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MaKendra L. Umstead Date

Aurora Kinase A Promotes Oncogenic Signaling Through Novel Protein-Protein Interactions

By

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Graduate Division of Biological and Biomedical Science
Cancer Biology

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Aurora Kinase A Promotes Oncogenic Signaling Through Novel Protein-Protein Interactions

By

MaKendra L. Umstead
B.S., North Carolina Central University, 2011

Advisor: Haian Fu, Ph.D.

An abstract of
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Abstract

Aurora Kinase A Promotes Oncogenic Signaling Through Novel Protein-Protein Interactions

By MaKendra L. Umstead

Cancer is a collection of diseases driven by genomic changes that alter normal protein-protein interactions, induce aberrant cellular signaling, and drive cellular transformation. Aurora Kinase A (Aurora A), a mitotic kinase that is amplified in several cancer types, has emerged as a compelling target for cancer therapy. While increased expression of Aurora A correlates to a worse prognosis for cancer patients, the impact of Aurora A overexpression on protein-protein interactions, oncogenic signaling, and cancer development remains unclear. This work describes the discovery and characterization of the novel interaction of Aurora A with two important mediators of cancer growth and development: H-Ras and Forkhead box transcription factor, FOXO1.

The Ras-mitogen activated protein kinase (MAPK) signaling cascade is a critical pathway for sustained cell growth and proliferation in cancer. We validated the interaction of Aurora A and H-Ras and determined that the kinase domain of Aurora A and the N-terminal Switch I and II domains of H-Ras are involved in binding. Aurora A positively regulates this pathway by forming a protein complex with H-Ras and Raf-1, the Ras effector that mediates MAPK signaling. Aurora A stabilizes the H-Ras/Raf-1 protein complex and enhances MAPK signaling in a H-Ras-dependent manner. We also determined that the kinase activity of Raf-1 also functions to enhance binding of the Aurora A/H-Ras/Raf-1 protein complex.

Aurora A also promotes oncogenic signaling through negative regulation of the tumor suppressor, FOXO1. In response to cell stress, FOXO1 localizes to the nucleus to initiate transcription of pro-apoptotic genes. Aurora A was found to interact with FOXO1, promoting exclusion of FOXO1 from the nucleus and inhibition of cell death.

Overall, this work demonstrates that through novel protein-protein interactions, Aurora A functions as a positive regulator of oncogenic Ras-MAPK signaling and as a negative regulator of the tumor suppressive activity of FOXO1. This provides two potential therapeutic protein-protein interaction targets for cancers with Aurora A overexpression, as inhibition of either the Aurora A/H-Ras or Aurora A/FOXO1 interactions may reduce pro-growth signaling and induce cell death. Ultimately, understanding the role of Aurora A in cellular signaling will provide new opportunities to develop targeted therapies for cancer.

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Chapter 1: Introduction

1.1 Cancer and its characteristics

Cancer is defined as a collection of diseases in which normal cells undergo a step-wise transformation that leads to uncontrolled growth, proliferation, and metastasis [1]. As tumor growth at the primary and metastatic sites can interrupt the function of essential organs and ultimately lead to death, the detection and treatment of cancer is vital to patient survival. In the United States, 2016 estimates show that approximately 1.6 million people will be diagnosed with cancer (14 million cases diagnosed worldwide) [2, 3]. This makes cancer a leading cause of death in the United States, second only to heart disease [2]. Early diagnosis and evolving treatment options have improved the survival rate for cancer, yet 6 hundred thousand individuals are estimated to die from the disease in the United States in 2016 (8.2 million deaths worldwide) [2, 3]. To increase the survival rate for patients diagnosed with cancer, the continued development of new and effective therapeutic options is imperative. Growing knowledge of the molecular characteristics associated with cancer has aided the development of cancer therapies. Specifically, targeted drugs that are active against cancer-specific alterations, selectively killing cancer cells while leaving normal cells intact, are able to reduce disease burden and prolong survival in patients with less side effects than traditional chemotherapy [4].

In 2000, the potentially targetable molecular characteristics that are common to cancer cells were grouped and defined by Douglas Hanahan and Robert Weinberg as the "Hallmarks of Cancer [1]." This paradigm assigns phenotypic categories to the molecular alterations acquired by normal cells that support cellular transformation and the creation of a tumor microenvironment that is supportive of cancer growth [1, 5]. Of the ten hallmarks of cancer shown in Figure 1-1 (sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation,

activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, and deregulating cellular energetics), the ability for cancer cells to sustain proliferative signaling and evade growth suppression are perhaps the most fundamental to cancer development [5]**.** Normally, cells undergo tightly regulated growth and proliferation processes that are activated as a result of extracellular signals. Through genetic and epigenetic alterations that enable oncogenes and disable tumor suppressors, cancer cells acquire the ability to uncouple extracellular signals from intracellular proliferative responses [1]. Extensive research and increasingly sophisticated technology has allowed many of the molecular mechanisms that drive sustained proliferative signaling to be described. However, the rise of genomic sequencing of patient tumors continues to reveal more about tumor biology and uncover additional alterations whose importance remain to be characterized. Cancer treatment is at the forefront of precision medicine, in which patients are treated based on the specific mutations detected in the tumor. As such, defining these molecular alterations as actionable therapeutic targets will expand the arsenal of therapies available for patients, better equipping doctors and patients in the fight against cancer.

1.2.1 The role of protein-protein interactions (PPIs) in cancer

To gain insights into the biology of cancer, understanding the impact of genetic alterations on the function of proteins is critical. Proteins constitute the major building blocks in the signaling pathways that underlie all physiological processes. The interactions between proteins, whether transient or permanent, create signaling pathways through which information is communicated and commands are enacted throughout the cell [6]. Thus, protein-protein interactions (PPI) serve as the backbone of cell processes ranging from growth, metabolism, DNA replication, and cell-cell communication (Figure 1-1) [7, 8]. General

examples of PPIs that impact cell signaling include ligand-receptor interactions, enzyme-substrate activities, and homo- or hetero-dimerization [8].

Aberrant regulation of PPIs enables cancer cells to sustain proliferative signaling, evade growth suppression, and acquire additional cancer hallmarks [8]. For example, gain of function mutations in growth factor receptors such as epidermal growth factor receptor (EGFR) can induce activating dimerization and initiate downstream pro-growth signal transduction in the absence of extracellular ligand binding [9]. Mutations found in enzymatic proteins including protein kinases and guanosine triphosphate hydrolase enzymes (GTPase) also drive the misregulation of PPIs. Common mutations in oncogenic kinases result in hyperphosphorylation and subsequent activation of substrates involved in proliferative signaling pathways [10]. Mutations in GTPases, another family of enzymatic proteins, can alter the conformation of the protein, resulting in deregulation and enhanced binding and activation of effector proteins that promote cell cycle progression, growth, and motility [11-13].

Due to the prominent role of protein interactions in tumor biology, targeting PPIs is an attractive approach for the development of new therapies for cancer [7]. Protein kinases are of the most targeted molecules in cancer drug discovery [14]. Inhibiting the catalytic activity of kinases reverses the upregulated phosphorylation of substrates and blocks the resulting PPI-mediated kinase cascades. Several classes of monoclonal antibodies and small molecule inhibitors have been successfully developed to target activating mutations in growth factor receptors either by blocking dimerization or the inhibiting the enzymatic activity of receptor tyrosine kinases [15, 16]. However, not all PPIs are mediated through enzymatic activity and the targeting of many critical PPIs pose a much greater challenge. PPI interfaces are often large and hydrophobic, contain non-contiguous binding sites, lack deep binding pockets, and exist independently of enzymatic activity [7]. For example, Ras GTPases are among the most

highly mutated oncogenic drivers, yet remain elusive to current targeting strategies due to their small size and lack of distinct binding pockets [17].

Despite the aforementioned challenges, several drugs that are currently in clinical development provide evidence for the importance of identifying and targeting novel PPIs. Inhibitors of the Mouse Double Minute 2 homolog (MDM2)/p53 interaction release the tumor suppressor, p53 from binding to a hydrophobic pocket on MDM2, block degradation of p53, and promote p53-mediated cell death [7, 18]. In another example, inhibition of the interaction of X-linked Inhibitor of Apoptosis (XIAP) and pro-apoptotic Caspase-9 by a peptide mimetic releases Caspase-9 to carry out its tumor suppressive activity [19].

The examples given here along with others found in literature demonstrate the feasibility of PPI-targeted therapies. Although challenging, the identification of novel interactions provides a ripe opportunity to inhibit cancer progression, either by blocking interactions that sustain oncogenic signaling or releasing interactions that inhibit growth suppression.

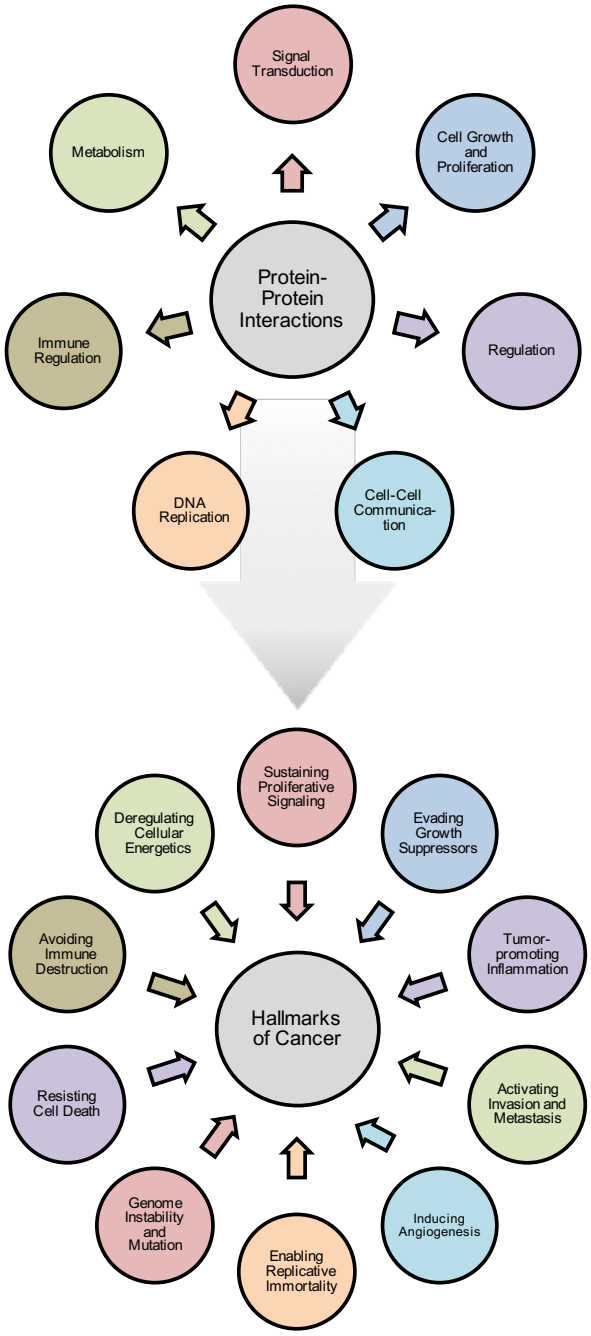

Figure 1-1. Protein-protein interactions and cancer.

Protein-protein interactions (PPIs) drive the physiological and phenotypic cellular processes underlying the hallmarks of cancer. Adapted from [5, 20].

1.2.2 PPI network mapping to unravel tumor biology

To understand how genetic alterations affect PPIs, advances in technology provide useful methods to identify new interactions and new connections between proteins and pathways that were previously unknown. The availability of genomic sequencing tools facilitated large-scale characterization of the molecular alterations found in cancer patients across tumor types. Our laboratory and the Emory Chemical Biology and Discovery center set out to utilize this information to establish the PPI landscape for different cancer types. By assaying PPIs using cancer-associated proteins in a high-throughput format, cancer genomics-informed PPI network maps that establish the PPI landscape in cancer cells and identify PPI hubs as putative nodes for therapeutic targeting can be revealed. To do this, a high-throughput PPI screen was conducted by collecting a gene library of cancer drivers, tumor suppressors, and other cancer-associated genes and testing their binary interactions using lysate-based Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) and the cell-based Renilla luciferase Protein Fragment Complementation Assay (PCA).

Interestingly, Aurora Kinase A (Aurora A), a serine/threonine kinase involved in mitosis, was identified as a PPI hub among the cancer-associated genes that were tested. To probe for potential therapeutic targets for cancer, we then sought to evaluate the function of specific interactions that were identified. Taken together, the evidence presented supports the disruption of these novel Aurora A PPIs as a potential mechanism for targeting Aurora A in cancer.

1.3.1 Aurora kinases

The finding of Aurora Kinase A (Aurora A) as a hub from our PPI network mapping was compelling due to the rise of Aurora A as a target for cancer therapy in recent years. First

discovered in the late 1980s using Drosophila as a model to search for cell cycle regulatory genes, scientists found that a homozygous mutation in a specific gene exhibited mitotic defects in centrosome separation and the formation of monopolar spindles [21]. The resulting pattern resembled the Aurora borealis observed at the North Pole, thus receiving the name Aurora. Soon following, paralogs were found in *Xenopus*, *Saccharomyces cerevisiae* (Aurora/Ipl1), *Caenorhabditis elegans* (AIR-1 and AIR-2), mouse, and rat (Aurora and Ipl1-like midbody-associated protein, AIM-1) [22, 23]. In human cells, three isoforms were identified: Aurora A, Aurora B, and Aurora C. Because of the conserved nature of the protein throughout evolutionary species, Aurora A was thought to serve a critical role in organismal development. Additional work revealed that Aurora kinases are serine/threonine kinases, and that this activity is critical to mitosis. Further elucidating the role of Aurora kinases in the cell cycle of mammalian cells, specific knockdowns of the Aurora isoforms revealed that the different Aurora proteins initiate distinct processes during mitosis and cooperate to facilitate centrosome function, chromatid separation, and cytokinesis [24]. Overall, Aurora A and Aurora B are the most well characterized, partially due to their abundance in cellular tissues (Aurora C expression is primarily in the testes) [24].

1.3.2 Aurora A protein structure

The domains of Aurora family proteins can be separated into the N-terminal regulatory domain, the kinase domain, and a C-terminal domain (Figure 1-2) and the N-terminal varies in length and amino acid sequence between the three isoforms found in humans. In total, Aurora A is 403 amino acids and 46 kDa in size. The approximately 130 amino acid long N-terminal domain is unstructured compared to the kinase domain which largely occupies the remainder of the protein [25, 26]. Within the N-terminal domain of Aurora

A is the A-Box (also called the D-box-activating-box), QRVL, which is involved in the degradation of the protein. A KEN box, named for its core sequence, is also located within this region but does not contribute to directly contribute to Aurora A degradation [27-30].

The family of Aurora proteins share a conserved catalytic domain and C-terminal domain. In fact, Aurora A and B share 71% homology [31]. Resolution of the crystal structure of the catalytic domain revealed that like most kinases, this region of Aurora A contains two lobes that are joined by a hinge [32]. The N-terminal lobe of the kinase domain, made up of five-stranded β-sheet and an α-helix, binds adenosine triphosphate (ATP) through the α-helix. Lysine 162 in Aurora A is critical for ATP binding, as mutations in this site (K162R) abolish Aurora A kinase activity [33]. The C-terminal lobe of the kinase domain, made up of seven α-helices and two β-sheets, contains the activation loop and substrate binding regions. To be catalytically active, Aurora A requires phosphorylation at a threonine in the activation loop (T288). Both p21-activated-kinase (PAK) and protein kinase A (PKA) are candidate kinases for phosphorylation at this site [34-36].

Figure 1-2. The structure of Aurora A.

The N-terminal domain of Aurora A contains a KEN box and an A-Box that is involved in protein degradation. The kinase domain spans the majority of the protein, and phosphorylation at T288 is critical for kinase activity. The D-box is located in the C-terminal region of Aurora A and is the site of degradation by the anaphase promoting complex (APC/C) [25, 26].

1.3.3 Aurora A regulation

In normal cells, Aurora A protein expression levels vary throughout the cell cycle. Protein expression increases starting at the S-phase and peaks during the G2/M phases. Transcription factors identified to promote Aurora A gene expression include: the ETS family of transcription factors downstream of Ras-mitogen activated protein kinase (MAPK) signaling hypoxia-inducible factor 1-alpha (HIF-1α), signal transducer and activator of transcription 5 (STAT5), and GATA binding protein 3 (GATA3) [37].

The kinase activity of Aurora A is regulated through PPIs and post-translational modifications. Aurora A kinase activity also peaks at the G2/M transition and is enhanced by binding to Ajuba and targeting protein for Xklp2 (TPX2). The protein phosphatase PP1 serves as a negative regulator of Aurora A, dephosphorylating the protein at the T288 site. As the predominant activator of Aurora A during mitosis, TPX2 binds to the C-terminal region of Aurora A (amino acids 130-403) and induces a conformation that protects against T288 dephosphorylation [24]. Additionally, the N-terminal domain of Aurora A is inhibitory and helps to maintain the kinase in an inactive state. Binding partners, such as TPX2, that interact with the C-terminal region of Aurora A displace the N-terminal domain [41]. As mitosis ends, Aurora A is degraded via ubiquitination and proteasome targeting by the anaphase promoting complex in complex with cdh1 (APC/C^{Cdh1}) in a process that involves recognition of the N-terminal A-box and D-box [27-29, 42, 43]. The D-box, RXXL, is located in the far C-terminal domain of all Aurora isoforms and is also involved in degradation. Like the N-terminus, this region also has a disordered tertiary structure [25, 26].

The N-terminal domain of Aurora A is also involved in regulation its localization. Using fluorescently-tagged proteins, the presence of Aurora A has been tracked throughout

the cell cycle. Prior to mitosis, low levels of Aurora A are detected in the pericentriolar material around the centrosomes as they separate during the S-phase/G2 transition. Aurora A expression is then upregulated prior to mitosis and maintains localization at the centrosomes where it associates with microtubules near the spindle poles [24].

1.3.4 Aurora A in tumorigenesis

In humans, the Aurora A gene is located on chromosome 20q13, which is often amplified in a variety of cancer types, including breast, ovarian, lung, prostate, and glioblastoma [25, 44]. It is overexpressed at the DNA, RNA, and protein level in tumors and correlates to a worse prognosis for patients. For example, studies into Aurora A protein expression and brain cancer found that Aurora A levels increased according to the severity of brain cancer, with Glioblastoma having the highest expression levels [45].

Whether Aurora A is a bona-fide cancer driver gene remains controversial. Although Aurora A facilitates transformation in NIH 3T3 and Rat1 fibroblasts, the ability for Aurora A misregulation to lead to tumor formation likely depends on other accommodating mutations in the cell [46, 47]. Due to its role in mitosis, over- or under-abundance of Aurora A is detrimental to normal cell function [25]. Contributing to genomic instability in cancer, centrosome amplification often results when overexpressed Aurora A leads to cytokinesis failure [24]. In normal cells, this defect would be recognized at the mitotic checkpoint. In cancer cells, however, co-occurrence of loss of the p53 tumor suppressor allows cells to proceed through another cell division in these conditions [48].

1.4.1 Non-canonical functions of Aurora A

Although the majority of Aurora functions previously characterized are related to mitosis, research is beginning to reveal a myriad of additional roles for Aurora A (Figure 1-3). The findings give way to a more comprehensive understanding of the oncogenic roles of Aurora A in cancer that are mediated through PPIs. Selected Aurora A functions that are relevant to this dissertation are described here.

1.4.1.i Aurora A PPIs with GTPases and their regulators

GTPases function as molecular switches, regulating pathways that are integral to several cellular process. Aurora A interacts with a number of GTPases and their regulators, leading to both regulation of Aurora A activity and modulation of GTPase hydrolysis. In yeast two-hybrid experiments, Aurora A was identified to interact with RasGAP, a Ras GTPase activating protein [49, 50]. Further work revealed that RasGAP also inhibits Aurora A kinase activity *in vitro* [49, 50]. Aurora A also directly interacts with and phosphorylates RalA (Ras-like protein A), altering its membrane localization. The Aurora A/RalA interaction activates both RalA and its effector protein, RalBP1 [51]. Activation of RalA promotes tumorigenesis through conferring the ability for anchorage-independent growth, translational modulation, and vesicle trafficking, thus providing a mechanism by which Aurora A promotes tumor development [52]. The positive regulator of RalA is the guanine exchange factor (GEF), RalGEF, which also serves as a substrate for Aurora A. Aurora A also forms a complex with and activates Rap-1A, a GTPase that is often deregulated in cancer and plays a role in cell proliferation and adhesion [53]. These studies establish a precedent for the interplay between Aurora A, GTPases, and GTPase associated proteins and suggest that these PPIs are important in cancer progression.

1.4.1.ii Aurora A PPIs with transcription factors

Aurora A also interacts and regulates several transcription factors, controlling genetic programming in cells. For example, Aurora A acts to destabilize p53 through phosphorylation at S215, which prevents p53 from binding DNA and initiating transcription of pro-apoptotic genes like p21 [54]. Phosphorylation of p53 by Aurora A at a distinct site, S315, promotes MDM2 binding, ubiquitination and degradation [55]. In another example, Aurora A acts to stabilize oncogenic N-Myc [56, 57]. Aurora A blocks ubiquitin-dependent degradation of N-Myc by FBxw7, promoting N-Myc stability and increased tumor burden [58]. Interestingly, allosteric inhibitors that effectively destabilize the interaction of Aurora A and N-Myc have been already been developed [56]. Thus, targeting the interaction of Aurora A with transcription factors also represents a viable mechanism for counteracting the oncogenic effects of Aurora A overexpression in cancer.

1.5.1 Novel Aurora A interaction partners

The work presented in this dissertation describes the validation and characterization of the functional effects of oncogenic Aurora A interactions with two novel partners: H-Ras, a GTPase, oncogene, and promoter of cell growth, and FOXO1, a transcription factor and key tumor suppressor involved in regulating cell death. These Aurora A PPIs were interesting due to the prominent role of H-Ras and FOXO1 in tumor progression and development. The identification of a novel regulator for both proteins furthers our understanding of tumor biology that can be utilized for the development of new therapeutics.

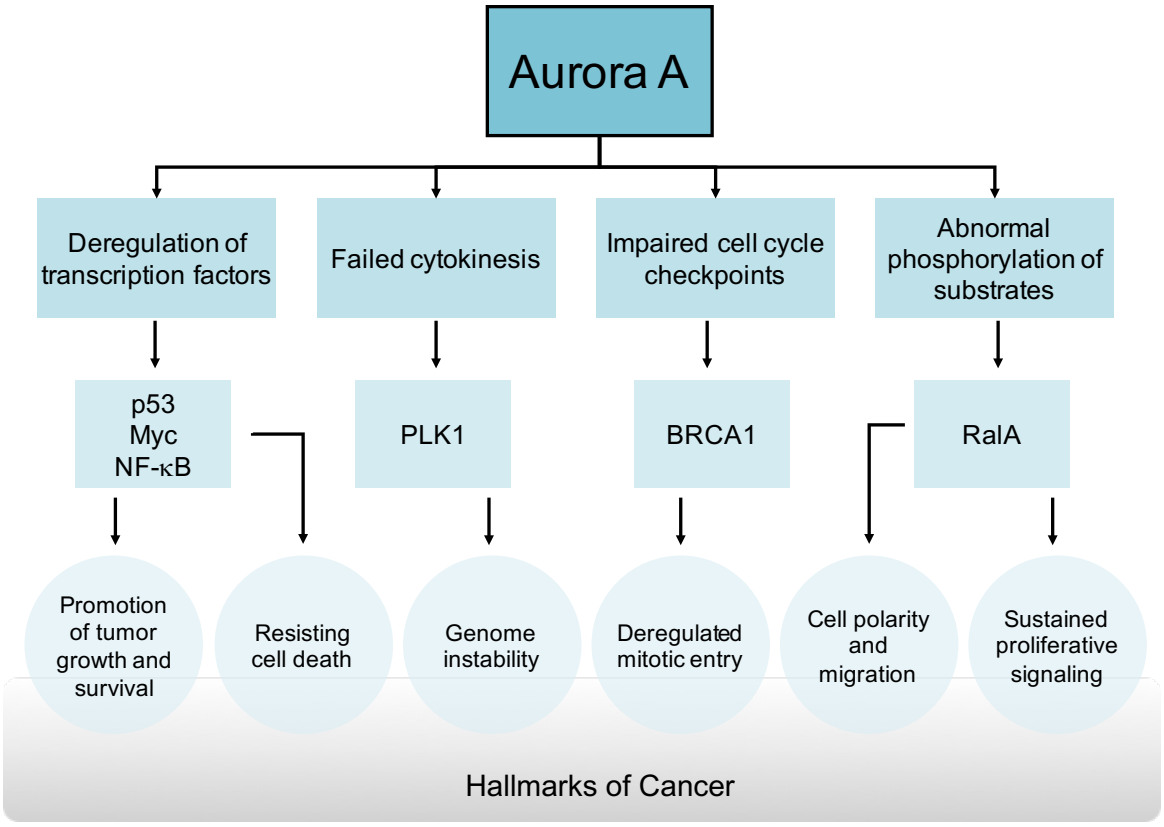

Figure 1-3. Aurora A signaling in cancer.

Overexpression of Aurora A in cancer affects multiple pathways that contribute to the development of cancer. Adapted from **[25]**.

1.5.2 Ras

Biochemical and genetic studies have revealed Ras as a central regulator of signal transduction and oncogenic transformation. Acting as a molecular switch, Ras plays a central role in transmitting cell growth from extracellular stimuli to cellular responses [59].

1.5.2.i Ras protein structure

The family of Ras proteins are 21 - 25 kDa in size, and contain three major isoforms, H-Ras, K-Ras, and N-Ras, and two lesser-characterized isoforms, M-Ras and R-Ras [60-62]. Ras proteins belong to a larger family of GTPases, which function as binary switches, catalyzing the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) [63]. This hydrolysis allows GTPases to cycle between active and inactive states, respectively, in response to activation signals. When GTP is bound, Ras proteins maintain a conformation that allows for binding to effector proteins and the initiation of several signaling pathways that are influential in cancer progression [64].

Like all GTPases, Ras proteins contain a G-domain, including two key structural domains, switch I and II, which change in conformation when Ras is in the active, GTP-bound or inactive, GDP-bound bound state [64]. This region is identical between H-Ras, K-Ras, N-Ras [64]. Ras effector binding domains also lie within this region. Lastly, the hypervariable C-terminal is critical for Ras localization to the plasma membrane, which is an essential step for Ras activity and function (Figure 1-4).

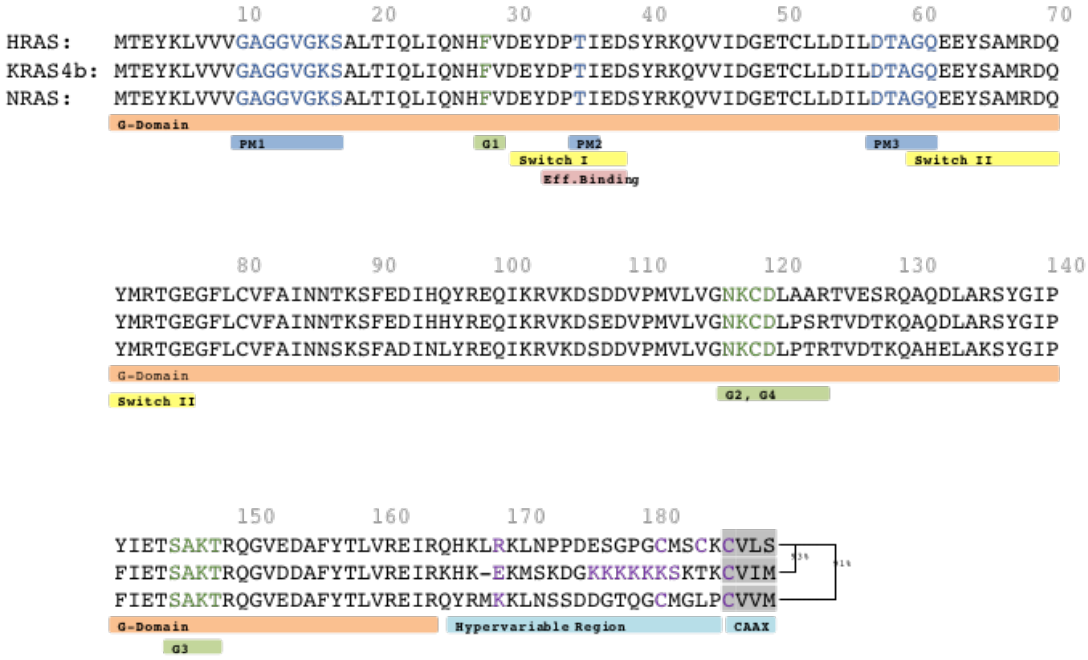

Figure 1-4. The structure of Ras proteins.

For Ras family proteins, there are six conserved sequence motifs: PM1, G1, PM3, G2, G4, and G3. PM motifs are involved in phosphate/magnesium binding, while G motifs mediate guanine base binding. Also located within the G-domain are two key structural domains: switch I and II. These domains, amino acids 30-38 and amino acids 60-76, respectively, change in conformation when Ras is GTP or GDP bound. The G domain also contains the effector-binding domain (amino acids 32-40). This region mediates binding of Ras proteins to various effectors. The specificity of Ras binding is determined by the amino acids surrounding this region. The G-domain of the Ras family members is identical in sequence. The C-terminal domain of Ras proteins is the hypervariable region which is responsible for anchoring Ras to the plasma membrane.

1.5.2.ii Ras regulation

Ras functions as an intracellular mediator of extracellular mitogenic stimuli that bind to growth factor receptors on the cell. Therefore, receptor tyrosine kinase (RTK) signaling is a major pathway that leads to Ras activation. Through a series of docking proteins, RTKs influence the direct regulators of Ras activity: GTPase activating proteins (RasGAPs) and guanine exchange factor (RasGEFs). RasGAPs facilitate GTP hydrolysis by Ras and RasGEFs enhance GTP binding of Ras in cells [65]. These proteins help control Ras cycling and enhance Ras activity levels in cells compared to the intrinsic Ras activity that is observed *in vitro* [65]. When growth factors bind RTKs, the dimerization and transphosphorylation of these receptors recruit growth factor receptor-bound 2 (GRB2) to docking sites on the intracellular surface of the receptor. GRB2 also binds and recruits the RasGEF, Son of sevenless (SOS), to the complex at the plasma membrane. SOS then promotes Ras activation and the initiation of downstream signaling by Ras (Figure 1-5) [66].

Post-translational modifications are also essential for Ras activity. Farnesylation of Ras at the C-terminal hypervariable region (HVR) allows trafficking and tethering of Ras to the plasma membrane [66]. At the endoplasmic reticulum, the CAAX sequence undergoes processing in which farnesyl transferase adds a farnesyl group to the cysteine residue and proteolysis removes the –AAX. This is followed by carboxyl methylation [67]. In addition to farnesylation, phosphorylation events have been found to drive regulation of K-Ras. Phosphorylation by protein kinase C (PKC) regulates the association of K-Ras4b with the plasma membrane [68]. Other lesser characterized post-translational modifications including ubiquitylation, nitrosylation, isomerization, ribosylation, and glucosylation events have also been identified as modifiers of Ras proteins and their functions [66].

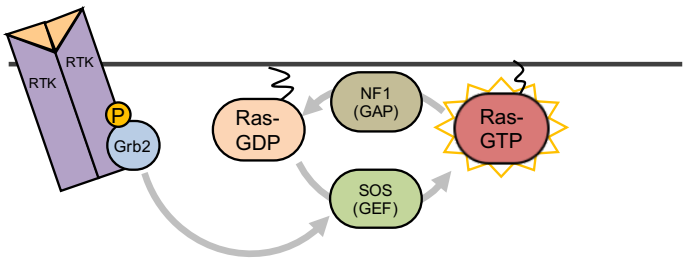

Figure 1-5. Ras activation by receptor tyrosine kinases (RTKs).

After binding of a mitogenic stimulus to RTKs, autophosphorylation of tyrosines in the intracellular tail of the receptor allows docking of Grb SH2 adapter proteins. A RasGEF, son of sevenless (SOS), binds to phosphorylated RTKs through the GRB2 adapter protein and serves as a GEF for Ras, enhancing GTP binding and activity.

1.5.2.iii Ras in tumorigenesis

Ras mutations occur in 30% of all cancers [69]. When Ras is not mutated, it is often upregulated by alterations present in its regulators such as mutations in RTKs, or deletions of RasGAP proteins [70-72, 73]. In total, 97-99% of all RAS mutation in cancer occur in codons 12, 13, and 61 [69]. Both H-Ras and K-Ras are commonly mutated at codon 12 and 13. These hotspot genetic point mutations found in H- and K-Ras substitute a glycine for valine or aspartate (G12V or G12D). This results in a change in the overall conformation of Ras that mimics the active, GTP-bound form [69, 74, 75]. When active, Ras recruits three distinct effectors: Raf, PI3K, or RalGEF [76-78]. Specifically, pro-growth and pro-proliferative signaling is sustained through activation of the mitogen-activated protein kinase (MAPK) signaling pathway [79]. This signaling cascade, involving Ras-Raf-MEK-ERK, promotes the transcription of genes directly involved in growth and proliferation, including cyclin D, Myc, and, interestingly, Aurora A (Figure 1-6).

Although its role in cancer has long been established, attempts to Ras have been largely unsuccessful [80]. The clinical failures of farnesyltransferase inhibitors (FTIs) for the treatment of cancer revealed a key difference between H-Ras and K-Ras modifications: H-Ras, N-Ras, and K-Ras 4A require palmitoylation at the Golgi; however, K-Ras4B does not [67]. As a result, cancers driven by K-Ras4B were not sensitive to FTIs [81]. Nucleotide analogs and small molecule binding inhibitors have also stopped short as viable approaches to target the protein [80]. Thus, targeting of downstream Ras effectors rather than the protein itself has become the approach of choice to mitigate the oncogenic role of Ras in cancer [80]. Understanding how Ras is regulated through novel PPIs may provide new methods to effectively target Ras directly.

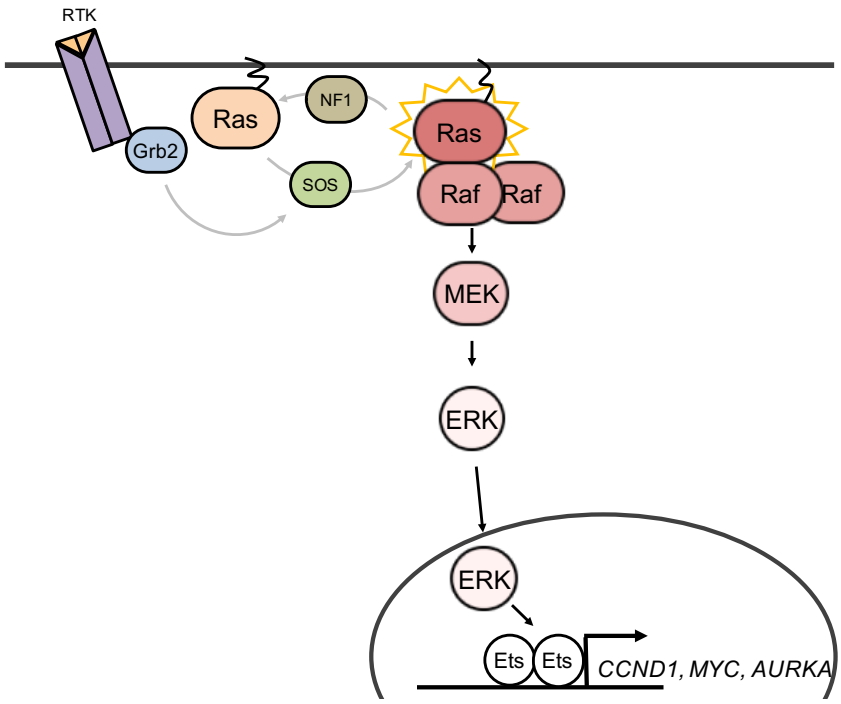

Figure 1-6. The mitogen-activated protein kinase (MAPK) signaling pathway.

Activated Ras recruits Raf (MAPKKK) proteins to the plasma membrane. Here Raf proteins dimerize, allowing transformation that actives Raf kinases. Activation of Raf then spurs the MAPK signaling cascade, amplifying the signaling through phosphorylation of MEK (MAPKK) and ERK1/2 (MAPK). When ERK is activated, the protein translocates to the nucleus. Nuclear translocation of ERK allows ERK to phosphorylate transcription factors (Ets, c-Fos and c-Jun) for genes directly involved in growth and proliferation, including but not limited to: CCND1 (cyclin D, progression through the G1/S restriction point), MYC (Myc, transcription factor targeting several proliferative genes), and AURKA (Aurora Kinase A, facilitates mitosis).

1.6 Forkhead box protein of the O class (FOXO1)

FOXO1 belongs to a large family of transcription factors (over 41 genes identified in humans) that contain a winged-helix DNA-binding domain that is termed the forkhead box due to the observed phenotype resulting from mutations in this gene in *Drosophila melanogaster* [82, 83]. The O class has 4 members: FOXO1, FOXO3, FOXO4, and FOXO6. From apoptosis, metabolism, differentiation, longevity, and migration, alterations in FOXO proteins have diverse and significant impacts on the cell, and have been linked to the development of cancer and other diseases [83]. As transcription factors, FOXO proteins activate transcriptional programs to direct physiological processes in response to cellular signaling (Figure 1-8). FOXO1, specifically, is a tumor suppressor that is most active at the G1/S-phase entry and G2/M transition and has an inhibitory effect on the cell cycle.

Insights on the regulation of FOXO1 can be derived from its protein structure. FOXO proteins contain a forkhead domain and nuclear localization sequence (both at the N-terminal half of the protein), as well as a nuclear export signal and a transactivation domain (at the C-terminal half) [84]. Post-translational modifications at or near these sequences alter the localization, function, and degradation of FOXO1 [85]. Phosphorylation, acetylation, and ubiquitination all play a role in FOXO1 regulation and are responsive to cellular growth or stress signals in the cell. For example, growth factor signaling promotes activation of AKT. AKT can phosphorylate FOXO1 at sites that promote 14-3-3 binding and exclusion from the nucleus. As a result, FOXO1's ability to transcribe pro-apoptotic genes is inactivated [85]. In cancer cells this axis is often co-opted as a mechanism to resist cell death. AKT is one of several kinases that can phosphorylate and inhibit FOXO1. More recently, casein kinase 1, inhibitor of kappa B (IkB), and cyclin-dependent kinase 2 (CDK2), and polo-like kinase 1 (PLK1) have been identified to promote exclusion of FOXO1 from the nucleus [85, 86].

Due to its role as a tumor suppressor, FOXO1 is an attractive therapeutic target for cancer. In fact, FOXO1 is a critical mediator of indirect response to current cancer therapies. Some chemotherapeutic agents, such as paclitaxel, have been shown to reduce tumor growth by upregulating FOXO3, a close relative of FOXO1 [87]. In addition, the efficacy of Alisertib, an Aurora A kinase inhibitor, in acute myeloid leukemia (AML) models was mediated through upregulation of FOXO3a expression [88]. Reconstitution of a tumor suppressor that is mutated or deleted in cancer is a challenging task; however, inactivation of a tumor suppressor through PPIs presents a potentially actionable PPI target for therapy.

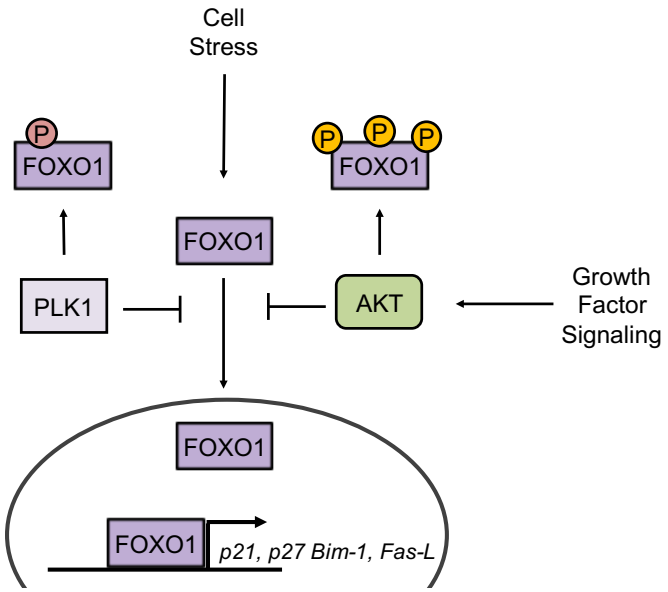

Figure 1-7. FOXO1 signaling in cancer.

In response to cell stress, FOXO1 initiates the transcription of genes that drive cell cycle arrest at apoptosis, including p21, 27, Bim-1, Fas-L, and others. Pro-growth signaling attenuates FOXO1 activity. Pictured here, AKT, a mediator of survival signaling, phosphorylates FOXO1 thereby promoting 14-3-3 binding and exclusion from the nucleus. In addition, mitotic kinase, PLK1, phosphorylates FOXO1 leading to nuclear exclusion and inactivation.

1.8.1 Aurora A and MAPK in cancer

Crosstalk between the Aurora A and Ras signaling pathways has been implied in various cancer models. For example, Aurora A and Ras co-expression potentiates oncogenic transformation in oral cancer models [89]. In addition, inhibition of Aurora A in nasopharyngeal cancer cells reduced Ras-MAPK activation and blocked cell invasion [90]. Cumulatively, these studies suggest that integration of the Aurora A and Ras pathways occurs in cancer; however, no previous work has described a mechanism by which this phenomenon occurs.

1.8.2 Aurora A and FOXO1 in cancer

Research that addresses the interplay between Aurora A and FOXO1 signaling in cancer is not as extensive as that of Aurora and MAPK signaling. However, Aurora A has an inverse relationship with FOXO1: overexpression of Aurora A suppresses transcription of FOXO1 [91]. In the converse, inhibition of Aurora A was found to upregulate FOXO1 expression [92]. Thus far, transcriptional regulation has been the primary hypothesis for the link between Aurora A and FOXO1. We propose a mechanism that is mediated through protein-protein interactions.

1.9 Scope of the dissertation

This dissertation explores the impact that Aurora A PPIs have on Ras and FOXO1 function in cancer. First, we address the promising hypothesis that Ras is also regulated by Aurora A, forming a novel signaling node and positive feedback loop that controls cell growth and oncogenic transformation. In Chapters 2 and 3, we show that the interaction of Aurora A and Ras enhances oncogenic signaling by forming a complex with H-Ras and Raf-1 and

potentiating Ras-MAPK signaling. We detail the mechanism by which this may occur, which uncovers the role of Raf-1 kinase activity in stabilizing the Aurora A/H-Ras/Raf-1 protein complex. Second, in Chapter 4, this dissertation provides preliminary evidence that the Aurora A/FOXO1 PPI may contribute to FOXO1 deregulation in cancer. We demonstrate that Aurora A interacts with FOXO1, reduces nuclear translocation in response to cellular stress, and inhibits FOXO1-induced cell death. Both studies further our understanding of the impact of Aurora A overexpression in cancer and provide a viable alternative approach to perturbing oncogenic Ras and FOXO1 signaling in cancer.

Chapter 2: Aurora Kinase A interacts with H-Ras and potentiates Ras-MAPK signaling

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2.1 Abstract

In cancer, upregulated Ras promotes cellular transformation and proliferation in part through the activation of oncogenic Ras-MAPK signaling. While directly inhibiting Ras has proven challenging, new insights into Ras regulation through protein-protein interactions may offer unique opportunities for therapeutic intervention. Here we report the identification and validation of Aurora Kinase A (Aurora A) as a novel Ras binding protein. We demonstrate that the kinase domain of Aurora A mediates the interaction with the N-terminal domain of H-Ras. Further, the interaction of Aurora A and H-Ras exists in a protein complex with Raf-1. We show that binding of H-Ras to Raf-1 and subsequent MAPK signaling is enhanced by Aurora A, and requires active H-Ras. Thus, the functional linkage between Aurora A and the H-Ras/Raf-1 protein complex may provide a mechanism for Aurora A's oncogenic activity through direct activation of the H-Ras/Raf-1-MAPK pathway.

2.2 Introduction

The Ras family of proteins (H-, K-, and N-Ras) are well characterized oncogenic drivers in a variety of cancer types [93, 94]. Ras proteins are GTPases, which function as molecular switches to activate molecular signaling cascades in response to extracellular signals. Ras binds to and hydrolyzes GTP, cycling between active, GTP-bound and inactive, GDP-bound states [95, 96]. Structurally, the switch I and II domains of Ras also change in conformation when either GTP or GDP is bound [59]. Ras activity is facilitated by GTPase-activating proteins (GAPs) that facilitate hydrolysis of GTP to GDP, thereby returning Ras to an inactive state [97-99]. Conversely, guanine exchange factor proteins (GEFs) bind to GDP-bound Ras, and help to exchange GDP for GTP and activate Ras. Point mutations in critical regions of Ras also affect activity. The oncogenic Ras G12V mutation prevents hydrolysis of

GTP, locking the protein in an active conformation [100-102]. This mutation is commonly observed in patients and is known to drive cancer progression. In contrast, the dominant negative Ras S17N mutant inhibits RasGEF activity, maintaining inactive Ras [103].

Active Ras recruits distinct effector proteins to initiate cellular signaling cascades and physiological processes [76-78]. Specifically, Ras sustains pro-growth and proliferative signaling through activation of the Ras-Raf-MEK-ERK (mitogen activated protein kinase, MAPK) pathway [104]. Hyperactive Ras-MAPK signaling increases the transcription of genes that drive the cellular growth and survival required for cancer progression [79].

Ras-MAPK signaling can upregulate transcription of the mitotic kinase, Aurora Kinase A (Aurora A) [37]. Aurora A belongs to a family of serine/threonine kinases (Aurora A, B, and C) that function in different spatial and temporal points in the cell to facilitate mitosis [22, 105]. Aurora A expression is upregulated during mitosis, where it facilitates alignment of microtubules to the centromeres, then quickly degraded during mitotic exit [23, 106]. Amplification of Aurora A occurs at the DNA, RNA, and protein levels in several cancer types, such as breast, glioblastoma, pancreatic, and bladder cancers [44, 45, 107, 108]**.** Recent literature has revealed details about the physiological impact of Aurora A overexpression beyond its canonical role in the cell cycle. For example, Aurora A can aid in oncogenic processes through forming different protein-protein interactions with many proteins, including GTPases [57, 109]. This may be facilitated by the observed mis-localization of Aurora A to both the cytoplasm and nuclear compartments in tumor tissue [110, 111]. Clinical data further support the role of Aurora A in cancer since overexpression of Aurora A is correlated with a worse prognosis and patient outcome [111, 112].

Interestingly, literature provides evidence of cooperation between MAPK signaling and Aurora A beyond transcriptional regulation. Aurora A amplification co-occurs in several

cancer types with deregulated Ras signaling [113-116]. Also, Aurora A can enhance the transformation of fibroblasts harboring activating Ras mutations [116], while knock-down of Aurora A correlates to decreased MAPK signaling [90]. Although these studies point towards a function for Aurora A upstream of MAPK signaling, how Aurora A engages the MAPK pathway is critical to further elucidate its role as an oncogene in cancer.

Here we report that the protein-protein interaction of Aurora A and H-Ras is a mechanism by which Aurora A functions upstream of H-Ras to promote MAPK signaling. The Aurora A and H-Ras interaction validated in this study provides a critical link and potential positive feedback loop between two oncogenic proteins known to drive proliferation and survival in cancer. Blocking this interaction may have promising therapeutic potential to inhibit Ras-MAPK activity in cancer.

2.3 Materials and methods

Cell culture

HEK 293T and MCF7 cells were utilized in the described experiments (American Type Culture Collection, Manassas, VA). HEK 293T and MCF7 cells were cultured in DMEM (Corning, MT10013CV, Manassas, VA) with 10% FBS (Sigma, F6178, St. Louis, MO) and 1% pen/strep at 5000I.U/ml penicillin and 5000μg/ml streptomycin (Corning, 30-001-Cl, Manassas, VA). Between passages, cells were trypsinized with 0.25% Trypsin with 2.21mM EDTA (Corning, 25-053-Cl, Manassas, VA). All cells were maintained at 37°C in a humidified atmosphere of 5% CO2.

Antibodies

Primary antibodies used for western blotting include Flag M2 at 1:3000 (Sigma; F3165), Flag-HRP at 1:1000 (Sigma; A8592), GST Z-5 at 1:3000 (Santa Cruz Biotechnology; sc-459), rabbit GST-HRP at 1:1000 (Sigma; A7340), Aurora Kinase A at 1:500 (Cell Signaling; 4718), rabbit pERK and ERK (Cell Signaling; 4370, 9102, respectively), pMEK and MEK (Cell Signaling; 9154, 4694, respectively), pRaf-1 (Cell Signaling; 9427) and Raf-1 (Santa Cruz; sc-133) all at 1:1000. Secondary antibodies include goat anti-rabbit IgG (Santa Cruz, sc-2004, Dallas, TX) and goat anti-mouse IgG (Santa Cruz, sc-2005, Dallas, TX) and were used at either 1:2500 or 1:5000 dilutions.

Pharmacological inhibitors

Sorafenib p-Toluenesulfonate Salt (S-8502) and U0126 (U-6770) inhibitors were obtained from LC Laboratories (Woburn, MA). Compounds were dissolved in dimethyl sulfoxide (DMSO) as 10mM stock and stored at -20°C. Cells were treated for 24 hours with 10μM of compounds diluted in DMSO and the indicated doses.

Serum starvation

MCF7 cells were plated in a 24-well plate at $1x10^5$ cells per well and cultured in 600μl of complete medium (as described, DMEM with 10% FBS and 1% Penicillin/Streptomycin). Cells were then transfected 24 hours after plating. Complete media was replaced with DMEM media without FBS supplementation (serum free media) 24 hours after transfection. Samples were collected for the 0 minutes-post serum stimulation time point following 24 hours in serum-free media. Then, serum (10% FBS) was added to all remaining wells. Remaining cells were collected at time points 5, 10, 15, 45, and 90 minutes after serum stimulation. Cells were

collected directly into 70µl SDS loading buffer, boiled for 5 minutes, and subjected to SDS-PAGE and western blotting.

Transfections

For experiments with ectopically expressed proteins, HEK 293Ts were transfected using X-tremeGENE (Roche, 06366546001, Basel, Switzerland). MCF7 cells were transfected with FugeneHD (Promega, E2312, Madison WI). Plated cells were transfected at a density of 60-80% confluency and performed with a ratio of 3µl transfection reagent to 1µg DNA to 100µl of serum-free media. DNA was mixed at appropriate concentrations prior to the addition of serum-free DMEM. Transfection reagent was then added and incubated at room temperature for 15 and 20 minutes (X-tremeGENE and FugeneHD, respectively). Transfection complexes were then added drop-wise to plated cells.

Plasmid construction

All plasmids of full length and truncated proteins were constructed using Gateway® technology (Invitrogen, Waltham, MA) according to the manufacturer's protocols. For GST-tagged and Venus-Flag tagged plasmids used for Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) and Glutathione-S-Transferase (GST) pull-downs, pDEST27 and pFUW vectors were used as destination cloning vectors, respectively. Amino (N-Venus) and carboxy (C-Venus) plasmids used for Venus Protein-Fragment Complementation Assay (PCA) were generated previously in the lab. Aurora A or H-Ras cDNA was PCR amplified and inserted into the pDONR201 (Invitrogen) vector using a BP reaction to generate entry cloning vectors. A LR reaction was used to clone the desired DNA into the appropriate destination vectors. Constructs were verified by restriction digest using BSRGI (NEB, Catalog,

City, State) or FastDigest Bsp1407I (Thermo Scientific, FD0933, City, State), both cutting at the T^GTACA attB1 and attB2 (entry clone) or attR1 and attR2 (destination vector) recombination sites, and DNA sequencing. Clones in pDEST-27 (GST) vectors were sequenced with forward primer 5'-AAGCCACGTTTGGTGGTG-3' and the standard T7 reverse primer. Clones in pFUW (Venus-Flag) vectors were sequenced with primer #1 5'-CGATCACATGGTCCTGCTG-3' and the standard SP6 reverse primer.

Site-directed mutagenesis

Site-directed mutagenesis was performed on the GST H-Ras vector to create the catalytically-inactive mutant (S17N) using the QuikChange™ Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene). The H-Ras S17N mutant was generated using the oligonucleotide forward primer 5'-GGCGGTGTGGGCAAGAATGCGCTGACCATC-3' and reverse primer 5'-GATGGTCAGCGCATTCTTGCCCACACCGCC-3'. Successful mutagenesis was confirmed by DNA sequencing as described previously.

Protein-Protein interaction studies

TR-FRET assay

TR-FRET was performed in 384-well black solid bottom plates (Corning Costar Cat. #3654) in a total volume of 30 μL in each well. Briefly, HEK 293T cells were transfected as described above. Cells were lysed using 0.5% NP-40 lysis buffer (0.5% NP-40, 150mM NaCl, 10mM HEPES, and Phosphatase Inhibitor Cocktail (Sigma, P5726) and Protease Inhibitor Cocktail (Sigma, P8340)). Lysates were collected and centrifuged at 13,500g for 10 minutes at 4°C to remove cellular debris. Cleared cell lysates were serially diluted in FRET buffer (20mM Tris, pH 7.0, 0.01% Nonidet-P40, and 50mM NaCl) in a 384-well plate, bringing the final volume

of diluted cell lysate to 15µL per well. Then, 15µL of diluted anti-GST-Terbium antibody (Cisbio US Inc, 61GSTTLB, Bedford, MA) was added to all wells at a final dilution of 1:1000. The TR-FRET signals were detected with an EnVision Multilabel plate reader (PerkinElmer) with laser excitation at 337 nm, emission1 at 486 nm and emission2 at 520 nm. TR-FRET signal is expressed as ratio and calculated by the following equation: TR-FRET signal = $F520/F486 \times 10^4$, where F486 and F520 are fluorescence counts at 520 nm and 486 nm for Venus and terbium emission signal, respectively. Data were presented as mean with standard deviation calculated from duplicate samples (Fig. A-1).

GST pull-down

Cells were seeded in to a 6-well plate and allowed to reach 60-80% confluency. Cells were then harvested by adding 200µL of 0.5% NP-40 lysis buffer to each well, scraping to collect cells and transfer to an eppendorf tube, and incubated at 4°C for 30 minutes. Lysis buffer components consisted of 0.5% NP-40, 150mM NaCl, 10mM HEPES lysis buffer, and Phosphatase Inhibitor Cocktail (Sigma, P5726) and Protease Inhibitor Cocktail (Sigma, P8340) at 1:1000. After incubation, lysates were centrifuged to remove cellular debris. After removing 20µl of the lysate for an input control and the debris pellet, 20µl of a 50% glutathione-conjugated sepharose bead slurry (Glutathione Sepharose 4B, Fisher Scientific, 50197956, Atlanta, GA) was added to the remaining lysate and incubated by slowly rotating for 3-4 hours at 4°C. Beads were then washed three times in 0.5% NP-40 lysis buffer by inverting 8 times with 200µl of fresh lysis buffer added each time. GST-bound protein complexes were then eluted by the addition of 20µl of 2x SDS loading buffer, boiled for 5 minutes, resolved by SDS-PAGE subjected to western blotting along with input controls.

Venus protein-fragment complementation assay

Cells were seeded into 24 well plates and transfected at 60% confluency with N-Venus or C-Venus constructs. After 24 hours, cell nuclei were stained with the addition of Hoechst 33342 (at 5µg/ml). Images were then acquired using the ImageXpress^{Micro} automated imaging high-content imaging system (Molecular Devices) with 20X objective. The standard filter set for FITC (excitation 482/35 nm and emission 536/40 nm) and DAPI (excitation 337/50 nm and emission 447/60 nm) was used for Venus and Hoechst 33342 imaging, respectively. The number of green (Venus) and total cells (Hoechst 33342) from the images were calculated using the Metamorph Analysis Cell Scoring module and presented as percent of Venus positive cells compared to the total number of cells (Fig. A-2).

Western blotting

Cell lysates were subjected to western blot analysis following protein separation by SDS-PAGE (10% acrylamide gels) and subsequent transfer to PVDF membranes at 100V for 1.5 hours. Membranes were blocked in TBST (50 mM Tris, 137 mM NaCl, 0.05% Tween, pH 7.6] containing 5% dry milk for 30 minutes – 1 hour at ambient temperature, then incubated at 4°C or ambient temperatures with primary antibodies diluted in 5% milk in TBST for appropriate times. After primary antibody incubation, membranes were washed three times with TBST for 5 minutes each prior to incubating with secondary antibody for 1 hour at ambient temperatures. For HRP conjugated antibodies, membranes were washed three times with TBST for 10 minutes each after blocking with milk prior to incubating with GST-HRP or Flag-HRP for 1 hour. Membranes were then washed three times with TBST for 10 minutes each and chemiluminescent signal (West Pico, West Dura (ThermoScientific, PI34080 or PI34076, respectively) or ECL, Amersham, 84-839, San Diego, CA) was added for 5 minutes

prior to developing by autoradiography. Proteins with the Venus-Flag epitope tag were detected by blotting with anti-Flag antibody.

2.4 Results

2.4.1 Aurora A is a novel H-Ras binding partner

To gain insight into Aurora A signaling pathways and oncogenic activities, we tested whether Aurora A directly interacted with a variety of signaling proteins, including Ras. We first used the homogeneous, solution-based time-resolved Föster resonance energy transfer (TR-FRET) assay to detect binding[117]. The assay has a stringent distance requirement (<10 nm) between two interacting partners for the generation of TR-FRET signals. Therefore, TR-FRET signals in this assay format indicate the interaction between two proteins. To monitor the interaction of Aurora A and H-Ras, TR-FRET was performed using HEK 293T cell lysate with co-expressed GST H-Ras and Venus-Flag Aurora A. Co-expression of GST H-Ras and Venus-Flag Aurora A led to the generation of TR-FRET signals in a dose-dependent manner (Fig. 2-1A). As background controls, no TR-FRET signal was detected with GST H-Ras or Venus-Flag Aurora A expression alone. Such a specific increase of the TR-FRET signal supports the direct interaction between Aurora A and H-Ras.

To confirm the Aurora A/H-Ras interaction detected by TR-FRET, a GST pull-down was performed as a secondary affinity-based binding assay. GST pull-downs were conducted with lysates from HEK 293T cells co-expressed with GST H-Ras with Venus-Flag Aurora A. Aurora A was found to pull down with GST H-Ras complex, but not in control lanes with GST (Fig. 2-1B), demonstrating the association of Aurora A with H-Ras and confirming the previous TR-FRET results.

TR-FRET and GST pull-down assays are both *in vitro* cell lysate-based assays, thus, we further validated the interaction of Aurora A with H-Ras *in vivo* by utilizing a fluorescence (Venus)-based protein-fragment complementation assay (PCA). In this assay, N-Venus or C-Venus fragments are fused to two interacting proteins. The association of these proteins leads to functional reconstitution of Venus and allows the detection of green fluorescence signal using imaging. For this purpose, Aurora A and H-Ras were fused with N-Venus and C-Venus, respectively, and co-expressed in HEK 293T cells. The percentage of cells with positive protein-protein interactions (reconstituted Venus) was revealed by fluorescence imaging. Co-expression with N-Venus or C-Venus established background (Fig. 2-1C). Co-expression of N-Venus Aurora A and C-Venus H-Ras resulted in an increase in the number of fluorescent cells compared to the expression of N-Venus Aurora A or C-Venus H-Ras with negative controls. Reconstitution of the Venus signal resulting from the interaction of Aurora A and H-Ras validates the presence of the interaction in living cells. The interaction was also detected in Cos7 fibroblast cells, MCF7 breast cancer cells, and 8-MG-BA glioblastoma cells (data not shown).

Overall, the Aurora A/H-Ras interaction was confirmed by three complementary approaches for monitoring protein-protein interactions, supporting Aurora A as a binding partner of H-Ras. Thus, the binding of Aurora A and H-Ras may provide a new mechanism for Ras regulation.

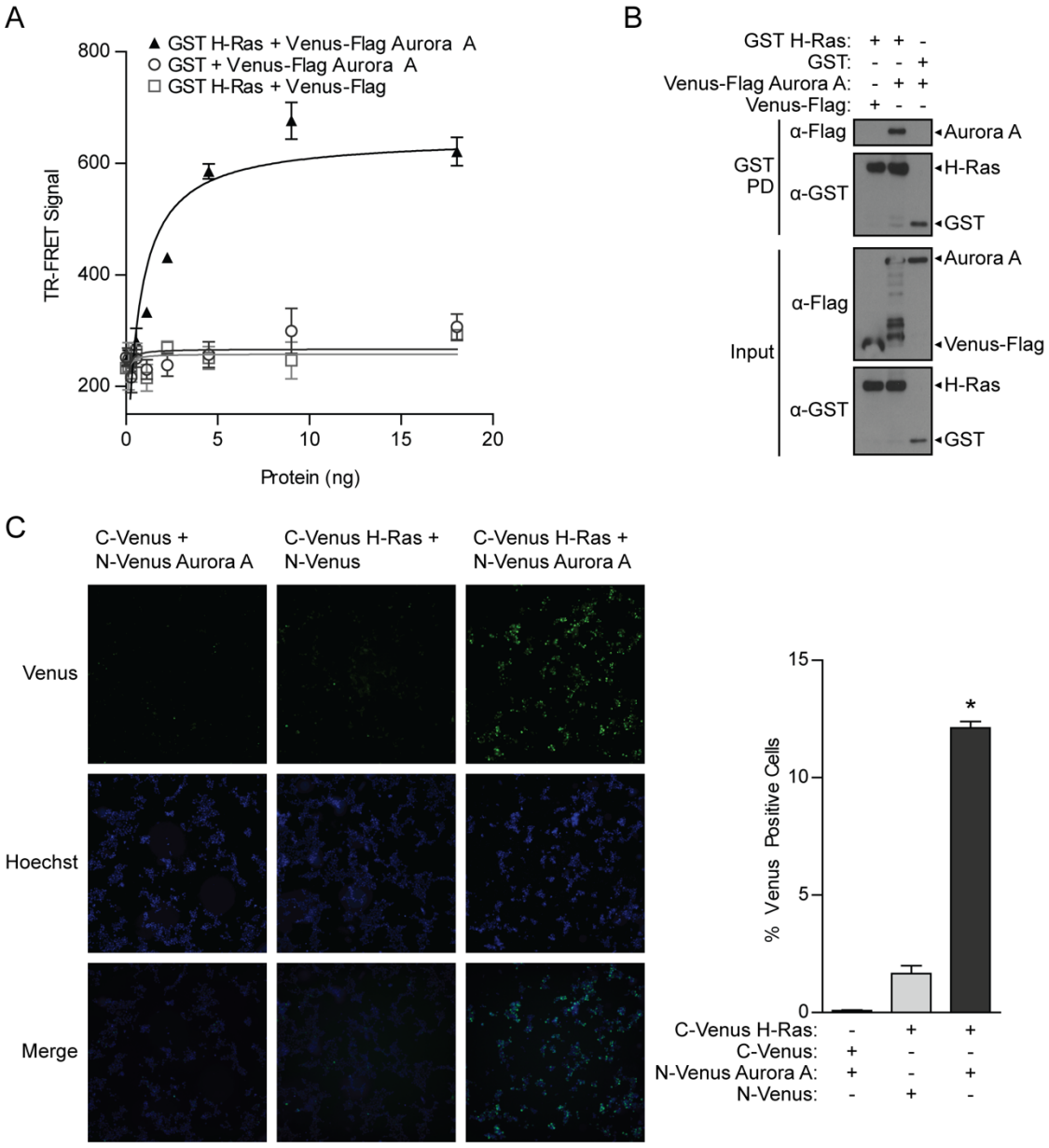

Figure 2-1. Detection of the Aurora A/H-Ras interaction.

(A) TR-FRET assay performed using lysates from HEK 293T cells in which GST H-Ras was co-expressed with Venus-Flag Aurora A or negative controls. TR-FRET signal calculated as X/Y*Z; Tb ex 340 nm; Tb em 486 nm (X); Venus em 520nm (Y); Z = 10^4). TR-FRET signals were recorded using an EnVision multilabel plate reader. Data shown are average signals with SD from duplicate samples. (B) GST pull-down assay conducted after GST H-Ras complexes

were isolated from HEK 293T cell lysates with co-expressed Venus-Flag Aurora or appropriate controls. The presence of Venus-Flag Aurora A in the GST H-Ras protein complex (GST PD) and protein expression levels in the cell lysate (Input) was detected by Western blotting using anti-Flag or anti-GST antibody, respectively. (C) A Venus protein-fragment complementation (Venus PCA) assay was conducted in living HEK 293T cells co-expressing N-Venus Aurora A and C-Venus H-Ras or vector controls. Interaction between tagged proteins allowed reconstitution of fluorescent Venus protein. The percentage of Venus positive cells was quantified by fluorescence imaging and scoring from triplicate samples. The percentage represents the number of cells with positive interactions compared to the total number of cells (determined by Hoechst staining). Representative images: Venus (positive protein-protein interaction), Hoechst (nucleus), Merge (overlap of Venus and Hoechst signals). Significance was determined using a two-tailed, two-sample equal variance Student's t-test $p<0.05$).

2.4.2 Aurora A interacts with H-Ras through the switch I and II regions

Ras proteins contain several key conserved regions that are involved in protein binding and oncogenic activity. To further characterize the Aurora A/H-Ras interaction, we next determined the structural domains that mediate binding using deletion analysis coupled with GST pull-downs. H-Ras truncations were generated and tested for their ability to bind Aurora A. The GST H-Ras truncations tested for binding are shown in Fig. 2-2A: a region that includes the switch I and II domains (SI&II, amino acids 1-66), deletion of the switch I domain (ΔSI, amino acids 36-189), deletion of the switch I and II domains (ΔSI&II, amino acids 66-189). Our results show that when co-expressed in HEK 293T cells, binding of Aurora A was detected with full-length H-Ras but not with GST (Fig. 2-2B). Aurora A was detected in complex with H-Ras SI&II and ΔSI truncations. In contrast, Aurora A was not detected in complex with H-Ras ΔSI&II. These data suggest that the N-terminal of H-Ras (amino acids 1-66) are necessary for the interaction with Aurora A since deletion of this region abrogates binding (Fig. 1-2B).

The N-terminal of H-Ras is highly conserved between the H-, K-, and N-Ras proteins. To test if Aurora A may also interact with other Ras proteins, we conducted a GST pull-down assay with the three Ras isoforms. Indeed, binding of Aurora A was detected with K-Ras and N-Ras as well as H-Ras (Fig. 2-2C).

2.4.3 The kinase domain of Aurora A mediates the H-Ras interaction

To characterize the domains of Aurora A that mediate binding to H-Ras, truncations of Aurora A were generated and tested for binding by GST pull-down. Venus-Flag Aurora A truncations are shown in Fig. 2-2D: the N-terminal and kinase domains of Aurora A (NK, amino acids 1-383), the N-terminal fragment of Aurora A (N, amino acids 1-130), the kinase

domain alone (K, amino acids 130-383), and the C-terminal domain (C, amino acids 383-403). Full length Aurora A binds to H-Ras, but not to GST (Fig. 2-2E). Binding of the NK and K truncations of Aurora A to H-Ras was detected. Conversely, no binding of Aurora A truncations lacking the kinase domain (N and C) to H-Ras was observed. This binding pattern suggests that the region of Aurora A that interacts with H-Ras lies within the kinase domain of Aurora A.

Aurora B is an isoform of Aurora A that is also linked to cancer and can enhance the transformation of fibroblasts with the H-Ras G12V mutation [89]. The kinase domains of Aurora A and Aurora B are 53% homologous [22]. To determine if Aurora B is also able to bind to H-Ras, we conducted a GST pull-down assay to test their interaction. Indeed, Aurora B was capable of binding to H-Ras (Fig. 2-2F).

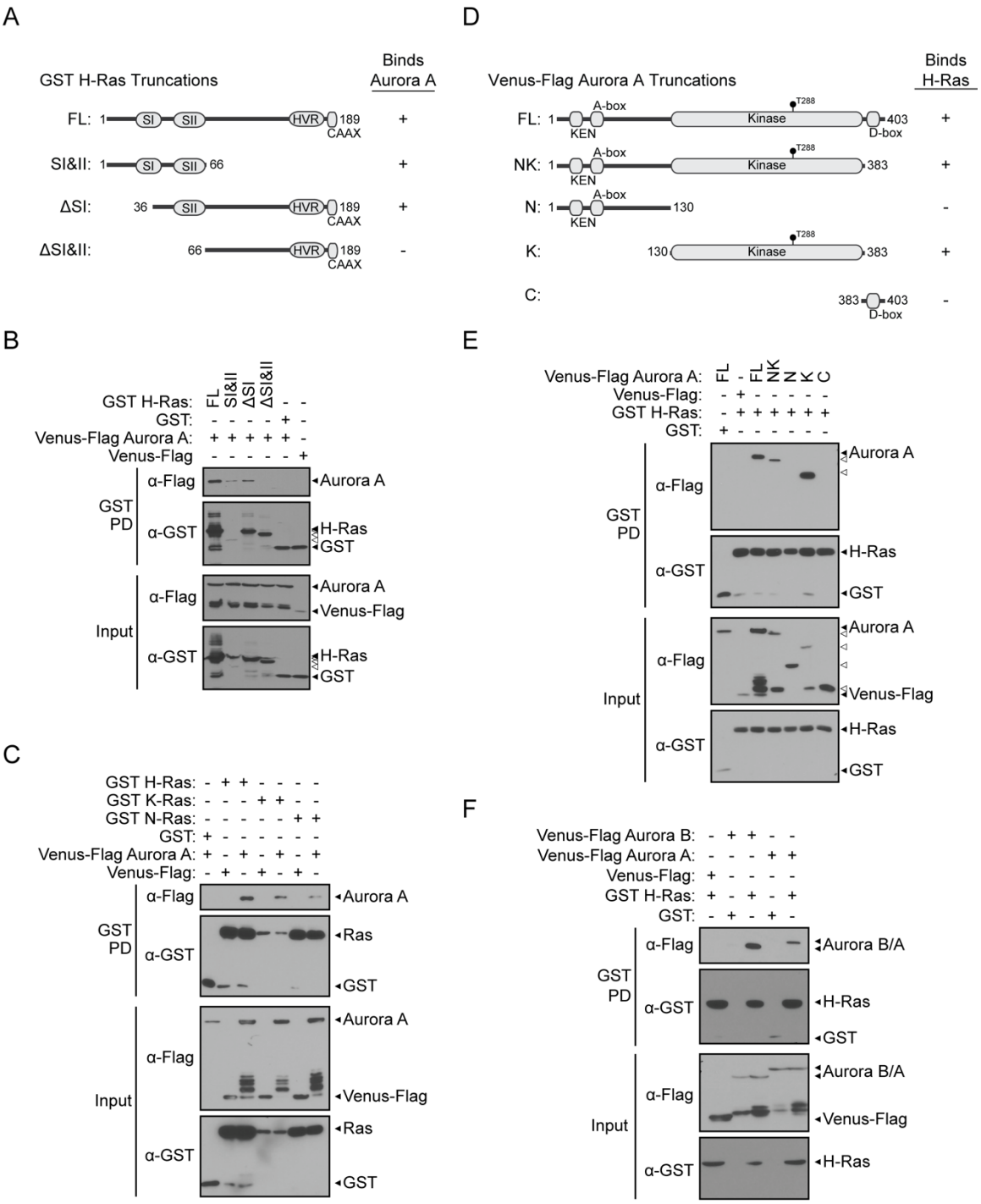

Figure 2-2. Interactions between Aurora and Ras proteins are mediated through conserved domains.

(A) Diagram of GST H-Ras protein domains and truncations used for deletion analysis: FL (amino acids 1-189), SI&II (amino acids 1-66), ΔSI (amino acids 36-189), ΔSI&II (amino acids 66-189). (B) Characterization of the H-Ras protein domain responsible for binding to Aurora A. GST pull-down conducted from HEK 293T cells co-expressing GST H-Ras truncations and Venus-Flag Aurora A. Western blotting using anti-Flag or anti-GST antibody allowed detection of GST H-Ras peptides that were able to isolate full-length Aurora A. Full-length Aurora A/H-Ras protein binding was used as a positive control. (C) Aurora A exists in protein complexes with H-, K-, or N-Ras. Binding of Aurora A as detected in GST pull-downs conducted from HEK 293T cells expressing GST H-Ras, GST K-Ras, or GST N-Ras and Venus-Flag Aurora A along with vector controls. (D) Characterization of the H-Ras binding domain on Aurora A. Diagram of Aurora A protein domains and truncations used for deletion analysis: FL (amino acids 1-403), NK (amino acids 1-383), N (amino acids 1-130), K (amino acids 130-383), C (amino acids 383-403). (E) GST pull-down conducted from HEK 293T cells co-expressing full-length GST H-Ras and Venus-Flag Aurora A truncations and analyzed by western blotting. Binding between full-length proteins served as a positive control. (F) Aurora B interacts with H-Ras. Like Aurora A, Aurora B can be isolated in a protein complex with H-Ras. Binding of Aurora B as detected in GST pull-downs conducted from HEK 293T cells expressing GST H-Ras and Venus-Flag Aurora B along with vector controls was identified by western blotting using anti-Flag or anti-GST antibodies.

2.4.4 Aurora A enhances ERK phosphorylation

Aurora A interacts with a region of H-Ras that mediates effector engagement and oncogenic signaling. Downstream from Ras proteins, MAPK signaling is a critical pathway for sustained proliferative signaling in many cancers. Therefore, we sought to examine the functional impact of the Aurora A/H-Ras interaction on the MAPK pathway. We first used western blotting to evaluate the impact of co-expressed Aurora A and H-Ras on ERK phosphorylation as a readout for MAPK signaling. As shown in Fig. 2-3A, no detectable effect on ERK phosphorylation was observed when Aurora A was expressed alone, while the expression of H-Ras alone induced ERK phosphorylation. Interestingly, co-expression of Aurora A and H-Ras further enhanced ERK phosphorylation compared to H-Ras alone. By conducting a GST pull-down in parallel, we confirmed that the observed increase in ERK phosphorylation correlated with the interaction of Aurora A and H-Ras (Fig. 2-3A).

Since Aurora A enhanced ERK phosphorylation when co-expressed with H-Ras in HEK 293T cells, we next sought to determine if Aurora A also affected ERK phosphorylation in cancer cells. With the sustained activation that occurs in cancer, ERK translocates to the nucleus to promote the transcription of genes that drive cell cycle progression [118, 119]. Therefore, we also tested if Aurora A was able to sustain ERK phosphorylation in a temporal, serum-dependent manner.

To do this, we utilized breast adenocarcinoma-derived MCF7 cells. Aurora A has been previously investigated as a therapeutic target in breast cancer and overexpression of Aurora A and robust ERK levels occur in this cell line [120]. Our results show that in conditions without H-Ras expression, serum starvation blocks ERK phosphorylation and serum stimulation induces ERK phosphorylation in a temporal manner (Fig. 2-3B). However, in serum starved cells expressing H-Ras, ERK phosphorylation levels are elevated in the presence

of Aurora A compared to the vector control (Fig. 2-3B, lane one of panels three and four). Lastly, after serum release, Aurora A prolongs ERK activation when co-expressed with H-Ras compared to expression of H-Ras alone (Fig. 2-3B, panels three and four).

Together, these data show that the co-expression of Aurora A and H-Ras enhances and sustains ERK phosphorylation.

2.4.5 Aurora A-induced ERK phosphorylation requires Ras-MAPK signaling

To clarify if the enhanced ERK phosphorylation observed in the presence of Aurora A and H-Ras requires Ras-MAPK signaling, we first employed site-specific inactivating or activating H-Ras mutants [121]. An activating mutant that mimics GTP-binding (GST H-Ras G12V) and a dominant negative GDP-binding preferred mutant (GST H-Ras S17N) were tested for the ability to interact with Venus-Flag Aurora A by GST pull-down in HEK 293T cells. When H-Ras WT or G12V were expressed in cells, ERK phosphorylation was stimulated (Fig. 2-3C). In contrast, H-Ras S17N effectively blocked ERK phosphorylation. Co-expression of Aurora A potentiated ERK phosphorylation in the presence of H-Ras WT and G12V, but not H-Ras S17N. In the GST pull-down, we observed that although Aurora A requires active H-Ras to potentiate ERK phosphorylation, Aurora A was able to bind the WT, G12V, and S17N forms of H-Ras (Fig. 2-3C). These data also suggest that the activity and conformation of H-Ras minimally impacts the ability of Aurora A to bind H-Ras; however, increased ERK phosphorylation requires active H-Ras.

To further validate that Ras-MAPK signaling is required for ERK phosphorylation, we took an alternative approach, employing pharmacological inhibitors to probe the involvement of Raf-1 and MEK in the effect of Aurora A on ERK phosphorylation. If Aurora A acts through MAPK signaling to activate ERK, pharmacological inhibition of the MAPK

pathway would block this effect. Following Aurora A and H-Ras co-expression in HEK 293Ts, cells were treated with Raf-1 and MEK kinase inhibitors. While expression of H-Ras was able to induce MEK and ERK phosphorylation in DMSO-treated cells, inhibition of Raf-1 and MEK by Sorafenib and U0126, respectively, inhibited ERK phosphorylation (Fig. 2-3D). We then tested if the ERK phosphorylation triggered by Aurora A co-expression also requires active Raf-1 and MEK. Indeed, these inhibitors were able to block ERK phosphorylation induced by Aurora A. In this model, serum starvation was unable to reduce ERK phosphorylation [122]. Collectively, these results support the hypothesis that Aurora A potentiates ERK phosphorylation through the Ras-MAPK signaling.

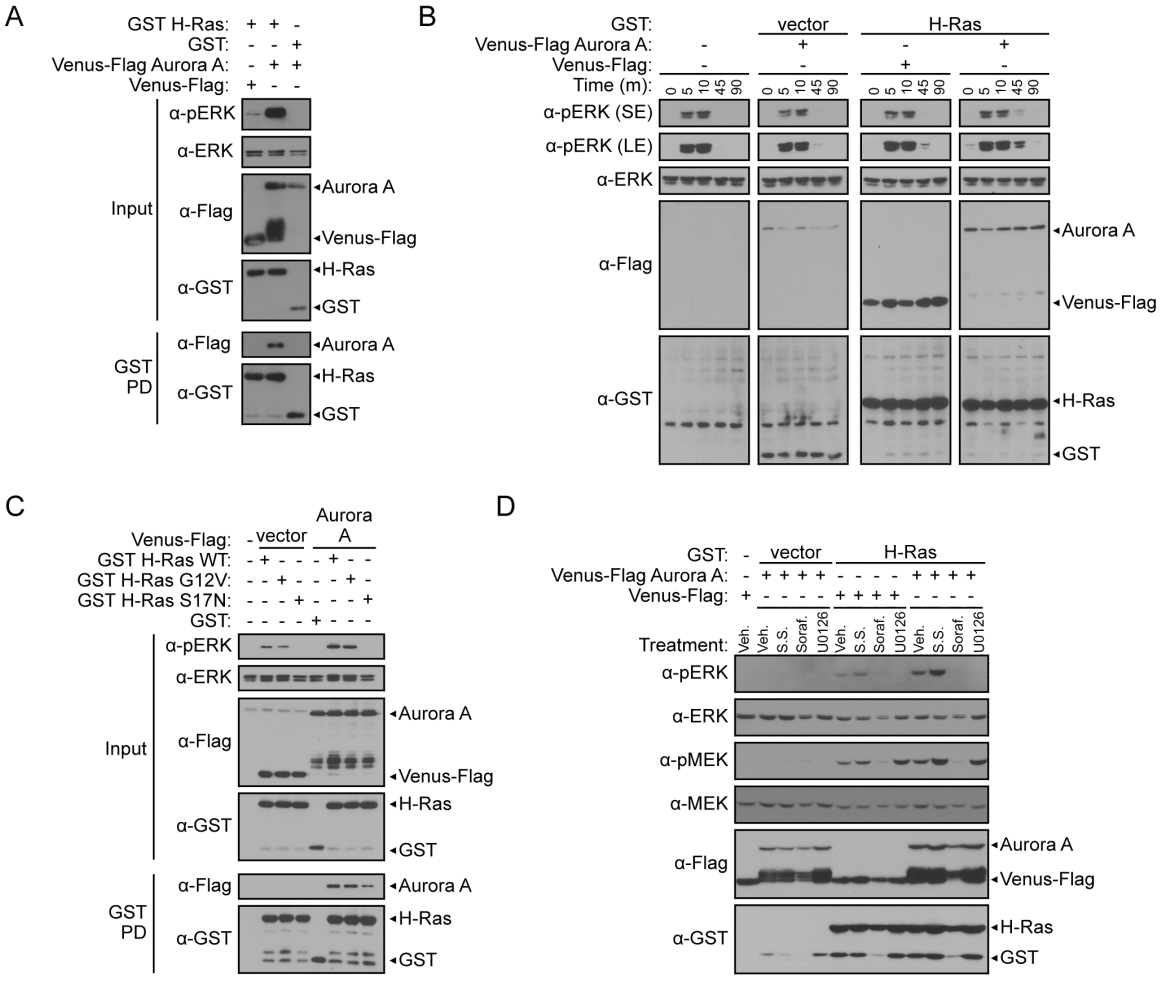

Figure 2-3. Aurora A potentiates ERK activation via H-Ras.

(A) Detection of the Aurora A/H-Ras interaction correlates with enhanced pERK. GST pull-down (described in Figure 1B) between GST H-Ras and Venus-Flag Aurora A with corresponding western blot analysis of cell lysate inputs to assess changes in pERK compared to total ERK 48-hours post-transfection in HEK 293T cells. (B) Aurora A sustains pERK levels in MCF-7 breast cancer cells. MCF-7 cells were either untransfected, transfected with Venus-Flag Aurora A or GST H-Ras with appropriate controls, or transfected with GST H-Ras and Venus-Flag Aurora A. As detected by western blotting, changes in pERK induced by

co-transfected plasmids was assessed after cells were stimulated with serum for 0, 5, 10, 45, and 90 minutes after 24-hours of serum starvation. A short exposure (SE) and longer exposure (LE) of pERK is shown. (C) H-Ras activity is required for potentiation of pERK by Aurora A. GST pull-down comparing binding and signaling changes between co-expression of GST H-Ras (WT), GST H-Ras G12V activating mutant, or GST H-Ras S17N dominant negative mutant with Venus-Flag Aurora A in HEK 293T cells. Western blot analysis of inputs to assess changes in pERK compared to total ERK 48 hours post-transfection. (D) Use of a pharmacological probe for the MAPK signaling pathway in HEK 293T cells co-expressing Aurora A and H-Ras alone or in combination. 24-hours post transfection, cells were treated with DMSO vehicle control (Veh.), serum starvation (S.S.) Sorafenib (Soraf.) or U0126 at 10μm then subjected to a GST pull-down and western blot analysis. Western blotting was conducted using anti-Flag, anti-GST, anti-pMEK, anti-MEK, anti-pERK, and anti-ERK antibodies.

2.4.6 Aurora A forms a protein complex with H-Ras and Raf-1 and acts through H-Ras to enhance MAPK signaling

To initiate MAPK signaling, GTP-bound Ras must recruit Raf-1 to the plasma membrane to dimerize, transphosphorylate, and initiate the kinase cascade. As we have demonstrated that Aurora A engaged with the N-terminal domain of H-Ras that contains the effector binding domain and enhances MAPK signaling, we next sought to determine if Aurora A also associated with the Ras effector, Raf-1. TR-FRET results in HEK 293T cells showed that GST Raf-1 and Venus-Flag Aurora A exhibit a dose-dependent increase in TR-FRET signal compared to the negative controls (Fig. 2-4A), providing evidence of an interaction of Aurora A with Raf-1. The binding of Aurora A and Raf-1 was also confirmed by GST pull-down (Fig. 2-4B).

Since Aurora A interacts with both H-Ras and Raf-1, one mechanism by which Aurora A may enhance Ras-MAPK signaling is by stabilizing the H-Ras/Raf-1 protein complex. To test this hypothesis, we conducted a GST pull-down assay testing the binding of both Venus-Flag Aurora A and Flag Raf-1 to either GST H-Ras WT or GST H-Ras S17N. Results from HEK 293T cells revealed that H-Ras WT forms a protein complex with Aurora A and Raf-1 (Fig. 2-4C). In addition, binding of both Aurora and Raf-1 to H-Ras WT is enhanced and ERK phosphorylation is strongly increased when all three proteins are co-expressed. In contrast to H-Ras WT, Raf-1 does not bind H-Ras S17N. Further, this inactive H-Ras mutant maintains the ability to interact with Aurora A, but Aurora A/H-Ras S17N binding was not enhanced as is observed with H-Ras WT.

An assessment of signaling changes demonstrates that ERK phosphorylation levels are tightly linked to Aurora A/H-Ras/Raf-1 protein complex formation. Aurora A further

enhances the ERK phosphorylation stimulated by H-Ras or Raf-1. Further, ERK remains inactive when Aurora A or Raf-1 are expressed with H-Ras S17N (Fig. 2-4C).

Together, these data demonstrate that Aurora A forms a protein complex with both H-Ras and Raf-1, stabilizes the H-Ras/Raf-1 interaction, and promotes MAPK signaling (Fig. 2-5). We also reveal that although Aurora A interacts with both H-Ras and Raf-1, H-Ras activity is required for the ability of Aurora A to enhance MAPK signaling.

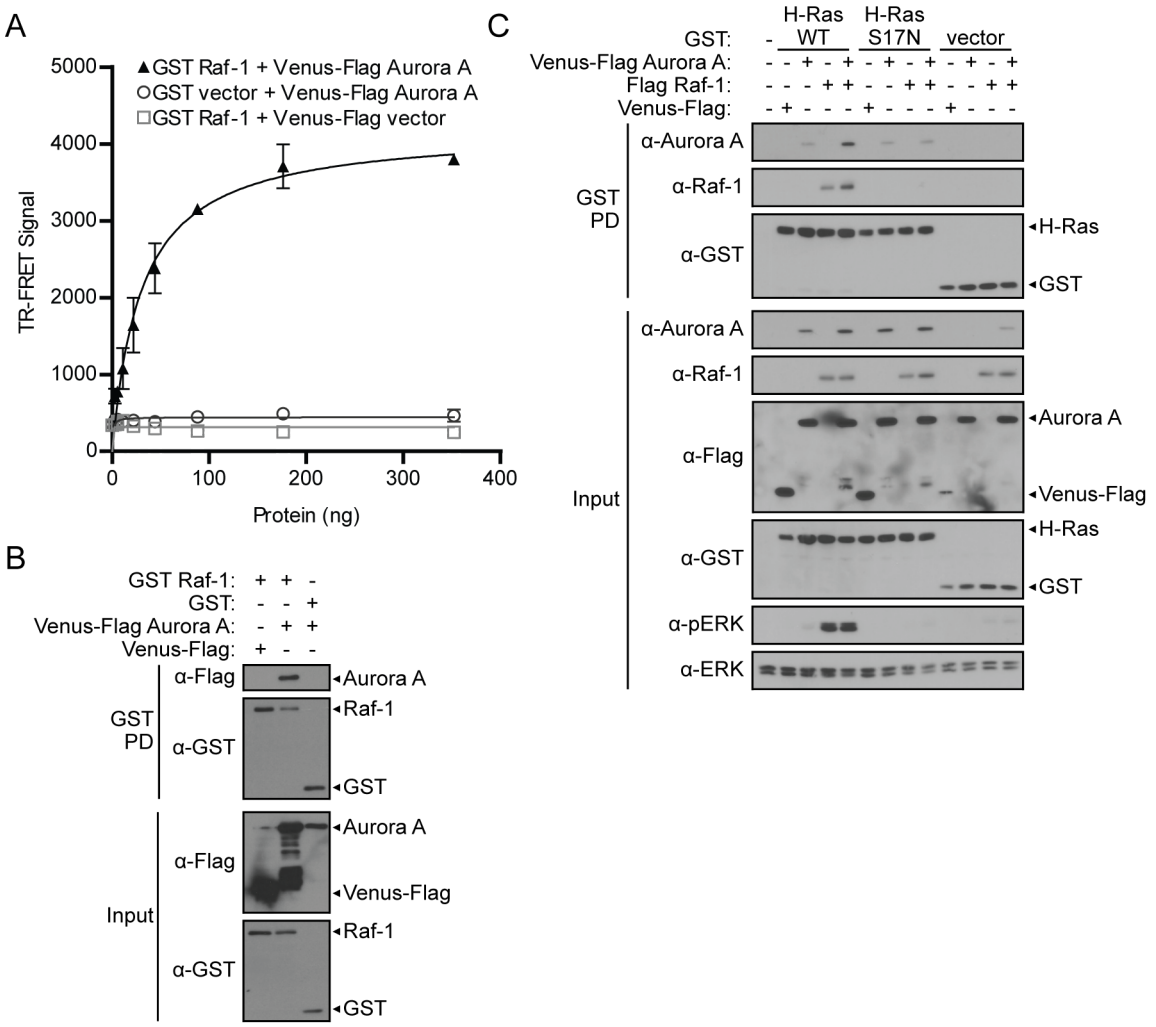

Figure 2-4. Aurora A forms a complex with H-Ras and Raf-1, acting through H-Ras to enhance ERK activation.

(A) Aurora A directly interacts with Raf-1. TR-FRET was performed using HEK 293T lysates in which GST Raf-1 and Venus-Flag Aurora A along with vector controls were co-expressed. TR-FRET signals were recorded using an EnVision multilabel plate reader. Data shown are average signals with SD from duplicate samples. (B) Aurora A associates with Raf-1. GST pull-down (as described in Figure 1B) between GST Raf-1 and Venus-Flag Aurora A with

corresponding western blot analysis of inputs to assess changes in pERK compared to total ERK 48-hours post-transfection in HEK 293T cells. (C) Aurora A/H-Ras/Raf-1 interactions stabilize the protein signaling complex. GST pull-down comparing the ability of wild-type (H-Ras WT) or dominant negative (H-Ras S17N) H-Ras to isolate either co-expressed Aurora A, Raf-1, or both proteins. Western blot analysis demonstrates binding of Aurora A or Raf-1 to H-Ras and the induced effect on pERK. Since both epitope-tagged proteins resolve around the same size, anti-Aurora A and anti-Raf-1 antibodies were used instead of anti-Flag. GST-tagged H-Ras WT and H-Ras S17N were detected using anti-GST antibody. Changes in pERK were detected using anti-pERK antibody.

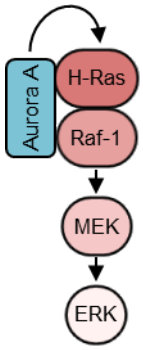

Figure 2-5. Proposed model for the role of Aurora A in the Aurora A/H-Ras/Raf-1 oncogenic signaling complex.

Aurora A interacts with H-Ras and enhances Ras-MAPK signaling. The Aurora A/H-Ras interaction is mediated by the kinase domain of Aurora A and the N-terminal domain of H-Ras. H-Ras is required for Aurora-mediated MAPK signaling, as the dominant negative mutant, H-Ras S17N blocks this effect. To enhance MAPK signaling, Aurora A also interacts with Raf-1 and stabilizes the H-Ras/Raf-1 protein complex. Thus, Aurora A forms a protein complex with H-Ras and Raf-1 to enhance oncogenic Ras-MAPK signaling.

2.5 Discussion

The family of Ras proteins (H, K, and N-Ras) function as oncogenic drivers in many cancer types by transmitting pro-growth and proliferative signals through the Ras-MAPK pathway. In this study, we identify a novel protein-protein interaction between Aurora A and Ras that provides a mechanism by which Aurora A acts as a regulator of Ras-MAPK signaling [113-116].

Using complementary protein-protein interaction assays, we demonstrated that Aurora A interacts with H-Ras and Raf-1, functioning upstream of Ras in the MAPK pathway to potentiate Ras-mediated MAPK signaling. Cooperation between Aurora A and the Ras-MAPK signaling pathway is implicated in various cancer models. For example, Aurora A overexpression and Ras alterations co-occur in pancreatic, colon, and bladder cancers [113-116]. Additionally, modulation of ERK activity and the ETS promoter alters Aurora A expression, indicating that MAPK signaling regulates transcription of Aurora A [37]. Other studies place Aurora A upstream of MAPK signaling, enhancing H-Ras G12V transformation [116, 123] Similarly, knockdown of Aurora A in nasopharyngeal cancer cells reduced invasion by reducing activation of Ras pathway components [90]. Our work, taken together with independent studies by other research groups [37, 51, 89, 115, 116, 123-131], suggests that Aurora A may form a positive feedback loop that contributes to cell growth and proliferation. In characterizing the structural domains that mediate the Aurora A/H-Ras interaction, we identified that a region within the kinase domain of Aurora A (amino acids 130 – 383) interacts with the N-terminal domain of H-Ras (amino acids 1-66). Although most of our characterization was done with H-Ras, the fact that Aurora A is able to bind the three isoforms of Ras and could bind the H-Ras G12V mutant suggests that the functional role of Aurora A

in MAPK signaling may be expanded to cancers with different predominant isoforms or mutation status.

Our finding that Aurora A interacts with Ras isoforms adds to previous reports of binding between Aurora family proteins and other GTPases and Ras-binding proteins. Aurora A interacts with RalA [51], Aurora A and B bind Ras GAP [49, 50], and Aurora B binds MgcRacGAP [132]. Both Aurora A and B have been implicated in cancer, thus the confirmation that Aurora B is also able to interact with H-Ras also expands the implications of this work and compliments studies in which Aurora B was found to associate with Survivin and RasGAP, and to stabilize Ras expression [131]. At this state, it remains unclear if Aurora B interacts with H-Ras while in complex with Survivin, or independently.

Raf-1 was identified to associate with Aurora A and other cell cycle machinery during mitosis [133] [134]. This led to the idea that Raf-1 may also exert MAPK-independent roles. Our finding reveals that Aurora A forms a protein complex with H-Ras and Raf-1, also placing the Aurora A/Raf-1 interaction in the context of Ras-MAPK signaling. The association of Aurora A with H-Ras does not appear to compete with H-Ras/Raf-1 binding and, in fact, enhances the protein complex. Since the Aurora A/H-Ras/Raf-1 complex does not form and MAPK signaling is not stimulated without active H-Ras, we were also able to show that H-Ras activity is required for Aurora A-induced Ras-MAPK signaling.

Beyond the interactions we discovered, how Aurora A leads to enhanced Ras-MAPK signaling remains to be established. It is possible that the Aurora A/H-Ras interaction may increase GEF activity, prevent GAP activity, or induce an active conformation of H-Ras. Ras G12V is a mutant of Ras that binds GAP but is unable to hydrolyze GTP. Because we were able to demonstrate that co-expression of Aurora A and H-Ras G12V also enhances ERK

activation, the mechanism of action of Aurora A may be Ras-GAP independent despite reports that both Aurora A and Aurora B both associate with Ras GAP [49, 135].

Attempts to directly target Ras proteins for cancer treatment have been largely unsuccessful in the clinic [75, 135]. Another opportunity to inhibit Ras signaling is by targeting protein-protein interactions that affect the regulation of Ras. Therefore, our identification of the novel interaction between Aurora A and H-Ras as a mechanism by which Aurora A can activate Ras-MAPK signaling opens the way for studies into perturbation of the Aurora A/H-Ras interaction and the effect on Ras-MAPK signaling. Evidence from these future studies would suggest that the interactions between Aurora A and Ras may serve as a therapeutic target in cancer.

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Chapter 3: The impact of kinase activity on the Aurora A/H-Ras/Raf-1 protein complex

3.1 Introduction

Aurora Kinase A (Aurora A) is a serine/threonine kinase that mediates oncogenic signaling through both mitotic and non-mitotic functions. Chapter 2 discussed our work to identify Aurora A as a binding partner of H-Ras. H-Ras is a GTPase that activates several oncogenic signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway. We found that Aurora A forms a protein complex with H-Ras and Raf-1, a Ras effector protein that mediates Ras-MAPK signaling.

Characterization of the domains involved in the interaction of Aurora A and H-Ras revealed that the kinase domain of Aurora A mediates the interaction. The kinase activity of Aurora A is essential for a number of oncogenic functions, including facilitating mitosis and degrading p53 [22, 44]. Interestingly, Aurora A is able to phosphorylate other GTPases and proteins involved in Ras signaling. Aurora A phosphorylates and positively regulates Ras family protein, RalA, promoting RalA association with the plasma membrane and anchorage-independent growth [51, 136]. Aurora A also phosphorylates RalGDS, a RalGEF that activates RalA. Although there are Aurora A kinase inhibitors are in clinical development, previous compounds failed in the clinic due to intolerable toxicity in patients [137].

We observed that Raf-1 enhances the interaction of Aurora A and H-Ras in the Aurora A/H-Ras/Raf-1 protein complex. Raf-1 belongs to a family of serine/threonine kinases (with A-Raf and B-Raf) that are recruited to the plasma membrane and activated by Ras [138]. Upon activation, Raf proteins begin phosphorylation events in a kinase cascade involving MEK and ERK. Mutations in Raf-1 are relatively rare in human cancer (less than 2%), but do occur in patients with Noonan Syndrome, a genetic disorder that affects physical and mental development [139, 140]. Structurally, Raf-1 has three distinct regions that are involved in its function. The N-terminal half of Raf-1 contains conserved regions (CR), CR1 and CR2. CR1

consists of the Ras-binding domain (RBD) and the cysteine-rich domain (CRD). CR2 consists of inhibitory phosphorylation sites, namely S259 which induces inhibitory 14-3-3 binding [141]. Notably, activating mutations in Raf-1 at the S259 site have been identified in colon and ovarian cancer [142]. The C-terminal half contains CR3, the catalytic domain and sites of activating phosphorylation including S338 within the activation loop of the kinase and S621 which allows activating 14-3-3 binding [138]. Although clinical development of Sorafenib as a Raf kinase inhibitor also revealed details about Raf dimerization and clinical resistance [143], several Raf targeted inhibitors are successfully used in the clinic for the treatment of cancer.

Due to the prominent role of kinase in mediating cellular signaling, we hypothesized that kinase activity may play a role in the formation of the Aurora A/H-Ras/Raf-1 protein complex. By using a combination of site-specific mutants and pharmacological inhibition, we reveal that the kinase activity of Raf-1 plays a role in stabilizing the Aurora A/H-Ras/Raf-1 protein complex. Lastly, we show that Sorafenib, an FDA-approved Raf-1 inhibitor also acts to disrupt the Aurora A/H-Ras interaction.

3.2 Materials and methods

Cell culture

HEK 293T (American Type Culture Collection, Manassas, VA) cells were cultured in DMEM (Corning, MT10013CV, Manassas, VA) with 10% FBS (Sigma, F6178, St. Louis, MO) and 1% pen/strep at 5000I.U/ml penicillin and 5000μg/ml streptomycin (Corning, 30-001-Cl, Manassas, VA). Between passages, cells were trypsinized with 0.25% Trypsin with 2.21mM EDTA (Corning, 25-053-Cl, Manassas, VA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2.

Antibodies

Primary antibodies used for western blotting include Flag M2 at 1:3000 (Sigma; F3165), Flag-HRP at 1:1000 (Sigma; A8592), GST Z-5 at 1:3000 (Santa Cruz Biotechnology; sc-459), rabbit GST-HRP at 1:1000 (Sigma; A7340), Aurora Kinase A at 1:500 (Cell Signaling; 4718), rabbit pERK and ERK (Cell Signaling; 4370, 9102, respectively), pMEK and MEK (Cell Signaling; 9154, 4694, respectively), pRaf-1 (Cell Signaling; 9427) and Raf-1 (Santa Cruz; sc-133) all at 1:1000. Secondary antibodies include goat anti-rabbit IgG (Santa Cruz, sc-2004, Dallas, TX) and goat anti-mouse IgG (Santa Cruz, sc-2005, Dallas, TX) and were used at either 1:2500 or 1:5000 dilutions.

Pharmacological inhibitors

Sorafenib p-Toluenesulfonate Salt (S-8502) was obtained from LC Laboratories (Woburn, MA) and dissolved in dimethyl sulfoxide (DMSO) as 10mM stock and stored at -20°C. Cells were treated for 24 hours with compounds diluted in DMSO and the indicated doses.

Transfections

For experiments with ectopically expressed proteins, HEK 293Ts were transfected using X-tremeGENE (Roche, 06366546001, Basel, Switzerland). Plated cells were transfected at a density of 60-80% confluency and performed with a ratio of 3µl transfection reagent to 1µg DNA to 100µl of serum-free media. DNA was mixed at appropriate concentrations prior to the addition of serum-free DMEM. Transfection reagent was then added and incubated at room temperature for 15 minutes. Transfection complexes were then added drop-wise to plated cells.

Plasmid construction

All plasmids of full length and truncated proteins were constructed using Gateway® technology (Invitrogen, Waltham, MA) according to the manufacturer's protocols. For GST-tagged and Venus-Flag tagged plasmids used for Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) and Glutathione-S-Transferase (GST) pull-downs, pDEST27 and pFUW vectors were used as destination cloning vectors, respectively. Full-length Aurora A, H-Ras, and full-length and truncated Raf-1 plasmids were previously generated in the lab. For the Aurora A truncations, cDNA was PCR amplified and inserted into the pDONR201 (Invitrogen) vector using a BP reaction to generate entry cloning vectors. A LR reaction was used to clone the desired DNA into the appropriate destination vectors. Constructs were verified by restriction digest using BSRGI (NEB, Catalog, City, State) or FastDigest Bsp1407I (Thermo Scientific, FD0933, City, State), both cutting at the T^GTACA attB1 and attB2 (entry clone) or attR1 and attR2 (destination vector) recombination sites, and DNA sequencing. Clones in pDEST-27 (GST) vectors were sequenced with forward primer 5'-AAGCCACGTTTGGTGGTG-3' and the standard T7 reverse primer. Clones in pFUW (Venus-Flag) vectors were sequenced with primer #1 5'-CGATCACATGGTCCTGCTG-3' and the standard SP6 reverse primer. Flag only constructs were generated previously in the laboratory.

GST pull-down

Cells were seeded in to a 6-well plate and allowed to reach 60-80% confluency. Cells were then harvested by adding 200μL of 0.5% NP-40 lysis buffer to each well, scraping to collect cells and transfer to an eppendorf tube, and incubated at 4°C for 30 minutes. Lysis buffer components consisted of 0.5% NP-40, 150mM NaCl, 10mM HEPES lysis buffer, and

Phosphatase Inhibitor Cocktail (Sigma, P5726) and Protease Inhibitor Cocktail 2 (Sigma, P8340) at 1:1000. For phosphatase inhibitor experiments only, the addition of the tyrosine phosphatase inhibitor, Phosphatase Inhibitor Cocktail 3 (Sigma, P0044) was used at 1:1000 also. After incubation, lysates were centrifuged to remove cellular debris. After removing 20µl of the lysate for an input control and the debris pellet, 20µl of a 50% glutathione-conjugated sepharose bead slurry (Glutathione Sepharose 4B, Fisher Scientific, 50197956, Atlanta, GA) was added to the remaining lysate and incubated by slowly rotating for 3-4 hours at 4°C. Beads were then washed three times in 0.5% NP-40 lysis buffer by inverting 8 times with 200µl of fresh lysis buffer added each time. GST-bound protein complexes were then eluted by the addition of 20µl of 2x SDS loading buffer, boiled for 5 minutes, resolved by SDS-PAGE subjected to western blotting along with input controls.

TR-FRET assay

TR-FRET was performed in 384-well black solid bottom plates (Corning Costar Cat. #3654) in a total volume of 30 µL in each well. Briefly, HEK 293T cells were transfected as described above. Cells were lysed using 0.5% NP-40 lysis buffer (0.5% NP-40, 150mM NaCl, 10mM HEPES, and Phosphatase Inhibitor Cocktail (Sigma, P5726) and Protease Inhibitor Cocktail (Sigma, P8340)). Lysates were collected and centrifuged at 13,500g for 10 minutes at 4°C to remove cellular debris. Cleared cell lysates were serially diluted in FRET buffer (20mM Tris, pH 7.0, 0.01% Nonidet-P40, and 50mM NaCl) in a 384-well plate, bringing the final volume of diluted cell lysate to 15µL per well. Then, 15µL of diluted anti-GST-Terbium antibody (Cisbio US Inc, 61GSTTLB, Bedford, MA) was added to all wells at a final dilution of 1:1000. The TR-FRET signals were detected with an EnVision Multilabel plate reader (PerkinElmer) with laser excitation at 337 nm, emission1 at 486 nm and emission2 at 520 nm. TR-FRET

signal is expressed as ratio and calculated by the following equation: TR-FRET signal = F520/F486 × 10^4, where F486 and F520 are fluorescence counts at 520 nm and 486 nm for Venus and terbium emission signal, respectively. Data presented as mean with standard deviation calculated from duplicate samples (Fig. A-1).

Western blotting

Cell lysates were subjected to western blot analysis following protein separation by SDS-PAGE (10% acrylamide gels) and subsequent transfer to PVDF membranes at 100V for 1.5 hours – 2 hours. Membranes were blocked in TBST (50 mM Tris, 137 mM NaCl, 0.05% Tween, pH 7.6] containing 5% dry milk for 30 minutes – 1 hour at ambient temperature, then incubated at 4°C or ambient temperatures with primary antibodies diluted in 5% milk in TBST for appropriate times. After primary antibody incubation, membranes were washed three times with TBST for 5 minutes each prior to incubating with secondary antibody for 1 hour at ambient temperatures. For HRP conjugated antibodies, membranes were washed three times with TBST for 10 minutes each after blocking with milk prior to incubating with GST-HRP or Flag-HRP for 1 hour. Membranes were then washed three times with TBST for 10 minutes each and chemiluminescent signal (West Pico, West Dura (ThermoScientific, PI34080 or PI34076, respectively) or ECL, Amersham, 84-839, San Diego, CA) was added for 5 minutes prior to developing by autoradiography. Proteins with the Venus-Flag epitope tag were detected by blotting with anti-Flag antibody.

3.3 Results

3.3.1 The kinase domains of Aurora A and Raf-1 mediate their interaction

To determine which regions of Aurora A are able to bind Raf-1, we tested the binding of Aurora A to Raf-1 by conducting a GST pull-down with GST Raf-1 and Venus-Flag Aurora A truncations co-expressed in HEK 293T cells. Venus-Flag Aurora A truncations spanned key protein domains: the N-terminal and kinase domains of Aurora A (NK, amino acids 1-383), the N-terminal fragment of Aurora A (N, amino acids 1-130), the kinase domain alone (K, amino acids 130-383), and the C-terminal domain (C, amino acids 383-403) (Fig. 3-1A). GST pull-down results shows that as a positive control, full length Aurora A binds to Raf-1, but not to GST (Fig. 3-1B). Binding of Aurora A NK and K truncations to Raf-1 was also detected, suggesting that the kinase domain of Aurora A is both necessary and sufficient for Raf-1 binding. Interestingly, we previously determined that the kinase domain of Aurora A also mediates binding to H-Ras.

To evaluate the domains of Raf-1 that mediate binding to Aurora A, we used TR-FRET to test the binding of key Raf-1 domains. The GST Raf-1 truncations used are shown in Fig. 3-1C: N-terminal half of Raf-1 (N, amino acids 1-321) and the C-terminal half (C, amino acids 321-648). GST Raf-1 or Raf-1 truncations were co-expressed with Venus-Flag Aurora A or Venus-Flag. Full length Aurora A binds to Raf-1, but not to GST (Fig. 3-1D). Dose-dependent increases in TR-FRET signals were detected when Aurora A was co-expressed with the C-terminal half of Raf-1. Binding of the N-terminal half of Raf-1 was comparable to negative controls. The kinase domain is the primary component of the C-terminal half of Raf-1; therefore, this data suggests that the kinase domain of Raf-1 is involved in the Aurora A/Raf-1 interaction.

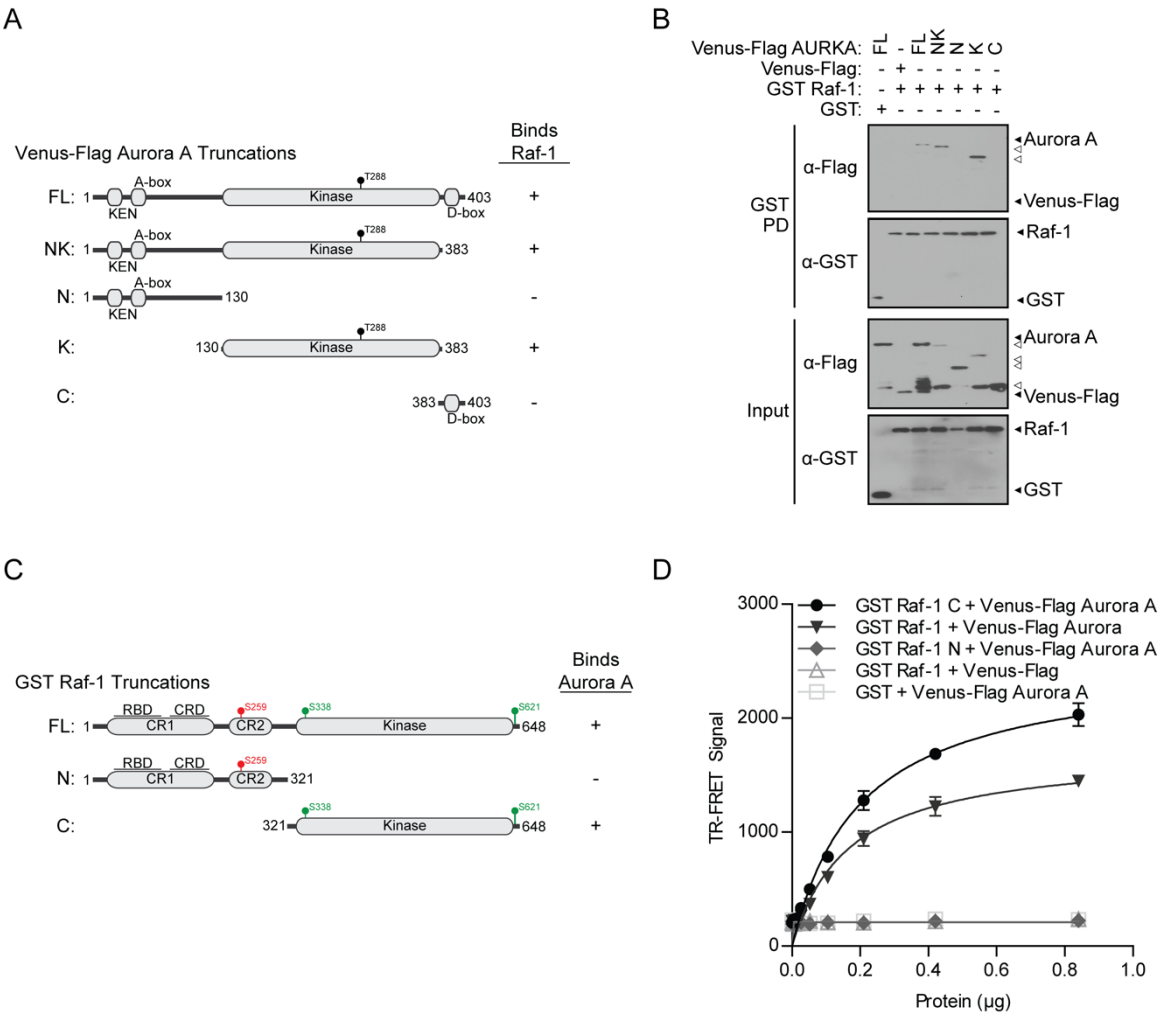


Figure 3-1. The kinase domains of Aurora A and Raf-1 are involved in the interaction.

(A) Characterization of the Raf-1 binding domain on Aurora A. Diagram of Aurora A protein domains and truncations used for deletion analysis: FL (amino acids 1-403), NK (amino acids 1-383), N (amino acids 1-130), K (amino acids 130-383), C (amino acids 383-403). (B) GST pull-down conducted from HEK 293T cells co-expressing full-length GST Raf-1 and Venus-Flag Aurora A truncations. Binding between full-length proteins served as a positive control. The presence of Venus-Flag Aurora A truncations in the GST H-Ras protein complex (GST PD) and protein expression levels in the cell lysate (Input) were detected by Western blotting

using anti-Flag or anti-GST antibody, respectively. (C) Characterization of the Aurora A binding domain on Raf-1. Diagram of Raf-1 protein domains and truncations used for deletion analysis: FL (amino acids 1-648), N (amino acids 1-321), C (amino acids 321-648). (D) TR-FRET assay performed using lysates from HEK 293T cells in which GST Raf-1 truncations were co-expressed with Venus-Flag Aurora A or negative controls. TR-FRET signal calculated as X/Y*Z; Tb ex 340 nm; Tb em 486 nm (X); Venus em 520nm (Y); $Z = 10^4$). TR-FRET signals were recorded using an EnVision multilabel plate reader. Data shown are average signals with SD from duplicate samples.

3.3.2 The Aurora A/H-Ras interaction is phosphorylation independent

Aurora A and Raf-1 are kinases that regulate protein function through phosphorylation of substrates. Phosphorylation events can direct cellular processes from binding to degradation to translocation. In our previous work, we determined that Aurora A acts through H-Ras to activate Ras-MAPK signaling; without functional H-Ras, Aurora A was unable to promote MAPK activation. Considering the importance of the Aurora A/H-Ras interaction, we sought to test whether the interaction was modulated by phosphorylation. To do this, we co-expressed GST H-Ras and Venus-Flag Aurora A in HEK 293T cells and conducted a GST pull-down in the presence or absence of phosphatase inhibitors. If the interaction was modulated by kinase activity, the removal of phosphate from key phosphorylation sites on either protein in the lysate would influence binding observed by GST pull-down. First, we confirmed the binding of Venus-Flag Aurora A to GST H-Ras but not GST in our typical GST pull-down conditions using phosphatase inhibitor (Fig. 3-2). We also observe that the presence of Aurora A in the H-Ras protein complex is unaffected in the absence of phosphatase inhibitors. However, the activating phosphorylation of ERK at T202/204 is lost when phosphatase inhibitors were removed. Thus, the Aurora A/H-Ras interaction may be independent of phosphorylation events since the Aurora A/H-Ras interaction is maintained in the presence of active phosphatases.

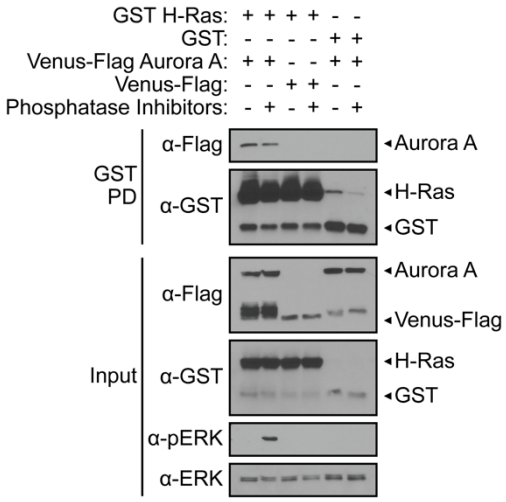


Figure 3-2. The Aurora A/Raf-1 interaction is phospho-independent.

GST pull-down conducted in the presence or absence of phosphatase inhibitors from HEK 293T cells co-expressing full-length GST H-Ras and Venus-Flag Aurora A and analyzed by western blotting. The presence of Venus-Flag Aurora A in the GST H-Ras protein complex (GST PD) and protein expression levels in the cell lysate (Input) were detected by Western blotting using anti-Flag or anti-GST antibody, respectively.

3.3.3 Constitutively active Raf-1 enhances Aurora A/Raf-1 binding

Although the interaction of Aurora A and H-Ras appears to be phosphorylation independent, we previously determined that expression of Aurora A enhanced the H-Ras/Raf-1 interaction. Likewise, binding of Aurora A to H-Ras in the same protein complex was also enhanced due to the presence of Raf-1. As Aurora A is able to bind Raf-1 directly, we next sought to evaluate the importance of Raf-1 kinase activity in the Aurora A/Raf-1 interaction. Raf-1 is a dynamically regulated protein in which post-translational modification and conformation strongly influence the kinase activity. Phosphorylation of Raf-1 at S259 and S621 allows 14-3-3 binding, maintaining Raf-1 in an auto-inhibitory conformation. Activated Ras recruits Raf-1 to the plasma membrane. Following Ras/Raf binding, removal of the phosphorylation at S259 by PP2A (but not S621) in combination with the phosphorylation of S338 by PAK releases kinase inhibition by 14-3-3 and exposes the fully active kinase domain of Raf-1.

Since the kinase domain of Raf-1 mediates binding to Aurora A (Fig.3-1D), we next determined if Raf-1 kinase activity impacted the Aurora A/Raf-1 interaction directly. To do this, we employed a site-specific activating mutant of Raf-1. Raf-1 S259A mimics PP2A activity in removing an inhibitory Raf-1 phosphorylation site. GST Aurora A and Venus-Flag Raf-1 WT or Venus-Flag Raf-1 S259A were co-expressed in HEK 293T cells and subjected to a GST pull-down. We observed a drastic increase of binding of Raf-1 S259A to Aurora A and ERK phosphorylation compared to Raf-1 WT (Fig. 3-3A). Interestingly, Aurora A further potentiated ERK phosphorylation when co-expressed with Raf-1 S259A compared to expression of Raf-1 S259A alone. This data suggests that constitutively active Raf-1 has enhanced ability to bind Aurora A and to promote ERK activation.

3.3.4 Kinase activity is required for Raf-1 to enhance the Aurora A/H-Ras binding

To assess if Raf-1 kinase activity impacts the interaction of Aurora A and H-Ras, we compared the impact of wild-type and kinase dead Raf-1 on the Aurora A/H-Ras interaction. Two kinase-dead versions of Raf-1 were employed. Raf-1 S259A/S621A is a phosphodeficient mutant that abrogates 14-3-3 binding. Raf-1 K375M mutant prevents ATP binding, thereby inhibiting kinase activity. A GST pull-down assay was used to compare the binding levels of Aurora A and Raf-1 mutants isolated in complex with H-Ras from HEK 293T cells. Combinations of Venus-Flag Aurora A, Venus-Flag Raf-1 WT, Flag Raf-1 AA, Flag Raf-1 K375M and GST H-Ras were expressed in HEK 293T cells. Results confirm that the presence of Raf-1 WT enhances the Aurora A/H-Ras interaction compared to co-expression of Aurora A and H-Ras alone (Fig. 3-3B). ERK phosphorylation levels increase accordingly. Interestingly, although also able to bind H-Ras, neither Raf-1 AA or Raf-1 K375M are able to enhance the Aurora A/H-Ras interaction. Both kinase dead mutants also greatly reduce ERK phosphorylation in the presence of Aurora A and H-Ras. The fact that kinase dead Raf-1 is unable to enhance the Aurora A/H-Ras interaction and inhibits Aurora A-enhanced ERK phosphorylation corroborates our finding that specifically, the kinase activity of Raf-1 may influence the Aurora A/H-Ras/Raf-1 interaction. This also reiterates that Aurora A acts upstream of Ras-MAPK signaling to enhance ERK phosphorylation

3.3.5 Sorafenib attenuates the Aurora A/H-Ras interaction

Raf-1 kinase activity is also crucial for mediating oncogenic Ras-MAPK signaling. Several Raf-targeted inhibitors have been developed and are used in the clinic. Because of our elucidation of the role of Raf-1 kinase activity in the Aurora A/H-Ras/Raf-1 protein complex, we investigated whether pharmacological inhibition of Raf-1 by Sorafenib is a clinically used

multi-kinase inhibitor with highest potency against Raf-1 (6nM)[144], had an effect on the Aurora A/H-Ras interaction.

HEK 293T cells with co-expressed GST H-Ras and Venus-Flag Aurora A were treated with increasing doses of Sorafenib for 24 hours prior to conducting a GST pull-down. Results showed that Sorafenib disrupts the Aurora A/H-Ras interaction in a dose-dependent manner (Fig. 3-3C). Further Sorafenib was able to block Aurora A-enhanced ERK phosphorylation. The use of a clinically available inhibitor in this study reveals an additional mechanism of action for Sorafenib, specifically in cancers with over-expression of Aurora A.

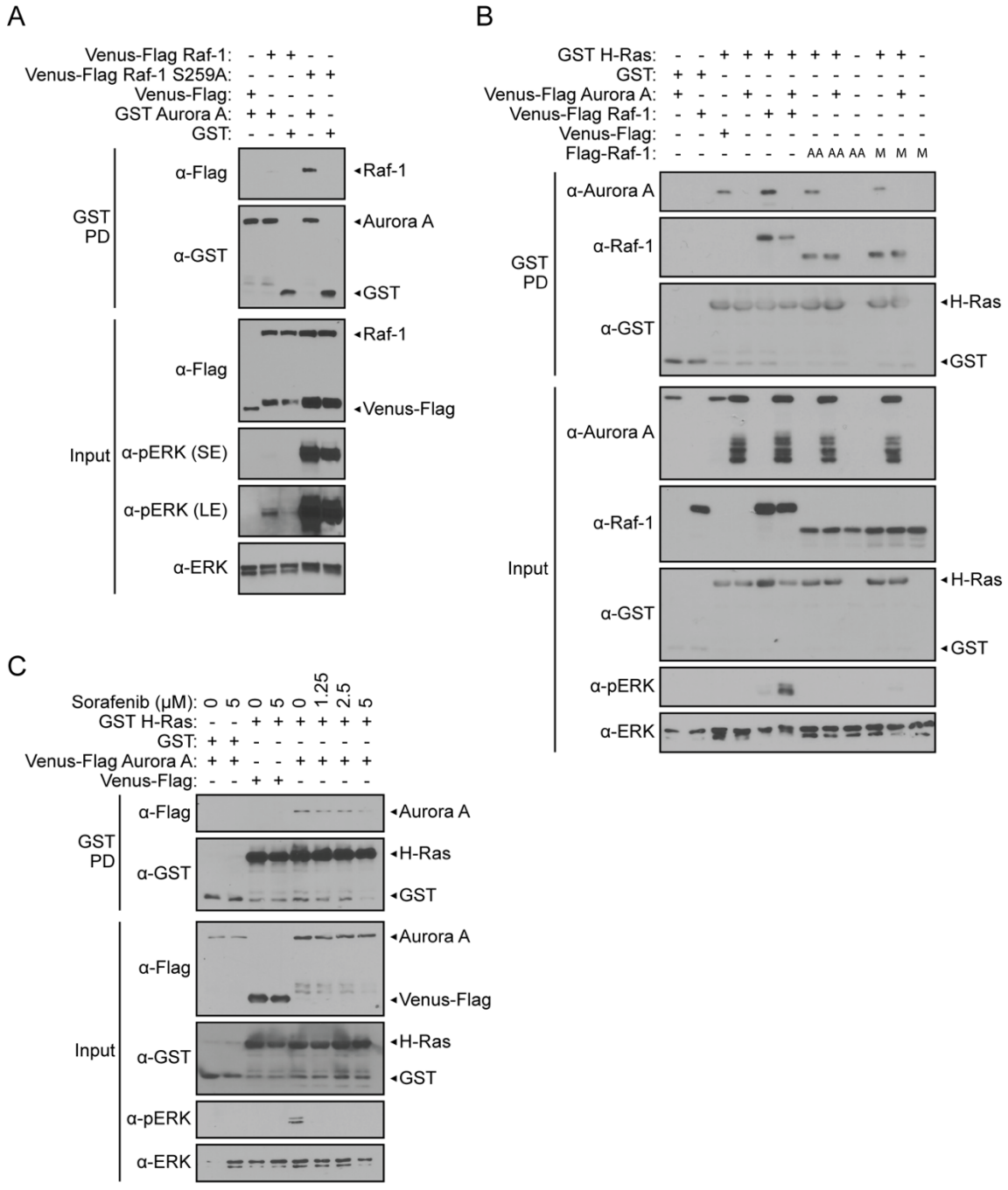


Figure 3-3. The role of Raf-1 in the Aurora A/H-Ras/Raf-1 protein complex.

(A) GST pull-down conducted from HEK 293T cells co-expressing full-length GST Aurora A and Venus-Flag Raf-1 WT or the catalytically active Venus-Flag Raf-1 S259A mutant and analyzed by western blotting. The presence of Venus-Flag Raf-1 in the GST Aurora A protein

complex (GST PD) and protein expression levels in the cell lysate (Input) were detected by Western blotting using anti-Flag or anti-GST antibody, respectively. Changes in pERK to total ERK were detected using anti-pERK and anti-ERK antibodies, respectively. A short exposure (SE) and longer exposure (LE) of pERK is shown. (B) GST pull-down comparing binding and signaling changes between co-expression of GST H-Ras, Venus-Flag Aurora A, and Venus-Flag Raf-1 WT or Flag Raf-1 kinase dead mutants: Raf-1 S259A S621A (AA) or Raf-1 375M (M). Western blot analysis of the GST H-Ras protein complex (GST PD) and protein expression levels in the cell lysate (Input) was conducted as follows: Venus-Flag Aurora A was detected with anti-Aurora A antibody; Venus-Flag Raf-1, Flag Raf-1 S259A S621A (AA), and Flag Raf-1 375M (M) were detected using anti-Raf-1 antibody; GST H-Ras and GST were detected using anti-GST antibody. Comparison of pERK compared to total ERK were detected using anti-pERK and anti-ERK antibodies, respectively. The size difference in Raf-1 WT and mutants is due to the respective expression vectors. (C) HEK 293T cells were used to co-express GST H-Ras and Venus-Flag Aurora A with vector controls or in combination. Cells were treated with DMSO vehicle control (0μM) or different concentrations of Sorafenib (1.25μM–5μM) for 24 hours, then subjected to a GST pull-down and western blot analysis. Western blotting was conducted using anti-Flag, anti-GST, anti-pERK, and anti-ERK antibodies.

3.4 Discussion

Aurora A, H-Ras, and Raf-1 are all oncoproteins that contribute to the development of cancer. The recent discovery of PPIs that connect Aurora A to H-Ras and Raf-1 and enhanced Ras-MAPK signaling exposes a potential signaling node worth therapeutic exploration. The kinase activity of both Aurora A and Raf-1 have been extensively targeted in pre-clinical and clinical development due to the important roles for kinase activity in their oncogenic functions.

In our investigation of the role of kinase activity in the Aurora A/H-Ras/Raf-1 protein complex, we found that, indeed, the kinase domains of Aurora A and Raf-1 mediate their interaction. Surprisingly though, the Aurora A/H-Ras interaction was not dynamically regulated by phosphorylation. As a caveat, proteins in this experiment were ectopically expressed in cells. The possibly that overexpression decreased the sensitivity of the interaction to manipulation by phosphatase inhibitors remains to be tested. However, a kinase-independent Aurora A/H-Ras interaction indicates that inhibiting Aurora A kinase activity alone may not inhibit all oncogenic functions of the protein. This finding may impact the clinical effectiveness of Aurora A kinase inhibitors like Alisertib in the clinic.

Through this work, we demonstrated that Raf-1 modulates the Aurora A/Raf-1 interaction, the Aurora A/H-Ras/Raf-1 protein complex formation, and subsequent oncogenic signaling. Aurora A interacts with the C-terminal half of Raf-1, which contains the kinase domain. In the cycle of Raf-1 activation, two events (binding to active Ras and removal of inhibitory phosphorylation at S259) are required to expose the kinase domain of Raf-1, allow dimerization, and activate kinase activity. By using two site-specific mutants with different mechanisms of inhibiting Raf-1 activity, we discovered that while kinase dead Raf-1

was detected in the complex with H-Ras and Aurora A, kinase dead Raf-1 lacks the ability to enhance protein complex binding compared to Raf-1 WT.

It is interesting to note that our previous work demonstrated that Aurora A was able to interaction with H-Ras S17N, a dominant negative Ras mutant that is unable to bind Raf-1. This suggests that Aurora A/Raf-1 binding is secondary to Aurora A/H-Ras binding. In fact, H-Ras S17N dissociated Raf-1 from the protein complex, but not Aurora A. Thus, Aurora A interacts with H-Ras in the absence of Raf-1. One explanation for the effect of Raf-1 on the Aurora A/H-Ras/Raf-1 protein complex may be that Raf-1 protein kinase domain stabilizes the complex. Raf-1 S259A creates a constitutively active kinase. The Raf-1 RBD is exposed in Raf-1 S259A as 14-3-3 can no longer bind this site. As a result, the ability for Ras binding is enhanced. Increased H-Ras/Raf-1 binding may then enhance the Aurora A/Raf-1 interaction. As a result, the complex is stabilized and oncogenic signaling through Ras-MAPK is enhanced. Surprisingly, unlike Raf-1 kinase dead mutants, treatment with 10μM of Sorafenib, a multikinase inhibitor with highest potency against Raf-1 kinase activity, was able to disrupt the Aurora A/H-Ras interaction. While the simplest explanation is that disruption of Raf-1 kinase activity disrupts the Aurora A/H-Ras interaction, the inability for kinase dead Raf-1 to disrupt the Aurora A/H-Ras interaction challenges this conclusion. Therefore, the disruption of the Aurora A/H-Ras interaction by Sorafenib at such high doses may be due to Raf-1 kinase inhibition or to non-specific inhibition of additional kinases that may indirectly regulate complex formation.

Taken together, our study shows that Raf-1 plays a role in enhancing the Aurora A/H-Ras/Raf-1 protein complex. Our proposed model, shown in Figure 3-4, is that Aurora A interacts with H-Ras which, when activated, recruits and activates Raf-1. In turn, Raf-1 interacts with Aurora A and helps to stabilize the Aurora A/H-Ras/Raf-1 protein signaling

complex. Importantly, the mechanism we discovered may be an important therapeutic target in colon and ovarian cancer patients with activating mutations at Raf-1 S259.

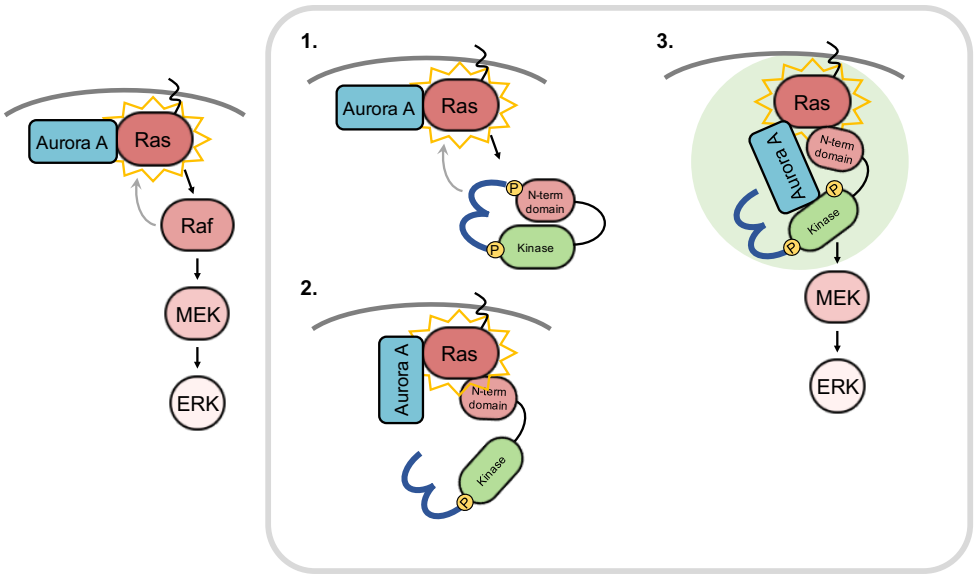


Figure 3-4. Proposed model for the role of Raf-1 in the Aurora A/H-Ras/Raf-1 signaling complex.

(1) Raf-1 is kept in an inactive state by phosphorylation at S259 and S621, and 14-3-3 binding. (2) Upon Ras activation, Raf-1 is first recruited by Ras to the plasma membrane where it binds through the N-terminal domain. PP1 then acts to dephosphorylate Raf-1 at the S259 site. (3) The resulting conformation of Raf-1 exposes its kinase domain to the kinase domain of Aurora A, allowing binding and stabilization of the Aurora A/H-Ras/Raf-1 protein complex. Phosphorylation at S338 fully activates Raf-1 and initiates the MAPK signaling cascade.

Chapter 4: Aurora Kinase A interacts with FOXO1 and inhibits FOXO1-induced apoptosis

MaKendra Umstead, Valentina González-Pecchi, Haian Fu

4.1 Introduction

The Forkhead box protein O1 (FOXO1) transcription factor belongs to a large, evolutionarily conserved family of Forkhead box proteins [83] . The most abundant of the Forkhead box proteins, FOXO1, transcribes genes with tumor suppressive functions such as the cell-cycle inhibitors, p21 and p27, and the apoptotic protein, BIM [92]. FOXO1 alterations confer the ability of a cancer cell to evade apoptosis in response to cellular stresses [145]. FOXO1 localization is critical for its function as a transcription factor, and is regulated in part by PI3K/AKT pro-survival signaling [146-148]. Phosphorylation of FOXO1 by AKT at three distinct sites (T24, S258, S319) excludes FOXO1 from the nucleus, sequestering the protein in the cytoplasm and preventing transcription of pro-apoptotic genes [149, 150].

Recently, the Polo-like kinase (PLK) family member PLK1 was also found to inactivate FOXO1 [86]. PLK1 is a serine/threonine kinase that regulates key mitotic functions such as mitotic entry, centrosome maturation, spindle assembly, and chromatin segregation [151-153]. Interestingly, during the G2/M transition, PLK1 interacts with and phosphorylates FOXO1, causing nuclear export to the cytoplasm and inhibiting transcriptional activity in an AKT independent manner [86]. Similarly, PLK1 is also able to phosphorylate, thereby activating, the transcriptional activity of pro-growth FOX family member, FOXM1 [154].

The functions of Polo-like kinases have significant overlap with another family of mitotic kinases: the Aurora kinases. Aurora Kinase A (Aurora A) belongs to family of kinases that play integral functions during mitosis [44]. Aurora A, Aurora B, and the lesser characterized Aurora C facilitate centrosome maturation, chromosome alignment, and cytokinesis [21]. Aurora A also interacts directly with PLK1: Aurora A phosphorylates PLK1 to allow progression through the G2/M checkpoint [155]. Inhibition of either PLK1 or Aurora A interrupts chromosome alignment and centrosome maturation [44].

Expression of Aurora A and FOXO1 have an inverse relationship in cancer cells. Aurora A levels are highest at the G2/M transition [156]. Loss of Aurora A upregulates FOXO1 and stimulates p53-dependent apoptosis during this phase [92]. Additionally, inhibition of Aurora A by Alisertib decreases inhibitory FOXO3a phosphorylation and induces transcription of p27 and BIM, and potentiates the effect of the chemotherapeutic cytarabine (ara-C) in acute myeloid leukemia (AML) cells [88]

One proposed mechanism of Aurora A crosstalk with FOXO1 function is by transcriptional regulation [86, 88]. However, the recent finding that PLK1 regulates FOXO1 through direct phosphorylation suggests that Aurora A, a mitotic kinase with shared functions, could also impinge on FOXO1 at the protein-level [150]. Our PPI mapping of cancer-associated proteins revealed that Aurora A is a novel interaction partner of FOXO1. Here, we verified the interaction of Aurora A and FOXO1, and describe several features of the Aurora A/FOXO1 interaction. Aurora A binds both wild-type, and AKT phospho-deficient FOXO1 *in vitro. In vivo,* Aurora A interacts with FOXO1 in punctate sites throughout the cytoplasm as well as in larger clusters within the nucleus. We also provide preliminary evidence that that Aurora A suppresses cell death triggered by FOXO1 in untransformed cells. Lastly, we show that Aurora A inhibits the nuclear translocation of FOXO1 in response to serum starvation, leading to the inhibition of cell death.

The discovery of the interaction between Aurora A and FOXO1 reveals yet another pathway through which Aurora A may enable tumor development and growth: the deregulation of a tumor suppressor with direct roles in enabling apoptosis in cancer cells. Future steps to define the mechanism by which Aurora A deregulates FOXO1, either through phosphorylation as with PLK1 or another mechanism, will provide valuable insight. Lastly,

disrupting the Aurora A/FOXO1 interaction may have therapeutic potential in cancers with overexpressed Aurora A and in-tact FOXO1.

4.2 Materials and methods

Cell culture

HEK 293T and Cos7 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM (Corning, MT10013CV, Manassas, VA) with 10% FBS (Sigma, F6178, St. Louis, MO) and 1% pen/strep at 5000I.U/ml penicillin and 5000μg/ml streptomycin (Corning, 30-001-Cl, Manassas, VA). Between passages, HEK 293T and Cos7 cells were trypsinized with 0.25% Trypsin with 2.21mM EDTA (Corning, 25-053-Cl, Manassas, VA). HBEC-3KT cells (American Type Culture Collection, Manassas, VA) were cultured in airway epithelial cell basal medium (ATCC, PCS-300-030, Manassas, VA) supplemented with a bronchial epithelial cell growth kit (ATCC, PCS-300-040) which contained HLL Supplement (ATCC, PCS-999-012, Manassas, VA), L-glutamine (ATCC, PCS-999-015, Manassas, VA), Extract-P (ATCC, PCS-999-009, Manassas, VA), and airway epithelial cell supplement (epinephrine, transferrin, T3,hydrocortisone, EGF, and insulin) (ATCC, PCS-999-035, Manassas, VA). Between passages, HBEC-3KT cells were trypsinized with trypsin-versene mixture (Lonza, 17-161E, Allendale, NJ), then neutralized with trypsin neutralizing solution (Lonza, CC-5002, Allendale, NJ). All cells were maintained at 37°C in a humidified atmosphere of 5% CO2.

Antibodies

Primary antibodies used for western blotting include Flag M2 at 1:3000 (Sigma; F3165), Flag-HRP at 1:1000 (Sigma; A8592) and GST Z-5 at 1:3000 (Santa Cruz Biotechnology; sc-459).

Secondary antibodies include goat anti-rabbit IgG (Santa Cruz, sc-2004, Dallas, TX) and goat anti-mouse IgG (Santa Cruz, sc-2005, Dallas, TX) and were used at 1:2500 dilution.

Plasmid construction

All plasmids of full length and truncated proteins were generated previously in the lab, and constructed using Gateway® technology (Invitrogen, Waltham, MA) according to the manufacturer's protocols. For GST-tagged and Venus-Flag tagged plasmids used for Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) and Glutathione-S-Transferase (GST) pull-downs, pDEST27 and pFUW vectors were used as destination cloning vectors, respectively. Amino (N-Venus) and carboxy (C-Venus) plasmids used for Venus Protein-Fragment Complementation Assay (PCA) were generated previously in the lab.

Transfections

For experiments with ectopically expressed proteins, HEK 293Ts and Cos7s were transfected using X-tremeGENE (Roche, 06366546001, Basel, Switzerland). HBEC-3KT were transfected with FugeneHD (Promega, E2312, Madison WI). Plated cells were transfected at a density of 60-80% confluency and performed with a ratio of 3µl transfection reagent to 1µg DNA to 100µl of serum-free media. DNA was mixed at appropriate concentrations prior to the addition of serum-free meida (DMEM for HEK 293T and Cos7; OptiMEM (Gibco, 31985088, Grand Island, NY) for HBEC-3KT). Transfection reagent was then added and incubated at room temperature for 15 or 20 minutes (X-tremeGENE or FugeneHD, respectively). Transfection complexes were then added drop-wise to plated cells.

GST pull-down

Cells were seeded in to a 6-well plate and allowed to reach 60-80% confluency. Cells were then harvested by adding 200µL of 0.5% NP-40 lysis buffer to each well, scraping to collect cells and transfer to an eppendorf tube, and incubated at 4°C for 30 minutes. Lysis buffer components consisted of 0.5% NP-40, 150mM NaCl, 10mM HEPES lysis buffer, and Phosphatase Inhibitor Cocktail (Sigma, P5726) and Protease Inhibitor Cocktail (Sigma, P8340) at 1:1000. After incubation, lysates were centrifuged to remove cellular debris. After removing 20µl of the lysate for an input control and the debris pellet, 20µl of a 50% glutathione-conjugated sepharose bead slurry (Glutathione Sepharose 4B, Fisher Scientific, 50197956, Atlanta, GA) was added to the remaining lysate and incubated by slowly rotating for 3-4 hours at 4°C. Beads were then washed three times in 0.5% NP-40 lysis buffer by inverting 8 times with 200µl of fresh lysis buffer added each time. GST-bound protein complexes were then eluted by the addition of 20µl of 2x SDS loading buffer, boiled for 5 minutes, resolved by SDS-PAGE subjected to western blotting along with input controls.

Venus protein-fragment complementation assay

Cells were seeded into 24 well plates and transfected at 60% confluency with N-Venus or C-Venus constructs. After 24 hours, cell nuclei were stained with the addition of Hoechst 33342 at 5µg/ml (Fisher Scientific, H1399, Atlanta, GA). Images were then acquired using the ImageXpress^{Micro} automated imaging high-content imaging system (Molecular Devices) with 20X objective. The standard filter set for FITC (excitation 482/35 nm and emission 536/40 nm) and DAPI (excitation 337/50 nm and emission 447/60 nm) was used for Venus and Hoechst 33342 imaging, respectively. The number of green (Venus) and total cells (Hoechst 33342) from the images were calculated using the Metamorph Analysis Cell Scoring module

and presented as percent of Venus positive cells compared to the total number of cells (Fig.A-1).

Cell health assay for HBEC-3KT cells

HBEC were seeded in a 384-well plate at 2000 cells per well and transfected using FugeneHD and diluted in Opti-MEM after 24 hours. After 72 hours, cells were stained with Propidium iodide (PI) at 5µg/ml (Fisher Scientific, BDB550825, Atlanta, GA), TO-PRO3 at 0.5µM (Fisher Scientific, T3605, Atlanta, GA), and Hoechst 33342 at 5µg/ml (Fisher Scientific, H1399, Atlanta, GA), and immediately imaged using the ImageXpress^{Micro} (Molecular Devices) with 20X objective. Analysis of cell health images was completed using a custom Metamorph Analysis module were the number of cells that stained positive with fluorescence signals for each wavelength (Hoechst, PI, and TO-PRO3) were counted and calculated as a percentage of the total cells, defined by Hoechst-stained nuclei.

Nuclear translocation and cell health multiplex assay for HEK 293T cells

HEK 293T cells were seeded at $4x10^6$ cells per well into 6 well plates with complete medium (DMEM with 10% FBS and 1% Penicillin/Streptomycin) and transfected 24 hours after plating. 48 hours after transfection, cells were stressed by replacing the complete media with DMEM media without FBS supplementation (serum free media) for an additional 24 hours. Cells were stained with Hoechst, PI, and TO-PRO3 and imaged as described above.

For nuclear translocation, the Metamorph Analysis translocation module was used to define the average intensity for the nuclear compartment, as defined by Hoechst 33342 staining and for the cytoplasmic compartment for four sites per well. The data is plotted as a ratio of nuclear

to cytoplasmic intensity (N:C). Data are presented as the mean and standard deviation from the four sites imaged for each sample. For cell health, a custom Metamorph Analysis module where number of cells that stained positive with fluorescence signals for each wavelength (Hoechst, PI, and TO-PRO3) were scored and calculated as a percentage of the total cells, defined by Hoechst-stained nuclei.

4.3 Results

4.3.1 Validation of the Aurora A/FOXO1 interaction

The Aurora A/FOXO1 interaction was identified in a high-throughput protein-protein interaction screen that used two methods to detect the presence of PPIs: TR-FRET and Renilla Luciferase PCA. High-throughput screening methodologies inherently pose risks of potential false positive or false negative signals. Therefore, an affinity-based GST pull-down was used to validate the interaction between Aurora A and FOXO1. GST FOXO1 and Venus-Flag Aurora A were co-expressed together, or with Venus-Flag or GST, respectively, in HEK 293T cells. Pull-down results from cell lysates showed that Aurora A was detected when isolated by GST FOXO1, but not GST (Fig. 4-1A). A positive interaction between Aurora A and FOXO1 provided validation of our high-throughput PPI screen.

4.3.2 Aurora A interacts with FOXO1 independently of AKT phosphorylation

AKT phosphorylation induces FOXO1 translocation from the nucleus to the cytoplasm. Recent studies revealed that PLK1, a mitotic kinase, also modulates FOXO1 localization through phosphorylation at distinct sites from AKT phosphorylation [86]. To test whether Aurora A also interacted with FOXO1 independently from AKT phosphorylation, we also tested the binding of Aurora A with a FOXO1 phospho-deficient mutant at AKT

phosphorylation sites T24, S258, S319 ($FOXO1^{AAA}$). Co-expression of GST FOXO1 compared to GST $FOXO1^{AAA}$ with Venus-Flag Aurora A in HEK 293T cells and subjected to GST pull-down revealed no difference in binding between FOXO1 or $FOXO1^{AAA}$ (Fig. 4-1B). Thus, Aurora A interacts with FOXO1 independent of phosphorylation by AKT.

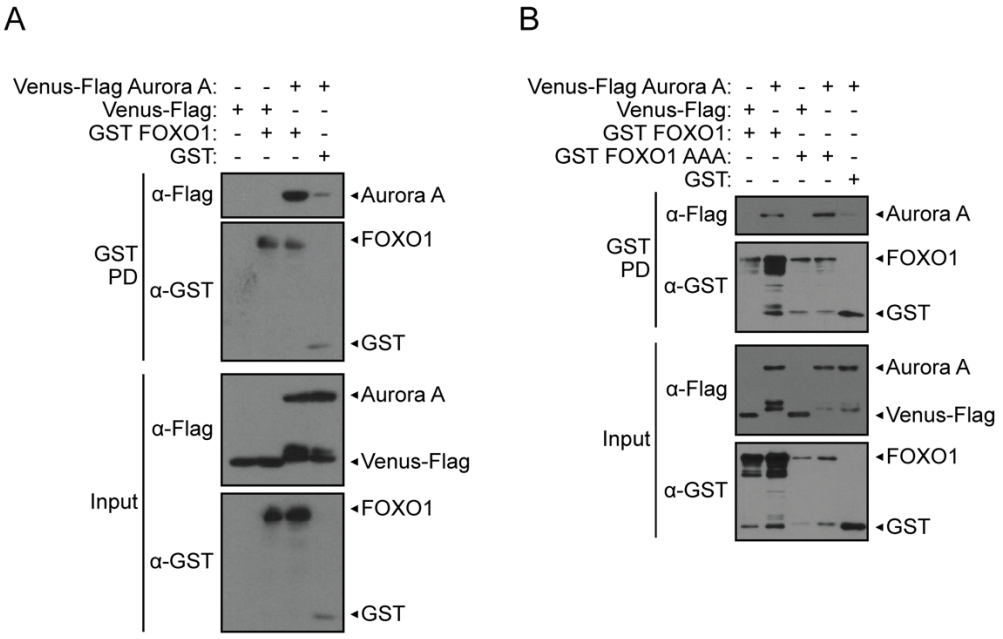
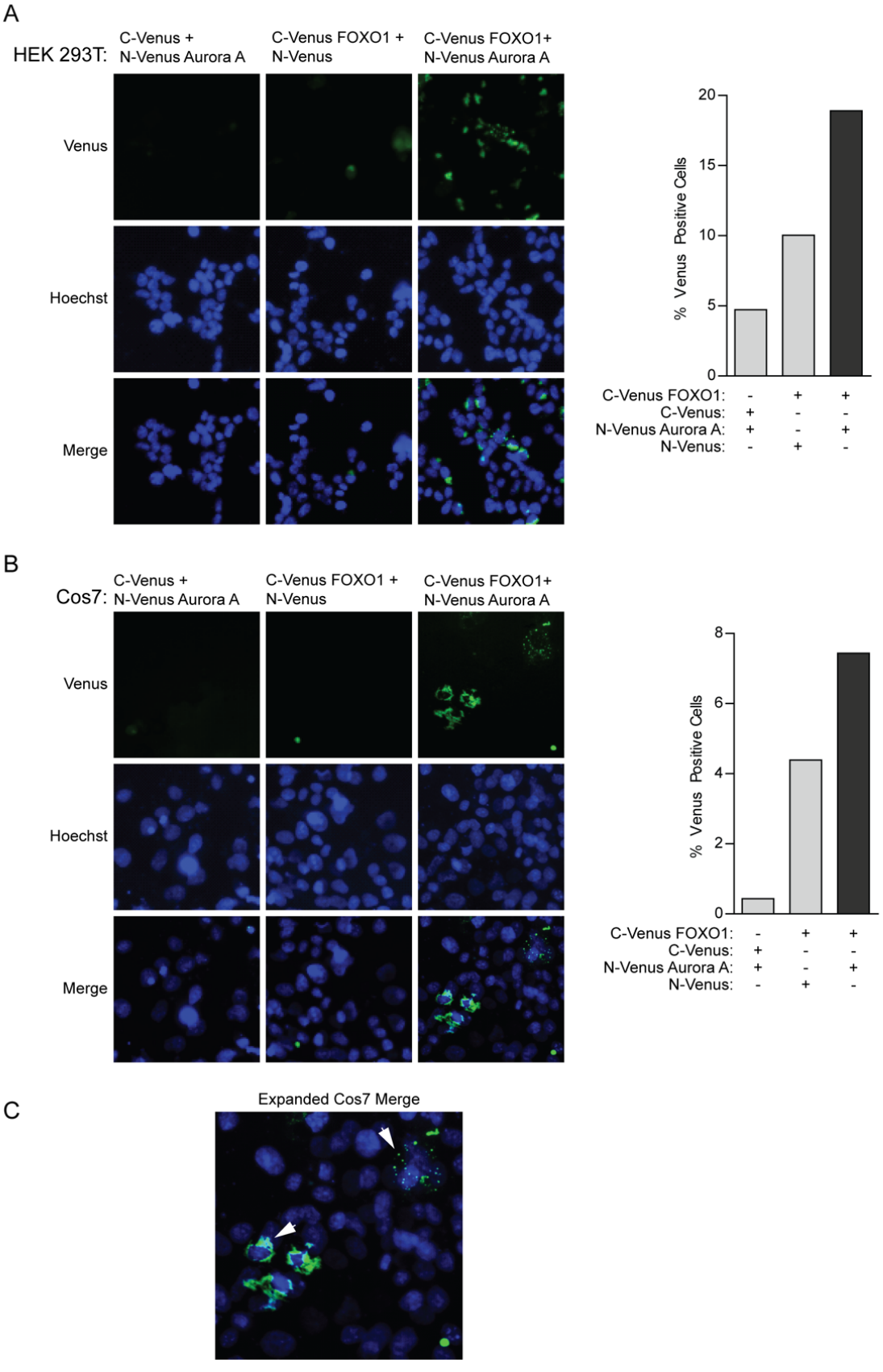


Figure 4-1. Validation of the Aurora A/FOXO1 interaction.

(A) GST pull-down assay conducted after GST FOXO1 complexes were isolated from HEK 293T cell lysates with co-expressed Venus-Flag Aurora or appropriate controls. The presence of Venus-Flag Aurora A in the GST FOXO1 protein complex (GST PD) and protein expression levels in the cell lysate (Input) was detected by Western blotting using anti-Flag or anti-GST antibody, respectively. (B) Aurora A interacts with AKT phospho-deficient FOXO1 mutant. GST pull-down assay conducted after GST FOXO1 or GST FOXO1^{AAA} complexes were isolated from HEK 293T cell lysates with co-expressed Venus-Flag Aurora or appropriate controls.

4.3.3 Aurora A binds nuclear and cytoplasmic FOXO1 in a distinct pattern

FOXO1 localization is critical for its transcriptional activity. Aurora A has been detected in both the nuclear and cytoplasmic cellular compartments. AKT or PLK1 inhibited FOXO1 localizes to the cytoplasm. To visualize the cellular localization of the Aurora A/FOXO1 interaction, we utilized the Venus PCA assay. This assay captures the functional reconstitution of N-Venus or C-Venus fragments fused to two interacting proteins by fluorescence imaging in living cells. Our results show that co-expression of N-Venus Aurora A and C-Venus FOXO1 in asynchronous HEK 293T or COS7 cells yielded two distinct interaction patterns (Fig. 4-2). In a subset of cells, the interaction appears in larger regions in and near the nucleus. In another subset of cells, the interaction appears at distinct, punctate regions throughout the cytoplasm. Quantitatively, the overall Aurora A/FOXO1 interaction is represented by an increase in the number of fluorescent cells compared to the expression of N-Venus Aurora A or C-Venus FOXO1 with negative controls. These data provide further validation of the Aurora A/FOXO1 interaction, supporting the TR-FRET and GST pull-down data. Further, identifying the localization of the interaction may point towards a potential function of the Aurora A/FOXO1 interaction in deregulating FOXO1.


A
HEK 293T:
C-Venus + N-Venus Aurora A
C-Venus FOXO1 + N-Venus
C-Venus FOXO1+ N-Venus Aurora A
Venus
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15
10
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C-Venus: + - -
N-Venus Aurora A: + - +
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C-Venus FOXO1: - + +
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N-Venus Aurora A: + - +
N-Venus: - + -
C
Expanded Cos7 Merge

Figure 4-2. Localization of the Aurora A/FOXO1 interaction.

Venus protein-fragment complementation (Venus PCA) assay conducted in living (A) HEK 293T and (B) Cos7 cells co-expressing N-Venus Aurora A and C-Venus FOXO1 or vector controls. Interaction between tagged proteins allowed reconstitution of fluorescent Venus protein. The representative data shown represents the Venus positive cells that were quantified from fluorescence imaging of 6 sites per well. The percentage represents the number of cells with positive interactions compared to the total number of cells (determined by Hoechst staining). Representative images: Venus (positive protein-protein interaction), Hoechst (nucleus), Merge (overlap of Venus and Hoechst signals). (C) Expanded view of the Venus and Hoechst merged image of the Aurora A/FOXO1 interaction in Cos7 cells. White arrows indicate distinct localization patterns of the interaction.

4.3.4 Aurora A inhibits FOXO1-induced apoptosis

The transcriptional activity of FOXO1 targets genes that have direct roles in cell cycle inhibition and apoptosis. Therefore, FOXO1 access to the nucleus promotes cell cycle arrest and cell death. To test the effect of Aurora A on FOXO1-induced apoptosis, we conducted a cell health assay in the human bronchial epithelial cell line, HBEC. Aurora A and FOXO1 were co-expressed together or with appropriate negative controls and stained for apoptosis markers after 5 days in culture (Fig. 4-3). A slight decrease in cell viability is observed with Aurora A expression compared to Venus-Flag alone. In contrast, expression of FOXO1 results in a striking decrease in cell viability. Interestingly, co-expression of Aurora A with FOXO1 rescues cell viability to the level of cells expressing Aurora A alone. Therefore, this suggest that Aurora A is able to rescue FOXO1-induced apoptosis in non-transformed epithelial cells.

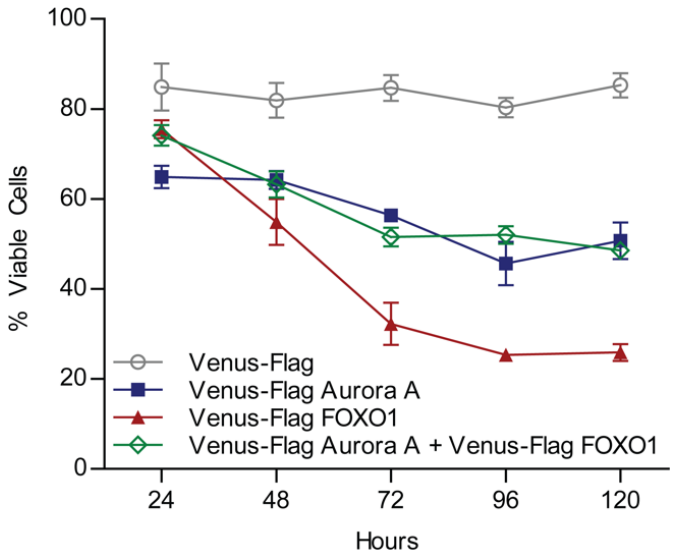


Figure 4-3. Aurora A inhibits FOXO1-induced cell death in non-transformed cells.

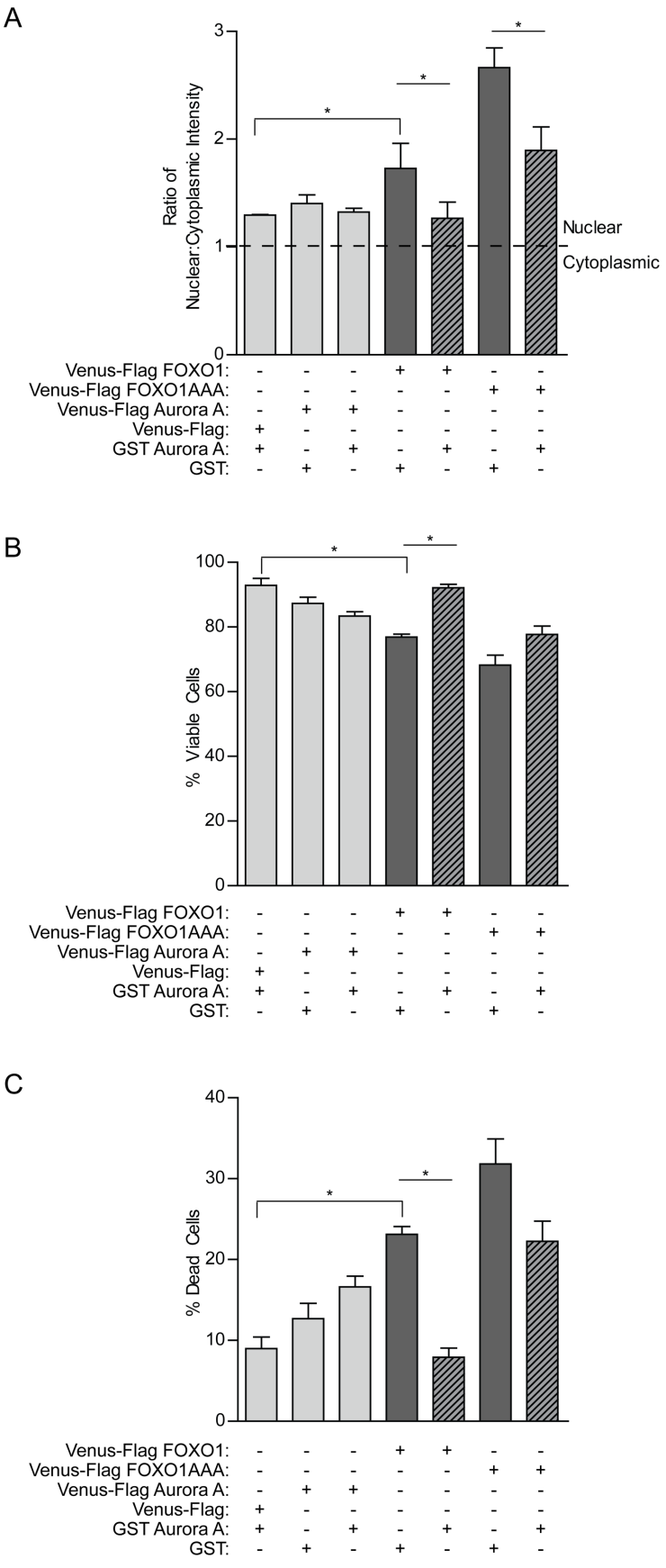
Cell health assay performed using HBEC-3KT cells co-expressing either Venus-Flag negative control, Venus-Flag Aurora A, Venus-Flag FOXO1, or the combination of Venus-Flag Aurora A and Venus-Flag FOXO1. Data presented as percent viable cells of total cells at 24, 48, 72, 96, and 120-hours post-transfection. Error bars represent the standard deviation among triplicate wells.

4.3.4 Aurora A suppresses FOXO1-induced apoptosis by inhibiting the nuclear localization of FOXO1

In response to stress signaling from stimuli such as nutrient deprivation, TNF-α stimulation, or Lipopolysaccharides (LPS), activated FOXO1 accumulates in the nucleus to initiate transcription of genes related to cell cycle inhibition and apoptosis [157]. Both AKT and PLK1 inactivate FOXO1 by preventing FOXO1 nuclear localization. To determine if Aurora A acts in a similar manner, we evaluated the impact of Aurora A on FOXO1 nuclear localization. In a multiplexed assay to determine nuclear translocation and cell viability, we utilized Venus fluorescence by co-expressing Venus-Flag FOXO1 or the constitutively nuclear AKT phospho-deficient mutant Venus-Flag FOXO1^{AAA} with either GST Aurora A or GST in HEK 293T cells, serum starving cells for 24 hours, and adding fluorescent dyes to stain cell nuclei and apoptotic markers. Co-expression of GST Aurora A and Venus-Flag Aurora A tested for comparison. The ratio of nuclear to cytoplasmic fluorescence intensity was used to quantify nuclear translocation in each condition. Our results show in serum starved conditions, both FOXO1 and FOXO1^{AAA} were primarily nuclear (Fig. 4-4aA). In contrast, co-expression with Aurora A greatly reduced nuclear localization of FOXO1. The same effect was observed, although to a lesser extent with FOXO1^{AAA}.

In the same serum starved samples samples, we assessed cell viability using a cell health assay. This multiplex assay relies on three dyes: Hoechst, TO-PRO3, and PI to evaluate the number of apoptotic or necrotic cells. The percent of viable cells in each condition as calculated. Results show that FOXO1 expression decreases cell viability compared to the expression of Aurora A of Venus-Flag; however, co-expression of Aurora A with FOXO1 recues cell viability. Similarly, FOXO1^{AAA} greatly reduces cell viability, but this effect is also rescued by co-expression with Aurora A (Fig. 4-4aB). In quantifying the number of early

apoptotic, late apoptotic, or necrotic cells in this same assay, the total percentage of dead cells was determined. In this data, the ability of Aurora A to reduce cell death induced by FOXO1 and FOXO1^{AAA} is also observed (Fig. 4-4aC). Representative images of Venus-Flag tagged protein nuclear or cytoplasmic localization after serum starvation are provided in Fig4-4b. Taken together, these results suggest that in the absence of a pro-growth stimulus like growth factors that are present in serum, the interaction of Aurora A and FOXO1 may inhibit FOXO1-induced apoptosis by excluding FOXO1 from the nucleus and thereby inhibit FOXO1 activity.


A
Ratio of Nuclear:Cytoplasmic Intensity
3
2
1
0
Nuclear
Cytoplasmic
*
*
*
Venus-Flag FOXO1: - - - + + - -
Venus-Flag FOXO1AAA: - - - - - + +
Venus-Flag Aurora A: - + + - - - -
Venus-Flag: + - - - - - -
GST Aurora A: + - + - + - +
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% Viable Cells
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Venus-Flag FOXO1: - - - + + - -
Venus-Flag FOXO1AAA: - - - - - + +
Venus-Flag Aurora A: - + + - - - -
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GST Aurora A: + - + - + - +
GST: - + - + - + -

Figure 4-4a. Aurora A inhibits FOXO1 nuclear translocation and rescues FOXO1-induced cell death.

(A) FOXO1 nuclear translocation assay conducted after GST Aurora A and Venus-Flag FOXO1 or Venus-Flag FOXO1^{AAA} were co-expressed in HEK 293T cells and serum starved for 24 hours. Nuclear and cytoplasmic fluorescence intensity was determined using fluorescence imaging and Metamorph analysis software. The ratio of nuclear to cytoplasmic intensity (Nuclear:Cytoplasmic) was calculated and plotted per condition. Data are presented as the mean and standard deviation from four sites imaged for each well. (B) Simultaneous staining for apoptotic markers was used to determine cell viability in the same samples. The percentage of viable cells compared to the total cells determined by Hoechst staining was calculated using fluorescence imaging and Metamorph analysis software. Data are presented as the mean and standard deviation from four sites imaged for each well. (C) Quantification of the percent of dead cells (included early apoptotic, late apoptotic, and necrotic cells) as calculated by fluorescence imaging and Metamorph analysis software. Data are presented as the mean and standard deviation from four sites imaged for each well. For all comparisons (A-C), Statistical significance was determined using a one-way analysis of variance (ANOVA) and Bonferroni multiple comparisons post-test ($p<0.05$).

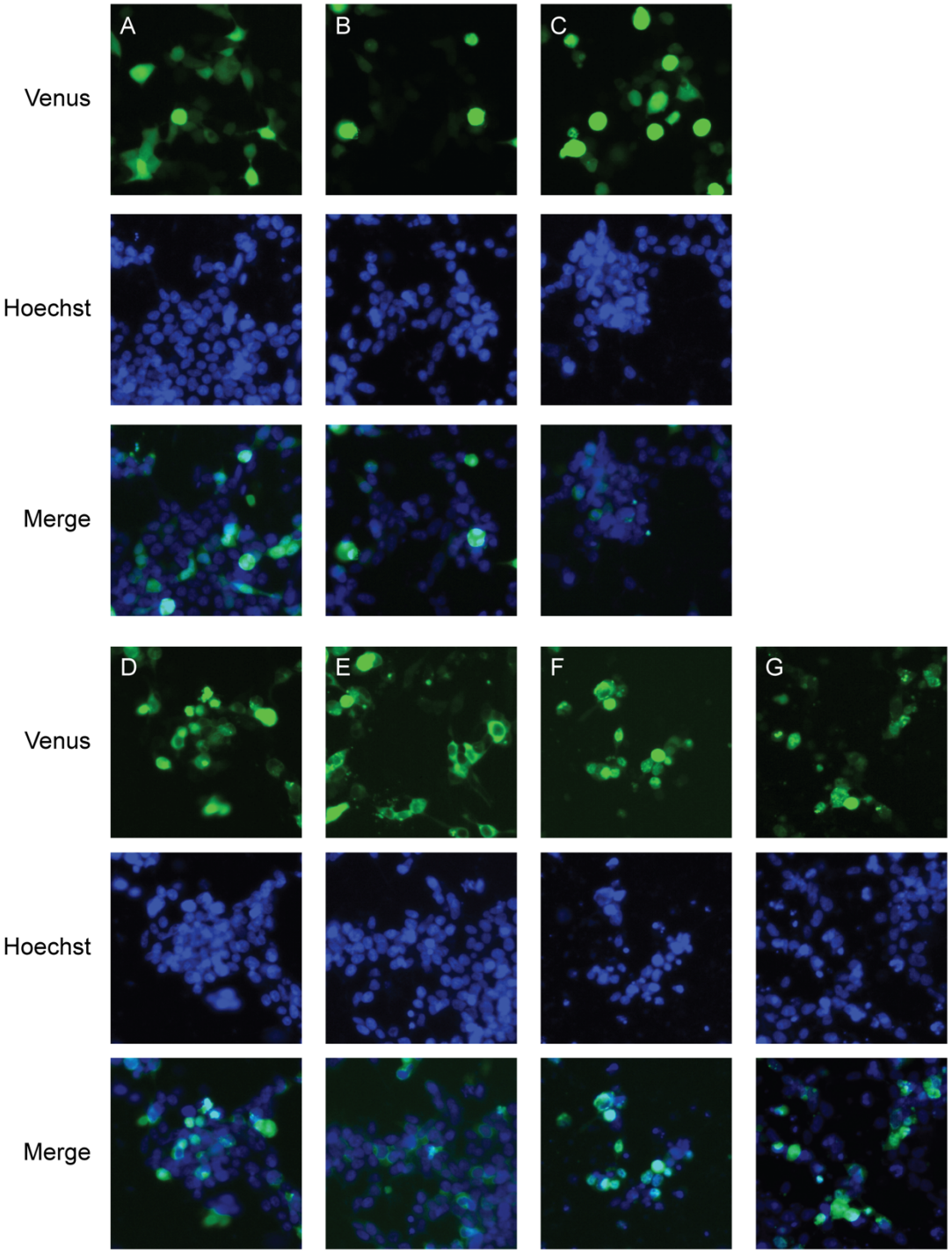
A
B
C
Venus
Hoechst
Merge
D
E
F
G
Venus
Hoechst
Merge

Figure 4-4b. Aurora A inhibits FOXO1 nuclear translocation.

Representative Venus, Hoechst (nuclear dye), and merged images from each condition are shown: (A) GST Aurora A + Venus-Flag (B) GST + Venus-Flag Aurora A (C) GST Aurora A + Venus-Flag Aurora A (D) GST + Venus-Flag FOXO1 (E) GST Aurora A + Venus-Flag FOXO1 (F) GST + Venus-FOXO1^{AAA} (G) GST Aurora A + Venus-FOXO1^{AAA}.

4.4 Discussion

Normal cells acquire the ability to evade apoptosis as a result of genetic and epigenetic alterations that result in cancer development. As an important mediator of cellular responses to stress, the FOXO1 transcription factor activates transcriptional programming that mediates cell death events including apoptosis and autophagy. Cancer cells utilize a number of mechanisms to deregulate FOXO1 at the transcriptional, translational, and functional level. Our work demonstrates that Aurora A, a kinase that is overexpressed in several cancer types, interacts with and functionally deregulates FOXO1.

Protein-protein interactions are the basis by which Aurora A mediates oncogenic signaling. Identifying these interactions may provide novel therapeutic targeting options for the treatment of cancer. For example, we identified that Aurora A forms a protein complex with H-Ras to promote Ras-MAPK signaling. Others have identified functional interactions of Aurora A specifically with transcription factors: Aurora A prevents degradation of oncogenic transcription factors like N-Myc in a kinase-independent manner [57], while it can also phosphorylate and promote degradation of critical tumor suppressive transcriptions factors like p53 [109]. These interactions have potential as therapeutic targets, as allosteric inhibition of the Aurora A/N-Myc interaction releases N-Myc from Aurora A and promotes Myc degradation [56].

One well-characterized mechanism of FOXO1 regulation is phosphorylation by AKT. Phosphorylation of FOXO1 by AKT excludes FOXO1 from the nucleus and leads to cytoplasmic sequestering by 14-3-3 protein. We observed that Aurora A interacts with both wild-type FOXO1 and the AKT phospho-deficient mutant, $FOXO1^{AAA}$, suggesting that Aurora A interacts with FOXO1 in a manner that is not regulated by phosphorylation from AKT. Because $FOXO1^{AAA}$ is a constitutively nuclear mutant [147], these results suggested that

the Aurora A/FOXO1 interaction may occur within the nuclear compartment of the cell. Indeed, imaging to reveal the localization of the Aurora A/FOXO1 demonstrated that the interaction takes place in and around the nucleus, but also in distinct regions in the cytoplasm. Interestingly, cytoplasmic Aurora A/FOXO1 interactions occurred a punctate regions throughout the cytoplasm, resembling that of P-bodies or the LC3 marker of autophagy [158, 159]. The fact that Aurora A is localized in both the nucleus and cytoplasm may facilitate the ability for Aurora A to alter FOXO1 localization.

When we tested this, we found that indeed Aurora A is able to exclude FOXO1 from the nucleus in serum starved cells. Although to a lesser extent, Aurora A was also able to exclude $FOXO1^{AAA}$ from the nucleus in serum starved cells. This data, as well as our data that shows Aurora A interacts with $FOXO1^{AAA}$, provides evidence that Aurora A may act in a mechanism that is independent from AKT phosphorylation. Lastly, our observation that Aurora A rescues FOXO1-induced apoptosis in bronchial epithelial cells highlights a potential functional effect of the Aurora A/FOXO1 interaction, whereas Aurora A reverses the ability for FOXO1 to induce apoptosis in these cells.

The exact mechanism by which the Aurora A/FOXO1 interaction deregulates FOXO1 remains unknown (Fig. 4-5). Over the years, additional kinases have been identified to also exclude FOXO1 from its site of functional activation in the nucleus. CDK5 and SGK1 both phosphorylate FOXO1 [160, 161]. Additionally, PLK1 phosphorylates FOXO1 at a site distinct from AKT. Aurora A, a kinase with shared functions as PLK1, may also deregulate FOXO1 through direct phosphorylation. In their investigation of the relationship between Aurora A and FOXO3a, another research group was unable to detect an interaction between Aurora A and FOXO3a, although FOXO3a expression was induced as result of treatment with the Aurora kinase inhibitor, Alisertib [88]. Since Aurora A activates the kinase activity of

PLK1, an alternative explanation is that Aurora A may serve to promote PLK1 kinase activity in complex with FOXO1. Lastly, Aurora A may deregulate FOXO1 through interactions with other shared partners such as SIRT, which impacts FOXO1 degradation through acetylation.

The functional consequence of the Aurora A/FOXO1 interaction is critical to our understanding of the ability for Aurora A to inhibit tumor suppression and aid in the development of cancer. FOXO1 also serves as critical mediator of innate immune responses to bacterial infection of the airway by indirectly initiating transcription of inflammatory cytokines and stimulating immune response [162]. Nuclear localization of a closely related FOXO family member, FOXO3a, is observed in response to a multitude of cellular stresses including smoking, cystic fibrosis, acute respiratory distress syndrome (ARDS), pneumonia, and chronic obstructive pulmonary disease (COPD) [162]. This stress-induced induction of cell death is crucial to maintaining organ homeostasis and the prevention of cancer growth. Therefore, our findings reveal that Aurora A may negate the tumor suppressive function of FOXO1 and aid in the progression of cancer and other diseases, thereby supporting the interaction as a viable target for therapeutic discovery.

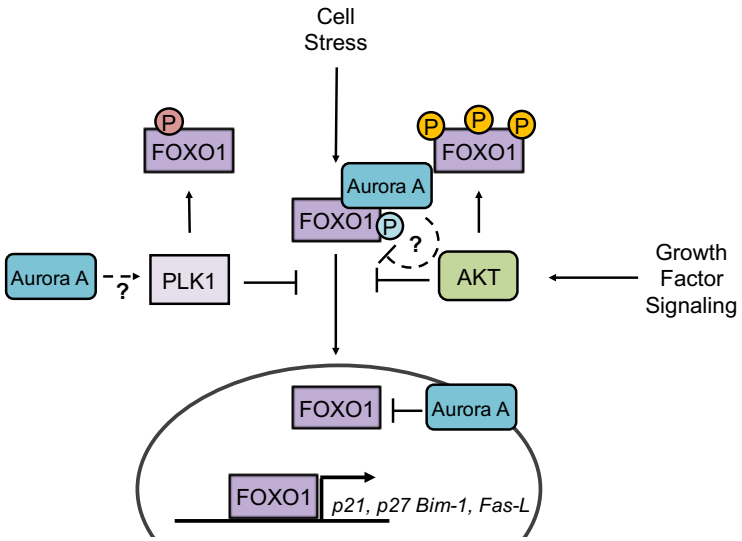

Figure 4-5. Potential mechanisms for negative regulation of FOXO1 nuclear translocation and inhibition by Aurora A protein-protein interactions.

Aurora A is able to inhibit nuclear translocation and subsequent cell death induced by both FOXO1 and to a lesser extent, the AKT phospho-deficient mutant, FOXO1^{AAA}, thus, Aurora A may induce FOXO1 nuclear exclusion in an AKT-independent manner. Aurora A was found to interact with FOXO1 both around the nucleus in some cells, and in the cytoplasm in others. Aurora A may associate with known negative regulator, PLK1 to inhibit FOXO1. Aurora A may also directly phosphorylate FOXO1 and interfere with its nuclear localization signals. Additionally, Aurora A interactions in and around the nucleus may interfere with FOXO1 DNA binding and transcriptional activity.

Chapter 5: Discussion

5.1 Novel functions for Aurora Kinase A (Aurora A) are revealed through protein-protein interactions

A human cell is well-orchestrated symphony of biomolecules, functioning in harmony to maintain a homeostatic, normal state. The framework for this symphony is a protein-protein interaction (PPI) network through which instructions about cellular processes from growth, movement, and even death are communicated and executed. The genetic and epigenetic alterations that occur in cells are reflected through changes in PPIs and eventually changes in phenotype. These alterations rewire the PPI network and deregulate cellular signaling pathways that are critical for regulation, leading to disease. Cancer is a collection of diseases that result from uncontrolled cellular growth and proliferation. Thus, the rewired PPI network that exists in cancer cells allows the acquisition of cancer hallmarks [1, 5]. Most significantly, the ability to sustain proliferative signaling and to evade growth suppression are crucial for cancer development [1].

The data presented in this thesis reveal new mechanisms by which the genetic alterations found in cancer cells promote growth and avoid death through novel functions of Aurora Kinase A (Aurora A). Aurora A has emerged as a viable therapeutic target in cancer [163]. Though much of the oncogenic function of Aurora A is linked to its canonical role in cell cycle progression, evidence is mounting for non-mitotic activities of Aurora A that promote tumor progression. Aurora A is overexpressed and mis-localized in many cancers, and protein levels remain high beyond M-phase into interphase [21, 163]. The over-abundance of protein is thought to contribute to the oncogenic nature of Aurora A, potentially placing Aurora A in cellular contexts in which it would not normally be found [24, 164-167]. Thus, Aurora A overexpression contributes to a rewiring of the PPI network in cancer. The identification of novel Aurora A PPIs in this dissertation supports this notion. We found that

Aurora A impinges on two well-known pathways involved in cancer. First, we discovered that Aurora A sustains proliferative signaling by forming a protein complex with H-Ras and Raf-1, thereby promoting Ras-MAPK signaling in a Ras-dependent manner (Chapters 2 and 3). Second, we revealed that Aurora A contributes to the evasion of apoptosis by interacting with and functionally inhibiting FOXO1, as evidenced by the ability to rescue FOXO1-induced apoptosis (Chapter 4). Taken together, we demonstrated novel functions for Aurora A in cancer cell signaling (Figure 5-1).

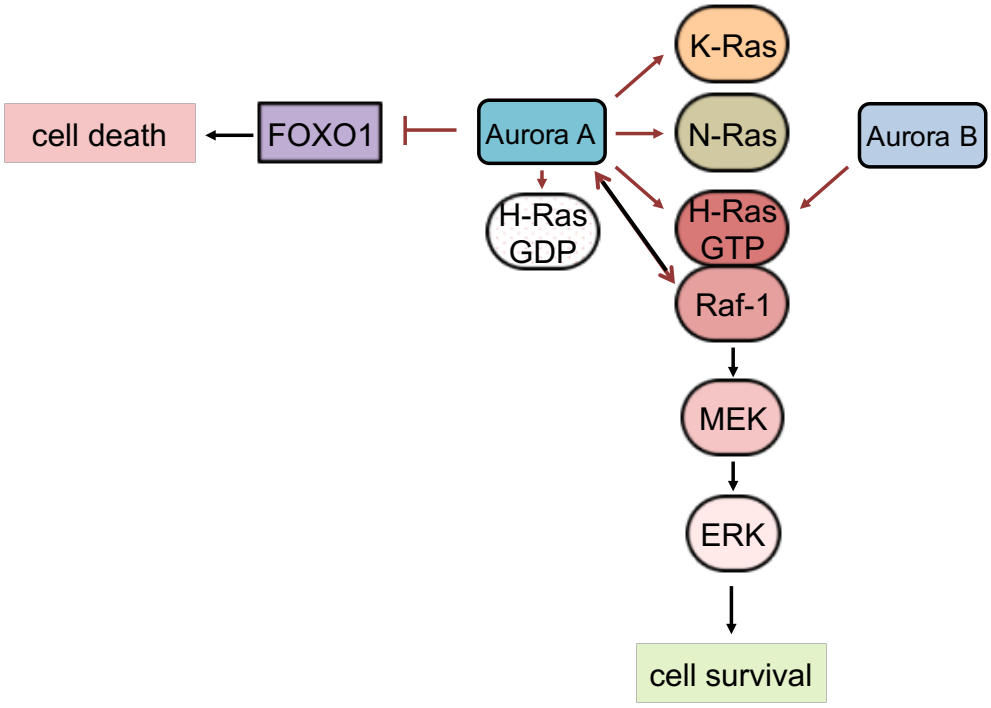

Figure 5-1. Novel Aurora A interactions and their function in cancer.

In this dissertation research, we identified that Aurora A interacts with H-Ras, K-Ras, and N-Ras. Further Aurora B also interacts with H-Ras. We demonstrated that Aurora A forms a protein complex with Raf-1 to enhance oncogenic Ras-MAPK signaling. We also identified that Aurora A interacts with FOXO1. We demonstrated that Aurora A inhibits FOXO1 nuclear translocation and cell death. The Aurora A interactions and functional effects that were elucidated through this work may serve as potential therapeutic targets to release the ability for Aurora A to sustain oncogenic signaling and to evade cell death in cancer.

5.2 Aurora A interacts with H-Ras, forming a positive feedback loop that sustains enhanced oncogenic MAPK signaling

The discovery that Aurora A interacts with H-Ras and forms a protein complex with H-Ras and Raf-1 provides a potential mechanism by which Aurora A promotes Ras-MAPK signaling in cancer. Support for crosstalk between Aurora A and MAPK signaling has been provided in previous work by several groups; yet, a mechanism for this phenomenon remained unknown. Aurora A is a downstream transcriptional target of of Ras-MAPK signaling. Aurora A is upregulated by MAPK signaling, and several Ras driven cancers are found to have amplified Aurora A [37, 124, 127]. Further, targeting of Ras by farnesyltransferase inhibitors decreases Aurora A expression [124, 129]. Aurora A is also thought to function upstream of MAPK signaling. Both Aurora A and Aurora B potentiate transformation by H-Ras G12V in mouse fibroblasts [115, 116, 123]. In addition, knock-down of Aurora A decreases MAPK signaling and inhibits the epithelial to mesenchymal transition (EMT) in nasopharngeal cancer cells [90]. Our work places Aurora A upstream of Ras-MAPK signaling and provides additional evidence that Aurora A may establish a positive feedback loop between pro-growth and cell cycle proteins in cancer.

We discovered that along with H-Ras, Aurora A interacts with K-Ras and N-Ras. Considering that Ras alterations drive 30% of cancers, the ability for Aurora A to interact with the three major Ras isoforms provides a basis for the impact of Aurora A overexpression in cancers regardless of which isoform is more dominant. This finding has significant implications for the role of the Aurora A/Ras PPI in a variety of cancers. The potential Aurora A enhanced Ras signaling mediated by K-Ras and N-Ras may induce positive regulatory circuits that can be therapeutically exploited. For example, K-Ras was found to induce expression of Aurora A and Aurora B, and inhibition of these kinases reduced cell growth in

K-Ras mutant lung cancer cells [168]. Further, the combination of Aurora A and MEK kinase inhibitors arrests cells at G2/M in colorectal cancer cells with K-Ras and PI3K mutations [169]. As additional evidence for the potential impact of disrupting the cooperation between Aurora A and Ras-MAPK signaling in a variety of cancers, Aurora A kinase inhibitors used in combination with B-Raf and MEK inhibitors exhibited enhanced efficacy in inhibiting melanoma cell growth [170].

Our work establishes a function of Aurora A upstream of Ras-MAPK signaling to enhance the activation of ERK. ERK is a key mediator of many cellular outcomes, including cell cycle progression, growth, proliferation, survival, and migration [171]. Importantly, we demonstrate that Aurora A activates ERK through MAPK signaling as Aurora A requires an in-tact Ras-Raf-MEK-ERK signaling pathway to activate ERK. Dominant negative H-Ras, kinase dead Raf-1, and pharmacological inhibition of Raf or MEK can all block the ability of Aurora A to enhance ERK phosphorylation. In this Aurora A-MAPK positive feedback loop, upregulated Ras-MAPK signaling drives the overexpression of Aurora A. In return, Aurora A overexpression upregulates Ras-MAPK signaling. Significantly, we demonstrate that this positive feedback loop may also be uncoupled from extracellular stimulation; in MC7 breast cancer cells, Aurora A enhances ERK phosphorylation even in serum starved conditions. This rewired circuitry may enable cell proliferation, ensuring that adequate protein levels of cell cycle mediators and pro-proliferative molecules are abundant during tumor development. The serum-independent effect of Aurora A on ERK activation also suggests that overexpressed Aurora A may promote activation of Ras in a mechanism that is either independent of or complimentary to growth factor signaling.

5.3 Structural domain characterization provides insight into functional outcomes of Aurora A interactions

In these studies, the functional domains that mediate binding for the interactions of Aurora A with H-Ras and Raf-1 were characterized. Identifying the binding regions not only informs the functional relevance of the interaction, it also provides a basis for structure-based design of peptide or small molecule PPI inhibitors to use as functional probes or potential therapeutics.

5.3.1 Aurora A domains

We generated Aurora A truncations around three distinct functional regions: the N-terminal domain (amino acids 1-130), the kinase domain (amino acids 130-383), and the C-terminal domain (amino acids 383-403). The N-terminal domain is a flexible, unstructured region that serves as a regulatory domain for localization, degradation, and binding [172]. It houses sites of recognition by the APC/C called the KEN domain (amino acids 6-8) and D-box-Activating Domains/A-boxes (amino acids 42-53) [28, 29]. This region is also thought to localize Aurora A to the centrosomes during interphase [166]. The kinase domain of Aurora A, which has been crystalized, contains an activating phosphorylation site (T288) and a centrosome targeting sequence [173, 174]. The D-box (amino acids 363-382) [41] is where APC/C ubiquitinates Aurora A targeting it for proteasome-mediated degradation and is located near the C-terminal domain (amino acids 383-403) [29]. Imaging of the Venus-Flag tagged Aurora A truncations confirmed the localization observed in previous work other groups [172, 174]. Full-length Aurora A, N, and C truncations were all found in both the nucleus and cytoplasm when co-expressed with H-Ras. In contrast, Aurora A K and NK truncations exhibited visibly distinct localization when co-expressed with H-Ras, presumably

at the centrosomes during mitosis (Fig. A-3). It is important to note that Ras is a driver of centrosome amplification, in which Aurora A localization to the centrosome would be vital and also contributory to chromosome instability in cells [175].

Both H-Ras and Raf-1 interacted with the NK and K, but with much less affinity if not at all for the N or C truncations. Thus, the kinase domain of Aurora A is both necessary and sufficient for binding. Interestingly, binding of K was consistently higher than that of NK. The N-terminal region of Aurora A contains an inhibitory region (amino acids 64-128) that was found to bind amino acids 240-300 in the Aurora A kinase domain; deletion of this region enhances Aurora A catalytic activity [41]. Deletion of the N-terminal domain of Aurora A may also provide a more accessible binding site within the kinase domain for H-Ras and Raf-1. While this finding suggests that the kinase activity plays a role in the H-Ras and Raf-1 interactions, we were not able to establish this conclusion through our work. Because both H-Ras and Raf-1 interact with the kinase domain, and Aurora A enhances Ras/Raf effector binding, we propose the kinase domain of Aurora A serves a protein scaffold for H-Ras and Raf-1 binding, allowing enhanced Ras effector engagement in Aurora A overexpressed cancers. Whether Aurora A kinase inhibitors modulate the Aurora A/H-Ras/Raf-1 protein complex remains to be confirmed. However, the exciting development of an allosteric inhibitor of the Aurora A/N-Myc interaction provides evidence that even a kinase-independent role of Aurora A can be effectively targeted and disrupted [56].

5.3.2 H-Ras domains

Aurora A interacts with the N-terminal domain of H-Ras. Amino acids 1-66 were sufficient to bind Aurora A; however, deletion of amino acids 1-36 also retained binding ability. Thus, Aurora A may minimally require a region within amino acids 36-66 of H-Ras for

binding. The N-terminal domain of Aurora A lies within the G-domain, which is responsible for GTPase activity [69]. The Switch I and II domains are also located here and change in conformation when GTP is bound or unbound. Lastly, the effector binding domain of H-Ras, specifically for Raf-1 binding (amino acids 32-40) are located within this region [176]. It is therefore unsurprising that Aurora A has an impact on the H-Ras/Raf-1 interaction. Although one may think that Aurora A binding may compete with Ras effectors, the opposite was true. We found that by binding the N-terminal of H-Ras, Aurora A actually enhances the binding of H-Ras and Raf-1. Due to the identical sequences in the N-terminal domain of H-Ras, K-Ras, and N-Ras, Aurora A can interact with these isoforms. However, the ability for Aurora A to enhance signaling through these isoforms remains to be tested.

5.4 Dual roles for the Aurora A/Raf-1 interaction

Our studies reveal that Raf-1 also associates with Aurora A and H-Ras. We determined that the kinase domain of Aurora A mediates the interaction with the kinase domain of Raf-1. Notably, although Aurora A and Raf-1 interact, we demonstrated that H-Ras activity is required for enhanced MAPK signaling. This suggests that the Aurora A/Raf-1 interaction does not circumvent the role of the Aurora A/H-Ras interaction. Raf-1 kinase activity does, however, play a role in the Aurora A/H-Ras/Raf-1 protein complex and subsequent MAPK signaling. Constitutively active Raf-1 (S259A) enhanced binding to Aurora A as well as binding of Aurora A to H-Ras. Treatment with Sorafenib 5μM was able to block Raf-1 kinase activity and to disrupt the Aurora A/H-Ras interaction. Interestingly, kinase-dead Raf-1 did not enhance nor dissociate the complex.

Aurora A was previously found to associate with Raf-1 and PLK1 and Raf-1 at the centrosome during mitosis [134]. PLK1 interacts with both Raf-1 WT and K375M, and that

allosteric inhibition (but not catalytic inhibition) of Raf-1 (S338D/K375M) interfered with PLK1 localization at the centrosome during mitosis [134]. A MEK-independent role of Raf-1 was proposed, suggesting that Raf-1 associates with Aurora A and PLK1 at the centrosome to enhance PLK1 activity and mitotic progression. Inhibition of Raf-1 with Sorafenib at 5μM did not inhibit PLK1 activation by Raf-1.

Whether the Aurora A/H-Ras/Raf-1 complex also functions to enhance PLK1 activation and promote tumor growth remains to be tested. This work suggests a dual role for the association of Aurora A and Raf-1, both in mitosis and MAPK signaling.

5.5.1 Functional impacts of the Aurora A/FOXO1 interaction

Our work also reveals a novel interaction between Aurora A and FOXO1. Previous work from other groups established Aurora and FOXO1 interplay through transcriptional regulation. Aurora A overexpression decreased FOXO1 transcription in a p53-mediated manner [92]. Another study into gene expression profiles in psoriasis patients found that Aurora A gene expression was upregulated and FOXO1 expression was repressed [91]. TNF-α stimulates apoptosis by activating FOXO1 [157]. Kayal et al found that patients treated with a TNF-α agonist had increased FOXO1 expression and decreased Aurora A gene expression [91]. Adding to the regulation at a transcriptional level, we provide evidence that Aurora A may also deregulate FOXO1 and the protein-level. In our observations, we captured the interaction of Aurora A and FOXO1 at two distinct localizations. These interaction locations may correlate with cell cycle stages. Nuclear and perinuclear Aurora A/FOXO1 interactions resemble the distribution of Aurora A during prometaphase [177, 178]. Cytoplasmic Aurora A/FOXO1 interactions resemble the distribution of p-bodies or autophagosomes [179]. We show that in cells that are stressed by serum starvation, Aurora A blocks translocation of both

FOXO1 WT and AKT phospho-deficient FOXO1^{AAA}. Although the results from our apoptosis assay strongly suggest that Aurora A interaction with FOXO1 results in FOXO1 inhibition, characterization of the associated cellular processes is a necessary step for understanding the function of the interaction.

5.5.2 Postulated mechanisms for FOXO1 deregulation by Aurora A

Aurora A could contribute to FOXO1 inhibition in a number of manners that remain to be understood. Aurora A may act similarly to PLK1, which has been recently identified to phosphorylate FOXO1 to exclude access to the nucleus for transcription of pro-apoptotic genes [86, 180]. Aurora A and FOXO1 also share the ability to bind SIRT1. SIRT1 is a class III histone deacetylase that can promote cell viability by reducing activating FOXO1 acetylation [181-183]. A mitotic kinases mix, including Aurora A and PKL1, promoted activating CDK1 phopshorylation of SIRT1. Thus, in addition to the proposed regulation of FOXO1 through a direct interaction, Aurora A may act in complex with SIRT1 or PLK1 to inhibit FOXO1.

5.6 Future directions and translational implications

The ability to improve the survival rate for patients diagnosed with cancer is directly linked to our ability to provide new therapeutic options. As the field has progressed in our understanding that cancer represents a complex, heterogeneous group of diseases, the importance of precision medicine, in which the key genetic alterations that contribute to the development of an individual's cancer is targeted through therapy, is truly underscored. Our work brings to the forefront the importance of understanding how genetic alterations also

change the protein-protein interaction landscape and signaling pathways that contribute to the hallmarks of cancer.

Specifically, we identified novel functions for Aurora A that are mediated through newly discovered protein-protein interactions with Ras and FOXO1. Ras is a key driver in cancer, yet efforts to target Ras directly have failed in the clinic. Our work has advanced the understanding how Ras is regulated and provided insights into how cell growth is controlled. Thus, our work may lead to new ways to target Ras for therapeutic discovery by targeting its positive regulator. The interaction of Aurora A and H-Ras provide a novel therapeutic target that could have great potential in cancers with overexpressed Aurora A and that rely on MAPK signaling to promote sustained proliferative signaling. Unfortunately, early clinical investigation of Aurora A kinase inhibitors met unexpected challenges in the clinic, where the efficacy of cell cycle inhibition was limited by toxicity in patients [163]. This work has also highlighted importance of targeting non-enzymatic functions of kinases. This may help to overcome drug resistance and toxicity also resulting from kinase inhibition.

The impact of Aurora A on FOXO1 function in HBECs also directs us to consider that Aurora A may play a role in normal cell function and other diseases. Additional investigation into this relationship may provide novel therapeutic options for other FOXO1-mediated processes such as immunity, longevity, and apoptosis [184].

Future directions stemming from this work would progress our findings towards the clinic. First, it is important to determine if Aurora A also enhances Ras-MAPK signaling in cancers mediated by other Ras isoforms. We show that Aurora A further enhances ERK activation by H-Ras G12V, and the same may be true for prominent K-Ras and N-Ras mutations found in different cancer types. Next, delving deeper into the mechanism, beyond protein scaffold that we propose, of how Aurora A upregulates Ras would increase our

understanding of the function of Aurora A in tumor biology. An evaluation of the impact of Aurora A on the ability for Ras to bind GTP, interact with RasGAPs or RasGEFs, or anchor to the membrane would elucidate this mechanism. In a similar manner, there is more to discover about the mechanism by which Aurora A deregulates FOXO1. Testing whether Aurora A phosphorylates FOXO1, enhancing its cytoplasmic localization or degradation, would clarify this point and potentially enable therapeutic targeting using kinase inhibitors. Lastly, developing small molecule inhibitors for the Aurora A/H-Ras and Aurora A/FOXO1 interaction would allow a tool for disrupting the interactions. Success in preclinical models would allow the clinical evaluation of these PPI inhibitors for the treatment of cancer.

Overall, the work presented in this dissertation provides a foundation for the development of PPI inhibitors for the oncogenic interactions described. New approaches to inhibiting the oncogenic activity of Aurora A were revealed by interrogating its interactions. Targeting of these interactions, especially in combination with established effected therapies has the potential to benefit patients and improve prognosis. The prevalence of Aurora A overexpression in GBM, breast, pancreatic, colon, bladder, and oral cancer heightens the importance of this study as beneficial for broadly applicable advances in cancer therapy.

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Appendix

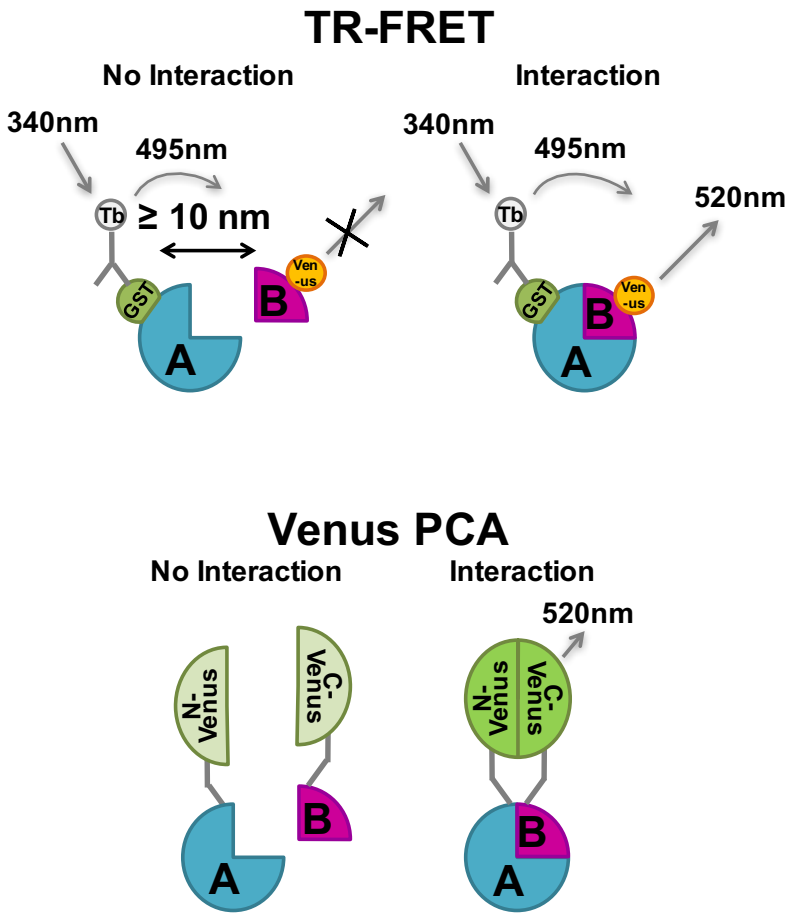

Figure A-1. Schematic representation of TR-FRET and Venus PCA assays.

Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) assay and Venus Protein-fragment Complementation Assay (Venus PCA) are two methods to detect protein-protein interactions.

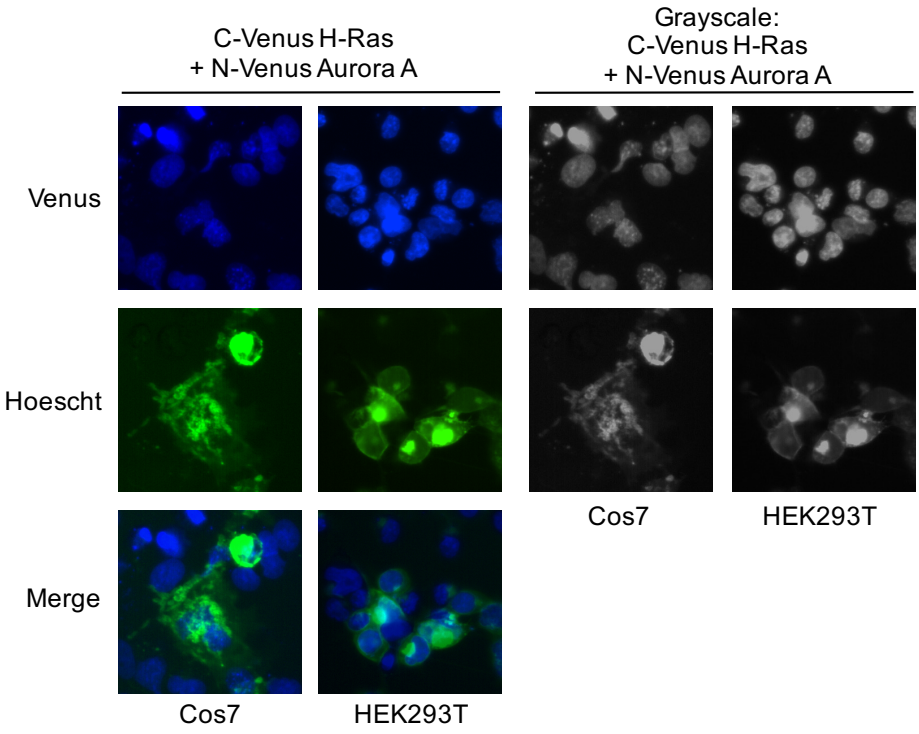

Figure A-2. The Aurora A/H-Ras interaction localizes to the plasma membrane and possibly the Golgi apparatus in HEK 293T and Cos7 cells.

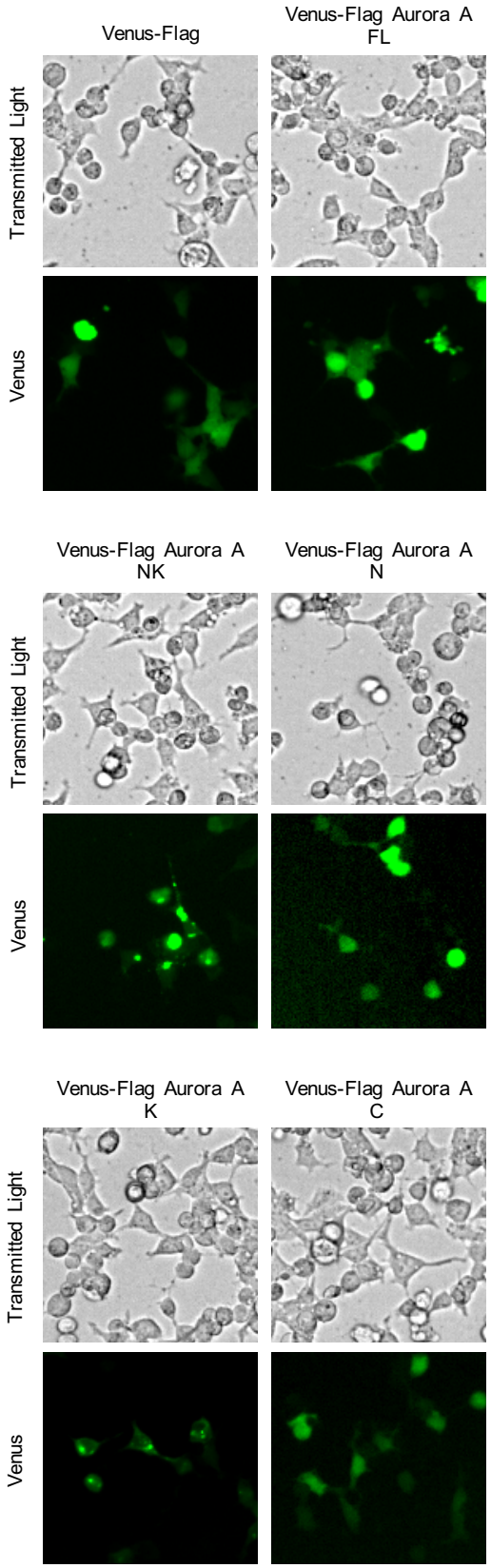

Figure A-3. Localization of Venus-Flag Aurora A truncations in HEK 293T cells.