubes for 5 minutes, then the diluted transfection reagent and siRNAs were mixed. The mixture was incubated at room temperature for 20 min. The siRNA/transfection reagent mixture was then added drop-wise to the cells. After 24 hrs of transfection, growth media with 10% serum only was added until the desired time of transfection.

**Western blotting**

Cells were rinsed with ice cold PBS twice and lysed on the plate using lysis buffer containing 20mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-
100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1ug/mL leupeptin, and 1 mM PMSF. The lysate was harvested by scraping and the debris was removed by centrifuging at 13000g for 13 min. The protein content was measured using Bradford protein assay. Samples were resolved using 12.5% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5% milk in TBST (Tris-Buffered Saline and Tween 20) and then probed with the desired antibodies. The enhanced chemiluminescence blotting system (PerkinElmer Inc, Waltham, MA) was used for antibody reaction.

**Result**

*Depletion of Ral but not Raf or Akt reduces survivin expression in cancer cells with mutant K-Ras.*

We have previously found that mutant K-Ras is required for the maintenance of high levels of survivin in cancer cells with mutant but not those with wild type K-Ras\(^\text{30}\). However, the downstream effectors of Ras critical for this regulation are not known. Although more than 20 downstream effectors of Ras are known so far, the RalGDS/Ral, PI3K/AKT, Raf/Mek/Erk pathways were extensively studied for their role in Ras-driven signaling and malignant phenotypes. We therefore, determined which of the 3 pathways mentioned above is involved in the regulation of survivin in the context of mutant K-Ras. To this end, ovarian cancer cells that harbor mutant K-Ras (ovcar-5) and those that harbor wild-type K-Ras (Skov-3) were transfected by siRNA to deplete K-Ras, RalA, RalB, Akt, or c-Raf, and the effects of these depletions were determined by western blotting as described under Methods. Figure 13 shows that depletion of RalA or K-Ras reduces survivin in ovcar-5, whereas in in Skov-3 cells depletion of none of these genes
reduced survivin levels. This suggests that RalA but not RalB, c-Raf or Akt1/2 is critical for survivin expression in OVCAR-5 cells which harbor mutant K-Ras.

**Figure 13. Depletion of RalA but not RalB, Raf or Akt reduce survivin in OVCA-5 cells which harbor mutant K-Ras.** Ovcar-5 and Skov-3 cells were transfected with the indicated siRNA for 48hrs and the cell lysates were processed for western blotting where the membranes were probed with the indicated antibody as described in the material and methods section.

**Depletion of RalA or RalB fails to reduce survivin in pancreatic cancer cells.**

Persistent activation of RalGTPases has been reported in many tumors where mutations of K-Ras are prevalent. Since activated RalA and RalB are common in tumor of pancreatic origin\(^\text{15}\), we determined if depletion of these genes could reduce survivin in pancreatic cancer cells with and without mutant K-Ras. Our results (Figure 14) show that while siRNA to K-Ras, reduced survivin levels, RalA or RalB failed to reduce survivin levels in all human pancreatic cancer cells whether they harbor mutant or wild type K-Ras. Therefore, we conclude that the presence of mutant K-Ras alone is not sufficient to predict if RalA or RalB can regulate survivin expression levels.
Depletion of RalA or RalB reduce survivin in subset of lung cancer cell lines

Since we observed that RalA can regulate survivin expression levels in ovcar-5 but not in any of the pancreatic cancer cell lines tested, we speculate that RalA contribution in survivin expression could be tissue specific. To this end, we took lung cancer cell lines with and without mutant K-Ras and investigate the effect of RalA or RalB depletion on survivin expression. Our result (Figure 15) show that, while depletion of RalA or RalB reduced survivin in H460 and A549 cells, depletion of Ral failed to reduce survivin levels in a other human lung cancer cell lines such as Calu-1 as well as those that harbor wt-K-Ras such as HOP-92.

Figure 14. Depletion of RalA or RalB fails to reduce survivin levels in pancreatic cancer cells. The indicated pancreatic cancer cell lines were transfected with siRNA to NT, RalA, RalB or K-Ras for 48hrs and then process for western blot analysis.
While in H460, A549 and Ovcar-5 depleting RalA and/or RalB reduce
donald levels, in calu-1 and all the pancreatic cell lines we tested, depleting Ral had no
significant effect on survivin levels, yet all these cell lines harbor mutant K-Ras. Our
finding suggests that the regulation of the survivin expression levels by Ral proteins is
not tissue specific nor it is exclusively associated with the K-Ras mutation status of the
cells. This suggests that other factors may be involved in this regulation of survivin by
Ral. To this end, we looked at the mutation status of different genes to see if other
additional mutations can potentially affect the ability of Ral to regulate survivin
expression. Our analysis shows that, all cell line where we observe Ral involvement in
survivin expression, had in common mutant K-Ras and wild type p53 (see table 2). We

**Figure 15. Depletion of K-Ras, RalA or RalB reduces survivin in subset of lung cancer cell lines.** Lung cancer cells with or without mutant K-Ras were transfected with the indicated siRNA for western blot analysis and membrane were probed with the indicated siRNA.
therefore conclude that, in addition to the K-Ras mutation status of the cell, the p53 status of a cell may determines the ability of Ral to regulate survivin.

**Table 2.** Mutation analysis reveals that, involvement of Ral in survivin expression is mutation- but not tissue-specific

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell line</th>
<th>Mutation status</th>
<th>Does depletion of Ral affect survivin?</th>
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</thead>
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<tr>
<td>Lung</td>
<td>A549</td>
<td>MT WT</td>
<td>RalB</td>
</tr>
<tr>
<td>Lung</td>
<td>H460</td>
<td>MT WT</td>
<td>RalA</td>
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<td>MT Deleted</td>
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<td>Lung</td>
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<td>WT MT</td>
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<td>SKOV-3</td>
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<tr>
<td>pancreas</td>
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*The ability of Ral to regulate survivin depends on the presence of p53 and mutant K-Ras.*

p53 is well known transcription factors that affect the expression of different genes that are known to be involved in apoptosis and cell cycle regulation. Since previous studies have shown negative regulation of survivin expression by wild-type p53 and lower survivin levels in tumors with wild-type p53, we speculate that K-Ras or Ral GTPases influence survivin expression in a p53-dependent manner. We therefore hypothesize that, depletion of K-Ras or RalA or RalB may lead to increases in p53 expression levels and subsequently the induced p53 will interfere with survivin expression. To determine if p53 plays a role in Ral regulated survivin, we co-deplete K-Ras, RalA or RalB with siRNA to p53 and ask if co-depletion of the genes rescue survivin expression. For this, we took A549 cells that have mt-K-Ras and WT-p53 and used siRNA to K-Ras, RalA or RalB alone or in combination with p53. Our result (Figure
show that, while siRNA to K-Ras or RalB reduced survivin levels, co-depletion of K-Ras or RalB with p53 rescued from the reduction of survivin following K-Ras or RalB depletion in A549 cells.

Figure 16: The ability of Ral to regulate survivin depends on p53 and on mutant K-Ras. A549 cells were transfected either with siRNA to NT, K-Ras, RalA or RalB alone or in combination with p53 for 48hrs and membrane was probed with the indicated antibody. The graph on the right panel shows the normalized densitometry values of p53 from the western blot in the left panel.

Discussion

The role of Ras in malignant transformation is well documented and the contributions of different proteins in Ras-induced malignant transformation have been the focus of intense scrutiny by different groups. To date, there are no FDA approved Ras inhibitors in clinic and hence researchers have been working at identifying alternative ways to disable Ras-driven malignant transformation. Some of these approaches include identifying targets downstream of Ras that are drugable.
Recently, we have demonstrated that K-Ras is required for the maintenance of the high levels of survivin in human cancer cells that harbor mutant K-Ras but not in human cancer cells where Ras is wild type and Chapter 2 of this thesis. These studies also showed that depleting survivin reduces mutant K-Ras-driven anchorage-independent growth and invasion. However, the K-Ras downstream effectors involved in survivin regulation are not known. Here we investigated the role of three known downstream effectors of Ras in the maintenance of high survivin levels in the context of mutant K-Ras. The Raf/Mek/Erk, Pi3K/Akt, and RalGDS/Ral pathways are three known canonical downstream effectors that have been extensively studied with regards to their roles in mediating Ras-driven malignant transformation. Although, initially the Raf/Mek/Erk and Pi3K/Akt pathways were thought to be the most critical, more recently the RalGDS/Ral pathway has been shown to be more important in mediating Ras-driven malignant transformation in some cancer cells.

To investigate the role of these three downstream pathways in regulating survivin protein levels in the context of mutant K-Ras, we individually depleted c-Raf, Akt, RalA and RalB by siRNA in two ovarian cancer cell lines, one that harbors mutant K-Ras (ovcar-5) and the other that harbors wild type Ras (skov-3). Our study shows that depleting K-Ras and RalA but not RalB, Akt, or c-Raf reduces survivin in ovcar-5 but not in skov-3 cell line. Our study suggests that RalA but not the other downstream effectors of Ras were critical to K-Ras regulation of survivin. Previous studies have used pharmacological inhibitors to explore the role of Raf/Mek/Erk and Pi3K/Akt in survivin expression. The studies however show inconsistent findings when different cell lines were used. While cell type differences remain a possible reason for these inconsistencies,
it is also possible that off target effects from the drugs could explain these inconsistencies. We therefore used a genetic approach, such as depletion of the genes by siRNA to reduce off-target effect and to investigate the pathways of interest in a more precise and defined pathway.

Persistently activated, GTP-bound Ral has been found in many tumors where mutation of K-Ras is also prevalent\textsuperscript{15,18}. Since depletion of RalA reduces survivin in the mutant K-Ras harboring ovarian cancer Ovcar-5 cells, we investigated the role of Ral in human pancreatic cancer cells where previous studies show the presence of activated Ral. Surprisingly, despite the presence of mutant K-Ras in these cancer cell lines, depletion of RalA, RalB, Akt and c-Raf did not reduce survivin levels. These results suggest that the involvement of Ral could be cell/tissue specific. Indeed previous studies have shown differential role of Ral in cancers from different tissues of origin\textsuperscript{15,18}. Although Ral proteins share approximately 80% sequence identity overall, and 100% sequence identity at their effector domains \textsuperscript{19}, these small GTPases have been shown to bind to different effectors and mediate different malignant phenotypes in different tissues. Lim et al have shown that in pancreatic cancer RalA promote anchorage-independent growth and in vivo tumor growth, RalB was shown to be involved in apoptosis and migration\textsuperscript{15}. In colorectal carcinoma however, while RalA promote similar phenotypes as in pancreatic cancer, RalB actually antagonizes the effector RalA\textsuperscript{18}. The differential effect observed in these cell lines were partly due to their binding to different effectors. While RalA binds to Exo-8 to promote the above-mentioned phenotypes, RalB binds to sec-5 to antagonize the effect driven by RalA. We therefore speculated that Ral GTPase regulation of survivin expression could be tissue specific. However, our depletion studies in several lung cancer
cell lines argued against a tissue-specific effect. Indeed, depleting RalA and/or RalB reduced survivin levels only in a subset of lung cancer cell lines tested.

Consistently throughout our study, we have found that regardless of the tissue of origin, depletion of Ral proteins fails to affect survivin levels in wt-K-Ras harboring cancer cell lines. Interestingly, in this study we have also observed that despite the differences in tissue of origin, depletion of RalA and/or RalB decreased survivin levels only in cell lines that harbor mutant K-Ras. Furthermore, in pancreatic cancer cell lines where p53 is mutated as well as in the lung cancer calu-1 cells where p53 is deleted, depletion of RalA or RalB fails to reduce survivin levels. However, in other lung cancer cell lines, H460 and A549, depletion of RalA and/or RalB reduces survivin levels. We conclude that neither tissue of origin or mutation of K-Ras is sufficient to predict whether depletion of Ral proteins will result in reduction of survivin levels, and that other factors may be involved.

A closer look at the mutation status of these cell lines lead us to discover that depletion of Ral proteins reduces survivin levels only in human cancer cell lines that harbor mutant K-Ras and wild type p53, but not in those cell lines with wilt type K-Ras and/or mutant p53. This observation is consistent with previous reports that showed that WT but not mutant p53 regulates the expression of survivin. Our recently published work demonstrated that depletion of K-Ras reduces survivin levels only in human cancer cells that harbor mutant K-Ras, regardless of the mutation status of p53. Furthermore, depletion of K-Ras in calu-1 cells where both p53 alleles are depleted reduced survivin levels further supporting a p53-independent mechanism. However, in A-549 cells where p53 is wild type, siRNA to p53 prevented the reduction in survivin following K-Ras
depletion (see chapter 4 of this thesis), suggesting that K-Ras may regulate survivin in p53 dependent and independent manner. Similar mode of regulation for the expression of p21 has been demonstrated previously. While K-Ras appears to regulate survivin in p53-dependent and -independent manner, Ral proteins seem to dependent on wild type p53 and mutant K-Ras to regulate Survivin. Consistent with this conclusion is the observation that depleting Ral proteins failed to reduce survivin in all cell lines that harbor mutant p53 despite the presence of mutant K-Ras. Similarly, Ral depletion failed to reduce survivin levels in cell lines that express WT-K-Ras and WT-p53. More direct evidence for the dependence on p53 comes from the observation that siRNA to p53 rescues from survivin reduction following Ral depletion. We therefore conclude that Ral involvement in the regulation of survivin depends on the mutation status of the cells and requires the presence of mutant K-Ras and WT-p53.

In summary, our study used a large number of cell lines to investigate the pathways downstream of K-Ras that are critical for the regulation of survivin expression in the context of mutant K-Ras. Our study took advantage of using cancer cell lines that harbor mutant K-Ras naturally instead of relying on artificial over expression that would potentially cause stress on the cells and alter their normal behavior. Furthermore, our studies lead to the discovery of the involvement of Ral GTPases, but not Akt or Raf, in the regulation of survivin, a known protein that is involved in resistance to apoptosis and promotion of cell cycle progression. An important finding of this work is the dependence on mutant K-Ras and wild type p53 for the Ral regulation of survivin.
References


Chapter 4

Ral GTPase down regulation stabilizes and reactivates p53 to inhibit malignant transformation

Abstract

Ral GTPases are critical effectors of Ras, yet the molecular mechanism by which they induce malignant transformation is not well understood. Here we show that down regulation of K-Ras, RalA and RalB, but not Akt1/2 and c-Raf, leads to p53 Ser-15 phosphorylation and a significant increase in p53 half-life. This increased stability results in a p53-dependent up-regulation of p21waf and mdm2 and down-regulation of survivin expression. Furthermore, depletion of Ral GTPases inhibits cell cycle progression, anchorage-independent growth and/or invasion in a p53-dependent manner. Thus, expression of Ral proteins is critical to maintaining low levels of p53, and down regulation of Ral GTPases reactivates p53 by significantly enhancing its stability, and this contributes to suppression of malignant transformation.

Introduction

Malignant transformation is a complex process that involves constitutive activation of proto-oncogenes and inactivation of tumor suppressor genes. The Ras family members K-, N- and H-Ras are proto-oncogenes that are mutated and constitutively activated in one third of all human cancers, with K-Ras mutations being the most prevalent. Mutant Ras proteins induce malignant transformation by persistently
activating several down stream pathways \(^3\). Among the most thoroughly studied
downstream effectors of Ras proteins are those mediated by the Raf/Mek/Erk, the
Pi3K/Akt, and the RalGDS/Ral pathways. These 3 pathways have been shown to be
required for Ras proteins to induce certain hallmarks of cancer such as uncontrolled
proliferation, apoptosis evasion, angiogenesis, migration and invasion \(^3\). In addition to
activating pathways that promote oncogenesis, mutant Ras proteins also antagonize
tumor suppressive pathways to transform cells.

The tumor suppressor p53 is the genome guardian that prevents malignant
transformation by inducing cell cycle arrest, senescence and apoptosis in response to
stress signals such as oncogene activation \(^4, 5\) and DNA damage \(^6, 7\). p53 is a transcription
factor that induces or represses the expression of many genes including those involved in
cell cycle progression and cell survival \(^8\). Most human tumors contain non-functional
p53, either because of p53 mutations or inactivation of p53-dependent pathways \(^4, 9\). One
mechanism by which tumors with wild type p53 inactivate it is by over expressing its
negative regulator mdm2, an E3 ligase that induces p53 degradation \(^10\). Another
mechanism by which wild type p53 is inactivated is by loss of the mdm2 antagonist ARF
\(^11-13\). Therefore, for oncogenes to transform cells with wild type p53 they must trigger
pathways that lead to the inactivation of p53. In the case of the Ras proteins this is
isoform-dependent \(^14-17\). Cells challenged with mutant H-Ras or mutant N-Ras protect
them selves by inducing the expression of ARF \(^18-20\) which antagonizes MDM2 function
either by sequestering MDM2 in the nucleoli \(^13\) or by directly inhibiting its ubiquitin
ligase activity \(^11\). This leads to increased p53 levels which in turn lead to senescence and
apoptosis \(^5\). In contrast, mutant K-Ras suppresses p53 \(^16\) but the mechanism involved is
not well understood. One proposed mechanism involves the activation of the E3 ligase SNAIL which leads to ubiquitination of p53 and its proteasomal degradation\textsuperscript{16}.

Although not thoroughly investigated, some studies reported on the regulation of p53 by downstream effectors of Ras such as Raf and Akt but not Ral proteins. For example, in Ras-transformed cells, Raf promotes the degradation of p53 by inducing mdm2 and this leads to resistance to p53-dependent apoptosis following DNA damage\textsuperscript{21}. Furthermore, Akt phosphorylates mdm2 on S186 which leads to ubiquitination and degradation of p53\textsuperscript{22}. Whether Ral proteins regulate p53 and whether this contributes to malignant transformation have not been investigated.

RalA and RalB GTPases are molecular switches that are on (active) when bound to GTP and off (inactive) when bound to GDP\textsuperscript{23}. RalGEFs such as RalGDS displace GDP for GTP to activate Ral proteins\textsuperscript{24}. Ral proteins can be activated by Ras as well as by other pathways that are independent of Ras\textsuperscript{25, 26}. The interest in Ral proteins has recently increased following the demonstration that in some cancers Ral proteins are more critical than Raf and Akt in mediating Ras-driven malignant transformation\textsuperscript{27}. RalA and RalB share \textasciitilde82\% sequence identity, yet they have been shown to have different contributions to malignant transformation processes, but this is cancer cell type specific\textsuperscript{28-32}. For example, in pancreatic cancer cells, RalA promotes anchorage-independent growth in soft agar and tumor growth in-vivo while RalB promotes cell survival, invasion and migration\textsuperscript{29}. In colon cancer cells, Ral A has similar functions as in pancreatic cancer cells but RalB antagonizes RalA-driven anchorage-independent growth\textsuperscript{30}. The reasons for these divergent effects are not known but differences in localization\textsuperscript{32} and post-translational modifications\textsuperscript{28, 31, 32} could be contributing factors.
Although Ral proteins have been shown to induce many hallmarks of cancer such as anchorage-dependent and -independent growth, migration and invasion\textsuperscript{33, 34}, the molecular mechanism by which they accomplish this is not well understood. In addition, whether Ral proteins antagonize tumor suppressive pathways to transform cell is not known. For example, whether Ral proteins regulate the levels of the tumor suppressor p53 and whether this contributes to their ability to induce malignant transformation are not known. In this manuscript, we demonstrate that depletion of Ral proteins increases p53 ser-15 phosphorylation and leads to a significant increase in p53 half-life and stability. In addition, depletion of Ral proteins inhibits malignant transformation in a p53-dependent manner. Taken together, these results suggest that down regulation of Ral GTPases leads to stabilization and reactivation of p53 to inhibit malignant transformation of cancer cells that harbor wt p53 and mt K-Ras.

\textit{Material and methods}

\textbf{Cell culture}

The human tumor cell lines were grown in their respective media: H460, MCF-7, Ovcar-5 and BxPC-3 in RPMI, (Invitrogen, Carlsbad, CA), A549 in Kaighn's modification medium (F12K) (Sigma Aldrich, St. Louis, MO), Calu-1 in McCoy’s 5a medium modified (Sigma-Aldrich, St. Louis, MO), Panc-1, HCT116 and HKH2 in Dulbecco’s modified minimal essential medium (DMEM) (Invitrogen, Carlsbad, CA). All cells were grown in their respective media containing 10% FBS and 1% penicillin streptomycin at 37\textdegree C in a humidified incubator at 5% CO\textsubscript{2}.
siRNAs and antibodies

Non-targeting (NT), K-Ras, RalA, RalB, p53 and p21, c-Raf siRNA were purchased from Dharmacon (Lafayette, CO) and Akt 1,2,3 siRNA were purchased from Cell Signaling (Danvers, MA). Survivin antibody was purchased from Abcam (Cambridge, MA). p21, c-Raf, p53 (DO-1), and Akt1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP and phospho-p53 (Ser-15) antibodies were purchased from Cell Signaling. RalB and MDM2 antibody was purchased from Millipore (Billerica, MA). β-actin, vinculin, α-tubulin and GAPDH antibodies were purchased from Sigma-Aldrich. K-Ras (OP-24) antibody was purchased from Calbiochem (Billerica, MA). RalA antibody was purchased from BD Biosciences, (Bedford, MA), Peroxidase-conjugated rabbit anti-goat IgG, goat anti-mouse IgG, and mouse anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

siRNA transfection

All cells used in this manuscript were transfected with a similar siRNA transfection protocol using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA). The cells were plated overnight to reach ~40-50% confluence at the time of transfection. Opti-MEM reduced serum medium (Invitrogen) and Lipofectamine RNAiMAX were mixed in one tube and a mixture of Opti-MEM and siRNA was prepared in a separate tube, and the content of the two tubes were mixed after 5 minutes of incubation at room temperature. Following this, the mixture was incubated for 20 minutes at room temperature and then added to the cells drop-wise. After 24 hours of
transfection, appropriate media with 10% serum only was added until the desired time of transfection.

**DNA transfection**

Cells were plated overnight to reach 70-80% confluence and transfected with DNA using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, Lipofectamine 2000 reagent (1.5μl/μg of DNA) was diluted in Opti-MEM and allowed to equilibrate for 5 minutes at room temperature. DNA was diluted in Opti-MEM in a separate tube. The contents of the two tubes were mixed and incubated for 20 minutes to complex at room temperature, then added drop wise to cells that contain only 10% FBS containing regular growth media. The cells were transfected for 6 hours before changing the medium to 10% FBS only regular growth media.

**Western blotting**

Cells were rinsed with ice-cold PBS twice and lysed on the plate in lysis buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1μg/mL leupeptin, and 1 mM PMSF. The lysate was collected by scraping and centrifuging at 13000xg for 13 minutes to remove the debris. The protein content was measured using Bradford protein assay. Samples were resolved using SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% milk in Tris-buffered saline and Tween-20 (TBST) and then probed with the desired antibodies. Western Lightning ECL (PerkinElmer Inc, Waltham, MA) was used for antibody detection.
**Caspase activation assay**

Caspase-3 activity was measured in cell lysates using a fluorogenic substrate. The assay measures the cleavage of AMC groups from a Caspase-3 specific substrate (Ac-DEVD-AMC) (Biomol Research Labs, Inc., Plymouth Meeting, PA). Briefly, cells were lysed in a protease inhibitor-free lysis buffer (50 mM Tris, pH 8, 5 mM EDTA, 150 mM NaCl, and 0.5% Igepal). The lysate was collected by scraping. After clearing the lysate by centrifugation, protein was measured. 20 μg of protein and 20 μM caspase-3 substrate were added into 50 mM Tris, pH 7.5 reaction buffer in triplicate in a 96-well plate. The reactions were incubated for 1 hour at 37°C and liberated AMC groups were quantified by a fluorometer (WALLAC Victor 1420 Multilabel counter, Perkin Elmer Life Sciences, Turku, Finland) with 355 nm excitation and 460 nm emission filters.

**Soft agar colony formation assay**

A549 and H460 cells were transfected with siRNA for 48 hours, then the cells were trypsinized, counted and 2,000 cells/well were seeded in triplicate in 12-well culture plates in 0.3% top agar over 0.6% bottom agar layer as previously described. Cultures were incubated for two weeks (H460) or three weeks (HCT116). Plates were scanned after overnight incubation with 2 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Calbiochem), in phosphate buffered saline. The colony numbers were visually determined and quantified.

**Cell invasion assay**

Analysis of cell invasion was performed using 24-well Matrigel invasion chambers (8 μM pore size, BD Biosciences, Bedford, MA) according to the manufacturer’s instructions. Briefly, the inserts were rehydrated by adding warm serum-
free medium for 2 hours at 37°C, 5% CO₂ atmosphere. The medium was then removed from the inserts, and 750 µl of medium containing 20%FBS was added to each well of the plate and the inserts were placed on top of the medium. Transfected A549, Panc-1, and H460 (40,000 cells in 500 µl of serum free-media) and HPNE cells (20,000 cells) were added to the inserts. The cells were incubated for 48 hours at 37°C. Cell invasion was determined by staining the bottom of the insert with crystal violet (0.5% crystal violet in 25% methanol). The porous membrane was removed and placed on the slide. The number of cells that invaded were counted in three high-power microscope (40X) fields and averaged as the mean number of invaded cells per field for each membrane.

**Promoter-reporter transcriptional assay**

To determine the effect of K-Ras, RalA or RalB depletion on p21 promoter activity, 300,000 H460 or A549 cells were plated overnight, in triplicate, in 6-well plates followed by 3 µg of p21 and (1:50 b-gal) transfection using Lipofectamine 2000 (as described above). 12hrs after DNA transfection, the cells were then transfected with different siRNAs using Lipofectamine RNAiMax or using Lipofectamine 2000 as described above. Following 48 hours of siRNA or DNA transfection, cells were rinsed with PBS and then lysed on the plate using Reporter Lysis Buffer (RLB) as described by the manufacturer (Promega BioScience, San Luis Obispo, CA). Cells were then freeze-thawed for efficient lysis. The lysate was centrifuged at 12,000g for 2 minutes to remove debris. The p21 promoter activity was detected by incubating the lysate with the assay substrate. The luciferase activity was detected using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) and b-galactosidase was read at 420nm wave length using Biot-
Tek plate reader The p21 promoter activity was normalized to the respective b-galactosidase reading.

**Determination of p53 half-life**

H460 or A549 cells were transfected with siRNA. Following 48 hours of transfection, cells were treated with 50 µg/mL of cyclohexamide for 0, 0.5, 1, 2, or 4 hours and then harvested and processed for western blot analysis as indicated above.

**Cell cycle analysis**

A549 cells were transfected with different siRNAs for 48 hours. Following completion of siRNA transfection, cells were trypsinized and then centrifuged at 200xg for 5 minutes to harvest the cells. The cells were then rinsed with PBS twice and then re-suspended in ethanol overnight. Following overnight fixation, cells were then centrifuged. The supernatant was removed and rinsed with PBS. The cells were stained with propidium (PI) iodide stain that contain 20µg/mL PI, 0.1%(v/v) Triton in PBS and 0.2mg/mL DNAase free RNAase A for 2 hours at room temperature. Cells distribution on different cell cycle phase was determined using a flow cytometer.

**Cell lines**

The human cancer cell lines Calu-1, Panc-1, A549, BxPC-3, H460, HCT116, MCF-7 and Ovcar-5 were all obtained from ATCC but none of the cell lines have been authenticated.

**Results**

*Expression of K-Ras, RalA or RalB, but not AKT1/2 or c-Raf, is required for the maintenance of low levels of p53 in human cancer cells that harbor mutant K-Ras and wild type p53*
About half of human cancers lack the tumor suppressive activity of p53 due to gene deletions or mutations\(^9,^{35-38}\). The other half that express wild type (wt) p53 harbor aberrant pathways that either inactivate p53 or suppress its expression levels \(^9,^{35-38}\). Mutant (mt) K-Ras is known to down regulate wt p53 levels but little is known about the contributions of its down stream effectors. For example, Ral GTPases are critical mediators of K-Ras-driven malignant transformation but whether they regulate wt p53 is not known. To address this important question, we first determined whether depletion of Ral GTPases affects the expression levels of p53 in A549 and H460 cell lines that harbor mt K-Ras and wt-p53. Since Akt and Raf are also critical to Ras malignant transformation, we also investigated the effects of their depletion on the expression levels of p53. To this end, A549 and H460 cells were transfected with either non-targeting siRNA or siRNAs to K-Ras, RalA, RalB, AKT1/2 or c-Raf and the cells were processed for western blotting as described under Experimental Procedures. Figure 1 shows that in both A549 and H460 cells depletion of K-Ras, but not Akt1/2 and c-Raf, resulted in increased p53 levels. Depletion of RalB in both cell lines also increased p53 and this increase was more pronounced (3.5-fold for both A549 and H460) than with K-Ras depletion (1.6 (H460) and 2.4 (A549)-fold). Furthermore, depletion of RalA increased the levels of p53 in H460 but had little effect in A549 (Figure 1). To determine if the increase in p53 levels is dependent on the mutation status of K-Ras and p53, we used other cell lines with mt K-Ras and wt p53 (HCT116 colon cancer cells and OVCAR5 ovarian cancer cells), wt K-Ras and wt p53 (MCF7 breast cancer cells and HCT116 cells where the mutant K-Ras was deleted by homologous recombination (HKH2 cells), wt K-Ras and mt p53 (BXPC3 pancreatic cancer cells), and mt K-Ras and mt p53 (Panc1
pancreatic cancer cells). Figure 18 shows that depletion of K-Ras, RalA and RalB increased p53 levels in cells with mt K-Ras and wt p53 (HCT116 and OVCAR5) but not in cells with either wt K-Ras and/or mt p53 (MCF7, BXPC3 and Panc1). Furthermore, depletion of K-Ras, RalA and RalB in the isogenic cell line HCT-116 (HKH-2) where the mutant K-Ras allele was deleted had little effect on the levels of p53 (Figure 18). Therefore, our results suggest that in human cancer cells that harbor mt K-Ras and wt p53, the expression of K-Ras, RalA and/or RalB is required for the maintenance of low levels of p53.

![Figure 17. Depletion of RalA and/or Ral B increases p53 protein levels in cancer cells with WT-p53 and mt-K-Ras.](image)

A. Depletion of RalA and/or Ral B but not AKT1/2 or c-Raf affects p53 expression in cells with mt-K-Ras and wt-p53. H460 and A549 cells were transfected with the indicated siRNA for 48hrs and processed for western blotting analysis as described in the material and method section.
Depletion of K-Ras, RalA and RalB leads to up-regulation of p21 and down-regulation of survivin expression in a p53-dependent manner

p53 controls cell cycle progression and cell survival by regulating at the transcriptional levels the expression of several genes that are involved in cell cycle progression (e.g. p21) as well as those involved in apoptosis (e.g. survivin) \(^{39-42}\). Therefore, we next determined whether Ral GTPases are involved in the regulation of these genes and whether this is dependent on p53. Figure 19A shows that in A-549 cells, depletion of K-Ras and RalB increased the protein levels of p53 and p21. Consistent with this, depletion of K-Ras and RalB also increased the p21 promoter transcriptional
Figure 19. Depletion of K-Ras, RalA and Ral B regulate survivin and p21 expression in a p53-dependent manner. A. (A549) and C. (H460) cells were transfected with NT, K-Ras, RalA or RalB siRNA alone or in combination with p53 siRNA for 48hrs. Cells were harvested for western blot analysis as described in the material and method section. B. (A549) and D. (H460) cells were transfected with p21 promoter-reporter constructs followed by transfection with the indicated siRNA. Cells were harvested and analyzed for p21 promoter activity as described in the Experimental Procedures sections. E. Calu-1 cells were transfected with the indicated siRNA and processed for western blotting analysis as described in the Experimental Procedures section. Extra lane on p53 and p21 blot were from a control lysate (Panc-1 cells) used as a control for the western blot analysis. Data are representative of three independent experiments except for Calu-1 cell experiments that were done twice.
activity (Figure 19B). Furthermore, Figure 19A also shows that depletion of K-Ras and RalB decreased the levels of survivin. In H460 cells, depletion of K-Ras, RalA and RalB increased p21 expression at the protein (Figure 19C) as well as the transcriptional (Figure 19D) levels. We next determined whether this increase in p21 and decrease in survivin levels are dependent on the expression of p53. To this end, we took two approaches. First, since depleting K-Ras, RalA and RalB increased p53 levels, we reasoned that co-depleting p53 should rescue if the effects are p53-dependent. Therefore, we transfected A549 and H460 cells with K-Ras, RalA or RalB siRNAs alone or in combination with p53 siRNA and evaluated the levels of p53, p21 and survivin as described under Experimental Procedures. Figures 19A and 19C show that K-Ras, RalA and RalB siRNAs were unable to decrease survivin and/or increase p21 protein levels in the absence of p53. Similar results were obtained with p21 promoter transcriptional activity (Figures 19B and 19D). In the second approach we used a cell line where K-Ras is mutated and both p53 alleles are deleted (human lung cancer calu-1 cells) and determined the effects of depleting K-Ras, RalA and RalB. Figure 19E shows that depletion of RalA and RalB failed to increase p21 and to decrease survivin expression levels. In contrast, depletion of K-Ras decreased the levels of survivin but was unable to increase the levels of p21, suggesting that in calu-1 cells the ability of K-Ras to regulate p21 but not survivin is p53-dependent.

**Depletion of RalA and RalB increases p53 Ser-15 phosphorylation and p53 stability and half life**

Figures 17,18 and 19 demonstrated that depletion of Ral GTPases increased the amount of functional p53 protein in human cancer cells with mt K-Ras and wt p53, and
that this resulted in the modulation of p53-regulated genes such as p21 and survivin. We next determined whether the increase in p53 levels is due to increased stability of the p53 protein. To this end, H460 cells were transfected with either non-targeting (NT) siRNA or siRNA to K-Ras, RalA or RalB for 48 hours followed by treatment with cyclohexamide (CHX) for 0, 0.5, 1, 2 or 4 hrs and the cells processed for western blotting as described under Experimental Procedures. Figure 20 and Figure 21 show that in cells transfected with NT siRNA, the protein levels of p53 decreased steadily after treatment with CHX with a half-life between 30 and 60 minutes.

**Figure 20. Depletion of K-Ras, RalA and RalB increases p53 protein stability.** H460 cells were treated with non targeting, K-Ras, RalA or Ral B siRNA for 48 hrs followed by cyclohexamide treatment for the indicated lengths of time. Cells were harvested and processed for western blotting analysis as described in the Experimental Procedures section. Data are representative of two independent experiments.
In cells transfected with RalA, RalB or K-Ras siRNA, p53 protein levels decreased after CHX treatment but at a much slower rate with half-lives greater than 4 hrs (RalA and RalB siRNAs) and greater than 2 hrs (K-Ras siRNA) (Figure 20 and Figure 21). Thus, in the absence of RalA and RalB, p53 half-life was increased by

Figure 21. Densitometer values of p53 and MDM2 proteins. Densitometer values represent the expression levels of p53 and MDM2 following depletion of K-Ras, RalA or RalB. The expression levels of p53 and actin was measured at the indicated time points and the relative p53 expression level was determined by dividing p53 by actin densitometry values. The value at the 0hr time point was set as one and the rest of the values at different time points were normalized against the 0 hr value.
greater than 6 fold and in the absence of K-Ras it was increased by greater than 2 fold. Similar results were obtained in A-549 cells where RalB depletion also increased the half-life of p53 from 30 min to 4 hours Figure 22. Depletion of K-Ras, RalA and RalB also increased the levels of mdm2, but in contrast to p53, it had little effect on its half-life (about 30 min with and without K-Ras, RalA and RalB siRNAs). This suggests that depletion of K-Ras, RalA or RalB stabilizes p53 which in turn induces the expression of mdm2, a known target for p53. Consistent with this, the ability of RalB siRNA to increase mdm2 levels was rescued by p53 siRNA suggesting

Figure 22. Depletion of RalB increases p53 stability in A549 cells. A. A549 cells were transfected with the indicated siRNA for 48hrs followed by cyclohexamide treatment for the indicated length of time and processed for western blot analysis as indicated in the material and method section. B. p53 and mdm2 from (A) were quantified as described under Figure 1S legend. C. A549 cells were transfected with the indicated siRNA for 48hrs and then processed for WB analysis. Data are representative of 3 independent experiments.
that RalB depletion up-regulates mdm2 expression in a p53-dependent manner Figure 22). Phosphorylation of p53 at ser-15 has been extensively used as a marker for stability of p53 \(^{43, 44}\). Therefore we next determined the effects of RalA, RalB and K-Ras depletions on p53 Ser-15 phosphorylation. Figure 20 shows that deletion of RalA and RalB in H460 cells resulted in increased p53 Ser-15 phosphorylation. Similar results were seen with A549 cells where depletion of RalB also increased p53 Ser15 phosphorylation, Figure 22.

*Depletion of RalB inhibits cell cycle progression in a p53- and p21-dependent manner by decreasing the proportion of cells in S phase*

Cells exposed to DNA-damaging agents induce p53 which in turn induces, depending on the extent of the damage, either cell cycle arrest or apoptosis by regulating the transcription of specific genes \(^{39-42}\). Since p53 was induced and up-regulated p21 expression following depletion of RalA, RalB or K-Ras, we next determined whether this increase in p53 has functional consequences on cell cycle progression. To this end, we transfected A549 cells with siRNA to K-Ras, RalA or RalB and processed the cells for cell cycle analysis as described under Experimental Procedures. Figures 23 show that depletion of RalA had little effect on cell cycle progression. In contrast, depletion of RalB decreased the percentage of cells in S-phase by 57\% (Figure 23 and Table 3A) to 70\% (Figure 23 and Table 3A) whereas K-Ras increased the percentage of cells in G2M phase by 4 to 6.7 fold (Figures 23/ Table 3A ). To determine if these effects are p53-dependent, we co-transfected the cells with siRNA to p53 along with siRNAs to either K-Ras, RalA or RalB and determined the effects on cell cycle progression as described.
under Experimental Procedures. Figure 23 and Table 3A show that depletion of p53 inhibited RalB and K-Ras siRNAs from affecting cell cycle progression.

Since p21 is known to be up-regulated by p53 and to be involved in G1/S transition, we next determined if the p21 induction by p53 following depletion of RalB is required for RalB siRNA to decrease the percentage of cells in S phase. To this end, we co-transfected the cells with

**Figure 23. Effects of Depletion of K-Ras, RalA and Ral B on cell cycle progression.** A549 cells were treated with the indicated siRNA for 48 hrs and processed for cell cycle analysis as described in the Experimental Procedures section. The percentage of cells in each cell cycle phase relative to NT is shown. (A) Depletion of P53 rescue. (B) Depletion of p21 rescue.
siRNA to p21 along with siRNAs to either K-Ras, RalA or RalB and determined the effects on cell cycle progression as described under Experimental Procedures. Figure 23 and Table 3B shows that, co-depletion of p21 completely rescued from the effects of RalB siRNA on cell cycle progression suggesting that the ability of RalB siRNA to decrease the proportion of cells in S phase depends on p53-induced p21.

Table 3. Percent of cells at different phases of cell cycle.

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**Effects of Depleting RalA and RalB on apoptosis**

As mentioned above another important function of p53 is to promote apoptosis. Furthermore, depletion of RalB but not RalA has been shown to induce apoptosis but the molecular mechanism by which this occurs is not known. Therefore, we next determined whether depletion of RalB induces apoptosis and whether this is p53-dependent. To this end, we transfected A549 and H460 cells with siRNA to K-Ras, RalA or RalB in the presence or absence of siRNA to p53 and processed the cells for western blotting and for Caspase 3 activation by a fluorescence assay as described under...
Experimental Procedures. Figure 24 shows that in A549 cells, depletion of K-Ras and RalB but not RalA induced apoptosis with K-Ras depletion having significantly higher effects (7-fold from 200 to 1400) than RalB depletion (3-fold from 200 to 600). Figure 24 also shows that siRNA to p53 inhibited K-Ras and RalB siRNA from inducing apoptosis. These results suggest that depletion of K-Ras and RalB induced apoptosis by a p53-dependent mechanism in A549 cells. In H460 cells, however, depletion of K-Ras but not that of RalA and RalB significantly induces apoptosis Figure 24, and depletion of p53 rescues apoptosis.

*Depletion of RalB and to a lesser extent RalA inhibits anchorage-independent growth and invasion in a p53-dependent manner*

The involvement of RalA and RalB in malignant transformation has been well documented. While RalA is believed to be involved in promoting anchorage-independent growth in soft agar, RalB is believed to promote invasion and migration, although this is cell type-dependent. Since p53 is a potent tumor suppressor, we reasoned that Ral proteins may affect some of these hallmarks of transformation in a p53-dependent manner. To this end we depleted K-Ras, RalA or RalB in the presence or absence of p53 siRNA and processed the cells for soft agar and invasion assays as described under Experimental Procedures. Figure 25 shows that depleting K-Ras or RalB but not RalA inhibited soft agar growth of H460 and HCT116 cells. The inhibition of soft agar growth following RalB depletion was rescued fully by siRNA to p53. In contrast, siRNA to p53 only partially rescued from the inhibition of soft agar growth following K-Ras depletion. Another critical hallmark of cancer is invasion; we therefore investigated the effects of depleting these 3 proteins on invasion. Figure 26 shows that
depletion of K-Ras, RalB and to a lesser extent RalA inhibited invasion of all 3 cell lines, A549, HCT116 and H460. Co-depletion with p53 rescued from this inhibition of invasion

These results suggest that depletion of K-Ras, RalA and RalB requires p53 to inhibit invasion of A549, H460 and HCT116 cells.

**Figure 24. Effects of depleting K-Ras, RaLA and RaLB on apoptosis.** H460 and A549 cells were transfected with siRNA for 48 hrs and then harvested for western blot analysis (left panel) or caspase-3 activation assay (right panel) as described under Experimental Procedures. Data are representative of 2 independent experiments.
Figure 25. Effects of Depletion of K-Ras, Ral A and Ral B on anchorage-independent growth. H460 or HCT116 cells were transfected with the indicated siRNA for 48hrs before plating in soft agar as described in Experimental Procedures section. Results are shown as average % inhibition of four independent experiments (H460) or three independent experiments (HCT116). NT and NT+p53 siRNA were set as 100%. For H460 Mean number of colonies for NT was 323+/-8.5 and for NT+p53 siRNA was 232+/-66.22; and for HCT116 mean number of colonies for NT was 469 +/- 7.2 and for NT+p53 siRNA was 484 +/- 6. Error bars show SE. Student t-test was used to compare the means; and p-values are shown for tests that show significant differences (*, p<0.05). Top panel shows representative soft agar pictures for HCT116 cells.
Among the three most studied downstream effector pathways of Ras, Raf/Mek/Erk, Pi3K/Akt and RalGDS/Ral, recently the latter has gained more attention as it has been shown to be more critical in mediating Ras-driven malignant transformation.

Discussion

Figure 26. Effects of Depletion of K-Ras, Ral A and Ral B on invasion. A549, H460 or HCT116 cells were transfected with the indicated siRNA for 48 hrs, then equal number of cells were seeded for invasion assays as described in the material and method section. The cells were then photographed and counted to determine the mean number of invading cells. Top panel shows representative image of invading cells of H460, while bottom panels show average number of invading cells. P values for H460 are K-Ras 0.02, RalA 0.02 RalB 0.03. P values for HCT116 are K-Ras 0.006, RalB 0.007. P values for A549 are K-Ras 0.004 and RalB 0.0006)
in several cancers \(^27,46\). Specifically RalA has been implicated in anchorage-independent growth whereas RalB has been implicated in apoptosis evasion, migration and invasion, although this is cell-type-dependent \(^28-32,39\). The mechanism by which RalA and RalB mediate these effects is not well understood. Furthermore, mt K-Ras suppresses p53 levels but it is not known which of its downstream effectors mediate this effect. In this manuscript we have demonstrated that the expression of K-Ras, RalA and RalB but not c-Raf and Akt1/2 is required for maintaining low levels of p53 in human cancer cells that harbor mt K-Ras and wt p53. The observation that depletion of K-Ras increased p53 levels is novel and consistent with observation that K-Ras can suppress p53 levels \(^16\). Indeed, while mt H-Ras and mt N-Ras were shown to induce p53 levels and senescence, mt K-Ras was shown to suppress p53 levels \(^11,14,16-20\). Therefore, it is not surprising that, unlike cells that harbor mt H-Ras and mt N-Ras, many human cancer cells that harbor mt K-Ras also express wt p53, suggesting that mt K-Ras triggers pathways capable of overcoming the tumor suppressive activity of p53. Our studies suggest that RalA and RalB could be among those pathways that may be used by mt K-Ras to suppress p53 levels. In contrast, pathways mediated by c-Raf and Akt1/2 do not appear to be required since their depletion did not increase p53 levels. While our studies demonstrated that depletion of Raf or Akt does not increase p53 levels, others have shown that forced expression of Raf or Akt induces p53 degradation \(^21,22\). This suggests that while Raf and Akt can induce degradation of p53, their expression is not required to maintain low levels of p53 in some human cancer cells. It is important to note that depletion of RalA and RalB increased p53 levels only in human cancers cells that harbor mt K-Ras and wt p53, suggesting that RalA and RalB may mediate the ability of mt K-Ras to suppress p53.
However, the fact that depletion of RalB was more potent at increasing the levels of p53 than depletion of K-Ras (Figure 19) coupled with the fact that depletion of RalB and K-Ras affected cell cycle progression differently (Figure 23) suggest that RalB may also regulate p53 in a K-Ras-independent manner.

In addition to increasing p53 levels, depletion of RalA and RalB also increased the expression levels of p21 and mdm2 and decreased the expression levels of survivin, all 3 genes known to be transcriptionally-regulated by p53. The p53 siRNA rescue from these effects suggests that the ability of siRNA to RalA and RalB to increase p21 and mdm2 and to decrease survivin levels is dependent on their ability to increase the levels of p53. This also indicates that the p53 induced following RalA or RalB depletion was functional and able to regulate the transcription of its target genes. Furthermore, the ability of Ral siRNAs to regulate the expression of the p21, mdm2 and survivin genes in a p53-dependent manner has important consequences on the ability of Ral GTPases to contribute to malignant transformation (see more below). In cells that lack both p53 alleles such as Calu1 cells, depletion of RalA or RalB failed to affect p21 and survivin expression, further confirming the dependency of the Ral proteins on p53 to regulate the expression of these genes. Our results also suggest that K-Ras regulates the expression of p21 in a p53-dependent manner. However, the effects of K-Ras depletion on survivin appear to be p53–independent in Calu-1 cells.

Depletion of RalA and RalB as well as K-Ras resulted in a significant increase in its half life. This increased p53 stability is specific in that depletion of K-Ras, RalA and RalB increased the mdm2 protein levels with little affects on its half life. Consistent with this, depletion of RalA and RalB resulted in an increase in the levels of p53 Ser-15
phosphorylation which has been associated with p53 stability\textsuperscript{43, 44}. Taken together, these observations suggest that depletion of RalA and RalB results in increased p53 Ser-15 phosphorylation and a more stable p53 that was able to induce the expression of p21 and mdm2 and down regulate the expression of survivin. Depletion of these GTPases could also affect other phosphorylation sites on p53 that affect its stability. Indeed, cellular stress and DNA damage induce p53 phosphorylation at Ser-15 as well as Ser-20 and Ser-37 which affect p53 stability by preventing its ubiquitination and degradation\textsuperscript{43, 44}. Therefore, investigations determining the effects of RalA and/or RalB depletion on p53 post-translational modifications other than Ser-15 are warranted to further understand the mechanism by which depletion of RalA and RalB stabilizes p53. Finally, mt K-Ras was shown to decrease the stability of p53 by activating the E3 ligase SNAIL which ubiquitinates p53 and targets it to proteasomal degradation\textsuperscript{16}. It is therefore possible that depletion of RalA and/or RalB inhibits SNAIL as well as mdm2 E3 ligases leading to less ubiquitination of p53.

The effects of depletion of the Ral GTPases on anchorage-dependent and -independent growth, invasion, apoptosis and proliferation have previously been studied\textsuperscript{29, 33}. However, the mechanisms involved are not known and the contribution of p53 to these effects is not known. Here we show that depletion of RalB inhibited cell cycle progression by decreasing the proportion of cells reaching S phase and by increasing the proportion of cells in both G1 and G2/M phases of the cell cycle. The ability of RalB depletion to decrease the proportion of cells in S phase is rescued by p53 siRNA as well as p21 siRNA suggesting that RalB depletion induces p53 and p21 which contribute to inhibition of cell cycle progression. Furthermore, depletion of K-Ras induces G2/M cell
cycle arrest in a p53-independent manner. Therefore, the fact that depletions of K-Ras or RalB resulted in an increase in p53 levels, yet the consequences on cell cycle progression were different suggest that, in addition to affecting common genes such as p53, each specific depletion also differentially affected other genes. This is not surprising as it is known that Ral GTPases are regulated in a Ras-dependent and -independent manner, and that RalA and RalB themselves mediate different cellular effects. In addition to the cell cycle effects, depletion of RalB and to a lesser degree RalA inhibits anchorage-independent tumor cell growth and invasion which were also rescued by p53 siRNA. This suggests that in the absence of RalB the stabilized p53 is not only able to inhibit cell cycle progression and to inhibit invasion but also contributes to inhibition of anchorage-independent tumor cell growth. Unlike cell cycle progression, depletion of K-Ras induces apoptosis and inhibits anchorage-independent tumor cell growth and invasion in a p53-dependent manner.

In summary, we have uncovered a novel molecular mechanism by which Ral GTPases affect transformation. Furthermore, we have provided a biochemical link between Ral GTPases and the tumor suppressor p53 by demonstrating that the expression of Ral GTPases is required for maintaining low levels of p53, and that down regulation of the Ral proteins results in the stabilization and reactivation of p53.

References


Chapter 5

Summary and Conclusions

K-Ras and Ral GTPases have been extensively studied for their role in driving malignant transformation and maintaining various malignant phenotypes. Similarly, down regulation of the tumor suppressor p53 and up regulation of the anti-apoptotic protein survivin are prevalent in human cancer and contribute to various malignant phenotypes. In this thesis, we investigated the role of K-Ras, RalA and RalB GTPases in the regulation of survivin and p53 and the functional consequence of this regulation.

First, we demonstrated that the expression of K-Ras is required for the maintenance of the high survivin levels in human cancer cell lines that harbor mutant K-Ras but not in human cancer cell lines that express wild type Ras. This observation was made in cancer cell lines from different lineages, suggesting that the involvement of K-Ras in survivin regulation is mutation- but not tissue-specific. Knocking down K-Ras expression not only reduced the basal levels of survivin but it also inhibited drug-induced survivin, suggesting that K-Ras may mediate resistance to apoptotic or anti-proliferative drugs partly by up-regulating the expression of anti-apoptotic proteins such as survivin.

While depletion of K-Ras reduced survivin levels, ectopic expression of constitutive active (CA) K-Ras failed to induce survivin levels. This is somewhat counter intuitive but it suggested that while the expression of K-Ras is required for maintaining high levels of survivin, mutant K-Ras alone is not sufficient to drive the expression of survivin. Though additional studies are required to reach a definitive conclusion, we
speculate that one possible reason for this is that the cancer cell lines as well as the immortalized cell lines that we used in these experiments already harbor many genetic alterations, and that ectopic expression of CA K-Ras was unable to further increase the levels of survivin. For example, the HPNE cells that were used in this project were immortalized by hTERT and E6/E7/st, which are known to antagonize p53, and induce the expression of survivin. The other possible explanation is that mutant K-Ras is one of several key nodes of complex signaling networks that are all required for maintaining high survivin levels in cancer cells. Therefore, removing one of the nodes such as depleting K-Ras would compromise the integrity of the network and lead to reduction in survivin levels. Consistent with explanation are recent studies that show that only co-expression of K-Ras with β-catenin was able to induce survivin expression. Therefore, we conclude that mutant K-Ras is required for maintaining high survivin levels, but alone is not sufficient to drive survivin expression.

Our studies also demonstrated that in T24 cells, a human bladder cancer cell line with mutant H-Ras, depletion of H-Ras reduced survivin expression. In contrast, depletion of H-Ras in the human breast cancer MDA-MB-468 cells that do not harbor mutant Ras did not reduce the levels of survivin. The limited number of cell lines used precludes making generalized conclusion but it appears that mutant H-Ras may also be involved in the regulation of survivin. These results warrant further investigation to determine whether only mutant H-Ras and mutant K-Ras are required for the maintenance of high survivin levels, and whether reduction in survivin levels could be used as a read out or surrogate biomarker to measure inhibition of mutant H- Ras and mutant K-Ras.
Our studies also demonstrated that the reduction in survivin levels following depletion of K-Ras was rescued by the proteasome inhibitor bortezomib. This suggested that the depletion of K-Ras leads to the proteasome degradation of survivin. The mechanism by which depletion of K-Ras leads to proteasome mediated degradation of survivin is not clear. We first reasoned that depletion of K-Ras might affect survivin phosphorylation at Thr-34 and/or Ser-20 and leads to proteasome degradation. Phosphorylation of survivin at Ser-20 has been shown to interfere with the binding of XIAP to survivin. Since XIAP binding to survivin has been suggested to stabilize survivin, we speculated that depletion of K-Ras might increase Ser-20 phosphorylation and hence decrease the stability of survivin by interfering with the binding of XIAP to survivin. Similarly, dephosphorylation of survivin at Thr-34 has been shown to reduce the stability of survivin. We generated S20A, S34A and S20A/S34A non-phosphorylatable survivin mutants as well as S20D, S34D and S20D/S34D phospho-mimic survivin mutants and demonstrated that depletion of K-Ras was as efficient at reducing the levels of all these mutants as it was at reducing wild type survivin levels (data not shown). These results suggest that the ability of K-Ras depletion to decrease the stability of survivin may not depend on the phosphorylation of survivin at Thr-34 and/or Ser-20.

Depletion of K-Ras has been shown to induce apoptosis in many, but not all, cancer cell line that harbor mutant K-Ras. Furthermore, in one study using the HCT-116 isogenic colon cancer cell lines with and without mutant K-Ras, depletion of survivin resulted in induction of apoptosis and cell cycle arrest selectively in the isogenic cell line that harbors mutant K-Ras. We found that the presence of mutant K-Ras does not predict that depletion of survivin will result in apoptosis. For example, while depletion of
survivin induced apoptosis in A549 and H460 cells it fail to produce similar effect in panc-1, miapaca-1 or sw1990 cells, yet all of these cell lines harbor mutant K-Ras. Furthermore, depletion of K-Ras decreases the levels of survivin and induced apoptosis in panc-1 as well as miapaca-2 cells. Yet, in these cell lines depletion of survivin itself does induce apoptosis, suggesting that the decrease in survivin levels does not contribute to apoptosis induction following K-Ras depletion in panc-1 and miapaca-2 cell lines. We found that depletion of K-Ras also reduced the levels of XIAP, and this may contribute to the induction of apoptosis following K-Ras depletion. Pro- or anti-apoptotic members of the Bcl2 family do not appear to be involved since their levels were not affected by depletion of K-Ras. Future study using gene expression profiling or proteomics that focus on other survival and apoptotic proteins could give further insight about the molecular mechanism by which depletion of K-Ras induces apoptosis.

Although survivin was dispensable for apoptosis in some human cancer cell lines, we have showed that it plays critical role in other K-Ras-driven malignant phenotypes. To this end, reduction of survivin following depletion of K-Ras might be beneficial to inhibit K-Ras-driven invasion and metastasis, therefore future studies are warranted to study the role of survivin inhibitors at inhibiting K-Ras driven malignant phenotypes.

While K-Ras has been shown to have multiple downstream effectors, the three canonical downstream effectors Raf/Mek/Erk, Pi3K/Akt and RalGDS/Ral have been extensively studied for their role in Ras-driven malignant transformation. Our studies demonstrated that depletion of RalA and/or RalB but not Akt1/2 or Raf-1 decreased the levels of survivin, and this suggested that Ral proteins may mediate the effects of K-Ras on survivin. While the presence of mutant K-Ras in a human cancer cell line predicted
whether depletion of K-Ras would result in decreased survivin levels, mutation of K-Ras alone was not sufficient to predict the same for Ral protein depletion. We found that both mutant K-Ras and wild type p53 are required for Ral depletion to result in decreased survivin. This suggested that Ral regulation of survivin could be mediated by p53. Indeed, in H460 and A549 cells, co-depletion with p53 prevented the decrease in survivin levels following RalA and/or RalB depletion. This is an important finding that not only gave evidence that the ability of Ral proteins to regulate survivin may depend on p53, but it also suggested a novel role for Ral as a potential regulator of p53. Our follow up studies gave further support to this suggestion (see more below).

Although depletion of Ral A and Ral B individually did not affect the levels of survivin in certain cells (wt Ras and/or mt p53), it is possible that the combined depletion of Ral A and Ral B may affect survivin levels. Studies have shown that depletion of RalA induces activation of RalB and vice-versa. Therefore, a compensatory mechanism could be responsible for the lack of effect on the survivin levels when the Ral proteins are depleted individually.

We also found that depletion of Akt1/2 or c-Raf by siRNA was unable to reduce survivin levels suggesting that the Pi3K/Akt1/2 and c-Raf/Mek/Erk pathways are not required for maintaining high survivin levels. However, it is important to point out that Pi3K could still be involved since it is upstream of Akt1/2 and it has additional effector than Akt1/2. Therefore use of siRNA to Pi3K may help to delineate if Pi3K is required for maintaining survivin levels in mutant K-Ras harboring cells. In contrast to this, since we saw reduction of Erk activation following depletion of c-Raf, we believe that the Raf/Mek/Erk pathway is not required to maintain high levels of survivin.
Our studies that demonstrated that the decreased survivin levels following Ral depletion required the expression of p53 (see above) suggested that Ral proteins may regulate p53 levels. Indeed, we have found that depletion of Ral GTPases increases the levels of p53 and this is majorly by affecting the stability of the protein. RalA and/or RalB depletion leads to significant increase in the half-life of p53 as well as phosphorylation of p53 at Ser-15. Phosphorylation of p53 has been used as a marker of p53 stability and we believe that Ral GTPase may affect the stability of p53 by affecting this phosphorylation site. While we used Ser-15 as a marker, our studies do not rule out the possibility of other phosphorylation sites and other post-translational modifications that are known to affect p53 stability. Therefore, future studies that investigate how depletion of Ral proteins leads to increased p53 protein stability could give further insight about the molecular mechanism.

The depletion of the Ral proteins resulted in increased levels of p53, and this p53 appears to be functional, as demonstrated by modulation of its target genes p21 and survivin. Indeed, depletion of Ral proteins led to increased expression of p21 and decreased expression of survivin in a p53-dependent manner. Depletion of RalA and/or RalB increased p53 levels in some cell lines more than did the depletion of K-Ras. Furthermore, functionally while depletion of K-Ras interfered with cell cycle progression at G2/M in a p53- independent manner, depletion of RalB affect cell cycle progression at S phase in a p53-dependent manner. Therefore, we conclude that Ral GTPase may regulate the levels of p53 in a K-Ras-independent manner in addition to K-Ras-independent manner.
In this thesis, we also showed that depletion of Ral GTPases inhibited anchorage-independent growth and invasion in p53-dependent manner. Despite previous studies that showed the importance of RalA but not RalB in anchorage-independent growth and in-vivo tumor formation, in our studies we found anchorage-independent growth to be potently inhibited in cells where RalB was depleted. Finally, We have recently shown that ectopic expression of CA K-Ras V12, RalA 72L and RalB 72L down regulated the expression of ectopically expressed p53 and inhibited its ability to up regulate the transcriptional activation of the promoter of its target gene p21 in normal mouse embryo fibroblasts. In contrast, ectopic expression of constitutive active RalB 72L in A549 cells that already harbor mutant K-Ras, failed to reduce p53 levels (data not shown).

In summary, in this thesis we have provided evidence that some human cancer cells require the expression of K-Ras and Ral GTPases to maintain high levels of survivin and low levels of p53, and this appears to be mediated at least in part by mechanisms that regulate the stability of both survivin and p53. Using several human cancer cell lines from different lineages, we have discovered that in cells that harbor mt K-Ras and WT p53, down regulation of K-Ras, RalA, RalB but not c-Raf and Akt1/2 leads to p53 Ser15 phosphorylation and a significant increase in its half life and stability as well as regulation of p53 target genes (i.e. p21, mdm2, survivin) and inhibition of cell cycle progression, soft agar growth and invasion. Our data suggest that K-Ras and Ral GTPases suppress p53 levels and their depletion increases p53 stability and reactivates it to suppress malignant transformation. The following working model for the role of K-Ras and Ral in the maintenance of survivin and p53 protein levels is proposed.
Figure 27. Role of K-Ras, RalA and RalB in the regulation of p53 and survivin. In cancer cells that harbor wt-Ras, regardless of the mutation status of p53, depleting K-Ras, RalA or RalB by siRNA fails to affect the protein levels of p53 or survivin. In cells that harbor mt-K-Ras and mt-p53, depleting K-Ras reduces survivin protein levels but fails to affect the levels of p53. On the other hand, in mt-K-Ras/wt p53 harboring cells, depleting RalA, RalB or K-Ras induces p53 and reduces survivin protein levels.
About the Author

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