The Pennsylvania State University

The Graduate School

College of Medicine

CHARACTERIZATION OF FOXO3A AS A SUPPRESSOR OF LUNG ADENOCARCINOMA

A Dissertation in

Genetics

by

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ABSTRACT

Lung tumor development is believed to occur through a step-wise series of molecular changes that influence cell growth and survival. This process can be facilitated by tobacco smoke, which contains numerous carcinogens that are known to induce mutations and chromosomal instability (CIN). The FOXO family of transcription factors has been shown to function in cell cycle arrest, apoptosis, and response to various physiologic and pathologic stresses, such as DNA damage and oxidative stress. Because these transcription factors have been implicated as tumor suppressors, genetic inactivation of these genes could contribute to the development of cancer. Here, I examined the status of the FOXO3a gene in human lung tumors from patients with early stage non-small cell lung cancer (NSCLC). Using qPCR, allelic loss of 80% or higher was detected in 8 out of 33 (24.4%) mostly-early stage lung adenocarcinoma (LAC) samples in smokers suggesting bi-allelic or homozygous deletion (HD) of FOXO3a. The remaining 60.6% of these tumors had losses of FOXO3a not reaching the level of HD, a loss of between 40-79% (hereafter referred to as sub-HD). Consistent with the status of the FOXO3a gene, there were corresponding decreases in its mRNA and protein levels in LAC samples. Squamous cell lung carcinomas (LSqCC) were also analyzed for allelic loss of FOXO3a. No HDs were detected in these samples, but 47% contained sub-HD loss of FOXO3a. Although a broader analysis of FOXO3a’s activity (either through mutations, protein expression, or cellular localization) in all samples is needed in order more fully assess FOXO3a’s inactivation in both LSqCC and LAC, our results suggest that FOXO3a inactivation occurs more frequently in LAC and that it’s inactivation plays a role in the development of LAC tumors.
FOXO3a’s ability to stimulate apoptosis or cell cycle arrest in response to carcinogens was investigated in H1299 and A549 LAC cell lines as a potential role in suppressing lung carcinogenesis. Here we have demonstrated that FOXO3a is functionally activated and augments caspase-dependent apoptosis in cells exposed to (+)-anti-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), a DNA-damaging carcinogen present at high concentrations in tobacco smoke. Consistent with this result was the FOXO3a-dependent upregulation of pro-apoptotic effectors BIM, BNIP3 and FASL with treatment. These results implicate FOXO3a in the elimination of carcinogen-damaged cells, a role consistent with the suppression of LAC carcinogenesis, and one frequently lost through gene deletion in LAC development.

Docetaxel and vinorelbine are common anti-mitotic drugs used in second line therapy of several cancer types, including lung adenocarcinoma. We investigated the role of FOXO3a in the cellular response to anti-mitotic agents in LAC cells, as this function would be lost in LAC as a consequence of gene deletion. MTS assays revealed that FOXO3a transfection causes a significant decrease in the number of LAC cells (both A549 and H460 cell lines) upon treatment with either of two different anti-mitotics, docetaxel and vinorelbine. These results indicate a potential pro-therapeutic response by FOXO3a in response to anti-mitotics. Unlike treatment with BPDE, annexin-PE and western blot analysis of various caspases showed only a modest stimulation of apoptosis in response to anti-mitotics in LAC cells expressing exogenous FOXO3a. Consistent with this result, RT-PCR expression analysis of known pro-apoptotic effectors of FOXO3a (BIM, BNIP and FASL) showed no increase in mRNA levels following treatment. Interestingly, cell cycle analysis of A549 and H460 LAC cell lines treated with anti-mitotic agents
showed a pronounced reduction in G2/M fraction in cells expressing FOXO3a exogenously compared with cells that did not express FOXO3a. RT-PCR analysis of known FOXO3a cell cycle effectors showed no change in Cyclin-D2 or p27Kip1 expression levels in response to treatment. However, analysis of CDC14A mRNA levels in treated cells show evidence of a pronounced FOXO3a-dependent derepression of this gene’s expression following treatment. CDC14A is known to stimulate exit from mitosis. This potentially accounts for the observed decrease in the G2/M fraction in FOXO3a transfectants treated with anti-mitotic agents. These results suggest a potential pro-therapeutic role for FOXO3a in LAC, which involves the stimulation of mitotic exit of tumor cells with therapeutically induced mitotic spindle defects. Together these results suggest that the loss of the FOXO3a gene in LAC is likely to contribute to lung carcinogenesis and lessen the response to anti-mitotics used to threat these tumors.
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<td>NFκB essential modulator</td>
<td>NEMO</td>
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<td>Non-fat milk</td>
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<tr>
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<td>Vinyl carbamate</td>
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<td>Von Hippel–Lindau tumor suppressor</td>
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<td>X-ray repair complementing defective repair in Chinese hamster cells</td>
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To Patience: for being there in seemingly unending quantities

For Josh Miller: day in and day out, the best part of coming to lab.
CHAPTER 1: Literature Review

1.1 INTRODUCTION

Cancer arises due to a disruption of the balance between cell proliferation and apoptosis (Hanahan and Weinberg 2000). There are several theories presented as to how cancer arises. One proposes that cancer arises from a single cell having acquired cancerous genetic properties and replicating into a cancerous mass. Another prominent theory suggests that cancers arise from a body of heterogeneous cells, having each acquired unique mutations that contribute to its evolution and selection into a fully developed cancer. A more recent theory suggests that cancers arise from a field of initiated cells that undergo independent selection and clonal expansion with the most successful ones becoming dominant in a tumor’s cell population (Hanahan and Weinberg 2000, Twombly 2005). All these theories involve a form of evolution or Darwinian selection and acquisition of genetic alterations, such as oncogene activation and tumor suppressor gene inactivation. Oncogenic activation and tumor suppressor inactivation are thought to occur at an abnormally high frequency in cancer due to the inherent genetic instability of cancer cells (Beckman and Loeb 2000, Fisher 1958, Rangarajan et al. 2004).

More than 80% of tumors arise from the epithelia, which are sheets of cells that line the walls of cavities or channels, or skin (Berman et al. 2004). Epithelia have two major biological functions. Some epithelia seal the channel which they are lining and protect the cells in the underlying populations, others also contain specialized cells that secrete substances that contribute to the function of the lining (e.g. absorption, gas exchange, etc.) into the cavities or ducts that they line (Berman et al. 2004). The most common type of cancer – carcinoma – arises
from epithelial cells. Carcinomas include tumors that arise from the gastrointestinal tract (mouth, esophagus, stomach, small and large intestines), skin, mammary gland, pancreas, lung, liver, ovary, gall bladder, and urinary bladder. Carcinomas are classified as either in situ (a local growth that has not invaded adjacent tissue) or invasive (tumors that have spread to nearby tissue or have metastasized) (Berman et al. 2004, Tsikitis et al. 2006).

Most carcinomas fall under two categories – squamous cell carcinomas or adenocarcinomas (Weinberg 2006). Squamous cell carcinomas arise from the epithelial cells lining ducts or channels (for example skin or esophageal cancers), while adenocarcinomas arise from epithelial cells that also contain specialized cells, such as the glandular epithelium of the lung, stomach, uterus, and cervix. Cancer also arises from non-epithelial tissue. These include sarcomas, which are derived from connective tissues, and angiosarcomas which arise from precursors of epithelial cells (Weinberg 2006). Other forms of non-epithelial cancers are ones that constitute blood-forming tissue. These hematological malignancies may derive from either of the two major blood cell lineages: myeloid and lymphoid cell lines. The myeloid cell line normally produces granulocytes, erythrocytes, thrombocytes, macrophages and mast cells; the lymphoid cell line produces B, T, NK and plasma cells. Lymphomas, lymphocytic leukemias, and myeloma are from the lymphoid line, while acute and chronic myelogenous leukemia, myelodysplastic syndromes and myeloproliferative diseases are myeloid in origin. Cancer also arises from neural tissue, such as gliomas and neuroblastomas (Berman et al. 2004, O’Donnel et al. 2009).
Cancer develops through successive stages of increasing aggressiveness. The earliest, most benign stage is hyperplasia, which is an abnormal increase in cell proliferation. These growths contain cells that appear similar to their normal surrounding tissue, except in excessive numbers (Bodey et al. 2005). These cells still retain the ability to assemble into a specific tissue (for example, ductal epithelium of mammary glands). Metaplastic growths appears most frequently in transition zones of epithelia where one type meets another (for example the junction of the cervix and the uterus, or the esophagus and the stomach), and is characterized by a disruption in the transition zone. In other words, at this stage, the cells appear completely normal but are essentially present in the wrong space. Dysplasia occurs with increased abnormalcy. Dysplastic cells do not have the appearance of normal cells, and can vary in properties such as nucleus size and shape, and lack of features present in differentiated cells of the tissue from which they arose. Dysplasia is considered the transition state between benign and malignant growth. When this growth invades the underlying tissues, it is called a carcinoma and referred to as neoplasia. Finally, metastasis occurs when carcinomas spread throughout the body via the vasculature, homing and establishing new sites of aggressive malignant growth. The accepted model of progression is that cancer evolves into a malignant entity as follows: normal tissue → hyperplastic → dysplastic → neoplastic → metastatic (Bodey et al. 2005, Kerr 2001).
1.1.1 **Genetic changes underlie cancer development: oncogenes**

Endogenous genes can be altered in such a way that they contribute to the malignant transformation of normal cells. These genes are called oncogenes (Fearon 1997).

Seminal work by Dr. Peyton Rous characterized the first viral oncogene (Rous 1911). In his experiments, Dr. Rous successfully transmitted a sarcoma in the breast muscle of a hen by homogenizing a small fragment of this tumor and injecting the filtrate into another chicken of the same breed. These chickens would later also develop tumors at the site of injection, and the infectious agent came to be called Rous sarcoma virus (RSV) (Rous 1911). When infected with RSV, chicken embryo fibroblasts appeared transformed, proliferated at a higher rate, lost contact inhibition (and produced RSV-induced foci), and displayed a rounded morphology as opposed to the flattened morphology of normal cells (Bute 2000). In 1976, Dr Michael Bishop and Dr. Harold Varmus used Southern blotting with a \( v\text{-src} \)-specific probe to determine that the \( src \) sequence was also present in wild type uninfected avian cells (Stehelin et al. 1976). This sequence belonged to a cellular gene (named \( c\text{-src} \)) that is structurally similar to \( v\text{-src} \). \( c\text{-src} \) encodes a tyrosine-specific kinase that associates with the plasma membrane. The protein consists of three domains, an N-terminal SH3 domain, a central SH2 domain, and a tyrosine kinase domain (Varmus et al. 1989). When phosphorylated, the SH2 and SH3 domains function as auto-inhibitors of \( c\text{-src} \)'s kinase domain. When active, \( c\text{-src} \) functions to signal cellular proliferation through other kinases such as RAS and Akt (Varmus et al. 1989). \( v\text{-src} \) lacks the inhibitory phosphorylation site (Tyr527), and the protein is therefore constitutively active (Snyder et al. 1985). These findings implicated that cancers can arise from genetic changes in a
single gene which can elicit cellular changes in shape, metabolism and growth behavior (Weinberg 2006).

Further evidence that cancers arise from genetic changes in such genes was uncovered with studies like the ones by Croce et al. who found C-MYC mRNA transcript to be expressed at higher levels in Burkitt lymphoma cells than in normal cells (Croce et al. 1983). These studies found that the cause of this overexpression was a translocation of an immunoglobulin light chain locus (which contains enhancer elements) to a region 3’ of the C-MYC gene from chromosome 22q to 8q. Untranslocated C-MYC (in normal cells) was found to remain silent, while in Burkitt’s lymphoma cells it was activated (Croce et al. 1983, Croce et al. 2008). The highly active immunoglobulin promoter in this cell type resulted in the overexpression of translocated C-MYC. Malignant lymphocytes have very frequently been shown to carry this translocation, which indicated that high level of MYC expression contributed to the formation of lymphoma (Croce et al. 2008).

The transforming nature of oncogenes was also demonstrated by transfecting DNA from human cancer cells into immortalized mouse fibroblasts (Shih et al. 1979). In these experiments, the fibroblasts (NIH-3T3 cells) formed foci more readily than untransfected cells, and in fact, when DNA from these foci was subsequently transfected into fibroblasts, these also formed foci at higher rates (Shih et al. 1979, Malumbres and Barbacid 2003). Dr. Robert Weinberg’s lab went on to demonstrate by Southern, northern, and western analysis that human oncogenic KRAS from the tumors was responsible for the transformation of the mouse fibroblasts (Parada and Weinberg 1983). DNA sequence analysis revealed that a single base
substitution of G → T was the only difference between KRAS in normal tissue and cancer tissue. This substitution resulted in the incorporation of valine instead of glycine at codon 12 (RAS’ active site), which would impede its interaction with other catalyzing proteins, keep RAS in its activated state (a GTP-bound protein), and continue a growth-promoting signal (Krengel et al. 1990, Reddy et al. 1982).

We have discussed oncogenes that arise from abnormally high expression levels (C-MYC) and mutations that lead to structural changes to proteins (RAS). Oncogenes also arise through formation of hybrid proteins with oncogenic properties. This was first uncovered from work on Chronic Myeloid Leukemia (CML), and the identification of a chromosomal translocation between chromosome 9 and 22 (discovered by karyotyping) which fused the Abl1 (Abelson murine leukemia) gene with the BCR (breakpoint cluster region) gene (Nowell 1962). This translocation, t(9;22)(q34;q11), appears as an elongated chromosome 9 and truncated chromosome 22 (the Philadelphia chromosome) (Kurzrock et al. 2003, Nowell 1962). Analysis of the protein content of CML cells revealed that a larger 210 kd fragment was identified in these cells using Abl-specific antibodies, which provided evidence that the fused genes also code for a fused protein (Goffen et al. 1984). The BCR-Abl protein has elevated Abl1 tyrosine phosphokinase activity, is constitutively active, and activates cell proliferation pathways (Kurzrock et al. 2003).
1.1.2 Genetic changes underlie cancer development: tumor suppressor genes

The proteins that oncogenes encode can overrule the normal function of proliferation signaling pathways and bring about malignant transformation of cells. Oncogenes are therefore considered to encode a dominant transforming phenotype (Finlay et al. 1989). Cancers can also arise from recessive mutations, and these genes are referred to as tumor suppressors. Tumor suppressor genes are classically defined as negative regulators of tumorigenesis which sustain loss-of-function mutations in the development of cancer (Haber and Harlow 1997). A classical demonstration of this was in the case of the retinoblastoma gene (RB). Retinoblastoma is a cancer of the retina that can be inherited by germline transmission of RB mutants (Friend et al. 1986). This was first postulated by Dr. Alfred Knudson, who observed the inheritance pattern of the disease. His observation led him to postulate that the onset of the disease resembled a recessive disorder involving the loss of function of two alleles of a single gene (Knudson 1971). Genetic aberrations on chromosome 13q14 were visualized in retinoblastoma patients using karyotyping, and the RB gene localized to the site of allelic loss by restriction fragment length polymorphism (RFLP) loss of heterozygosity (LOH) experiments (Friend et al. 1986). RFLP analysis is a process by which a restriction enzyme’s cleavage site is lost due to a germline polymorphism within the restriction site. Restriction digestion of normal DNA produces a heterozygous pattern when this is the case. Consequently, loss of one or the other allele can be visualized in matching tumor samples. This is referred to as loss of heterozygosity (LOH). This technique is now performed by PCR or comparative genomic hybridization of heterozygous sequences. Retinoblastoma samples showed a loss of heterozygosity (LOH) on 13q14. Eventual positional cloning of the RB gene was done in 1986 (Friend et al. 1986). Subsequent Southern
blotting of restriction digested retinoblastoma samples showed large biallelic deletions within 13q14 that specifically affected the RB gene and that one allele was typically altered in the normal parental DNA (Friend et al. 1986). This indicated inheritance of a defective RB allele was likely responsible for the inheritance of these tumors. Functional analysis of RB identified it as a key suppressor of G1 to S-phase transition through inhibition of E2F transcription factors (Sherr 2004). This provided evidence for the theory that a null copy of RB was inherited, and afterward the other wild type copy underwent deletion, which would be later called a two-hit hypothesis (Knudson 1971, Knudson 1996).

The most widely mutated tumor suppressor gene in human cancer is p53. Linzer and Levine first identified p53 in mouse fibroblasts infected with SV40 (Simian Virus 40) and mouse embryonal carcinoma cells as a 54 kd precipitate that co-reacted with the large T antigen (Linzer and Levine 1979). This version of p53 was transformative and was expressed at approximately 100 fold higher levels than observed in normal cells, which led to the original theory that p53 was an oncogene that was overexpressed (Finlay et al. 1989, Oren et al. 1981). However, wild type p53 cloned from normal cells, failed to transform rat fibroblast cells when transfected with RAS, and actually repressed the formation of foci. This confusion was resolved in 1988 when the original clone of p53 that had transformative properties was analyzed and found that a single amino acid change at position 132 (Cys to Phe) gave p53 oncogenic properties (Eliyahu et al. 1984, Finlay et al. 1988). It was found that p53 was a homotetrameric transcription factor and this mutated version of p53 was a dominant negative mutant. It bound poorly to p53 promoter binding sites and could not activate the reporter gene, but bound with wild type p53 inhibiting its function (Vogelstein and Kinzler 1992).
p53’s ability to suppress tumor cells was established by Baker et al. who transfected wild type or mutant p53 in colorectal cancer cell lines which were p53 null (Baker et al. 1990). Cells transfected with wild-type p53 contained ten times fewer malignant colonies as those transfected with mutant p53 or a vector control (Baker et al. 1990). Interestingly, analysis of p53 expression levels in the wild type transfectants that formed colonies after prolonged selection showed that the wild type p53 in these colonies had been deleted or “rearranged” (shown through restriction digest analysis that was specific for wild type p53) (Baker et al. 1990). Loss of function mutations of p53 have since been reported in most cancer types (Weinberg 2006).

Numerous studies have now characterized the function of p53 and its role as a tumor suppressor. p53 was first shown to induce G1 arrest in response to gamma radiation (Kastan et al. 1991). p53 is responsive to DNA damage, eliciting cell cycle arrest, senescence or apoptosis depending on the cellular context and the severity of the stress (Kastan et al. 1991, Sherr 2004). p53 also suppresses oncogenic stimulation. Wild-type p53 has been shown to suppress the oncogenic effects of mutant RAS. This was shown in co-transfection experiments in which p53 induced senescence or apoptosis in cells forced to express oncogenic RAS (Hollstein et al. 1994). p53 is considered the most frequently inactivated gene in human cancer, shown to be mutated in over 50% of some cancer types (Hollstein et al. 1994, Sherr 2004). Germline mutations of p53 also predisposes to the L-Fraumeni cancer syndrome that is characterized by heritable breast, colon and brain tumors.
In addition to DNA loss and mutation, promoter methylation is a frequent mechanism for inhibiting tumor suppressors (Greger et al. 1989). This was first described in RB in retinoblastoma tumors by using methylation-sensitive restriction enzyme digestions of the 5’ region of RB in tumor samples and comparing these to the restriction digest of unmethylated RB promoter used as a control (Greger et al. 1989). Methylation of CpG islands of gene promoters was discovered to be a mechanism of control expression. Whereas hypermethylation resulted in gene silencing hypomethylation enabled expression. However, it wasn’t until five years later that promoter methylation correlated with gene silencing of tumor suppressor genes. Herman et al. observed methylation of the Von Hippel Lindau (VHL) tumor suppressor gene promoter in renal carcinomas in 5/26 of samples studied (Herman et al. 1994). The group went on to demonstrate that the samples with methylated VHL promoters also did not express VHL RNA in a northern blot (Herman et al. 1994). This was reversed by treating the cells with 5-azacytidine, an analogue of cytidine that cannot be methylated. Therefore, these findings demonstrated tumor suppressors could be inactivated by epigenic promoter silencing. Of all tumor suppressors, p16\textsuperscript{INK4a} stands out as being the tumor suppressor most frequently found to undergo promoter hypermethylation in a variety of human cancers (Esteller et al. 2001). p16\textsuperscript{INK4a} is part of the INK4a family of proteins that function as inhibitors of CDK4 (inhibitor of CDK4) and G1 to S cell cycle progression (Kusy et al. 2004).
1.1.3 Tumorigenesis is a multi-step process

Mounting evidence had indicated that oncogenes and tumor suppressor genes contribute to cancer development and cancer development appeared to be an evolutionary process occurring slowly in a step-wise manner demarked by phenotypic change over time (Vogelstein et al. 1988). This led to the postulation that cancers develop by the step-wise progressive acquisition of genetic changes, each conferring an advantage for malignant growth. This was first demonstrated in colon cancer, which provided the clearest example of a multi-step process in tumor development due to the distinct stage-specific phenotypic characteristics of lesions that had been extracted by colonoscopy and were available for study. In 1988, Dr. Bert Vogelstein’s group conducted an analysis of the molecular changes in the various stages of colon cancer demonstrating that there was indeed some order to the process of tumorigenesis (Vogelstein et al. 1988). Their analysis revealed a general sequence of stage-specific genetic changes suggesting a context in which each change contributes to colon cancer development. The genetic changes throughout colon cancer progression were shown to follow a sequential stage-specific order starting with loss of heterogygosity (LOH) in 5q, then activation of KRAS, then LOH in 18q, followed by LOH of p53 (Vogelstein et al. 1988). Previous studies has indicated that loss of chromosomes 5q, 18q and 17p (p53), and mutation of KRAS were typical of colon carcinoma. They investigated whether these defects occurred at defined stages of colon cancer development. Approximately 30% of early stage adenomas displayed LOH on 5q (Vogelstein et al. 1988). Genetic linkage analysis of familial colon cancer characterized by a predisposition to adenomatous polyposis coli (APC) then led to the identification of the APC gene on 5q21 as the candidate tumor suppressor gene in that region. The losses on 5q were
subsequently shown to target this gene for inactivation, occurring most often early in the benign adenotomatous stage of sporadic colon cancer (Bodmer et al. 1987, Nishisho et al. 1991). KRAS mutations were found to be more prevalent in lesions that already contained 5q LOH. Analysis of KRAS mutations revealed that this genetic aberration occurred in the samples that were in the intermediate adenoma stage (observed in almost 60% of samples) and very infrequently in early adenomas (approximately 7%) (Fearon and Vogelstein 1989). To conduct this experiment, DNA from tumor samples at different stages were hybridized with radio-labeled probes specific to mutant codons 12 and 61 of KRAS. Colorectal carcinomas and adenomas greater than 1 cm in size contained mutations of KRAS in as many as 50% of samples, while fewer than 10% of adenomas smaller than 1 cm in size contained KRAS mutations (Bos et al. 1987, Vogelstein et al. 1988). This provided evidence that KRAS mutations contributed to an increase in mass of the adenomas, but were probably not the earliest event in colon cancer development.

Inactivation on chromosome 18q appeared to occur most frequently after KRAS mutations (Fearon and Vogelstein 1989). In fact, the second most frequent allelic loss in this study occurred on 18q (after 17p) occurring in more than 70% of carcinoma and almost 50% of late adenomas. The Deleted in Colon Cancer gene (DCC) has been identified as the likely target of LOH as it was also found to be expressed in normal mucosal cells, but to a much lesser extent or not at all in colon carcinoma samples. This reduced expression was later linked with direct mutation of the DCC gene in colon carcinoma (Fearon and Vogelstein 1989).
p53 inactivation was found to frequently occur after DCC (Fearon and Vogelstein 1989). An analysis of chromosomal loss in the different stages of colon cancer formation showed that 17p loss was rare in early stages (<5% in early adenoma) but increased in frequency in later-stage colon cancer samples (10% in intermediate adenomas, 30% in late adenomas, and 75% in carcinomas). The high frequency of 17p allelic loss in the later stage samples and low frequency in early stages argued that 17p loss contributed to the late stage progression of these tumors (Fearon and Vogelstein 1989). The p53 tumor suppressor gene is located on chromosome 17p and mutations of this gene were identified in the colon tumors with 17p LOH, implicating p53 inactivation as a contributing factor to late stage progression of colon cancer (Fearon and Vogelstein 1989). p53 has been shown to stimulate apoptosis in response to oncogenic stimuli, which specifically includes KRAS mutagenic activation (Eliyaho et al. 1984, Lin and Lowe 2001). It is thought that the successive accumulation of such changes in these tumors activate this function of p53 which often drives the selection for its loss at this stage of colon cancer.

Further study by Volgelstein and Kinzler confirmed these initial findings, demonstrating that colon cancer develops through a somewhat orderly succession of genetic changes as the tumors progress toward malignancy (Figure 1.1). This was the first indication that the changes selected for in cancer are not independent of other changes that occur, but evidently interact or cooperate with prior changes which together drive tumorigenesis forward. Subsequent studies have demonstrated a multi-step process for other cancer types including squamous cell carcinoma of the lung (Wistuba and Gazdar 2006).
Figure 1.1. A, A genetic model for colon tumorigenesis. B, Mutations accumulate as tumor stage advances. Four genetic alterations (RAS mutation, or allelic loss on chromosome 5p, 17p, and 18q) were analyzed in colon cancer samples at different stages. Adapted from Fearon and Vogelstein 1990.
A multi-step process of molecular events has been described for colorectal cancer. Analysis of their roles can provide an understanding of the step-wise interactions of altered functions that lead to cancer.

Allelic loss at 5q in colorectal cancer patients has been tightly linked specifically to 5q21 and the APC gene (Bodmer et al. 1987). The majority of APC mutations in colorectal cancer patients involve a premature stop codon, leading to a truncated APC protein. Wild type APC binds and negatively regulates β-catenin. When active, β-catenin stimulates the Wnt signal transduction pathway, which promotes cellular proliferation (Robbins et al. 2002, Takayama et al. 2006). Therefore, inactivation of APC leads to a constitutively active Wnt signal transduction pathway (Alberici and Fodde 2006). Interestingly, cells of the colon undergo migration, differentiation and degradation within 3-4 days. Loss of APC function has been linked to impaired cellular turnover in the colon (Robbins et al. 2002). APC inactivation therefore promotes cellular longevity and proliferation and the possibility that these cells will acquire additional mutations (Robbins et al. 2002). This is evident in the fact that germline APC mutations predispose to a type colon cancer characterized by numerous benign polyps.

KRAS mutations in colorectal cancer most typically involve activating mutation in codon 12, but also occur with less frequency at codons 13 and 61 (Jen et al. 1994, Robbins et al. 2002). Mutated KRAS activates a variety of effector pathways such as RAF/MAPK/ERK, JNK, and PI3K/Akt that promote cell proliferation (Robbins et al. 2002). Interestingly, KRAS mutations confer an advantage for colorectal carcinoma progression in cooperation with APC inactivation. In an analysis of 54 hyperplastic and dysplastic lesions, KRAS mutations were detected in 22%
hyperplastic specimens and APC mutations in 0% of the specimens. However, dysplastic lesions contained KRAS mutations in 25% of specimens and APC mutations in 82% of specimens examined (Jen et al. 1994). This study suggested that KRAS activation promotes the progression of colon cancer in lesions that also contain APC inactivation. In this study, APC truncating deletions were detected by PCR and electrophoresis, and KRAS mutations detected by Southern blot using mutation specific probes. The DCC gene (deleted in colorectal cancer) has been proposed as a candidate tumor suppressor gene on 18q21 (Cho et al. 1994). DCC undergoes allelic deletion in ~60% of colorectal carcinomas, and it has been shown that 90% of colorectal cancers that undergo allelic loss at 18p, lose DCC (Cho et al. 1994, Carethers 1996). DCC encodes the main receptor for the neuronal navigation cue netrin-1 (Mehlen et al. 1998). It has been shown that DCC belongs to the so-called family of dependence receptors. Such receptors induce apoptosis when their ligand is absent, thus conferring a state of cellular dependence on ligand availability. It’s been proposed that DCC is a tumor suppressor because it induces the death of tumor cells that grow in settings of ligand unavailability (Furne et al. 2006). Loss of DCC evidently promotes the survival of premalignant colon lesions.

p53 is inactivated in ~50% of high-grade dysplastic colorectal tissue and ~75% of colorectal carcinomas (Robbins et al. 2002). Therefore it is thought to play a role in acquisition of malignancy. p53 is considered the guardian of the genome, and is upregulated in response to various stresses (such as DNA damage, radiation, and hypoxia) and minimizes the propagation of genotoxic mutations through apoptosis and inhibition of growth (Carethers 1996). As mentioned previously, it has been shown that KRAS’ oncogenic properties are suppressed by wild type p53 in rat fibroblasts (Eliyahu et al. 1984). This implies that KRAS oncogenic signaling
forces selection pressure on p53 which would contribute to the inactivation of p53 in more advanced tumors.

Based on these findings, a scheme has been proposed for colorectal cancer and the role these genes play in its progression. APC deletion increases the pool of premalignant cells that can acquire additional changes sufficient for tumor progression, such as KRAS mutations. KRAS mutations drive this progression by constitutively signaling for proliferation. DCC and p53 loss enable the survival and continue progression of these lesions to malignancy. These are the major changes observed in colon cancer. However, additional changes and alternatives are also evident. For example, Wnt and β-catenin upregulation have been shown to be alternatives to APC inactivation. Also, MDM2 upregulation has been shown to substitute for p53 loss. Lastly, p16\textsuperscript{INK\textsubscript{4a}} hypermethylation is also common in colon cancer. However, the establishment of this model predates the discovery of p16\textsuperscript{INK\textsubscript{4a}} inactivation in these tumors (Croce et al. 2008, Esteller 2002, Osada and Takahasi 2002).

1.1.4 Cancer development occurs through Darwinian evolution

Studies on colon cancer progression suggested that the multi-step progression of cancer occurs by Darwinian evolution (Boland and Ricciardiello 1999). According to this model, a random initiating mutation affecting a critical cellular function produces a cell with some growth advantage. This cell and its descendants proliferate more effectively than its neighbors. Through a process of clonal expansion, this mutated population expands, crowding out genetically less favored cells. As this clonal population expands, the likelihood that additional
changes will occur that are favorable for continue malignant growth is increased. This doubly mutated cell is able to survive or proliferate more effectively, and spawns a new subclonal population able to outcompete the previous one (Hanahan and Weinberg 2000, Nowell 1976) (Figure 1.2). A third mutation is then acquired and the process of Darwinian selection of changes advantageous for malignant progression continues (Boland and Ricciardiello 1999).

Studies suggest that four to six such successions or steps explain the progression of cancer at each stage (Hanahan and Weinberg 2000, Nowell 1976, Weinberg 2006). This process is evident in the step-wise progression of colon cancer.

**Figure 1.2.** Darwinian evolution of cancer occurs through clonal expansions. Adapted from Weinberg 2006.
1.1.5  **Carcinogenesis occurs by initiation, promotion, and progression**

Numerous studies in rodent models of human cancer have demonstrated that carcinogens cause many cancer types (Goertler et al. 1980, Paul and Hecker 1969). This process is referred to as chemical carcinogenesis and has classically been shown to occur through 3 broadly defined stages. This concept predates the evidence for the multi-stage process of the Darwinian evolution of cancer. However, each of these concepts compliments the other. The model of skin carcinogenesis in rodents classically defined the stages of chemical carcinogenesis. One such experimental protocol involved repeated paintings of mouse skin with 7,12-dimethylbenz[a]anthracene (DMBA: one of many carcinogenic tar constituents). Daily paintings of mice with DMBA on a patch of skin led to carcinoma development after several months (Goertler et al. 1980, Paul and Hecker 1969). However, a single painting of DMBA, followed by repeated paintings with 12-O-tetradecanoylphorbol (a skin irritant also known as TPA) led to the appearance of papillomas (benign epithelial growths) sometimes in as soon as 4 weeks. However, repeated painting with just TPA failed to cause papilloma formation. Interestingly, if a patch of skin was treated with DMBA once and left to rest for a year, subsequent paintings with TPA would still lead to papilloma formation. Finally, the formed papilloma would grow into a carcinoma if TPA treatments continued for months, and disappeared if TPA treatments stopped. However, if the papilloma was painted with a second dose of DMBA, carcinoma formation would be accelerated (Goerttler et al. 1980, Paul and Hecker 1969).
The following model for chemical carcinogenesis was proposed based on these findings. DMBA behaved as an “initiator” that left a prolonged mark on the treated cells (possibly a genetic alteration). TPA was coined a “promoter” because it prompted proliferation as long as DMBA had left its mark. The continued localized promotion of cells that had undergone initiation would eventually produce a papilloma. Discontinued TPA treatments would cause a reversion of the tumor, which suggested that TPA exerted its effect through a reversible, non-genetic mechanism. The second painting of DMBA on the papilloma constituted a third step in tumor development: “progression.” This step involved the introduction of additional irreversible changes or genetic mutations that could also substitute for the effects of repeated TPA exposure (Goerttler et al. 1980, Paul and Hecker 1969).

Some of the genes and pathways that play a role in initiation, promotion and progression have been identified in the mouse skin cancer model described above. These are consistent with the multistep development of colon cancer described earlier. Initiation is very frequently the result of mutations in the HRAS gene (Bizub et al. 1986). This was demonstrated in DMBA-induced primary papillomas which not only contained higher HRAS expression (demonstrated through northern blotting), but also contained HRAS activating mutations on codon 12 and 61 in up to 90% of tumors analyzed. In these studies, mutations were identified by Southern blotting with mutant vs. wild-type DNA probes and direct sequencing of these codons (Bizub et al. 1986, Quintanilla et al. 1986). These methods relied on prior knowledge of the mutational hotspots of the RAS genes. Nelson et al. presented substantial evidence for HRAS as an initiating mutation when DMBA-treated skin tissue was subjected to mutation-specific PCR one week after treatment and detected mutated HRAS in these cells (Nelson et al. 1986).
Also, activated HRAS genes were introduced into epidermal cells in vivo by direct application of retroviruses to mouse skin, and this area subsequently treated with TPA (Brown et al. 1986). Similar to the DMBA treatments, papillomas were induced after 8 weeks of continuous treatment of TPA (Brown et al. 1986). Therefore, the initial step in skin carcinogenesis in this mouse model was shown to involve a genetic lesion, namely HRAS mutagenic activation.

Promoting agents do not damage DNA, but reversibly stimulate the growth of initiated cells. Although the precise mechanism is still unclear, protein kinase C (PKC) is believed to play a role in the promotion phase of skin tumor development in mice (Yuspa 1994). For one, TPA is a phorbol ester which is known to stimulate PKC activity (Yuspa 1994). PKC activity has been shown to be elevated in mouse fibroblasts infected with retroviruses containing activate HRAS. Also, PKC inhibitors have been shown to inhibit TPA promotion of papilloma induction in vivo, in one study by reducing the average number of papillomas per mouse from 20 to 3 (Yamamoto et al. 1989, Yuspa 1994). As mentioned before, the effects of promoters are reversible as papillomas disappear when TPA treatment stops. Therefore, it is likely that tumor promoters (like TPA) can function as important agents in the multi-step process of tumorigenesis, even though they do not directly affect the genome.

If a papilloma is exposed to a mutagenic initiating agent a second time, a second genetic lesion is more likely to occur. This second genetic lesion leads to the process of progression (Ruggeri et al. 1991). p53 inactivation is an example of such a change, which again is analogous to the multi-stages of colon cancer (Hahn and Weinberg 2002). This was demonstrated by
Ruggeri et al. who monitored p53 expression levels in papillomas from mice induced with
DMBA and TPA at various stages of progression (Ruggeri et al. 1991). These experiments
showed that p53 inactivation occurred mainly in skin carcinomas, but not papillomas. p53
status was assessed in these experiments by Southern blotting to detect loss of p53.
Importantly, these tumors also retained HRAS mutations (Ruggeri et al. 1991). These studies
showed that the deletion or inactivation of p53 (a second gene) is able to produce a population
of cells that is independent of the promoting agent. The evidence that p53 has been shown to
play the role of this second gene implies that later stage papillomas can develop into
carcinomas through the loss of p53 function in cells incurring DNA damage and expressing
oncogenic stimulation.

1.1.5.1. Carcinogenesis often involves alteration of several functions: DNA repair

mechanism

The previous section describes studies showing that carcinogens can cause cancer by
inducing mutations in genes. This implies that DNA damage caused by a carcinogen is likely
responsible for these events. Mammalian cells have developed elaborate DNA repair
mechanisms to maintain the integrity of the genome and prevent cancer causing mutations.
Cancer causing mutations induced by carcinogenic DNA damage implies that these mechanisms
can be overwhelmed by extensive carcinogen exposure. Some of these mechanisms will be
discussed in this section.
The simplest form to maintain the integrity of the genome is a reversal of the chemical change that initially alters the DNA (Kaina et al. 2007). An example of this process is provided by the O⁶-methylguanine DNA methyltransferase gene (MGMT), which removes methyl and ethyl adducts at the O⁶ position of guanine, and glutathione-S-transferases (GSTs), which function to link electrophilic compounds (such as reactive oxygen species) with glutathione, thereby detoxifying these compounds (Kaina et al. 2007). Another gene that plays a role in methyl/ethyl group removal is AlkB, which oxidizes these groups when they attach to DNA bases. Biochemically, these lesions are epoxidized and hydrolyzed into glycol which is then excreted (Delaney et al. 2005).

Newly synthesized DNA is essentially monitored for miscopied DNA sequences by the mismatch repair (MMR) mechanism (Duckett et al. 1996). MMR repair occurs after DNA polymerases have proofread the newly synthesized DNA strand and is critical for regions that contain high amounts of mononucleotide and dinucleotide repeats because they are prone to mismatching. Strand slippage occurs when the strand slips out of alignment when DNA polymerase is copying it. This occurs at a relatively high frequency in stretches or mono- and dinucleotide repeats (Duckett et al. 1996). The MMR apparatus is highly sensitive to bulges or loops created by polymerase slippage, bulky adducts, or misincorporated nucleotides (Duckett et al. 1996). In eukaryotic cells, MutS scans for DNA mismatches and MutL scans for single strand nicks and triggers the degradation of the defective segment of the strand. DNA polymerase and ligase then fill in the missing strand (Figure 1.3) (Hoejmakers 2001). This repair occurs after the mistake has been made.
Figure 1.3. In eukaryotic cells, MutS and MutL collaborate to remove mismatched DNA. Adapted from Weinberg 2006.

When chemically altered bases are present in the genome, DNA repair mechanisms are triggered in the form of base-excision repair (BER) or nucleotide excision repair (NER) (Hang 2010). While BER tends to repair DNA lesions that are derived from endogenous sources (such as reactive oxygen species) that do not distort the DNA double helix, NER repairs lesions caused by exogenous sources that do distort the double helix (such as UV or chemical carcinogens such as benzo[a]pyrene) (Hang 2010).

BER is initiated by a group of glycosylases which recognize a chemically altered base and cleave its covalent bond with deoxyribose. APE (apurinic/apyrimidic endonuclease) then
removes the baseless deoxyribose cleaving it on the 5’ side. AP lyase then cleaves the 3’ side, liberating the deoxyribose. The resulting single strand gap is repaired by DNA polymerase (often polymerase β), and ligated by DNA ligase (Figure 1.4A) (Hooeijmakers 2001).

NER, on the other hand, is accomplished by a large multiprotein complex that is composed of almost two dozen subunits. In order for this complex to form, the DNA double helix requires significant distortion (e.g. by the incorporation of a bulky chemically altered base). Once this defect is sensed and the complex has formed, the complex cleaves the damaged strand leaving a single-stranded fragment that is 25-30 nucleotides in length. DNA polymerases (usually δ or ε) and DNA ligase then fill in the resulting gap (Figure 1.4B) (Hoejmakers 2001).
Figure 1.4. Base excision repair (BER) and nucleotide excision repair (NER). A, BER is achieved by enzymes that recognize chemically altered basepairs caused by endogenous sources that produce minimal helix distortion. B, NER repairs bulky, helix-distorting lesions caused by exogenous sources. Adapted from Weinberg 2006.
Double strand breaks (DSB) unrelated to DNA adducts or basepair mismatches are repaired by a specific mechanism (Schwartz et al. 2005). DNA breaks can occur by several mechanisms, such as repeated interruption of DNA replication or chromosome breakage resulting from defective chromosome segregation. The repair of such DSBs involves the recruitment of the homologous sequence in an undamaged sister chromatid (Schwartz et al. 2005). This homology-directed repair (HDR) involves the resection of a DNA strand at each end of the DSB, and pairing with the unwound undamaged sister chromatid. Each single-stranded DNA strand is elongated in a 5’ to 3’ fashion by DNA polymerase using the sister chromatid strands as template. These extended strands then pair with one another and DNA ligase fills the gaps (Figure 1.5) (Schwartz et al. 2005).
Figure 1.5. Homology-directed repair (HDR) of a double-strand break. HDR begins with the resection of the DNA strands. The resulting single-stranded DNA strands then align with the double helix of the undamaged sister chromatid and use its sequence as a template for elongation. Adapted from Weinberg 2006.

Mutations in genes that play a role in DNA repair are often found in cancer cells. For example, MGMT gene silencing by promoter methylation is found in approximately 40% of colorectal tumors and gliomas and 25% of NSCLC (Weinberg 2006). As an example, mice overexpressing MGMT in thymic glands were shown to have increased resistance to
methylNitrosourea (MNU) -induced lymphoma formation for up to 400 days of repeated MNU treatment. GSTs have also been shown to be silenced by promoter methylation in as many as 90% of human prostate cancers (Pegg 2000).

Defective MMR, BER, and NER also increase susceptibility to cancer. This has been demonstrated in individuals who suffer from xeroderma pigmentosum due to mutations in the genes that form the multiprotein complex in NER (Shiloh 2003). Mutations in these genes make individuals more susceptible to exogenous carcinogens such as UV radiation (Shiloh 2003). These individuals have a 1000-fold increased risk of developing skin cancer, and most begin to develop cancer as early as 10 years of age. Mutations in at least one of four MMR genes (MSH2, MSH6, PMS2 and MLH1) is also associated with hereditary non-polyposis colon cancer (HNPCC) characterized my increased MIN or a high frequency of mutations in genes that contain microsatellite repeats in their sequences (Loeb 2001).

Finally, mutations in the BRCA family of proteins have been linked with chromosomal aberrations caused by double-strand breaks (Greenberg et al. 2006). Specifically, cells lacking BRCA1 and BRCA2 function show compromised HDR. BRCA1 and BRCA2 act as scaffolds that assemble a cohort of DNA repair proteins which assist in HDR (Shiloh 2003). One of these is RAD51, which binds single-stranded DNA and allowing for their pairing with sister chromatids. BRCA2 has eight BRC domains which may allow for a string of RAD51 proteins to bind single-stranded DNA (Sherr 2004, Shiloh 2003). Also, it has been demonstrated in immunohistochemical experiments where BRCA1 protein localized to the sites of double-strand breaks introduced by focal doses of radiation (Greenberg et al. 2006). Furthermore, although
BRCA2 null mice are embryonic lethal, BRCA2 heterozygous mice are susceptible to chromosomal aberrations and lymphoma (particularly, abnormal chromosomal fusions) (Weinberg 2006). Finally, cells deficient in BRCA1 or BRCA2 display a high amount of CIN that appears to arise due to double-strand breaks (Heinen et al. 2002).

Mutations are relatively infrequent in normal human somatic cells compared to cancer cells (Bielas and Loeb 2005). A comparison of mutation rates in skeletal muscle, renal tissue, and colon tissue showed much higher mutation rates in adjacent cancer tissue (Bielas et al. 2006). Specifically, random mutations in somatic cells were estimated to occur at a rate of <1-4 X 10^{-8} mutations/basepair/generation compared with a rate of 65-475 X 10^{-8} mutations/basepairs/generation in cancers (Bielas et al. 2006). Mutation rates were measured by a technique called random mutation capture (RMC). Briefly, this assay involves digestion of DNA using Taq1, followed by qPCR of the genomic DNA across the Taq1 restriction site. This PCR will only amplify DNA molecules that contain a mutation in the Taq1 restriction site. A second PCR adjacent to the Taq1 site then quantifies every DNA molecule in a sample, and the ratio between mutated strands amplified and total strands is calculated (Vermulst et al. 2008) (Figure 1.6).
Figure 1.6. The concept of random mutation capture (RMC). DNA is digested with Taq1. Sites with mutated Taq1 sites remain uncut (denoted as red). Mutated sites are amplified using PCR near the Taq1 site (red) and compared to total DNA amplified in the sample (black). Adapted from Vermulst et al. 2008.

Furthermore, the random rate of mutation that a normal cell is able to acquire during their lifetime does not account for the large amount of genes found mutated in cancers (Loeb 2001). Therefore, in 1974 Loeb et al. proposed a mechanism for cancer development where an error in the DNA repair/replication machinery would confer an increase in mutation frequency, the results of which would lead to a “mutator phenotype” and increased susceptibility to develop cancer (Loeb et al. 1974, Loeb 2001). This mutator phenotype would be advantageous for cancer development by increasing the rate of selection of cancer-causing mutations.
Loeb’s mutator phenotype is based on various observations. In addition to the observations that mutations occur at a much higher rate in cancer cells than in normal cells, there are many hereditary cancers that involve mutations in DNA repair genes, for example BRCA proteins, ATM, and the XP family (Fearon 1997). Cancers are believed to arise after decades of molecular changes, yet individuals that inherit disrupted BRCA, ATM, DNA polymerase, and XPs develop cancer at an early age, which shows that cancer occurs in cells with a higher rate of mutation, which is dramatically evident when repair processes are defective (Bielas and Loeb 2005, Fearon 1997). The theory of the mutator phenotype has progressed through demonstration that cancer cells are genetically unstable- i.e. they are characterized by genetic instability.

Numerous studies have shown that most tumor cells exhibit widespread chromosomal aberrations, such as translocations, whole chromosome and intrachromosomal copy-number changes, and rearrangements (Breivik 2005). The increased rate of these defects in cancer relative to normal cells is referred to as chromosomal instability (CIN), which is a type of genetic instability prevalent in cancer. For example, comparative genomic hybridization analysis of 371 lung cancer tumors exhibit wide fluctuations in copy number of various segments of chromosomes (Weir et al. 2007). CIN, as a mutator phenotype, is a high rate of chromosomal defects which serves as a source of genetic variation where changes advantageous for malignant growth are selected for (Breivik 2005). The potential causes of CIN are considered numerous and various considering the number of genes that protect the genome from structural defects. This is supported by evidence showing that genes involved in chromosome maintenance increase susceptibility to cancer when germline mutated. However, the same
genes are rarely mutated in sporadic human cancer (Beckman and Loeb 2005). Hence, numerous genes may redundantly contribute to CIN, with each being altered in a given cancer type at a relatively low frequency.

In contrast, mutations in mismatch repair genes have been shown to predispose individuals to colon cancer with microsatellite instability (MIN), another characterized type of genetic instability in cancer. MIN is a high rate of mismatch mutations that are most prevalent in dinucleotide repeat sequences or microsatellites and has only been associated with defects in mismatch repair genes (Bardelli et al. 2001). MIN is also evident in some sporadic tumors of the colon (~15%) and endometrium as a result of somatic mutations in mismatch repair genes (Huang et al. 1996). Therefore, by acquiring chromosomal/microsattelite instability during their development, cancer cells evidently scramble their genomes and speed up the rate at which they arrive at genetic configurations that are more favorable for neoplastic growth.

1.1.5.2 Carcinogenesis often involves alteration of several functions: Cell cycle progression

The cell cycle clock progression is executed through the expression of several protein kinases. These proteins are called cyclin dependent kinases (CDKs) which are serine/threonine kinases (Malumbres and Barbacid 2001). When cyclins associate with their CDK partner, they activate the CDK molecule through stereochemical shifts of its catalytic site and direct the catalytically activated complex to its substrate for phosphorylation (Weinberg 2006). Abnormal regulation of CDKs and the cell cycle are typical of cancer cells.
The different cyclin/CDK/CDC complexes will be discussed briefly here. In G1 of the cell cycle phase, CDK4 and CDK6 associate with D-type cyclins (Cyclin-D1-3). In late G1, the E-type cyclins (E1 and E2) associate with CDK2 to enable phosphorylation of appropriate substrates required for S-phase entry. As cells enter S-phase, the A-type cyclins (A1 and A2) replace the E-cyclins as the partners of CDK2 and allow S-phase progression. In late S-phase, the A-type cyclins dissociate with CDK2, and they associate with CDC2 or CDK1 instead. As G2 begins, CDC2 then associate with Cyclin-B. Finally, at M-phase onset, CDC2/Cyclin-B complex triggers many of the events of the prophase, metaphase, anaphase and telophase program (Figure 1.7A) (Malumbres and Barbacid 2001). The specific activating associations between CDKs and cyclins are largely determined by stoichiometric changes in the expression levels of the different cyclins at specific phases of the cell cycle (Figure 1.7A).
Figure 1.7. Cell cycle progression involves pairing and fluctuation of various kinases. A, Pairings of various cyclins with cyclin-dependent kinases. B, Fluctuation of cyclin levels during the cell cycle. Adapted from Weinberg 2006.

Cyclin proteins fluctuate in their expression levels as the cell-cycle progresses, going through a gradual accumulation followed by quick degradation. This allows the cell cycle to
move in one direction (Figure 1.7B) (Reed 2003). The D-type family of cyclins is interesting in regard to cancer because they are controlled by various mitogenic growth factors and external signals. For example, Cyclin-D1 mRNA levels were shown to increase in macrophages as soon as 2 hours after they were exposed to nutrients (Matsushime et al. 1991). It has therefore been proposed that Cyclin-Ds function to inform the cell-cycle of environmental conditions outside the cell (Matsushime et al. 1991). Cyclin-Ds (activated by mitogenic signals) then associate with CDK4/6, which phosphorylates and inactivates RB. Inactivated RB dissociates from E2F, and allows E2F to carry out its transcriptional function to support DNA replication (Massague 2004).

Cyclin-CDK complexes are inhibited by a class of proteins referred to as cyclin dependent kinase inhibitors. \(p27^{\text{Kip1}}\) and \(p21^{\text{cip1}}\) are two of these proteins and bind both CDK2 and CDK4/6 (Hunter and Pines 1999). Interestingly, when \(p27^{\text{Kip1}}\) and \(p21^{\text{cip1}}\) bind Cyclin-D-CDK4/6 complexes they actually stimulate the activity of the complex. Inhibition of these complexes occurs as \(p15^{\text{INK4b}}\) or \(p16^{\text{INK4a}}\), which specifically inhibit Cyclin-D-CDK4/6, displace \(p27^{\text{Kip1}}/p21^{\text{cip1}}\). However, \(p27^{\text{Kip1}}/p21^{\text{cip1}}\) inhibit the activity of Cyclin-E/CDK2 complexes. As Cyclin-D/CDK4/6 complexes accumulate and bind \(p27^{\text{Kip1}}/p21^{\text{cip1}}\), more Cyclin-E/CDK2 complexes remain dissociated from \(p27^{\text{Kip1}}/p21^{\text{cip1}}\) and trigger passage of the cell cycle from G1 into S-phase as a result of sustained E2F activity (Hunter and Pines 1999).

\(p16^{\text{INK4a}}\) and \(p15^{\text{INK4b}}\) inhibit Cyclin-D/CDK4/6 and therefore block transition from G1 to S (Massague 2004). This occurs in the absence of mitogenic stimulation when Cyclin D levels are low. These inhibitors act stochiometrically by binding CDK4/6 when in excess of Cyclin-D preventing association of CDC4/6 with Cyclin-D. In the absence of \(p16^{\text{INK4a}}\), Cyclin-D/CDK4
complex activity is elevated, which leads to continuous RB phosphorylation and E2F accumulation (Massague 2004).

Cyclin-D1 expression has been shown to be induced in response to excess RAS/ERK activity, which further promotes CDK4/6 activity and cell cycle progression (Massague 2004). Cyclin D1 is commonly over-expressed in cancer as a consequence of RAS/ERK hyperactivity. Also, the p16 gene is frequently inactivated by deletion, mutation, or by promoter hypermethylation in several cancer types, including NSCLC (Hunter and Pines 1994, Massague 2004). These findings suggest that enhanced CDK4/6 activity confers a significant advantage for tumor growth. The primary substrates of CDK4/6 and CDK2 in G1 progression are members of the retinoblastoma protein family (RB, p107, p130), which function as a docking site for other proteins that play a role in the cell cycle. For example, RB binds the E2F family of transcription factors and inactivates them during M and G0. When active, E2F proteins activate the expression of genes required for transition into S-phase (such as DNA polymerases) and subsequent cell cycle events (such as Cyclin-B) (Massague 2004).

Cell cycle progression is often disrupted in carcinogenesis by several mechanisms. For example, RB inactivation and RAS activation are frequently reported in tumors (Yamasaki and Pagano 2004). Inactivated RB allows for a constitutively free E2F to facilitate cell cycle progression through S-phase, and activated RAS stimulates Cyclin-D expression (through AP-1) which also stimulates S-phase progression (Weinberg 2006). Interestingly, mice overexpressing Cyclin-D1 in mammary glands have been shown to develop mammary hyperplasia and carcinomas (Yamasaki and Pagano 2004). Also, p16-null mice are prone to develop melanoma,
and exposure of these mice to the mutagen DMBA further increased the incidence of melanoma (Krimpenfort et al. 2001). Therefore, these results highlight the importance of cell cycle control in deterring cancer development.

1.1.5.3 **Carcinogenesis often involves alteration of several functions: Cell death and apoptosis**

The loss of a fully functioning apoptotic program is a trait of malignant cancer cells (Hanahan and Weinberg 2000). Apoptosis is triggered by various cellular stresses, such as excessive calcium within a cell, excessive oxidants, DNA-damaging agents, microtubule and mitotic spindle disruption, and viral infection (Igney and Krammer 2002, Sunters et al. 2003). In mammalian cells, apoptosis occurs through an intrinsic or extrinsic pathway.

The intrinsic pathway by definition does not involve external stimuli, but involves the mitochondria and cytochrome c as central actors. Cytochrome c normally resides in the space between the inner and outer mitochondrial membranes where it functions to transfer electrons as part of oxidative phosphorylation (Igney and Krammer 2002). Under apoptosis signaling, the outer mitochondrial membrane becomes depolarized and cytochrome c is released into the cytoplasm. Once in the cytoplasm, cytochrome c triggers an apoptotic cascade triggering apoptotic death. Through specialized channels, the Bcl-2 protein family controls the flow of cytochrome c to the cytoplasm (Hengartner 2000).
Although the exact mechanism is still unknown, different members of the Bcl-2 family are known to play different roles in the process of cytochrome c release. For example, Bcl-2 itself and Bcl-X\textsubscript{l} work to keep the cytochrome c channels closed and thereby keeping cytochrome c in the mitochondria (Hengartner 2000). In ways that still remain unclear, pro-apoptotic Bcl-2 family members: Bax (which is activated by p53), Bad, Bak, and Bid function oppositely and apparently displace Bcl-2 causing these channels to leak cytochrome c into the cytoplasm. This has been demonstrated in experiments where different Bcl-2 members were overexpressed and cytochrome c’s localization in cells was then monitored (Hengartner 2000, Weinberg 2006). Several mechanisms have been proposed for Bcl-2 family function. One such mechanism is that Bcl-2 family members form mitochondrial channels with or without other proteins with a role of pro-apoptotic Bcl-2 proteins in inducing the rupture of the outer mitochondrial membrane (Hengartner et al 2000). Evidence also shows that Bad can maintain the mitochondrial channels in an open configuration, allowing for cytochrome c leakage into the cytoplasm. Although the role of Bcl-2 proteins is still not fully understood, the relative balance between pro- and anti-apoptotic proteins within each channel has been consistently shown to determine whether cytochrome c will be retained in the mitochondria or not (Hengartner 2000, Reed 2000).

Once released into the cytoplasm, cytochrome c molecules associate with the Apaf-1 protein and form a structure called the apoptosome. This apoptosome then cleaves pro-caspase 9 (a member of the family of cysteine aspartyl-specific proteases) into active caspase 9 (Igney and Krammer 2002). Caspase 9 then cleaves caspase 3 and the signaling cascade of cleaved caspases continues until the cleavage of death substrates such as ICAD which
fragments chromosomal DNA, and actin, plectin, vimentin and gelsolin which leads to collapse of the cytoskeleton and formation of apoptotic bodies (Igney and Krammer 2002). While cytochrome c release into the cytoplasm acts as an activator of caspases, Smac/DIABLO are also released into the cytoplasm to inhibit anti-apoptotic proteins. These anti-apoptotic proteins block caspase action either by binding to them and preventing their cleavage or by marking them for ubiquitination and proteosomal degradation (Danial and Korsmeyer 2004).

Since the aforementioned series of apoptotic events occur entirely within the cell, it is called the intrinsic apoptosis program. Apoptosis can also be triggered through an alternative extrinsic route which is also called the receptor activated apoptotic pathway. These death receptors include TNF-α, FAS/CD95, and TRAIL, and are members of the tumor necrosis factor (TNF) family of proteins (Hengartner et al. 2000). As many as 30 members of these proteins are encoded by the human genome. Once activated by ligand binding (such as FAS/CD95 ligand or Apo2L), these proteins bind and activate an associated protein called FADD (FAS-associated death domain protein). The resulting complex is termed the “death inducing signaling complex” (DISC) and it summons and cleaves caspases 8 and sometimes 10 (Hengartner et al. 2000). Cleaved caspases 8 and 10 then activate the executioner caspases 3, 6, and 7, where the process then parallels that of the intrinsic pathway. c-Flip, on the other hand, has been shown to interact with FADD and inhibit apoptosis when coexpressed in cells (Irmler et al. 1997). Interestingly, the extrinsic signal can be amplified by Bid, which is cleaved by caspase 3 and then migrates to the mitochondrial channel and facilitates its opening (Reed 2000). Hence, cells can trigger their own death through two pathways: an internal process involving cytochrome c
and the mitochondria, and through secretion of a ligand that will activate its death receptors on
the cell surface (Hengartner 2000) (Figure 1.8).

Tumor cells can acquire resistance to apoptosis by overexpressing anti-apoptotic genes
and repressing pro-apoptotic proteins. For example, Bcl-2 overexpression in leukemia (through
chromosomal translocations to the immunoglobulin heavy chain locus) and high levels of c-Flip
in melanoma and in Kaposi’s sarcomas is often reported (Igney and Krammer 2002). In addition,
lack of Apaf-1 expression is frequent in melanoma and chromosomal deletion of 8p21-22
(where TRAIL-R2 is located) occurs often in NSCLC (Igney and Krammer 2002).

In addition, p53 stimulates apoptosis through a transcriptional program involving up-
regulation of numerous genes involved in apoptosis such as Apaf-1, Bax, and Diablo, among
others (Levine 1997). p53 is functionally activated by phosphorylation of specific serine residues
within its amino terminus by kinases involved in recognizing specific cellular stresses such as
DNA damage, oncogenic stimulation and oxidative stress, all of which are typical of cancer cells.
Furthermore, studies have shown that restoration of p53 function in cancer cells in culture
typically results in a robust apoptotic response indicating a critical role in averting cancerous
Figure 1.8. Apoptosis occurs through an intrinsic program involving mitochondria and cytochrome c or extrinsic program involving cell surface receptors and their ligands. Adapted from Hengartner 2000.
1.1.5.4. **Carcinogenesis often involves alteration of several functions: Acquisition of immortality**

A characteristic of cancer cells is an acquisition of a limitless lifespan or immortalization. Telomere extension has been shown to greatly contribute to this process. Briefly, telomeres are located at the ends of chromosomes and in vertebrates contain a GT-rich nucleotide repeat sequences of 5’TTAGGG3’ (Neumann and Reddel 2002). In humans they are 5-15kb in length and end in a 30-2000 nucleotide 3’ overhang. This overhang has been shown to form a “T-loop” by partly attaching to the double-stranded portion of the nearing DNA (Figure 1.9A). During DNA replication, the 3’ end of the DNA helix that undergoes leading strand DNA synthesis is only partly replicated as DNA primers are placed several nucleotides away from the edge of the 3’ strand (Figure 1.9B). Due to this “end-replication problem,” telomeres lose 20-100 basepairs during each generation, and if this process continues, cells enter a “crisis” stage of senescence or apoptosis (Neumann and Reddel 2002, Tian et al. 2010).
Figure 1.9. Telomeres and their replication during DNA replication. A, Structure of the telomeric T-loop. B, DNA replication from the parental 3’ strand is incomplete due to primer placement on telomeres. Adapted from Weinberg 2006.
However, telomeres can be regenerated through elongation with the telomerase enzyme. Telomerase consists of many distinct subunits. Two of these subunits are a reverse transcriptase (TERT) and an RNA template 451-nucleotides in length. The telomerase enzyme attaches to the 3’ overhang and extends the G-rich strand by six nucleotides. By repeating this process, telomeres can be extended by thousands of nucleotides (Neumann and Reddel 2001).

Interestingly, telomeres can also be extended without telomerase. This was concluded from immortalized cancer cell lines which showed increased telomere length but did not express telomerase (Dunham et al. 2000). This telomerase-independent mechanism is called the alternative lengthening of telomeres (ALT). Although not well understood, it has been proposed that using a complimentary telomeric strand (either inter-chromosomally or intra-chromosomally), the DNA sequence can be primed on the template to which the invading strand is annealed (Dunham et al. 2000). This model was demonstrated by Dunham et al. who inserted a neomycin resistance gene in the telomeric region of immortalized skin fibroblasts using homologous recombination. Early passages of this neo cell line contained the insert in the telomeric region of 2 chromosomes. However, after 40 population doublings, in situ hybridization showed the neo resistance gene in 5 chromosomes’ telomeric regions (Dunham et al. 2000). Therefore, the telomeric region of the neo-containing chromosomes appears to have been used as a template to extend the telomeres of other chromosomes.

Telomerase and ALT activity have been shown to be important for tumor cell proliferation and elevated in cancer cells. For example, transfection of HeLa cells with telomerase siRNA caused growth arrest of these cells in culture after 23 days (Kurvinen et al. 2006). Furthermore, telomerase has been shown to be overexpressed in 90% of all tumors.
(Neumann and Reddel 2002). Hence, increased telomere stability is an immortalizing change common to cancer cells.

1.1.5.5. Carcinogenesis often involves alteration of several functions: Invasion and metastasis

In order for tumors to become metastatic, cancer cells must undergo a transformation where they acquire motility and invasiveness. This involves a process known as epithelial-mesenchymal transition (EMT). EMT, which normally occurs extensively during embryogenesis when epithelial/neural crest cells translocate, involves a loss by epithelial cells of their characteristic morphology surface proteins and gene expression pattern and an acquisition of mesenchymal properties and surface proteins (Savagner 2001).

Proteins that play a role in EMT transition have been elucidated. For example, cells undergoing EMT transformation typically repress the expression of epithelium proteins, E-cadherin and cytokeratins, and instead express mesenchymal proteins such as vimentin, N-cadherin, and secrete fibronectin (Weinberg 2006). E-cadherin forms a transmembrane dimer that assists in cellular adherence and its loss is believed to play a chief role in tumor cell invasion and metastasis. For example, E-cadherin expression is often lost in tumors and cancer cell lines, and when re-expressed, these cells have been shown to lose invasive/metastatic properties (Savagner 2001). Finally, mouse xenograft experiments have proven helpful in demonstrating this phenotypic conversion. In xenografts, immunohistochemical staining
showed a loss of cytokeratin in the cells that were near the mouse stroma, which instead were vimentin-positive indicating a conversion to a more mesenchymal phenotype (Savagner 2001).

This EMT conversion is believed to occur through secretions from the stroma, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), and transforming growth factor β (TGF-β), which induces EMT through RAS signaling (Brabletz et al. 2005). Based on observations made in RAS oncogene-expressing MECs (mammary epithelial cells), these cells express E-cadherin in their junctions with other cells. However, when exposed to TGF-β, E-cadherin expression was greatly reduced and vimentin expression became visible. Interestingly, once EMT occurred, these RAS-MECs began expressing their own TGF-β (Janda et al. 2002). This indicated that TGF-β signalling could conspire with RAS oncogene to cause or maintain EMT in human carcinoma cells.

It has been proposed that cancer cells alternate between EMT and MET (mesenchymal-epithelial transition) during metastasis. For example, metastases often display an epithelial phenotype, which means a reversion must occur at some point in the mesenchymal-like tumor cells (Rubio et al. 2007). In addition, immunohistochemical experiments show evidence of this transition as primary colorectal tumors were shown to express very little basement membrane protein (an epithelial marker) at their invasive edge. In subsequent metastasis of these colorectal tumor cells to the liver, they expressed basement membrane protein once again (Thiery 2002). Furthermore, the aforementioned EMT-RAS-MEC that expressed their own TGF-β, changed in appearance after 10 days from mesenchymal spindle-like cells to a more epithelial cobblestone appearance (Oft et al. 1996). Therefore, it is believed that cells acquire
the ability to break through the epithelium and mobilize (through the blood- or lymph system) to another location where they must once again acquire adhesive properties in order to form a tumor in a different location (Thiery 2002).

1.2 LUNG CANCER

Lung cancer is the leading cause of cancer death worldwide. According to the latest statistics from the American Cancer Society (ACS), lung cancer death rates quantify at 70% in males and 40% in females in the US. In fact, the incidence of lung cancer deaths in males is higher than deaths from all other forms of cancer combined (ACS Cancer Facts and Figures 2010).

Lung cancer staging is based on an assessment of various factors such as tumor size, whether it has spread to various structures within the lung, and whether the lung tumor has undergone metastasis. A more detailed table with lung cancer staging criteria is shown in Table 1.1.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Contained within lung</td>
</tr>
<tr>
<td>A</td>
<td>smaller than 2cm across</td>
</tr>
<tr>
<td>B</td>
<td>between 2 and 3 cm across</td>
</tr>
<tr>
<td>II</td>
<td>Tumor is either:</td>
</tr>
<tr>
<td></td>
<td>- Between 3 and 7cm across.</td>
</tr>
<tr>
<td></td>
<td>- Has grown into the largest airway (the main bronchus) more than 2cm below the branch point where it divides to go into each lung.</td>
</tr>
<tr>
<td></td>
<td>- The tumor has grown into the inner lining of the chest cavity (the visceral pleura).</td>
</tr>
<tr>
<td></td>
<td>- The tumor has made part of the lung collapse.</td>
</tr>
<tr>
<td>A</td>
<td>5cm or smaller</td>
</tr>
<tr>
<td>B</td>
<td>larger than 5cm</td>
</tr>
<tr>
<td>III</td>
<td>Tumor is either:</td>
</tr>
<tr>
<td></td>
<td>- Larger than 7cm.</td>
</tr>
<tr>
<td></td>
<td>- Has grown into one of the following structures.</td>
</tr>
<tr>
<td></td>
<td>o The chest wall,</td>
</tr>
<tr>
<td></td>
<td>o The central lining of the chest cavity (the mediastinal pleura)</td>
</tr>
<tr>
<td></td>
<td>o The muscle at the bottom of the chest cavity (the diaphragm)</td>
</tr>
<tr>
<td></td>
<td>o The phrenic nerve</td>
</tr>
<tr>
<td></td>
<td>o The outer covering of the heart (the pericardium)</td>
</tr>
<tr>
<td></td>
<td>- The tumor has made the whole lung collapse.</td>
</tr>
<tr>
<td></td>
<td>- There is more than one tumor nodule in the same lobe of the lung.</td>
</tr>
<tr>
<td>A</td>
<td>Spread to lymph nodes on same side of the chest as primary tumor</td>
</tr>
<tr>
<td>B</td>
<td>Cancer spread to lymph nodes on other side of chest where primary tumor is located. Spread to heart and esophagus.</td>
</tr>
<tr>
<td>IV</td>
<td>The tumor has grown into one of the following structures - the area between the lungs in the middle of the chest (the mediastinum), the heart, a major blood vessel, the windpipe (trachea), the area where the main airway divides to go to each lung, the food pipe (oesophagus), a spinal bone, the nerve that controls the voicebox, or there are tumor nodules in more than one lobe of the same lung. Metastasis is present in other regions of body.</td>
</tr>
</tbody>
</table>

Many factors contribute to such high mortality from lung cancer. For one, methods for detecting small, early-stage lung cancer are ineffective. Even though chest radiography is considered standard procedure for lung cancer detection, their use has been shown to be only marginally effective at detecting early, small lesions. Also, many lung cancers are detected at later stages of development when patients are symptomatic (experiencing pain). However, very late stage tumors have fewer treatment options (ACS 2010, IARC 2004). For example, surgery, although effective, is frequently not an option at a late stage when cancers have already become highly invasive and metastatic. Taken together, current methods of diagnosis and treatment account for a 5-year survival rate of only 15% (ACS 2010, Jemal et al. 2006, Manser et al. 2004).

Lung cancer typically presents itself in one of two forms of broad categorization; small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (Herbst et al. 2008, Travis et al. 1995), with SCLC accounting for 15% and NSCLC accounting for 85% of all lung cancer cases. NSCLS is further subcategorized into squamous cell carcinomas, large cell carcinomas, and adenocarcinomas. Adenocarcinomas, which arise from glandular cells, are the most common sub-type, accounting to 40% of all lung cancers (Travis 2002). All forms of lung cancer are caused mainly by exposure to carcinogens in cigarette smoke. However, each is a pathogenetically and clinically distinct disease type.
1.3 CAUSES OF LUNG CANCER

It is well established from both epidemiological and laboratory studies that exposure to environmental carcinogens is the major cause of lung cancer, with those in tobacco smoke being predominant (Hecht 1999, Hecht 2006, Parkin et al. 2005). In fact, 85% of lung cancers in men and 47% in women are attributed to tobacco smoke (Parkin et al. 2005, Sun et al. 2007). Tobacco smoke has been shown to contain over 60 known carcinogens. Among these, polycyclic aromatic hydrocarbons (PAH) and tobacco-specific nitrosamines (TSA) are well-characterized in terms of their metabolic activation, mechanism of action, and potency (Hecht 2003).

Mechanistically, PAHs and TSAs have been shown to cause DNA damage, leading to genetic mutations and chromosomal aberrations (Hecht 1999). As already described, if this damage persists unrepaired (through an overwhelming of DNA repair mechanisms, or avoiding of apoptosis), permanent genetic changes such as mutations of oncogenes and tumor suppressing genes occur (Beckman and Loeb 2005, Breivik and Gaudernack 2004, Hecht 1999). In addition, it was recently demonstrated that carcinogens are a cause of chromosome instability (CIN) in tumor cells. A consequence of CIN is errors in chromosome maintenance, that lead to a higher rate of aberrant mitotic recombination, mitotic nondisjunction, chromosomal breakage, and rearrangement that affect oncogenes and tumor suppressor genes (Loeb 1998, Macleod 2000, Weinberg 1995). Such changes contribute to the development of cancer. For example, the RAS oncogene and p53 tumor suppressor gene are often mutated in different types of lung cancer with specific mutation signatures or patterns that have been
correlated with exposure to tobacco carcinogens (Sato et al. 2007, Sekido et al. 2003). Also, lung cancer of smokers has more extensive CIN than those of never-smokers (Sanchez-Cespedes 2003). Hence, carcinogens increase the likelihood that affected cells will develop into cancer by directly inducing mutations, and by increasing the mutation rate of the cells by inducing a mutator phenotype.

1.3.1 Tobacco Smoke: A major cause of lung cancer

Exposure to tobacco smoke carcinogens is the leading cause of lung cancer (HHS 2009). These carcinogens form a link between nicotine addiction and lung cancer in humans. While nicotine itself is not considered to be a carcinogen, tobacco smoke that is the vessel for nicotine intake contains over 60 known lung carcinogens, tumor promoters, and co-carcinogens (HHS 2009, Hecht 2003). Addiction to nicotine renders the smoker to a lifetime of direct carcinogen exposure. These carcinogens belong to various classes of chemicals: polycyclic aromatic hydrocarbons (PAHs) (10 compounds), aza-arenes (3), N-nitrosamines (8), aromatic amines (4), heterocyclic amines (8), aldehydes (2), volatile hydrocarbons (4), nitro compounds (3), miscellaneous organic compounds (12), and metals and other inorganic compounds (9) (Pfeifer et al. 2002).

The scientific link between lung cancer and smoking was first established in a landmark epidemiological study by Ernest Wynder and Evarts Graham, who in 1950 published their findings on 684 lung cancer cases and their relationship to smoking. In this study, questionnaires were used to compare the smoking histories of lung cancer patients to those
without lung cancer. Wynder and Graham found that the majority (85-93%) of the lung cancer patients were moderate-heavy smokers and that only a small percentage of lung cancers occurred in non-smokers (~14%) (Wynder and Graham 1950). Furthermore, the authors concluded that “excessive and prolonged use of tobacco, especially cigarettes, seems to be an important factor in the induction of bronchiogenic carcinoma” (Wynder and Graham 1950). After these findings, the association between smoking and lung cancer continued to be investigated using other study designs. For example, large groups were questioned on their smoking habits and then observed for several years for data on mortality and cause of death. These studies also found that not only did individuals who smoked have a ten times higher risk of dying of lung cancer, but that lung cancer death rates increased with increased smoking (Hammond and Horn 1958). These results provided further support for the hypothesis that cigarette smoke caused lung cancer.

This proposed link between tobacco smoke and lung cancer has since been extensively studied and has been demonstrated in experiments where cigarette smoke condensate caused tumor formation when applied to mouse skin and implanted in the rodent lung (IARC 1986). Also, inhalation experiments were conducted where hamster and A/J mice exposed to environmental tobacco smoke developed preneoplastic lesions, tumors of the larynx, and lung tumors (IARC 1986, Witschi 2000). For example, A/J mice exposed to tobacco smoke for 5 months were 1.5-3 times more likely to develop lung tumors (Witschi et al. 1997). In rats, Mauderly et al. demonstrated an increase in lung tumors in male and female F344 rats exposed to cigarette smoke for up to 30 months (16% as opposed to 0% in controls) (Mauderly et al. 2004). PAHs and tobacco-specific nitrosamines (TSA) are two potent and well-characterized
classes of carcinogens present in tobacco smoke (Hoffmann and Hoffmann 1997, Hecht 1998). These carcinogens have also been shown to induce lung tumors in A/J mice, demonstrating a direct link between exposure to carcinogens isolated from tobacco smoke and lung cancer.

PAHs are chemical compounds that are produced as by-products of hydrocarbon combustion and as such are environmentally pervasive. They occur in oil, coal, tar deposits, and a variety of cooked foods, and have been elucidated to be carcinogenic and mutagenic (Larsson et al 1983). Of all known PAH compounds, benzo[a]pyrene (BaP) is notable for being the first chemical carcinogen to be discovered in tobacco smoke. PAHs generally contain four- to seven-member rings and once present in the cell are metabolized to their ultimate carcinogenic structure. In the case of BaP, it is metabolically activated via a three-step process. First, cytochromes P450 1A1 and 1B1 catalyze BaP into (7R,8S)-epoxy-7,8-dihydrobenzo[a]pyrene (BaP-7,8-oxide). This structure is then converted by epoxide hydrolase to (7R,8R)-dihydroxy-7,8-dihydrobenzo[a]pyrene (BaP-7,8-diol). BaP-7,8-diol then undergoes another oxidation step, catalyzed by cytochromes P450 and other enzymes, producing mainly (7R,8S)-dihydroxy-(9S,10R)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) (Boysen and Hecht 2003). BPDE is highly carcinogenic and reactive with DNA, forming a major adduct at the N² position of deoxyguanosine (Figure 1.10). This can result in mutations by perturbing the double-helical DNA structure, disrupting normal DNA replication, and resulting in misreading and mutation (Larsson et al. 1983, Pfeifer et al. 2002). When these mutations occur in oncogenes or tumor suppressor genes, they contribute to cancer development.
**Figure 1.10.** The major established pathway of metabolic activation of BaP leading to the formation of BPDE-N2-dG shown as the nucleoside obtained by enzymatic hydrolysis of DNA. The adduct results from the reaction with DNA of BPDE. BPDE is one of four possible tereoisomers of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. BPDE is the most carcinogenic of these isomers and the most reactive with DNA. (Boysen and Hecht 2003)

Numerous studies have demonstrated that BPDE forms DNA adducts. However, one study demonstrated that these adducts actually form in lung tissue from exposure to cigarette smoke. BPDE-DNA adducts were detected in the lung tissue of smokers with lung cancer, but not in non-smokers patients (Alexandrov et al. 1992). Using HPLC, Alexandrov et al. detected BPDE-DNA adducts in 9 of 11 non-tumorous lung parenchymal tissue (at levels of 0.6-9.9 adducts/10^8 nucleotides). These results provided evidence that BaP levels present in tobacco smoke are sufficient to cause BPDE-DNA adduct formation in lung tissue.

Carcinogens in tobacco smoke have also been linked to mutations in oncogenes and tumor suppressor genes. As mentioned previously, BaP is a PAH and a potent carcinogen found in tobacco smoke. BPDE is a highly DNA-reactive by-product of BaP metabolism that causes
DNA adducts and mutations (Hecht 2006). A study of lung tumor samples from 106 patients showed that 38% of these samples contained a mutation in KRAS (Ahrendt et al. 2001). Interestingly, KRAS mutations were only found in smokers, which indicates that KRAS mutations were induced by tobacco smoke carcinogens. Ninety percent of the KRAS mutations were found to be G \rightarrow T or A mutations in codon 12 (Ahrendt et al. 2001). Metabolically activated PAHs react primarily with the N2 position of guanine, which implicates PAHs as a probable cause of these mutations found in KRAS (Hecht 2006). A more direct role for PAHs and BPDE in tumorigenesis is provided by Denissenko et al. who treated HeLa cells with varying concentrations of BPDE and then extracted DNA from these cells for sequencing of the p53 gene (Denissenko et al. 1996). This study showed that BPDE forms adducts preferentially to the p53 sequence at various guanine residue hotspots such as codons 157, 158, 245, 248, and 273 which are frequently mutated in human lung cancers (Denissenko et al. 1996). Hence, studies have shown that exposure to cigarette smoke and specific carcinogens such as BaP induce specific mutations in key cancer genes.

The metabolic activation of carcinogens occurs by a mechanism evolved to detoxify and eliminate potentially harmful compounds from the body. Carcinogen exposure triggers a metabolic detoxification mechanism involving cytochrome P450 enzymes that catalyze addition of an oxygen atom to the carcinogen, which increases its water solubility and allows the carcinogen to be excreted more readily (Pfeifer et al. 2002). It is in this process that carcinogens undergo inadvertent metabolic activation by which cytochrome P450s produce intermediate compounds that contain electrophilic (electron-deficient) centers, which are reactive with
nucleophilic base motifs of nucleotides. This results in the formation of DNA adducts (Hecht 1999, Pfeifer et al. 2002).

Carcinogen-DNA adducts, such as those formed between BPBE and N2 of guanine are normally repaired by elaborate DNA repair systems which have evolved in order to eliminate such damage. These include the nucleotide excision repair (NER) pathway which is the system used to repair PAHs. The role of NER in this regard was determined in an Escherichia coli model using a plasmid with site-specific BPDE adducts that required repair for expression in transfected cells (Zou et al. 1995). uvrABC mutant cells were unable to express a BPDE-damaged reporter plasmid, indicating that uvrABC mutant cells were unable to repair the BPDE-damage because the uvrABC (NER) proteins were absent (Zou et al. 1995). Furthermore, Zou et al. demonstrated uvrABC protein interaction with with oligonucleotide sequences containing BPDE adducts through pulldown methods and DNA footprinting (Zou et al. 1995). The role of NER in adduct removal was also investigated in cancer cells. Cells that were deficient in NER (due mutant XP genes) were unable to excise BPDE adducts (Gunz et al. 1996, Pfeifer et al. 2002). Therefore, a well-functioning DNA repair mechanism is important for the removing of tobacco carcinogens and maintaining the integrity of the genome.

Studies also have shown that DNA repair deficiency was associated with lung cancer. For example, a 2000 study that assessed DNA repair capacity in 316 lung cancer patients observed that lung cancer patients with lower DNA repair efficiency were at an increased risk of developing lung cancer (Wei et al. 2000). Specifically, in this study lymphocytes were isolated from lung cancer patients and their ability to repair plasmids with BPDE adducts was assessed. On average, lung cancer patients were able to repair BPDE adducts at a lower rate (~17%) than
control patients (Wei et al. 2000). These findings suggested that reduced DNA repair efficiency can play a role in lung cancer formation.

TSAs are derived from nitrites and secondary amines formed under circumstances when proteins are subjected to high heat or acidity. As such, cooking conditions such as frying and curing greatly enhance their presence in foodstuffs. To date, seven tobacco specific nitrosamines have been identified in tobacco products that are formed during tobacco curing (Hecht 2008). The most abundant of these are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN). These carcinogens affect users of smoked and smokeless tobacco products, causing mainly lung cancer in the former and cancers of the head and neck in the latter. As with PAHs, TSAs are metabolized by cytochrome P450 1A1 and 1B1 resulting in their conversion to DNA-reactive carcinogens. In the case of NNK, it is metabolized to α-hydroxyNNKs by cytochrome P450 and further metabolized to other intermediates including methanediazohydroxide, which is highly reactive with DNA. These reactions with DNA produce 7-methyguanine, O⁶-methylguanine, and relatively bulky pyridyloxobutyl DNA adducts that are both mutagenic and carcinogenic (Hecht 1998, Hecht 2003, Hecht 2008) (Figure 1.11).

The potency of NNK as a carcinogen has been determined in rodent models where doses of NNK (ranging from 0.1-50 mg/kg) were shown to induce a significant increase in lung tumors in a dose dependent manner (Belinsky et al. 1990). In this experiment, O⁶-methylguanine adducts were detected in lung tissue and tumors of NNK-treated animals, indicating NNK-induced DNA damage (Belinsky et al. 1990). The relation between NNK exposure and DNA adduct formation has also been demonstrated in experiments where pyridyloxobutylating adducts (in the form of 4-hydroxy-1-(3-pyridyl)-1-butanone) were
detected in lung DNA of smokers and rodents treated with NNK (Hecht 2008). Also, NNK metabolites have been reported in urine of people exposed to tobacco smoke (Hecht 2003). As with other mutagenic carcinogens, unless repaired, NNK-derived DNA adducts become potential sites of cancer causing mutation.

Studies have shown that both $O^6$-methylguanine and the $O^6$- (pyridyloxobutane-1-yl) deoxyguanine adducts are repaired by $O^6$-alkylguanine-DNA alkyltransferase (AGT). The mechanism by which AGT repairs these adducts is via the transfer of methyl and pyridyloxobutyl moieties to a cysteine residue located in AGT active site, rendering the protein inactive. After alkylation, AGT is thought to undergo a conformational change, exposing the site needed for interaction with the ubiquitin-conjugation system. Ubiquitination of AGT occurs at cystein 145, and this protein is then rapidly degraded (Pegg 2000). While the exact role of BER in repair of nitrosamine-derived DNA damage has not been established, certain observations suggest that BER may be important. For example, NNK treatment conferred increased toxicity in hamster ovary cells deficient in XRCC1 (a scaffolding protein in BER) (Li et al. 2009). In these experiments, Li et al. exposed XRCC1-deficient cells and controls to NNK. XRCC1-deficient mutants showed an increased mutation in the HPRT gene. Loss of function mutation of HPRT is an indirect measure of mutation rate, which can be enumerated as surviving colonies in media containing 6-thioguanine. The mutants also had increased amounts of $O^6$- (pyridyloxobutane-1-yl) deoxyguanine adducts in their DNA (Li et al. 2009). HPRT activity is important for DNA synthesis, and the use of a toxic nucleoside analog 6-thioguanine allows for the selection of HPRT mutants (Li et al. 2009). Therefore, these observations provide evidence that BER is involved in the repair of nitrosamine-derived damage (Petersen 2010).
Figure 1.11. DNA adduct formation from NNK. α-Hydroxylation of the NNK (1) methyl group leads to intermediate 2 which spontaneously loses formaldehyde yielding (3). α-Hydroxylation at the methylene group of NNK produces intermediate 4 which spontaneously decomposes to methanediazohydroxide (5). Methanediazohydroxide (5), formed in these reactions from NNK,
reacts with DNA to produce the well known DNA adducts —, $O^6$-methylguanine ($O^6$-methyl-dGuo) and 7-methylguanine (7-methyl-dGuo). 4-(3-pyridyl)-4-oxobutanediazohydroxide (3) is further metabolized into 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), and pyridyloxobutyl (POB)-DNA adducts. Adapted from Hecht 2008.

This culmination of evidence has led to the understanding that DNA adducts are formed through continuous exposure to tobacco smoke carcinogens which can overwhelm cellular repair mechanisms. These mechanisms include direct base repair by alkyltransferases, base excision repair, nucleotide excision repair, or double-strand break repair. If DNA adducts persist through an overwhelming or malfunction of the repair mechanisms, DNA polymerase enzymes can process these adducts incorrectly and cause mutations. If these mutations are caused in genes that control cellular growth or apoptosis, function of these genes may be altered resulting in abnormal survival and proliferation of damaged cells increasing their likelihood of developing into a malignancy of highly carcinogen-exposed tissue (Figure 1.12) (Hecht 2008).

**Figure 1.12.** Scheme linking nicotine addiction and lung cancer via tobacco smoke carcinogens and their induction of multiple mutations in critical genes. (Hecht 1999)
Although base mutations evidently are a major mechanism of carcinogenesis, another mechanism by which carcinogens produce cancer-causing damage to DNA is by inducing chromosomal instability (CIN). CIN is an increase in the rate of numerical and structural defects to chromosomes (Masuda and Takahashi 2002, Beckman and Loeb 2005, Breivik and Gaudernack 2004). CIN is a mutator phenotype typical of cancer cells. Genetic instability, such as CIN, is a source of genetic variation and a higher rate of genetic change that drives Darwinian selection in tumor development (Beckman and Loeb 2005). It was proposed that carcinogens can cause CIN as a secondary outcome of the disrepair of bulky DNA adducts, such as those caused by PAHs and TSAs (Bishop and Barlow 2000, Herzog et al. 2004, Michel et al. 2001). CIN was shown to coincide with cellular tolerance to carcinogenic damage (Bardelli et al. 2001). This was demonstrated using FISH analysis of clones resistant to PhiP (a heterocyclic hydrocarbon carcinogen common in western diets). FISH analysis showed a higher rate of chromosomal rearrangement in cells lines that had acquired resistance to PhiP cytotoxicity (Bardelli et al. 2001). The genetic basis of CIN is unclear, but is thought to involve defects in any of several mechanisms with roles in DNA repair, as described earlier. Carcinogens apparently exert selection pressures on such functions by inducing extensive DNA damage. Up to a certain threshold the extent of CIN is thought to be advantageous for tumor development by conferring an increase in the rate of genetic change that drives tumor development. That carcinogens actually induce CIN in tumor development was ultimately demonstrated in mice where NNK-induced lung tumors displayed much more extensive CIN than lung tumors of the same histology developing in the absence of carcinogen exposure (Herzog et al. 2004). Consistent with this, studies have shown that NSCLC of smokers (highly carcinogen exposed)
have more extensive CIN than those of never-smokers, who may in general be less exposed than smokers to carcinogens (Sanchez-Cespedez et al. 2001). Thus, evidence has shown that carcinogens in tobacco smoke cause cancer through direct induction of mutations, and the induction of CIN, which is a permanent increase in mutation rate.

1.3.2  **Lung cancer in non-smokers**

Approximately 15% of all lung cancer and 25% of lung adenocarcinoma cases worldwide occur in never-smokers (people who have never smoked). Although no predominant factor has emerged as the cause for this form of lung cancer, epidemiological studies have suggested that genetic susceptibility, environmental exposure to air pollution, occupational exposure to carcinogens and asbestos, and dietary factors can significantly contribute to risk (Parkin et al. 2005, Sun et al. 2007).

Environmental tobacco smoke (occurring through second-hand smoke) plays a significant role in lung cancer risk. It is known that the carcinogen dose from second-hand smoke is far lower than that in active smokers, however detection of urinary metabolites of TSAs in non-smokers exposed to environmental tobacco smoke provides biological evidence for their role in lung cancer development in non-smokers (Hecht 2008). Epidemiological studies have concluded that environmental tobacco smoke exposure increases lung cancer risk ~3-fold compared to ~15-fold in smokers, and is therefore a cause of lung cancer in never-smokers (IARC 2004, Stayner et al. 2007). The mechanism of action of carcinogen exposure is assumed to be the same as described for smokers, but with lower levels of exposure. Another
environmental factor contributing to lung cancer is exposure to fumes from cooking. For example, cooking methods using oils at high heat with poor ventilation result in exposure to fume emissions containing volatile substances, such as PAHs. This historically is fairly prevalent in Asia where lung adenocarcinoma incidence in women never-smokers is relatively high (Chiang et al. 1997).

Genetic factors have also been reported as contributors to lung cancer risk in both smokers and never-smokers. For example, recent studies have shown that individuals with a family history of lung cancer are associated with a 1.5-fold increased risk of lung cancer (Matakidou et al. 2005). In fact, epidemiological studies have suggested that individuals with heritable polymorphisms in CYP1A1, GSTM1, and DNA-repair-related proteins such as ERCC1, XRCC, and cell cycle regulatory genes p53 and MDM2 are all at higher risk of lung cancer development ranging from 2-10 fold (Goode et al. 2002, Hung et al. 2003, Schwartz et al. 2007, Zhang et al. 2006, Zhou et al. 2003). Recently, a large linkage analysis study of inherited lung cancer in 52 families identified a major susceptibility gene on chromosome 6q23-25, RGS17. Germline mutations of this gene were shown to be strongly linked for association with lung cancer, with a LOD (logarithm of odds) of 4.27 (LOD scores above 3 represent a strong likelihood of association) (Bailey-Wilson et al. 2004, You et al. 2009).

Finally, hormonal factors also play a role in lung cancer of non-smokers. For example, a study of NSCLC stage I-III in 301 patients detected ERβ (estrogen receptor β) overexpression (through immunohistochemistry) in mostly samples from non-smokers (53.5%) rather than smokers (36.6%) (Wu et al. 2005). ERβ overexpression was also observed more so in females
(54.3%) than males (39.7%) (Wu et al. 2005). The role of estrogen in cancer development is not fully understood, but it has been shown that estrogen stimulates cellular proliferation through activation of its receptor. This was demonstrated in in vitro experiments where NSCLC cell lines were shown to express ERβ protein at higher levels than lung fibroblast cell lines with NSCLC cells proliferating at a faster rate than lung fibroblasts after treatment with estradiol β (shown through BRDU assays) (Stabile et al. 2002).

Estrogen also been shown to stimulate EGFR ligand release and activate EGFR and MAPK pathways in lung cancer cells (Stabile et al. 2005). EGFR is a strong inducer of cellular growth, and known to be upregulated in lung cancer in non-smokers (Sun et al. 2007). Estrogen may therefore exert oncogenic stimulation through activation of EGFR/MAPK. Finally, estrogen catechols (a metabolic intermediate of estrogen) have been shown to form DNA adducts and DNA single strand breaks in the liver when injected in hamsters, though at high doses (Yager and Leihr 1996). Whether or not these adducts actually lead to mutations is still under investigation. Therefore, hormone levels may also play a role in lung carcinogenesis in non-smokers, whether indirectly by activating cellular proteins involved in growth or directly by causing DNA adducts that induce mutations in cancer-causing genes.

Although tobacco smoke is the main contributor to lung cancer, the incidence of lung cancer in non-smokers is also significant. This year alone approximately 250,000 individuals are expected to die from this disease (Parkin et al. 2005). A number of possible causes of lung cancer in non-smokers have now been identified.
1.4 LUNG CANCER: Forms of Treatment

There are three main methods for treating lung cancer patients: surgery; radiotherapy; and chemotherapy.

Surgery is most effective, but is usually reserved for lung cancer in patients with contained tumors (Stage I-IIIA), which has not spread to surrounding tissue or is still contained in one lobe of the lung. Typically, it is reserved for patients who will be able to tolerate the procedure and have cancer that is completely resectable. Procedures for pulmonary cancer removal include wedge resection (partial removal of one lobe), segmentectomy (removal of an anatomic division of a lobe), lobectomy (removal of one or several lobes), or pneumonectomy (removal of an entire lung). As technological advancements have allowed for increasingly less invasive methods of resection, surgery has become more tolerable and more widely implemented. It is important to note, however, that patients who have undergone surgery typically undergo post-surgical chemotherapy or radiotherapy in order to increase the efficiency of cancer obliteration (ACS 2010, Molina et al. 2008).

Radiation therapy is very effective for the treatment of highly localized tumors, or cancer that has not spread to surrounding tissues. Its mode of action involves the induction of double-strand breaks of cells within the tumor. Cells that are unable to repair the damage caused by the particles of radiotherapy undergo cell death (Prise and O’Sullivan 2009). Radiotherapy is used for both small cell and non-small cell lung cancers and most commonly involves external beam radiation therapy (EBRT) or brachytherapy. Radiotherapy is also applied to patients who have tumors that cannot be surgically removed due to size, location, or poor
patient health. EBRT procedures focus radiation delivered from the outside of the patient’s body onto the internal location of the cancer. Brachytherapy involves placement of small radioactive material in the vicinity of the cancer in order to shrink its size. Dosage usually ranges between 60 to 80 Gy for solid tumors and is fractionated over several small doses over a period of time. Typically, adult patients receive approximately 1.8 to 2 Gy per day five days a week depending on the cancer type. These radiotherapies are usually used in conjunction with chemotherapy, as this regimen has been shown to significantly increase patient survival: 1 year compared with 111 days with placebo (Lally et al. 2006, Molina et al. 2008, Wagner 1998).

One distinction between radiotherapy and chemotherapy is that radiotherapy can be delivered locally to a very specific part of the body (Kepka et al. 2010). As such, it can be used for a number of purposes. For example, it can be used pre-surgically to reduce tumor size and increase effectiveness of tumor removal, post-surgically to treat any remaining tumor cells after surgery, or to the brain in order to reduce any metastasis. It is also frequently used to palliate symptoms of lung cancer, such as clearance in the endobronchial or central airway (ACS 2010, Chella et al. 2000, Kepka et al. 2010).

Close to 70% of lung cancer patients are diagnosed as locally advanced or metastatic. In these patients (as with patients with resected lung cancer), chemotherapy is standard regimen for treatment (Pfister et al. 2003). The most commonly used chemotherapy agents involve topoisomerase II inhibitors (etoposide), platinum-based drugs (cisplatin, carboplatin), folate antimetabolite pemetrexed, taxanes (paclitaxel/docetaxel), and vinca-alkaloids
(vinorelbine/gemcitabine). These agents generally exploit the proliferative aspect of tumor cells (ACS 2010).

Etoposide is a semisynthetic drug used mainly for lung cancers, testicular cancers and glioblastoma multiforme. Its mechanism of action is topoisomerase-II enzyme inhibition, thereby preventing re-ligation of DNA, causing errors in DNA synthesis, which ultimately leads to programmed cell death via apoptosis. Also called “alkylating-like drugs,” the platins function by binding to deoxyguanine and causing interference with DNA repair, leading to programmed cell death. Platins are commonly used to treat ovarian, lung, and head and neck cancers (Aruajo et al. 2009, Azim and Ganti 2007, MacCallum and Gillenwater 2006, Murray and Turrisi 2006).

Pemetrexed is a folate antimetabolite mainly used to treat mesothelioma and NSCLC. Its mechanism of action involves inhibition of three enzymes used in purine and pyrimidine synthesis. These include thymidylate synthase, dihydrofolate reductase and glycinamide ribonucleotide formyltransferase. Inhibition of these enzymes by pemetrexed prevents de novo nucleotide synthesis and therefore inhibits DNA and RNA synthesis, which are required for cell growth and survival (Clegg et al. 2002, Exinger et al. 2003).

Taxanes and vinca-alkaloids are microtubule-disrupting or anti-mitotic agents. Currently, these agents are used in combination with other chemotherapeutic agents (such as alkylating agents), and have been shown to improve overall survival in patients (ACS 2010). Taxanes bind to β-tubulin resulting in enhanced tubulin polymerization and a disturbance in tubulin dynamics causing stabilization (and preventing depolymerization) in microtubule structures. Interestingly, the binding site of taxanes is only present on assembled microtubules (Abal and Andreu 2003).
Vinca-alkaloids are microtubule-destabilizing agents that bind to the vinca domain on microtubules, and cause an inhibition of microtubule assembly (Zhou et al. 2005). Once bound, both of these anti-mitotic agents block the transition from metaphase to anaphase causing mitotic arrest, which when sustained also ultimately leads to programmed cell death (Jordan and Wilson 2004, McGrogan et al. 2008).

EGFR is overexpressed or mutated in 40-80% of NSCLC patients, and has been shown to correlate with poor survival, disease progression, poor response to standard therapy, and development of resistance to cytotoxic drugs (Bencardino et al. 2007). As such, various small molecules able to inhibit EGFR have been developed to treat this cancer type. Two of these agents that have been recently approved by the US Food and Drugs Administration are gefitinib and erlotinib. These drugs are most effective against tumors with mutant EGFR, which is most prevalent in NSCLC of non-smokers. Demographically, Japanese patients and women have shown slightly better response rates (Bencardino et al. 2007, Molina et al. 2008). Interestingly, mutations in KRAS and MET are associated with reduced response rate to these EGFR inhibitors (De Luca and Normanno 2010). This is not surprising as RAS signaling lies downstream of EGFR.

As such, genetic screening of predictive factors such as KRAS/MET mutations is important to determine sensitivity or resistance to EGFR inhibitors in tumors and increase treatment efficiency.

Typical treatment regimen for advanced stage NSCLC lasts 3-4 weeks and involves platinum-based drugs (usually cisplatin or carboplatin for 4-6 cycles) in combination with taxanes or vinca-alkaloids (commonly paclitaxel or vinorelbine). Depending on patient response
to this first-line treatment regimen, second line treatment is sometimes administered using a 
single drug; often docetaxel, pemetrexed, or sometimes erlotinib (ACS 2010).

1.5 MOLECULAR CHANGES IN NON-SMALL CELL LUNG CANCER

Although a significant number of lung cancer cases are diagnosed as SCLC 
(approximately 15%), the focus of this thesis pertains to our findings in NSCLC. As such, this 
background review will focus on the molecular aberrations found in NSCLC and genes that 
contribute to NSCLC development.

Gene mutation and changes in gene copy number are typical of all cancer types, and 
these molecular alterations in NSCLC have been studied extensively. These alterations consist of 
inactivating mutations of tumor suppressors and activating mutations of oncogenes. Whereas 
loss of DNA copy number inactivates tumor suppressor genes, increases in copy number 
commonly increase the function of many oncogenes (Niklinski et al. 2000). Our current 
understanding of cancer holds that an accumulation of these molecular changes drives cancer 
development (Loeb 1998, Macleod 2000, Weinberg 1995). It is evident that, as cancers develop, 
tumor-inhibiting functions are continuously selected against, and functions that enhance tumor 
growth and survival are selected for. Squamous cell lung carcinoma (LSqCC) and lung 
adenoarcinoma (LAC) are the most common histotypes of NSCLC. Studies have shown that 
LSqCCs and LACs develop through distinct pathogenic pathways (Sekido et al. 2003, Singhal et 
al. 2008).
Whole genome analyses have led to the identification of chromosomal defects typical of LAC and LSqCC. Analysis of karyotypic changes in NSCLC specimens and cell lines in previous studies has identified numerous chromosomal losses and gains that occur in lung carcinogenesis. These include frequent allelic loss of regions of chromosome 9 (84% of tumors analyzed) and loss of chromosomal arms in 3p (~75%), 6q, 8p, 9q, 13q, 17p, 18q, 19p, 21q, and 22q (all lost in at least 60% of tumors analyzed), and chromosomal gains on 7p and 7q (also observed in approximately 60% of cases) (Balsara and Testa 2002). A high resolution mapping analysis of the short arm of chromosome 3 on lung carcinomas preneoplastic lung tissue, showed allelic loss in 95% of squamous cell carcinomas, and 71% of adenocarcinomas (Wistuba et al. 2000). These losses were also observed in preneoplastic lesions indicating that loss of chromosome 3p could be an early event in both major types of NSCLC development.

A closer inspection of allelic loss on 3p has led to the identification of putative oncogenes and tumor suppressor genes. For example, restriction fragment length polymorphism analysis and deletion mapping of NSCLC DNA mapped specific regions of DNA loss to 3p14, 3p21, and 3p24 (Sekido et al. 1998, Wistuba and Gazdar 2006). Positional cloning of the 3p14 region led to the identification of the FHIT at the minimum region of deletion. Intragenic deletions of the FHIT gene were later found to occur in several cancer types (Croce et al. 1999). FHIT’s characterization provides a good example of how LOH and deletion mapping can be used to identify target genes, followed by functional analysis to determine their potential role in lung cancer. As such, a more detailed description of FHIT’s characterization as a putative tumor suppressor gene now follows.
LOH studies of FHIT in 59 lung cancer indicated LOH in 76% of samples (Sozzi et al. 1996). Sozzi et al. further observed LOH of FHIT in 41 of 52 tumors from smokers (80%) but only in 9 of 40 tumors of nonsmokers (22%), indicating that LOH of FHIT was potentially facilitated by carcinogens in tobacco smoke (Sozzi et al. 1997). In addition, immunohistochemical studies in 473 stage I NSCLC samples demonstrated a strong staining of FHIT in normal tissue, whereas 73% of tumor tissue cells stained negative (Croce et al. 1999). Exogenous expression of FHIT in tumor cell lines also corresponded to a loss of ability to form subcutaneous tumors in mice (Croce et al. 1999). The 1-2 Mb FHIT gene is composed of 10 exons, five of which code for protein. The gene encodes a small mRNA (1.1kb) and a small protein (16.8 kd) and contains the FRA3B fragile site (a region frequently rearranged in cancer). Disruption of the FRA3B site has been shown to coincide with rearrangements within the FHIT gene (Croce et al. 1999). Comparison of FHIT transcripts in cancer cells and normal cells showed frequent absence of whole FHIT coding exons in cDNAs PCR-amplified from lung, colon, breast and stomach carcinomas (Croce et al 1999). Furthermore, FHIT is a member of the histidine triad (HIT) gene family which encodes a diadenosine 5’,5’’-P1,P4-tri- phosphate (Ap3A) asymmetrical hydrolase, which cleaves the Ap3A substrate into ATP and AMP (Sozzi et al. 1996). Interestingly, a decrease of free Ap3A levels has been associated with apoptosis in human cultured cells (Kisselev et al. 1998), and studies in lung cancer cell lines have demonstrated that restoring FHIT expression significantly increased apoptosis and G1 cell cycle arrest through upregulation of p21 (Sard et al. 1999).

Finally, Fhit -/- mice exhibited higher tumor burden when subjected to DMBA, than Fhit +/+ mice (Zanesi et al. 2001). Zanesi et al. subjected Fhit +/+, +/-, and -/- mice to a single dose
administration of DMBA and after 29 weeks, 89.5% of Fhit -/- mice exhibited tumors of the forestomach and squamocolumnar junction, while 78% of Fhit +/- mice and 7.7% of Fhit +/+ mice exhibited tumor burden (Zanesi et al. 2001). Spontaneous tumors also developed in untreated mice after 1.5 years of age carrying null alleles of Fhit. Specifically, Fhit -/- mice exhibited tumor burden at a rate of 0.76 tumors (of various types) per mouse and Fhit +/- mice exhibited a rate of 0.94 tumors per mouse (Zanesi et al. 2001). Wild type mice exhibited no tumor burden. These results suggested that FHIT had tumor suppressing function that when lost contributed to NSCLC.

1.5.1 Molecular changes of squamous cell lung carcinoma

Preneoplastic lesions are frequently extensive and multifocal throughout the lung of tobacco smokers and rare in never-smokers (Wistuba et al. 1999, Wistuba et al. 2006). In LSqCC these include changes in mucosal cells that depict metaplasia (replacement of mucus-secreting ciliated simple columnar respiratory epithelial cells with stratified squamous epithelium), squamous dysplasia (expansion of premature cells with a corresponding decrease of mature cells), and carcinoma in situ. The study of these preneoplastic lesions in lung cancer has proven informative in uncovering a multi-step sequence of genetic alterations that play a role in tumor progression and pathogenesis.
Studies have assessed the relative order in which known molecular abnormalities occurred in early stage LSqCC by examining distinct preneoplastic stages of its development. The earliest event, determined in hyperplastic tissue, was shown to be allelic loss at multiple 3p loci (FHIT/SEMA3B/RASSF1A are the proposed tumor suppressor genes in this region), followed by 9p21 allele loss (p16\(^{INK4a}\)) and potential telomerase disregulation. Later changes in dysplastic tissue occurred on 8p (which harbors candidate metastasis suppressor genes PSD3 and LPL), 13q14 (RB), followed by loss of 17p (which harbors the p53 gene), and loss of 5q (Boelens et al. 2009, Sekido et al. 2003, Wistuba et al. 1999, Wistuba and Gazdar 2006).

There have also been several studies to assess molecular changes in malignant LSqCC. These studies have reported DNA amplification in 3q24-26 (56% of LSqCC samples analyzed), 7p15 (40% of samples), 17q21 (45%), 17q25 (52%) and allelic loss in 4p15 (29%), 13q21 (24%), and 17p12-13 (36%) among others (Shen et al. 2008). As these studies were conducted on LSqCC at stages I-IV, it is uncertain when or if these lesions occur in any particular preneoplastic stage.

1.5.2 Molecular changes of lung adenocarcinoma

Adenocarcinomas are characterized as cancers arising from epithelium of glandular tissues and have a peripheral location in the lung (closer to the alveoli), as opposed to LSqCC which are more centrally located and of the larger airways (ACS 2010, Westra 2000). So far, there has been only one morphological entity described as a precursor to adenocarcinomas: atypical adenomatous hyperplasia (AAH) (Wistuba and Gazdar 2006). AAHs are discrete
parenchymal lesions arising in alveoli near terminal and respiratory bronchioles. These lesions retain their alveolar structure, but their walls may be slightly thickened by collagen, fibroblasts, and lymphocytes. There is evidence supporting the concept of AAH as a precursor for LACs. For example, AAH is mostly detected in lungs of patients who develop adenocarcinomas (as many as 40%) than do LSqCC (11%) (Sekido et al. 2006). Also, molecular changes such as KRAS-activating mutations on codon 12 (which are more prevalent in LACs) have been shown to be present in as many as 39% of AAHs (Westra et al. 1996). This suggests KRAS activation as an early event in LAC development.

Other molecular alterations in AAH are overexpression of Cyclin-D1 (in approximately 70% of samples studied using immunohistochemistry in one study), mutant p53 (28% of samples), and increased Survivin mRNA levels (48% of AAH lesions) (Nakanishi et al. 2003, Wistuba and Gazdar 2006). Also, bi-allelic loss and decreased immunohistological staining of LKB1 (in approximately 35% of AAH samples studied), LOH in chromosome 3p (18%), 9p (p16\textsuperscript{INK4a}), 9q (53%), and 17q have been reported (Ghaffari et al. 2003, Kitaguchi et al. 1998). Since these alterations occur in AAHs which are precursors to LAC, they are considered early perturbations in the development of adenocarcinomas.

Studies have found many more molecular alterations occurring in LAC. Some of these include DNA gains in 1q (35%), 3q24-26 (41% of samples), and 8q24 (47%) (Shen et al. 2008, Yan et al. 2007). LACs were also reported to contain frequent allelic losses at 3p (33% of samples), 9p (where p16\textsuperscript{INK4a} is located), 13q (which might perturb BRCA2), and DNA loss on 17p12-13 in
41% of samples (potentially targeting p53 for deletion), and 22q in 29% of samples (MYO18B is a proposed gene) (Sekido et al. 2003, Shen et al. 2008, Yen 2007).

A large scale study of 371 lung adenocarcinoma samples (stages I-IV) for genome-wide copy-number alterations also identified numerous molecular changes (Weir et al. 2007). These included amplifications in 8q24 (c-MYC is a candidate oncogene in this region), 14q13, and allelic loss in 5q11, 9p21, and 10q23 among others (Weir et al. 2007). As was mentioned in the case of LSqCC samples, these modifications were found in malignant samples. Therefore, it is not certain whether they occur in preneoplastic stages of LAC or are precursors for LAC.

Compared to LSqCC, LACs typically contain more frequent mutations in codon 12 of KRAS (87% of LACs studied), deletion the tyrosine kinase domain (in exon 19) of EGFR (in 40% of LAC studied), and overexpression of 2-3 fold of Her2/neu (determined by immunohistochemistry of 238 samples) (Ahrendt et al. 2001, Brose et al. 2002, Hirsch et al. 2002, Shigematsu et al. 2005, Shigematsu et al. 2006, Weir et al. 2007, Wistuba and Gazdar 2006). The high prevalence of EGFR mutations also in AAH lesions has led to some consideration that EGFR expression could serve as a potential biomarker for early detection of LACs (Kurie et al. 1996). LSqCC tend to have higher incidences of deletion of 17p13 (where p53 is located), 13q14 (RB), p16^{INK4a} inactivation through hypermethylation, and several regions of chromosome 3p affecting the FHIT, SEMA3B and RASSF1A genes (Wistuba and Gazdar 2006) (Table 1.2).

Expression changes also have been identified that are more common to either LSqCC or LAC. For example, microarray experiments comparing the expression profile of 22,283 genes in
58 LACs and 53 LSqCC found 25 genes that are differentially expressed between these two histotypes of lung cancer (Chang et al. 2010). Therefore, these studies suggest that LSqCC and LAC develop distinct recurrent chromosomal and epigenetic aberrations that contribute to their development.
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**Table 1.2.** Molecular changes frequently occurring in NSCLC. Bold represents increase in copy number/activation. Allelic loss/inactivation not bold. Molecular changes associated with squamous and adenomatous tissue are underlined. Data collected from Balsara and Testa 2002, Duarte et al. 2006, Garnis et al. 2006, Shen et al. 2008, Weir et al. 2007, Wistuba and Gazdar 2006, Yen et al. 2007)
1.5.3 **Molecular changes in LAC of smokers vs. non-smokers**

Genome-wide analyses of LAC in smokers and non-smokers have shown several differences. Overall copy number changes are more numerous in those of smokers, which indicates a greater degree of CIN in these tumors. One copy number change that appears unique to LAC of never-smokers is copy number gain on 16p (in 59% of samples) (Wong et al. 2002). Certain findings have suggested that LAC in smokers and non-smokers develop through distinct pathogenetic changes. For example, available evidence indicates that p53 mutations occur more frequently in tobacco-associated lung cancer than in never-smokers. p53 mutations were detected in 47.5% tumors of never smokers and in 77.4% of current smokers (Le Calvez et al. 2005). The most significant difference between these two forms of cancer is that smoking-related LACs associate more with KRAS mutations and nonsmoking-related LACs associate more with EGFR mutations. For example, EGFR mutations (in exons 18-21; the tyrosine kinase domain) were detected in 45% LAC of of never-smokers, but only 7% of smokers in a study looking at 2,128 carcinomas (Shigematsu and Gazdar 2006). KRAS mutations on codon 12 were also found in 43% (out of 106 cases) in smokers and in 0% of non-smoker cases (Subramanian et al. 2010). EGFR and RAS changes are of a common signal transduction pathway and appear to occur in mutual exclusion to one another (Subramanian et al. 2008, Wistuba and Gazdar 2006, Yen et al. 2007). The selective roles of these mutually exclusive changes in LAC is not yet known. However, EGFR mutations have a broader effect on signal transduction than KRAS mutations as there are 3 branches of the EGFR pathway with KRAS representative of one of them. Therefore, other changes to the broader EGFR network are likely to occur in LAC of
smokers. One such change of the PI3K/AKT branch that is the topic of this dissertation is FOXO3a gene deletion, which may be a selective occurrence in LAC of smokers.

To summarize, evidence indicates that LSqCC and LAC in smokers and never-smokers develop with numerous and distinct genetic changes. Many candidate target genes have been proposed as the target of these genetic changes. This thesis describes the identification of the FOXO3a gene as a site of recurrent gene deletion on chromosome 6q21 in NSCLC, occurring more frequently in LAC, and the to-date characterization of its role in the development of these tumors. A discussion of FOXO proteins now follows.

1.6 FOXO TRANSCRIPTION FACTORS

The forkhead family of proteins was first identified in Drosophila melanogaster in 1989, when mutated forkhead proteins caused homeotic transformations of the gut structures into head-derived elements. Therefore, the gene was called fork head (Weigel et al. 1989). The identification in fork head of a winged helix DNA binding domain encompassing about 110 amino acids has led to the identification of a wide range of over 100 fork head family members in organisms ranging from yeast to human (Kaufmann and Knochel 1996). This DNA binding domain consists of three α-helices, three β-sheets and two loops or “wings” which form a helix-turn-helix-like motif (Myatt and Lam 2007). Based on amino acid sequence alignment, the human FOX gene family currently contains 43 sub-families consisting of FOXA-Q (Katoh and Katoh 2004). All 43 sub-families are further categorized into 2 classes: class 1 which have a basic C-terminal of their DNA binding domain (containing Arg and Lys amino acids) and class 2 which
do not. Members of FOX subfamilies A-G, I-L, and Q are grouped into class 1, and subfamilies H, M-P are grouped into class 2 (Katoh and Katoh 2004).

FOXOs are the Forkhead box type-O family of transcription factors. The first member of the O subclass of forkhead proteins was originally characterized in the nematode worm Caenorhabditis elegans as DAF-16. DAF-16 was shown to regulate dauer formation, which is a developmental stage which ensures survival under poor nutrient conditions (Thomas 1993). DAF-16 was found to extend life in C. Elegans when continually active (Thomas 1993).

FOXOs contain a DNA binding domain (DBD) (i.e. the forkhead box), a transactivation domain located in the N-terminal and C-terminal parts of the protein, a nuclear localization signal (NLS) and a nuclear exclusion signal (NES) (Obsil and Obsilova 2008) (Figure 1.13A). The DNA binding domain resembles a winged-helix and consists of three α-helices (H1, H2, and H3), three β-strands (S1, S2, and S3) and two wing-like loops (W1 and W2) (Figure 1.13B).
Figure 1.13. FOXO’s domain structures and topology of the FOXO domain. A, schematic representation of domain structures in FOXO transcription factors. FOXOs are comprised of a DNA binding domain (DBD), nuclear localization sequence (NLS), nuclear exclusion signal (NES), and a transactivation domain (TD). B, topology of the FOXO domain. Adapted from Obsil and Obsilova 2005.
There are four functional mammalian FOXO family members encoded by four different genes. FOXO1, FOXO3 and FOXO6 proteins all have similar lengths of approximately 650 amino-acid residues, while FOXO4 has a shorter sequence of approximately 500 amino-acid residues. A sequence alignment between these proteins shows that there is a sequence homology of approximately 39% across FOXOs and that several regions are conserved among members (Figure 1.14). These regions include the N-terminal region surrounding the first Akt/protein kinase B (PKB) phosphorylation site, the forkhead DBD, the NLS and part of the C-terminal transactivation domain. FOXO2 is identical to FOXO3a, but is characterized as a processed pseudogene, as is FOXO3b. FOXO5, on the other hand, is an ortholog solely expressed in zebrafish (Anderson et al. 1998, Greer and Brunet 2005, Kaestner et al. 2000).

Expression of the different FOXOs, although ubiquitous, has been shown to vary greatly between different tissues and cell types. For example, FOXO1 mRNA is highly expressed in ovarian and adipose tissue, FOXO3a in neuronal tissue, bone-marrow and lung, FOXO4 is abundant in placenta and skeletal, and FOXO6 expression appears to be restricted to the brain (Figure 1.15) (http://www.BioGPS.org, Jacobs et al. 2003).

The Forkhead family of transcription factors have been shown to be functionally active and implicated in a variety of cellular processes including development, metabolism, apoptosis, cell cycle, and proliferation (Kaestner et al. 2000, Kaufmann and Knochel 1996). Together, evidence has implicated the FOXO family in both tumor suppression and aging.
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<td>351 SKLAQVDGCQPTEAPSSLVPTSMIAFQPMASQIPKAG----------</td>
</tr>
<tr>
<td>FOXO6</td>
<td>369 APSRALAHPSMPEGGSAEAPP----GSAFPQGGG----------</td>
</tr>
<tr>
<td>FOXO3a</td>
<td>564 SLGSAKHQQQSPQTSMQTSPEPLLQSSSLYSSSSN---FPVQIYFSDLDLLMENCSL</td>
</tr>
<tr>
<td>FOXO1</td>
<td>559 ---PPLPHPSPAFAKSFVSYCAYGRTHFBNREGSQW葛METER</td>
</tr>
<tr>
<td>FOXO4</td>
<td>396 ---PPLTHGLPHNSTPQPKKSSQWLVQGMPHDQQW</td>
</tr>
<tr>
<td>FOXO6</td>
<td>409 ---EPLYSLPFAALPAGAPAAGAAGSPGLAAGAPPDPPLGL</td>
</tr>
</tbody>
</table>

**Figure 1.14.** Sequence alignment of FOXO proteins. FOXO proteins share a sequence homology of ~39% as determined by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).
Figure 1.15. FOXO3a mRNA expression pattern in various tissues. Expression pattern was acquired from BioGps (http://biogps.gnt.org). Expression values are from Affymetrix chips related to fluorescence intensity. FOXO1 and FOXO4 expression patterns are included in Figure A.1.
FOXOs activate or repress transcription through binding to their response element. All FOXOs bind to a common Forkhead binding element (FHBE), RYAAAYA. The affinity of individual FOXOs with different promoters is likely affected by the actual FHBE sequence and sequence variation within the DNA binding domain of the different FOXOs. Several hundred effector genes are expected to contain functional FHBEs. Many of these genes have shown to be regulated by FOXOs with functions ranging from control, apoptosis, cell cycle regulation, glucose uptake and metabolism, and oxidative stress response. The transcriptional activity of FOXOs also includes interactions with other transcription factors in the co-regulation of gene expression (Greer and Brunet 2005, Tran et al. 2003, Yang et al. 2009).

It has been shown that FOXOs and Smad4 interact in the transcriptional response to TGF-β, which activates Smad4 via adaptors Smad2/Smad3 Smad activity (Seoane et al. 2004). TGF-β is a growth factor that has been shown to regulate a number of cellular processes including cellular proliferation, differentiation and apoptosis. In this study, RNAi against FOXO1, FOXO3a and FOXO4 and Smad4 inhibited expression of a set of genes induced by TGF-β. These genes were found to have both Smad4 and FOXO response elements suggesting that full expression in response to TGF-β utilized both elements. A subsequent report suggested that FOXO3a activation by TGF-β involved transcription upregulation of the Runx1 transcriptional co-activator by TGF-β. Co-immunoprecipitation, electrophoretic mobility shift assays, and chromatin immunoprecipitation assays demonstrated that Runx1 did not bind directly to the
identified FHBE site, but bound FOXO3a, which occupied the FHBE site. RNAi knockdown of Runx1 or FOXO3 also was shown to decrease TGF-β-induced BIM expression, demonstrating the cooperation between Runx1 and FOXO3a in the expression of a FOXO3a effector (Massague et al. 2004, Seoane et al. 2004, Wildey and Howe 2009).

Mouse knockout models for FOXOs have proven to be very informative in elucidating their role in mammals. Hosaka et al. conducted a study where each individual FOXO gene was disrupted in mice using a promoter trapping vector, which allows for the selection of a neo cassette incorporated within the FOXO genes by homologous recombination-driven FOXO gene rearrangement (Hosaka et al. 2004). Hosaka et al. uncovered distinct phenotypes when each individual FOXO gene was disrupted. Foxo1-/- mice were embryonic lethal due to defective vascular development. In contrast, no distinct phenotype was observed in Foxo4-/- knockouts (Hosaka et al. 2004). Foxo3a-/- knockout female mice contained unusually formed ovaries (due to incomplete follicle development) and could not produce offspring when mated (Hosaka et al. 2004). Conditional knockout of all FOXOs generated a progressive cancerous phenotype that included thymic lymphomas and hemangiomas (Paik et al. 2007). These mice were generated using an inducible Mx-Cre transgene to achieve a widespread somatic deletion of FOXO in adult tissue. The Mx1 promoter is activated by polyinosine-polycytidylic acid, and mice were injected at 4-5 weeks of age in order to induce widespread Cre expression (Paik et al. 2007). In mammals, FOXOs have tissue specific effects and appear to be redundant with regard to general tumor suppression. FOXO members confer an effect on cancer in the cell that is largely similar (Brenkman and Burgering 2003, Castrillon et al. 2003, Furuyama et al. 2000).
1.6.1 Regulation of FOXO proteins

FOXO transcription factors are known to be regulated at three levels: a) sub-cellular translocation, b) DNA binding, and c) protein degradation. All three of these levels of regulation are effected through post-translational modification of FOXO proteins.

It was first proposed that FOXOs are regulated by Akt when it was found that *C. elegans* FOXO ortholog (DAF16) was negatively regulated when Akt was active. This was done by observations that activated Akt mutants in *C. elegans* suppressed dauer arrest. Dauer phenotype was again achieved when *C. elegans* were injected with Akt RNAi in order to knock out the gene, but this dauer phenotype could not be achieved when Akt activity was suppressed in DAF16 mutant *C. elegans* (Ogg et al. 1997, Paradis and Ruvkun 1998). Therefore, it was proposed that Akt acted as a DAF16 repressor which could induce dauer state formation. Analysis of the DAF16 sequence also revealed that three sites in the protein sequence conform to the consensus site phosphorylated by Akt (RXRXXS/T) (Alessi et al. 1996, Brunet et al. 1999). FOXOs were later shown to be phosphorylated by Akt which caused its cytoplasmic retention. This was demonstrated by Brunet et al. in *in vitro* transfection experiments with wild type FOXO3a and constructs that contained mutated Akt phosphorylation sites (Brunet et al. 1999). Western blot analysis showed an increase of phospho-FOXO3a when cells were transfected with the wild type construct, but not in the mutant construct. Confocal microscopy of cells transfected with wild type FOXO3a or Akt phosphorylation site triple mutant verified wild type
FOXO3a presence in the cytoplasm under conditions of starvation, but a nuclear retention of the triple mutant FOXO3a (Brunet et al. 1999).

Akt inactivates FOXOs through direct phosphorylation of specific residues that results in 14-3-3 binding and subsequent inactivation by nuclear exclusion. This has been demonstrated, for example, by FOXO3a coimmunoprecipitation assays that failed to show interaction with 14-3-3 ζ when thr32 and ser253 (Akt phosphorylation sites) were mutated to alanine. Although, the ser315 mutant of FOXO3a was still able to coimmunoprecipitate with 14-3-3 ζ, mutation of these phosphorylation sites (T32, S253, and S315) and prevented nuclear export of FOXO3a (as shown by immunohistochemistry) even under presence of growth factors (Brunet et al. 1999, Brunet et al. 2002).

As transcription factors, FOXOs are functional in the nucleus, and inactive when in the cytoplasm. In response to growth factor stimulation, IKK and ERK also phosphorylate FOXOs at different phosphorylation sites, enhancing its interaction with 14-3-3 proteins, and promoting FOXO protein nuclear export to the cytoplasm where it is inhibited (Greer and Brunet 2005, Potente et al. 2005, Tsai et al. 2007). The exact mechanism of FOXO nuclear export has not been elucidated, although it has been proposed that FOXO phosphorylation causes an association with 14-3-3 protein that inhibits DNA binding and causes a conformational change that exposes the NES for interaction with Crm1. Support for this model was provided by Brunet et al. who showed an accumulation of 14-3-3 and FOXO3a protein in the nucleus as a result of leptomycin B (a Crm1 inhibitor) treatment, while untreated controls showed 14-3-3 and
FOXO3a protein accumulating in the cytoplasm (Brunet et al. 2002). Brunet et al. also showed FOXO3a accumulating in the nucleus when its NES was mutated (Brunet et al. 2002).

Although FOXO phosphorylation has typically been shown to cause its nuclear exclusion through 14-3-3 association, recent findings have also shown that, in one case, FOXO phosphorylation can also repress 14-3-3 binding and activate FOXO. This has been demonstrated for one FOXO3a phosphorylation site so far: S207 (Lehtinen et al. 2006). Lehtinen et al. demonstrated this by showing FOXO3a localization to the nucleus and induction of apoptosis in response to oxidative stress when phosphorylated at this site. It was demonstrated that this site was a MST1 (macrophage stimulating 1) phosphorylation site. In this study, wild type MST1 (but not a kinase dead MST1) interacted with FOXO3a and was able to phosphorylate FOXO3a on S207 in response to oxidative stress, as demonstrated by co-immunoprecipitation and western analysis. Immunoprecipitation experiments further demonstrated that MST1 phosphorylation inhibited FOXO3a interaction with 14-3-3 protein and induced cell death (Lehtinen et al. 2006) (Figure 1.16).
**Figure 1.16.** Phosphorylation of FOXO3a. Figure shows confirmed phosphorylation sites in FOXO3a and the kinases involved. AKT, ERK, and IKK negatively regulate FOXO3a, while MST has been shown to be an activator. DBD: DNA binding domain, NLS: nuclear localization sequence, NES: nuclear export signal, TAD: transactivation domain. Adapted from Miranda et al. 2010.

FOXOs also contain ERK and IKK regulate phosphorylation sites, which like AKT phosphorylation lead to its cytoplasmic localization and ubiquitination and proteosomal degradation (Yang et al. 2008). This was fist elucidated by investigating if the purported IKK phosphorylation sequence (D$\Psi$xxS/T) was present in FOXO3a. Hu et al. confirmed that this site was present at S644 (Hu et al. 2004). ERK-specific phosphorylation sites on FOXO were elucidated by Yang et al. who analyzed phosphorylated FOXO3a through mass spectrometry and found three such sites located on S294, S344, and S425, and led to the suggestion of a purported ERK phosphorylation site on FOXOs (P/XXS/TP) (Yang et al. 2008). It has been proposed that FOXO phosphorylation by ERK facilitates its physical interaction with E3 ubiquitin ligase, MDM2 (Fu et al. 2009, Yang et al. 2008). This is based on results obtained by Yang et al. which first demonstrated an interaction between ERK and FOXO3a (determined by immunoprecipitation), and an increase in phosphorylated FOXO3a protein at putative ERK
phosphorylation sites (determined by MS) (Yang et al. 2008). The interaction between FOXOs and MDM2 was then demonstrated by immunohistochemistry which found FOXO1 and FOXO3a co-localizing with MDM2 in MEFs and by coimmunoprecipitation experiments which showed strong interaction between FOXO1 and FOXO3a with MDM2. Furthermore, it was found that knockdown experiments of MDM2 and ERK led to increased protein levels of FOXO3a and FOXO1, and western analysis determined that overexpression of MDM2 caused a decrease in FOXO3a protein and an increase in Ub-FOXO3a protein (Fu et al. 2009, Yang et al. 2008). Western analysis also demonstrated that with a constitutively active p-ERK, not only were FOXO3a protein levels undetectable in 293T cells, but FOXO3a apoptosis and cell cycle effectors (p27 and BIM) were also not expressed at the protein level (Yang et al. 2008). While FOXO3a inactivation by ERK appears to occur through MDM2, Akt phosphorylation promoted degradation through an alternate E3 ubiquitin ligase, namely Skp2. This was demonstrated in transfection experiments in human prostate cancer cells which showed FOXO1 protein degradation when Skp2 protein was exogenously expressed and an increase of FOXO1 protein levels when Skp2 was knocked down with siRNA (Huang et al. 2005). Furthermore, FOXO1 did not undergo degradation when an Akt-specific phosphorylation site was mutated (S264A), nor did immunoprecipitation experiments detect interaction with Skp2 in this S264A mutant (Huang et al. 2005). Interestingly, it was found that Akt positively regulates Skp2 and that phosphorylation of Skp2 by Akt promotes 14-3-3/Skp2 interaction (Gau et al. 2009, Vogt et al. 2005). This was determined by western analysis in HeLa cells which demonstrated decreased Skp2 protein levels when Akt was knocked down, and an interaction between Akt with Skp2 when Skp2 was overexpressed (determined by immunoprecipitation) (Gau et al. 2009, Lin et al. 2005).
When cells were transfected with an active form for Akt, confocal microscopy experiments showed Skp2 associating with 14-3-3 β and both proteins localizing to the cytoplasm (Lin et al. 2009). Therefore, these experiments indicated that Akt phosphorylation of Skp2 promotes its cytoplasmic localization with the help of 14-3-3 protein. Once in the cytoplasm, Skp2 may then bind to FOXO proteins and target them for degradation.

Although the FOXO ubiquitination discussed so far typically leads to its degradation, FOXOs are also regulated by mono-ubiquitination. For example, FOXO4 was shown to undergo monoubiquitination as a result of oxidative stress. FOXO4 and Ub were shown to colocalize in response to the same treatment in a fibroblast cell line (van der Horst et al. 2006). Mono-ubiquitination was also shown to increase the nuclear localization of FOXO4, and when removed (by overexpressing de-ubiquitinating enzyme USP7/HAUSP), repress its transcriptional activity. Protein monoubiquitination has been found to induce receptor internalization and degradation, DNA repair by stimulating interaction of PNCA with damage tolerant polymerases through a yet-unknown mechanism in yeast, viral budding, and even nuclear export (Hicke 2001, Sun and Chen 2004). It has been speculated that FOXO monoubiquitination possibly impedes 14-3-3’s interaction allowing for FOXOs nuclear localization and transcriptional activity (van der Horst and Burgering 2007). However, more study is needed to further understand the mechanism by which monoubiquitination controls FOXO activity. Hence, it appears that although FOXO activity is enhanced by mono-ubiquitination, but its mechanism requires further study (van der Horst et al. 2006).
In summary, translational modification of FOXO is a means of regulating its activity. FOXOs have been shown to be phosphorylated by Akt, ERK, and IKK, and phosphorylation by these kinases leads of functional inactivation through cytoplasmic localization and its subsequent degradation. In contrast, phosphorylation by MST1 has been shown to promote FOXO activity by inhibiting its interaction with 14-3-3. Although the E3 ubiquitin ligases that mediate Akt and ERK dependent degradation as a result of polyubiquitination have been described, that for IKK has not. Evidence also shows that mono-ubiquitination of FOXO enhances its activity.

1.6.2 Akt, ERK, and IKK pathways are frequently disrupted in cancer

Elements in the Akt, ERK, and IKK pathways are frequently disrupted in cancer, and a brief discussion of each individual pathway and relevancy to NSCLC now follows.

Akt is regulated by the phosphatidylinositol-3-kinase (PI3K) pathway. PI3K was first identified as a kinase by Lewis Cantley who found polyoma T antigen to associate with certain elements in the membrane in order for it to have its oncogenic effect. Using immunoprecipitates made with polyoma T antisera, these precipitates were found to have a strong ability to phosphorylate phosphatidylinositols (PIs) \textit{in vitro}. These proteins were subsequently named phosphatidylinositol kinases (Whitman et al. 1985).

PI3K is a kinase responsible for phosphorylating the hydroxyl group of phosphatidylinositol (PI), which are membrane bound. One of these inositols is called PIP\textsubscript{2}. 
When phosphorylated by PI3K, PIP\textsubscript{2} is converted to PIP\textsubscript{3}. Through its PH (pleckstrin homology) domain, PIP\textsubscript{3} is able to attract a number of downstream kinases including Akt. Once docked with PIP\textsubscript{3}, Akt is then doubly-phosphorylated by PDK1 and PDK2 (phosphoinositide-dependent kinase). The three major biological effects that active Akt has on the cell are 1) repressing apoptosis, 2) stimulating cellular proliferation, and 3) stimulating cell size growth. Apoptosis is repressed through inhibition of BAD, caspase 9, MDM2, and FOXOs. These proteins are inactivated by Akt phosphorylation. Activation of GSK-3B (glycogen synthase kinase 3B) activation by Akt is proliferative. Growth is stimulated through activation of the mTOR (mammalian target of rapamycin) kinase, which promotes protein translation by inhibiting 4E-RP (translation inhibition factor 4E binding protein) (Brazil and Hemmings 2001, Vivanco and Sawyers 2002).

PI3K is activated by various growth factor receptors such as EGFR. It is also activated by other signaling mitogens such as interleukin-3 (IL3), and by extracellular attachment of integrins. In the case of EGFR, EGFR receptors undergo homodimerization when bound to ligands such as EGF or TGF-β. Once this occurs, EGFR dimerization stimulates an intrinsic intracellular protein-tyrosine kinase activity and autophosphorylation c-terminal tyrosines. Phosphorylated EGFR attracts the binding of the p85 subunit of PI3K either directly or with the help of IRS as an adaptor. Binding of p85 then stimulates binding of the p110 subunit and this forms the complete PI3K heterodimer (Markman et al. 2010). It should be noted that RAS has also been associated with PI3K activity by allowing its close proximity to the plasma membrane (where PIs are locate) when it binds RAS. RAS’ ability to regulate PI3K was demonstrated by Rodriguez-Viciana et al. who showed RAS interaction with PI3K (through immunoprecipitation).
PI3K activity was calculated by measuring the amount of free PIP₃ (using western analysis) in cells infected with wild type or constitutively inactive RAS. Rodrigues-Viciana et al. found that PIP₃ levels were decreased 2-3 fold when cells expressed inactive RAS protein (Rodriguez-Viciana et al. 1994). Therefore, evidence indicated that PI3K activity may be directly or indirectly (by attracting PI3K to the cellular membrane) upregulated by RAS. On the other hand, the PI3K/Akt pathway is also under tight control by several phosphatases. One of these is PTEN which removes the 3’ phosphate group on PIP₃ that has been previously attached by PI3K. As such, PIP₃ is unable to form and Akt’s docking site becomes unavailable (Brazil and Hemmings 2001, Vivanco and Sawyers 2002).

PI3K/Akt hyperactivity is common in many forms of cancer. PI3K is hyperactive in almost one third of human colorectal cancers (caused by mutation in its regulatory subunit p110). Inactivation of PTEN also frequently occurs, such as in glioblastoma (25-50%) and prostate cancers (40-50%). Finally, Akt protein levels are elevated in several cancer types including ovarian and breast carcinomas (Brazil and Hemmings 2001, Cheng et al. 1992, Vivanco and Sawyers 2002).

The PI3K/AKT pathway is constitutively active in LAC due to mutation or elevated expression of EGFR. PTEN does not appear to be a frequent target of inactivation (some studies have shown mRNA levels to be reduced in only 8% of lung cancer samples analyzed), but Akt has been shown to be present in constitutively phosphorylated active form in 94% of NSCLC cell lines under low serum conditions (Brognard et al. 2001, Forgacs et al. 1998).
ERK (extracellular signal-regulating kinase) is regulated by the RAS kinase pathway, which lies downstream of EGFR signaling. Briefly, when active EGFR recruits docking proteins GRB2 and SOS (which bind through their SH2 and SH3 domains respectively), SOS then removes GDP from the inactive GDP-bound RAS. RAS is then able to bind GTP, which renders the protein functionally active. Mutations of RAS at codon 12 and 61 are common in cancer; they maintain RAS in a GTP bound active state. Active RAS activates RAF by directly involving RAS’ effector loop. Activated RAF phosphorylates MEK, and this activated MEK phosphorylates ERK. This signaling cascade has been given the generic name MAPK (mitogen-activated protein kinase) pathway, and is one of many pathways activated by GTP-bound RAS. When active, ERK has been shown to regulate various cellular processes such as transcription, and as we have discussed, FOXO cellular localization. ERK activates the c-FOS transcription factor which stimulates proliferation by associating with c-JUN and forming the activating protein complex AP-1. ERK has been shown to activate the MYC transcription regulator and oncoprotein, which is a potent stimulator of cellular proliferation (Blume-Jensen and Hunter 2001, Hazzalin and Mahadevan 2002, Johnson et al. 2001, Malumbres and Barbacid 2003).

Disruption of the RAS/RAF/ERK pathway occurs frequently in various types of cancer. KRAS activation has been documented in various types of cancer such as pancreas (75-90%) and colon (~50%), and in 43% of LAC of smokers (Shigematsu and Gazdar 2006). B-RAF activation (by mutation in exons 11 and 15) has been found in 66% of melanomas, and ERK activation in 75% of primary ovarian cancers (Brose et al. 2002, Ventura et al. 2010). As mentioned, activation of EGFR in LAC is another mechanism by which this pathway is activated in LAC. RAS/ERK and PI3K/Akt pathways lie downstream of EGFR. EGFR in a membrane protein kinase
which has been shown to be overexpressed or mutated in 40-50% of NSCLC. This implicates both pathways and their downstream elements as contributors to lung cancer development (Bencardino et al. 2007, Ding et al. 2008).

IkB kinase (IKK) is the third negative regulator of FOXOs discussed. IKK functions as an inhibitor of IkB in the NFkB pathway. Originally identified as a protein that binds to the κB site of the immunoglobulin enhancer, NFκB was later found to be a heterodimeric transcription factor composed of a p65 and p50 subunit. Interestingly, NFκB’s p50 subunit is a member of the REL (reticuloendotheliosis) family of proteins, which is an oncoprotein of the REV-T retrovirus. This was elucidated in 1990, when Ghosh et al. cloned and sequenced the p50 subunit of NFκB. To do this, Ghosh et al. developed PCR primers elucidated from the protein sequence of the p50 subunit. Interestingly, it was uncovered that the mRNA transcript of the p50 subunit is twice as large as expected, and appears to be processed down to a 50 kd size. Analysis of the sequence revealed 45% sequence homology to V-REL/C-REL oncoproteins (Ghosh et al. 1990, Gilmore 2006).

IkB binds NFκB in the cytoplasm, which inhibits its function. In response to activating signals, IKK phosphorylates IkB, which causes its degradation. This allows the unbound NFκB to carry out its function as a transcription factor. Once in the nucleus, NFκB can activate the expression of anti-apoptotic genes Bcl-2, and IAP, but also proliferative factors such as MYC and Cyclin-D. NFκB also turns on the expression of its own repressor IkB, which binds NFκB and creates a negative auto feedback loop (Gilmore 1990, Gilmore 2006).
IKK is activated by several signals, including tumor necrosis factor α and IL1β (inflammatory response factors), lipopolysacharides (a sign of bacterial infection) and reactive oxygen species. When active, IKK phosphorylates two serine residues on IκB, which signals its degradation by 26S proteasome (Karin and Ben-Neriah 2000, Weinberg 2006).

The NFκB pathway has been linked to many forms of cancer, including breast cancer and colon cancer. Immunohistochemistry and western analysis of breast tumors and adjacent tissue found increased expression level of the p50 NFκB subunit in tumors. RT-PCR analysis of these tumor samples also showed increased expression levels of both NFκB subunits and known NFκB effectors Bcl-2, and Cyclin-D1. In addition, one immunohistochemical study of 45 colon cancer samples showed increased expression levels of phosphorylated IκB (which causes its degradation) in all samples analyzed (Cogswell et al. 2000, Vandoros et al. 2006).

Studies have shown that expression of NFκB and phosphorylated IκB correlated with a reduced survival rate of more than 50% among NSCLC patients. Increased activity of NFκB has also been shown in NSCLC, as determined by immunohistochemistry. In this study, a comparison of 88 stage I and II NSCLC tumor samples and adjacent tissues showed nuclear staining of NFκB in 46.6% of tumor samples compare to 26% of normal adjacent tissue (Jin et al. 2008).

In summary, Akt, ERK, and IKK are part of complex signaling pathways that influence apoptosis, survival, and cell cycle. These pathways are upregulated in various cancer types. All 3 have been shown to be upregulated in NSCLC FOXOs are also part of the Akt, ERK, and IKK signaling pathways, and are negatively regulated by these proteins. FOXO protein activation by
stress is able to override negative regulation by Akt, ERK and IKK. FOXO inactivation in tumor cells may therefore facilitate the activity of these pathways and contribute to cancer development (Figure 1.16).
Figure 1.16. Basic mechanism of FOXO proteins. FOXOs are known to be phosphorylated by Akt, ERK, or IKK. When activated, FOXOs are known to regulate apoptotic/cell-cycle proteins such as FASL, BIM, and p27Kip1. Once phosphorylated, FOXOs then localize to the cytoplasm where their activity is suppressed. Adapted from Yang and Hung 2009.
1.6.3 Functions of FOXO proteins

FOXO proteins are transcriptionally activated by a variety of physiologic stresses, suggesting complex physiological roles. In general, activation of FOXOs has been shown to override the negative regulation imposed by Akt, IKK and ERK during mitogenic stimulation. Compared to the mechanisms known to inhibit FOXO function, surprisingly much less has been elucidated over the past decade since the initial discovery of FOXO1 with regard to the post-translational modifications that override this inhibition and activate FOXO transcriptional activity. Here we discuss the different stresses that have been shown to activate FOXOs, and the cellular response to these stresses.

1.6.3.1 Stimuli that activate FOXOs

FOXOs have been shown to be activated by various stimuli, including oxidative stress, nutrient deprivation, irradiation/DNA damage, and cytotoxic chemotherapies. A discussion of these findings now follows.

FOXOs have been shown to be activated in response to elevated reactive oxygen species. In response to this stress, FOXO activity has been linked with increased cellular protection against reactive oxygen species. Kops et al. demonstrated this in D23 colon carcinoma cell lines that expressed a constitutively active (due to mutation of Akt phosphorylation sites) form of FOXO3a upon treatment with hydrogen peroxide. Cells expressing this form of FOXO3a showed a decreased amount of oxidative stress (Kops et al.)
Also, cells expressing active FOXO3a had increased mRNA levels of MnSOD and increased survival (as measured by MTS assay) (Kops et al. 2002). MnSOD is a member of the manganese superoxide dismutase family that binds manganese and catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Superoxide is a reactive oxygen species (ROS) that causes oxidative damage to macromolecules and membranes, and causes DNA damage (Fridovich 1995). Therefore, with their findings Kops et al. demonstrated that FOXOs can play a role in oxidative damage response.

Nutrient and growth factor deprivation has also been shown to activate FOXOs, resulting in apoptosis. This was initially shown by Brunet et al. who monitored cellular localization of FOXO3a under serum starved conditions (no FCS for 20 hours). Immunohistochemistry experiments showed that FOXO3a was localized to the nucleus in ~90% of serum starved fibroblasts analyzed, while nearly 0% of cells had nuclear FOXO3a localization when incubated with 10% FCS (Brunet et al. 1999). Treatment with IGF and EGF similarly decreased FOXO3a presence in the nucleus. The effect of serum and growth factors on FOXO3a localization was found to be Akt-dependent. FOXO3a transcriptional activity was also shown to be inhibited by serum and growth factors as a decrease in FOXO3a-mediated luciferase reporter activity also was observed in the presence of these growth stimuli. Furthermore, cotransfection of a constitutively activated Akt and the reporter vector showed significant reduction of luciferase activity (Brunet et al. 1999). These experiments provided evidence for the hypothesis that FOXOs are able to induce expression of effectors under conditions of serum and growth factor withdrawal starvation. This effect was likely the consequence of Akt functional downregulation in the absence of growth stimulation.
FOXOs have also been shown to stimulate apoptosis in response to ionizing radiation in a study in osteosarcoma cell lines exposed to ionizing radiation. Again FOXO3a was shown to localize to the nucleus upon treatment. In addition, FOXO3a was shown to increase activity in a luciferase reporter assay when cotransfected with a construct containing the FASL promoter region (an established FOXO3a effector gene). Finally, increased FOXO3a levels and pro-apoptotic BIM protein (FOXO3a effector), and cellular apoptosis was evident in response to exposure to ionizing radiation (Yang et al. 2006).

FOXOs also have been implicated in DNA repair. In these experiments rat fibroblasts were transfected with a constitutively active FOXO3a construct showed increased DNA repair, and Growth Arrest and DNA Damage 45 A (GADD45A) mRNA levels (quantitated using qPCR) and protein levels following UV irradiation (Tran et al. 2002). This study demonstrated that GADD45A is an effector of FOXO3a (by luciferace reporter assay) activated by UV-induced DNA damage. GADD45A is a stress sensor protein that modulates the response to DNA damage including repair. Increased signal levels were obtained in a luciferase assay when cells were cotransfected with a constitutively active FOXO3a gene and construct with the intact GADD45A promoter. When cells were cotransfected with a FOXO3a gene that contained mutated DNA-binding sites, luciferase signal was almost undetectable (Tran et al. 2002). Also, GADD45A constructs that contained mutated FOXO3a binding sites were unable to activate the luciferase reporter (Tran et al. 2002).

Tran et al. also measured DNA repair by a host cell reactivation assay, which measures the amount of luciferase expression from a vector damaged by UV irradiation. Luciferase
expression in these vectors is only possible if the vector is repaired. With this assay, Tran et al. demonstrated that DNA repair and luciferase expression was elevated when cells were cotransfected with a constitutively active mutant of FOXO3a and repressed when cells expressed a FOXO3a construct with deleted DNA binding domain (Tran et al. 2002). These results showed that FOXO3a is directly able to upregulate GADD45A expression (a protein involved in DNA repair) in response to UV irradiation, which causes DNA damage. FOXO3a also stimulated DNA repair. Together these results suggest that a mechanism by which FOXO3a elicits DNA repair is through GADD45A upregulation.

FOXOs are also activated by different chemotherapeutic stresses. In one study, paclitaxel induced FOXO3a-mediated apoptosis in MCF-7 breast cancer cells. This response was also found to be BIM dependent as the response was lost upon RNAi knockdown of BIM (Sunters et al. 2003). In colon cancer cells, FOXO3a was shown to localize to the nucleus in response to cisplatin treatment (Fernandez de Mattos et al. 2008). In addition, MTS assays and FACS analysis showed reduced cell number and increased apoptosis when treated with cisplatin. When FOXO3a levels were knocked down using siRNA, cell viability was almost at the level of untreated control cells and apoptotic cells were reduced from 37% to 10% (Fernandez de Mattos et al. 2008) These results suggest that FOXOs are activated in response to specific chemotherapeutic stresses (microtubule inhibition and DNA damage) and confer a pro-therapeutic response in the form of cell cycle arrest and apoptosis.

In summary, studies have shown that FOXOs are activated by a broad range of cellular stress stimuli. These stimuli include oxidative damage, nutrient deprivation, ionizing radiation,
DNA damage, and chemotherapeutic drugs. In response to these stresses, FOXOs have been shown to regulate genes involved in oxidative damage response, apoptosis, DNA repair, and cell cycle arrest.

1.6.3.2 Cellular functions of FOXO proteins

FOXOs have been shown to upregulate Manganese dependent superoxide dismutase (MnSOD) expression as an apparent measure to detoxify ROS and protect quiescent cells from ROS-induced apoptosis (Kops et al. 2002). MnSOD detoxifies superoxide ROS species. Luciferase assay of the wild-type MnSOD promoter and that with the putative FHBE (RYAAAYA) mutated indicated FOXO3a responiveness in cells expressing a 4-hydroxy-tamoxifen (4-OHT) inducible FOXO3a. Chromatin immunoprecipitation (ChIP) of FOXO3a at the FHBE confirmed binding by FOXO3a. FOXO3a transfected MnSOD-deficient MEFs were sensitive to ROS-induced apoptosis under conditions of quiescence (growth factor withdrawal) compared to wild-type MEFs (Kops et al. 2002). MnSOD upregulation by FOXO3a therefore was shown to confer protection against ROS induced apoptosis during quiescence. Previous studies had shown that Akt conferred protection against ROS-induced apoptosis in proliferating cells. This subsequent study showed that FOXO3a elicited protection through MnSOD upregulation in the absence of Akt stimulation with growth factors.

In addition, further study has indicated that FOXO3a upregulates a battery of genes with roles in ROS detoxification through apparent direct binding with peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) (Olmos et al. 2009). These include: MnSOD,
catalase, thioredoxin, thioredoxin reductase 2, peroxiredoxin 3, peroxiredoxin 5, glutaredoxin 2a, and glutathione peroxidase 4. All of these genes were shown to have functional FHBEs by ChIP analysis. However, their expression was diminished in PGC-1α-deficient cells, as well as in FOXO3a-deficient cells. Co-immunoprecipitation demonstrated binding of FOXO3a with PGC-1α. Transfection with either gene enhanced luciferase expression in the construct with the wild-type MnSOD promoter with an intact FHBE (Olmos et al. 2009).

As mentioned, FOXO3a has been shown to upregulate the expression of DNA damage repair protein GADD45. This has been demonstrated in response to UV damage in colon cancer cell lines (Tran et al. 2002) and also in MEFs and HeLa cells in response to hydrogen peroxide (oxidative stress) (Furukawa-Hibi et al. 2002). There is also some evidence linking the NER protein, DNA-damage binding protein 1 (DDB1), to FOXO-mediated DNA damage repair. In this study, DDB1 levels and DNA repair were shown to decrease when FOXO3a was inactivated by Akt (Chen et al. 2008). In this study Akt was activated by latent membrane protein 1 (LMP1), which is an Epstein-Barr virus (EBV) oncoprotein that mimics a constitutively activated tumor necrosis factor receptor and activates various signaling pathways, including PI3K/Akt. Chen et al. demonstrated that LMP1 expression caused inhibition of FOXO3a by Akt phosphorylation. This was demonstrated by western blot and confocal microscopy immunofluorescence showing cytoplasmic localization of FOXO3a upon LMP1 transfection. In addition, DDB1 protein levels were shown to decrease in correlation with FOXO3a inactivation (Chen et al. 2008). Furthermore, Chen et al. measured DNA repair by a host cell reactivation assay, as already described. Using this assay, Chen et al. demonstrated that DNA repair was repressed when cells expressed LMP1, but rescued when cells expressed a constitutively active form of FOXO3a.
Repair was also partially restored when LMP1 expressing cells were transfected with DDB1. Although this study did not directly demonstrate that DDB1 is regulated by FOXO3a, it showed that FOXO3a and DDB1 downregulation correlated with defective NER. These studies further demonstrated that FOXO3a transcriptional activity can stimulate DNA repair.

FOXOs have also been shown to play a role in the negative regulation of the cell cycle. In one study, mouse B-cells transfected with a constitutively active FOXO3a were shown to arrest in G1 with elevated p27\textsuperscript{Kip1} levels. Luciferase assay demonstrated FOXO3a activation of the p27\textsuperscript{Kip1} promoter (Dijkers et al. 2000). In addition, FOXO3a’s ability to induce G1 arrest was diminished in p27\textsuperscript{Kip1} deficient fibroblasts (Kops et al. 2002). FOXO’s role in cell cycle arrest was further established when mouse fibroblasts and human colon cancer cells showed decreased Cyclin-D1/Cyclin-D2 mRNA and protein levels as a consequence of FOXO3a or FOXO4 transfection. Also, analysis of FOXO showed decreased levels of phosphorylated RB and had a significantly reduced replication rate (analyzed by BRDU incorporation and colony formation) (Schmidt et al. 2002). These studies therefore suggest that FOXOs are able to induce cell cycle arrest through the regulation of known cell cycle regulators p27\textsuperscript{Kip1} and Cyclin-D1 and Cyclin-D2.

However, FOXOs have also been shown to directly upregulate Cyclin-G2 expression and bind to FHBE in its promoter. Cyclin G2 blocks cell cycle entry by inducing G0. FOXOs upregulated Cyclin-G2 mRNA in NIH-3T3 cells following transfection with active forms of FOXO3a or FOXO4 (Martinez-Gak et al. 2004). Electrophoretic mobility shift assays demonstrated FOXOs ability to bind the FHBE sequence present in the Cyclin G2 promoter.
Also, microarray experiments of transfected FOXO3a cells showed increased levels of Cyclin-G2 expression (Tran et al. 2002).

While FOXOs have typically shown to induce cell cycle arrest, it has also been demonstrated that FOXO3a and FOXO4 are able to regulate Cyclin-B and Polo-like kinase (Plk) and control completion of mitosis (transition from M to G1) (Alvarez et al. 2001). Alvarez et al. showed that when transfected with a constitutively active form of p110 (PI3K), mouse MEFs accumulated in G2 and completed mitosis more slowly. Cells also accumulated in G2 when inactivated FOXO3a was expressed in these cells, but had a normal cell cycle distribution when expressing a constitutively active FOXO3a. Cyclin B is required for entry into mitosis and its degradation which is enhanced by Plk1 is required for mitotic exit. Both proteins were increased in cells transfected with constitutively active FOXO3a or FOXO4, but were decreased in cells expressing transcriptionally inactive forms or with PI3K. FHBEs were found to be present in Cyclin-B and Plk promoters and luciferase assays, and ChIP confirmed FOXO activation of these sites (Alvarez et al. 2001). These studies suggested that FOXOs could impede PI3K/Akt stimulated transition through G1, and promoted execution of mitosis and cytokinesis. The effects of FOXOs on cell cycle were demonstrated in the absence of stress and showed their ability to counter PI3K/Akt stimulated events. Studies have demonstrated FOXOs’ ability to stimulate apoptosis in response to various stresses through their upregulation of different pro-apoptotic genes (e.g. BIM, FASL, BNIP3 and TRAIL) (Greer and Brunet 2005). As mentioned previously, Brunet et al. demonstrated a FASL-dependent induction of apoptosis by FOXO3a in response to starvation conditions in human embryonic kidney cells (Brunet et al. 1999). FASL binds death receptor FADD, which triggers extrinsic pathway apoptosis via caspase 8 activation.
FOXO3a was shown to directly upregulate the FASL promoter by luciferase reporter assay. Furthermore, Brunet et al. showed an increase in apoptosis in cells when transfecting with a FOXO3a triple mutant (constitutively active) and a decrease when transfected with a functionally inactive form of FASL (Brunet et al. 1999). Wild-type FOXO3a did not increase apoptosis in these experiments indicating that apoptosis mediated by FOXO3a in these cells required bypass of Akt and activation of FASL.

FOXO-mediated apoptosis correlating with BIM upregulation was first demonstrated by Dijkers et al. who demonstrated FOXO3a-mediated apoptosis after cytokine withdrawal. Expression of FOXO3a mutant (containing mutated Akt-phosphorylation sites) was sufficient to increase apoptosis and BIM expression (Dijkers et al. 2000). As mentioned, RNAi against BIM was shown to block FOXO3a-mediated apoptosis in response to paclitaxel in breast cancer cells (Sunters et al. 2003). Similarly, constitutively active FOXO3a was sufficient to increase apoptosis and TRAIL mRNA in colon cancer cells (Modur et al. 2002). TRAIL was also shown to be a target gene of FOXO3a (through luciferase assays) in these experiments (Modur et al. 2002). Finally, microarray studies of rat fibroblast cells transfected with a constitutively active form of FOXO3a showed an increase in BNIP3 (1.4 fold) and GADD45 (2.1 fold) mRNA levels (Tran et al. 2002). Therefore, studies have shown that FOXOs are able to induce apoptosis through direct upregulation of a variety of pro-apoptotic gene, including FASL, BIM, and TRAIL. All of the genes cited here possess active FHBEs (Greer and Brunet 2005).

Studies have demonstrated that FOXO acetylation status controls the balance of FOXO function towards either stress response and survival or apoptosis. This has been shown to occur
through direct acetylation by CBP, p300, and PCA in *C. elegans*. In this study, CBP-mediated acetylation of FOXO4 was detected at three of its lysine residues through western analysis. Mutation of these lysine residues enhanced FOXO4 transcription of p27^Kip1 expression (Fukuoka et al. 2003). The Sirt1 deacetylase has been shown to deacetylate these analogous sites, which in FOXO1 promoted the expression of p27^Kip1 and MnSOD (Daitoku et al. 2004). Under stress condition such as nutrient deprivation and oxidative stress, SIRT1 activation was shown to counteract CBP acetylation of FOXO1, 3a, and 4. This resulted in an attenuation of apoptosis, and increased cell cycle arrest (Brunet et al. 2004, Greer and Brunet 2005). It has been proposed therefore that since the FOXO acetylation sites are located in the DBD, that acetylation/deacetylation is a means of altering FOXO/DNA interaction and modifying transcriptional activity (Greer and Brunet 2005).

Sirt2 expression in yeast, worms and flies has been shown to extend longevity. In mammalian cells, FOXOs and SIRT1 have been shown to interact in response to oxidative stress, as has been demonstrated in immunoprecipitation experiments of FOXO3a transfected kidney cells treated with hydrogen peroxide (Brunet et al. 2004). In these experiments, FOXO3a was shown to be deacetylated by SIRT1 in response to hydrogen peroxide (but not UV damage or heat shock). Furthermore, FOXO3a/SIRT1 activation correlated with an increase in G1 cell cycle arrest and decreased apoptosis (Brunet et al. 2004). This has led to speculation that SIRT1 deacetylation tips the balance of FOXO functions away from cell death and towards stress resistance (Greer and Brunet 2005).
FOXOs have also been implicated in cellular differentiation. This was demonstrated by Nakae et al. who investigated FOXO1’s activity during adipocyte differentiation, which occurs over a 10-day period in vitro where pre-adipocytes undergo morphological changes (Nakae et al. 2003). This study found that FOXO1 was highly phosphorylated at early stages of differentiation, and that adenoviral infection of pre-adipocytes with an active mutant of FOXO1 inhibited the differentiation process (until removal of the adenovirus) and stimulated the expression of cell cycle inhibitors p27^{Kip1} and p21^{Cip1} (Nakae et al. 2003). Hence, inactivation of FOXO1 promoted differentiation in these cells. In contrast, FOXOs have also been shown to stimulate differentiation in erythropoiesis (Bakker et al. 2004). This was supported by findings demonstrating that FOXO3a directly upregulated the B cell translocation gene (BTG1). BTG1 is a negative regulator of the cell proliferation and highly expressed during erythrocyte differentiation. Cell differentiation, as determined through cellular morphology and hemoglobin content, was assessed in cells expressing either inactive or constitutively active FOXO3a. Whereas expression of active FOXO3a was shown to stimulate differentiation, expression of inactive FOXO3a suppressed it. Also, cells expressing active FOXO3a expressed BTG1 mRNA at much higher levels than cells expressing inactive FOXO3a (Bakker et al. 2004). Finally, FOXO3a binding sites were detected in the BTG1 promoter region and FOXO3a regulation of BTG1 was confirmed in a luciferase assay. Therefore, there are contrasting roles for FOXOs in differentiation. While FOXO1 seems to inhibit differentiation as was determined in adipocytes, FOXO3a is able to stimulate cellular differentiation in erythrocytes.

Finally, FOXOs have been linked with increased longevity in *C. elegans*. Expression of daf-16 (FOXO ortholog) in these organisms promoted a developmentally arrested dauer state.
(Ogg et al. 1997). This normally occurs under conditions of nutrient unavailability. Mutants of the daf-2 gene were found to live twice as long wild-type worms. This effect was suppressed by null-mutants of the daf-16 gene. Daf-16 was later found to transduce insulin-like metabolic and longevity signals from daf-2 (Ogg et al. 1997). Daf-16 therefore was found to promote a longevity phenotype in worms.

Daf-16/FOXO protein’s ability to regulate stress response and in particular to protect against stresses such as ROS and nutrient deprivation is thought to be another mechanism promoting longevity. This hypothesis is supported by studies that show the longevity gene daf-16 is activated (by nuclear localization) in *C. elegans* in response to nutrient deprivation, oxidative stress, heat shock, and UV damage (Henderson and Johnson 2001). These stresses lead to SIRT1 modulation of FOXO in mammalian cells in favor of cell survival, suggesting a resistant consistent with longevity.

The potential role of FOXOs in human longevity also has been investigated. These studies involved genotyping of candidate longevity genes (chosen for their relation to insulin signaling pathways) in 600 individuals, and found that genetic variations in FOXO3a to strongly correlate with a longevity phenotype (being a centenarian). Specifically, certain FOXO3a SNPs were strongly associated with longer lifespan (mean age of 98 years) in individuals of Japanese and German descent in both men and women (Flaschsbart et al. 2009, Willcox et al. 2008). These studies showed that certain FOXO3a SNPs would appear more frequently in long lived individuals (a frequency of ~0.35) than average aged individuals (81 years old: frequency of ~0.25). The individuals who carried these SNPs also had lower glucose and insulin levels, and a
lower incidence of disease (measured as prevalence of coronary heart disease, stroke, cancer, Parkinson’s disease, pulmonary disease, and type 2 diabetes in these subjects) (Willcox et al. 2008). Therefore, these studies implicated that FOXO3a may also play a role in human longevity, which is possibly due to its role in stress response and oxidative damage.

To summarize, FOXOs have been shown to respond to a variety of stresses including DNA damage, reactive oxygen species and under deprived growth conditions. When activated, FOXO proteins then accumulate in the nucleus and induce cellular programs as a response to these triggers. The responses range from apoptosis, such as transcriptional upregulation of BIM, BNIP3 and FASL, which encode specific pro-apoptotic proteins, oxidative stress detoxification (through MnSOD or catalase), or a temporal cell cycle arrest through p27\textsuperscript{Kip1} upregulation and Cyclin-D down regulation to name a few, or DNA repair involving GADD45 or DDB1. In addition, FOXOs have been implicated in cellular differentiation by regulating p21\textsuperscript{Cip1} and BTG1. Finally, a role for FOXOs in longevity has also been proposed due to its role in \textit{C. elegans} in dauer state formation and lifespan, and due to the association of certain FOXO3a SNPs with longevity in human. FOXO3a is believed to contribute to longevity through its role in cellular stress response.

1.6.4 \textbf{FOXOs are tumor suppressors}

The observations that FOXOs were able to elicit cell-cycle arrest and apoptosis suggested that FOXO proteins may act as tumor suppressors, based on evidence that such roles are typical of many known tumor suppressors (e.g. p53, p16\textsuperscript{INK4a}). For example, comparisons
have been made with the p53 tumor suppressor gene because p53, like FOXO proteins, has been shown to induce cell cycle arrest and apoptosis in response to cellular stress (Farnebo et al. 2010). As such, on a purely mechanistic evaluation, this similarity implicated FOXOs as potential tumor suppressors.

The research supporting this theory is present on many levels. For one, FOXO3a inactivation is associated with poor prognosis for breast cancer and liver cancer (Hu et al. 2004, Lu et al. 2009). In these studies, immunohistochemistry analysis that investigated FOXO3a localization in 131 breast cancer tumors and 91 liver cancer tumors found that FOXO3a was expressed in lower amounts or was localized to the cytoplasm more often in tumors than in normal tissue. Furthermore, a Kaplan-Meier overall survival curve of cancer patients showed that the group that predominantly expressed cytoplasmic FOXO3a had a lower survival rate than the group expressing nuclear FOXO3a (Hu et al. 2004, Lu et al. 2009). Also, prostate cancer cell survival and xenografts tumor volume in nude mice decreased when either active FOXO3a or FOXO1 was over-expressed in these cells. Furthermore, both FOXOs were shown to bind to the TRAIL promoter and stimulate its expression (shown with luciferase assays) (Modur et al. 2002). These studies suggested that FOXOs were able to induce apoptosis as a protective function against cancer development.

The evidence suggesting a correlation between disrupted FOXO expression and tumorigenesis is also substantial. For example, translocations of FOXOs have been observed in human cancer resulting in PAX3/7-FOXO1 and MLL-FOXO4 fusion proteins. Both of these
chimeras occurred in acute myeloid leukemia, suggesting that FOXO disruption might contribute to this cancer type (So and Cleary 2003).

As mentioned previously, Akt, ERK, and IKK are all known to be oncogenic proteins commonly upregulated in human cancers (Brunet et al 1999, Greer and Brunet 2005). Since these proteins are known negative regulators of FOXO activity, their upregulation suggests that FOXO downregulation contributes to tumorigenesis.

More recently, direct examination demonstrated FOXO’s role as a tumor suppressors in knockout mice. Foxo1, Foxo3, and Foxo4 conditional triple knockouts showed susceptibility to mainly lymphoma and hemangioma (similar to p53 knockout models) (Paik et al. 2007). However, this study showed only moderate susceptibility of Foxo3 and Foxo4 knockouts to tumorigenesis, and Foxo1 knock out alone conferred no increase in susceptibility to tumor formation. These results suggested that FOXOs may act redundantly in cancer resistance in mice.

Finally, the site of the FOXO3a gene on human chromosome 6q21 has been shown to be frequently affected by LOH in several cancer types. For example, array CGH studies have found allelic loss to frequently occur in 6q or regions overlapping 6q21 in ~60% of NCLC tumor cases (Balsara and Testa 2002). Allelic losses that overlap FOXO3a on 6q21 have also been reported in several other studies including B-cell lymphoma (25% of cases) and breast cancer samples (33% of cases), which implicates FOXO3a as a candidate tumor suppressor gene in a wide range of cancers (Bayani et al. 2002, Bea et al. 2005). A role for FOXO1 as a tumor suppressor has also been implicated based on similar findings in human cancer. Dong et al. demonstrated frequent
Allelic loss of 13q14 (where FOXO1 is located) in primary prostate cancer and cell lines. qPCR analysis in these samples showed that the allelic loss frequently affected the FOXO1 gene (34% signal reduction) compared to flanking markers (9% signal reduction in each) (Dong et al. 2006). Furthermore, an analysis of FOXO1 expression in prostate cancer cell lines and tumors showed decreased mRNA expression of FOXO1. Finally, transfection of FOXO1 in prostate cancer cell lines with reduced FOXO1 expression led to the growth of fewer colonies and decreased tumor cell survival (determined by clonogenic and MTS assays) (Dong et al. 2006). Allelic loss in the region of the FOXO1 gene has also been found in LAC (22% of samples in studies involving female smokers) and late stage ovarian cancer (14% of cases) (Partheen et al. 2004, Sy et al. 2003). On the other hand, FOXO4 and FOXO6 sites in the genome (Xq13.1 and 1p34.1 respectively) have so far not been reported to be frequent sites of allelic loss in any studies involving copy number changes in human cancer samples. In fact, both sites have instead been reported as frequent sites of amplification in human cancer (Balsara and Testa 2002, Paulson et al. 2009, Zielenska et al. 2001). Because allelic loss is a common mechanism of tumor suppressor gene inactivation in cancer, current findings implicate FOXO1 and FOXO3a as putative human tumor suppressors. However, the findings to date do not preclude such a role for the other FOXO family members.
In this literature review, we have stressed the impact of lung cancer on men and women worldwide, and we highlighted how the discovery of genetic changes has advanced our understanding of the different disease sub-types. A culmination of evidence has revealed that tumor development occurs through progressive or step-wise selection of genetic changes that are advantageous for tumorigenesis.

The majority of lung cancer patients are smokers, and tobacco smoke contains numerous carcinogens that have been causally linked to lung cancer. Exposure to these carcinogens has been shown to lead to the development of a distinct sub-type of LAC with a profile of molecular changes different from that of LAC that arise from non-smoking etiology. In this regard, a previous study from our lab demonstrated that FOXO3a deletions specifically occur in carcinogen-induced LAC (Herzog et al. 2009). In that study, LOH was found to occur on mouse chromosome 10 at a frequency of 48% in various genotoxic carcinogen induced tumors and in 23% of those occurring in the absence of carcinogenic induction (spontaneous tumors). Deletion mapping ultimately led to the identification of the FOXO3a as a focus of deletion on this chromosome. Specific analysis of this gene showed the loss of at least one allele in 51% of carcinogen-induced LAC versus 10% in the spontaneous LAC, indicating that this event was largely carcinogen induced.

Molecular studies have shown that mouse models of human cancer types accurately predict the genetic changes in the human tumors, including LAC (Herzog et al. 1997). We therefore hypothesized that FOXO3a loss would also be a frequent novel event in human LAC,
particularly of smokers, whose tumors are caused by exposure to high levels of genotoxic carcinogens. Our results, as presented in Chapter 2, support this hypothesis, showing that FOXO3a, and not other FOXO family members, is the frequent site of deletion in LAC of smokers. These losses were shown to occur in the earliest malignant stage of LAC.

FOXO3a encodes a transcription factor activated by various stresses on cellular homeostasis, including DNA damage. When activated, FOXO3a elicits apoptosis, cell cycle arrest, or DNA repair, with the particular response evidently being stress and cell type specific. These functions have implicated FOXO3a and the other FOXO family members as potential tumor suppressors, as analogous functions are typical of known tumor suppressors (e.g. p53). These functions also seemed particularly relevant in lung cancer. As we have described in this chapter, DNA damage induced by tobacco-derived carcinogens is cited as a major cause of lung cancer. PAHs (BaP) and TSAs (NNK) are well-characterized DNA damaging carcinogens in tobacco smoke. With this information, we surmised that FOXO3a may prevent the tumorigenic effects of DNA damage in suppressing LAC. We therefore hypothesized that FOXO3a would be functionally activated and elicit some protective, anti-carcinogenic response to tobacco carcinogens. In testing this hypothesis, we have shown that FOXO3a indeed activates a pro-apoptotic transcription program and cellular response to DNA damaging metabolites of NNK and BaP.

Cellular stress caused by various chemotherapeutic agents has also been shown to lead to FOXO3a functional activation and response suggesting that FOXO3a may contribute to the
therapeutic effect of these drugs. Microtubule inhibitors are used to treat LAC and other cancer types. In this class of drugs, paclitaxel, a taxane, was shown to induce FOXO3a-mediated apoptosis in breast cancer cells. We hypothesized that a similar response would be induced by taxanes and possibly vinca alkaloids in LAC cells. Taxanes and vinca alkaloids are two mechanistically distinct sub-classes of microtubule inhibitor/anti-mitotics. The rationale for this analysis was that the loss of FOXO3a in LAC may influence treatment response to these drugs if FOXO3a is functionally activated in response to their effects on the cell. Somewhat unexpectedly, we observed that although FOXO3a was indeed activated in cells treated with both drug types, it elicited a response distinct from that reported in breast cancer cells. Instead of eliciting apoptosis, FOXO3a was observed to mitigate the anti-mitotic effect of these drugs while stimulating cytostasis. Although this mechanism requires further elucidation our results indicate that this response includes the upregulation of CDC14A by FOXO3a. As discussed earlier, CDC14A has been shown to block entry into mitosis as well as to stimulate mitotic exit, which is what was observed in FOXO3a expressing cells exposed to the microtubule inhibitors. These results support our overall hypothesis that FOXO3a is a suppressor of LAC with a role in preventing carcinogen-induced tumorigenesis. The loss of such a function may also influence how well these tumors respond to treatment with microtubule inhibitors.

Our hypotheses were tested through the following Specific Aims:
HYPOTHESIS

Hypothesis 1: FOXO3a gene is deleted in lung adenocarcinoma (LAC) and possibly lung squamous cell carcinoma (LSqCC) of smokers.

Specific Aim 1.1: Determine whether and at what frequency the FOXO3a gene is inactivated by deletion or mutation in LAC and LSqCC.

Hypothesis 2: FOXO3a is activated by and elicits a protective cellular response to DNA damage caused by BPDE in LAC cells.

Specific Aim 2.1: Determine FOXO3a’s functional activity in response to DNA damage caused by BPDE in LAC cells.

Specific Aim 2.2: Determine whether FOXO3a induces a cytostatic or cytotoxic response to BPDE treatment in LAC cells.

Specific Aim 2.3: Determine the transcriptional response of FOXO3a to BPDE treatment in LAC cells.

Hypothesis 3: FOXO3a stimulates growth arrest or apoptosis in response to microtubule disruption in LAC cells.

Specific Aim 3.1: Determine FOXO3a’s functional activity in response to anti-mitotic agents in LAC cells.

Specific Aim 3.2: Determine whether FOXO3a reduces cell viability in response to treatment with mitotic agents in LAC cells.
Specific Aim 3.3: Determine whether FOXO3a stimulates a cytostatic or cytotoxic response to anti-mitotic agents in LAC cells.

Specific Aim 3.4: Determine the transcriptional response of FOXO3a to anti-mitotic agents in LAC cells.
CHAPTER 2: FOXO3a encodes a carcinogen-activated transcription factor frequently deleted in early-stage lung adenocarcinoma

ABSTRACT

The FOXO family of transcription factors induces cell cycle arrest and apoptosis in response to various physiologic and pathologic stresses relevant to sporadic cancer, such as DNA damage and oxidative stress. Although implicated as tumor suppressors, FOXO genetic inactivation has not been observed in human cancer. In an investigation of the two major types of non-small cell lung cancer (NSCLC) here we identify the FOXO3a gene (FOXO3a) as a novel target of deletion in human lung adenocarcinoma (LAC). Using RT-PCR, allelic loss of 80% or higher was detected in 8 out of 33 (24.4%) mostly-early stage lung adenocarcinoma (LAC) samples in smokers suggesting bi-allelic or homozygous deletion (HD) of FOXO3a. Another 60.6% of these tumors had losses of FOXO3a not reaching the level of HD we had set (gene dose loss >80%), and instead had 40-79% loss in gene dose level (hereafter referred to as sub-HD). In contrast, no HD of FOXO3a (gene dose loss >80%) was observed in any of the 19 lung squamous cell carcinoma (LSqCC) analyzed. Consistent with the deletion of FOXO3a were corresponding decreases in its mRNA and protein levels in LAC.

The potential role of FOXO3a loss in LAC was also investigated. The carcinogen (+)-anti-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) is strongly implicated as a cause of human lung cancer. Here we demonstrate that FOXO3a undergoes nuclear localization and augments the level of caspase-dependent apoptosis in cells exposed to this DNA-damaging
carcinogen. These results implicate FOXO3a as a suppressor of LAC carcinogenesis, a role potentially lost through gene deletion.
2.1 INTRODUCTION

Lung cancer exceeds all cancer types as the leading cause of cancer death world-wide (ACS 2008). Eighty seven percent of these cases are classified as non-small cell lung cancer (NSCLC), among which lung adenocarcinoma (LAC) and squamous cell carcinoma (LSqCC) are major histotypes (ACS 2008). The majority of lung cancer is causally linked to chronic exposure to air-borne carcinogens (Dipple 1995, Hecht 2003, IARC 2004). In particular, epidemiological and experimental studies together have established that carcinogens present in cigarette smoke account for ~85% of all human lung cancer (Dipple 1995, Hecht 2003, IARC 2004).

LAC and LSqCC develop through distinct pathogenetic pathways, and they respond differently to treatment (Sekido et al. 2003, Wistuba et al. 2002). Another level of complexity to the underlying biology of these tumors is that those of smokers and never-smokers also have some distinct molecular and clinical characteristics and as such can be viewed as separate diseases (Sun et al. 2007). For example, lung tumors in smokers are more commonly associated with KRAS activation, while those in non-smokers more frequently contain EGFR activation (Wistuba and Gazdar 2006). Also, compared to LAC, LSqCC tend to have higher incidences of p53 inactivation and allelic losses on chromosomes 13q14 (RB), 9p21 (p16/NK4a), 8p21-23, and several regions of 3p. In LAC, mutations of KRAS, Epidermal growth factor receptor (EGFR) and Her2/neu, and allelic losses on 6q and 19p are more typical (Ahrendt et al. 2001, Paez et al. 2004, Pao et al. 2005, Petersen et al. 1997, Virmani et al. 1998).

Comprehensive genome-wide studies have also revealed many unidentified genes that are recurrently altered in and likely to contribute in some way to the development of these
tumor types (Chitale et al. 2009, Goeze et al. 2002, Weir et al. 2007). The identification and characterization of these unknown participants should further our understanding of NSCLC and, ultimately, lead to improved therapies.

Functions that contribute to the inhibition or suppression of cancer are often selectively inactivated in tumors by gene deletion. This can occur as a consequence of any of a number of errors in chromosome maintenance, such as mitotic recombination, mitotic nondisjunction, chromosomal breakage, or rearrangement (Lengauer 1998, Loeb 1998, Macleod 2000, Weinberg 1995). Quantitative PCR (qPCR) is a validated tool to quantify the relative copy number of specific DNA sequences that result from these defects (Cairns et al. 1995, Herzog et al. 1994 Jung et al. 2000, Loda 1994). With the availability of highly dense databases of the annotated human and mouse genomes, PCR now essentially has single nucleotide resolution for detecting genetic targets of copy number changes. We recently demonstrated by qPCR that FOXO3a is deleted in carcinogen-induced LAC of mice and in human NSCLC cell lines (Blake et al. 2010, Herzog et al. 2009). This suggests that FOXO3a loss contributes to NSCLC pathogenesis.

The proposition that FOXO3a is capable of functioning as a ‘tumor suppressor’ is supported by several lines of evidence. FOXO3a is one of four related FOXO transcription factors that protect cells against a wide range of physiologic stresses (Tran et al. 2002). In particular, FOXO3a has been shown to play a role in DNA repair, growth arrest and apoptosis in response to DNA damage and oxidative stress (Furukawa-Hibi et al. 2002, Greer and Brunet 2005, Kops et al. 2002, Tran et al. 2002). We recently demonstrated that FOXO3a activates a
pro-apoptotic transcription program and cellular response to the human lung carcinogen NNK (Blake et al. 2010). FOXOs are negatively regulated at the protein level by the kinases Akt/ERK/IKK (Brunet et al. 1999, Greer and Brunet 2005, Kops et al. 2002, Nakamura et al. 2000, Yang et al. 2009). These kinases phosphorylate FOXOs on specific residues that promote 14-3-3 binding and result in its cytosolic retention and transcriptional inactivation (Brunet et al. 1999, Kops et al. 2002, Nakamura et al. 2000). Akt and ERK are activated by EGFR-PI3K signaling, a pathway frequently up-regulated in NSCLC (Sekido et al. 2003, Paez et al. 2004). Under certain stress conditions the negative control of FOXOs by Akt and ERK is overcome through both phosphorylation and acetylation of FOXOs, which results in their transcriptional activation (Daitoku and Fukamizu 2007, Greer and Brunet 2005). Finally, the tumor suppressor roles of FOXOs has been confirmed in mice where it was shown that germline inactivation of Foxo1, Foxo3a, and Foxo4 (Foxo6 was not examined) predisposed mice to lineage-specific tumorigenesis (Paik et al. 2007). Here we show that FOXO3a is selectively deleted in human LAC. We also demonstrate that FOXO3a is functionally activated by, and induces a pro-apoptotic response to BPDE, a human lung carcinogen. The findings of this study implicate FOXO3a as an anti-carcinogenic suppressor of LAC.

### 2.2 MATERIALS AND METHODS

#### 2.2.1 Tissues and Cell Lines

Human lung tissues were obtained from the tumor and tissue bank of the Penn State Cancer Institute (IRB protocols 24072EP and 29448EP). Tumor stage was assigned using standard TNM criteria. Normal lung tissue was obtained from the Sun Health Research...
Institute’s Brain and Body Donation Program for which the autopsy results showed normal lungs. Samples were excluded for any type of abnormality including gross and microscopic abnormalities such as bronchopneumonia, lung cancer, or metastatic cancer. The majority of the tumors, both LAC and LSqCC, examined in this study were early stage carcinoma (see Fig 1D). Genomic DNA was isolated from frozen patient samples using previously published methods (Herzog et al. 1994). H358, A549, H1299 cell lines were obtained from the American Type Culture Collection (ATCC). All cell lines were grown in RPMI medium supplement with 10% FBS, 2mM glutamine, 100μg/ml penicillin and 100 mg/ml streptomycin in a humidified incubator at 37°C, 20% O₂ and 5% CO₂.

2.2.2 Deletion Analysis

Gene deletions were determined by quantitative PCR (qPCR) of genomic DNA, as previously reported (Herzog et al. 1994, Herzog et al. 2009). PCR was carried out as follows: 95°C for 2 min followed by 22-25 cycles (i.e. within exponential phase) at 95°C, 55-60°C, and 72°C each for 30s. PCR products were resolved in 2% agarose gels stained with ethidium bromide. Deletions were determined by calculating the ratio of target intensity/control intensity in the tumors and cell lines normalized by that value obtained from normal DNA.

GAPDH and β-Actin were used as controls. Several primer pairs were used in the deletion analysis of FOXO3a and its flanking genes, ARMC2 and LACE1. GADPH and β-Actin were used as control. Primers for FOXO3a used in this study are listed in table 2.I. Additional primer pairs used include (5´-3´): ARMC2-1: forward, ATACACTCTGCGAACTGAAGGGGTGCT; reverse, TGCTGAGCTTTCTTTCTCCGAT; LACE1-1: forward, GTGTCAAAATTCAGCCAACAGGCA; reverse,
TGCTGCAAGGAGAGCAAGTGTCTA; β-ACTIN: forward, GCACCACACCTTCTACAATGAG; reverse, CTTTCATGAGGTAGTCAGTG; GAPDH: forward, GTATTGGGCGCCTGGTCACC; reverse, CAGTGGACTCCACGACGTAC; FOXO1: forward, TATGAACCGCCTGACCCAAGTGAA; reverse, TCGTTGTCTTGACACTGTGGGA; FOXO4: forward, AGTTCAAATGCCAGCAGTGTCAGC; reverse, TGCTGCAAAGAGAAGCCAGAGAGA. Primers used were designed based on the available gene sequences from NCBI and Ensembl genome browsers and purchased from Integrated DNA Technologies, Coralville, IA. Oligos were designed to avoid known single nucleotide polymorphisms that may affect gene dose quantitation.

Quantification was carried by densitometry using UVP Imaging and Analysis System and LabWorks software (UVP, Inc.). Gene dose was quantified as follows: test gene intensity/control gene intensity of tumor DNA divided by that of normal (non-cancerous) tissue DNA as previously described (Herzog et al. 2009). To account for genetic and cellular heterogeneity of the tumor samples gene HD was defined as a reproducible gene dose reduction of ≥80% relative to normal DNA. A gene reduction of 40-80% relative to control levels was considered a hemizygous or sub-clonal homozygous loss. For deletion scoring, a minimum of two contiguous, non-overlapping PCR products within FOXO3a must have met the threshold of HD or sub-HD. The approximate locations of the FOXO3a primers used are shown in Table 2.1 and Figure 2.1B.

2.2.3 **Mutation Analysis**

Examination of tumors and cell lines for mutations within the coding region of human FOXO3a (i.e. exons 2 and 3) was carried out by direct sequence analysis of PCR amplified
fragments. Sequencing was performed by the Genomics Core Facility of the Penn State University using an ABI Hitachi 3730XL DNA Analyzer using protocols provided by the manufacturer.

2.2.4 RNA extraction and cDNA synthesis

Total RNA was extracted from tissue samples using RNeasy Mini Kit (Qiagen) according to manufacturer’s protocol. Samples were subjected to on-column DNAse I digestion during extraction to prevent confounding of the results by genomic DNA contamination. RNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer, and RNA purity was assessed by absorbance ratios A260/A280 (> 1.9) and A260/A230 (>1.8). Reverse transcription was performed using Superscript First Strand cDNA synthesis kit (Invitrogen) with 1µg of starting RNA per sample. A negative control without RNA and a negative control without enzyme were analyzed in parallel.

2.2.5 Real-time quantitative reverse transcription-PCR

FOXO3a expression in lung tissue was assessed using Taqman gene expression assays (Applied Biosystems). Expression levels were normalized to GAPDH content. cDNAs were run in quadruplicate and amplified in a 10µl reaction containing 5µl 2X Taqman Universal PCR Master Mix, 0.5 ml 20x primer/probe mix, and 25ng of cDNA. Relative quantification of expression was calculated using the ΔΔCt method. Briefly, ΔCt was calculated as the Ct value of the target gene (FOXO3a) minus the Ct of the control gene (GAPDH). The ΔCt was then calculated as the ΔCt of
the sample minus the ΔCt of a calibrator sample, in this case the highest expressing adjacent normal tissue. Relative quantification was then determined with the formula $2^{(-\Delta\Delta C_t)}$. FOXO3a mRNA expression was also analyzed by normalization to three additional control genes, RPLP0, HPRT1, and PES1. These genes were found to be stably expressed in a subset of matched lung tumor and adjacent normal samples (data not shown).

For qPCR data, statistical analyses were done using GraphPad Prism version 5.00. Expression of FOXO3a in matched tumor and adjacent normal tissue was compared using a Wilcoxon signed rank test with significant $p$-value < 0.05. Nonparametric analysis of unpaired samples was analyzed using a Mann-Whitney test with significant $p$-value < 0.05.

2.2.6 Transfections and treatment of cells

Expression vector of FOXO3a cDNA was generated from RNA isolated from human buccal cells. Reverse transcription was carried out on 1μg total RNA using 200 units of MMLV reverse transcriptase for 1h at 37°C. High fidelity PCR amplification of full-length FOXO3a cDNA used Hi-Fi Platinum Taq DNA polymerase (Invitrogen). Amplified FOXO3a cDNA was cloned into the pCR3.1 mammalian expression vector (Invitrogen), and wild-type clones were confirmed by direct sequence analysis. Transfections of A549 and H358 cells were carried out using lipofectAMINE reagent (Invitrogen) with conditions optimized for 1μg of plasmid DNA in 35mm² dishes. 1 X 10^5 cells were seeded into 6 well plates (35mm²/well) for transfection. Cells were treated with BPDE (0.4-0.7μM) for 1h following transfection. Twenty-four hours following transfection, cells were plated in selective medium (containing G418). MTS assays were carried out according to manufacturer’s recommendations (Promega) to determine the extent of cell
death caused by these treatments. Cells treated with BPDE were also collected at the indicated time points for Western blot analysis, as described below. The pan-caspase inhibitor, Z-VAD-FMK (Sigma-Aldrich), was used at a concentration of 15µM. Treatment with the inhibitor commenced one hour prior to treatment with BPBE.

2.2.7 Reverse-transcription PCR

RNA was isolated using Trizol Reagent and was subjected to reverse transcription with MMLV reverse transcriptase. PCR was performed on 50-100ng of high quality cDNA to determine the expression levels of reported FOXO3a effector genes. This was carried out essentially as described above for deletion analysis. Quantitation of target genes was determined by calculating target PCR intensity/control PCR intensity in the test (treated) sample relative to that of the control (untreated) sample. GAPDH and BACTIN were used as control genes for test gene expression quantitation. Quantification was carried by densitometry using UVP Imaging and Analysis System and LabWorks software (UVP, Inc.). Oligonuclotide primers used in this study were designed based on the available gene sequences from NCBI and Ensembl genome browsers and purchased from Integrated DNA Technologies, Coralville, IA.

2.2.8 Immunocytochemistry

Formalin-fixed, paraffin-embedded human lung tissue was sectioned at 5µm and placed on glass slides. A low-temperature antigen retrieval procedure was applied to deparaffinized and rehydrated tissue sections using Antigen Unmasking Solution (Vector Laboratories) at low
pH for 1 hr at 80°C Immunocytochemistry was performed using rabbit monoclonal primary antibody anti-FOXO3A (Epitomics) diluted 1: 50 and incubated overnight at 4° C. The ImmPRESS™ polymerized reporter enzyme staining system for rabbit antibodies was used (Vector Laboratories). ImmPACT™ DAB (Vector Laboratories) was used as the chromagen followed by Vector® Methyl Green counterstain (Vector Laboratories) or a blush of Eosin Y/Phloxine (Sigma-Aldrich) in order to visualize cytoplasm. Endogenous peroxidase was inhibited by treating the sections with hydrogen peroxide. Negative control slides were immunostained in the absence of primary antibody. A set of slides matching those stained for FOXO3a were also stained with hematoxylin and eosin (Fig. 2.6).

2.2.9 Immunofluorescence

H1299 cells were grown on chamber slides in completed RPMI medium. At ~50% confluence the cells were synchronized with 1mM hydroxyurea in completed medium for 12h. Cells were washed 2X with culture medium and treated with 0.7μM BPDE in the same medium for 2, 4, and 8h. Cells were fixed with 4% paraformaldehyde for 10 min. on ice, washed and permeabilized with PBS/0.02% triton X-100, and then blocked with PBS/3% BSA. Primary FOXO3a (H-144) and 14-3-3 antibodies were applied to the cells at a concentration of 50 μg/ml and incubated overnight at 4°C. Cy5- and Cy3-conjugated and Cy5-secondary antibodies (Jackson ImmunoResearch Laboratories) were applied following washing at a concentration of 10 μg/ml and incubated at room temperature for 2 hrs. Nuclei were stained with Hoechst stain at a dilution of 1:10 for 1 hour at room temperature. Images were acquired with a Leica
confocal microscope (TCS SP2 AOBS, Leica Microsystems), using a 488 nm laser for the Cy2, a 543 nm laser for the Cy3, and 633 laser for the Cy5 fluorophores. The fluorophores were imaged using a sequential line scan, with detection bands set at 420-475 nm for Hoechst stain and 554-640 for Cy3. Each image was saved at a resolution of 1024 x 1024 pixel image size. The optical sections were reconstructed by maximum projection with the Leica software. This work was done in collaboration with the Microscopy Core Facility at the Penn State College of Medicine.

2.2.10 Western Blotting

Typically 25-40µg of whole cell lysates were denatured in 1X Laemmeli sample buffer, electrophoresed, and transferred onto nitrocellulose membranes. Membranes were blocked with either 5% milk or 5% bovine serum albumin (BSA) in TBS with 0.05% Tween 20 for 2hrs. Incubations with primary antibodies were for 2 hrs at 4°C, and with secondary antibodies for 45 min at room temperature. Antibodies were diluted in 5% milk or 5% BSA in TBS. Rabbit polyclonal anti-FOXO3a (H-144) was used at a dilution of 1:200 (Santa Cruz Biotechnology). Mouse monoclonal anti-β-actin antibody was used at a dilution of 1:3000 (Sigma-Aldrich). Antibodies used, their sources and concentrations were as follows: mouse monoclonal anti-caspase 7, anti-caspase 8, anti-caspase 9 were used at dilutions of 1:500 (Stressgen). Rabbit polyclonal anti-α-tubulin was used at a dilution of 1:500 (Santa Cruz). Secondary antibodies were conjugated with horseradish peroxidase and detected by chemiluminescence (Pierce).
2.3 RESULTS

2.3.1 FOXO3a is frequently deleted in human LAC

Homozygous deletion (allelic loss >80%) of FOXO3a was detected in 8 out of 33 (24.2%) LAC, but in none of the LSqCC examined (Fig. 2.1). Sub-HD (allelic loss between 40-80%) was detected in 20 out of 33 (60.6%) primary LAC and in 9 out of 19 (47.4%) LSqCC (Table 2.2). The use of several primers within FOXO3a enabled the identification of exon 2 as the primary site of deletion in the tumors examined (Fig. 2.1 C and D). Most deletions included FOXO3a primer sets 1-4, which span an area of 1238bp in exon 2 and extend into intron 2-3 (Fig. 2.1 B and D, Table 2.1). Primer sets 6 and 7, which encompass sets 1-3, were used to confirm deletions affecting exon 2 (data not shown). Of note, the qPCR analysis was of synonymous or homozygous DNA, which could identify loss of heterozygosity.

The nearest flanking genes of FOXO3a were also analyzed for deletion in order to define more specifically the focus of the observed deletions. Although sub-HDs affected these genes (ARMC2 and LACE1) at a high frequency, none of the HDs of FOXO3a extended into either gene (Fig. 2.1 D). Similar results are shown for the Sub-HD losses of FOXO3a in the LSqCC. These results identify FOXO3a as a focus or target deletion in these tumors.

The FOXO3a coding region (exons 2 and 3) also was examined by direct sequence analysis for intragenic mutations in several of the LAC and LSqCC. This alternative mechanism of gene inactivation was not detected in any of the tumors examined. Other mechanisms of gene inactivation in cancer were not addressed in this study. For example, it is possible FOXO3a to have undergone inactivation through promoter mutation/silencing by hypermethylation or
malfunctioning translational mechanism. These possibilities would not affect FOXO3a gene copy number and may be active in cases showing retention of FOXO3a alleles. We propose to conduct future studies addressing these alternative mechanisms of inactivation in our tumor samples.
**Figure 2.1.** FOXOα deletions in human NSCLC. A, Diagrammatic representation of the human FOXO3α locus. B, Relative locations of the FOXO3α sequences analyzed by qPCR. C, Representative qPCR analysis of LAC DNA demonstrating deletions within FOXO3α. D, Summary of qPCR data of FOXO3α and flanking genes. Values shown are gene doses in tumors relative to
normal DNA normalized with either GAPDH or βACTIN. Values are the means of several experiments, which resulted in minimal standard deviation. Black, HD; dark gray, sub-HD DNA loss in the range of 60-79% decrease; light gray, sub-HD DNA loss in the range of 40-59% decrease; white, no DNA loss.

Table 2.1. FOXO3a RT-PCR Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Length</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXO3a set 1</td>
<td>CGGCCAGAGGAGGAATGTT</td>
<td>TGGTGCTGTCGCCCTATGCTT</td>
<td>172 bp</td>
<td>n555–727 exon 2</td>
</tr>
<tr>
<td>FOXO3a set 2</td>
<td>AGATCTACGATGATGATGATGCTT</td>
<td>AAGCGGACTCCGACGAGATCCGAGA</td>
<td>169 bp</td>
<td>n668 exon 2–n86 introns 2–3</td>
</tr>
<tr>
<td>FOXO3a set 3</td>
<td>TCTCGATTGTCTGTTGAGTGCGCTT</td>
<td>TCCAGDAATGACTCTCTGTCCGTG</td>
<td>207 bp</td>
<td>n86–292 introns 2–3</td>
</tr>
<tr>
<td>FOXO3a set 4</td>
<td>TGGATTCCGGCTCTTTTTAGGAG</td>
<td>TGATACCCAACGTGACAGAAGTC</td>
<td>281 bp</td>
<td>n768–1049 introns 2–3</td>
</tr>
<tr>
<td>FOXO3a set 5</td>
<td>TGACTGTTATGCGAGGACCCAGATGA</td>
<td>TTCTCTTGAGTGCTGCTGAGG</td>
<td>232 bp</td>
<td>n508–740 exon 3</td>
</tr>
<tr>
<td>FOXO3a set 6</td>
<td>AAGTGGAGCTGAGAGCCGAGGAGGTT</td>
<td>TGCTGTGCGCACTTACCTTTAAG</td>
<td>558 bp</td>
<td>n166–724 exon 2</td>
</tr>
<tr>
<td>FOXO3a set 7</td>
<td>AAGATGGAGAGGAGAGGCCAGGGCTT</td>
<td>AAGCGGACTCCGACGAGATCCGAGA</td>
<td>710 bp</td>
<td>n142–n86 introns 2–3</td>
</tr>
</tbody>
</table>

Table 2.2. FOXO3a Deletion Frequency in NSCLC

<table>
<thead>
<tr>
<th>Tumor</th>
<th>HD</th>
<th>Sub-HD</th>
<th>Total deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>8/33 (24.2%)</td>
<td>20/33 (60.6%)</td>
<td>28/33 (84.8%)</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>0/19</td>
<td>9/19 (47.4%)</td>
<td>9/19 (47.4%)</td>
</tr>
</tbody>
</table>

Evidence in mice suggests that Foxos may act redundantly as suppressors of some cancer types (Paik et al. 2007). Therefore, as potential additional targets of selective gene loss in NSCLC, we examined other members of the FOXO gene family by qPCR analysis. We observed that, unlike FOXO3a, neither FOXO1 nor FOXO4 was deleted in any of the tumors of this study (data not shown).
2.3.2 *Decreased FOXO3a mRNA expression in LAC*

Real-time reverse transcription PCR was conducted on 17 blindly selected NSCLC, surrounding matched non-cancerous tissue, and 10 lung samples of cancer-free donors. Relative expression levels were first studied in normal and adjacent tissue in order to determine the level of FOXO3a expression in both control sets. Expression of FOXO3a was normalized to GAPDH and was very similar in these two sets of tissues. The mean RQ expression values were 0.32±0.08 measured in tumor-free tissue and 0.30±0.06 in adjacent normal tissue (Fig. 2.2).

Expression was then compared between matched tumor and adjacent normal tissue samples (n=17). The mean FOXO3a RQ value in the tumor tissue was 0.16±0.02, and there was a trend towards lower FOXO3a expression in tumor tissue compared to matched adjacent normal tissue (p=0.0829, data not shown). However, when stratified by histology, FOXO3a expression in LAC (n=11) was found to be significantly lower than expression in matched adjacent normal tissues (p =0.0292) (Fig. 2.2). The mean RQ values for FOXO3a expression were 0.34±0.08 in adjacent normal tissue and 0.14±0.03 in LAC tissue. FOXO3a expression in LSqCC (n=5) was not significantly different from matched adjacent normal tissue (p=0.7150). As described in the Materials and Methods, FOXO3a expression was also normalized to three experimentally determined control genes, HPRT1, PES1, and RPLP0, with similarly significant results obtained (data not shown).
Figure 2.2. Decreased mRNA expression of FOXO3a in LAC. Real-time RT-PCR shows a significant decrease in FOXO3a mRNA in LAC relative to adjacent non-cancer lung tissue and lung tissue of non-cancer donors. ANOVA was used to statistically compare the groups of tissues. *Relative quantitation (RQ).

2.3.3  FOXO3a protein loss corresponds with gene deletion in LAC

To further investigate the inactivation of FOXO3a in LAC, a sampling of these tumors and non-cancerous lung tissue was analyzed for FOXO3a protein expression by immunocytochemistry. Tumors 6784 is shown to stain strongly positive for FOXO3a (Fig. 2.3 A). This is consistent with the qPCR results showing that tumor 6784 has a full complement of FOXO3a. Relatively strong nuclear staining of FOXO3a is evident in the non-cancerous aveoli (Fig. 2.3 B). In contrast, tumors 6379 and 6498 display evidence of FOXO3a loss, which is consistent with the qPCR results indicating HD of FOXO3a in these tumors (Fig. 2.3 C and D). Heterogeneous staining of FOXO3a is evident in tumor 2621. Areas of both negative and positive staining of FOXO3a were observed on the same slide obtained from this sample (Fig.
2.3 E). This also is consistent with the qPCR evidence of sub-clonal deletion of FOXO3a in this tumor sample. Hematoxylin and eosin stained sections of these tumors are shown in Figure 2.6.

Figure 2.3. Immunocytochemistry of FOXO3a protein in LAC. A, tumor 6784; B, non-cancerous lung alveoli; C, tumor 6379; D, tumor 6498; E, tumor 2621. Panel E-5, negative control lacking primary antibody.
2.3.4  **FOXO3a is transcriptionally activated in cells exposed to BPDE**

FOXO3a loss in LAC suggests a role in the suppression of these tumors. As a stress activated transcription factor FOXO3a may protect against the tumorigenic effects of carcinogens in the pathogenesis of LAC. BPDE is a carcinogenic metabolite of the polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P), which is an environmentally pervasive human lung carcinogen (Dipple 1995, Hecht 2003, IARC 2004). We next investigated the functional activation of FOXO3a in cells exposed with BPDE. Using immunofluorescence and confocal microscopy, endogenous FOXO3a was observed to localize from the cytosol to the nucleus indicating its transcriptional activation within 4h of exposure of H1299 cells to 0.7µM BPDE (Fig. 2.4A).

The transcriptional activation of FOXO3a in response to BPDE was further investigated by qRT-PCR of a set of FOXO3a effector genes (Bakker et al. 2007, Blake et al. 2010, Furukawa-Hibi et al. 2002, Greer and Brunet 2005, Kops et al. 2002, Tran et al. 2002). For these experiments, FOXO3a expression was restored in A549 cells, which have abnormally low levels of endogenous FOXO3a as a result of gene deletion (Blake et al. 2010). FOXO3a and empty vector transfected cells were selected for 3 days with G418 to eliminate cells that failed to transfect. Cells were then treated with 0.7µM BPDE and harvested 18h later. A FOXO3a-dependent increase in the expression of GADD45B, BIM, BNIP3, and FASL was observed
(Fig. 2.4B). Each of these FOXO3a effectors has been implicated in FOXO3a-mediated stress response involving DNA repair (GADD45) and apoptosis (BIM, BNIP3, FASL).
Figure 2.4. Transcriptional activation of FOXO3a in response to BPDE. A, Nuclear localization of endogenous FOXO3a is induced by BPDE in H1299 cells treated with 0.7μM BPDE for the indicated times. Immunofluorescence was performed as described in the Materials and Methods showing localization of FOXO3a to the nucleus 4h after BPDE exposure. B, Representative RT-PCR of FOXO3a effectors in A549 cells transfected with FOXO3a or control vector and treated with 0.7 μM BPDE. Cells were collected at the times shown post-treatment.
for analysis. Results were confirmed by repeat experiments. Numbers represent quantification of band intensity normalized to controls.

2.3.5 FOXO3a stimulates apoptosis in response to BPDE in LAC cells

Two different LAC cell lines expressing low endogenous levels of FOXO3a (A549 and H358) were transfected with FOXO3a or empty vector and selected in medium containing G418. Under these conditions, wild-type FOXO3a caused a significant decrease in the number of cells one week following transfection (Fig. 2.5 A). Wild-type FOXO3a is transcriptionally active under these conditions, as we have previously reported, resulting in suppression of cell growth (Blake et al. 2010). The response to BPDE was investigated by treating the selected cells with increasing concentrations (0, 0.4 or 0.7µM) of BPDE and harvesting after 18-36h for cell cycle analysis, and after 5 days to determine the effect on relative cell number. Exogenous FOXO3a was observed to cause a significant decrease in the relative fraction of surviving cells following exposure to 0.7µM BPDE (Fig. 2.5 B). In contrast, there was no noticeable FOXO3a-dependent change in the cell cycle in response to BPDE (Fig. 2.7). These results suggest that FOXO3a augments the sensitivity of LAC cells to BPDE treatment.

Since we previously showed that FOXO3a elicited apoptosis in response to NNK-induced DNA damage in LAC cells, we next investigated whether FOXO3a also stimulated apoptosis in response to BPDE (Blake et al. 2010). Experiments were again carried out as described above and after 24h of exposure to 0.7µM BPDE apoptosis was measured by FACS selection and quantitation of annexin V-PE stained cells. The results showed a significant increase of annexin V-PE-positive cells in FOXO3a compared to control-transfectants following BPDE treatment.
(Fig.2.5 C and D). This analysis was also conducted on stable FOXO3a-expressing clones of A549 cells with similar results (Fig. 2.8). Of note, similar results were obtained from both high and low FOXO3a expressing clones (data not shown). Stable independent clones were isolated by dilution following transfection with FOXO3a and clonal expansion during long-term (several weeks) selection with G418.
The role of caspase activation in BPDE-induced apoptosis was examined next. A marked decrease in BPDE-induced FOXO3a-dependent apoptosis was observed when cells were co-treated with 15µM Z-VAD-FMK, a pan-caspase inhibitor (Fig. 2.5E). This indicated that FOXO3a-dependent apoptosis involved caspase activation. Direct analysis of several caspases by Western blot showed evidence of increased caspase 9, 8, and 7 cleavage activation in FOXO3a-relative to control-transfectants following treatment with BPDE. These results together with the RT-PCR results suggest that FOXO3a stimulates apoptosis involving both intrinsic (FASL/caspase 8) and extrinsic (caspase 9) caspase-dependent apoptotic pathways in response to BPDE. Similar caspase activation was obtained in both A549 (p53-positve) and H358 (p53-negative) LAC cell lines (Fig. 2.9).
2.4 DISCUSSION

The results of this study show that *FOXO3α* is a novel target of somatic HD in early stage LAC. This is supported by RT-PCR and immunocytochemistry results showing concomitant losses of both FOXO3α mRNA and protein in these tumors. Our immunocytochemistry results revealed heterogeneous loss of FOXO3α in individual stage I LAC. Loss of *FOXO3α* in several tumors of this early stage, including heterogeneous loss within individual tumors, suggests that *FOXO3α* inactivation may play a selective role in this stage of malignancy.

Our results also suggest that *FOXO3α* loss may play a role in early stage LSqCC. Although LSqCC and LAC are pathogenetically and phenotypically distinct types of NSCLC they do share some molecular changes, such as *p53* mutation and loss of the *CDKN2 (p16^{INK4a})* tumor suppressor gene (Sekido et al. 2003). Our analysis of LSqCC detected a decrease in FOXO3α gene dose of greater than 60% in 7/19 (37%) of samples, and a gene dose loss greater than 70% in 5/19 (26%) of the patient samples. Although these losses did not meet our conservative threshold for HD they nevertheless suggest that FOXO3α loss is also a selective occurrence in the development of this type of NSCLC. These results suggest that FOXO3α loss plays a more significant role in LAC than in LSqCC. However, FOXO3α’s protein expression and cellular localization in LAC and LSqCC samples must be determined in order to more fully assess whether FOXO3α inactivation is specific to LAC. For example, mutations of FOXO3α in its known phosphorylation sites that would mimic constitutive phosphorylation (such as serine to glutamate mutations) would cause inactivation of FOXO3α undetectable with RT-PCR.
Determining FOXO3a’s cellular localization in our LSqCC would prove helpful in determining whether FOXO3a is further inactivated in this tumor type.

In contrast, FOXO1 and FOXO4 genes were not deleted in any of the NSCLC examined in this study. Foxo1, Foxo3a, and Foxo4 knock-out mice have an increased susceptibility for the development of specific cancer types, including thymic lymphomas and hemangeomas. This finding has implicated all of the FOXO genes as tumor suppressors (Paik et al. 2007). However, the absence of FOXO1 and FOXO4 deletions indicates that among these three FOXO genes FOXO3a is uniquely targeted for deletion in NSCLC.

Several pieces of evidence suggest that the mechanism of FOXO3a inactivation in NSCLC may be causally linked with the structure of the FOXO3a locus and its susceptibility to disruption. Here we show that FOXO3a HDs tumors of smokers are specifically located in a region of the gene lying 5’ to exon 3. It has been reported that active regions of the FRA6F fragile site are located within this part of human FOXO3a (Morelli et al. 2002). Fragile sites are inherently prone to breakage and consequently are sensitive to DNA damaging agents, such as genotoxic carcinogens (Arlt et al. 2006, Glover et al. 2005). FRA6F has been implicated as a cause of DNA losses at its location on chromosome 6q22-21 in human cancer (Arlt et al. 2006). LOH at this location also is higher in LAC of smokers compared with those of never-smokers (Sanchez-Cespedes et al. 2001, Sy et al. 2003, Wong et al. 2002). Interestingly, fragile sites are well-conserved among mammals, and the same pattern of FOXO3a deletion was observed in mouse LAC, occurring predominantly in tumors induced by carcinogens (Herzog et al. 2009).
The structure of the FOXO3a locus may therefore be prone to carcinogen-induced disruption resulting in the occurrence of relatively precisely positioned FOXO3a HDs in LAC.

An underlying cause of lung cancer is exposure to polycyclic aromatic hydrocarbons (PAHs) such as BaP, which are among the most environmentally pervasive human lung carcinogens (IARC 2004). Also a component of cigarette smoke, BaP is metabolically activated by cytochrome P450s to BPDE, a highly DNA reactive and mutagenic diolepoxide (Dipple 1995, IARC 2004). Whereas the effects of such DNA-damaging carcinogens are most noticeable in tumor initiation, their effects are not limited to any stage of carcinogenesis, but are expected to impact cells throughout tumor development so long as there is exposure. Taking this into account, we examined the response of FOXO3a to BPDE by restoring its function in LAC cells that apparently had selectively lost FOXO3a through gene deletion during tumor development. We show that FOXO3a is functionally activated as a transcription factor in cells treated with BPDE, and this activation leads to caspase-dependent apoptosis. In this response, we observed up-regulation of three known pro-apoptotic FOXO3a effector genes FASL, BIM, and BNIP3. We previously reported similar results in LAC cells exposed to a DNA reactive metabolite of NNK, also a human lung carcinogen present in tobacco smoke (Blake et al. 2010). Thus, FOXO3a increases the sensitivity of LAC cells to the effects of genotoxic lung carcinogens. The stimulation of apoptosis in these cells suggests a role in eliminating carcinogen damaged cells as a means of suppressing LAC. The loss of this function may then increase the likelihood that LAC will result from carcinogen exposure.
We previously demonstrated that bulky DNA adduct-forming carcinogens (including NNK) induce extensive chromosome instability (CIN) as a causal mechanism in LAC of mice (Herzog et al. 2004, Herzog et al. 2006). BPDE also forms bulky DNA adducts, but was not investigated in these previous studies. CIN is a characteristic of most cancers (Lengauer et al. 1998, Loeb 1998). It is an increase in the rate of chromosomal defects that is considered to be a necessary source of genetic variation acted on by selection pressures in the development of most sporadic cancers (Lengauer et al. 1998, Loeb 1998). The loss of ‘caretaker’ functions, which protect the genome from damage, has been shown to underlie CIN in cancer (Gisselsson et al. 2003, Rajagopalan et al. 2004). Based on our findings, FOXO3a may act as a ‘caretaker’ whose loss can enable CIN causing DNA damage to accumulate or persist. Therefore, FOXO3a loss may also contribute to the emergence of CIN in LAC. This requires further study.

Consistent with this role is evidence that FOXO3A contributes to the repair of damaged DNA. A role in repair of UV-damaged DNA has been associated with its up-regulation of GADD45 (Tran et al. 2002). We have shown that GADD45 is also up-regulated by FOXO3a in response to NNK- (Blake et al. 2010) and BaP-damaged DNA damage (Fig. 2.4). Repair of DNA damage caused by lung carcinogens may be another means by which FOXO3a suppresses LAC, and possibly protects against CIN induction.

Upon stress activation, FOXO function overrides its negative control by EGFR/P13K/Akt, resulting in growth arrest or apoptosis. Abnormalities of the EGFR signaling network drive the oncogenesis of LAC, and to a lesser extent LqSCC (Ahrendt et al. 2001, Pao et al. 2005, Sekido et al. 2003, Sun et al. 2007, Wistuba et al. 2002). For example, numerous components of this
network have been implicated in LAC including *EGFR* and *KRAS* activation and Akt overexpression (Ahrendt et al. 2001, Dutu et al. 2005, Pao et al. 2005, Sekido et al. 2003, Sun et al. 2007, Wistuba et al. 2002). FOXOs are immediately downstream of Akt and are negatively regulated by Akt under physiological conditions suitable for growth and proliferation (Daitoku and Fukamizu 2007, Greer and Brunet 2005). However, stress-activation of FOXOs overrides the pro-survival and oncogenic signaling of Akt resulting in cell cycle arrest or apoptosis (Bakker et al. 2007, Blake et al. 2010, Furukawa-Hibi et al. 2002, Greer and Brunet 2005, Kops et al. 2002, Tran et al. 2002). The frequent deletion of *FOXO3a* would permit unchecked EGFR/P13K/Akt signaling in the face of DNA damage. This combination of selection pressures may drive *FOXO3a* inactivation in LAC.
Figure 2.6. Lung tissue samples stained with hematoxylin. Slides shown matched those stained for FOXO3a.
Figure 2.7. Cell cycle distribution of FOXO3a transfectants treated with BPDE (0.7μM). No FOXO3a-dependent change in the cell cycle was detectable in response to treatment.
Figure 2.8. FOXO3a augments apoptosis induced by BPDE in LAC cells stably expressing FOXO3a. A, Representative FACS analysis selecting for annexin-PE and 7-AAD in stable FOXO3a-expressing cells treated with 0.7μM BPDE. Cells were collected for analysis at the times shown following BPDE administration. B, Quantitation of annexin-PE. C, Western blot of stable expressing clones showing expression levels of FOXO3a.
Figure 2.9 Western blot of caspases in stable-expressing clones. For Western blot, cells were harvested 24h post-treatment with 0.7μM BPDE.
CHAPTER 3: FOXO3a evokes cytostasis with diminished mitotic arrest in response to microtubule inhibitors in lung adenocarcinoma cells

ABSTRACT

Microtubule inhibitors such as taxanes and vinca alkaloids disrupt cellular tubulin polymerization and cause mitotic arrest. These drugs are currently used in first-line combination therapy of lung adenocarcinoma (LAC). FOXO3a transcription factor is functionally activated by various cancer chemotherapeutics, suggesting a potential role in treatment response. In addition, FOXO3a has been shown to induce apoptosis in response to microtubule inhibitors in breast cancer cells. Here we investigated the response to microtubule inhibitors elicited by FOXO3a in LAC cells. We observed evidence of functional activation in H1299 non-small cell lung cancer cells treated with docetaxel (taxane) or vinorelbine (vinca alkaloid) as indicated by the localization of FOXO3a from the cytosol to the nucleus. Next, A549 and H460 LAC cell lines with restored FOXO3a by transfection showed a substantial decrease in relative cell number compared to control vector transfected cells following treatment with docetaxel or vinorelbine. However, only a modest degree of cell death was observed in these cells. FACS analysis showed significantly fewer FOXO3a transfected cells arrested in mitosis following treatment with either drug. Investigating this effect further, expression microarray and quantitative RT-PCR expression analysis gave results consistent with the cellular response to microtubule inhibitors, showing no FOXO3a-dependent change in expression of known pro-apoptotic FOXO3a effectors (BIM, BNIP and FASL) following treatment with either drug. Interestingly, a FOXO3a-dependent increase in expression of CDC14A was observed following
these treatments. CDC14A is a dual specificity kinase that has been shown to both block entry into mitosis and to promote mitotic exit. These results suggest that FOXO3a only weakly promotes cell death, but instead elicits cytostasis in response to microtubule inhibitors that is characterized by a diminished level of drug-induced mitotic arrest and a corresponding upregulation of CDC14A expression.
3.1 INTRODUCTION

Lung cancer is the leading cause of cancer death in the U.S. and worldwide killing more people in the U.S. than breast, colon, prostate, and pancreatic cancers combined (ACS 2010, IARC 2004, Zhao et al. 2007). Approximately 85-90% of lung cancers are of non-small cell lung cancer (NSCLC) histology, with adenocarcinoma being the major sub-type (ACS 2010, Alvarez et al. 2007). Lung cancer has a 5-year survival rate of only ~15%, which has improved only marginally over the past several decades (ACS 2010, IARC 2004). Major factors contributing to this outcome are persistent difficulties in detecting more treatable early-stage tumors, and ineffective treatment of more aggressive advanced stage tumors (ACS 2010).

Current treatment of advanced stage LAC includes a microtubule inhibitor in combination with platinum-based drugs in first line-therapy. This combination has been shown to confer some improvement in overall survival and increased drug tolerability of patients with stage IV LAC (ACS 2010). Microtubule inhibitors such as taxanes and vinca alkaloids are anti-mitotics. These drugs act on tubulin structures within the cell, leading to an inhibition of microtubule polymerization and function. This disrupts mitotic spindles, blocking transition from metaphase to anaphase, and causing mitotic arrest (Jordan and Wilson 2004, McGrogan et al. 2008). It has been proposed that cancer cell death caused by microtubule inhibitors occurs by mitotic catastrophe leading to either apoptosis or necrosis depending on the molecular profile of the cell. Although the mechanism of mitotic catastrophe is not fully understood, it can result from abnormal spindle function leading to the appearance of

Taxanes and vinca alkaloids have different mechanisms of action, but both lead to mitotic arrest and cell death. The mechanism of action of taxanes is through binding to β-tubulin, which results in rigidity and dysfunction of polymerized microtubules, such as mitotic spindles (Abal and Andreu 2003). Mitotic arrest induced by the taxane paclitaxel has been shown to lead to activation of the mitochondrial apoptotic pathway in MCF-7 breast cancer cells (Hoffmann et al. 2008, Janssen et al. 2007, Kroemer et al. 2007, Sunters et al. 2003). Vinca-alkaloids are microtubule-destabilizing agents that bind to the vinca domain of β-tubulin dimers blocking their assembly into microtubule polymers, causing an inhibition in mitotic spindle formation (Zhou et al. 2005). As with taxanes, unless the drug is cleared, cells undergo sustained or chronic mitotic arrest and cell death (McGrogan et al. 2008, Jordan et al. 2002).

The forkhead box O3a gene (FOXO3a) is part of the forkhead family of transcription factors characterized by their distinct forkhead domain (Furukawa-Hibi et al. 2002, Greer and Brunet 2005, Kops et al. 2002, Tran et al. 2002). Studies have shown that FOXOs are inactivated by Akt, ERK and IKK kinases during mitogenic or oncogenic stimulation. Inactivation occurs by phosphorylation of specific serine and threonine residues and consequential sequestration in the cytosol by 14-3-3. However, this effect is overridden under cellular stress conditions, including oxidative stress, DNA damage, and cellular exposure to various chemotherapeutic agents, including taxanes (Fernandez de Mattos et al. 2008, Furukawa-Hibi et al. 2002, Greer and Brunet 2005, Kops et al. 2002, Sunters et al. 2003, Tran et al. 2002). Most of these studies
have been carried out on FOXO3a, but functional redundancy is evident among all FOXO family
members. Upon activation, FOXO3a elicits a protective cellular response that has been shown
to include DNA repair, cell cycle arrest, and apoptosis. The response appears to be dependent
on the type of stress (Furukawa-Hibi et al. 2002, Greer and Brunet 2005, Kops et al. 2002, Tran
et al. 2002).

In addition to the studies of FOXO function, genetic and molecular studies also have
implicated them as tumor suppressors. It was shown that Foxo1, Foxo3, and Foxo4 knockout
mice were prone to lineage-specific tumorigenesis (Paik et al. 2007). More recently, we
reported the frequent deletion of the FOXO3a gene in human LAC of smokers and carcinogen-
induced mouse LAC (Blake et al. 2009, Mikse et al. 2010). We also showed that FOXO3a is
functionally activated by and induces a pro-apoptotic transcription program and cellular
response to DNA-damaging metabolites of the human lung carcinogens benzo[a]pyrene and
nicotine-derive nitrosaminoketone. Hence, FOXO3a is a likely suppressor of LAC whereby its
loss may enable the persistence of carcinogen-damaged lung epithelium, increasing the
likelihood of malignancy. FOXO3a also was shown to be inactivated in breast cancer as a
consequence of Akt and IKK upregulation, which caused aberrant cytosolic retention of FOXO3a
by 14-3-3 proteins (Hu et al. 2004). Although there have been no reports to date that other
FOXO genes are inactivated in cancer, the knockout study by Paik et al. demonstrated genetic
redundancy in suppressing susceptibility to cancer, suggesting that all FOXOs may exhibit
context-specific tumor suppressor functions (Paik et al. 2007).
As a stress activated transcription factors, FOXO3a appears to have an effect on tumor cell response to chemotherapeutics. Reports have shown that FOXO3a mediates BIM-dependent apoptosis in MCF-7 breast cancer cells treated with paclitaxel, and in colon cancer cell lines treated with cisplatin (Sunters et al. 2003, Fernandez de Mattos et al. 2008). FOXO3a was also found to elicit cell cycle arrest and cell death to gefitinib, an EGFR inhibitor (Krol et al. 2007). This reportedly resulted from dephosphorylation of Akt phosphorylation sites and consequential activation of FOXO3a function. The breadth of the physiologic and pathologic stresses to which FOXOs respond suggests a potential role as mediators of cancer cell response to various therapies.

We observed the loss of FOXO3a in both early and late stage LAC indicating that this molecular event persists throughout LAC malignancy (Mikse et al. 2010). Its status may then have an effect on the response of late stage tumors to standard therapy. Here we have investigated FOXO3a’s role in the cellular response to two different classes of microtubule inhibitors commonly used to treat advanced LAC. Here we investigated the potential role of FOXO3a in the response of LAC cells to these drugs.

Distinct from its reported role in breast cancer cellular response to paclitaxel, we report that FOXO3a elicits mainly a cytostatic response in LAC cells. This response includes mitigation of docetaxel and vinorelbine induced mitotic arrest, upregulation of CDC14A, and a substantial decrease in cell number without a corresponding increase in cell death. CDC14A is a dual specificity phosphatase, which has been shown to block entry into mitosis by inactivating CDC25, and may also promote exit from mitosis through CDK1 inactivation (Vasquez-Novelle et
al. 2010, Trautmann et al. 2002). The results suggest that CDC14A may be a novel transcriptional target of FOXO3a whose upregulation may contribute to a FOXO3a-dependent cytostasis and mitigation of microtubule inhibitor-induced mitotic arrest in LAC cells.

3.2 MATERIALS AND METHODS

3.2.1 Cell Culture

A549 and H460 LAC cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI medium supplemented with 10% FBS, and 100 µg/ml penicillin/streptomycin as recommended by ATCC. Cells were grown in a humidified incubator at 37 °C and 5% CO₂.

3.2.2 Transfections and cell treatments

Expression vector of FOXO3 cDNA was generated from RNA isolated from human buccal cells. Reverse transcription was carried out with 1 µg of total RNA using 200 units of Moloney murine leukemia virus (MMLV) reverse transcriptase for 1 h at 37°C. High-fidelity PCR amplification of full length FOXO3 cDNA used Hi-Fi Platinum Taq DNA polymerase (Invitrogen). Amplified FOXO3 cDNA was cloned into the pCR3.1 mammalian expression vector (Invitrogen), and wild-type clones were confirmed by direct sequence analysis.

A549 and H460 lung adenocarcinoma cell line transfections were carried out using lipofectAMINE reagent (Invitrogen) with conditions optimized for 1 µg of plasmid DNA in 35
mm² dishes. 1 X 10⁵ cells were seeded into 6-well plates (35 mm²/well) for transfection. Cell
treatments used 3-25 nM docetaxel and 6-50 nM vinorelbine based on our empirical
determination of cytotoxicity in the cells used. MTS colorimetric assays were carried out
according to manufacturer’s instructions (Promega, St. Louis, MO). Briefly, after overnight
transfection cells were collected and counted using a hemocytometer. Cells were then plated in
96-well plates in medium containing 500 µg/ml G418 at equal densities and allowed to select
overnight. Cells then had their medium replaced in medium containing docetaxel or vinorelbine
(n=5). After 24 hours, treatment was removed and cells were incubated in 100-250 µg/ml G418
for approximately 5 days.

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-
tetrazolium) is reduced to a purple-colored formazan product in living cells when in the
presence of PMS (phenazine methosulfate). MTS and PMS were combined in completed
medium at final concentrations of 333 µg/ml and 25 µmol, respectively, and cells were
incubated in this solution. The absorbance of the colored solution was then measured by
spectrophotometry at a wavelength of 490 nm. Several readings were made to assure
absorbance was a representation of relative cell number.

3.2.3 **Immunofluorescence**

H1299 NSCLC cells were plated onto chamber slides and synchronized in G1 phase of
the cell cycle by treating with 1 mM hydroxyurea for 12 h. The cells were then treated with 25
nM docetaxel. At several time points following treatment, cells were fixed in the chamber slides
with 4% paraformaldehyde for 10 min on ice. Cells were then washed and permeabilized with PBS/0.02% Triton X-100, and blocked with PBS/3% BSA. Primary FOXO3a (H-144) antibody (Santa Cruz) was applied to the cells at a concentration of 50 µg/ml and incubated overnight at 4°C. Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) was applied at a concentration of 10µg/ml following washing and incubated at room temperature for 2 h. Nuclei were stained with Hoechst at a dilution of 1:10 for 1 h at room temperature. Images were acquired with a Leica confocal microscope (TCS SP2 AOBS, Leica Microsystems), using a 488 nm laser for the Cy2, a 543nm laser for the Cy3, and 633 laser for the Cy5 fluorophores. The fluorophores were imaged using a sequential line scan, with detection bands set at 420–475 nm for Hoechst and 554–640 nm for Cy3. Each image was saved at a resolution of 1024×1024 pixel image size. The optical sections were reconstructed by maximum projection with the Leica software.

3.2.4  **Cell Cycle Analysis**

Transfected A549 and H460 cells were selected with G418 and treated for 24 and 48 hours at concentrations of 3-25 nM docetaxel and 6-50 nM vinorelbine. Cells were then collected, washed with PBS, and fixed by resuspending in 70% EtOH for 12-24 hours at -20°C. Afterwards, fixed cells were centrifuged and resuspended in PBS/propidium iodine solution (5 µg/ml) containing RNAse A (250 µg/ml). Cells were incubated in the dark for 30 minutes at room temperature prior to FACS analysis. FACS analysis was performed using a FACSCalibur
flow cytometer (Becton Dickinson) with pulse processing on FL2 for doublet discrimination with cell cycle histogram analysis using ModFit LT 2.0 software (Verity Software House).

3.2.5 **Apoptosis Analysis**

Apoptosis was quantitated using Annexin-PE Apoptosis Kit I (BD Pharmingen). During apoptosis, phosphotidylserines translocate from the inside of the cellular membrane to the outside. Annexin-PE (annexin that has been conjugated with phycoerythrin) has a high affinity to phosphotidylserines and binds to them when they are located on the outer leaflet of the plasma membrane. 7-AAD (7-Aminoactinomycin D) is a fluorescent chemical marker that has a high affinity for DNA. It intercalates in DNA with a higher affinity for GC-rich regions. 7-AAD does not readily pass through intact membranes, and only does so when cells that have disrupted or compromised membranes. As such, 7-ADD labeling is an indicator of dead or dying cells.

To conduct the assay, transfected cells were trypsinized and washed with DPBS. Labeling was done by treatment with 5µl each of 7-AAD and Annexin-PE for final concentrations of 1µg/ml, and incubated for 30 min prior to FACS analysis. Experiment was conducted in triplicates and the average calculated in percentage of cells in apoptotic quadrants. Two sided student t-test was performed between samples.
3.2.6  **Western Blotting**

Western blotting involves gel electrophoresis to separate native or denatured proteins by the length of the polypeptide and detection with antibody-coupled chemiluminescence. Briefly, cell lysates of different treatment groups were prepared by incubating cells in a solution containing detergents SDS, NP-40, and Triton X, and protease/phosphatase inhibitors. Lysates were then incubated with Laemmli buffer (a reducing agent) to break down their secondary and tertiary structures. Cells were incubated in this solution for ~2 hours and run on a 4-20% polyacrylamide gel containing SDS for ~3 hours at 100 volts.

Transfectants were selected and treated with docetaxel at 25 nM or vinorelbine at 50 nM for 24 hours or left untreated as controls. Western blotting was performed by preparing 30-50 µg of whole cell lysates denatured in 1X Laemmli sample buffer, electrophoresed, and transferred onto nitrocellulose membranes. Membranes were blocked with either 5% non-fat milk or 5% bovine serum albumin (BSA) in TBS with 0.1% Tween 20 for 2 hours. Membranes were incubated with primary antibodies overnight, followed by secondary antibody (directed at the species-specific portion of the primary antibody) incubation for 45 minutes. Anti-FOXO3a (H-144) was used at a dilution of 1:200 (Santa Cruz Biotechnology) in BSA. Anti-caspase 3 and 9 were used at a dilution of 1:500 in milk. Anti-caspase 7 and 8 were used at a dilution of 1:500 in BSA. Anti-β-actin antibody was used at a dilution of 1:3000 (Sigma-Aldrich) in BSA. Anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase were used at a concentration of 1:2000 and detected by chemiluminescence on x-ray film.
3.2.7  Quantitative Reverse Transcription-PCR Analysis

RNA was isolated using Trizol Reagent and was subjected to reverse transcription with MMLV reverse transcriptase in order to produce cDNA. PCR was performed on 50-100 ng of high quality cDNA to determine the expression levels of reported FOXO3a effector genes. Samples were then run on a 2% agarose gel. Signal strength of target genes was determined by calculating target PCR intensity/control PCR intensity in the test (treated) sample relative to that of the control (untreated) sample. As a control, β-ACTIN was used for quantification of test gene expression. Quantification was carried by densitometry using UVP Imaging and Analysis System and LabWorks software (UVP, Inc.). Oligonucleotide primers used in this study were designed based on the available gene sequences from NCBI and Ensembl genome browsers and purchased from Integrated DNA Technologies, Coralville, IA. The following gene-specific primer pairs (5’-3’) were designed and used in analysis. p27: forward, AGCTTGCCGGAGTTCTACTACA; reverse, TTTGGGTCCACAAATGCGTG; Cyclin-D2: forward, TTTGCGGTGGACACAGGTGA; reverse, TGCCAAGGGCCACATTACCTTT; CDC14A: forward, GTTACTGCAAAGCTGGTCTTGGA; reverse, ATGTCTCCTTTGGACCACAACG; BIM: forward, TGAAGGCAATCAAGGAGGTGAA; reverse, AATACCCACTGGAGGATCGAGACA; BNIP: forward, ACCACAGTACAAACAGGGCT; reverse, TTCATGACGCTCCTGCTTCCTCA; FASL: forward, TCAGGGGTGGCAGCGGTAGT; reverse, AGTAAACCGGTTGCTGGGGCT; βACTIN: forward, GCACCACACCTCTACAATGAG; reverse, CTTGAGGTAGTCAGTCAGG.
3.2.8 Expression Microarray Analysis

Microarrays consist of thousands of unique DNA oligonucleotides or “probes”, each complimentary to specific mRNA sequences arrayed as spots on a glass slide. RNA from the experimental samples is converted to cDNA and then *in vitro* transcribed into cRNA. During cRNA synthesis a biotin tag is incorporated into each cRNA. The cRNA is then hybridized to the arrayed probes. After hybridization of the cRNA and subsequent washing to remove non-specific binding, fluorescently conjugated streptavidin is incubated with the slide to provide a fluorescent signal. The microarray is scanned with a laser beam and the fluorescent signal for each spot is collected. The approach of using cRNA-oligonucleotide binding has two advantages. The first is the linear amplification of the cRNA synthesis, which allows for detection of low abundance transcripts. Second, cRNA-oligonucleotide dimers have stronger binding than DNA-DNA dimers, allowing for more stringent washing and reduced non-specific hybridization.

Here we used the CodeLink Bioarray system with arrays representing the fully transcribed human genome (~40,000 probes). After transfection of A549 cells with FOXO3a (vs. empty vector control), cells were selected, and treated with docetaxel (25 nM) for 6 hours of exposure. Control group was treated with DMSO vehicle using the same volume as treated cells (~1/1000 of total volume). Afterwards, cells were collected and processed for microarray expression analysis. Experimental triplicates were analyzed for each of the four treatment groups.

Sample preparation followed standard protocols and conditions. Total RNA was isolated using Trizol reagent (Life Technologies, Rockville, MD), and purified on Qiagen columns (Qiagen,
Valencia, CA). cDNA synthesis was performed using reverse transcription of 2µg of RNA and the resulting cDNA product was isolated by Qiaquick (Qiagen) spin columns. For cRNA synthesis, T7 reaction buffer, T7 NTPs, 10 mM biotin-11-UTP, and T7 polymerase were then added to the purified cDNA and incubated at 37°C for 14 h. Biotin labeled cRNA was then purified using RNeasy columns (Qiagen), fragmented, and denatured before hybridization for 18 h at 37°C. Alexa Fluor 647 labeled steptavidin was added to the slides followed by washing.

An Axon 4000B scanner with GenePix4 v4.0 software at a 5µm resolution at 635 nm with laser power at 100%, PMT voltage at 600 V, focus position 0µm, and lines to average - 1 was used to scan microarrays. Images were imported into CodeLink Expression Analysis Software v4.1 (GE Healthcare, Piscataway, NJ). Initial quality control (positive and negative controls), exclusion of manufacturing defects (MSR spots), background subtraction, and intra-array normalizations were performed and results exported to GeneSpring 7.3 (Agilent Technologies, Palo Alto, CA). Arrays were normalized to the 50th percentile, and individual genes normalized to the median. Potential differential expression was determined with a one-way ANOVA (variances not assumed to be equal) and filtered for p < 0.05 differences in expression with a cutoff 1.4-fold change in expression.
3.3 RESULTS

3.3.1 FOXO3a is transcriptionally activated in cells exposed to microtubule inhibitors

FOXO3a is localized to the nucleus upon functional activation in the presence of various cellular stresses; FOXO3a is otherwise cytosolic. We examined whether FOXO3a localized to the nucleus in NSCLC cells treated with docetaxel, a synthetic taxane used in chemotherapy. Using immunofluorescence against FOXO3a and confocal microscopy for imaging, endogenous FOXO3a was observed to localize from the cytosol to the nucleus within 7 hours of exposure of H1299 cells to 25 nM docetaxel indicating functional activation in response to this drug (Figure 3.1).
Figure 3.1. Transcriptional activation of FOXO3 in response to docetaxel. Nuclear localization of endogenous FOXO3 is induced by docetaxel in H1299 cells treated with 25 nM docetaxel after 7h. Immunofluorescence was performed as described in Materials and Methods showing localization of FOXO3 to the nucleus 7h after docetaxel exposure.

3.3.2  FOXO3a elicits a cytostatic response to docetaxel and vinorelbine

We next examined the cellular response elicited by FOXO3a in response to docetaxel. To do this, A549 cells (an LAC cell line that expresses low levels of FOXO3a) were transfected with a FOXO3a expression vector. Cells were also transfected with empty vector as a control. Positively transfected cells were selected with G418 and treated with increasing concentrations of docetaxel (3-25 nM). Relative cell growth and viability were measured using MTS assay 5 days after treatment with docetaxel. Our results show that FOXO3a significantly decreases the relative number of A549 cells treated with docetaxel in the dose range of 3-12.5 nM. For
comparison, cells were also treated with vinorelbine, a chemotherapeutic microtubule inhibitor with different mode of action from taxanes. Similar results were obtained for vinorelbine (Figure 3.2). FOXO3a-expressing A549 cells were found to have significantly increased sensitivity to docetaxel and vinorelbine compared with vector controls ($p < 0.01$). The experiments were also performed on H460 cells, another LAC cell line that expresses low levels of FOXO3a, yielding similar results (Figure A.2).

**Figure 3.2.** FOXO3a reduces surviving cellular fraction in response to docetaxel and vinorelbine in LAC cells. MTS assay of transfected A549 cells following treatment with docetaxel (left) and vinorelbine (right) for 48 hours. Surviving fractions are relative to vehicle (DMSO) treated cells transfected with the same vector. Statistical analysis used was two-tailed Student’s t-test. * $p<0.01$
FOXO3a stimulates an apoptotic response to paclitaxel in breast cancer cells (Sunters et al. 2003). We next examined whether the decrease in relative LAC cell number mediated by FOXO3a in response to the microtubule inhibitors may be explained by an increase in apoptosis. Apoptosis and necrotic cell death were analyzed on treated transfectants using FACS analysis of Annexin-PE and 7-AAD double-stained cells. Observed was only a modest degree of apoptosis in both A549 and H460 cells treated with either docetaxel or vinorelbine at concentrations that caused a substantial decrease in relative cell number after 48 hours (based on our MTS results) (Figure 3.3, Figure A.2, Figure A.3). This was only slightly augmented by FOXO3a after 24h, but no difference between FOXO3a and vector control transfected cells was evident at 48h of treatment (Figure 3.3). We also observed no evidence of necrosis, as very few cells stained positively for 7-AAD and negatively for Annexin-PE (data not shown).

Figure 3.3. FOXO3a promotes modest apoptosis in LAC cells treated with docetaxel and vinorelbine. Annexin-PE assay of transfected A549 cells following treatment with docetaxel (left) and vinorelbine (right) for 24 hours. Apoptotic fractions are relative to vehicle (DMSO)
treated cells transfected with the same vector. Average of 3 experiments is shown. Statistical analysis used was two-tailed Student’s t-test; * p < 0.05.

Examination of several caspases for cleavage activation also indicated a moderate degree of apoptosis induction by relatively high doses of docetaxel and vinorelbine (Figure 3.4). Modest levels of cleavage of caspase 9, caspase 8, caspase 7, and caspase 3 were evident in both A549 and H460 after 24h of treatment with 25 nM docetaxel and 50 nM vinorelbine. Also consistent with Annexin-PE analysis, cleavage of caspases was only slightly augmented by FOXO3a (Figure 3.4).
Figure 3.4. FOXO3a elicits a modest apoptotic response to anti-mitotics. Western blot of caspases in A549 cells transfected with either FOXO3a or control vector. For Western blot, cells were harvested 24 hours post-treatment with docetaxel (25 nM) or vinorelbine (50 nM). Results are representative of several experiments.

We further investigated the apparent modest level of apoptosis induced by microtubule inhibitors by RT-PCR of reported pro-apoptotic effectors of FOXO3a. Consistent with our FACS analysis, FOXO3a did not elicit an increase in expression of BIM, BNIP3, and FASL in transfected A549 and H460 cells following treatment with 6-25 nM docetaxel and 12-50 nM vinorelbine (Figure 3.5). Cells were treated for 6-24h with no increases in expression evident at any time point.
**Figure 3.5.** Representative RT-PCR of FOXO3 effectors in A549 cells transfected with FOXO3 or control vector and treated with docetaxel (25 nM) and vinorelbine (50 nM). Cells were collected at 24 hours post-treatment for analysis. Results were confirmed by repeat experiments. Similar results were obtained for H460 cells and lower treatment concentrations (data not shown). Numbers represent quantification of band intensity normalized to controls.
3.3.3 **FOXO3a mitigates mitotic arrest caused by docetaxel and vinorelbine in LAC cells.**

The effect of the anti-mitotics on cell cycle distribution in FOXO3a and vector control transfected A549 cells also was investigated. Cell cycle distribution was determined by FACS analysis of propidium iodide stained cells after 24 hours of treatment with docetaxel (25 nM) and vinorelbine (50 nM). These drugs are known to induce mitotic arrest as a result of disruption of mitotic spindle function (McGrogan et al. 2008). As expected, this response was observed in the vector control transfected cells following treatment with either drug. In contrast, the mitotic fraction was significantly decreased in the treated FOXO3a transfected cells (Figure 3.6). With continued treatment, a similar degree of sub-G1 (a potential indicator of cell death) for both FOXO3a and control vector transfected cells was apparent after 48h as indicated by a similar increase in sub-G1 fraction. This is consistent with our observation of a similar decrease in cell viability of both groups after 48h of treatment with these drug concentrations as determined by MTS assay (Figure 3.2).
Figure 3.6. Cell cycle analysis of A549 and H460 LAC cell lines treated with docetaxel and vinorelbine. A, A549 and H460 cells treated with docetaxel (25 nM) and vinorelbine (50 nM). Peaks representing G1 and G2/M phase of cell cycle are indicated by arrows. B, Western blot analysis of FOXO3a status in A549 and H460 transfectants.

3.3.4 **FOXO3a stimulates a transcription program in response to docetaxel that includes derepression of CDC14A.**

FOXO3a has been shown to stimulate G1 arrest through p27 up-regulation and Cyclin-D2 down-regulation, both of which are reported effectors of FOXO3a (Tran et al. 2002, Kops et al. 2002). We therefore examined whether expression of either of these G1 regulators
potentially contributed to the apparent shift of the FOXO3a transfected cells from mitosis to G1 following treatment with anti-mitotics. Semi-quantitative RT-PCR was used to examine whether their expression was altered by FOXO3a during treatment with either anti-mitotic. Our data shows no effect on their expression during the treatment that produced the FOXO3a-dependent change in cell cycle (Figure 3.7).
Figure 3.7. Representative RT-PCR of FOXO3 effectors in A549 and H460 cells transfected with FOXO3 or control vector and treated with docetaxel (25 nM) and vinorelbine (50 nM). Cells were collected at 24 hours post-treatment for analysis. Results were confirmed by repeat experiments. Numbers represent quantification of band intensity normalized to controls.

In an effort to better understand the response elicited by FOXO3a to anti-mitotics in LAC cells, we performed an expression microarray analysis of FOXO3a and vector control
transfected A549 cells treated with 25 nM docetaxel. After 6 hours of treatment there was evidence of a FOXO3a-specific response as 385 genes were uniquely differentially expressed in this group (Figure 3.8). In vector control transfected cells, docetaxel induced changes in expression of 245 genes that were unique to this group. In the absence of treatment, FOXO3a elicited changes in expression of 95 genes. Significant changes were identified by filtering for p < 0.05 differences in expression with a cutoff of 1.4-fold change. These array data were then sorted by function to ascertain the likely cellular effect of the transcription program elicited by FOXO3a in response to docetaxel. Ingenuity Systems Pathway Analysis software was used to sort the changes according to biological process (Table 4.1).

The array data are consistent with our RT-PCR results with regard to showing essentially no effect on apoptotic genes, and surprisingly little effect on cell cycle (Figure A.4). The biological processes most significantly altered by FOXO3a transcriptional activity in response to docetaxel were cell-cell signaling, cell morphology, cellular movement, and cellular assembly and organization (Table 4.1). As indicated in Figure 3.8B and Table 4.1, docetaxel induced a distinct response in the absence of transfected FOXO3a. From these results, we observed an apparent FOXO3a-dependent increase in the level of CDC14A expression in response to docetaxel relative to the vector control treated cells (Figure A.4). This was confirmed by RT-PCR, which showed evidence of derepression by FOXO3a. CDC14A expression was clearly decreased in the vector control cells treated with either docetaxel or vinorelbine. However, this did not occur in the presence of transfected FOXO3a (Figure 3.7).
Examination of ~5 kb of the immediate 5´-flanking region of \textit{CDC14A} revealed several consensus Forkhead binding elements (FHBE) suggesting direct regulation of expression by activated FOXO3a (Figure A.5). Mammalian CDC14A is a dual action phosphatase that blocks G2/M transition by inactivating CDC25 and preventing Cdk1-Cyclin-B1 activation (Vazquez-Novelle et al. 2010). Anti-mitotic-activated FOXO3a may therefore promote the FOXO3a-mediated bypass of mitotic arrest induced by these drugs by sustaining CDC14A expression.
Figure 3.8. Modulation of gene expression by FOXO3a and docetaxel. A, Gene expression profiles for (left to right) A549 cells 24 h following transfection with FOXO3a vs. control vector; control vector cells with and without 25 nM docetaxel treatment; FOXO3a transfected cells with and without docetaxel treatment. Treatments were for 6h. Data points reflect the mean value for replicate arrays. Y-axis, normalized gene expression levels in which the expression value for each treatment of a gene is divided by the average of all treatments for that gene. B, Differentially expressed genes between pair-wise comparisons shown in A.
### A

<table>
<thead>
<tr>
<th>Biological processes modulated by FOXO3a in response to docetaxel in A549 cells</th>
<th>No. of genes</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell-to-cell signalling and interaction</strong>&lt;br&gt;ADAM28, ALOX5, BCAP31, CD97, CNP, DCX, HCK, MLLT4, PCDHA6, PREX1, PSCD3, RAP2A, RASGRF2, TIAM1, TIMP3, USH1C</td>
<td>16</td>
<td>3.88E-6 - 4.72E-2</td>
</tr>
<tr>
<td><strong>Cell morphology</strong>&lt;br&gt;ALOX5, CNP, DCX, HBP1, HCK, MAP7, MLLT4, PSCD3, RAP2A, RASGRF2, SYPL2, TIAM1, TIMP3</td>
<td>13</td>
<td>6.21E-4 - 4.98E-2</td>
</tr>
<tr>
<td><strong>Cellular movement</strong>&lt;br&gt;ALOX5, CD97, CHI3L1, CNP, DCX, HBP1, HCK, MLLT4, PREX1, RAP2A, TIAM1, TIMP3</td>
<td>12</td>
<td>2.68E-3 - 4.98E-2</td>
</tr>
<tr>
<td><strong>Cellular assembly and organization</strong>&lt;br&gt;ALOX5, CNP, DCX, HBP1, HCK, MAP7, PSCD3, RAE1, TIAM1, USH1C</td>
<td>10</td>
<td>2.68E-3 - 4.98E-2</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Biological processes modulated by docetaxel in absence of FOXO3a in A549 cells</th>
<th>No. of genes</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular movement</strong>&lt;br&gt;CNR2, GIT1, HARS, HMMR, LLP, MAPK8IP3, MYO10, PDGFA, PDLIM2, PITX2, PREX1, PTEN, RANBP9</td>
<td>13</td>
<td>1.20E-5 - 4.94E-2</td>
</tr>
<tr>
<td><strong>Molecular transport</strong>&lt;br&gt;ABCB10, ABCG1, ANKHCBS, ARG2, CBS, EXOC4, GIT1, IRS2, KCNJ3, PSAP, PTEN, SLC25A10</td>
<td>12</td>
<td>2.03E-3 - 4.27E-2</td>
</tr>
<tr>
<td><strong>Small molecule biochemistry</strong>&lt;br&gt;ABCB10, ABCG1, ANKH, B3GALNT2, CBS, EXOC4, GIT1, IRS2, KCNJ3, PSAP, PTEN, SLC25A10</td>
<td>12</td>
<td>2.03E-3 - 4.27E-2</td>
</tr>
<tr>
<td><strong>Lipid metabolism</strong>&lt;br&gt;ABCG1, CBS, LLP, PSAP, PTEN, SLC25A10</td>
<td>6</td>
<td>2.03E-3 - 4.27E-2</td>
</tr>
</tbody>
</table>

**Table 4.1.** Most significantly altered biological process in response to docetaxel treatment. A, biological pathways modulated by FOXO3a in response to treatment. B, biological pathways modulated by treatment in absence of FOXO3a overexpression. Biological pathways and corresponding genes were determined using Ingenuity Systems Pathway Analysis software.
All genes were transcriptionally upregulated except for genes in bold which underwent downregulation. Those most significantly altered by FOXO3a transcriptional activity in response to docetaxel were cell-to-cell signaling, cell morphology, cellular movement, and cellular assembly and organization.

3.4 DISCUSSION

Previous studies have shown that the FOXO transcription factors are activated by various physiologic and pathologic stresses including DNA damaging carcinogens and chemotherapeutics (Greer and Brunet 2005, Mikse et al. 2010, Tran et al. 2002). Typically, FOXOs induce either growth arrest or apoptosis depending on the cellular context upon activation by cell-damaging stress. FOXO3a frequently undergoes allelic loss in LAC (Mikse et al. 2010). Here we show that FOXO3a elicits primarily a response of growth inhibition to two different classes of therapeutic microtubule inhibitors, taxanes and vinca alkaloids, in LAC cell lines. We observed a substantial FOXO3a-dependent decrease in the relative number of cells after treatment with either docetaxel or vinorelbine. This involved exposure to the drugs for 48h followed by maintenance in culture without the drug for an additional 5 days. However, during this time, FOXO3a elicited only a modest augmentation in apoptosis including limited caspase activation and no transcriptional upregulation by FOXO3a of described pro-apoptotic effectors (i.e. BIM, BNIP3 and FASL). The level of apoptosis mediated by FOXO3a could not account for the degree to which FOXO3a elicited a decrease in relative cell number upon treatment with docetaxel and vinorelbine. Our findings are in contrast with a study showing a FOXO3a-mediated increase in apoptosis in the MCF-7 breast cancer cell line treated with
another taxane, paclitaxel, a response that was shown to be BIM-dependent (Sunters et al. 2003). Docetaxel and paclitaxel have no reported differences in their mode of action. Therefore, the differential effect elicited by FOXO3a to taxanes in breast and LAC cell lines is most likely attributable to the specific molecular characteristics of the different cell types.

Evident from our results is that the LAC cell lines used in this study may be relatively resistant to apoptosis induced by the therapeutically relevant concentrations microtubule inhibitors used, but these cells are not insensitive to FOXO3a-stimulated apoptosis. This is evidenced by our previous studies showing a robust FOXO3a-mediated apoptotic response to DNA reactive metabolites of the human lung carcinogens, nicotine-derived nitrosaminoketone (NNK) and benzo[a]pyrene (Blake et al. 2010, Mikse et al. 2010). Expression microarray experiments involving NNK also showed that FOXO3a stimulates a pro-apoptotic transcription program in response to NNK (Blake et al. 2010). Consistent with the cellular response to the microtubule inhibitors mediated by FOXO3a, the expression microarray results in A549 cells treated with docetaxel did not show evidence of apoptosis induction by FOXO3a.

Microtubule inhibitors disrupt mitotic spindle function resulting in mitotic arrest. Expectedly, LAC cells were inhibited in mitosis upon treatment with either microtubule inhibitor. However, this effect was greatly diminished in cells that had been transfected with wild-type FOXO3a. The response elicited by FOXO3a was the same for an inhibitor of microtubule polymerization (vinorelbine) and an inhibitor of the functional dynamics of polymerized microtubules (docetaxel) suggesting that FOXO3a is generally sensitive to loss of microtubule function. Analysis of the expression microarray results gave a clue as to the
potential mechanism underlying this effect. We found that the \textit{CDC14A} gene was transcriptionally elevated by FOXO3a in response to docetaxel. Upon further investigation we observed that \textit{CDC14A} transcript levels in fact decreased precipitously in vector control transfected cells treated with either docetaxel or vinorelbine. However, in FOXO3a-transfected cells, \textit{CDC14A} expression was sustained during treatment, a response that suggests that FOXO3a overrides the downregulation of this gene by other factors in response to the drugs. Analysis of the 5´- upstream region identified five potential FHBES, implicating \textit{CDC14A} as a novel effector of FOXO3a.

\textit{CDC14A} is a dual specificity phosphatase that has been shown to block entry into mitosis by inactivating CDC25 phosphatases (Vasquez-Novelle et al. 2010). CDC25s activate CDK1, which is required for G2/M transition. CDC14A also promotes mitotic exit by facilitating the inactivation of CDK1. Together these roles suggest that increased CDC14A levels could produce fewer cells in mitosis where they would be inhibited by microtubule inhibitors. Hence, upregulation of CDC14A by FOXO3a in response to microtubule inhibitors may diminish the anti-mitotic effect of docetaxel and vinorelbine. This potential role of CDC14A remains to be demonstrated; however, its known cellular roles are suggestive of such involvement. Such a role could also contribute to the evident FOXO3a-mediated growth inhibition in response to these drugs. This effect may then be anti-therapeutic, if inhibition of spindle function is essential for the therapeutic effect of microtubule inhibitors.

The transcription program activated by FOXO3a in response to docetaxel after 6 hours of treatment appeared to be a stress-specific response reflecting the cellular damage caused by
microtubule inhibitors. Ingenuity Systems Pathway Analysis software was used to sort the changes according to biological process. Those most commonly altered by FOXO3a transcriptional activity in response to docetaxel were cell-cell signaling, cellular movement, and cellular assembly and organization, which would be altered by disruption of microtubule function throughout the cell (Table 4.1).

Microtubule inhibitors are used in combination first-line and second-line treatment of LAC among other solid tumor types (Dubey et al. 2008, McGrogan et al. 2008, Simon 2008). The results of this study suggest an unexpected and novel role of FOXO3a in LAC response to these drugs. They show that FOXO3a elicits a cytostatic effect to sub-lethal concentrations of both taxanes and vinca alkaloids. By mitigating the effect of these drugs on mitosis, FOXO3a would appear to have an anti-therapeutic effect if the effect on mitosis is necessary for their therapeutic effect. However, the induction of cytostasis by FOXO3a may offset this effect if the cells become senescent or terminally growth arrested. Docetaxel has been shown to induce senescence in NSCLC in combination with carboplatin, and whether this is a FOXO3a-dependent effect requires further study (Ewald et al. 2010).

At the highest doses of the drugs used in this study, a high degree of apoptosis was observed in both FOXO3a and vector control transfected cells, indicating that this effect was not mediated by FOXO3a. At lower doses we only observed modest induction of apoptosis by FOXO3a. However, at lower doses we also observed pronounced cytostasis. All concentrations used are in the therapeutic range of these drugs (ACS 2010). The findings of this study therefore indicate that FOXO3a mediates a cytostatic response to microtubule inhibitors in LAC cells. The detailed mechanism of this response requires further study.
We previously showed that FOXO3a stimulates an apoptotic response to DNA-damaging lung carcinogens in LAC cells suggesting a potential role in suppressing the carcinogenic induction of these tumors (Mikse et al. 2010). Precedence indicates that this change will persist throughout malignant progression (Wistuba and Gazdar 2006). FOXO3a has been shown to be activated by various chemotherapeutics including paclitaxel, cisplatin, and gefitinib, eliciting a pro-therapeutic apoptotic response to each (Fernandez de Mattos et al. 2008, Krol et al. 2007, Sunters et al. 2003). Hence, loss of FOXO3a in LAC may have an effect on response to certain therapeutics regardless of tumor stage. Our investigation is the first to address this potential role in LAC. Our results indicate that FOXO3a stimulates cytostasis, but also mitigates the anti-mitotic effect of microtubule inhibitors. Further investigation is needed to determine whether and how this might affect the outcome of LAC treated with these drugs, as FOXO3a status have an effect on this outcome.
4.1.1 Background: FOXO3a as a candidate tumor suppressor gene in LAC

The main objective of the work presented in this dissertation involves the identification of FOXO3a as a novel target of deletion in LAC and characterization of FOXO3a’s role in lung carcinogenesis as implicated by its relatively frequent loss in LAC. Previous work by our lab showed that carcinogen-induced LAC in mice displayed allelic loss in a region on proximal chromosome 10 (Herzog et al. 2009). A closer inspection of this region (chromosome 10B2) in these tumors using quantitative PCR (qPCR) showed that the deletions overlapped at the FOXO3a gene. This implicated FOXO3a as the focal point of deletion in these tumors. We conducted an analysis of the orthologous region (chromosome 6q21) in human adenocarcinomas, which also revealed frequent deletion of the FOXO3a gene in early-stage LAC of smokers. Homozygous deletions (HD) were detected in 24.2% of our LAC samples, and signal not reaching the level of complete gene loss in another 42.2% of these tumors. Precedence suggests that complete inactivation of a gene at a frequency of 24.2% in a given tumor type indicates some significant involvement in the development of those tumors (Wistuba and Gazdar 2006). We also examined several LSqCC, but no HDs were detected. However, sub-homozygous deletions of FOXO3a were evident in 47.4% of these tumors. The more frequent loss of FOXO3a at sub-HD levels (9/19 cases) than its immediate flanking genes (6/19 cases) suggests a potential role of FOXO3a inactivation in LSqCC. Mutation analysis of the FOXO3a gene by direct sequencing of tumor versus normal DNA revealed no mutations in 20 of the LAC not displaying HD. FOXO3a may also be inactivated in both LAC and LSqCC through other
mechanisms that have been cited for other tumor types, but were not investigated in this study. For example, retention of FOXO3a protein in the cytoplasm by means of Akt or IκB kinase upregulation has been reported in breast cancer (Hu et al. 2004). Consequently, the frequency of FOXO3a deletion may underestimate its involvement in NSCLC. The relatively frequent loss of FOXO3a in carcinogen-induced-LAC, but not in LAC occurring in the absence of carcinogenic induction, suggests some role in the development of tumors following carcinogen exposure.

4.1.2 FOXO3a’s cellular response to carcinogens present in tobacco smoke

Based on our identification of FOXO3a as a novel target of deletion in LAC, our initial step consisted of a literature study pertaining to FOXO3a and its known function. As we have described, previous work has shown that FOXO3a is a transcription factor primarily activated by stresses such as DNA damage and oxidative stress (Yang and Hung 2009). Upon activation, FOXO3a, like all FOXOs, activates a response that is protective against the perceived stress. Among its functions in this regard are a stimulation of cell cycle arrest, apoptosis, DNA repair, and the reversal of oxidative damage (Farnebo et al. 2001, Hu et al. 2004, Yang et al. 2008). FOXO genes have been implicated as tumor suppressors based on these functions and on a study in knock-out mice showing a propensity for mice deficient in FOXOs to develop lymphomas and hemangeomas (So and Clearly 2003, Paik et al. 2007). These results suggest no role for FOXO3a in lung tumor susceptibility. However, no reports to date have implicated or suggested FOXO3a’s involvement in lung carcinogenesis.
Based on our understanding of FOXO3a as a likely tumor suppressor and DNA damage-activated transcription factor, we initially hypothesized that FOXO3a is activated by DNA damage caused by carcinogens in tobacco smoke, stimulating a protective cellular response that could play a role in offsetting carcinogenesis.

Tobacco smoking is the leading cause of cancer death, accounting for approximately 85% of all lung cancers (HHS 2009). Tobacco smoke contains a wide variety of carcinogens such as PAHs and TSAs. Among PAHs, benzo[a]pyrene is a well characterized human lung carcinogen that exerts its tumorigenic effect by damaging DNA. Specifically, benzo[a]pyrene is metabolically activated to BPDE, which forms a very stable adduct with deoxyguanine causing mutations and secondary DNA strand breakage resulting from erroneous repair of this bulky adduct (Hecht 1999). Therefore as a model for lung carcinogen activation, we investigated whether FOXO3a was activated in LAC cells exposed to BPDE.

As a transcription factor, FOXO3a has been shown to localize from the cytosol to the nucleus upon stress-activation. We analyzed the localization of endogenous FOXO3ain cells exposed to BPDE. To do this, we used immunofluorescent confocal microscopy to observe FOXO3a’s cellular localization in cells treated with DNA-damaging concentrations of BPDE. The results showed evidence of FOXO3a localization to the nucleus within 4 hours of treatment with BPDE (Fig. 2.4). The same extent of nuclear localization was not evident in the absence of treatment. Transcriptional activation of FOXO3a target genes upon BPDE exposure was also evident in FOXO3a-transfected cells, also indicating functional activation. FOXO3a-deficient LAC cell lines (A549 and H358) were used in these experiments. We observed a more substantial
upregulation of known FOXO3a effectors in the FOXO3a-transfected cells than in the empty vector transfected cells following BPDE treatment. These results indicated FOXO3a functional activation in LAC cells treated with BPDE.

We next investigated the cellular effect of FOXO3a upon BPDE activation. In order to elucidate this role, we again conducted transfection experiments in A549 and H358 LAC cell lines. MTS cell survival assay on FOXO3a transfectants showed a significant decrease in the relative number of cells surviving BPDE treatment compared to cells transfected with the empty vector and subjected to the same treatment. Again, this was evident in both A549 and H358 cells (Fig. 2.5A). These results suggested further that FOXO3a is functionally activated by BPDE treatment. It also shows that the cellular response to this activation is either cell death or growth arrest in LAC cells subjected to BPDE.

We next investigated the BPDE-induced cellular response mediated by FOXO3a. FOXO3a has been shown to stimulate G1 arrest in cells subjected to stress, such as oxidative stress. However, cell cycle distribution as determined by FACS analysis showed that FOXO3a did not alter cell cycle distribution in response to BPDE (Fig 2.7). Consistent with previous reports, a slight increase in S-phase was observed in cells treated with BPDE, but this was independent of FOXO3a expression (Jeffy et al. 2000).

FOXO3a also has been reported to stimulate apoptosis in response to various cellular stresses such as DNA damage and various chemotherapeutics (Carter and Brunet 2005, Fernandez de Mattos et al. 2008, Sunters et al. 2003, Yang et al. 2008). We next investigated whether FOXO3a mediated an apoptotic response to BPDE as a potential means of protection
against lung tumor development. In this regard, our results showed that, although an increase in apoptotic fraction was detectable in both transfection groups after 24 hours of treatment with BPDE, the apoptotic fraction was significantly augmented in the FOXO3a-expressing transfectants (Fig. 2.5D). These data suggest that FOXO3a increases sensitivity of LAC cells to DNA damage caused by BPDE, which culminates in apoptosis. We decided to study in more detail the mechanism of FOXO3a-induced apoptosis occurring in response to BPDE.

Previous experiments studying FOXO3a’s apoptotic mechanism in breast and colon cancer cells have suggested that FOXO3a mediates apoptosis through FASL/caspase 8, BIM or BNIP3 that is likely dependent both on the type of stress and the cell type (Fernandez de Mattos et al. 2008, Sunters et al. 2003, You et al. 2006). First, to determine whether BPDE-induced apoptosis was operating through conventional caspase pathways, we exposed our treated and untreated transfectant groups to FMK, a pan-caspase inhibitor. If FOXO3a elicited caspase-dependent apoptosis, this would be decreased in the presence of FMK. Indeed, when cells were pre-treated with FMK, we detected a significant decrease in apoptosis (Fig. 2.5D). In fact, apoptosis was nearly inhibited completely by this treatment. In addition, we conducted western blot analysis of various caspase proteins. We detected activation of caspases 7, 8, and 9 in a FOXO3a-dependent fashion in response to BPDE (Fig. 2.5E). We also observed upregulation of pro-apoptotic effectors of FOXO3a, namely, BIM, BNIP3 and FASL when FOXO3a-transfected cells were treated with BPDE (Fig. 2.4). These results suggested that FOXO3a promotes caspase-dependent apoptosis as a consequence of DNA damage caused by BPDE in LAC cells via both intrinsic (caspase 9) and extrinsic (caspase 8) pathways that also
involve BIM and BNIP3. DNA-damage is known to activate the intrinsic pathway; however, the mechanism of caspase 8 activation by BPDE requires further study.

Our characterization so far of FOXO3a’s DNA-damage response in lung cancer cells, although significant, was limited to a short-term expression of FOXO3a through transfection of FOXO3a-deficient lines and selection of pooled positive transfectants. We were also interested in an analysis of FOXO3a’s apoptotic potential in a more stable FOXO3a-expressing model. To do this, we generated stable FOXO3a-expressing clones of A549 cell lines by restoring its function through transfection and continuous selection of clones expanded from individual colonies. Because FOXO3a is an endogenous protein activated in response to exogenous stress, these clones could be generated in the absence of exogenous stress. Our results showed that, in accordance with our short-term model, apoptosis as a result of BPDE exposure is also augmented by FOXO3a in stable-expressing clones (Fig. 2.8). Interestingly, similar results were obtained for both high and low FOXO3a-expressing clones. This implies that, in order to carry out its function, a threshold level of FOXO3a expression must be reached, and the low expressing clones have reached that threshold. Taken together, these results showed that FOXO3a increases the sensitivity of LAC cells to BPDE-induced apoptosis.

These results support our hypothesis that FOXO3a confers a protective cellular response to a carcinogen present in tobacco smoke. With essentially the same results obtained in our study of another DNA damaging lung carcinogen, NNK, these results suggest that FOXO3a plays a role in the elimination of DNA-damaged cells that could potentially develop into cancer (Blake et al. 2009). Based on our results, we propose a model in which DNA damage caused by
tobacco smoke acts as a selection pressure on FOXO3a resulting in its frequent deletion in early stage LAC carcinogenesis whereby the deletion of FOXO3a would serve to enable survival of DNA-damaged cells increasing their likelihood of becoming cancerous.

4.2 FOXO3a’s role in response to anti-mitotic chemotherapeutics

The first part of this dissertation has dealt with characterizing the potential role of FOXO3a as a suppressor of LAC carcinogenesis in a cell culture system. Our initial findings have suggested that FOXO3a may be a contributing factor in early stage LAC. Genetic changes such as homozygous deletion are irreversible and precedence suggests that such events that occur early in cancer development persist as the tumor advances to full malignancy. Therefore FOXO3a’s loss may also have an effect on the later stages of LAC, perhaps influencing tumor aggressiveness, prognosis, or treatment efficacy. LAC detected at any stage are most often subjected to chemotherapy. In advanced stage tumors this is often the only therapeutic option (ACS 2010). As a stress-activated transcription factor, FOXO3a’s presence/absence in tumors could potentially have an effect on patient outcome and response to chemotherapy. The work in this thesis also addresses this potential role for FOXO3a in LAC.

Studies have shown that FOXO3a is activated and elicits a pro-therapeutic response in cancer cells treated with therapeutic agents, including gefitinib, cisplatin and paclitaxel (Fernandez de Mattos et al. 2008, Krol et al. 2007, Sunters et al. 2003). In one study, the sensitivity of different breast cancer cell lines to paclitaxel was correlated with FOXO3a expression. In one cell line (MCF-7), paclitaxel-induced apoptosis was dependent of FOXO3a’s
transactivation of BIM (Sunters et al. 2003). Also, FOXO3a was shown to mediate the cytotoxic effects of cisplatin in different colon cancer cell lines (Fernandez de Mattos et al. 2008, Sunters et al. 2003). Similarly, FOXO3a was shown to mediate G1 cell cycle arrest and apoptosis in response to the EGFR inhibitor, gefitinib in different breast cancer cell lines (Krol et al. 2007). However, to date no such studies have been conducted on LAC cells. The same effect in LAC cells cannot be assumed because the underlying molecular aspects of these tumors that are expected to influence treatment response differ from those in breast and colon cancer. We therefore investigated the role of FOXO3a in the response of LAC cells to anti-mitotics, which are currently used to treat LAC, to determine whether FOXO3a alters or contributes to their therapeutic effect (ACS 2010).

Docetaxel and paclitaxel belong to the taxane family of anti-mitotic chemotherapeutics used to treat LAC among other solid tumor types (McGrogan et al. 2008). Evidence suggests that both inhibit cancer cells by the same mechanism of microtubule inhibition. Paclitaxel is naturally occurring and docetaxel is a synthetic analog (Mcgrogan et al. 2008). We initially tested whether FOXO3a was activated by docetaxel, using it as a representative taxane. Immunofluorescent confocal microscopy was again used to qualitatively determine whether FOXO3a was activated in LAC cells treated with this drug at therapeutically relevant concentrations. Indeed, we observed obvious docetaxel-induced localization to the nucleus within 7 hours of treatment, indicating functional activation by this drug (Fig. 3.1). Interestingly, confocal microscopy of cisplatin-treated cells did not show FOXO3a activation in NSCLC cells (data not shown). Cisplatin is a first-line therapeutic for LAC that was shown to induced FOXO3a-mediated cell cycle arrest and apoptosis in colon cancer cell lines (Fernandez de
Mattos et al. 2008). Given these results, we decided to pursue experiments designed to better understand FOXO3a’s response to microtubule-disrupting therapeutics in NSCLC. Included for comparison in the next experiments was a vinca alkaloid, vinorelbine, which is another class of microtubule inhibiting cancer therapeutic used to treat LAC (McGrogan et al. 2008). As described earlier, both vinca alkaloids and taxanes inhibit microtubule function by different mechanisms. Their cellular effect is most notably an induction of mitotic arrest of actively proliferating cells due to disruption of mitotic spindle function. These drugs, like most chemotherapy, exploit the proliferative aspect of tumor cells, inhibiting them in mitosis (McGrogan et al. 2008).

We again conducted FOXO3a transfection experiments and measured relative cell number in LAC cell lines following treatment with docetaxel or vinorelbine. MTS cell survival assays showed that, at various doses of docetaxel and vinorelbine, there were significantly fewer FOXO3a transfectants than vector control transfectants (Fig. 3.2). Next we proceeded to study whether a FOXO3a-dependent decrease in the relative number of living cells was a result of cell death or growth inhibition. As mentioned, FOXO3a was shown to induce Bim-dependent apoptosis in response to paclitaxel in breast cancer cells (Sunters et al. 2003). However, our results showed that FOXO3a elicited only a modest apoptotic response to both docetaxel and vinorelbine in two different LAC cell lines. This was observed at drug concentrations that caused a significant FOXO3a-dependent decrease in relative cell number (Fig. 3.3). Only at the highest concentrations tested was there pronounced apoptosis, but this was independent of FOXO3a. Caspase analysis and RT-PCR of pro-apoptotic effectors, BIM, BNIP3, and FASL, also indicated a very weak apoptotic response to these drugs (Fig. 3.4, Fig. 3.5). Necrotic cell death was also not
evident at any concentration tested (based on 7-AAD staining). These results were in contrast to those observed upon treatment with lung carcinogens in the same cell line, and suggested a distinct cellular response to anti-mitotics elicited by FOXO3a in these cells compared to that reported for breast cancer cells.

Based on these results we decided to study cell cycle distribution in our different experimental groups to gain a better understanding of the observed FOXO3a-dependent decrease in cell number following anti-mitotic treatment. FACS analysis showed an expected increase in mitotic fraction upon microtubule stress in vector control transfected cells. However, the FOXO3a transfectants displayed a substantial decrease in the percentage of cells in M phase with a corresponding increase in the percentage of cells in G1 phase under the same treatment (Fig. 3.6). This was evident in both A549 and H460 cells. This difference in cell cycle distribution suggested that FOXO3a may be mitigating the therapeutic effect (through mitotic arrest) of these drugs. This effect was strikingly distinct from that reported for breast cancer cells treated with paclitaxel. To assess FOXO3a’s ability to evoke this unexpected effect in LAC cells, we first analyzed mRNA expression levels of known FOXO3a effectors involved in cell cycle regulation, particularly G1, using RT-PCR. However, this analysis showed no change in expression levels of these effectors, which included Cyclin-D2 and p27^Kip1 (Fig. 3.7).

We next conducted a microarray expression experiment to gain a better understanding of the transcriptional and biological response elicited by FOXO3a to these drugs. Consistent with the modest apoptotic response of these cells to microtubule inhibitors, no changes in expression of known apoptotic effectors of FOXO3a were evident after treatment with
docetaxel (Figure A.4). Surprisingly, expression of very few cell cycle regulators was altered by the treatment as well. These results were starkly different from our microarray analysis of NNK treated LAC cells, which showed a broad FOXO3a-dependent pro-apoptotic and antiproliferative transcriptional response (Blake et al. 2010). Interestingly, our results did reveal a FOXO3a-dependent increase in CDC14A expression upon treatment with docetaxel. This was confirmed by RT-PCR, and was also evident with vinorelbine treatment in both A549 and H460 cells (Fig. 3.7). Because CDC14A was not a reported effector of FOXO3 we examined the 5’-region of the gene for FOXO binding elements (FBE). The CDC14A transcript has 6 splice variants that are encoded from a common promoter that contains five FBEs, suggesting that FOXO3a may directly regulate the expression of CDC14A in response to anti-mitotics.

CDC14A is a dual specificity phosphatase (DUSP) (Trautmann et al. 2002). DUSPs have the unique feature of being able to dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues within the same substrate. This is potentially explained by the DUSP catalytic pocket, which is wider than the one found in protein-tyrosine phosphatases (Mocciaro and Schiebel 2010).

CDC14A has been studied mostly in budding yeast or other non-mammalian models. However, human CDC14A shares 50% amino acid sequence identity with Cdc14 in budding yeast, and was able to fulfill all essential functions of budding yeast CDC14a in a rescue experiment (Vazquez-Novelle et al. 2010). This was demonstrated by transfection experiments in yeast that showed interaction of human CDC14A with CDC25 and an increase in dephosphorylated CDC25 (a known function of yeast CDC14A) when human CDC14A was
expressed in yeast (Vazquez-Novelle et al. 2005). In addition, when human CDC14A was expressed in CDC14-deficient mutant yeast, phenotypic defects of this mutant yeast were rescued. For example, the CDC14 mutant yeast grows normally at 30° C, but undergoes cell cycle arrest at 37° C. However, when yeast mutants expressed the human CDC14A, cell cycle progression at 37° was restored (Li et al. 1997). This suggests that some properties of CDC14 have been conserved between species.

In order to understand CDC14A’s function, we must discuss the cell cycle in a little more detail. As we mentioned earlier, cyclin-dependent kinases (CDKs) regulate the transition from one phase of the cell cycle to the next. For example, CDK1/Cyclin-B association and function is required for the transition of cells from G2 to mitosis and controls events in early mitosis such as chromosome condensation, nuclear envelope breakdown and assembly of the mitotic spindle. However, in metaphase, CDK1/Cyclin-B complexes must be depleted in order for cells to complete and exit mitosis (Weinberg 2006). So, CDK1 is essential for entry into mitosis, but must be inactivated for cells to complete and exit mitosis.

CDK1 is inactivated when it is phosphorylated on Thr14 and Tyr15 (Vasquez-Novelle et al. 2010). CDC25 activates CDK1 by dephosphorylating the protein at these sites (Vazquez-Novelle et al. 2010). CDC14A has been shown to inhibit CDC25 (through a yet-unknown mechanism), thereby negatively regulating CDK1 and blocking entry into mitosis (Vasquez-Novelle et al. 2010). Overexpression of CDC14A also promotes CDK1 inactivation and exit from mitosis. Western analysis also showed that expression of a wild type CDC14A coincided with decreased Cyclin-B levels (Vinistin et al. 2008). For example, a decrease of Cyclin-B protein was
found in human osteosarcoma cancer cells when CDC14A was overexpressed (Kaiser et al. 2002). These experiments also showed a decreased level of tubulin and cytokinesis (demonstrated by confocal microscopy) when CDC14A was overexpressed (Kaiser et al. 2002). The exact mechanism through which negative regulation of Cyclin-B occurs is unclear, but CDC14’s dephosphorylation of Cdh2 (an APC inhibitor) activates APC (anaphase-promoting complex), which has been shown to target Cyclin-B for ubiquitination and degradation (Bloom et al. 2007). These findings suggest that CDC14A inhibits entry into mitosis by inhibiting CDC25 activation of CDK1 and promoting Cyclin-B degradation. It also may promote the exit of mitotic cells by promoting CDK1 inactivation.

CDC14A’s function and regulation as of yet has not been characterized in great detail. However, immunohistochemistry experiments have indicated that CDC14A is inactive and present in nucleoli (and associated with Net1) during most of the cell cycle. However, during anaphase, FEAR (fourteen early anaphase release) and MEN (mitotic exit network) stimulate nuclear exit of CDC14A into the cytoplasm where it colocalizes with centrosomes (Bloom et al. 2010, Kaiser et al. 2002). The FEAR and MEN regulatory networks are comprised of several proteins including polo kinase, CDC5, separase ESP1, kinetochore-associating proteins SLK19 and SPO12, and DBF2 kinase. Although the regulation of CDC14A by FEAR and MEN is not well understood, current evidence suggests that FEAR initiates CDC14A release from the nucleolus during early anaphase, and MEN plays a role in localizing CDC14A to the cytoplasm (Bloom et al. 2007, Stegmeier and Amon 2004). In the FEAR network, it is believed that CDC5 is the ultimate effector, as CDC5 has been shown to interact with CDC14A/Net1, phosphorylate both proteins, and cause a dissociation of the two proteins. In the MEN network, DBF2 is then believed to
phosphorylate the freed Net1 protein to sustain its dissociation with CDC14A (Stegmeier and Amon 2004). Further work is required to elucidate how exactly CDC14A is regulated by FEAR and MEN.

Interestingly, at high levels of expression, CDC14A has been demonstrated to inhibit microtubule re-assembly from centrosomes, induce spindle defects, premature chromosomal segregation (in the form of micronuclei and fragmented nuclei), and promotion of cytokinesis as a result of CDC14A localization at the cleavage furrow (Kaiser et al. 2002, Mailand et al. 2002). Also, Mailand et al. demonstrated that CDC14A downregulation using RNAi caused failure of centrosome segregation and multinucleation (Mailand et al. 2002). Taken together these studies indicate that CDC14A plays a complex and significant role in promotion of mitotic completion and exit (Figure 4.1). However, this role is not certain as a colorectal carcinoma cell line with CDC14A deleted (Cre/Lox site specific recombination) had normal viability and cell cycle distribution (Mocciaro et al. 2010). However, this may be unique to this particular tumor type or cell line. Further studies will clarify the details of CDC14A’s cellular function.
Figure 4.1. CDC14A plays a complex role in mitotic exit/completion. CDC14A is sequestered in the nucleus by NET1. During anaphase, FEAR/MEN promote nuclear exit where CDC14A inhibits CDC25 function and stimulates a range of processes that block entry into mitosis and stimulate the exit of cells in mitosis.

As mentioned previously, over-expression of CDC14A can induce premature mitotic exit and cytokinesis of mammalian cells (Kaiser et al. 2002, Mailand et al. 2002). Interestingly, FOXO3a may then induce mitotic exit by up-regulating CDC14A expression in LAC cells. This would account for the decrease in mitotic fraction of cells treated with anti-mitotics. Notably, we observed no cell death prior to the FACs analysis of cells treated with the anti-mitotics in which the FOXO3a-augmented decrease in mitotic cells was observed. These results suggest
that FOXO3a may elicit this effect via CDC14A upregulation in LAC cells. The subsequent decrease in the number of cells in the absence of significant cell death suggests that these cells are growth inhibited or arrested either by a mechanism that involves inhibition of entry into mitosis and/or mitotic exit with damaged spindles. The results therefore suggest that a cytostatic effect is elicited by FOXO3a in LAC cells in response to these drugs that may be mediated by CDC14A. Further study is needed to confirm the role of CDC14A in the mitigation of mitotic arrest induced by anti-mitotics in LAC cells and to understand the mechanism of cytostasis stimulated by FOXO3a in response to these drugs.

Our results suggest that FOXO3a stimulates a cytostatic effect in LAC cells treated with anti-mitotic chemotherapeutics docetaxel and vinorelbine. Specifically, the results that show FOXO3a-dependent decrease in LAC cell number when treated with docetaxel and vinorelbine. The presence of FOXO3a in LAC may contribute to the efficacy of these drugs as it decreases the tumor cell population, which suggests a pro-therapeutic effect. As such, FOXO3a loss, occurring relatively frequently in LAC, may also result in a decrease in the efficacy of these drugs in tumors lacking FOXO3a function. Functions in addition to FOXO3a, also contribute to the efficacy of these drugs, such as MAD2 and BRCA1 which have been shown (through knockdown experiments) to sensitize gastric and breast cancer cell lines to paclitaxel and vinorelbine, and induce protein expression of cytochrome c, caspase 3, and apoptosis (Du et al. 2006, LaFarge et al. 2001). As such, the actual effect of FOXO3a on the efficacy of microtubule inhibitors requires further investigation in vivo. One such approach could be to use xenografts of LAC cell lines,
both with and without FOXO3a, in nude mice to determine whether the therapeutic effect of these drugs (tumor shrinkage) correlates with FOXO3a expression.

Finally, our findings suggest a potentially novel role for FOXO3a in cell cycle regulation. Specifically, we demonstrate that FOXO3a is able to mitigate mitotic arrest caused by docetaxel and vinorelbine in LAC cells. Our results suggest that this is not through conventional FOXO3a cell cycle effectors p27Kip1 and Cyclin-D2, but possibly through an upregulation of CDC14A. An approach to investigate the necessity of CDC14A in this effect would be to repeat the described experiments following RNAi inactivation of CDC14A. If the cell cycle effect is lost upon inactivation of CDC14A, it would indicate the necessity of CDC14A. If the effect is diminished when CDC14A is inactivated, a partial role of CDC14A would be indicated.

4.3 Future directions in the study of FOXO3a in LAC and cancer

We have identified FOXO3a as a novel site of frequent deletion in LAC of smokers. Precedence suggests that its inactivation contributes to LAC development. We went on to show that FOXO3a is functionally activated by and elicits an apoptotic response, via a transcriptional program, to lung carcinogens BPDE and NNK (Blake et al. 2010). This supports our hypothesis that FOXO3a plays a role in protecting against LAC by eliminating carcinogen-damaged cells, and that the selective loss of FOXO3a increases the likelihood that this damage will lead to LAC. Based on these findings, future studies are now needed to more directly test this hypothesis.
FOXO3a’s role in tumor formation could be investigated in a xenograft model focusing on FOXO3a as the tested variable. Mouse xenograft models are commonly used to assess the tumorigenic potential of cell lines whereby cells are injected subcutaneously in nude or immuno-compromised mice and tumor formation and tumor cell growth are measured. A simple approach to assess FOXO3a’s tumor-suppressing abilities would be to compare LAC cell lines that still express FOXO3a compared to those that do not. In concordance with our results, we would expect FOXO3a-deficient cell lines to more readily develop tumors compared to FOXO3a expressing cell lines. One limitation of this study is that the variation in the genetic makeup of the different cell lines would also have an effect on tumorigenesis. In other words, cancer cells acquire distinct molecular changes throughout their development in addition to FOXO3a expression, and the tumor growth could be dependent on molecular changes other than FOXO3a expression. To account for this variance, several cell lines may be needed to determine whether FOXO3a expression correlates with the effect on tumor burden. This is a common approach that has also been used to study the relationship between certain functions and other endpoints such as therapeutic efficacy of a drug, as cited earlier in the evaluation of FOXO3a’s effect on the efficacy of paclitaxel in breast cancer (Sunters et al. 2003).

A second approach that would lessen the effect of the variation in tumor burden conferred by genetic changes other than FOXO3a would be to knock down FOXO3a expression by RNAi in individual cell lines and compare their tumorigenic potential with and without FOXO3a. Alterations upstream (e.g. Akt) and downstream (e.g. BIM) would need to be accounted for in these cell lines as these alterations may be mutually exclusive with FOXO3a loss abrogating any effect of FOXO3a knockdown on tumor burden in cell lines that have
already acquired a malignant phenotype. Again, several cell lines would need to be used to account for such effects.

A third approach would be to introduce FOXO3a into cell lines that have undergone deletion of the gene at some point during malignancy. The loss of tumor burden would indicate that FOXO3a restoration has a mitigating or suppressing effect on tumor growth. Physiologic expression levels would be needed to accurately assess the effect of FOXO3a in this system. This could be accomplished by establishing stable clones, following transfection, that express FOXO3a at physiologic levels. This could be determined by comparing expression to that in non-cancerous lung epithelial cells. Another approach that would offer more information on FOXO3a’s role in lung carcinogenesis would be to investigate the effect in FOXO3a-knockout mice, which are commercially available (Paik et al. 2007). This more informative approach compared to the previous three would investigate the effect of FOXO3a loss on de novo tumor development. It would also enable the investigation of the suppressive effects of FOXO3a against specific human lung carcinogens against which we FOXO3a mounts a protective anti-carcinogenic apoptotic response. If there is a higher carcinogen-induced LAC burden in the FOXO3a knockouts compared to the wild-type, it would indicate a broader role in suppressing LAC beyond that of protecting against carcinogenic induction. Such a role might relate to its responsiveness to other cancer related physiologic stresses such as oxidative damage caused by reactive oxygen species, hypoxia, and nutrient deprivation all of which are encountered by developing solid tumors (Hanahan and Weinberg 2000). Many tumor suppressors are gene dose dependent in their effect, such that heterozygous loss confers some partial effect on tumorigenesis. This effect of FOXO3a could be investigated in the mouse model as well, which
would provide a better understanding of its role as a tumor suppressor. The relationship between smoking and other environmental exposure and FOXO3a loss in LAC could also be addressed directly in surgically resected tumors if history of smoking and other exposure is known.

The results of our analysis of FOXO3a loss do not preclude the inactivation of FOXO3a in both LAC and LSqCC by other mechanisms typical of cancer. Our analysis did not investigate other mechanisms of inactivation other than deletion and mutation in these tumors. Other known mechanisms of gene inactivation include gene promoter silencing by hypermethylation, and post-transcriptional degradation mediated by microRNA. It would also be informative to assess cellular localization of FOXO3a in samples that did not appear to undergo allelic loss of FOXO3a. FOXO3a is inactive as a transcription factor when present in the cytoplasm. An assessment of FOXO3a localization in all our tumor samples would further verify if samples that did not undergo allelic loss of FOXO3a in significant amounts are still able to express active FOXO3a protein. Since FOXO3a is an antagonist of the PI3K/Akt cell proliferation pathway, an upregulation in any proteins upstream of FOXO3a could lead to its inactivation, such as upregulation/hyperphosphorylation of EGFR, PI3K, Akt, ERK, or IKK. Interestingly, upregulation of several of these proteins is commonly reported in a variety of cancer tumor types, including LAC and LSqCC (Brunet et al. 1999, Greer and Brunet 2005, Sekido et al. 2003).

It would be informative to know whether FOXO3a inactivation interacts in LAC development with other known molecular changes in LAC, such as KRAS mutation, and p53 and p16\(^{INK4a}\) inactivation. This could be investigated directly in tumors to see whether such changes
may or may not cooperate in tumor development. This would provide additional insight into FOXO3a’s role in LAC development and broader understanding of how these tumors develop.

FOXO proteins are somewhat redundant in their function. This was shown in *in vitro* models where FOXO proteins share common target genes and are activated by similar stresses, such as oxidative stress and DNA damage (Greer and Brunet 2005). They have also been shown to have an additive effect on susceptibility to tumor development (Paik et al, 2007). Therefore, future studies are needed to determine whether other FOXOs are inactivated in LAC and LSqCC, contributing to their development.

To investigate the functional redundancy of FOXOs, expression profiles by microarray analysis of each FOXO transcription factor in response to individual stresses would be one informative approach. This might be accomplished in cells overexpressing one particular FOXO following transfection. Conversely, cell lines from single, double or triple FOXO knockout mice would provide data on functional redundancy as well. Although functional redundancy is already evident, the tissue and cell type specific differences in their expression and function should account for differences in their respective physiological roles. Therefore, of interest to us would be to conduct microarray experiments in lung cancer and non-cancerous lung epithelial cell lines using knockdowns of each FOXO protein to assess functional variation and redundancy as it pertains to LAC and NSCLC. This would address whether FOXO3a acts independently or not of other FOXOs in this type of lung cancer, and if not, what other FOXOs are likely involved.
Our findings indicate that FOXO3a loss not only plays a role in LAC development, but also how these tumors respond to therapy. Further study is needed to fully elucidate its role in response to anti-mitotics as well as other drugs used to treat LAC. As mentioned, it appears that FOXO3a elicits a cytostatic response and a modest cytotoxic response to different classes of anti-mitotics in LAC. A measurement of proliferation rate is needed to confirm this response. This is distinct from its reported role in other cancer types treated with these classes of anti-mitotics. This suggests a cell/tumor type dependent response determined by differences in their genetic and expressional composition and regulation of FOXO3a. To investigate the latter, SIRT1 expression/FOXO acetylation has been shown to shift FOXO’s function away from apoptosis and towards cell cycle regulation. As was previously mentioned, FOXO acetylation has been shown to occur through direct acetylation of FOXO4, which was detected at three of its lysine residues through western analysis. Mutation of these lysine residues enhanced FOXO transcriptional activity (p27^Kip1 expression) (Fukuoka et al. 2003). Sirt2 otholog expression in yeast, worms and flies has been shown to extend longevity. Consistent with this role, in mammalian cells, oxidative stress has been shown to induce FOXOs and SIRT1 interaction in response to oxidative stress as an apparent measure to reverse the damage through upregulation of MnSOD (Brunet et al. 2004). Therefore, investigating FOXO3a’s interaction with SIRT and acetylation would prove insightful because FOXO3a’s response to microtubule inhibitors seems similarly diverted away from apoptosis. This can be done by investigating known acetylation sites by western analysis using acetylation site specific antibodies that are commercially available. To investigate the role of SIRT, in the cytostatic response we would perform the same experiments as already described with and without SIRT1 targeted
knockdown by RNAi. Loss of the cytostatic response to the drugs would indicate an effect elicited by SIRT1.

The mechanism of cytostasis itself needs to be elucidated. Our results indicate that FOXO3a mediates a decrease in M phase with a corresponding increase in G1. This suggests two likely possibilities: quiescence or senescence. Cellular senescence is a permanent exit from the cell cycle in either G1 or G2 as a consequence of terminal differentiation. Quiescence is a temporary exit from G1 to G0 under conditions unsuitable for cell proliferation, such as lack of mitogens (Ewald et al. 2010). Interestingly, docetaxel in combination with carboplatin has been shown to induce senescence in lung cancer. Senescent cells can be detected by various means, due to their enlarged appearance and increased SA-β-galactosidase activity (Ewald et al. 2010). The cellular decision between apoptosis and senescence is dependent, in part, on the magnitude of stress, with lower levels triggering senescence. We observed evidence of FOXO3a-mediated cytostasis only at the lower microtubule inhibitor concentrations with apoptosis occurring at the highest concentrations. Further study is needed to determine whether this mechanism of cytostasis is senescence and whether it involves CDC14A upregulation.

Furthermore, in vitro studies have shown that RNAi of known spindle checkpoint proteins BUBR1, CENPE, and Aurora A confer resistance and reduced apoptosis in response to microtubule inhibitors in breast cancer, colon cancer, and lung cancer cell lines (Gascoigne and Taylor 2008, Harrison et al. 2009, McGrogan et al. 2008). BUBR1, CENPE, and Aurora A are expressed at high levels and localize to kinetochores in G2 cell cycle in order to block entry into
anaphase (McGrogan et al. 2008, Mussachio et al. 2007). Determining BUBR1, CENPE, and Aurora A mRNA and protein expression levels (and their localization on kinetochores using confocal microscopy) would further determine whether FOXO3a expression is inducing a cytostatic response to microtubule inhibitors through regulation of these spindle checkpoint proteins.

The detailed mechanism of FOXO3a’s response to anti-mitotics would be an obvious objective to pursue. This might be investigated through a more comprehensive expression microarray study. The microarray experiment discussed in this thesis was of a single time point (6h), and a single concentration of docetaxel, which may have missed important genes involved in the observed FOXO3a response to this stress. We essentially observed the same FOXO3a-mediated response to both vinorelbine and docetaxel and several different doses in two different cell lines. Not all FOXO3a effector genes are expected to be transcriptionally activated at the same time and within 6 hours of a treatment. The most valuable information to be gained from a microarray experiment would therefore be not to repeat what was done, but to investigate several time points. We would expect a more complete understanding of the observed cytostatic response to emerge from data obtained at both earlier (~2 hours) and later (12 and 24 hours) time points following treatment. A more specific analysis of FOXO3a effectors that might contribute to the FOXO3a-mediated cytostatic response would be to examine Cyclin-B, Cyclin-G2, and Plk1, each of which is a FOXO3a effector. Plk1 plays a role in mitotic exit by promoting chromosome separation, phosphorylating and activating CDC25 (which dephosphorylates and activates Cyclin-B/CDK1), and by localizing to centromeres and interacting with CDC14A (Rahal and Amon 2008, van de Weerdt and Medema 2006). Cyclin-B
positively regulates CDK1, which is required for transition from G2 to mitosis. FOXO3a has been shown to transcriptionally downregulate Cyclin-B, which could contribute to the observed decrease in mitotic cells following treatment with microtubule inhibitors (Alvarez et al. 2001). Cyclin-G2 has not been well characterized, but studies have shown it to be expressed at higher levels in mouse lung tissue than Cyclin-D2 and also that its upregulation is involved in G1 arrest by inhibiting transition to S-phase when cells were exposed to growth inhibiting molecule (Horne et al. 1996, Horne et al. 1997). FOXO3a has been shown to cause upregulation of Cyclin-G2 mRNA when transfected in mouse fibroblasts (Tran et al. 2002). Whether Cyclin-B, Cyclin-G2, or Plk1 are effectors of FOXO3a specifically in response to antimitotics remains to be determined.

The actual regulation of CDC14A expression by FOXO3a needs to be confirmed by either of two common approaches, chromatin immunoprecipitation (ChIP) or luciferase reporter assays using the 5′-upstream region that harbors putative FHBE sequences. These assays would directly measure FOXO3a binding (ChIP) to the FHBEs and increased transcriptional activity from these sequences. In the latter case, a luciferase reporter assay would be conducted in FOXO3a and control vector transfected cells to ascertain the effect of FOXO3a on increase reporter activity. Also, as already noted, it is of interest to determine whether CDC14A’s upregulation by FOXO3a in cells with disrupted mitotic spindles leads to or triggers cytostasis. CDC14A knockdowns followed by a cell cycle analysis using FACS would directly address this. An in vivo study would be helpful to establish the extent to which FOXO3a elicits any therapeutic effect in response to microtubule inhibitors. As alluded to earlier, this can be done with mouse xenografts using cell lines that express FOXO3a and cell lines that do not express endogenous

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FOXO3a, cells with FOXO3a knocked down, or with restored expression of FOXO3a. The mice would be treated with docetaxel/vinorelbine and xenografts tumor growth measured. Based on our results, we would predict that tumor growth would be inhibited in FOXO3a-expressing xenografts. Additionally, a study could be conducted in resected lung cancer samples from patients who have undergone this type of chemotherapy. FOXO3a status in these tumors could be ascertained using qPCR and patient chemotherapy regimen correlated with treatment response. Typically, both partial and complete response (PR and CR) to treatment are determined by specific criteria assessing changes in tumor volume/size over time. Response would then be correlated with FOXO3a deletion status to determine any relationship. Such an analysis would most probably require participation in a phase III clinical trial of LAC patients for taxanes and vinca alkaloids. As these trials have already been completed, retroactive examination of existing specimens would be required.

In addition, it is of interest to determine the response of FOXO3a to other drugs used to treat LAC, using the same approach as taken with the anti-mitotics. LAC is treated with different combinations of drugs, including platins, EGFR inhibitors, anti-metabolites, and topoisomerase inhibitors. Knowing FOXO3a’s response to these drugs would provide a more complete understanding of its role in therapeutic outcome.

4.4.1 A role for FOXO3a in lung carcinogenesis

Certain questions arise as to what role FOXO3a plays in lung carcinogenesis. For example, FOXO proteins have been shown to act redundantly. This has been shown in cell
culture where FOXO1, FOXO3a, and FOXO4 appeared to regulate common target genes and bind to the same target sequences (Brunet et al. 1999, Furuyama et al. 2000). In addition Paik et al. demonstrated in a mouse knockout model three FOXO genes (FOXO6 was not examined) required deletion in order for thymic lymphomas and hemangeomas to develop (Paik et al. 2007). Our analysis of lung adenocarcinomas determined that FOXO1 and FOXO4 were not deleted in tumors, but FOXO3a was at a relatively high frequency. As already noted, further analysis is needed to determine whether these other FOXO genes are inactivated by mechanisms other than deletion. Therefore, it appears that, out of all FOXO proteins, FOXO3a is the specific target for deletion in our samples. FOXO3a mRNA is expressed in the lung at a higher level than FOXO1 and FOXO4. Specifically, BioGPS (a website that compiles gene expression datasets of various genes across tissue types) reports that mRNA expression level of FOXO3a is almost three times higher than FOXO1 and FOXO4 in the lung (BioGPS: http://biogps.gnf.org/). This suggests a larger role for FOXO3a in this tissue type with its deletion perhaps having a relatively higher phenotypic impact on a cell and tumor development.

Perhaps facilitating FOXO3a’s deletion is also the presence of a fragile site (FRA6F) within the FOXO3a gene, with a reportedly active region in intron 2. Fragile sites are regions of the genome that are subject to breakage due to their composition that renders their replication difficult. Consequently, these sites are prone to incur deletions (Durkin and Glover 2007). Frequently, what is observed are large deletions of tens to hundreds of kilobases directly within a fragile site region. The mechanism is poorly understood, but evidence suggests that fragility results from a high content of AT repeats. These repeats may lead to secondary structure
formation (in the form of hairpin loops) that when encountered during replication causes stalling of replication machinery and leads to a higher rate of strand breakage and consequential deletions and rearrangements (Durkin and Glover 2007).

Fragile site stability is affected by several mechanisms of chromosome maintenance. Evidence has shown that disruption of the replication machinery can serve to “activate” fragile sites. For example, aphidicolin (an inhibitor of DNA polymerase α) is a well known inducer of fragile site activation when used at low concentrations that only partially inhibits the DNA replication machinery (Glover et al. 2005, Glover 2006). In addition, Ataxia-telangiectasia and Rad3-related (ATR) checkpoint kinase also plays a role in activation of fragile sites by directing a cell cycle checkpoint in response to stalled replication at fragile sites. Indeed, cells lacking ATR showed a dramatic increase in fragile site activation when treated with aphidicolin (Schwartz et al. 2005).

Interestingly, treatment of cells with low doses of camptothecin (a topoisomerase I inhibitor) almost completely prevented aphidicolin-induced fragile site breaks (Schwartz et al. 2005). Also, it was demonstrated that nonhomologous end joining (NHEJ) contributes to fragile site stability (Schwartz et al. 2005). In their experiments, Shwartz et al. observed that Rad51, DNA-PKCs, and Ligase IV NHEJ proteins formed foci at known fragile sites during aphidicolin treatment of HeLa cells. An increase in fragile site activation was then observed when any of these genes were knocked down using RNAi (Schwartz et al. 2005). Based on available data, Arlt et al. proposed a mechanism of fragile site activation whereby continuous stalling of the replication machinery causes unwinding of the DNA, formation of secondary structures in single
stranded DNA, and a higher rate of breakage (Arlt et al. 2006). Breakage at these sites is thought to occur as a result of several possibilities including increased focal nuclease activity, deficient repair, checkpoint escape, or mechanical force exerted at these sites by the replication machinery (Arlt et al. 2006).

Fragile sites are also more active in cancer cells due to inherent chromosome instability (CIN) caused by defects in chromosome maintenance and by exposure to DNA damaging carcinogens that have been shown to induce a CIN phenotype. Both can exacerbate fragile site instability directly or indirectly by causing mutations in genes that stimulate fragile site activity (such as ATR and BRCA1, which when knocked down, have been shown to activate fragile sites in cancer cells) (Arlt et al. 2004, Arlt et al. 2006). This increased fragile site activity has an effect on genes that contain them. Genes present at these sites are susceptible to deletion resulting from fragile site activation. Darwinian selection of these deletions can occur in cancer if the gene normally plays a role in suppressing malignant growth. A common example of this is the proposed tumor suppressor, FHIT. The \textit{FHIT} gene is composed of 10 exons distributed over 1.5 Mb. Two of its introns span several hundred kilobases where a fragile site (FRA3B) is located. \textit{FHIT} is frequently the site of biallelic loss due to deletions within the FRA3B region in various forms of cancer including gastric cancer, head and neck squamous carcinomas and lung cancer (Huebner and Croce 2001). Importantly, smokers have been shown to contain expressed fragile sites at various locations (including FRA3B) at higher rates than non smokers (Huebner and Croce 2001, Stein et al. 2002). Therefore, it appears that tobacco carcinogens can increase the rate of fragile site activity, which can be caused by replication delays as the cell attempts to repair DNA damage or adducts (perhaps by NJEH) incorporated in the fragile sites.
The FRA6F fragile site is a 1.2 Mb region that spans 6q21 and an active region within intron 2 of FOXO3a (Morelli et al. 2002). FRA6F has been implicated as a cause of DNA losses at its location on chromosome 6q21-22 in human cancer (Arlt et al. 2006). CGH analysis comparing LAC of smokers and non-smokers showed a higher frequency of allelic loss at 6q in tumors of smokers (Sy et al. 2003). Lung adenocarcinomas of smokers also have been shown to have more extensive CIN than those of non-smokers. Our lab has reported that NNK-induced lung adenocarcinoma have much more extensive CIN than the same tumors developing in the absence of an inducing agent (Herzog et al. 2004). Interestingly, fragile sites are also well-conserved among mammals, and the same pattern of FOXO3a deletion was observed in mouse LAC, occurring predominantly in tumors induced by carcinogens (Herzog et al. 2009). The structure of the FOXO3a locus may therefore be prone to carcinogen-induced disruption perhaps also mechanistically linked to CIN, resulting in the occurrence of relatively precisely positioned FOXO3a HDs that we observed in LAC.

Based on these findings, we propose a model in which continuous carcinogenic stress caused by tobacco smoke leads to extensive CIN and activation of FRA6F and deletion of the FOXO3a gene. In this model, we speculate that exposure to tobacco carcinogens leads to increased DNA adducts and secondary genetic/chromosomal damage which at fragile sites exacerbates their instability. Secondary chromosomal damage also has been shown to lead to CIN as a consequence of selective changes that increase cellular tolerance to the DNA damage (Lengauer et al. 1998). In this scenario, increased repair activity at FRA6F may lead to a higher rate of escape of unrepaired damage and secondary disruption of FRA6F via the mechanisms discussed above. For example, repair activity involving unwinding of DNA would lead to
secondary structure formation (e.g. hairpins) within the FOXO3a region, which further perturbs the replication process. Although many of these perturbations would be detected and repaired, the high volume would increase the likelihood that some could escape repair resulting in breakage and deletions within this region of the FOXO3a gene.

This could potentially be studied by using yeast artificial chromosome (YAC) probes that map to the FRA6F fragile site region and using FISH to quantitate signals in BPDE treated and untreated lung adenocarcinoma cell lines. This protocol has been used to successfully quanititate fragile site activity in HeLa and MCF cells treated with aphidicolin (Schwartz et al. 2005).

Based on our findings FOXO3a loss contributes to early stage LAC development. We found FOXO3a allelic loss in all stages of LAC. However, the majority of the tumors analyzed were stage I tumors. Of the tumors analyzed, 85% of LACs and 47% of LSqCC have loss of at least one allele of FOXO3a. This correlates to some degree with studies which have identified frequent allelic loss at 6q in LAC (36.7%) and LSqCC (28-40%) (Petersen et al. 1997, Sanchez-Cespedes et al. 2003). Without analysis of premalignant lesions it is only speculative to attempt to ascertain at what specific point of lung cancer development FOXO3a may actually be deleted and therefore contribute to tumor development. Our evidence therefore can be interpreted to indicate that FOXO3a is selectively deleted at stage I or prior. This suggests a potential primary role in tumor initiation, survival of cells with carcinogen-induced DNA damage and/or malignant conversion, but no primary role in metastasis, which occurs in subsequent stages of tumor development (stage III and IV). Interestingly, Goeze et al. found frequent allelic loss on 6q21
associated with an acquisition of metastatic phenotype (Goeze et al. 2002). Specifically, this study used CGH to compare chromosomal aberrations in 23 primary adenocarcinomas and their metastatic pairs, and found, among other genomic aberrations, allelic loss on 6q21-qter to associate highly with a metastatic phenotype (greater than 50% of samples had an allelic loss on 6q) (Goeze et al. 2002). Therefore, this study provides support for an allelic loss of 6q21 (and perhaps FOXO3a) to correlate with late events in cancer development e.g. the acquisition of metastatic properties. Our results suggest that either another gene on 6q21-ter contributes to the metastatic phenotype or that FOXO3a loss contributes to it secondarily. The latter is not supported by reports showing an absence of FOXO3a effectors with roles in tumor metastasis.

In regard to earlier stages of cancer development, it seems unlikely that FOXO3a plays a role in cancer susceptibility, as FOXO3a knockout mice do not appear to develop cancer of any kind. As for pre-neoplastic stages, Wistuba and Gazdar and Lantuejoul et al. have reviewed the findings pertaining to pre-neoplastic lesions of LAC, but no investigation of FOXO3a allelic loss or chromosome 6q (where FOXO3a is located) was cited (Lantuejuol et al. 2009, Wistuba and Gazdar 2006). A study specifically of FOXO3a allelic loss in pre-neoplastic tissues therefore is needed to directly address this issue. According to our findings FOXO3a mediates apoptosis in response to carcinogenic damage. FOXO3a could therefore conceivably contribute to tumor initiation or the emergence of a genetic instability phenotype, both of which occur in premalignant NSCLC (Lantuejuol et al. 2009, Wistuba and Gazdar 2006).

Based on our findings, allelic loss of FOXO3a is likely to occur at any stage leading up to stage I malignancy (Figure 4.2). It is tempting to speculate whether FOXO3a allelic loss and p53
inactivation may be mutually exclusive since both FOXO3a and p53 have similar functions (cell cycle arrest and apoptosis in response to DNA damage) and their inactivation occurs at similar frequency in LAC. This interesting possibility requires further study.

Our findings in LSqCC show that FOXO3a undergoes no homozygous deletions (allelic loss >80%) in stage I-IV, however only 19 tumors were examined. It is possible that the second allele of FOXO3a is inactivated at later stages of LSqCC development or that FOXO3a is inactivated through other means in this tumor type (such as mutations, translational defects, or cytoplasmic retention). A more thorough analysis of FOXO3a status in later stage tumors (stages II-IV) needs to be conducted before we can make more conclusive statements regarding FOXO3a status in LSqCC.
Figure 4.2. FOXO3a undergoes allelic loss at early stages of lung carcinogenesis. The accumulation of molecular abnormalities has been shown to involve many stage-specific changes.
Unfortunately, LAC pathogenesis has not been studied as extensively as LSqCC. So far, it is known that smokers more frequently develop activated KRAS mutations, and non-smokers develop mutations in EGFR (Lantuejoul et al. 2009, Wistuba and Gazdar 2006). These appear to be mutually exclusive as they affect a common signal transduction pathway. KRAS mutations have been detected in 20-30\% of premalignant AAH (atypical alveolar hyperplasia) (Wistuba and Gazdar 2006). Our human tumor samples are derived almost exclusively from smokers. Therefore, FOXO3a loss would appear to cooperate in some way with KRAS mutations in LAC. Again further study of premalignant lesions is needed before FOXO3a deletion can be accurately placed in any model of LAC development.

However, we also propose the following functions for FOXO3a that explain its deletion in stage I LAC. First, our results suggest that FOXO3a stimulates apoptosis in response to DNA-damaging tobacco carcinogens. Our expression microarray results also indicate a role in cell cycle inhibition and DNA damage (Blake et al. 2010). As such, FOXO3a seems to play a protective role in some early stage of LAC.

p53 and p16^{INK4a} inactivation are known to occur in late preneoplastic/early carcinoma stages (Lantuejoul et al. 2009, Wistuba and Gazdar 2006). FOXO3a is functionally similar to these proteins (apoptosis/cell cycle arrest in response to DNA damage/hypoxia response). p53 also has been shown to keep the oncogenic function of activated KRAS in check by eliciting growth arrest or apoptosis in cells with oncogenic KRAS (Johnson et al. 2001). This would appear to account for the occurrence of p53 inactivation in later stages than KRAS activation in lung carcinogenesis. It is therefore possible that FOXO3a inactivation functions as an alternative
to p53 and possibly p16 inactivation. In this regard FOXO3a potentially keeps activated KRAS’ oncogenic function in check.

FOXO3a is inactivated by EGFR/PI3K/Akt and KRAS/ERK. As mentioned, EGFR/PI3K/Akt and KRAS/ERK are frequently hyperactivated in mutual exclusion in LAC (Sekido et al. 2003, Wistuba and Gazdar 2006). When stress-activated, FOXO3a overrides this inhibition (Greer and Brunet 2005). Consequently, FOXO3a is able to keep the proliferative signal of EGFR/PI3K/AKT and KRAS/ERK in check by inducing cell cycle arrest and/or apoptosis in response to stress, much like p53 is able to keep activated KRAS in check. Therefore, FOXO3a deletion would enable DNA damage to persist while enabling the growth promotion of activated KRAS/ERK in LAC of smokers. In this sense, FOXO3a may be an alternative to p53 inactivation occurring after KRAS mutational activation in LAC. Examination of FOXO3a status in LAC of non-smokers is needed to ascertain whether it also obviates oncogenic stimulation through EGFR/PI3K/AKT.

4.4.2 FOXO3a interacts with co-factors in order to carry out its function

In the case of its protective cellular function in response to carcinogens, FOXO3a’s apoptotic function might be dependent on its interaction with other proteins. p53 is a potential candidate gene that plays a role in FOXO3a’s downstream signaling response. FOXO3a has been shown to promote p53 stability and may contribute to its apoptotic function. Induced expression of functionally active FOXO3a was shown to increase the half-life of p53 from 20 minutes to ~90 minutes (You et al. 2006). FOXO3a’s interaction with p53’s DNA binding domain (DBD) is thought to cause this extension of its half-life as p53 is ubiquitinated at the DBD and
FOXO3a’s location at the DBD could be inhibiting ubiquitination of p53 (Wang et al. 2008). Furthermore, You et al. demonstrated that cells that expressed active FOXO3a were able to induce apoptosis and the expression pro-apoptotic p53 effectors, PUMA and Bax in response to DNA damage (You et al. 2006). This required wild type p53 function. Finally, cytosolic fractioning showed that expression of active FOXO3a caused an increase in cytosolic p53 protein and its association with nuclear exporting protein CRM1 (demonstrated by immunoprecipitation) (You et al. 2006). These results indicate that under DNA damage, FOXO3a is able to interact with and regulate p53’s pro-apoptotic activity. These results also are consistent with the possibility that FOXO3a and p53 may be alternatively inactivated in LAC.

The mechanism by which DNA damage activates FOXOs has also been investigated and evidence suggests involvement of the DNA damage response protein ATM and CHK1 (Guo et al. 2002, Huang et al. 2006). FOXO3a has been shown to interact with ATM in response to DNA damage (Tsai et al. 2008). This was demonstrated in breast cancer cell lines where FOXO3a levels were knocked down using siRNA. These experiments showed that FOXO3a interacted with ATM (shown using confocal microscopy and immunoprecipitation) and that FOXO3a expression promoted ATM phosphorylation and activation, induced cell cycle arrest, and reduced DNA damage when exposing cells to ionizing radiation (Tsai et al. 2008). CHK1 is activated by ATM and is a checkpoint kinase that activates G1 cell cycle arrest in response to DNA damage. Specifically, in response to DNA damage, ATM/ATR activates CHK1 though phosphorylation, and activated CHK1 phosphorylates and inactivates CDC25. Inactivated CDC25 then induces mitotic arrest by inhibiting Cyclin-B/CDK1 complex (Kastan and Lim 2000, Sanchez et al. 1997). CHK1 has been shown to activate FOXO1 under conditions of DNA damage.
Specifically, during treatment with topoisomerase inhibitors, phosphorylated FOXO1 levels were shown to decrease in a CHK1-dependant manner in human prostate cancer cells (Huang et al. 2006). When CHK1 was knocked down, FOXO1 remained in phosphorylated form when exposed to topoisomerase inhibitors (Huang et al. 2006). Although FOXO3a’s activity was not analyzed, it has been shown that BPDE damage in human cells activates CHK1 (by phosphorylation) and induced S-phase arrest (Guo et al. 2002). Therefore, it is likely that CHK1 also plays a role in activating FOXO3a as a consequence of BPDE damage. Furthermore, Huang et al. did not analyze ATM/ATR’s role in this CHK1-dependent activation of FOXO1, but since both proteins are known activators of CHK1, they are likely candidates in DNA-damage signaling of FOXO.

Based on these findings we speculate that CHK1 (likely through ATM/ATR signaling) may activate FOXO3a through its dephosphorylation by some phosphatase activated by CHK1. Based on our findings, FOXO3a could then directly induce a protective cellular function in the form of DNA repair (through GADD45) and apoptosis. In some settings, FOXO3a may also interact with p53, prolonging its active state, and also inducing DNA damage response or apoptosis.

4.4.3 A role for FOXO3a in therapy response

Our results also implicate FOXO3a in anti-mitotic treatment response in LAC, however its exact role remains to be determined. Our results suggest that FOXO3a acts pro-therapeutically in LAC as its expression substantially decreases the number of LAC cells when treated with anti-mitotic drugs. This effect appears to affect cell proliferation more so than cell
death including apoptosis. We observed a similar fraction of cells following BPDE, docetaxel and vinorelbine treatment over the same period of time (7 days). However, only BPDE induced a robust FOXO3a-mediated apoptotic effect. Together with the very different expression profiles elicited by FOXO3a in response to carcinogens and microtubule inhibitors, our results indicate that FOXO3a’s responsiveness to stress is relatively stress-specific. Sunters et al. have investigated FOXO3a’s mechanism of action in response to microtubule inhibitors. In their studies, Sunters et al. found that microtubule damage caused by paclitaxel (a taxane like docetaxel) in breast cancer cell lines induced nuclear localization of FOXO3a, an increase in JNK1 levels and a decrease in Akt protein levels. Paclitaxel induced BIM-dependent apoptosis in these cells (Sunters et al. 2003, Sunters et al. 2006). However, when cells were treated with a JNK inhibitor, apoptosis was reduced and FOXO3a remained phosphorylated and inactive. Also, western analysis showed that when cells expressed active levels of JNK1/2 through transfection, phosphorylated FOXO3a decreased, and phosphorylated levels of C-JUN increased. These studies therefore indicate that, in response to microtubule damage, FOXO3a is activated through JNK1/2 in breast cancer cells. As we did not see the same response in LAC cells, JNK1/2 activation of FOXO3a may be impaired or inactive in these cells. Alternatively, JNK1/2 may invoke a different FOXO3a-mediated response in the lung or in LAC cells.

In contrast, we observed that FOXO3a has a pronounced effect on cell cycle distribution when treated with anti-mitotics, docetaxel and vinorelbine. FOXO3a mitigated mitotic arrest induced by these drugs, and this correlated with FOXO3a-mediated increase in CDC14A gene expression. As mentioned previously, this may contribute to a cytostatic FOXO3a-mediated effect to these drugs. As discussed, Cyclin-B, Cyclin-G2, and Plk1 involvement is possible in this
cytostatic response as all are transcriptionally regulated by FOXO3a and have an effect on CDC14A, mitotic exit, and G1-regulation. Plk1 localizes to centrosomes and associates with CDC14A to stimulate exit from mitosis (Rahal and Amon 2008). Cyclin-B associates with CDK1 and promotes mitosis, but must be depleted in order for exit from mitosis to occur (Malambres and Barbacid 2001). Down-regulation by FOXO3a may block entry into mitosis. Cyclin-G2 is also interesting because it has been shown to be expressed at higher levels in mouse lung tissue than Cyclin-D2 and also that its upregulation is involved in G1 arrest by inhibiting transition to S-phase (Horne et al. 1996, Horne et al. 1997). Our microarray analysis also showed FOXO3a-dependent increase in JUNB expression in response to docetaxel treatment (Figure A.4). JUNB is positively regulated by JNK1/2, and JUNB has also been shown to induce G1 cell cycle arrest by causing upregulation of p16INK4a in MEFs (Passegue and Wagner 2000). A FOXO3a-mediated increase in G1 was observed following treatment with anti-mitotics. However, no change in expression of G1 effectors (p27Kip1, Cyclin-D2) of FOXO3a was evident at several time points following treatment. Plk1, Cyclin-B, Cyclin-G2 and JUNB have not been examined, but are warranted as we have described. In addition, RNAi knockdown of Plk1, Cyclin-B, Cyclin-G2, and JUNB in our cell lines would help us to further determine whether they play a role in FOXO3a-dependent decrease in cell numbers or our observed cell cycle fraction differences.

The cellular response to microtubule inhibition is incompletely understood. However, in response to microtubule damage, cells have been shown to undergo a process known as ‘mitotic slippage’ when apoptosis does not occur. Mitotic slippage is defined as an exit from
mitosis and ‘slippage’ into G1 in order to escape cell cycle arrest or apoptosis (Gascoigne and Taylor 2008). This process involves the gradual degradation of Cyclin-B. To determine this, GFP-tagged Cyclin-B levels were monitored in various lung and colon carcinoma cell lines exposed to microtubule inhibitor paclitaxel (Gascoigne and Taylor 2008). Using immunohistochemistry, Gascoigne and Taylor found that, in order for cells to exit mitosis and undergo mitotic slippage, Cyclin-B levels had to decrease below a certain threshold (~30% of its expression level at the beginning of mitosis). If cells did not approach this threshold, they would die due to apoptosis. In addition, if Cyclin-B was overexpressed in paclitaxel treated cells, it would nearly double the amount of time cells remained in mitosis (Gascoigne and Taylor 2008). JNK has been shown to inhibit CDC25 activity (through its phosphorylation) which caused CDC25 to be unable to dephosphorylate and therefore activate Cyclin-B/CDK1 complex (demonstrated through western analysis of JNK, CDC25, and Cyclin-B/CDK1 phosphorylation levels in response to UV light), which suggests that JNK plays a role in exit from mitosis in response to cellular stress (Goss et al. 2003). This function of JNK possibly occurs in conjunction with its activation of FOXO3a and its subsequent upregulation of CDC14A and down-regulation of Cyclin-B in response to microtubule damage. Together, JNK and FOXO3a could promote mitotic slippage (Alvarez et al. 2001).

Our knowledge so far of FOXO3a in response to microtubule stress is very limited. However, we propose the following speculative model for FOXO3a’s role in anti-mitotic therapy of LAC. Treatment of LAC with docetaxel and/or vinorelbine causes disruption of the mitotic spindles and cells are unable to complete mitosis. JNK responds to this stress by activating FOXO3a and promoting Cyclin-B downregulation both functionally and via FOXO3a
transrepression. FOXO3a also upregulates CDC14A, which inactivates CDC25. CDC25 in turn is unable to activate CDK1 via dephosphorylation and cells are unable to enter mitosis. Cells in mitosis are stimulated to exit into G1 by CDC14A-dependent inactivation of CDK1 by a yet to be elucidated mechanism that results in centrosome separation, chromosome segregation, cytokinesis, and mitosis completion. This effect may entail microtubule inhibitor induced mitotic slippage. Cells that escape arrest or undergo mitotic “slippage” through FOXO3a’s upregulation are then arrested in G1 through FOXO3a-dependent upregulation of Cyclin-G2 and/or JUNB, the latter of which being activate by JNK1/2 and the former upregulated by FOXO3a (Figure 4.3).
Figure 4.3. FOXO3a encodes a cytostatic and weak cytotoxic cellular response stimulated by anti-mitotic therapy. Docetaxel and vinorelbine are anti-mitotic agents that promote G2/M arrest. However, under FOXO3a expression, docetaxel and vinorelbine stimulate FOXO3a activity via JNK1/2 which promotes cell cycle progression into G1 through Plk1, Cyclin-B, and CDC14A, and G1 cell cycle arrest through upregulation of Cyclin-G2 and JUNB.
4.5 Conclusion

The work presented in this dissertation suggests that FOXO3a loss plays a complex role in LAC malignancy. Our analysis suggests that FOXO3a induces a stress-specific cellular response. In response to DNA damage caused by tobacco smoke carcinogens FOXO3a stimulates a protective transcription program culminating in caspase-dependent apoptosis. In contrast, when exposed to anti-mitotic chemotherapy, FOXO3a mitigates mitotic arrest caused by these agents and promotes cytostasis with a modest degree of cell death. Our findings suggest a role for FOXO3a in suppressing tumor induction by carcinogens and possibly promoting therapeutic response in LAC. These distinct roles are both lost as a result of gene deletion in LAC. In early stage carcinogenesis, FOXO3a appears to play a role in eliminating DNA-damaged cells that could potentially develop into tumors. FOXO3a stimulates cytostasis following treatment with microtubule inhibitors, the exact nature of which requires further study. The loss of FOXO3a may then lessen the efficacy of this treatment. Our results are a first step in the characterization of FOXO3a's role in lung carcinogenesis, the future directions proposed above will further our understanding of this role. Our results also warrant the investigation of FOXO3a loss in other cancer types and analysis of other FOXOs as tumor suppressors in human cancer.
Figure A.1. FOXO1 (A) and FOXO4 (B) mRNA expression pattern in various tissues. Expression pattern was acquired from BioGps (http://biogps.gnt.org). Expression values are from Affymetrix chips related to fluorescence intensity.
Figure A.2. FOXO3a reduces cellular viability but induces modest apoptosis in response to microtubule inhibitors. A, FOXO3a reduces surviving cellular fraction in response to docetaxel in LAC cells. MTS assay of transfected H460 cells following treatment with docetaxel (left) and vinorelbine (right). Surviving fractions are relative to vehicle (DMSO) treated cells transfected with the same vector. B, Annexin-PE assay of transfected H460 cells following treatment with docetaxel (left) and vinorelbine (right). Apoptotic fractions are relative to vehicle (DMSO) treated cells transfected with the same vector. Western blot of caspases in H460 cells transfected with either FOXO3a or control vector. For Western blot, cells were harvested 24h post-treatment with docetaxel (25 nM) or vinorelbine (50 nM). Results are representative of several experiments. Statistical analysis used was two-tailed Student’s t-test; * p < 0.05, ** p<0.01
Figure A.3. FOXO3a promotes modest apoptosis in LAC cells treated with docetaxel and vinorelbine. A, Annexin-PE assay of transfected A549 cells following treatment with docetaxel (25 nM) and vinorelbine (50 nM) for 48 hours (n=3). Apoptotic fractions are relative to vehicle (DMSO) treated cells transfected with the same vector. B, Annexin-PE assay for transfected A549 cells following treatment with docetaxel and vinorelbine at varying doses for 48 hours (n=1). Statistical analysis used was two-tailed Student’s t-test.
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Figure A.4. Genes regulated by FOXO3a in response to docetaxel. Refer to Methods section for methodology.
CDC14A

**Ensembl Transcript ID:** ENST00000455467

-5023

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Figure A.5. FOXO3a binding sites on two human CDC14A transcripts. Sequences matching the consensus Forkhead binding element are highlighted. Number denotes bp upstream of start codon.
REFERENCES


Lafarge, S., V. Sylvain, et al. (2001). "Inhibition of BRCA1 leads to increased chemoresistance to microtubule-interfering agents, an effect that involves the JNK pathway." Oncogene 20(45): 6597-6606.


Westra, W. H., R. J. Slebos, et al. (1993). "K-ras oncogene activation in lung adenocarcinomas from former smokers. Evidence that K-ras mutations are an early and irreversible event in the development of


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Mentor: Christopher Herzog

M.S. 2003-2005
Genetics/Biological Sciences
Cal State San Marcos
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B.S., 2001-2003
Biological Sciences
Cal State San Marcos
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2003-2005
Volunteer
Laboratory of Dr. Donald Cleveland
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Volunteer
Laboratory of Dr. Denise Garcia
Supervisor: Suzanne Hizer
Cal State University San Marcos
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2000-2001
Volunteer
Biological Science Department
Miracosta College, Oceanside, CA

Courses Taught
2003
Teaching Assistant: Genetics Laboratory, BIOL 352

Honors and Awards
1999-2001
President’s list of outstanding students Miracosta College
2001-2003
Dean’s list of outstanding students Cal State San Marcos

Publications


Mikse OR, Freeman WM, Herzog CR. 2010. FOXO3a elicits a pro-therapeutic cellular response and derepression of CDC14A as a response to anti-mitotic chemotherapy. (In preparation)