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# Tyrosine Phosphorylation of p68 RNA Helicase Promotes Metastasis in Colon Cancer Progression

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# TYROSINE PHOSPHORYLATION OF p68 RNA HELICASE PROMOTES METASTASIS IN COLON CANCER PROGRESSION

by

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## ABSTRACT

The initiation of cancer metastasis usually requires Epithelial-Mesenchymal Transition (EMT), by which tumor cells lose cell-cell interactions and gain the ability of migration and invasion. Previous study demonstrated that p68 RNA helicase, a prototypical member of the DEAD-box RNA helicases, functions as a mediator to promote platelet-derived growth factor (PDGF)-induced EMT through facilitating nuclear translocation of β-catenin in colon cancer cells. In this context, p68 RNA helicase was found to be phosphorylated at the tyrosine 593 residue (referred as phosphor-p68) by c-Abl kinase, and this phosphorylation is required for the activation of β-catenin signaling and the consequent EMT. The phosphor-p68 RNA helicase-mediated EMT was characterized by the repression of an epithelial marker, E-cadherin, and the upregulation of a mesenchymal marker, Vimentin. E-cadherin, a major cell-cell adhesion molecule that is involved

in the formation of adherens junctions, has been shown to sequester β-catenin at the cell membrane and thus inhibit its transcriptional activity. The functional loss of E-cadherin is the fundamental event of EMT. Despite the role of phosphor-p68 RNA helicase in regulating nuclear translocation of β-catenin, whether phosphor-p68 is involved in the regulation of E-cadherin remains unknown. Here, our data indicated that phosphor-p68 RNA helicase initiated EMT by transcriptional upregulation of Snail1, a master transcriptional repressor of E-cadherin. The data suggest that phosphor-p68 RNA helicase displaced HDAC1 from the chromatin remodeling MBD3:Mi-2/NuRD complex at the Snail1 promoter, thereby activating the transcription of Snail1. In the xenograft tumor model, abolishing the phosphorylation of p68 RNA helicase by the expression of Y593F mutant resulted in a significant reduction of metastatic potential in human colon cancer cells. Analyses in the colon cancer tissues also revealed that the tyrosine 593 phosphorylation level of p68 RNA helicase is substantially enhanced in the tumor tissues comparing to that in the corresponding normal counterparts, suggesting a correlation of phosphor-p68 and tumor progression. In conclusion, we showed that tyrosine phosphorylation of p68 RNA helicase positively correlated to the malignant status of colon cancer progression. The molecular basis behind this correlation could be partly through the transcriptional regulation of Snail1.

INDEX WORDS: p68 RNA helicase, DEAD box helicase, Transcription regulation, Snail1, NuRD, HDAC1, EMT, Phosphorylation, Cancer metastasis

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# CHAPTER 1: GENERAL INTRODUCTION

## 1.1 Helicases and Nucleic Acid Translocases

Helicases are a highly diverse group of proteins that perform a variety of functions in cells. These proteins are capable of unwinding deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) duplex substrates by hydrolyzing nucleotide triphosphate (NTP), mainly adenosine triphosphate (ATP), to provide energy. The helicases exist in every living organism and virus, and they play a role in almost every process that involves nucleic acids, including DNA replication and repair, transcription, translation, ribosome synthesis, RNA maturation and splicing, and nuclear export processes. Originally, the helicases were identified by a series of conserved motifs that form a core domain to exert their ATP hydrolysis and unwinding activity (Koonin 1993b, Koonin and Deutscher 1993). With accumulative evidence over years, it has become clear that these motifs are actually characteristics of nucleic acid translocases that use ATP hydrolysis to move directionally along nucleic acid strands. Therefore, helicases are now classified as a subgroup of translocases (Berger 2008, Singleton et al 2007).

Based on the fundamental works by Gorbalenya, Koonin, and others, this large group of helicases/translocases is classified into six superfamilies (Erzberger et al 2006, Gorbalenya et al 1989, Koonin 1993a, Koonin 1993b, Korolev et al 1998, Mahdi et al 2003, Pause and Sonenberg 1992, Tanner et al 2003). For the largest two superfamilies (SF1 and SF2), their helicase/ATPase core domain shares several common motifs, summarized in Fig.1.1a. The available structural evidence suggests that the active interface (cleft) of the helicase/ATPase core domain in SF1 and SF2 is shaped by the amino-terminal and carboxyl-terminal recombinase A (RecA)-like domains shown in Fig.1.1b (Singleton et al 2007). The RecA-like domain is a universal structural feature of a large class of ATPases, including helicases and proteinases, and it is responsible for the

nucleotide binding and hydrolysis that provides energy in mechanical work, such as moving polypeptides or nucleic acids (Ye et al 2004).

## 1.2 RNA Remodeling Proteins/ RNA Helicases

RNA helicases are ubiquitous, highly conserved enzymes involved in most aspects of RNA metabolism, including transcription, pre-mRNA splicing, translation, mRNA decay, and microRNA biogenesis (Aratani et al 2001, Hilliker et al 2011, Jia et al 2011, Moore et al 2011). It is generally believed that RNA helicases modulate dynamic RNA structures and RNA-protein complexes in an ATP-dependent manner, by which the hydrolysis of the ATP drives the dissociation of RNA-RNA and/or RNA-protein interactions (Chen et al 2002, Jankowsky et al 2001, Linder et al 2001). With their important role in RNA metabolism, RNA helicases are found in all kingdoms of life, and many viruses express one or more of these proteins to execute their biological functions, for example, to replicate in the host (Jin and Peterson 1995, Wen et al 2009).

## 1.3 DEAD Box RNA Helicases

The family of DEAD box RNA helicases, one of the largest subdivisions of SF2 helicases, is characterized by the presence of an Asp-Glu-Ala-Asp (DEAD) motif and plays an essential role in almost every cellular event, including cell survival, growth, proliferation, and differentiation (Abdelhaleem 2005, Jankowsky 2011). Similar to all SF2 helicases, the DEAD box proteins share nine conserved sequence motifs (Fig.1.2) that build the helicase core of two nearly identical RecA-like domains. These sequence motifs encompass an approximate 300-400 amino acid region involved in ATP binding/hydrolysis and RNA binding/unwinding (Caruthers and McKay 2002, Cordin et al 2006, Fairman-Williams et al 2010, Singleton et al 2007).

The crystal structures of a number of DEAD-box proteins or domains have been solved, including eIF4A (Benz et al 1999, Caruthers et al 2000, Johnson and McKay 1999); the DEAD-box protein of *Methanococcus jannaschii* (Story et al 2001) and *Bacillus stearothermophilus* (Carmel and Matthews 2004); Vasa in *Drosophila melanogaster* (Sengoku et al 2006); DDX3X, the closely related form of the DDX3 protein (Hogbom et al 2007); yeast DEAD box protein Mss116p (Del Campo and Lambowitz 2009); as well as some human DEAD box proteins (Schutz et al 2010). According to the comparative structural analysis of DEAD-box RNA helicases, motifs I, II (DEAD box), VI, and the Q-motif participate in nucleotide binding, whereas motifs Ia, Ib, IV, and V contribute to the RNA binding process (Linder 2006, Schutz et al 2010). Moreover, the helicase core of the DEAD box proteins is flanked by variable N-terminal and C-terminal auxiliary domains, which are thought to be critical for the diverse functions of these enzymes by posttranslational modification or interacting with cofactors, as Fig.1.3 summarizes (Gustafson and Wessel 2010, Linder 2006).

The biological functions of DEAD box RNA helicases in eukaryotic cells are very diverse and range from transcription to the degradation of RNA (Fuller-Pace 2006, Linder 2006). How the single-folded enzymes perform such diverse functions in the cells remains elusive. *In vitro*, the vast majority of RNA helicases do not display sequence or structural preferences except for the polarity requirements of some enzymes for unwinding RNA duplexes. However, *in vivo* studies have revealed that different RNA helicases, even with a high structural similarity, perform distinct functions on various substrates (Fairman-Williams et al 2010, Franca et al 2007, Strohmeier et al 2011). For example, the eukaryotic translation initiation factor 4A (eIF4A, also known as DDX2A/B), a prototypic member of the DEAD box helicase family, has been shown to be recruited to the mammalian translation initiation complex 4F (eIF4F) to stimulate the

translation process (Hilbert et al 2011, Imataka and Sonenberg 1997). During translation initiation, eIF4A is capable of unwinding the secondary structure in the 5'-untranslated region of eukaryotic mRNAs, thus promoting ribosome binding (Hilbert et al 2011, Imataka and Sonenberg 1997, Ozes et al 2011). However, eIF4AIII (DDX48), which is structurally similar to eIF4A, has a distinct function in the exon junction complex (EJC). The EJC is a multi-protein complex deposited roughly 20 nucleotides upstream of exon-exon junctions during pre-mRNA splicing in higher eukaryotes (Le Hir and Andersen 2008, Tange et al 2004). Crosslinking and antibody inhibition studies suggested that eIF4AIII constitutes at least part of the platform that anchors other EJC components, such as nuclear-cytoplasmic shuttling proteins, Y14 and Magoh, to the spliced mRNAs. In mammalian cells, it is also known that eIF4AIII is essential for nonsense-mediated mRNA decay. Therefore, eIF4AIII may represent a new functional class of DEAD box proteins that act as RNA clamps for the sequence-independent attachment of additional factors to RNAs (Shibuya et al 2004).

Many DEAD box RNA helicases appear to participate solely in a single step of one cellular process. A multi-step process, such as ribosome biogenesis, requires a large number of DEAD box RNA helicases, suggesting a functional specificity for the proteins in each step (Burger et al 2000, Daugeron and Linder 2001, Hilliker et al 2011). Interestingly, several DEAD box proteins, including Ded1p (DDX3) and p68 (DDX5), were found to play a role in various multi-component complexes, such as chromatin remodeling, transcription, and splicing complexes (Carter et al 2010, Fuller-Pace and Moore 2011, Hilliker et al 2011, Mooney et al 2010a, Rosner and Rinkevich 2007). Therefore, understanding the role(s) of DEAD box RNA helicases in the formation and modulation of these complexes will help to elucidate possible mechanisms by which these proteins participate in different cellular processes in the cells.

## 1.4 DEAD Box Protein-p68 RNA Helicase

### 1.4.1 Gene and Structure of p68 RNA Helicase

Human p68 RNA helicase is encoded in *DDX5* gene located at chromosome 17q21 (Iggo et al 1989). The cDNA sequence of human p68 shows an open reading frame encoding a polypeptide of 614 amino acids (Hloch et al 1990). The p68 RNA helicase was first identified because of its immunological cross-reaction with the anti-SV40 large T monoclonal antibody DL3C4 (PAb204) (Crawford et al 1982a, Lane and Hoeffler 1980a). Sequence analysis of human p68 cDNA revealed the molecular basis for its cross-reaction with the SV40 large T antigen and its extensive homology to the eIF4A protein, both of which have nucleic acid unwinding activity (Pause and Sonenberg 1992, Scheffner et al 1989). Purified p68 protein has been shown to exhibit ATP binding, RNA-dependent ATPase, and RNA helicases activities *in vitro* (Hirling et al 1989, Iggo and Lane 1989b).

The p68 protein is evolutionarily conserved. The similarity of the amino acid sequence of mouse and human p68 is as high as 98% (Lemaire and Heinlein 1993). The amino acid sequence of the p68 protein contains multiple conserved motifs that place it in the family of DEAD box RNA helicases. Although the crystal structure of p68 has not yet been solved, the structure of eIF4A has been rendered (Benz et al 1999, Caruthers et al 2000, Johnson and McKay 1999). The crystal structure of the eIF4A helicase core resembles a dumbbell-like shape that consists of two RecA-like globular domains connected by a flexible linker (Fig.1.4) (Caruthers et al 2000). The structure of the helicase core reveals that the motifs conserved in the DEAD box family form a pocket within the two globular domains, providing the binding sites for ATP and RNA. The latest identified Q motif has been reported to regulate ATP binding and hydrolysis (Tanner et al 2003). Motif I (AxxGxGKT) and Motif II (DEAD) are Walker A and B motifs, respectively,

which are essential for ATP binding/hydrolysis in the family of DEAD box RNA helicases (Carmel and Matthews 2004, Lorsch and Herschlag 1998, Rocak et al 2005, Schneider and Hunke 1998). Mutations in the motif I affect ATP binding, whereas mutations in the motif II affect ATP hydrolysis that couples with the RNA helicase activity. Furthermore, the first residue in the motif VI (HRxGRxGR) is involved in ATP hydrolysis, and mutations in the motif III (SAT) abolish RNA unwinding while the binding of RNA and ATP is unaffected (Pause and Sonenberg 1992). The motif V (RGID) has been shown to contribute to the RNA binding process (de la Cruz et al 1999). Outside the helicase core, an IQ motif, identified in the C-terminal of the p68 protein, has been shown to contain the conserved protein kinase C phosphorylation site and the calmodulin binding site, both of which have negative regulatory effects on p68 ATPase activity (Buelt et al 1994, Yang et al 2004). An RGG (Arg-Gly-Gly) repeat region following the helicase core of the p68 protein has been suggested to regulate the protein function via posttranslational modification (Yang and Liu 2004b, Yang et al 2005d).

### 1.4.2 Expression of p68 RNA Helicase

The expression of the p68 RNA helicase has been shown in a wide range of vertebrate species and is essential for normal development and cell growth (Lane and Hoeffler 1980a, Lemaire and Heinlein 1993). The chicken homologue of p68 was found to be tightly associated with the 5-methylcytosine-DNA glycosylase complex in developing embryos. This glycosylase complex is involved in the active de-methylation of DNA on the CpG repeats, which is vital for the normal development of vertebrates (Jost et al 2001). In the Swiss 3T3 cells, the expression of p68 is detectable in quiescent cells, but is enhanced greatly by serum stimulation, suggesting a growth-related role of this protein (Stevenson et al 1998b). In adult rats and the human tissues, p68 mRNA and/or protein are reported to be expressed differentially in different cell/tissue

types; however, the expression levels do not correlate completely with the proliferation status, suggesting that the regulation of expression of this protein is more complicated in the tissue than in the cultured cells (Rossler et al 2000, Stevenson et al 1998b).

In the mouse brain, it has been shown that the expression level of the p68 RNA helicase is significantly lower in old males compared with adult males. Furthermore, the p68 RNA helicase in the mouse brain was found to interact with the transactivation domain of the estrogen receptor α (ERα), and this interaction is relatively lower in old mice of both sexes and even lower in male mice of the same ages compared with their counterparts. Such age- and sex-dependency of the p68-ERα interaction suggests a role for p68 in ERα-mediated brain function during aging (Ghosh and Thakur 2009).

In the screening of the differential cDNA library, nitric oxide (NO) stimulation in HaCaT human keratinocytes was found to induce the p68 RNA helicase. NO is known to pivotally drive epithelial movement and gene expression during skin repair. Wound healing experiments on mouse skin demonstrated that the p68 RNA helicase is essential for developing epithelium, possibly through stimulating keratinocyte proliferation and the expression of the vascular endothelial growth factor to accelerate the healing process (Kahlina et al 2004).

### 1.4.3 Posttranslational Modification of p68 RNA Helicase

Posttranslational modification is a process, in which proteins are added with other biochemical functional groups, such as acetates, phosphates, lipids, carbohydrates, and small polypeptides, to specific amino acid residue(s). The posttranslational modification changes the chemical nature of an amino acid and thus extends the range of the protein functions.

In the cells, phosphorylation is accomplished by transferring the γ-phosphoryl group of ATP to serine, threonine, or tyrosine residues of a protein, which is catalyzed by protein kinases (Berg et al 2007). Phosphorylation modification grants the host protein two extra negative charges, which are likely to induce a conformational change of the protein and thus alter its enzymatic activity, binding affinity to interacting factors, and/or cellular localization (Berg et al 2007). Also, a protein can be phosphorylated simultaneously or sequentially at multiple residues to perform its function precisely in a spatiotemporal manner. In addition, protein phosphorylation can be reversed by phosphatases, which confers a more flexible regulatory mechanism onto the cells in response to different stimulations. Therefore, kinases and phosphatases play a critical role in most, if not all, signaling pathways that are vital for regulating cellular behaviors, such as cell growth and survival. Imbalanced regulation of protein phosphorylation may cause severe diseases, such as cancer (Brognard and Hunter 2011).

A previous study from our laboratory demonstrated that the recombinant p68 RNA helicase purified from bacteria, *Escherichia coli*, has the phosphorylation modifications on serine, threonine and tyrosine residues (Yang and Liu 2004b). Biochemical analysis showed that the phosphorylation modification on the recombinant p68 RNA helicase affects its ATPase and RNA binding/unwinding activities, which may suggest an important mechanism in controlling the function of this protein at the posttranslational level (Yang and Liu 2004b). Further studies showed that the recombinant C-terminal domain of p68 RNA helicase is responsible for the binding of single stranded RNA, which is negatively regulated by protein kinase C (PKC). Phosphorylation of the C-terminal domain of p68 RNA helicase by PKC abolishes its RNA binding activity *in vitro* (Yang et al 2004). These data suggested that the C-terminal domain of p68 RNA helicase is the RNA binding site and that the enzymatic activities of this protein might

be regulated through phosphorylation by PKC in the cells. Another study also showed that the phosphorylation on tyrosine residue(s) of p68 RNA helicase is enhanced in tumor cell lines compared with their normal counterparts, suggesting a role of p68 in abnormal cell proliferation and cancer development (Yang et al 2005c). Furthermore, phosphorylation of p68 at threonine or tyrosine residues was found to respond differently to tumor necrosis factor α (TNF-α) treatment in HeLa cells (Yang et al 2005d). In the absence of TNF-α, p68 RNA helicase is phosphorylated on tyrosine but not on serine or threonine residue(s) in HeLa cells. In the presence of TNF-α, tyrosine phosphorylation of p68 RNA helicase is first enhanced and then replaced by threonine phosphorylation due to the activation of a serine/threonine kinase, p38 MAP kinase. The data also revealed that the threonine phosphorylation of p68 is essential for its ATPase and RNA unwinding activities, whereas the tyrosine phosphorylation attenuates its enzymatic activities and the functional role in the pre-mRNA splicing, suggesting that phosphorylation pattern of p68 RNA helicase is associated with different cellular functions (Yang et al 2005d).

In glioblastoma cells, a site-directed mutagenesis study specified that double phosphorylation of tyrosine 593 and 595 residues on p68 RNA helicase confers the cells with the resistance to apoptosis upon the treatment of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Yang et al 2007a). In colon cancer cells, p68 is phosphorylated at the tyrosine 593 residue by c-Abl kinase upon the stimulation of platelet-derived growth factor (PDGF)-BB. The tyrosyl (593)-phosphorylated p68 protects a transcription activator, β-catenin, from GSK3β-medicated protein degradation in cytoplasm, enriches the nuclear portion of β-catenin, and thus promotes epithelial to mesenchymal transition (EMT) (Yang et al 2006). The tyrosine phosphorylation of p68 also mediates the effects of PDGF on promoting cell proliferation by activating the transcription of cyclin D1 and c-Myc genes (Yang et al 2007b). Taken together,

phosphorylation of p68 RNA helicase on different residues may play an important role in regulating various cellular functions.

Ubiquitination is a posttranslational modification in which a small conserved peptide, ubiquitin or ubiquitin chains, are added to the target proteins, giving rise to mono or polyubiquitination (Busch and Goldknopf 1981, Schlesinger and Goldstein 1975). The process of ubiquitination involves a series of steps. First, an E1 ubiquitin-activating enzyme driven by ATP hydrolysis activates an ubiquitin. Second, the ubiquitin is transferred from E1 to the active cysteine of an E2 ubiquitin-conjugating enzyme. Finally, an isopeptide bond is created between a lysine of the target protein and the C-terminal glycine of ubiquitin, which is catalyzed by E3 ubiquitin ligase with substrate specificity (Scheffner et al 1995). The effect of the ubiquitination depends on the number and the position of ubiquitin appended to the protein. Polyubiquitination marks proteins for proteasome-mediated degradation to maintain the protein homeostasis in the cells (Busch and Goldknopf 1981), whereas monoubiquitination regulates proteins in multiple ways, including changing the cellular localization, stability, and activity. The monoubiquitination modification of proteins affects many cellular functions, such as membrane trafficking, chromatin remodeling, transcription regulation, DNA repair, and DNA replication (Sigismund et al 2004).

Sumoylation is another posttranslational modification, in which a small ubiquitin-like modifier (SUMO) is added to a protein (Hay 2005). Similar to ubiquitination, posttranslational modification of proteins by the SUMO-1 regulates their cellular localization, stability, and activity, with implications in many cellular processes, such as cell cycle control, DNA repair, and transcription activation/repression (Boyer-Guittaut et al 2005, Gill 2003, Johnson and Hochstrasser 1997, Pastushok and Xiao 2004).

It has been shown that the expression of p68 protein is correlated with the development of colorectal adenocarcinomas (Causevic et al 2001b). Interestingly, the increased level of p68 protein is accompanied by polyubiquitination modification, suggesting a possible defect in proteasome-mediated degradation and/or an unknown mechanism by which the polyubiquitinated p68 regulates the cellular proliferation in tumorigenesis.

The diverse effects on the transcriptional regulators by SUMO modification are often through altering their localization or interactions with other factors. The p68 RNA helicase has been shown to function as a transcriptional coactivator or corepressor in a context-dependent manner (Rossow and Janknecht 2003, Wilson et al 2004a). A recent review indicated that SUMO modification is a key in switching the transcriptional regulatory role of p68 between activation and repression (Fuller-Pace et al 2007). It has been shown *in vivo* that p68 is modified on its lysine 53 by the small ubiquitin-like modifier-2 (SUMO-2). This SUMO-2 modification of p68, catalyzed by the SUMO E3 ligase, PIAS1, enhances its transcriptional repression activity through altering chromatin structure by the recruitment of histone deacetylase 1 (HDAC1) (Jacobs et al 2007). The SUMO modification has been shown to increase protein stability by reducing polyubiquitination and the consequent proteasome-mediated protein degradation (Geiss-Friedlander and Melchior 2007). Interestingly, a bioinformatics study revealed that p68 contains one consensus sumoylation motif, ψKxE, where ψ is an aliphatic-branched amino acid and x is any amino acid. The SUMO modification of p68 increases its stability, whereas mutation of the consensus sumoylation site enhances its polyubiquitination and degradation (Mooney et al 2010b). Dysfunctional regulation of both ER and p53 signaling pathways has been correlated with breast cancer development (Pelosi et al 1994, Shirley et al 2009). A recent study showed that SUMO modification of p68 enhances its transcriptional coactivation activity on the

promoters containing ER response elements, whereas the p68 sumoylation status does not affect p53-dependent MDM2 transcription (Mooney et al 2010b). Therefore, this study suggests that the pleiotropic effects of SUMO modification of p68 may be a critical mechanism by which ER and p53 activities are regulated in breast cancer cells.

According to previous studies, it is obvious that p68 plays multiple roles in cells through different posttranslational modifications. Emerging evidence also suggests that the posttranslational regulation gains more weight in maintaining the normal function of cells and is associated with pathogenesis, such as tumorigenesis. Therefore, understanding the underlying mechanisms and the effects of posttranslational modification may shed light on its therapeutic purpose in cancer treatment.

## 1.5 Transcriptional Regulation

### 1.5.1 General Transcriptional Regulation

Transcriptional regulation is very important in living cells, as it responds to various cellular signals, and it involves a large number of proteins, including sequence-specific DNA binding factors, chromatin regulators, general transcription factors, RNA polymerase II (Pol II), and the coactivators or repressors (Li et al 2007, Orphanides and Reinberg 2002, Pugh 2000, Struhl et al 1998). The general view of transcriptional activation is initiated by the interaction of sequence-specific regulators and their cognate DNA motifs. The sequence-specific regulators further recruit coactivators to alter and expose the local chromatin structure, facilitate the assembly of the pre-initiation complex (PIC) consisting of general transcription factors (GTFs) and RNA polymerase II (Pol II), and eventually lead to gene transcription (Venters and Pugh 2009). In contrast to the transcriptional activation, genes may be repressed through the steric

hindrance mechanism by which chromatin is compacted with histone proteins into a dense nucleosome structure that limits its accessibility to transcription activators. Two groups of enzymes with antagonistic activities, called histone acetyltransferases (HATs) and histone deacetylases (HDACs), play a crucial role in activating or repressing gene transcription (Johnsson et al 2009). HATs, such as CBP/p300, acetylate histone and make the chromatin more accessible to transcription factors, whereas HDACs, such as HDAC1, function in a reverse way to repress gene expression. Another group of enzymes, called DNA methylases, also plays a role in transcriptional regulation, mainly in gene repression (Bhakat and Mitra 2003, Kang et al 1999). These regulatory enzymes can be recruited to the promoters by sequence-specific factors to provide additional active or repressive effects on gene transcription (Lande-Diner et al 2007, Li et al 2007).

Many sequence-specific regulators have been determined and granted with a crucial role in regulating gene transcription. Growing evidence indicates that the sequence-specific regulators orchestrate gene transcription in multiple aspects, including the recruitment of chromatin remodeling complexes, general transcription factors, chromatin modifying complexes, and RNA Pol II (Cosma et al 1999, Garbett et al 2007, Goldmark et al 2000, Green 2005, Larschan and Winston 2001, Neely et al 2002, Nourani et al 2004, Ranish et al 1999, Yudkovsky et al 1999).

It is evident that transcriptional activation involves the movement of nucleosome, which is driven by ATP hydrolysis catalyzed in chromatin remodeling complexes (Li and Reese 2001, Lohr 1997, Ravindra et al 1999). Based on sequence conservation, many identified chromatin remodeling complexes are classified into four families, SWI/SNF, INO80/SWR1, ISWI, and CHD, each of which plays a distinct role in regulating chromatin structure (Bao and Shen 2007).

The SWI/SNF family of chromatin remodeling complexes generally functions as a positive regulator through sliding or ejecting the nucleosomes, exposing the DNA biding sites to transcription factors, and thus activating gene expression (Liu et al 2011). Unlike the SWI/SNF family, the INO80/SWR1 family of chromatin remodeling complexes regulates transcription positively by affecting the composition of nucleosomes rather than their position. For instance, the SWR1 complex replaces the H2A with H2A.Z in an ATP-dependent manner in the promoter region, where the substitute histone variant destabilizes the nucleosome structure and increases its accessibility (Guillemette et al 2005, Raisner et al 2005, Zhang et al 2005). In contrast to the positive regulatory roles of SWI/SNF and INO80/SWR1 families, the ISWI (Initiation SWItch) family of chromatin remodeling complexes tends to repress gene transcription. Since the ISWI chromatin remodeling complexes lack a proven DNA binding domain, it has been suggested that they are recruited by sequence-specific repressors and may function in concert with the HDACs to repress gene transcription (Fazzio et al 2001, Goldmark et al 2000). The intracellular role of the CHD (Chromatin organization modifier, Helicase, and DNA-binding domains) family of chromatin remodeling complexes is less understood. The Chd1 protein is a component of the SAGA (Spt-Ada-Gcn5 Acetyltransferase) chromatin remodeling complex, and it interacts *in vitro* with methylated H3K4, a gene activating marker, suggesting that the CHD family may cooperate with other chromatin modifying complexes to regulate gene transcription (Pray-Grant et al 2005). Taken together, chromatin remodeling and modifying complexes play important roles in regulating gene transcription, and their functions rely largely on the sequence-specific factors.

While chromatin remodeling complexes keep the promoter region dynamic, the components of the PIC, including GTFs and Pol II, are assembled in a highly regulated fashion

mediated by the mediator complex. The mediator complex is composed of a large number of proteins involved in many aspects of transcription (Biddick and Young 2005). Step-wise assembly of PIC indicates that TBP (TATA-Binding Protein) and TAFs (TBP-Associated Factors) in the TFIID (Transcription Factor II D) complex first associate with the specific promoter region (Meisterernst et al 1990). The TFIIA and TFIIB join afterward and recruit Pol II, which is then stabilized by TFIIF (Chung et al 2003, Lagrange et al 1996). The further association of TFIIE, TFIIH, and other regulators controls Pol II activity and mediates the transition from transcription initiation to elongation, which is mainly through regulating the phosphorylation state of a subunit of Pol II, CTD (Carboxyl-Terminal Domain). It is now evident that Pol II CTD phosphorylation and isomerization regulate all stages in a transcription cycle, including initiation, elongation, and termination (Egloff and Murphy 2008, Meinhart et al 2005, Phatnani and Greenleaf 2006). As a result, controlling the activity and the configuration of Pol II plays a critical role in efficient and accurate regulation of gene transcription.

### 1.5.2 p68 RNA Helicase in Transcriptional Regulation

The first evidence indicating that p68 RNA helicase acts as a transcription coactivator is finding its interaction with nuclear estrogen receptor α (ERα) (Endoh et al 1999a). ERα is a ligand-activated transcription factor that contains two transactivation domains, AF1 (activating factor 1 containing N-terminal A/B domain) and AF2 (activating factor 2 containing C-terminal E (ligand binding) domain). The activity of AF1 is ligand-independent, whereas the binding of estrogen mediates AF2 activation (Nilsson et al 2001). It has been shown that p68 interacts with AF1 but not AF2 of human ERα. Moreover, mitogen-activated protein kinase (MAPK)-induced phosphorylation of ERα on Ser118 potentiates the interaction of p68 and AF1, which enhances the activity of AF1 and the estrogen-mediated transcriptional activation. Interestingly, the

helicase activity of p68 is dispensable as a coactivator of ERα-AF1. Specificity tests also showed that p68 has no effect on ERβ- AF1 and AF2. These findings suggest that p68 functions as a coactivator that is specific for ERα-AF1, and MAPK signaling regulates the interaction between p68 and ERα-AF1 (Endoh et al 1999a).

Other than ERα, p68 was found to interact with other ERα coactivators, such as steroid receptor coactivator 1 (SRC1) and steroid receptor RNA activator (SRA), suggesting that p68 might serve as a bridge to facilitate the interaction between ERα and its coactivators (Watanabe et al 2001). Indeed, some reports have strengthened this idea of p68 acting as a bridge. First, p68 was found to enhance CBP/p300-mediated transcriptional activation by interacting with CBP/p300 and RNA Pol II (Rossow and Janknecht 2003). Second, it was found that p68 interacts with Smad3, a member of the Smad family of transcriptional activators, and facilitates the formation of a transcriptionally active complex comprising smad3, p68, and CBP to enhance TGF-β mediated cellular responses (Warner et al 2004). Third, p68 was shown to serve as an adapter between the CCCTC-binding factor (CTCF), which is a DNA-binding protein, and its interacting factors (Yao et al 2010). Therefore, these findings suggest that p68 may serve either as a bona fide coactivator through its ATPase/helicase activities or as an adaptor to regulate gene transcription in the cells.

A recent report demonstrated that p68 functions as a transcription coactivator of p53, a tumor suppressor that transcriptionally activates certain genes involved in cell cycle arrest, DNA repair, and apoptosis in response to stresses and DNA damage. p68 was found to be recruited to the p53-responsive promoters to stimulate transcription in a p53-dependent manner. Depletion of p68 inhibits the expression of p53 target genes, such as p21 (a cell cycle inhibitory protein), as well as p53-induced apoptosis, suggesting an essential role of p68 in p53-mediated response

(Bates et al 2005a). The coactivator activity of p68 in p53 signaling echoes the finding for p68 and ERα in response to estrogen, suggesting that p68 may regulate transcription through the recruitment of other transcription factors or coactivators, such as CBP, p300, SRC1, and RNA Pol II (Endoh et al 1999a, Rossow and Janknecht 2003, Watanabe et al 2001).

In contrast to functioning as a transcription coactivator, p68 has been suggested as acting as a transcription corepressor by interacting with HDAC1 (Wilson et al 2004a). A recent study showed that Drosophila homolog of human p68 RNA helicase, Dmp68, plays a role in RNA export and gene deactivation. Termination of gene transcription requires the maturation or destruction of pre-existing transcripts and the return of the chromatin structure to an inactive state. Mutation of Dmp68 revealed an accumulation of transcripts in the nucleus and a delayed gene shutdown (Buszczak and Spradling 2006a). Therefore, these findings suggest that p68 may regulate transcription at multiple stages, including early initiation and termination.

### 1.5.3 p68 RNA Helicase in Pre-mRNA Splicing

The precursor messenger RNAs (pre-mRNAs) in higher eukaryotes usually comprise multiple non-coding intervening sequences, so-called introns, which must be removed by the splicing process. The process of splicing pre-mRNAs involves the precise excision of the introns and the ligation of the flanking exons. Alternative splicing is a mechanism by which the introns in the pre-mRNAs are selectively excised to produce variants of mature mRNAs, thus increasing the complexity of the entire proteome. In the pre-mRNA splicing process, RNAs and proteins cooperate extensively as ribonucleoproteins (RNPs) to form a MegaDalton machine, called the spliceosome, where the pre-mRNAs are spliced to yield the mature mRNAs.

Extensive studies revealed that the spliceosome is composed of several major building blocks, including U1, U2, U4/U6, and U5 small nuclear (sn)RNPs (Jurica et al 2002). In addition to snRNPs, a core set of over 100 proteins with splicing activities is identified in this complex in a stage-specific fashion (Agafonov et al 2011). Due to the spliceosome's size and the dynamics of its assembly, analyses of the splicing kinetics and the conformational rearrangements are challenging. Recently, the basic molecular mechanism underlying the splicing process was proposed. During the splicing process, the 5'splicing site (5'ss) in the intron is recognized by the U1 snRNP complex that subsequently recruits the U2 snRNP to the 3' splicing site (3'ss) and the branching point sequence (BPS) in order to form a pre-spliceosome complex. The U4/U6 and U5 snRNPs are further recruited to the pre-spliceosome complex to form pre-catalytic spliceosome. In order to activate the splicing activity of the spliceosome, the U1 and U4 snRNPs have to be released from the complex with the assistance of some RNA-dependent ATPases/helicases to unwind RNA-RNA and RNA-protein interactions. At the end of the splicing process, the intron is removed from the pre-mRNA along with the rest of the snRNPs (Brow 2002, Wahl et al 2009). Within a single splicing cycle, numerous DEAD box RNA helicases have been identified to catalyze RNA-RNA rearrangements and RNP remodeling events (Forch et al 2003, Tseng et al 2011, Xu et al 2004).

The first clue linking the p68 RNA helicase to the pre-mRNA splicing process is supported by the comprehensive analysis of mass spectrometry that showed the existence of p68 in the spliceosome complex (Neubauer et al 1998). Using methylene blue-mediated crosslinking also showed that p68 interacts with the U1-5'ss duplex during spliceosome assembly. Depletion of the p68 RNA helicase does not affect assembly of the pre-spliceosome complex; however, it inhibits the dissociation of the U1 snRNP from the 5'ss, suggesting that p68 plays a role in

destabilizing the U1-5'ss interaction, which is essential in the transition from pre-spliceosome to spliceosome (Liu 2002). Abolishing the ATPase/helicase activities of the p68 RNA helicase does not affect the association of p68 to the splice sites, whereas it abrogates the dissociation of the U1 snRNP from the 5'ss. Also, it was found that p68 participates in the addition of U4.U6/U5 snRNPs, and this does not require the ATPase and helicase activities (Lin et al 2005). These data suggest that p68 may play multiple roles in the pre-mRNA splicing process and that some of them may require the ATPase/helicase activities.

Human Ras (H-Ras) plays a crucial role in controlling proliferation, differentiation, and apoptosis via coupling extracellular signals to intracellular networks. Alternative splicing has been found to process H-Ras pre-mRNA to render two proteins, p19 and p21 H-Ras, by either excluding or including the alternative intron D exon (IDX). This alternative splicing process is regulated by some cis- and trans-acting factors (Camats et al 2008). An intronic silencer sequence (rasISS1), acting along with IDX, has been characterized to regulate its upstream intron negatively. The p68 RNA helicase was shown to associate with both IDX and rasISS1. Depletion of p68 expression in HeLa cells results in an increase of IDX inclusion in the endogenous mRNA, indicating that p68 plays a role in regulating H-Ras alternative splicing (Guil et al 2003). Further studies revealed that the p68 RNA helicase promotes the exclusion of IDX from H-Ras pre-mRNA by unwinding the IDX-rasISS1 structure, preventing the binding of hnRNP H to IDX-rasISS1, and altering the dynamic localization of SC35, a splicing factor that facilitates IDX inclusion (Camats et al 2008).

Another example of the p68 RNA helicase involving in alternative splicing is the regulation of tau exon 10 splicing, which plays a crucial role in neurodegenerative diseases, such as Alzheimer's disease. The p68 RNA helicase was identified as an activator of tau exon 10

splicing. During the splicing process, p68 induces a conformational change at the 5'ss, thereby increasing the access of the U1snRNP to the 5'ss of tau exon 10. In concert with p68, RBM4, an intronic splicing activator, interacts with the splicing region to enhance the spliceosome activity (Kar et al 2011). Taken together, p68 was shown to play a role in the regulation of pre-mRNA splicing, as well as alternative splicing, by interacting with different splicing regulators.

### 1.5.4 p68 RNA Helicase in Ribosome Biogenesis

Ribosome is an essential component for protein biogenesis in all living organisms. It has been shown that some DEAD box RNA helicases are involved in the ribosome biogenesis (Burger et al 2000, Daugeron and Linder 2001, Jalal et al 2007, Lavoie et al 1993, Saporita et al 2011). The p68 RNA helicase and its homologue, p72, play a role in ribosome biogenesis and cell proliferation (Jalal et al 2007). In the case where the p68 gene is suppressed, p72 overexpression can rescue the cell proliferation, whereas co-silencing of both genes causes the disorder of nucleolar structure and cell death. Furthermore, suppression of both p68 and p72 reduces the nucleolytic cleavage of 32S pre-rRNA, which causes failure of pre-rRNA maturation and ribosome biogenesis. The p19ARF tumor suppressor has been shown to regulate ribosome biogenesis negatively in response to hyper-proliferative signals by interfering nucleolar protein or RNA polymerase I transcription factor, TTF-1 (Itahana et al 2003, Lessard et al 2010). Recently, the p68 RNA helicase was shown to play a role in ARF-mediated inhibition of ribosome biogenesis (Saporita et al 2011). This study demonstrated that ARF targets p68 and prevents its interaction with nucleophosmin (NPM) protein, rDNA promoter, and nuclear pre-ribosomes, ultimately reducing the ribosome output. Additionally, in ARF-deficient cells, suppression of p68 is sufficient to impair oncogene RasV12-driven colony formation *in vitro* and

tumor growth in mice. Taken together, these findings indicate that p68 RNA helicase is a target to regulate cell proliferation at least through participating in ribosome biogenesis.

### 1.6 p68 RNA Helicase in Cancer Development

Cancer, also known as a malignant neoplasm, involves unregulated cell growth. The unrestricted growth observed in neoplasms is generally due to a stepwise acquisition of genetic alterations, including the function gain of oncogenes and repression of tumor-suppressor genes (Hanahan and Weinberg 2000). In cancer, cells divide uncontrollably to form malignant tumors, which invade nearby tissues, and eventually spread throughout the body via the lymphatic system or bloodstream (Hanahan and Weinberg 2000). Not all tumors are malignant. Benign tumors do not grow unlimitedly, invade nearby tissues, or spread throughout the body. Therefore, understanding the mechanisms causing uncontrolled cell division and growth, as well as their dissemination ability, is a key to treating patients with cancers.

The p68 RNA helicase has been demonstrated to play an essential role in normal development involving cell growth and differentiation (Stevenson et al 1998b). Recently, a growing body of evidence links p68 to cancer development (Fuller-Pace and Moore 2011, Shin et al 2007, Wang et al 2011, Yang et al 2005c). It has been shown that the tyrosine phosphorylation of p68 is present in six different cancer cell lines and tumor tissues, but not in the corresponding normal cells/tissues. Induction of the apoptosis in cancer cells by some anticancer drugs, such as tumor necrosis factor α (TNF-α), tumor necrosis factor-related apoptosis-inducer ligand (TRAIL), and STI-571, diminishes the tyrosine phosphorylation of p68, suggesting that the tyrosine phosphorylation status of p68 is associated with abnormal cell proliferation and cancer development (Yang et al 2005c).

Another DEAD box RNA helicase, p72, highly related to p68, was discovered in 1996 (Lamm et al 1996). It was shown that p68 and p72 share a 90% identity across the conserved helicase core and can form heterodimers in cells to function in mRNA processing. Moreover, p68 and p72 can be found in a variety of protein complexes. The potential of p68 and/or p72 to coexist with other factors in different complexes may imply a wide range of functions for p68 and p72 (Ogilvie et al 2003). A recent study reported that the expression of p68 and p72 increases during the transition of polyp-->adenoma-->adenocarcinoma in the progression of colon cancer (Shin et al 2007). In this study, p68 and p72 form complexes with β-catenin to activate the expression of some proto-oncogenes, including c-Myc, Cyclin D1, c-jun, and fra-1. Moreover, double knockdown of p68 and p72 reduces the expression of these proto-oncogenes and enhances the transcription of a cell cycle inhibitor, p21 (WAF1/CIP1), whose expression is suppressed by c-Myc. Knockdown of p68 and p72 in colon cancer cells diminishes their ability to form tumors *in vivo*. Thus, p68 and p72 may participate in colon cancer development by activating proto-oncogenes directly and suppressing the cell cycle inhibitors indirectly.

The androgen receptor (AR) has been shown to play an important role in prostatic cancer (PCa) development (Jenster 1999, Lonergan and Tindall 2011). Recently, p68 RNA helicase was found to function as a coactivator in AR-mediated transcription activation in prostate cancer (Clark et al 2008a). The p68 protein was identified to interact with AR in a yeast two-hybrid screening, and coimmunoprecipitation in the PCa LNCaP cell line confirmed this interaction further. Chromatin immunoprecipitation (ChIP) analysis showed that AR and p68 coexist in the promoter region of the androgen responsive prostate-specific antigen (PSA) gene. Knockdown of p68 reduces AR-mediated PSA expression. Luciferase reporter assay revealed that tyrosine phosphorylation of p68 is required for the transcription activity of AR-regulated promoter,

wherein the ATP-binding is dispensable. Tissue microarray screening also showed an increased frequency and expression of p68 in prostate cancer compared with benign tissues. Therefore, the study suggests that the p68 RNA helicase may control gene expression in an AR-dependent manner to regulate prostate cancer development (Clark et al 2008b).

Although abundant evidence supports the role of the p68 RNA helicase in promoting cancer progression, recent studies in breast cancer revealed an opposite role of p68 as a tumor co-suppressor in a p53-dependent manner (Bates et al 2005a, Moore et al 2010). As mentioned earlier, p68 has been shown to interact with p53 to activate the expression of cell cycle inhibitor, p21, and other apoptosis-related genes in response to DNA damage (Bates et al 2005a). Also, a recent study showed that the expression of p68 and the Δ133p53 isoform(s), negative regulators of full-length p53, is inversely correlated in primary breast cancer (Moore et al 2010). Consistently, depletion of p68 in culture cells results in an increase of the Δ133p53 isoform(s) in response to DNA damage, and the increased Δ133p53α inhibits the p53-dependent transcription of p21. The data suggest that p68, p53, and Δ133p53α may form part of a complex to regulate p53-mediated transcription and may modulate the function of p53 in breast and other cancers that harbor wild-type p53.

Novel mechanisms of microRNAs (miRNA)-mediated gene regulation at the posttranscriptional level have emerged recently. MiRNAs regulate gene expression by targeting mRNAs for translational repression or mRNA degradation (Chen 2005). Growing evidence shows that many miRNAs are regulated in the normal development, and abnormal expression of specific miRNAs may contribute to cancer progression (Chen 2005). Studies in cultured cells and human tissues revealed that the expression of the p68 RNA helicase increases progressively during breast cancer development (Moore et al 2010, Wang et al 2011). In breast cancer cells,

p68 was found to upregulate a subset of miRNAs, including miR-21 and miR-182, which downregulate a tumor suppressor, PDCD4, and cytoskeleton regulators, cofilin and profilin, respectively (Wang et al 2011). Depletion of p68 leads to cytoskeleton reorganization and reduced cell proliferation (Wang et al 2011). These findings reveal a novel functional role of p68 via regulating microRNAs, and suggest a potential target, including p68 and its downstream microRNAs in the treatment of breast cancer.

The compound (-)-epigallocatechin-3-gallate (EGCG), a major catechin found in green tea, induces apoptosis and suppresses tumor growth by regulating a wide range of signaling pathways (Nagle et al 2006, Singh et al 2011). It has been found that EGCG can bind covalently to cysteine residues in proteins and thus regulate protein function. Recently, the p68 RNA helicase was identified as a novel EGCG-binding target in AZ521 human gastric cancer cells (Tanaka et al 2011). Treatment of AZ521 cells with EGCG reduces the protein level of p68 in dose and time-dependent manners. Furthermore, EGCG inhibits AZ521 cell proliferation by promoting protein degradation of β-catenin (Tanaka et al 2011), a transcription activator that was shown to be upregulated by p68 in tumor progression (Yang et al 2006). Taken together, the p68 RNA helicase seems to play an essential role in the progression of various types of cancers, which may strengthen the idea that targeting this protein could be an effective way to treat cancers.

### 1.7 Cancer Metastasis

Cancer metastasis is the spreading of primary tumor cells through the lymphatic system or bloodstream to distant organs. Metastasis causes most cancer deaths, yet the underlying mechanism controlling it remains one of the most enigmatic aspects of this disease. Accumulating evidence suggests that metastasis can be viewed as a two-phase process. The first

phase involves the escape of cancer cells from the original site to a distant organ. The second phase, known as colonization, involves the adaptation of the cancer cells and the development of micrometastasis at the new place. To start the metastatic cascade, cancer cells within the primary tumors need to gain an invasive phenotype. The invasive cancer cells then invade the surrounding tissues and migrate toward blood vessels, where they intravasate to enter the circulation system. The cancer cells travel through the circulation and exhibit anchorage-independent survival. Then, the circulating cancer cells leave the circulation via extravasation and invade the foreign tissue. At the foreign site, the cancer cells have to escape the innate immune response, adapt to the microenvironment, and initiate proliferation to form an active macrometastatic colony (Chaffer and Weinberg 2011). Whether the acquisition of malignant traits occurs as an inevitable consequence of primary tumor progression or as an accidental outcome remains a question. Therefore, much work is needed to uncover the mechanisms behind the entire metastatic cascade.

Critical thought from recent studies suggests that the cell population within individual tumors is heterogeneous. The intra-tumoral heterogeneity is uncovered in many types of carcinomas. The populations of cells within a tumor are arranged hierarchically like those in the normal tissue, with the scheme of self-renewing stem cells (SCs), partially differentiated progenitor cells, and fully differentiated end-stage cells (Ailles and Weissman 2007, Al-Hajj et al 2003). These cancer stem cells (CSCs), although not yet proved completely equal to the normal tissue SCs, are defined with enhanced tumor-initiating potential relative to other cancer cells within a tumor and should reveal the ability of self-renewal and producing non-CSC progeny (Ailles and Weissman 2007). Many of the biological traits, such as motility, invasiveness, and self-renewal, are central to the high-grade malignancy and have been traced specifically to the

CSCs' subpopulation in a tumor with larger populations of neoplastic cells. The traits that are ascribed to CSCs, self-renewal and tumor-initiating ability, seem to be the inextricable trigger of successful metastasis. Other traits in CSCs, such as notable motility, invasiveness, and the resistance to apoptosis, also contribute to metastasis (Charafe-Jauffret et al 2009, Marcato et al 2011, Pang et al 2010). This implies a multi-faceted biological program that empowers cancer cells within primary tumors to accomplish multiple steps of the metastasis process.

### 1.7.1 Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) is a biological program characterized by the loss of cell-cell adhesion, the loss of cell polarity, and the acquisition of migratory and invasive properties. The well-organized epithelial cells attach to their neighbors and establish an apical-basolateral polarity through various types of cell junctions, including tight junctions, adherens junctions, desmosomes, and gap junctions. In contrast, mesenchymal cells are organized loosely and have the abilities of migration and invasion (Alberts et al 2002). EMT and its reverse program, MET, (mesenchymal-epithelial transition) are involved in many processes of embryogenesis, including gastrulation, mesoderm formation, and neural crest maturation (Thiery et al 2009). Recently, migratory tumor cells that lose E-cadherin-mediated cell-cell adhesion and detach from the tumor mass and the surrounding stroma, have been observed, providing evidence of EMT at the invasive front of tumors (Brabletz et al 2001, Prall 2007). The phenotype and molecular changes during EMT are briefly illustrated in Fig.1.5.

Recent studies have shown that EMT stimulates non-CSCs to enter into a CSC-like state (Mani et al 2008, Reiman et al 2010). Activation of EMT endows non-CSCs with the set of CSC traits that empowers them to spread from primary tumors to distant sites (Thiery et al 2009). Moreover, EMT confers the carcinoma cells with the increased resistance to apoptosis, the

property that is critical to survive the rigors of the journey from primary tumors to sites of dissemination (Gal et al 2008). EMT may also contribute to the launch of new colonies during metastasis.

How EMT is activated during tumorigenesis is not fully understood. Activation of EMT during embryonic development and tumorigenesis requires signals from the neighboring stromal cells (Yang and Weinberg 2008). It has been suggested that a variety of cell types, including fibroblasts, myofibroblasts, granulocytes, macrophages, mesenchymal stem cells, and lymphocytes, are recruited to the surrounding stroma of advanced primary tumors to create an inflammatory microenvironment that releases EMT-inducing signals (Lopez-Novoa and Nieto 2009). The signals released from the stromal cells activate the expression of EMT-related transcription factors (EMT-TFs) that orchestrate EMT programs within certain cancer cells (Thiery et al 2009). Taken together, it suggests that two elements are required to activate the EMT program. First, certain cancer cells have to undergo genetic and epigenetic alterations in order to respond to EMT induction. Second, the reactive stroma has to release the inductive signals to cause the expression of EMT-TFs and thus activate the EMT program within these responsive carcinoma cells.

It seems that the EMT program is being induced in certain responsive carcinoma cells during tumorigenesis. However, there is still a possibility that some carcinoma cells have intrinsic metastatic ability (Chaffer and Weinberg 2011). Whether the metastatic ability is intrinsic or induced through the EMT program within these carcinoma cells remains largely unproved, due partly to the difficulties of capturing the transitory process in human cancer patients or distinguishing the cancer cells after EMT from stromal cells or tumor-associated fibroblasts. Moreover, although EMT is usually described as a bi-stable switch that causes cells

to flip from one state into the other, in many tumors, epithelial carcinoma cells seem to advance only partly toward the mesenchymal state, which yields cancer cells with concomitant expression of epithelial and mesenchymal markers and thus brings more complexity to understand the EMT program.

### 1.7.2 Downregulation of E-cadherin in Epithelial-Mesenchymal Transition

Loss of functional E-cadherin (encoded by *CDH1* gene) is a key step in the transition of epithelial to mesencymal phenotypes and is also considered a fundamental event in the progression of adenoma to invasive carcinoma (Perl et al 1998). E-cadherin, a member of the cadherin family of transmembrane proteins, serves as a major component in the formation of adherens junctions (Takeichi 1993) that facilitate cell-cell adhesion and establish cell polarity in epithelial cells (Shapiro et al 1995). The adherens junctions are stabilized by the interaction of the intracellular domains of E-cadherin with α, β, and γ catenins, which link E-cadherin to the actin filament cytoskeleton (Takeichi 1995). In epithelial cells, E-cadherin regulates not only cell-cell adhesion, but also cell growth by interacting with β-catenin, a multifunctional transcription factor that responds to Wnt signaling to promote cell proliferation (Nelson and Nusse 2004).

The function of E-cadherin can be regulated in multiple aspects, including genetic, epigenetic, transcriptional, and posttranslational levels (Guilford et al 1998). The loss-functional mutation of *CDH1* gene encoding E-cadherin protein is found less frequently in the majority of E-cadherin-negative carcinomas. On the other hand, epigenetic silencing of the *CDH1* promoter via hypermethylation of CpG islands has been found in various types of cancers (Chang et al 2002, Nass et al 2000, Yeh et al 2002, Yoshiura et al 1995). Moreover, evidence in breast cancer cells showed that the methylation of the *CDH1* promoter is dynamic and not stable, which may

contribute to the heterogeneous phenotype of tumor cells that drives metastasis (Graff et al 2000). Since downregulation of E-cadherin is a hallmark of epithelial-mesenchymal transition, it is reasonable that the transcriptional repressors that regulate E-cadherin expression during embryonic development also contribute to the tumor progression. Indeed, several *CDH1* transcription repressors, including the Snail family, the ZEB family, and some basic helix-loop-helix (bHLH) factors, are now thought of as potent EMT inducers during tumor progression (Peinado et al 2007).

The Snail family share some common features, including a C-terminal domain containing four to six zinc fingers for sequence-specific DNA binding, an N-terminal SNAG domain for transcriptional repression, and a central divergent region that is considered a regulatory domain for their activities and subcellular localization (Peinado et al 2007). Two representatives of the Snail family, Snail1 (aka Snail) (Batlle et al 2000, Bolos et al 2003b, Cano et al 2000) and Snail2 (aka Slug) (Bolos et al 2003b, Hajra et al 2002), are described as key regulators of EMT via repressing E-cadherin expression during both embryonic development and tumor progression (Nieto 2002). They bind to the E-box elements C/A(CAGGTG) of the E-cadherin promoter and recruit corepressors, such as Sin3A and HDAC1/2, to form a repressor complex, which causes chromatin remodeling and the consequent transcriptional repression of E-cadherin (Peinado et al 2004a). In some cases, the function of Snail1 and Snail2 is interchangeable (Barrallo-Gimeno and Nieto 2005). However, a distinct role for each of them was revealed in knockout mice experiments, where Snail1 knockout causes early embryonic death (Carver et al 2001) and Snail2 knockout has no apparent effect on early development (Jiang et al 1998). Previous studies in breast cancer and Xenopus embryos also revealed that Snail1 precedes Snail2 in the genetic expression cascade, supporting their distinct roles in both developmental and tumor-associated

EMT (Aybar et al 2003, Come et al 2006). Moreover, investigation on the role of Snail1 and Snail2 in tumorigenesis of mouse skin carcinoma cell lines in nude mice showed that Snail1 and Snail2 collaborate on tumor growth, while Snail1 is more determinant on local invasion and tumor dissemination (Olmeda et al 2008).

The ZEB family in vertebrates, including ZEB1 (aka δEF1) and ZEB2 (aka SMAD interacting protein 1 (SIP1)), are featured by the presence of two zinc finger clusters, one at each end, and a central homeodomain (Vandewalle et al 2009). ZEB1 and ZEB2 have been shown to play a crucial role in multiple EMT-associated processes that are essential for embryonic development (Vandewalle et al 2009, Zwijsen et al 2001). Emerging evidence also showed that the function of ZEB1 and ZEB2 in transcriptional repression of E-cadherin correlates to tumor malignancy (Comijn et al 2001, Eger et al 2005). Further insight into the molecular mechanism revealed that ZEB1 and ZEB2 bind to the E box-like sequence CACCT(G) in the E-cadherin promoter and recruit corepressors, including C-terminal binding protein (CtBP) and HDACs, to silence E-cadherin expression (Gheldof et al 2012).

The common feature for all bHLH families involves two parallel α-helices connected by a loop structure. This common feature is required for the hetero- or homo-dimerization of bHLH proteins, which is essential for their function in transcriptional regulation. In some cases, bHLH proteins can act as transcriptional activators or repressors by the recruitment of either HAT proteins, such as p300, or corepressors, such as SIN3A (Massari and Murre 2000). E12/47 and Twist belong to class I and class II bHLH factors, respectively. Class I bHLH factors are widely expressed and can function as homodimers or heterodimers with class II proteins. Class II bHLH factors are tissue-specific and function as heterodimers with class I factors. Despite different tissue specificity, all bHLH factors bind to consensus E-box (CANNTG) sites in the promoter

region to regulate gene transcription (Massari and Murre 2000). Recent studies showed that abnormal expression of E12/47 and Twist plays a role in cancer progression through repressing E-cadherin expression (Hwang-Verslues et al 2011, Thiery and Morgan 2004, Yuen et al 2007); however, the molecular basis behind this regulation remains largely unknown.

The evidence that Snail1, Snail2, ZEB1, and ZEB2 function as transcription repressors by recruiting specific chromatin modifying complexes provides a link between the transcriptional repression and the epigenetic modification on the gene silencing of *CDH1* during developmental and tumor-associated EMT (Peinado et al 2004b). Whether these repressors affect other genes by this mechanism remains to be studied.

### 1.7.3 Signaling Pathways toward Epithelial-Mesenchymal Transition

Many signaling pathways, including the transforming growth factor β (TGFβ) superfamily, Wnt, Notch, epidermal growth factor (EGF), hepatocyte growth factor/scatter factor (HGF/SF), fibroblast growth factor (FGF), and hypoxia-inducible factor (HIF), have been shown to contribute to the induction of EMT in both embryogenesis and cancer progression (Yang and Weinberg 2008). Most studies also suggested that the collaboration of different signaling pathways is essential to generate the specificities of EMT in various morphogenetic steps. For example, in cultured Madin-Darby canine kidney (MDCK) epithelial cells, activation of both TGFβ/Smad and Ras signaling pathways is required to promote a full EMT (Janda et al 2002).

TGFβ signaling is mediated by ligand-induced activation of type 1 and type 2 receptors, and it is transduced mainly by the Smad family of proteins (Ikushima and Miyazono 2010). TGFβ signals also regulate non-Smad pathways, including Erk, p38, JUN N-terminal kinase

(JNK), PI3K-Akt, and small GTPases (Moustakas and Heldin 2005, Zhang 2009). Various studies have implicated that TGFβ signals function as primary inducers of EMT during embryonic morphogenesis and cancer progression, although individual family members may induce different cellular responses through diverse mechanisms (Ikushima and Miyazono 2010, Yang and Weinberg 2008). TGFβ receptors are serine/threonine kinases that can be homo or heterodimers and are found at tight junctions, where they associate with regulators of epithelial cell polarity and tight-junction assembly, Par6 and Occludin (Barrios-Rodiles et al 2005, Dore et al 1998, Ozdamar et al 2005). Ligand-mediated activation of TGFβ type II receptors leads to formation of a hetero-tetrameric complex that phosphorylates Par6 and causes the loss of tight junctions and apical-basal polarity, thus promoting EMT (Ozdamar et al 2005). Induction of EMT by activating TGFβ signaling has been shown to be essential in mesoderm formation, neural crest maturation, and cardiac development in various vertebrate embryos (Correia et al 2007, Kimelman 2006, Mercado-Pimentel and Runyan 2007).

Interestingly, TGFβ signaling has been endowed with two opposite functions, tumor promotion and tumor suppression, which are performed through the context-dependent effects on cellular behaviors and tumor-stroma interactions (Blobe et al 2000). Loss of function mutations on type II TGFβ receptors and SMAD4 transducers are commonly found in several types of cancers, which provides evidence that TGFβ signaling functions as a tumor suppressor (Levy and Hill 2006). However, TGFβ signaling also plays a role in tumor promotion, with the evidence that the excessive expression of TGFβ1 is positively correlated with the progression and the metastasis of various cancer types, including breast, colorectal, and prostate cancers (Tsushima et al 1996, Walker and Dearing 1992, Wikstrom et al 1998). TGFβ1-induced activation of SMAD proteins mediates the transcription of several EMT promoting factors, such as the

repressors of cell-cell adhesion molecules, Snail1 and Snail2, and the actin filament remodeling proteins, thus increasing the metastatic potential of tumor cells (Medjkane et al 2009, Morita et al 2007).

The NFκB pathway is emerging as a regulatory mechanism of EMT in carcinoma cells through the induction of Snail1 (Julien et al 2007, Strippoli et al 2008). Inhibition of NFκB signals by introducing non-destructible IκB blocks the induction of EMT, confirming the essential role of this pathway in the regulation of EMT (Huber et al 2004). In colorectal cancer cells, PDGF has been shown to induce EMT through promoting nuclear translocation of β-catenin in a Wnt-independent manner (Yang et al 2006). PDGF signals may also function as an essential mediator of TGFβ-induced EMT (Gotzmann et al 2006). EGF signaling is known to promote EMT by enhancing E-cadherin endocytosis (Lu et al 2003) or by inducing the expression of Snail1 and Twist to repress E-cadherin transcription (Lee et al 2008, Lo et al 2007). Continuous activation of β-catenin-mediated Wnt signaling is viewed as an essential element in the initiation of the vast majority of colorectal cancers (Clevers 2006).

Many genes associated with the induction of EMT are β-catenin targets that are transcriptionally activated during cancer progression (Kim et al 2002). Inhibition of the Wnt-Frizzled (FZD) interaction suppresses tumor invasion and metastasis, further supporting the essential role of Wnt signaling in cancer malignancy (Vincan and Barker 2008). Notch signaling has been shown to function as an EMT promoter during heart development and neural crest formation, as well as tumor progression, partly through the induction of Snail1 and Snail2 to repress E-cadherin transcription (Cornell and Eisen 2005, Niessen et al 2008, Timmerman et al 2004). Studies of various types of cancers revealed that HGF/SF signaling stimulates EMT and promotes metastasis (Argast et al 2011, Copple 2010, Elliott et al 2002). In many cellular and

tumoral systems, hypoxia-induced activation of HIF1 transcription factor has been shown to promote EMT by inducing Snail1, and the concomitant expression of E12/47, ZEBs, and Twist is also observed, suggesting a correlation between hypoxia and the tumor metastasis (Lopez-Novoa and Nieto 2009, Yang and Weinberg 2008). During embryonic development, it is evident that FGF signaling is necessary for maintaining the expression of two Snail genes, Snail1 and Snail2, both of which are critical inducers of EMT (Barrallo-Gimeno and Nieto 2005, Billottet et al 2008).

Taken together, although many signaling pathways contribute to EMT, accumulating evidence indicates that theses diverse signals interplay with each other and converge to the induction of several EMT-associated transcription factors (EMT-TFs), such as Snail1/2, ZEB1/2, E12/47, and Twist (Thiery et al 2009). The EMT-TFs cause the loss of cell-cell adhesion and cell polarity in epithelial cells by downregulating epithelial markers, such as E-cadherin, and enhance the ability of cell migration and invasion by upregulating mesenchymal markers, such as vimentin.

### 1.7.4 Convergence of Signaling Pathways toward Snail1

The induction of Snail1 expression has been demonstrated as the convergence of several signaling pathways that promote EMT during embryonic development and cancer progression (Grunert et al 2003, Seton-Rogers and Brugge 2004, Seton-Rogers et al 2004, Siegel et al 2003, Ueda et al 2004, Xie et al 2004). As the functional loss of E-cadherin is a key event in the process of EMT, the transcriptional repressors mentioned above, including Snail1, Snail2, ZEB1/δEF1, ZEB2/SIP1, E47, and Twist, are implicated in tumor progression. Studies *in vitro* and in model organisms supported further that Snail1 acts as a master downstream effector in TGFβ-induced EMT, as knockdown of Snail1 reverts the loss of epithelial phenotypes (Peinado

et al 2007). Moreover, growing evidence indicated that Snail1 enhances the resistance to apoptosis in cancer cells (Franco et al 2010, Roy et al 2004), implicating its additional role in promoting cancer metastasis by increasing the survival of cancer cells in the circulating system. Also, studies in breast cancer suggested that Snail1 is involved in tumor recurrence by maintaining the self-renewal potential in cancer stem-cell-like cells (De Craene and Berx 2006). Therefore, the induction of Snail1 expression has been considered a key step in the initiation of EMT, as well as the following metastatic process in cancer progression.

### 1.7.5 Snail1 Expression and Cancer Metastasis

During the process of tumor metastasis, the increased migratory and invasive abilities of cancer cells are reminiscent of EMT that is associated with the downregulation of E-cadherin. Snail1, as a potent transcriptional repressor of E-cadherin, has been implicated to play a critical role in tumor metastasis. In the studies of various types of tumors, it has been found that the expression of Snail1 in the tumor cells correlates with poor prognosis in cancer patients. The expression of Snail1 is present at a substantial frequency in the breast carcinomas that have the appearance of lymph node metastasis (Blanco et al 2002). A comprehensive study in hepatocellular carcinomas (HCCs) revealed that over 50% of the primary HCCs have the expression of Snail1 (Yang et al 2009). A recent study demonstrated further the related molecular mechanism that Snail1 promotes HCC metastasis by downregulation of E-cadherin and upregulation of matrix metalloproteinase-2 (MMP2) (Chen et al 2011).

Pancreatic cancer is usually viewed as a deadly malignancy because of its tendency to metastasis and the resistance to chemotherapy. The expression of Snail1 has been found to correlate closely with lymph node invasion and distant metastasis in pancreatic cancers (Yin et al 2007). Studies using two human pancreatic cancer cell lines, Panc1 and BxPC3, revealed that the

expression of Snail1 endows the tumor cells with a highly malignant potential by triggering EMT (Nishioka et al 2010, Yin et al 2007). Moreover, Snail1 expression confers pancreatic cancer cells the chemoresistance to 5-fluorouracil and gemcitabine (Yin et al 2007).

Recently, it was proposed that Snai1l might serve as a tumor marker to predict tumor metastasis. A study conducted lately in head and neck squamous cell carcinomas (HNSCCs) revealed that Snail1-positive tumors are associated strongly with poor differentiation, lymph node invasion, and distant metastasis (Mendelsohn et al 2012). In addition to the epithelial tumor cells, the expression of Snail1 was also found in tumor endothelial cells and stromal myofibroblasts of pharyngeal squamous cell carcinomas (PSCC) (Jouppila-Matto et al 2011). The presence of Snail1 protein in the tumor microenvironment correlates positively with the advanced stage of tumors (stage III, IV) and the poor survival in PSCC patients, suggesting that Snail1 may play an additional role in remodeling the tissues that surround tumor mess, thus promoting metastasis. Taken together, these findings suggest that the expression of Snail1 is involved in tumor metastasis through the effects on the tumors and the surrounding tissues in various types of cancers. Targeting Snail1 could be a promising therapeutic strategy to prevent tumor metastasis.

### 1.7.6 Regulation of Snail1

Posttranslational Regulation

Emerging evidence has shown that subcellular localization and activity of Snail1 are regulated at the posttranslational levels. Although the reverse correlation between Snail1 and E-cadherin expression has been well established, in some cases, the mRNA of both genes co-exists in the same tissues or cancer cell lines (Dominguez et al 2003, Hajra et al 2002), suggesting that

Snail1 is not only regulated at the transcriptional level, but also at the translational and posttranslational levels.

Recently, a proposed model suggested that Snail1 is regulated by glycogen synthase kinase 3 β (GSK3β)-mediated phosphorylation. The model implicates a two-stage phosphorylation of Snail1 by GSK3β at two consensus motifs identified between residues 92 to 121 (Zhou et al 2004b). According to this model, nuclear GSK3β phosphorylates Snail1 at regulatory motif 2, thus inducing its nuclear export. In the second step, cytoplasmic GSK3β phosphorylates motif 1, which results in the association of Snail1 with β-Trcp E3 ubiquitin ligase and the subsequent polyubiquitination and protein degradation. This model provides an explanation that controls the protein stability of Snail1 and explains its short half-life (around 30 min) in some cell lines.

A recent study showed that two lysine residues (K98 and K137) are essential for the stability and activity of Snail1 protein, as double mutation of these two residues abolishes its function as a transcription repressor of E-cadherin (Peinado et al 2005). These two lysine residues are essential for Snail1 to interact with lysyl-oxidase homolog 2 (LOXL2), by which it attenuates GSK3β-mediated Snail1 degradation. Another study also showed that p21-activated kinase 1 (Pak1) modulates the repressor activity of Snail1 by phosphorylating its serine 246 residue. Phosphorylation of Snail1 by Pak1 facilitates its nuclear accumulation and thus enhances its repressor activity (Yang et al 2005e). Taken together, different posttranslational modifications play a role in determining the cellular localization and the activity of Snail1, suggesting a context-dependent regulatory mechanism in the cells.

Transcriptional Regulation of Snail1

Transcriptional regulation is considered a major mechanism to regulate Snail1 expression during the embryonic development and tumor progression. Many signaling pathways, as mentioned above, can stimulate the expression of Snail1. In contrast, studies in breast cancer cells revealed that activation of the ER pathway suppresses Snail1 expression via the involvement of the metastasis-associated gene 3 (MTA3), a subunit of the nucleosome remodeling and histone deacetylation (NuRD) repressor complex (Fujita et al 2003). The promoter study in Snail1 identified several conserved response elements, including two E-boxes, AP1, AP4, SMAD, and LEF1 binding sites (Barbera et al 2004, Peinado et al 2003). Snail1 was found to limit its own expression by binding to the E-box of the promoter, suggesting a delicate feedback mechanism of controlling Snail1 transcription. Although abundant studies demonstrated that Snail1 can be regulated at transcriptional level (Batlle et al 2000, Cano et al 2000, Cheng et al 2001, Jiao et al 2002), the molecular basis behind this mechanism remains elusive and needs to be explored.

Mi-2/Nucleosome Remodeling and Deacetylase (NuRD) Complex

The Mi-2/nucleosome remodeling and deacetylase (NuRD) complex is identified as a multi-subunit protein complex with the unique property of combining both histone deacetylase and chromatin remodeling ATPase activities (Tong et al 1998, Wade et al 1998, Xue et al 1998b, Zhang et al 1998). The ATPase activity of Mi-2/NuRD complex resides in Mi-2 α and/or β proteins, both of which belong to the SWI/SNF chromatin remodeling family that functions as transcription regulators through sliding or ejecting the nucleosome structure (Eisen et al 1995). The deacetylase subunits of Mi-2/NuRD complex comprise HDAC1 and/or HDAC2. Other subunits, such as the methyl CpG-binding domain (MBD) family of proteins, Rbbp4 and Rbbp7,

p66α and p66β, and the metastasis associated (MTA) protein family, are also identified in this complex (Wade et al 1999, Zhang et al 1999a). Two representatives of the MBD family of proteins, MBD2 and MBD3, have displayed a role in coupling chromatin remodeling and DNA methylation in Mi-2/NuRD-mediated gene regulation (Wade et al 1999, Zhang et al 1999a). The mutation analyses in mouse models and embryonic stem cells have provided evidence that MBD2 and MBD3 are critical for gene regulation during development (Hendrich et al 2001, Kaji et al 2006).

The MTA family is composed of three members: MTA1, MTA2, and MTA3. All three have been demonstrated to be components of the Mi-2/NuRD complex (Fujita et al 2003, Xue et al 1998b, Zhang et al 1999a). The function of MTA proteins is correlated intimately with the ER signaling in the development of both normal mammary gland and breast cancer (Manavathi et al 2007). MTA1 was originally identified in breast cancer cell lines with high metastatic potential (Toh et al 1994). A study at molecular level revealed that MTA3 gene is activated through the direct binding of ERα on its promoter. Furthermore, MTA3 protein serves as a corepressor in the Mi-2/NuRD complex to repress the transcription of Snail1, which in turn, suppresses the invasive growth in breast cancer cells (Fujita et al 2003). Interestingly, studies in transgenic mice demonstrated that MTA3 is implicated in suppression of ductal branching in the mammary gland (Zhang et al 2006), whereas MTA1 exhibits an opposite effect with more branching phenotypes (Bagheri-Yarmand et al 2004). These findings suggest that the involvement of different members of MTA proteins can direct the Mi-2/NuRD complex to achieve a unique function in a given cellular or tissue context. Based on the current understanding, a model was proposed to demonstrate a mechanism of transcription repression that involves Mi-2/NuRD complex, as Fig.1.6 shows. Although it is clear that the incorporation of different subunits is required for the

Mi-2/NuRD complex to perform its unique function, to what extent these molecules participate in transcription repression remains a question and needs further effort to uncover the mechanistic links.

### 1.8 Rationale and Aims of the Dissertation

Previous studies from our laboratory demonstrated that tyrosine phosphorylation of p68 on the Y593 residue is required for PDGF-mediated EMT in the colorectal cancer cell line, HT29 (Yang et al 2006). The tyrosyl-phosphorylated p68 facilitates the nuclear translocation of β-catenin in a Wnt-independent manner, subsequently activating the EMT-related genes. Insight into the molecular basis revealed that p68 is tyrosyl-phosphorylated by c-Abl kinase upon the stimulation of PDGF. The tyrosyl-phosphorylated p68 displaces Axin protein from the APC-Axin-GSK3β destructive complex and thus stabilizes β-catenin. The study also showed that the tyrosyl-phosphorylated p68 associates with β-catenin and promotes its nuclear translocation. In the epithelial cells, the transactivation function of β-catenin is also restricted by adherens junctions through interacting with E-cadherin. Whether tyrosyl-phosphorylated p68 plays a role in regulating E-cadherin or not remains a question.

In our preliminary experiment, we found that tyrosyl-phosphorylated p68 downregulated E-cadherin expression at both protein and mRNA levels. However, overexpression or knockdown of E-cadherin did not significantly affect the nuclear translocation of β-catenin, suggesting that tyrosyl-phosphorylated p68 may activate a more comprehensive signaling to trigger EMT. Given the fact that p68 has been identified to associate with several critical transcriptional regulators, such as RNA Pol II, CBP/p300, HDAC1, p53, ERα, and Smad3, it is very likely that the functional role of phosphorylated p68 is involved largely in transcriptional regulation.

Metastasis is the major cause of death in cancer patients. Despite intensive studies over decades, the molecular mechanisms that control this death-leading process are poorly defined. Growing evidence suggests that EMT is the process that confers carcinoma cells with metastatic potentials, such as increased migration and invasion abilities. It has been well established that downregulation of E-cadherin is the hallmark of EMT and that this phenomenon correlates to various types of high-grade tumors. In our preliminary experiments, we observed that phosphorylation of p68 at Y593 downregulates the expression of E-cadherin at both mRNA and protein levels, which is consistent with our previous report that phosphor-p68 promotes EMT. We also observed that the level of tyrosine-phosphorylated p68 is higher in metastatic cancer cell lines than in the corresponding non-metastatic counterparts. Therefore, we speculate that phosphorylation of p68 at Y593 may contribute to the process of cancer metastasis via regulating EMT.

In this dissertation, the aims can be divided into two parts. First, I study the molecular basis by which tyrosyl-phosphorylated p68 is involved in the regulation of E-cadherin expression in colon cancer cells. Second, I examine the correlation between tyrosyl-phosphorylated p68 and the metastasis potential in a xenograft tumor model and colorectal tumor tissues. Here, I demonstrate that the central role of tyrosyl-phosphorylated p68 in downregulation of E-cadherin is through upregulating Snail1 transcription in colorectal tumor cells. The tyrosyl-phosphorylated p68 displaces HDAC1 from the Mi-2/NuRD corepressor complex, which leads to the activation of Snail1 transcription. The Snail1 protein is a potent EMT inducer and is positively correlated with the metastatic potential in various types of cancers. Abolishing the phosphorylation by expressing Y593F mutant of p68 reduces the metastatic potential of a colorectal cancer cell line, SW620, in the xenograft tumor model. I also demonstrate that phosphorylation of p68 at the

Y593 residue is higher in human colorectal tumors than in their corresponding normal tissues. Additionally, the expression of tyrosyl-phosphorylated p68 is positively correlated with the expression of Snail1. Taken together, I discover a molecular mechanism by which tyrosyl-phosphorylated p68 regulates Snail1 transcription through modulating the protein composition in the Mi-2/NuRD corepressor complex. Regarding the expression of Snail1 as an indicator of cancer malignancy, I also link the tyrosyl-phosphorylated p68 to the metastatic potential in colorectal tumors, which may provide a new route as a therapeutic target in future cancer treatment.

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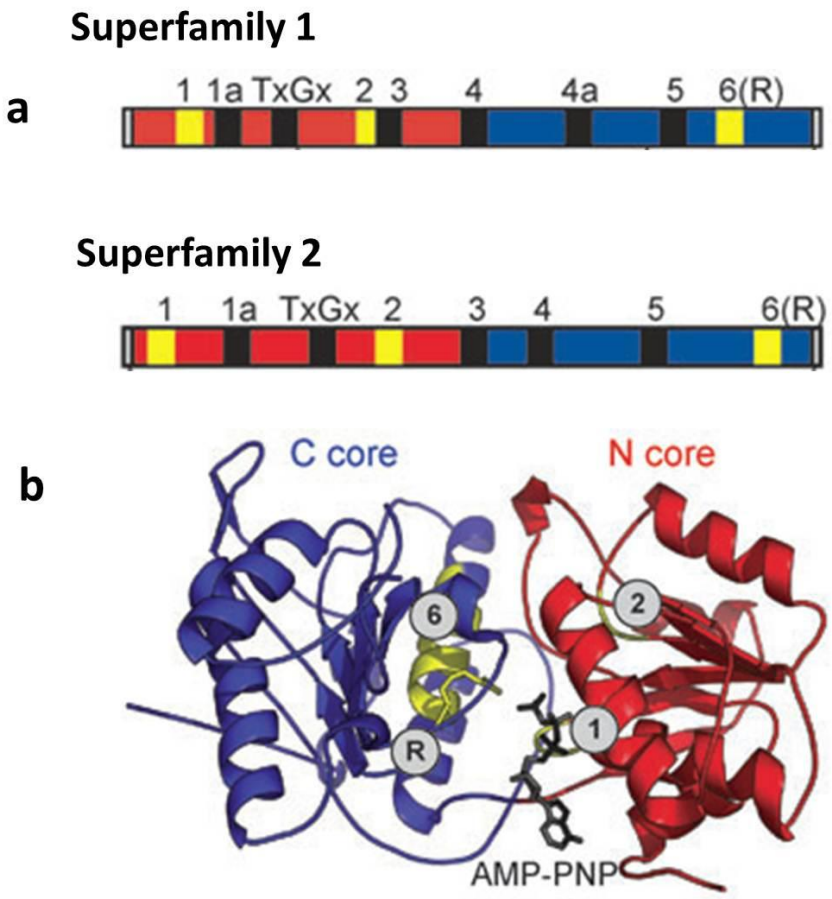


Figure 1.1 Classification of helicases and translocases

(a) The "core domains" of superfamily 1 and 2 (SF1 and SF2) of six helicase/translocases superfmailies are shown here. The positions of the nine signature motifs therein are shown for SF1 and SF2 classes of helicase. (b) The SF1 and SF2 enzymes contain a monomeric core formed from the tandem repeat of a RecA-like fold. The N- (red) and C-terminal (blue) RecA-like domains are shown. An NTP analogue (black) is bound at the interface of the core domains. Motifs 1 and 2, related to Walker A and B motifs, are located on the N-core side of the cleft. Motif 6, which contains an arginine finger residue, is contributed by the C-core domain. This representative structure is the core of PcrA helicase from SF1. Note that these core domains constitute the minimal translocation motor. (Singleton et al 2007)

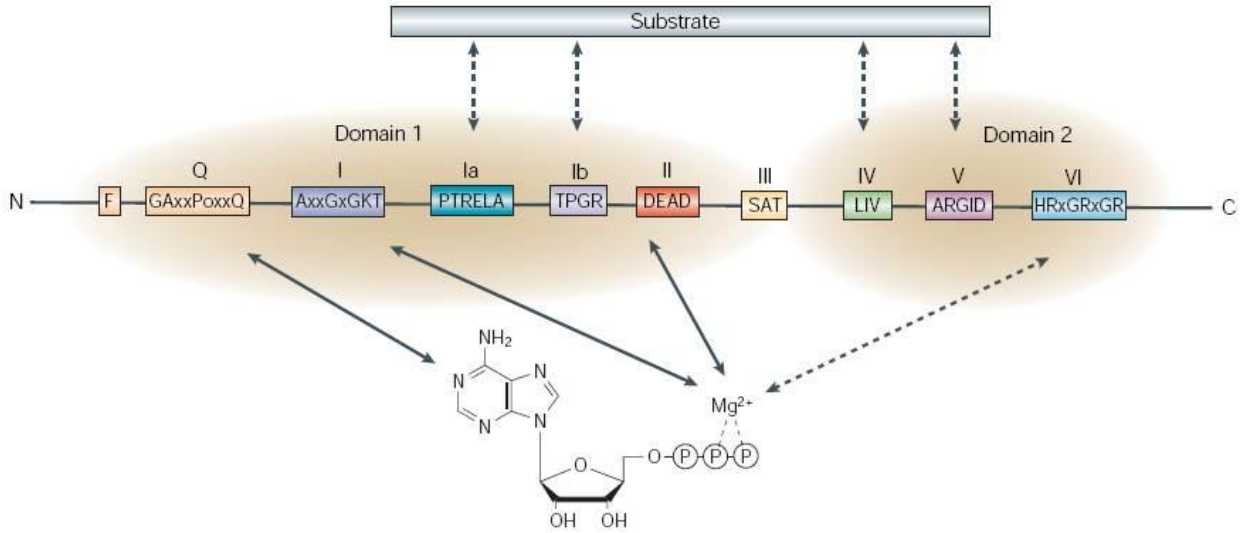


Figure 1.2 The conserved motifs of DEAD-box proteins and their interaction with ATP

A sequence analysis of the DEAD-box proteins has revealed nine conserved motifs in the helicase core domain, which is responsible for ATP binding/hydrolysis and RNA binding/ unwinding activities. The motifs Q, I, and II are required for ATP binding and hydrolysis. The motifs Ia, Ib, IV, and V are involved in the process of RNA binding and unwinding. (Rocak and Linder 2004)

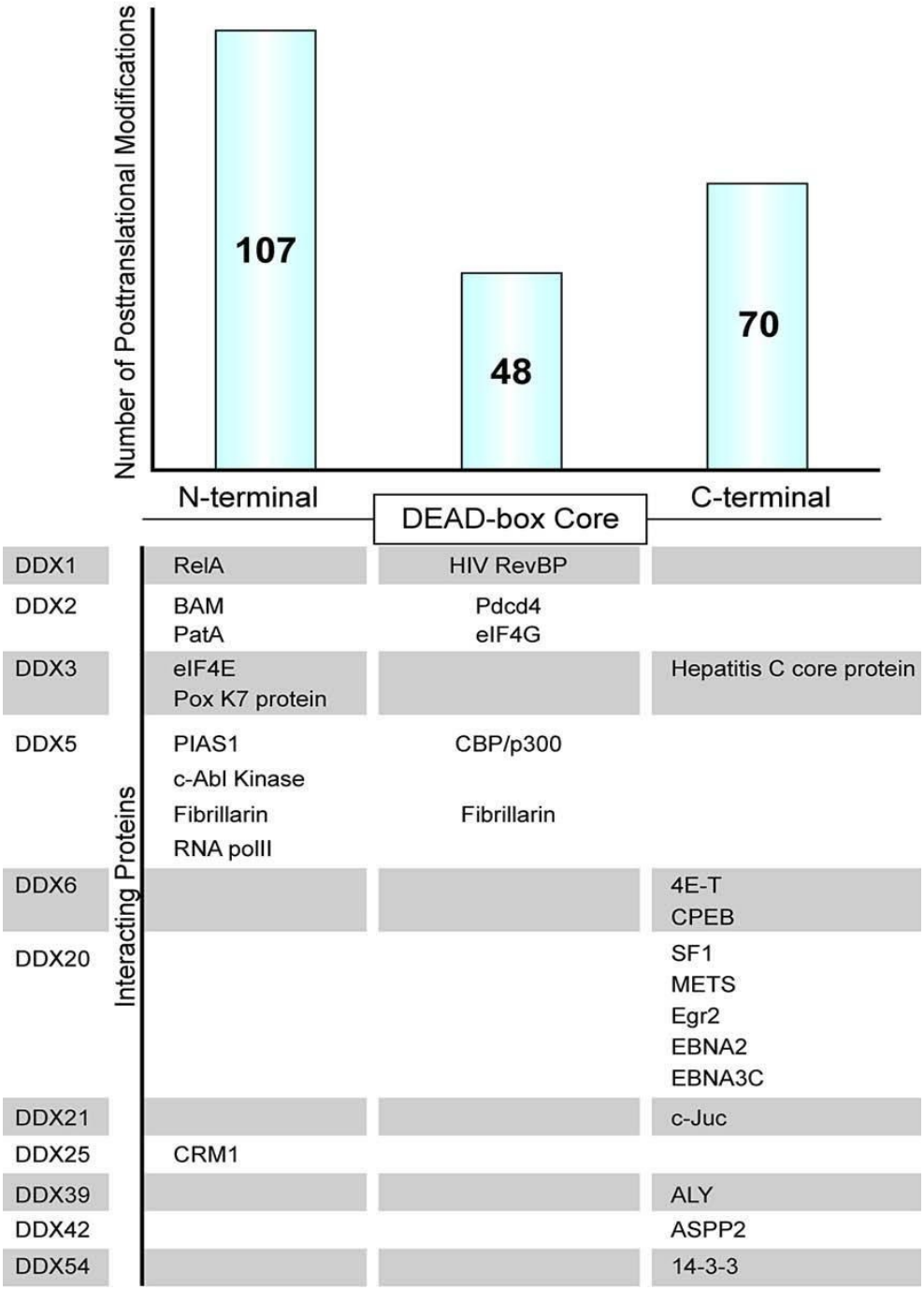


Figure 1.3 DEAD-box posttranslational modifications and protein-protein interactions

A schematic representation of the domain structure of the DEAD-box proteins illustrates the conserved central DEAD-box core region and the unique flanking N-terminal and C-terminal regions. The above shows the number of posttranslational modifications with respect to their location within the DEAD-box proteins. Below lists known DEAD-binding proteins, as well as where the interaction occurs on their respective open reading frame. (Gustafson and Wessel 2010)

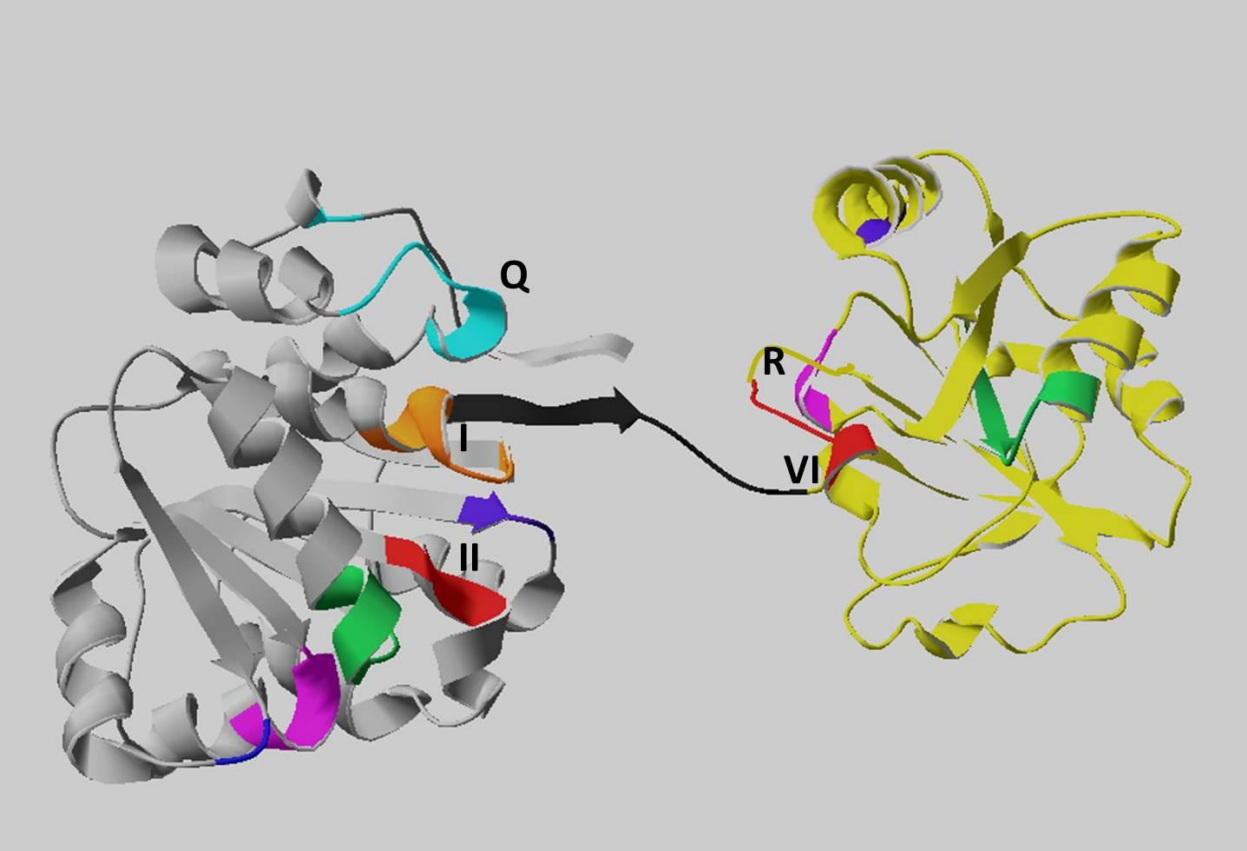


Figure 1.4 The crystal structure of conserved helicase core of DEAD box RNA helicase

N-terminal domain, Grey; linker, black; C-terminal domain, yellow

N-terminal motifs: Q motif, F(16)GFEEPSAIQ, cyan; motif I (Walker A motif), ASQSGTGKT, orange; motif Ia, PTRELA, green; GG, blue; motif Ib, TPGR, pink; motif II (Walker B motif DEAD, red; motif III, SAT, purple.

C-terminal motifs: motif IV, VIFCNTRR, green; "conserved R" motif, purple; motif V, RGID, pink; motif VI, HRIGRGGR, red.

Data Deposition: The atomic coordinates and structure factors were retrieved from the Protein Data Bank, www.rcsb.org (PDB ID codes: 1FUU and1FUK for full-length eIF4a and its carboxyl-terminal domain, respectively). Picture was generated by Swiss-PdbViewer software. (Caruthers et al 2000)

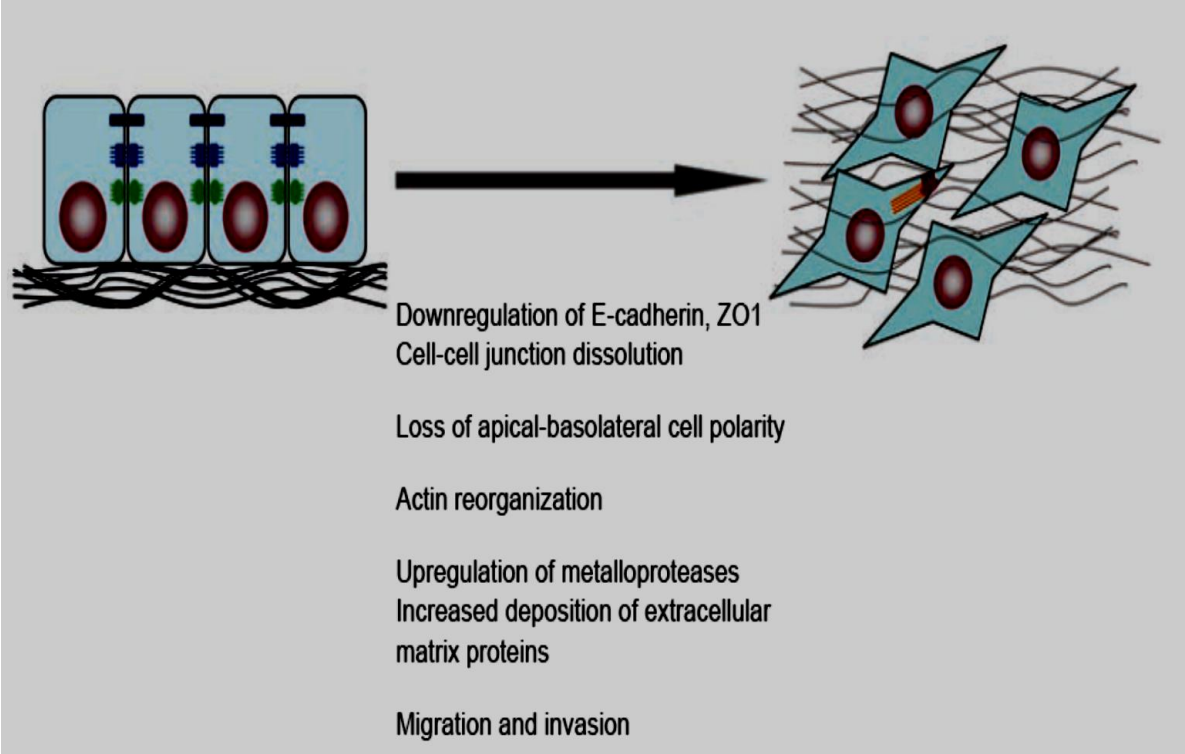


Figure 1.5 Epithelial-mesenchymal transition (EMT)

EMT is characterized by the loss of cell-cell junctions, including tight junctions (black), adherens junctions (blue), and desmosomes (green), which results in the collapse of apical-basolateral polarity in epithelial cells following by the acquisition of mesenchymal phenotypes, such as spindle-shape morphology, stress fiber formation (red), and the migration and invasion abilities. (Xu et al 2009)

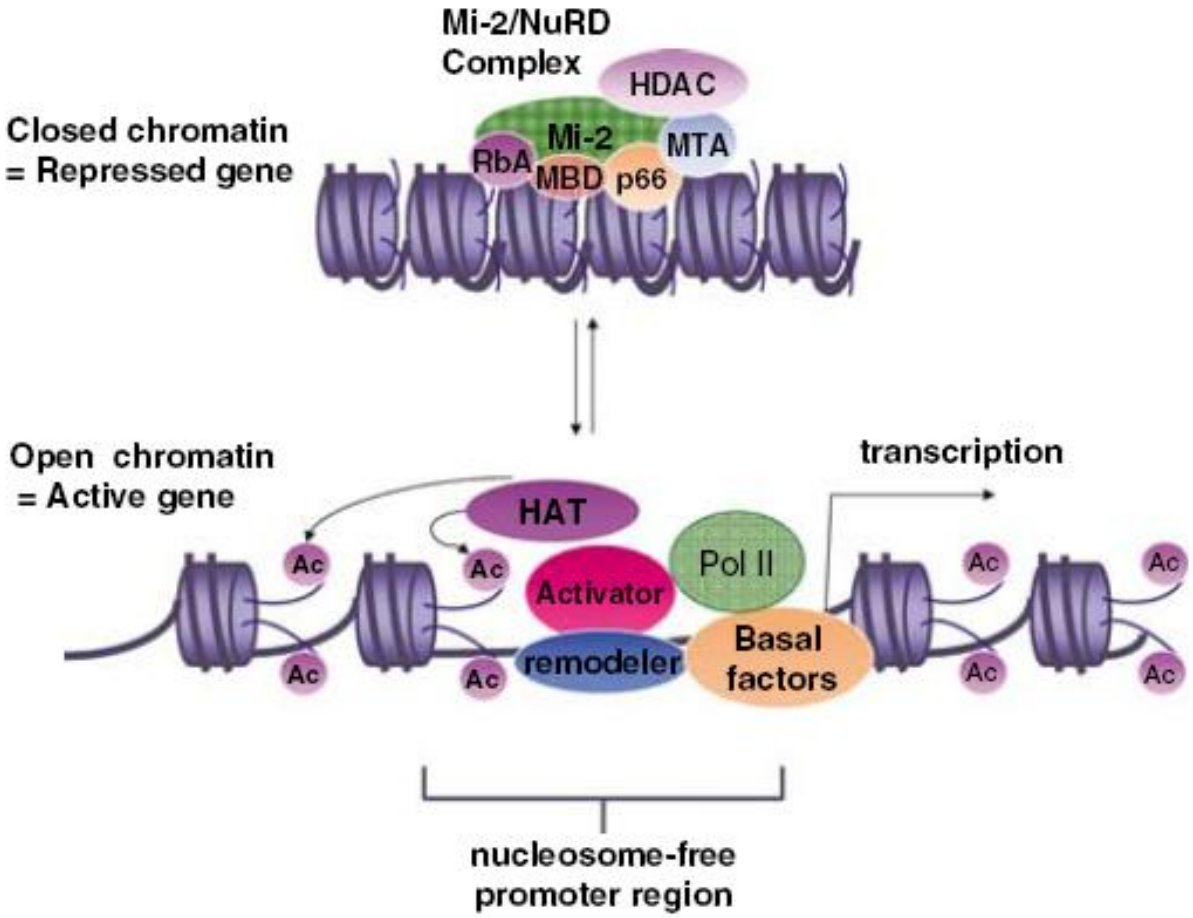


Figure 1.6 The molecular model describes the structural change of chromosome between transcription repression and activation.

The upper portion illustrates dense nucleosome and compacted chromatin in a repressed gene. The bottom portion illustrates the open, more relaxed chromatin and nucleosome-free structure of an actively transcribed gene. The Mi-2/NuRD complex has been shown to have a critical role in gene repression through nucleosome remodeling. (Denslow and Wade 2007)

# CHAPTER 2: PHOSPHORYLATED p68 RNA HELICASE ACTIVATES SNAIL1 TRANSCRIPTION BY PROMOTING HDAC1 DISSOCIATION FROM THE SNAIL1 PROMOTER[1]

[1] This chapter has been published in Oncogene (2010) 29, 5427–5436. I contributed equally to this work.

## 2.1 Abstract

The nuclear p68 RNA helicase is a prototypical member of the DEAD box family of RNA helicases. P68 RNA helicase has been implicated in cell proliferation and early organ development and maturation. However, the functional role of p68 RNA helicase in these biological processes at the molecular level is not well understood. We previously reported that tyrosine phosphorylation of p68 RNA helicase mediates the effects of PDGF in induction of EMT by promoting β-catenin nuclear translocation. Here we report that phosphorylation of p68 RNA helicase at Y593 up-regulates transcription of the Snail1 gene. The phosphorylated p68 activates transcription of the Snail1 gene by promoting HDAC1 dissociation from the Snail1 promoter. Our results showed that p68 interacted with the nuclear remodeling and deacetylation complex MBD3:Mi-2/NuRD. Thus, our data suggested that a DEAD box RNA unwindase can potentially regulate gene expression by functioning as a protein 'displacer' to modulate protein-protein interactions at the chromatin remodeling complex.

## 2.2 Introduction

E-cadherin, a prototypical member of the cadherin family, is the key component of the epithelial cell-cell adhesion junction. During embryonic development and tissue remodeling, the expression of E-cadherin is repressed. As a consequence, the strong adhesions of the epithelial cells are weakened. The cells adopt a fibroblast-like morphology. This is the so-called Epithelial-

Mesenchymal-Transition (EMT) process (Kang and Massague 2004). Loss of E-cadherin expression or function constitutes one main reason for epithelial carcinoma progression to an invasive metastatic status (Kang and Massague 2004, Rodrigo et al 1999). Both expression and function of E-cadherin are regulated at multiple levels (Bryant and Stow 2004, Davis et al 2003). A zinc-finger transcription factor, Snail1, and its closely related family have been demonstrated to play a key role in downregulation of E-cadherin gene transcription (De Craene et al 2005, Peinado et al 2004b). It was revealed that Snail1 mediates E-cadherin repression by recruiting histone deacetylase (HDAC) to the E-cadherin promoter (Peinado et al 2004b). Repression of E-cadherin by Snail1 leads to Epithelial-Mesenchymal Transition (EMT). As a master regulator for EMT, expression of Snail1 is stimulated by signaling pathways of a number of growth factors (De Craene et al 2005), such as, EGF, FGF and TGF-β (Ciruna and Rossant 2001, Lu et al 2003, Zavadil and Bottinger 2005). Cellular levels of Snail1 are regulated via a number of different mechanisms, including gene transcription and protein turn-over in cells (Barbera et al 2004, Zhou et al 2004a). Most recently, Fujita and co-workers demonstrated that MTA3, a member of the metastasis associated gene family, regulates Snail1 expression by targeting the nuclear remodeling and deacetylation complex MBD3:Mi-2/NuRD-HDAC1 to the Snail1 promoter in breast cancer cells (Fujita et al 2003).

The nuclear p68 RNA helicase (ref to as p68) is a prototypical member of the DEAD box family of RNA helicases (Crawford et al 1982b, Lane and Hoeffler 1980a). As an early example of a cellular RNA helicase, the ATPase and the RNA unwinding activities of p68 RNA helicase were documented (Ford et al 1988, Hirling et al 1989, Iggo and Lane 1989a). Expression of p68 correlates with cell proliferation and early organ maturation (Stevenson et al 1998a). The protein was also shown to potentially play a critical role in the tumorigenesis process (Causevic et al

2001a, Dubey et al 1997, Wei and Hu 2001). It has been demonstrated by numerous laboratories that p68 has a functional role in transcriptional regulation of a number of genes, including Estrogen Receptor alpha (ERα) (Endoh et al 1999b) and several p53-dependent genes (Bates et al 2005b). The protein was also shown to interact with p300/CBP and the RNA polymerase II holoenzyme (Rossow and Janknecht 2003). The molecular mechanism by which p68 is involved in transcriptional regulation is not clear. Interestingly, p68 was detected to interact with histone deacetylase 1 (HDAC1), indicating that the protein may have a functional role in regulation of gene expression by chromatin remodeling (Wilson et al 2004b). We previously reported that p68 is phosphorylated at multiple amino acid residues, including serine/threonine and tyrosine (Yang and Liu 2004a, Yang et al 2005a) Tyrosine phosphorylation of p68 correlates with tumor progression (Yang et al 2005b). In the present study, we present evidence to show that the phosphor-p68 represses E-cadherin expression by regulating transcription of the Snail1 gene. Phosphorylation of p68 at Y593 promoted dissociation of HDAC1 from Snail1 promoter. P68 RNA helicase interacted with the nuclear remodeling and deacetylation MBD3:Mi-2/NuRD complex, suggesting a potential role of phosphor-p68 in dissociating HDAC1 from the MBD3:Mi-2/NuRD-HDAC1 complex at the Snail1 promoter. Our study revealed a close correlation between the Snail1 expression levels and the phosphorylation levels of p68, both correlated closely with cancer metastasis.

## 2.3 Results

### 2.3.1 The Phosphor-p68 Repressed E-cadherin by Upregulating Transcription of Snail1

We previously reported that phosphorylation of p68 at Y593 mediates growth factor PDGF stimulated EMT by promoting β-catenin nuclear translocation (Yang et al 2006). The

phosphor-p68 represses expression of epithelial marker, E-cadherin. We sought to understand the molecular mechanism by which the phosphor-p68 regulates E-cadherin expression. We first tested whether the effects of p68 phosphorylation on the changes in E-cadherin expression was due to transcription of the E-cadherin gene. The transcription activity of the E-cadherin promoter in a metastatic colon cancer cell line SW620 was examined. We used a luciferase reporter fused to the E-boxes of the E-cadherin promoter (Fearon 2003) in cells in which p68 was knocked down and HA-p68s wild type (WT) or Y593F mutant was exogenously expressed in p68 knockdown cells ($SW620^{-p68/+wt}$ and $SW620^{-p68/+Y593F}$). E-cadherin transcription activity was not markedly affected by p68 knockdown but was substantially down-regulated in $SW620^{-p68/+wt}$ cells (Fig. 2.1A). On the other hand, E-cadherin transcription was significantly upregulated in $SW620^{-p68/+Y593F}$ cells (Fig. 2.1A). We tested whether phosphor-p68 regulated E-cadherin transcription directly or if the regulatory effects were mediated through other cellular factor(s). To this end, we examined whether phosphor-p68 interacted with the promoter of the E-cadherin gene by chromatin immunoprecipitation (ChIP). It was certain that neither p68 (WT) nor the Y593F mutant interacted with the E-cadherin promoter (Fig. 2.1B). Thus, it was likely that phosphor-p68 regulated E-cadherin expression indirectly.

Snail1/Slug and SIP1 are the master regulators that regulate E-cadherin transcription (Bolos et al 2003a, Fearon 2003, Thiery and Chopin 1999). In a ChIP-on-chip assay, we detected that p68 interacted with the Snail1 promoter (Data not shown). Thus, we reasoned whether the phosphor-p68 regulated E-cadherin through regulation of Snail1 expression. We examined the effects of p68 phosphorylation on the expression of Snail1. Immunoblotting showed that cellular levels of Snail1 were increased in the $SW620^{-p68/+wt}$ cells and decreased in the $SW620^{-p68/+Y593F}$ cells (Fig. 2.1C). The Snail1 upregulation correlated with the p68 expression and

phosphorylation at Y593 (Fig. 2.1C). Our results indicated that phosphorylation of p68 at Y593 may regulate the transcriptional activity of the Snail1 gene. Regulation of transcription of the Snail1 gene by phosphor-p68 was also confirmed by RT-PCR detection of the Snail1 mRNA in SW620, SW620$^{-p68/+wt}$, or SW620$^{-p68/+Y593F}$ cells (Fig. 2.1D). To test whether phosphor-p68 regulated Snail1 transcription directly, we conducted ChIP experiments with the Snail1 promoter using the antibody Pabp68. Clearly, p68 precipitated with the Snail1 promoter (Fig. 2.2A). The similar experiments were also carried out with exogenously expressed HA-p68 in SW620 cells using anti-HA antibody. P68 precipitated with the Snail1 promoter (Fig. 2.2B), indicating that p68 was directly involved in the transcriptional regulation of the Snail1 gene.

Whether the phosphor-p68 repressed E-cadherin through transcriptional regulation of the Snail1 gene, we reasoned that Snail1 expression must be required for the effects of the Y593 phosphorylation on the E-cadherin repression. To test this conjecture, we examined the effects of the p68 phosphorylation on the cellular level of E-cadherin in SW620 cells with/without Snail1 knockdown by RNAi. We found that Snail1 was upregulated, E-cadherin was repressed, and vimentin was upregulated by expression of wild-type p68 in SW620 cells. In contrast, expression of p68 Y593F mutant in SW620 cells led to Snail1 repression, E-cadherin upregulation, and vimentin repression (Fig. 2.2C). However, knockdown of Snail1 by RNAi abolished the effects of exogenous expression of HA-p68s (wild-type and Y593F mutant) on cellular levels of E-cadherin and vimentin (Fig. 2.2C). The results supported the conclusion that regulating the transcription of Snail1 mediated the regulatory effects of the phosphorylated p68 in repressing E-cadherin expression. Interestingly, we repeatedly observed that there was a very low level of expression of E-cadherin in cells where Snail1 was knocked down (Fig. 2.2C), suggesting that

there is an alternative mechanism which represses E-cadherin expression in Snail1 knockdown cells. This alternative mechanism is not regulated by phosphor-p68.

### 2.3.2 p68 Associates with the MBD3:Mi2/NuRD Complex

The preceding experiments suggested the role of phosphor-p68 in repression of E-cadherin through the regulation of transcription of the Snail1 gene. To further understand the molecular mechanism by which phosphor-p68 regulated transcription of the Snail1 gene, we attempted to probe the protein or protein complex that interacted with phosphor-p68 at the Snail1 promoter. Recently, Fujita and co-workers demonstrated that MTA3 targeted the nuclear remodeling and deacetylation complex Mi-2/NuRD to the Snail1 promoter, and directly regulated Snail1 gene transcription (Fujita et al 2003). Thus, we asked whether phosphor-p68 interacted with the MBD3:Mi-2/NuRD complex in SW620 cells. To this end, we carried out co-immunoprecipitation with the nuclear extracts made from SW620 cells using PAbp68. As a comparison, the co-immunoprecipitation experiments were also conducted with nuclear extracts made from SW480, a cell line derived from the tissue of the same patient from whom SW620 was derived. SW480, however, was derived from tissue of non-metastatic adenocarcinoma. The tyrosine phosphorylation of p68 was almost undetectable in SW480 cells (data not shown, also see Fig. 2.5A). It was certain that the antibody against p68 precipitated MBD3 and Mi-2 in the extracts made from both SW480 and SW620 cells (Fig. 2.3A). To confirm the co-immunoprecipitation results, we performed co-immunoprecipitation using antibodies against MBD3 and Mi-2. The co-precipitation of p68 with MBD3 and Mi-2 in the extracts made from SW480 and SW620 cells was clearly evident (Fig. 2.3B). These coimuunoprecipitation experiments suggested that p68 interacted with Mi-2/NuRD complex.

We next asked whether the interaction between p68 and MBD3:Mi-2/NuRD is required for the association of p68 and/or MBD3:Mi-2/NuRD with the Snail1 promoter. We conducted the ChIP experiments in SW620 cells in which either p68 or MBD3 was knocked down by RNAi. Immunoblotting showed an efficient knockdown of these two proteins (over 90%) (Fig. 2.3D and Fig. 2.1C). We then exogenously expressed HA-p68s (WT or Y593F mutant) in MBD3 and p68 knockdown cells. Anti-HA antibody did not precipitate the Snail1 promoter in MBD3 knockdown cells (Fig. 2.3D, lower panel). However, antibody against MBD3 did precipitate the Snail1 promoter in the p68 knockdown cells (Fig. 2.3C). Expression of p68 (WT or Y593F mutant) did not affect the MBD3-Snail1 promoter precipitation (Fig. 2.3C). The data suggested that association of p68 with the MBD3:Mi-2/NuRD is not required for the recruitment of MBD3:Mi-2/NuRD complex to the Snail1 promoter. In contrast, MBD3 was required for association of p68 with Mi-2/NuRD complex and the Snail1 promoter.

### 2.3.3 HDAC1 Dissociated from the Snail1 Promoter in the Presence of Phosphor-p68

An important cellular activity of Mi-2/NuRD complex is histone deacetylation via its associated HDAC1 and HDAC2 (Bowen et al 2004, Xue et al 1998a). To determine whether phospho-p68 plays a role in regulating the function of the Mi-2/NuRD complex at the Snail1 gene promoter, we first probed the association of HDAC1 with the Snail1 promoter in $SW620^{-p68}$, $SW620^{-p68/+wt}$, and $SW620^{-p68/+Y593F}$ cells by ChIP experiments using antibody against HDAC1. It was certain that p68 knockdown markedly enhanced the association HDAC1 with the Snail1 promoter. However, expression of HA-p68 (WT) in the p68 knockdown cells diminished the association of HDAC1 with the Snail1 promoter. The HDAC1 was associated with the Snail1 promoter in the cells expressing Y593F mutant after endogenous p68 knockdown but not expressing the p68 (WT) (Fig. 2.4A). The experiments suggested that the phosphor-p68

promoted HDAC1 dissociation from the Snail1 promoter. Interestingly, both wild-type and Y593F mutant of p68 interacted with the Snail1 promoter in the ChIP assay (Fig. 2.3D), suggesting that the dissociation of HDAC1 from the Snail1 promoter is phosphorylation-dependent. Examination of the association of CREB binding protein (CBP) with the Snail1 promoter in SW620$^{-p68}$, SW620$^{-p68/+wt}$, and SW620$^{-p68/+Y593F}$ cells revealed that dissociation of HDAC1 from Snail1 promoter facilitated by the p68 phosphorylation increased the binding of CBP to the Snail1 promoter (Fig. 2.4A), consistent with active transcription of the Snail1 gene. We previously showed that EGF treatment led to the increase in the p68 phosphorylation (Yang et al 2006). Thus, we probed the effects of EGF treatment on interaction between HDAC1 and the Snail1 promoter. ChIP assays indeed showed that EGF treatment led to a substantial decrease in HDAC1 and the Snail1 promoter interaction, while a strong increase in CBP and Snail1 promoter interaction (Fig. 2.4D).

We questioned whether the p68 phosphorylation at Y593 affected the HDAC1 activity at the Snail1 promoter. To this end, the HA-p68s (wt or Y593F mutant) were stably expressed in SW620 cells using a commercially available lentiviral system. The expression level of p68 WT/mutant was high as revealed by the immunoblot with the anti-HA antibody (Fig. 2.4B). The expressed p68s were immunoprecipitated from nuclear extracts by anti-HA. The deacetylase activity of the immunoprecipitates was analyzed by HDAC Activity Colorimetric Assay kit. It was evident that overexpression of p68 (WT) suppressed the deacetylase activities by over three folds (Fig. 2.4B, comparing LacZ/IP:HA to HA-WT/IP:HA). In contrast, overexpression of Y593F mutant led to the increase of deacetylase activities by over five folds (Fig. 2.4B, comparing LacZ/IP:HA to HA-Y593F/IP:HA) or by 17-folds (Fig. 2.4B, comparing HA-WT/IP:HA to HA-Y593F/IP:HA). The increase in the deacetylase activities was sensitive to

TSA (an HDAC inhibitor) treatment (Fig. 2.4B), supporting that an HDAC activity was co-precipitated with HA-Y593F but not with HA-WT. To further investigate whether the p68 phosphorylation affected the HDAC activity at the Snail1 promoter, using the Snail1 promoter/luciferase reporter construct, we measured the Snail1 promoter activity in the presence and absence of the HDAC inhibitor trichostatin A in SW620 cells in which p68 WT/mutant was overexpressed. The data indicated that the phosphor-p68 indeed affected the deacetylase activities at the Snail1 promoter (Fig. 2.4C).

### 2.3.4 p68 Phosphorylation Correlates with Snail1 Expression in Metastatic Cancer Cells

Snail1 expression has been shown to be associated with cancer metastasis in various different cancer types (Barrallo-Gimeno and Nieto 2005, Miyoshi et al 2005, Nyormoi and Bar-Eli 2003). We previously reported that p68 phosphorylation at tyrosine residues correlates with cancer progression. Thus, we sought to determine whether there is a correlation between phosphorylation of p68 at tyrosine 593 and Snail1 expression levels, and whether both will correlate with cancer metastasis. To this end, we used three pairs of cell lines that were derived from different cancer types. SW480 and SW620 are colon cancer cell lines derived from the same cancer patient. WM115 and WM266 are melanoma cell lines taken from the same patient. H146 and H460 are lung cancer cell lines derived from the same patient. Among these three pairs of cell lines, SW620, WM266, and H146 were derived from metastatic cancer tissue, while the other three were derived from cancer before metastasis had begun. Protein extracts and total RNA samples were made from these cells. Immunoprecipitation of p68 followed by immunoblot of the immunoprecipitates using antibody P-Tyr-100 showed that there were substantially higher p68 tyrosyl phosphorylation levels in the three cell lines that were derived from metastasis cancer than that in cell lines derived from corresponding non-metastatic cancer (Fig. 2.5A).

Examination of Snail1 mRNA levels in these three pairs of cell lines by RT-PCR indicated that the Snail1 mRNA levels were substantially higher in SW620, WM266 and H146 cells than in corresponding non-metastatic cells (Fig. 2.5B), correlating with the tyrosine phosphorylation of p68 in these cells. We further analyzed the cellular Snail1 levels in the extracts made from the three pairs of cells by immunoblot analyses using antibody against Snail1. It was clear that the cellular Snail1 levels in the metastatic cancer cells (SW620 and H146) were much higher than that of matched non-metastatic cells (SW480, H460) (Fig. 2.5C). In the pair of melanoma cells, the Snail1 levels are higher in metastatic cells than in non-metastatic cells. However, the difference was less significant (Fig. 2.5C). Thus, our experiments showed a close correlation between phosphorylation of p68 at tyrosine residues and Snail1 expression, and both the Snail1 expression and p68 tyrosyl phosphorylation correlated with cancer metastasis. To further extend correlation of Snail1 expression and p68 phosphorylation, SW480 cells were stimulated by EGF treatment. Phosphorylation of p68 and Snail1 expression were examined. Consistent with our previous study (Yang et al 2006), the p68 tyrosine phosphorylation responded well to EGF stimulations in the cells. Snail1 expression was also up-regulated by the growth factor treatment. The results further support a correlation between p68 tyrosine phosphorylation and Snail1 expression (Fig. 2.5E).

## 2.4 Discussion

In this report, we showed that phosphorylation of p68 RNA helicase repressed E-cadherin expression by up-regulating transcription of the Snail1 gene. The phosphor-p68 activates transcription of the Snail1 gene by dissociating HDAC1 from the Snail1 promoter. P68 RNA helicase has been implicated in transcriptional regulation of many genes (Bates et al 2005b, Endoh et al 1999b, Metivier et al 2003). However, it is not known how a DEAD-box RNA

helicase functions in transcriptional regulation. A recent study done by Allen C. Spradling's laboratory reveals that *Drosophila's* p68 may have a role in unwinding the RNA transcripts from its DNA template, facilitating a quick reset of the nucleosome structure, which is transcriptionally inactive (Buszczak and Spradling 2006b). Our observations may reveal another model for the functional role of p68 in transcriptional regulation. P68 RNA helicase may have a role in remodeling or re-arranging the protein complex that assembles at the gene promoter.

There were two possible explanations for the observed dissociation of HDAC1 from the Snail1 promoter in SW620 cells. (1) The unphosphorylated p68 recruited HDAC1 to the promoter. The phosphor-p68 could not function as a recruiter. (2) The phosphor-p68 may 'displace' HDAC1 from the Snail1 promoter. The unphosphorylated p68 could not function as a protein 'displacer'. Although we observed that the phosphor-p68 acquired β-catenin binding-dependent ATPase activity (data not shown), indicating a possibility that phosphor-p68 can use protein-binding as substrate to stimulate its ATPase activity for the protein 'displacement', additional experiments are required to prove that the phosphor-p68 can indeed displace HDAC1 from Mi-2/NuRD complex. Histone deacetylases (HDACs) are enzymes that modify chromatin structure and subsequently regulate gene expression. HDACs are usually recruited to a particular regulatory site with their associated multi-protein complexes, such as NuRD or Sin3 complex (Knoepfler and Eisenman 1999, Narlikar et al 2002). While most studies concentrated on the mechanism by which the HDAC activity and its associated complex are recruited to a specific gene promoter (Forsberg and Bresnick 2001, Kurdistani and Grunstein 2003, Neely and Workman 2002), our studies suggested a possible mechanism of action by which HDACs can be displaced from their associated complex by a DEAD-box helicase. Given that tyrosine phosphorylations of p68 were closely associated with cancer development (Yang et al 2005b), it

is tempting to speculate that displacement of HDACs by the phosphor-p68 is a dysregulated route for tumor progression through activation of specific genes. Whether p68 is a constitutive member of the NuRD complex is an open question. P68 was not identified in the originally isolated NuRD complex (Bowen et al 2004, Tong et al 1998, Xue et al 1998a, Zhang et al 1999b). Our experiments showed co-immunoprecipitation of p68 with the Mi-2 and MBD3, the components of NuRD complex. This seems to argue that p68 is a part of protein components of the NuRD complex. In contrast, knockdown of p68 did not affect the association of the MBD3:Mi-2/NuRD complex with Snail1 promoter, suggesting that p68 is not required for the NuRD complex to be recruited to the Snail1 promoter. This observation disagrees that p68 may not be a constitutive component of the NuRD complex. The best explanation is that p68 only associates with a subset of NuRD complexes. Most recently, p68 was detected to interact with HDAC1 in a promoter-specific manner (Wilson et al 2004b), which also seems to support the concept. It was not known whether the detected interaction of p68 with HDAC1 was direct or mediated by other protein factors. Knockdown of MBD3 abolished the association of p68 at the Snail1 promoter, suggesting that p68 was recruited to the Snail1 promoter by a component of the NuRD complex. Taken together, we speculated that p68 RNA helicase is recruited to the MBD3:Mi-2/NuRD complex in a promoter specific manner. An alternative explanation is that phosphorylated p68 RNA helicase may compete with HDAC1 to assemble to the Mi-2/NuRD complex. The unphosphorylated p68 and Y593F mutant are unable to compete. Thus, there may be two populations of Mi-2/NuRD complexes in SW620 cells. One contains phosphor-p68 and the other contains HDAC1.

## 2.5 Materials and Methods

*Reagents and Antibodies*

TSA was purchased from PeproTech. Both polyclonal antibody PAbp68 and monoclonal antibody p68-rgg against human p68 were raised against bacterially expressed His-tagged C-terminal domain of p68 (Invitrogen, Auburn University Hybridoma Facility). Commercial antibodies used in this study were purchased from Santa Cruz (Actin, against human □-actin, HDAC1, GAPDH against human GAPDH, ChIP grade monoclonal against HDAC1, Mi-2, SNAI 1 against Snail1), Cell Signaling Technology (p-Tyr-100, against phosphor-tyrosine, HDAC1, polyclonal against HDAC1, H2A, against human histone 2A), Imgenex (MBD3, ChIP grade), BD Bioscience (E-cadherin and Vimentin), Roche (12CA5, against HA-tag ChIP grade), Abcam (KAT3A/CBP, against CBP) and Upstate (HA, against HA-tag).

*Cell Culture and RNA Interference*

SW620 and SW480 cells were purchased from ATCC and grown by following the vendor's instructions. All DNAs or RNAs transfections were performed using Lipofectamine 2000 by following the manufacturer's instructions (Invitrogen). For the siRNA experiments, cells were grown to 50% confluence and transfected with siRNA (100 pM). The duplex RNA oligonucleotides for RNAi were purchased from Dharmacon siGENOME™ and SMARTpool®. A duplex RNA oligonucleotides with random sequence (non-targeting, NT) provided by the vendor was included in all siRNA knockdown experiments as negative controls. For transient expression of wild-type p68 or mutants in p68 knockdown cells, the cells were transfected with the indicated plasmid DNA 24 hours after the cells were transfected with siRNA and harvested after additional 48 hours incubation.

*Subcellular Extracts, Immunoprecipitation, Immunoblot*

All subcellular extracts or whole cell extracts were made freshly after appropriate treatments (indicated in figures). Subcellular extracts were prepared using commercially available cell extracting kit and by following the vendor's instructions (Active motif). The protein concentration of the extracts was determined using the Bradford assay (Bio-Rad). Immunoprecipitation experiments and immunoblot analyses were performed as described in previous studies . The blotting signals were detected using SuperSignal West Dura Extended Duration Substrate (Pierce).

*Luciferase Reporter Assays*

Before cells were appropriately treated (indicated in figures), cells were transfected with 1 μg of the indicated reporter plasmid and 0.01 μg of pRL null, which expresses Renilla luciferase from Renilla reniformis as an internal control. The total amount of plasmid DNA was adjusted with pcDNA3-β-Galactosidase. Firefly and Renilla luciferase activities present in cellular lysates were assayed using the Dual-Luciferase Reporter System (Promega, Madison, WI). Data were represented as Firefly luciferase activity normalized by Renilla luciferase activity.

*Expression of p68s by Lentiviral System*

Stable overexpression of HA-tagged p68 wild-type or Y593F mutant was carried out using the ViralPower lentiviral expression system (Invitrogen) by following the manufacturer's instructions. The ORFs of p68 wild type or Y593F mutant with N-terminal HA-tag were cloned into pLenti6/TOPO (Invitrogen). The infections of cells with the lentiviruses that carry pLenti6-

p68 were carried out in the presence of 6 µg/mL of polybrene and 10 mM HEPES. Following transduction, the cells were selected by 8 µg/mL of Blasticidin (Invitrogen).

*RT-PCR*

Cells were appropriately treated, and then total RNA was extracted using the total RNA extraction kit (Qiagen, Valencia, CA). The RNA was quantified and then converted to cDNA using the Improm II reverse transcription system (Promega, Madison, WI) following the manufacturer's protocol. The cDNA was then used in the final PCR reaction. The cycles were an initial denaturing of 94 ºC for 2 minutes followed by 25 cycles of 94 ºC for 15s, 55 ºC for 30s, and 72 ºC for 1m with an additional extension time of 5m added after the last cycle. Densitometry was performed using the ImageJ program. Primers used were: Snail1 (sense 5'-TCTAGGCCCTGGCTGCTAC-3' antisense 5'-GCCTGGCACTGGTACTTCTT-3'); B2M (sense 5'-TGCTGTCTCCATGTTTGATGTATCT-3') antisense 5'-TCTCTGCTCCCCACCTCTAAGT-3'). Primers for B2M were used as PCR and loading controls. Controls for the RT reaction (not shown) contained no template or no reverse transcriptase.

*Chromatin Immunoprecipitation (ChIP)*

The ChIP experiments were performed using ChIP-IT$^{TM}$ Kit (Activemotif). The precipitation of Snail1 or E-cadherin promoters was determined by PCR using primers spanning nt -680 – -541 of the Snail1 promoter (sense 5'-GGGTGCTCTTGGCTAGCTG-3'antisense 5'-CTGGAGAGCGTGGCATTG-3') or nt -164 – +49 of the E-cadherin promoter (sense 5'-GTCACCGCGTCTATGCGAGGCCG-3', Antisense 5'-GGACACTCGAACGCCTTCAGTCAAGT-3'). TFIIB/Pol II antibodies and mouse IgG were

used as control antibodies (included in the kit). ChIP-IT's negative control primers flank a region of genomic DNA between the GAPDH gene and CNAP1 gene. The primers were provided by the vendor (sense5'-ATGGTTGCCACTGGGGATCT-3'antisense 5'-TGCCAAAGCCTAGGGGAAGA-3').

*HDAC Activity Assay*

SW620 cells were lysed in RIPA buffer (Upstate) 48 hr after transfection as described above. The lysate was then diluted in RIPA buffer and HA-tagged proteins immunoprecipitated with anti-HA polyclonal antibody (Upstate). HDAC activities were determined by HDAC Activity Colorimetric Assay Kit (BioVision) according to manufacturer's instructions. Antibody-bound beads were washed in HDAC assay buffer prior to being added to the 96-well plate, to remove immunoprecipitation buffer. Reactions were incubated for 30 min at 37°C with or without the addition of 1 μM TSA. Samples were read in a VICTOR$^{3}$™ plate reader (PerkinElmer) at 405 nm. Typically each assay was performed 3 times.

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Figure 2.1 The phosphor-p68 down-regulated E-cadherin by upregulation of transcription of Snail1.

(A upper panel) Luciferase reporter of E-boxes (E-cadherin promoter) was cotransfected into p68 knockdown SW620 cells along with HA-p68s, wt or mutant (indicated). The luciferase activity was expressed as relative luciferase activity (numbers on top of bars) by comparing the luciferase activity of SW620 cells without p68 knockdown (NT) and without HA-p68s expression (define as 100). The lower panel shows the mRNA expression of E-cadherin under the same conditions. (B) Chromatin immunoprecipitations (ChIP) of the E-cadherin promoter by anti-HA antibody in SW620 cells with/without (p68/NT) p68 knockdown. The HA-p68s (WT or Y593F mutant) were exogenously expressed. The primers positions for PCRs were indicated by arrows. ChIP by mouse IgG and antibody against TFIIB, which binds to the GAPDH promoter, were used as controls. Inputs were PCR products from DNA extracts without ChIP. (C) & (D) Expression of Snail1 was examined by immunoblot of cell lysate (C) and RT-PCR of total RNAs (D) prepared from SW620 cells with/without (p68/NT) p68 knockdown. The HA-p68s (WT or Y593F mutant) was exogenously expressed. The expression and tyrosine phosphorylation of HA-p68s were examined by IB of immunoprecipitated HA-p68s (IP:HA). Total p68 level was detected by IB using monoclonal antibody p68-rgg (IB:p68). IB of histone 2A (H2A) was a loading control. RT-PCR detection of B2M mRNA in the RNA samples was a control for PCR reaction and loading.

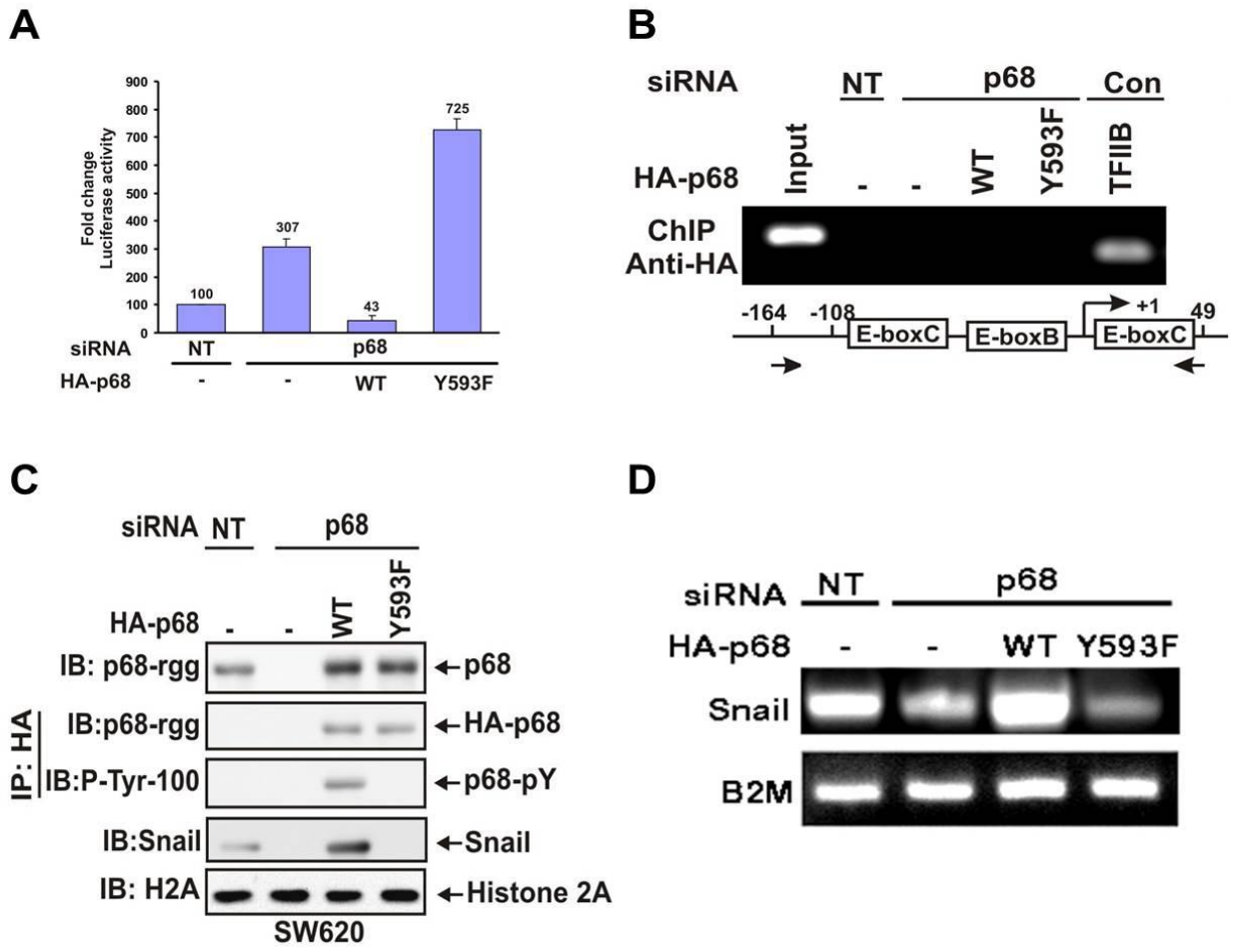
A
Fold change Luciferase activity
900
800
700
600
500
400
300
200
100
0
100
307
43
725
siRNA
NT
p68
HA-p68
-
-
WT
Y593F
B
siRNA
NT
p68
Con
Input
HA-p68
-
-
WT
Y593F
TFIIB
ChIP Anti-HA
-164
-108
E-boxC
E-boxB
E-boxC
+1
49
C
siRNA
NT
p68
HA-p68
-
-
WT
Y593F
IB: p68-rgg
←p68
IP: HA
IB:p68-rgg
←HA-p68
IB:P-Tyr-100
←p68-pY
IB:Snail
←Snail
IB: H2A
←Histone 2A
SW620
D
siRNA
NT
p68
HA-p68
-
-
WT
Y593F
Snail
B2M

Figure 2.1

Figure 2.2 p68 interacted with Snail1 promoter.

(A) & (B) Chromatin immunoprecipitations (ChIP) of the Snail1 promoter by antibody Pabp68 (A, Anti-p68) and by anti-HA antibody (B, Anti-HA) in SW620 cells with/without (p68/NT) p68 knockdown. The HA-p68s (WT or Y593F mutant) were exogenously expressed. The primer positions for Snail1 PCR were indicated by arrows. ChIP by mouse IgG and antibody against RNA polymerase II (POL II), which binds to the GAPDH promoter, were used as controls. Inputs were PCR products from DNA extracts without ChIP (use 10% of sample). The lower panel shows the quantization ChIP results by real-time PCR. The ChIP quantization is expressed as a percentage of the input by defining the input as 100. The error bars represent standard deviation of results from three independent measurements. (C) Cellular levels of E-cadherin (second panel from top) and vimentin (third panel from top) were detected by IBs of cellular extracts made from SW620 cells with/without (Snail1/NT) Snail1 siRNA knockdown and exogenous expression of HA-p68s (WT or Y593F mutant). Tyrosine phosphorylation of HA-p68s was analyzed by IB of anti-HA IPs via antibody p-Tyr-100 (fifth panel from top). IB of histone 2A (H2A) was loading control.

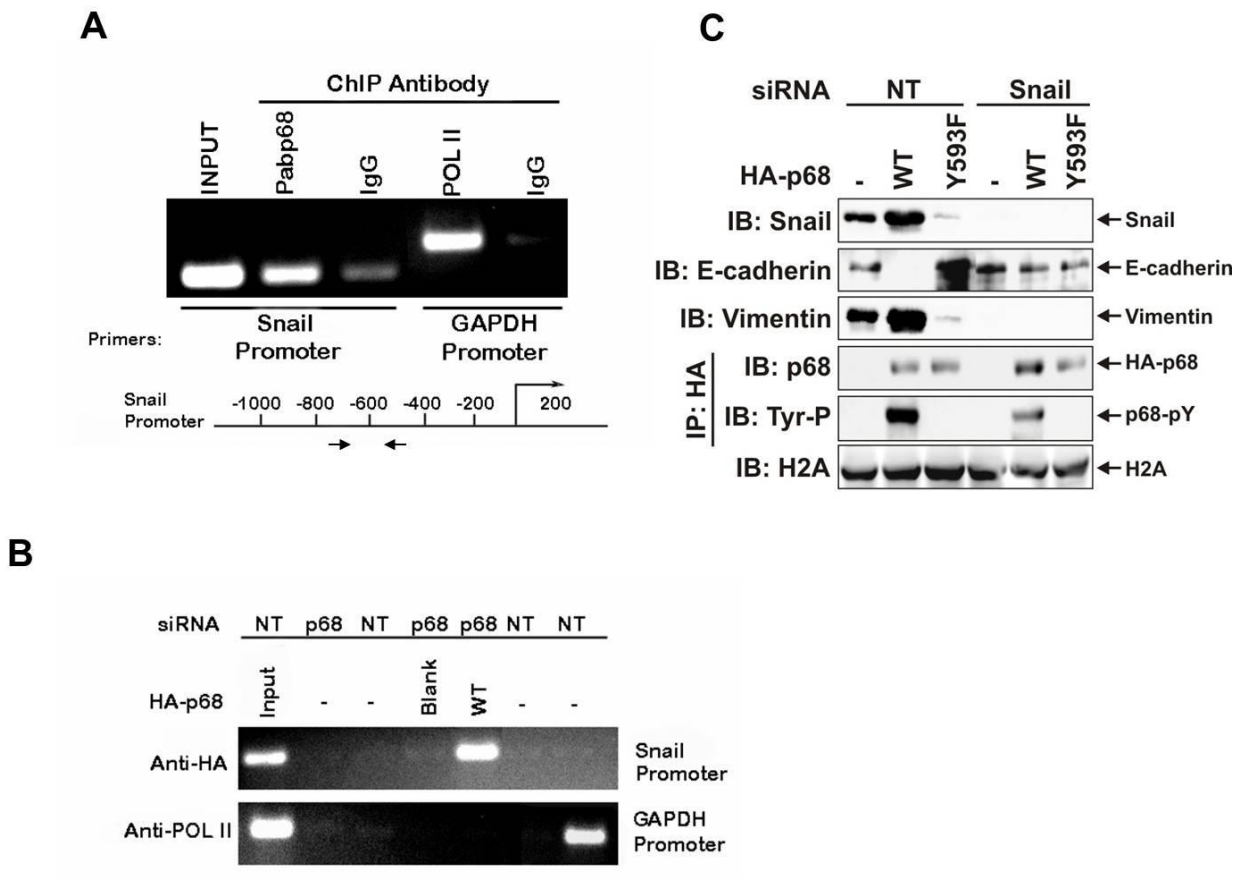
A
ChIP Antibody
INPUT
Pabp68
IgG
POL II
IgG
Primers:
Snail Promoter
GAPDH Promoter
Snail Promoter
-1000 -800 -600 -400 -200 200
C
siRNA
NT
Snail
HA-p68
-
WT
Y593F
-
WT
Y593F
IB: Snail
IB: E-cadherin
IB: Vimentin
IP: HA
IB: p68
IB: Tyr-P
IB: H2A
Snail
E-cadherin
Vimentin
HA-p68
p68-pY
H2A
B
siRNA
NT p68 NT p68 p68 NT NT
HA-p68
Input
-
-
Blank
WT
-
-
Anti-HA
Anti-POL II
Snail Promoter
GAPDH Promoter

Figure 2.2

Figure 2.3 P68 interacted with the MBD3:Mi-2/NuRD complex.

(A) Co-IPs of MBD3 and Mi-2 with p68 in SW480 and SW620 cells were detected by IB of p68 co-immunoprecipitates using appropriate antibodies (indicated). P68 was precipitated by polyclonal antibody Pab-p68. Rabbit IgG was used as a negative control IP antibody. Inputs were the IBs of extracts without IP. (B) Co-IPs of p68 with MBD3 and Mi-2 in SW620 (620) and SW480 (480) cells were detected by IBs of co-immunoprecipitates of antibodies (anti-MBD3 and anti-Mi-2) using monoclonal antibody p68-rgg. Mouse IgG was used as control IP antibody. The inputs were the IBs of extracts without IP. The tyrosine phosphorylation of p68 was detected by IB of PAbp68 immunoprecipitated p68 using antibody Tyr-100. (C) Upper panel, cellular levels of MBD3, HDAC1, and exogenously expressed HA-p68s (WT or Y593F mutant) were analyzed via IBs using appropriate antibodies (indicated). The IBs were performed with cellular extracts made from SW620 cells that were treated with p68 siRNA (p68) or non-targeting siRNA (NT). Lower panel, Interactions of MBD3 with Snail1 promoter in the cells treated as described in upper panels were detected by ChIP assays using anti-MBD3 antibody (Anti-MBD3). (D) Upper panel, cellular levels of MBD3 and exogenously expressed HA-p68s (WT or Y593F mutant) (without endogenous p68 knockdown) were analyzed via IBs using appropriate antibodies (indicated). The IBs were performed with cellular extracts made from SW620 cells that were treated with MBD3 siRNA (MBD3) or non-targeting siRNA (NT). IB of Actin was a loading control. Lower panel, Interactions of HA-p68 with Snail1 promoter in the cells treated as described in upper panels were detected by ChIP assays using anti-HA antibody (Anti-HA).

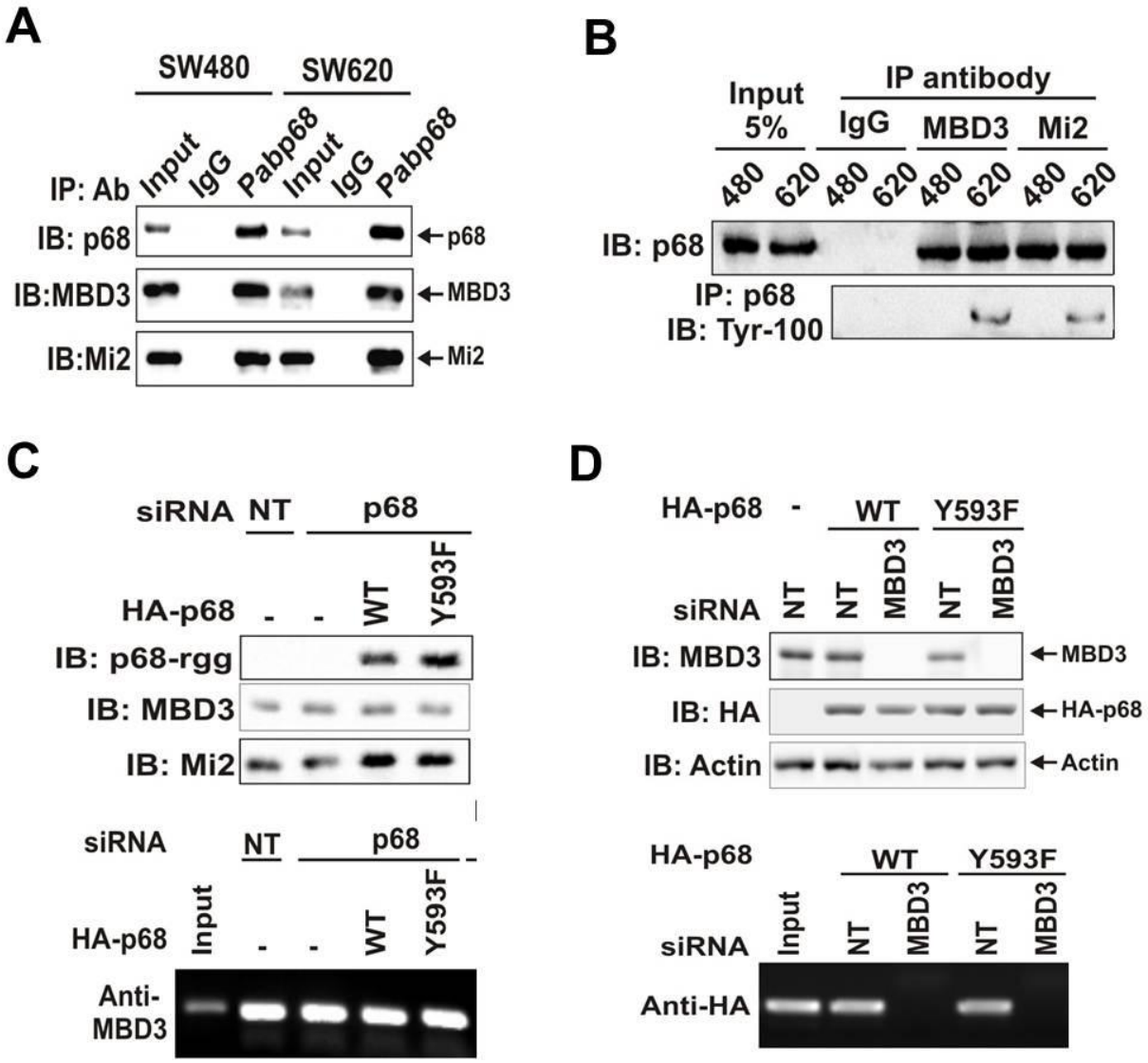
A
SW480
SW620
IP: Ab
Input
IgG
Pabp68
Input
IgG
Pabp68
IB: p68
p68
IB:MBD3
MBD3
IB:Mi2
Mi2
B
Input 5%
IP antibody
IgG
MBD3
Mi2
480
620
480
620
480
620
480
620
IB: p68
IP: p68
IB: Tyr-100
C
siRNA
NT
p68
HA-p68
-
-
WT
Y593F
IB: p68-rgg
IB: MBD3
IB: Mi2
siRNA
NT
p68
HA-p68
Input
-
-
WT
Y593F
Anti-MBD3
D
HA-p68
-
WT
Y593F
siRNA
NT
NT
MBD3
NT
MBD3
IB: MBD3
MBD3
IB: HA
HA-p68
IB: Actin
Actin
HA-p68
WT
Y593F
siRNA
Input
NT
MBD3
NT
MBD3
Anti-HA

Figure 2.3

Figure 2.4 Phosphor-p68 dissociated HDAC1 from the Snail1 promoter.

(A) ChIP of the Snail1 promoter using Anti-p68rgg (p68), anti-HDAC1 (HDAC1), and anti-CBP (CBP) antibodies in SW620 cells. SW620 cells were treated with p68 siRNA (p68) or non-targeting siRNA (Nt). HA-p68s (WT or Y593F mutant) or empty vector (Vec) was exogenously expressed in p68 knockdown cells. Inputs were PCR products from SW620 DNA extracts without ChIP. Quantization ChIP results by real-time PCR. The ChIP quantization is expressed as fold difference by defining the lowest ChIP value in each antibody group (αCBP or αHDAC1) as 1 after normalizing ChIP value in each antibody group to the inputs within the group. The error bars represent standard deviation of results from three independent measurements. NT/p68 means the cells were treated with non-target or p68 target RNAi. Vec, wt, or Y593F represents p68 wt, Y593F mutant, or vector was expressed in the cells. (B) Deacetylase activities of co-immunoprecipitates by mouse IgG (IgG, as control IP antibody) and anti-HA antibody (HA) from cellular extracts made from SW620 cells were analyzed. HA-p68s (WT or Y593F mutant) were stably expressed using Lenti-viral system. LacZ is a control expression protein. The immunoprecipitates were treated/untreated (+/-) with 100 ng/ml of TSA. The deacetylase activity (numbers on top of bars) was expressed as relative deacetylase activity by define the activity of co-IP by mouse IgG without TSA treatment without HA-p68 expression as 100. The upper panel is IB analyses of stable expression of HA-p68 in SW620 cells. IB of H2A is a loading control. (C) Luciferase reporter of the Snail1 promoter was transfected into SW620 cells in which HA-p68, wt or mutant (indicated) was stably expressed. Twenty four hours post transfection, cells were treated/untreated (filled bars/open bars) with TSA (100 ng/ml) overnight. Luciferase activities were then analyzed. The luciferase activity was expressed as relative luciferase activity (numbers on top of bars) by compared to the luciferase activity of SW620 cells without HA-p68s expression and TSA treatment (define as 100). (D) ChIP of the Snail1 promoter using anti-HDAC1 (HDAC1) and anti-CBP (CBP) antibodies in SW480 cells that were treated (+EGF) or untreated (-EGF) (EGF 15 ng/ml). ChIP using rabbit IgG was a control ChIP experiment. In (B) and (C), the error bars represent standard deviation of results from three independent measurements.

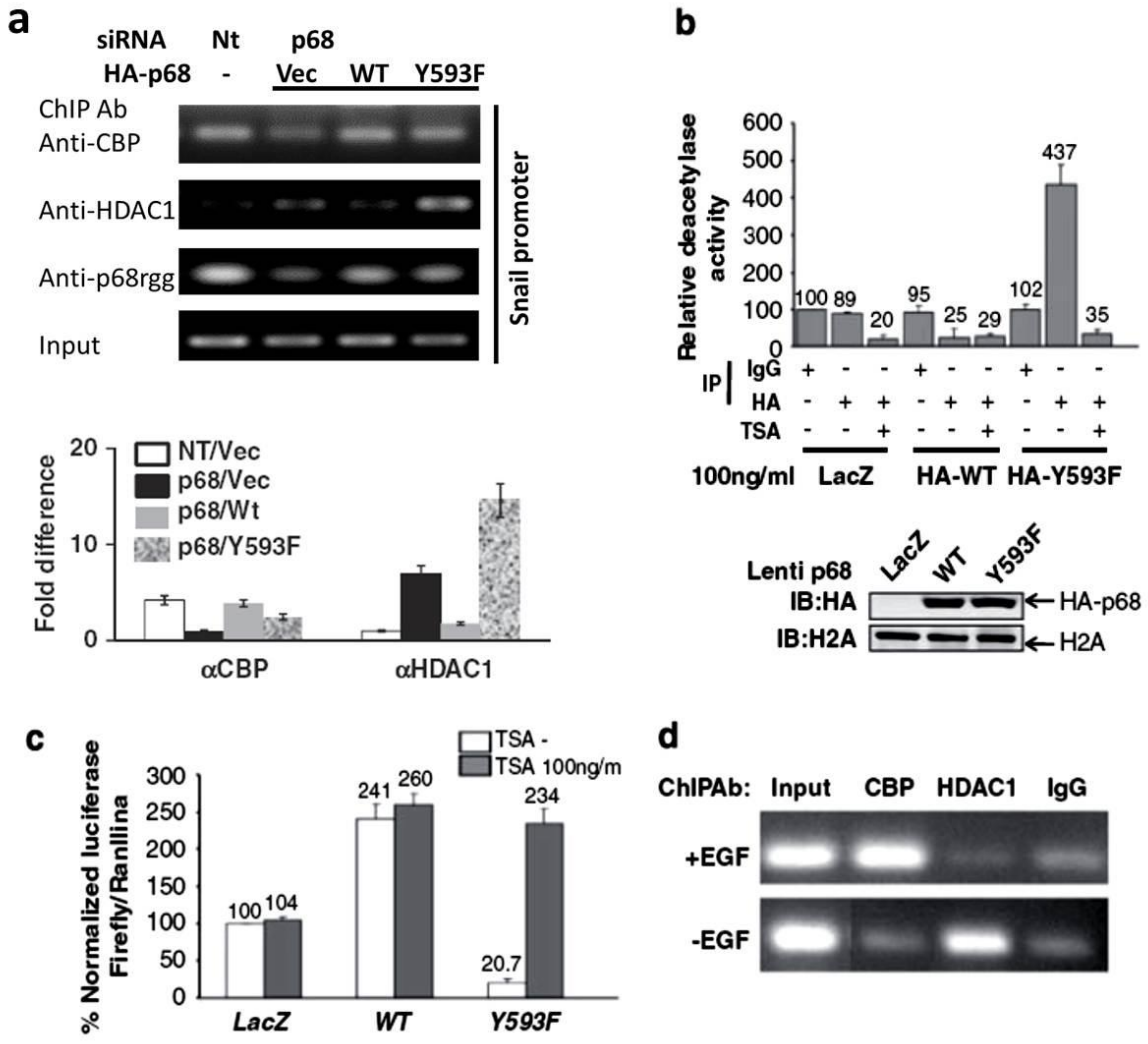
a
siRNA Nt p68
HA-p68 - Vec WT Y593F
ChIP Ab
Anti-CBP
Anti-HDAC1
Anti-p68rgg
Input
Snail promoter
Fold difference
NT/Vec
p68/Vec
p68/Wt
p68/Y593F
αCBP
αHDAC1
b
Relative deacetylase activity
100 89 20 95 25 29 102 437 35
IP
IgG
HA
TSA
100ng/ml LacZ HA-WT HA-Y593F
Lenti p68 LacZ WT Y593F
IB:HA
HA-p68
IB:H2A
H2A
c
% Normalized luciferase Firefly/Ranillina
TSA -
TSA 100ng/m
100 104 241 260 20.7 234
LacZ WT Y593F
d
ChIPAb: Input CBP HDAC1 IgG
+EGF
-EGF

Figure 2.4

Figure 2.5 P68 phosphorylation correlates with Snail1 expression in metastatic and non-metastatic cancer cells

(A) P68 tyrosine phosphorylation in three pair of cancer cell lines (indicated). The tyrosine phosphorylation of p68 was detected by immunoblot (via the anti-phosphor-tyrosine antibody; IB:P-tyr-100) of p68 that was immunoprecipitated from nuclear extracts of the cells using the antibody Pabp68 (IP:Pabp68). Immunoblot of p68 in the IPs using the antibody p68-rgg (IB:p68-rgg) was the loading control. (B) The expression levels of Snail1 mRNA in the three pair of cancer cells (indicated) were detected by RT-PCR of total RNAs isolated from the cells. The RT-PCR detections of mRNA of B2M (B2M) gene in the cells were the controls. (C) The cellular Snail1 protein levels in the three pair of cancer cells (indicated) were examined by immunoblot of the cell lysate prepared from the cells using antibody against Snail1 (IB:Snail1). IB of β-tubulin was used as the loading control. (D) is the quantification (average) of the immunoblot signals of Snail1 after normalizing to the loading control β-tubulin blots. The error bars represent standard deviation of results from three independent immunoblots. (E) (Left) Tyrosine phosphorylation of p68 in SW480 cells that were treated (+EGF) or untreated (-EGF) with EGF (25 ng/ml) was analyzed by Immunoprecipitation of p68 (IP:p68) from the cell lysates followed by immunoblot using the antibody P-y-100 (IB:P-y-100). Immunoblot of p68 (IB:p68-RGG) indicated the precipitated p68. (Right) Expression of Snail1 (Snail) in SW480 cells that were treated (+EGF) or untreated (-EGF) with EGF (25 ng/ml) was analyzed by RT-PCR of the total RNA isolated from cell lysates. RT-PCR analyses of B2M mRNA in the EGF treated or untreated cells are controls.

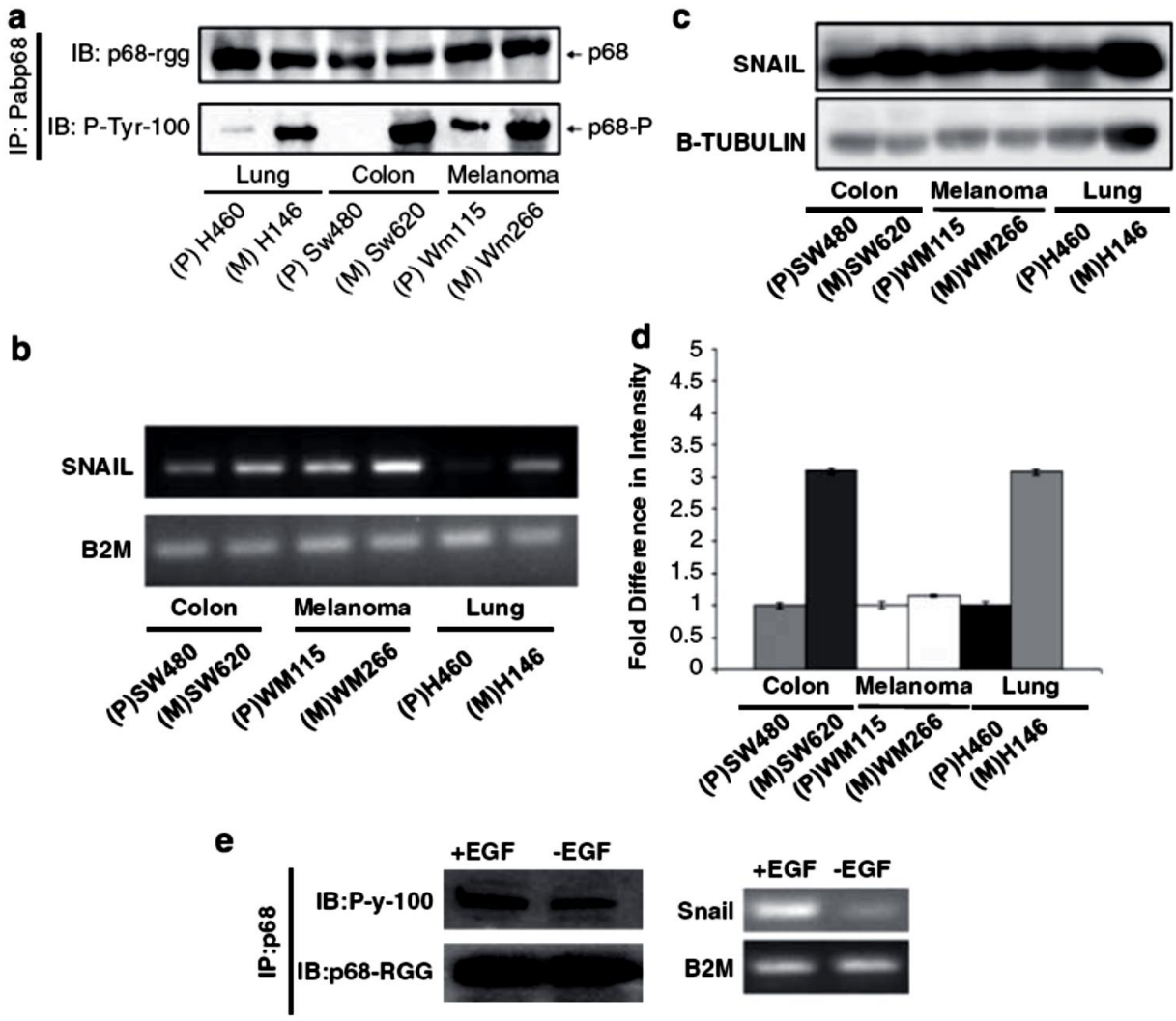
a
IP: Pabp68
IB: p68-rgg
IB: P-Tyr-100
← p68
← p68-P
Lung
Colon
Melanoma
(P) H460
(M) H146
(P) Sw480
(M) Sw620
(P) Wm115
(M) Wm266
c
SNAIL
B-TUBULIN
Colon
Melanoma
Lung
(P)SW480
(M)SW620
(P)WM115
(M)WM266
(P)H460
(M)H146
b
SNAIL
B2M
Colon
Melanoma
Lung
(P)SW480
(M)SW620
(P)WM115
(M)WM266
(P)H460
(M)H146
d
Fold Difference in Intensity
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Colon
Melanoma
Lung
(P)SW480
(M)SW620
(P)WM115
(M)WM266
(P)H460
(M)H146
e
+EGF
-EGF
IP:p68
IB:P-y-100
IB:p68-RGG
+EGF
-EGF
Snail
B2M

Figure 2.5

# CHAPTER 3: PHOSPHORYLATION OF p68 RNA HELICASE PROMOTES COLON CANCER METASTASIS

## 3.1 Abstract

Most cancer-related deaths are the consequence of cancer metastasis. Despite intensive studies over decades, the molecular mechanisms that govern this death-causing process are still poorly defined. We previously reported that phosphorylation of p68 RNA helicase at Y593 mediates the effects of PDGF in promoting EMT (Yang et al 2006). Here, we show that phosphorylation of p68 RNA helicase at tyrosine Y593 promotes cancer metastasis in nude mice xenograft model of human colon cancer cells. Analyses of tissue samples from colon cancer patients reveal a close correlation between phosphorylation of p68 at Y593 and cancer progression, supporting the notion that phosphorylation of p68 at Y593 plays an important role in tumorigenesis and cancer progression.

## 3.2 Introduction

The nuclear p68 RNA helicase is a prototypical DEAD box family of RNA helicase (Crawford et al 1982, Lane and Hoeffler 1980). Knock out experiments showed that p68 knockout is embryonic lethal indicating the critical function of the protein in the developmental programs. The protein is essential to maintain cell normal growth and differentiation (Janknecht 2010, Stevenson et al 1998). However, the cellular functions of the protein at the molecular level are not well understood (Schmid and Linder 1992). A number of independent studies have revealed that expression and/or posttranslational modifications of p68 are closely associated with cancer development and progression. It was first noted that overexpression and polyubiquitylation of p68 is associated with colon cancer development (Causevic et al 2001).

Association of p68 with colon cancer development and progression was further confirmed by observation from an independent study showing that expression of p68 was strongly increased during the polyp-->adenoma-->adenocarcinoma transition in the colon. It was believed that overexpression of p68 upregulated expression of a number of β-catenin-regulated genes (Shin et al 2007, Yang et al 2006). Our laboratory demonstrated that p68 is phosphorylated at multiple amino acid residues (Yang and Liu 2004). Phosphorylation of p68 at tyrosine closely correlated with cancer progression and metastasis (Yang et al 2005). Phosphorylation of p68 RNA helicase at Y593 mediates the effects of several growth factors in promoting EMT in colon cancer cells by promoting nuclear translocation of β-catenin (Yang et al 2006).

Epithelial-mesenchymal-transition (EMT) is a process by which epithelial cells undergo dramatic morphology changes marked by loss of cell polarity and cell-cell contacts. During tumor progression, substantial evidence suggests that EMT plays a critical role in tumor cells leaving organized epithelial layers and gaining property of invasiveness. Such changes often initiate cancer metastasis (Chaffer and Weinberg 2011, Rhim et al 2012). Phosphorylation of p68 at Y593 closely correlates with tumor progression (Yang et al 2005). Thus, it is speculated that the phosphorylation of p68 at Y593 may play an important role in cancer progression and metastasis. In this report, we provide evidence to demonstrate that phosphorylation of p68 RNA helicase at tyrosine Y593 promotes cancer metastasis in nude mice xenograft model of human colon cancer cells. Analyses of tissue samples from colon cancer patients reveal a close correlation between phosphorylation of p68 at Y593 and cancer development and progression.

## 3.3 Results

### 3.3.1 Characterization of Sublines with Endogenous p68 Knockdown and Exogenous p68 wt and Y593F Mutant Expressions

Phosphorylation of p68 at Y593 mediates the effects of PDGF on promoting EMT (Yang et al 2006). We speculated that the p68 phosphorylation may play a functional role in cancer metastasis. To test the potential role of the p68 phosphorylation at Y593 in promoting cancer metastasis, we examined whether expression of a non-phosphorylable p68 mutant Y593F would inhibit tumor metastasis of metastatic cancer cells in nude mice xenograft model. We chose a human colon cancer cell line SW620. This cell line was derived from colon cancer tissue of the patient with cancer metastasized to lymph nodes (the line was derived from lymph node metastatic cancer) (Leibovitz et al 1976). We first sought to establish a stable subline with endogenous p68 knockdown and expression of the p68 mutant Y593F. P68 was knocked down using shRNA by a retro-viral system. Immunoblot and RT-PCR analyses demonstrated that the endogenous p68 was largely knocked down (Fig. 3.1, A & B). P68 wild type and non-phosphorylable Y593F mutant were subsequently stably expressed in the p68 knockdown SW620 cells by plasmid DNA transfection followed with appropriate antibiotic selection. RT-PCR analyses showed that the exogenous HA-p68s, wt or Y593F mutant, were expressed and the expression levels were comparable with that of endogenous p68 in the cells (Fig. 3.1, C & A).

Several clones of sublines with p68 knockdown and HA-p68, wt and Y593F mutant, were selected. We carried out extensive characterizations of the obtained sublines. Firstly, to test whether knockdown of endogenous p68 and exogenous expression of HA-p68s, wt and Y593F mutant, led to significant changes in growth behaviors and other properties, cell proliferation of the established sublines were tested by a commercially available cell proliferation kit. It was

evident that knockdown of p68 resulted in a reduction in cell growth rate both in short-term (24hr) and long-term (6 days) testing periods (Fig. 3.2, A & B). Although the reduction in growth rate was not recovered by expression of HA-p68, wt and Y593F mutant in a 24-hour testing time (Fig. 3.2C), a gradual recovery of growth rate was observed on these cells in a 6-day measurement (Fig. 3.2D). We next analyzed the cell migration property by Boyden chamber assay. Evidently, p68 knockdown strongly reduced the cell migration (Fig. 3.3A). Expression of p68 wild-type completely recovered the migration property of the cells, whereas the expression of non-phosphorylable mutant p68 did not have a significant effect on cell migration (Fig. 3.3B). Since phosphorylation of p68 at Y593 promotes EMT (Yang et al 2006), we therefore examined the expression of epithelial and mesenchymal markers, E-cadherin and vimentin, in the p68 knockdown and wild-type/mutant expression cells, by RT-PCR. As expected, knockdown of p68 up-regulated E-cadherin and down-regulated vimentin expression (Fig. 3.3C). The pattern of E-cadherin expression could be restored by exogenous expression of wild-type p68, but not by expression of the Y593F mutant (Fig. 3.3D). We showed in our previous studies that phosphorylation of p68 at Y593 affected Snail1 expression (Carter et al 2010). Thus, we examined whether the Snail expression was affected in the selected clones of sublines. Clearly, Snail1 was down-regulated by p68 knockdown (Fig. 3.3C).

Examination of the selected clones of different sublines by phase contrast microscope revealed that expression of wild-type p68 in p68 knockdown cells led to increase in number of cells with spindle shaped morphology (Fig. 3.4B), while expression of the Y593F mutant in p68 knockdown cells led to strong increase in cell adhesion as demonstrated by substantial more cells clustered together in cultures (Fig. 3.4C). The cell-cell adhesion morphology observed in p68-Y593F expressing SW620 cells is comparable to that in its paired non-metastatic cell line,

SW480 (Fig. 3.4D), which is characterized with less or no invasive phenotypes. From these *in vitro* characterizations, we concluded that the established clones of sublines demonstrated that p68 phosphorylation at Y593 indeed support an invasive phenotype of SW620 colon cancer cells.

### 3.3.2 Knockdown of p68 and Expression of Y593F Mutant Significantly Reduced Cancer Metastasis

Next, we tested the metastasis of these established sublines in xenograft model. It is well established that the xenograft of SW620 will metastasize to lymph nodes, and the metastasis can be analyzed by examination of SW620 cells in the spleen (Liu et al 2003a). The established sublines of the SW620 cells were implanted at the right flank of nude mouse. The tumors were grown for 30 days. The growth of tumor was monitored by measuring the tumor volume and the tumor weight at end of experiments. In consistent with our observations with cultured cells, the growth rates of xenograft tumors were not significantly affected by expression of wild-type p68 or the Y593F mutant (Fig. 3.5A & B). We then analyzed the cancer metastasis of the xenograft tumors by examining the mRNA of human cytokeratin-18 (ref to as hCK-18) in the spleen of the tumor bearing mice. It was clear that knockdown of p68 led to no detection of hCK-18 mRNA in the spleen of any mouse within the test group. As comparison, we observed hCK-18 mRNA in three out of six test mice that were implanted SW620 cells without p68 knockdown (Fig. 3.5C, top-right panel). Expression of wild-type p68 resulted in observation of hCK-18 mRNA in three or four mice in the group of six mice (Fig. 3.5C, mid-panel). Expression of Y593F mutant led to detection of hCK-18 mRNA in only one mouse in each group of six mice (Fig. 3.5C, bottom-panel). Table 1 summarized the results of growth and metastasis tests of the xenograft of established sublines of SW620 cells. To ensure that the knockdown of p68 and expression of

HA-p68s were maintained in the xenograft tumors, we examined the endogenous p68 and exogenously expressed HA-p68 in the tumor tissues by RT-PCR using the primer pairs illustrated in Fig. 3.6A. It was clear that both knockdown of endogenous p68 and exogenous expression of HA-p68s were well maintained in the xenograft tumors (Fig. 3.6B). We also tested the expression of hCK-18 mRNA in the tumor tissues to ensure that the different detection of this protein from mouse spleen was not due to the varied expression level (Fig. 3.6B).

### 3.3.3 p68 is Phosphorylated at Y593 in the Colon Cancer Tissue Samples

Our results thus far showed that phosphorylation of p68 at Y593 promoted colon cancer metastasis in nude mouse xenograft model. We questioned whether p68 are indeed phosphorylated in the colon cancer tissues from cancer patients. To answer this question, we raised a polyclonal antibody using the peptide CNQQA[pY]AYPATA with Y593 phosphorylation as antigen in rabbit. Screening tests with the phosphorylated and unphosphorylated recombinant p68 and cell extracts indicated that the resultant rabbit anti-serum specifically recognized the Y593 phosphorylated p68 (Fig. 3.7A & B). Using the raised antibody in combination with an antibody against p68, we analyzed the levels of p68 and the Y593 phosphorylated p68 in the extracts prepared from 4 pairs of matched cancer and normal tissue samples (with tumor and normal tissues from the same patient in each pair) by immunoblots. It was evident that p68 was phosphorylated at Y593 in all the cancer tissues, but was not phosphorylated (or phosphorylated in very low levels in the corresponding normal tissues (Fig. 3.7C). Quantitative analyses of the immunoblots by normalizing the Y593 phosphorylated p68 to the total p68 levels in tissue extracts indicated that there is a remarkable increase of phosphorylation at Y593 in cancerous tissues compared to those of normal tissues (Fig. 3.7D), indicating that p68 is indeed phosphorylated at Y593 in colon cancers.

It was previously reported that expression of p68 is strongly increased in colon cancer cells (Causevic et al 2001). However, this increase was not observed in our immunoblot analyses. In fact, we observed a strong decrease in p68 levels in extracts made from some tumor tissues (Fig. 3.7C). We are curious if indeed there is a sharp decrease in cellular levels of p68 in colon cancer cells. To this end, we expand the areas of the immunoblots to the entire SDS-PAGE. It was evident that strong blot of a higher molecular weight band (~ 150 kDa) were observed. These higher MW band was not observed with extracts of normal tissues, neither with extracts of cultured colon cancer cells. Interestingly, this higher MW band was detected much stronger using the antibody against the Y593 phosphorylated p68 compared to that using an antibody against total p68 (Fig. 3.8).

## 3.4 Discussion

We show in our study that phosphorylation of p68 at Y593 promotes colon cancer metastasis with the nude mice xenograft model of SW620 cells. The results are in consistent with our previous observation that phosphorylation of p68 at Y593 mediates the effects of growth factors in promoting EMT (Yang et al 2006). Furthermore, we observed that p68 became strongly phosphorylated at Y593 in tissue samples of colon cancer patients, while the phosphorylation is not or very weakly detected in corresponding normal tissues, which indicates that the functional role(s) of the p68 phosphorylation is physiologically relevant to colon cancer development and progression.

How does phosphorylation of P68 RNA helicase at Y593 promote cancer metastasis? Clearly, our results showed that replacement of endogenous p68 with the Y593F mutant did not result in a significant change in tumor growth rates. On the other hand, the cell-cell attachments experienced strong increases with the Y593F mutant expression cells. In consistent with this

observation, we also observed a decrease in cell migration and an increased expression of E-cadherin with the mutant expression cells. Thus, it is clear that phosphorylation of p68 at Y593 promotes cancer metastasis by affecting cell morphology and migration/invasion capability.

Immunoblot analyses of p68 and Y593 phosphorylated p68 in cultured cells and tumor tissues revealed very different results. P68 were detected at around 68 kDa position by the antibodies against p68 and the phosphorylated p68 in the extracts of cultured colon cancer cells, while the majority of p68 were detected at very high molecular weight position in the extracts of tissue samples. This high molecular weight immunoblot band is not observed with normal tissue samples. The detection of p68 at 68 kDa became very weak in the tissue samples from cancer patients. The observation suggests that p68 is extensively modified in cancer tissues, and the modifications are lost in the cultured cells. One possibility is that the communications between cancer cells and stroma cells do not present in the case of cultured cancer cells. Alternatively, cancer cells in actual cancer mass are grown under hypoxia conditions, while the cells are usually cultured under normoxia condition. It is possible that the high MW modification of p68 is driven by hypoxia. An open question is whether this high MW modification of p68 plays a role in colon cancer progression. Since p68 with high MW modification was detected much stronger using antibody against the Y593 phosphorylated p68, it is possible that phosphorylation facilitates the posttranslational modification. The high molecular weight modification(s) was observed earlier by Causevic and co-workers. Based on the detection of the same mobility band by antibody against ubiquitin, it was suspected that p68 is ubiquitinated in cancer tissues (Causevic et al 2001). We attempted to pull-down the p68 by antibody against ubiquitin. We also attempted to immunoprecipitate p68 and followed by immunoblot using antibody against

ubiquitin. Both experiments were not successful. Thus, we could not confirm that p68 is ubiquitinated in cancer tissues.

## 3.5 Materials and Methods

*Antibodies, Reagents, and Cell Lines*

Puromycin and G418 antibiotics were purchased from Invivogen (San Diego, CA) and Sigma (St. Louis, MO) respectively. Both polyclonal PAbp68 and monoclonal p68-rgg antibodies were raised against bacterially expressed His-tagged C-terminal domain (a.a.437-614) of human p68 (Invitrogen, Carlsbad, CA, USA, Auburn University Hybridoma Facility). Polyclonal rabbit antibody against Y593 phosphorylated-p68 (pY593-p68) was generated by immunizing rabbits with KLH conjugated peptide CNQQA[pY]AYPATA, corresponding to residues 588-599 of the p68 protein (GenBank accession number NM_004396). Blood was taken 2 weeks after the fourth boost, and IgG was purified and tested for specific immune reactivity. Antibodies against β-actin, phosphor-tyrosine (pY20), E-cadherin, Vimentin, and HA-tag (12CA5) were purchased from Santa Cruz, BD Bioscience, and Roche Applied Science respectively.

Human colon cancer SW620 cells, human embryonic kidney HEK293 and 293T cells were obtained from ATCC and were cultured by following vendor's instruction. The SW620 derivatives including p68 knockdown, wild-type- and Y593F-p68 re-expressing cells were established in our laboratory. The SW620 cells and the derivatives were cultured in Leibovitz L-15 medium (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum and appropriate antibiotics, and maintained at 37°C in a humidified incubator (Thermo Scientific, Rockford, IL).

HEK293 and 293T cells were cultured in DMEM (Cellgro, Manassas, VA) supplemented with 10% FBS, and maintained at 37°C in a humidified incubator supplemented with 5% $CO_2$.

*Stable Knockdown of p68 by Retroviral System*

The pSIREN-RetroQ retroviral vector (Clontech, Mountain View, CA) containing short hairpin RNA (shRNA) targeting the sequence GAACTGCTCGCAGTACCAA of p68 (shRNA-p68) and the control vector (shRNA-conl) were gifted from Dr. Janknecht (Shin et al 2007). 293T cells were co-transfected with 15μg of ShRNA-p68 or shRNA-conl plasmids and two packing plasmids including 5μg of VSVG and 10μg of gag-pol using Lipofectamine 2000 (Life Technologies, Grand Island, NY) per manufacturer's instruction. The retroviruses were harvested 48-72 hours after transfection and used to infect SW620 cells according to standard procedures. The infected SW620 cells were selected in 10μg/ml puromycin (Invivogen, San Diego, CA) and the individual clones were isolated after 2-week selection. The knockdown efficiency of p68 in each clone was examined by Western blot (not shown) and RT-PCR. The SW620 clones stably expressing p68 shRNA (SW620-p68KD) were then used in this study.

*Transfection and Isolation of p68-expressing Stable Cell Lines*

The pHM6 vectors containing wild-type or Y593F human p68 cDNA (Yang et al 2006) were first undergone the site-directed mutagenesis on the p68 shRNA target site using QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), which resulted in the silent mutations and allow these constructs to express p68 proteins in p68 shRNA-expressing SW620 cells. The p68-knockdown SW620 cells were seeded in 6-well plates with 50 % confluence and transfected with 4μg of each construct using Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to manufacturer's instruction. After 48 hours of

transfection, the cells were plated in a 15-cm tissue culture dish and selected in 1mg/ml of G418 antibiotic for 4-6 weeks. The growing colonies were isolated individually and tested for the expression of p68 by Western blot (not shown) and RT-PCR. The G418 concentration used on the positive clones was gradually reduced to 200μg/ml during the routine culture, and the stability of p68 expression was tested. The positive clones were then used in this study. The empty pHM6 vector-transfected cells were undergone the same procedure and used as a control.

*RT-PCR Analysis*

Cells were properly treated, and then total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA was quantified and then converted to cDNA using the GoScript reverse transcription system (Promega, Madison, WI) following the manufacturer's instruction. The cDNA was then used in the final PCR reaction. PCR products were visualized after electrophoresis in a 1% agarose gel and stained by ethidium bromide (0.5 mg/ml). Densitometry was performed using ImageJ program. Primers used were: E-cadherin (sense 5'-AGAAGACAGAAGAGAGACTGGG-3' antisense 5'-TCCTCAACTGCATTCCCGTT-3'); HA-tagged p68 (HA-sense 5'-TACCCATAC GACGTCCCAGACTA-3' P68RV301-antisense 5'-ACATCCATGACATTTGCAGGGAA-3'); p68(endogenous) (sense 5'-TGGAAGAACTGCTCGCAGTACCAA-3' antisense 5'-TGGAACGACCTGAACCTCTGTCTT-3'); Snail1 (sense 5'-TCTAGGCCCTGGCTGCTAC-3' antisense 5'-GCCTGGCACTGGTACTTCTT-3'); Vimentin (sense 5'-AATGCGTCTCTGGCACGT-3' antisense 5'-ATTTCACGCATCTGGCGTTC-3'); GAPDH (sense 5'-GAGTCAACGGATTTGGTCGT-3' antisense 5'-TTGATTTTGGAGGGATCTCG-3'). Primers for GAPDH were used as PCR and loading controls. Controls for the RT reaction (not shown) contained no template or no reverse transcriptase.

The PCR primers (Liu et al 2003b) used in the metastasis study in xenograft tumor model included: human keratin-18 (sense 5'-GCCTACAAGCCCAGATTGCC-3' antisense 5'-GGTGGTCTTTTGGATGGTTTGC-3'); human β-actin (sense 5'-ATGGATGATGATATCGCCGCG-3' antisense 5'-CTAGAAGCATTTGCGGTGGAC-3'); mouse β-actin (sense 5'-ATTGTTACCAACTGGGACGACATG-3' antisense 5'-CTTCATGAGGTAGTCTGTCAGGTC-3'). The PCR parameters used were as following: 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for 30 cycles. Three primer sets were used for detecting exogenous expression of p68 including: 1. HA-tag+ P68RV301 (sequence listed above); 2. p68 601FW (sense 5'-ATCTACGGTGGTGCTCCTAA-3')+p68MutRTRV (antisense 5'-TTGGTGCTCCGCGCCGTT-3') with~700bp product; 3. p68 MutRTFW (sense 5'-AACGGCGCGGAGCACCAA-3')+ p68 (antisense 5'-TGGAACGACCTGAACCTCTGTCTT-3') with ~150bp product.

*Proliferation Assay*

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO) method was used to determine the cell proliferation. Cells with the proper treatment were seeded in a 96-well tissue culture plate with an amount of $10^4$ cells per well. On the second day, the culture cells were replaced with 100µl of MTT-containing media (10µl of MTT stock solution (5mg/ml) in 90µl of the culture medium without phenol red). The cells were returned to the incubator for 2 hours. After the incubation, the resulting formazan crystals were dissolved by adding 100µl of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) and mixed well by pipetting up and down. The absorbance at a wavelength of 570nm was measured by a spectrophotometer. The background absorbance was eliminated by measuring the absorbance at a wavelength of 690nm and subtracted from the 570nm

measurement. A blank containing complete medium without cells was included, and each group of cells was repeated in 8 wells for each independent experiment. The statistical result was based on three independent experiments.

*In Vitro Trans-well Assay*

The cell migration assays using trans-well system (BD Biosciences, Franklin Lakes, NJ) were performed on SW620 cells with proper treatments per manufacturer's instructions. The cells were serum-starved overnight, and $5x10^4$ of cells in 100µl were plated onto the top chamber of 24-well transwell plates with 8 µm pore size of trans-membrane in serum-free condition. The surface of the trans-membrane facing the bottom chamber was coated with $2µg/cm^2$ of human fibronectin (BD biosciences, Franklin Lakes, NJ), and the bottom chamber was filled with 400µl of complete culture medium. The trans-well plates were returned to the incubator and incubated for 48 hours. The cells trans-migrating to the bottom side of the membrane were fixed with 3.7% formaldehyde solution and stained with 0.5% crystal violet. The non-migrated cells inside the top chamber were removed by swabbing. The migrating cells with crystal violet staining were quantitated by solubilizing the dye into Sorenson's buffer (0.1 M sodium citrate and 50% ethanol, pH 4.2) and measuring the absorbance at 590nm wavelength.

*Analyses of Tissue Samples*

Frozen patient tissue specimens were obtained from University of Massachusetts Medical School Cancer Center (Worcester, MA). The specimens (200 ~300mg) were weighted and diced into small pieces using a clean razor blade. Collected pieces were immersed in liquid nitrogen and disrupted in a pre-chilled 1.5ml micro-centrifuge tube on ice using an electric pestle (VWR, Radnor, PA). Each disrupted specimen was lysed in 500µl of 1X RIPA buffer and further

homologized by using the pestle manually for 10 strokes. The lysate was rotated for 60 min at 4°C and then centrifuged at 1,2000rpm for 15 min at 4°C to remove debris. The supernatant was stored at –80°C until use.

*Preparation of Cell Lysates, Nuclear Extracts, Immunoprecipitation, and Immunoblot*

The experimental procedures for cell lysates and nuclear extracts, and for immunoprecipitation and immunoblot were similar to those described in our previous reports (Carter et al 2010, Yang et al 2007).

*Tumor Growth in Nude Mice*

All animal experiments were carried out in accordance with the guidelines of IACUC of Georgia State University. SW620 cells or its derivatives were inoculated subcutaneously into athymic nude mice (Harlan Laboratories, Indianapolis, IN) in the right flank with 2 X $10^6$ cells per mouse. Tumor growth was monitored by digital caliper in two dimensions, and the volume was calculated based on the formula of (width squared X length)/2. Four weeks after the tumor cell inoculation, spleens and tumors were isolated from each mouse and immersed in RNAlater RNA Stabilization Reagent (Qiagen, Valencia, CA) for later use of total RNA preparation. The RT-PCR based micrometastasis detection in mouse spleen was following the procedures reported previously and the primers were listed in RT-PCR analysis section (Liu et al 2003b).

*Statistical Analysis*

All statistical analyses were performed by using Analysis ToolPack in Microsoft Excel 2010. Two groups of samples were first analyzed by F-test to determine equal or unequal variances, and then analyzed by appropriate type of t-Test to determine the p value.

## 3.6 References

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Figure 3.1 P68/HA-p68 levels in sublines of SW620

Cellular levels of mRNA **(A)** and protein **(B)** of p68 in SW620 cells were analyzed by RT-PCR **(A)** and immunoblot **(B)** using antibody against p68 (IB:p68). The cells were infected with virus that carries non-targeting shRNA (NT) or shRNA target p68 (p68). The Clone# represents the selected sublines. RT-PCR **(A)** and immunoblot **(B)** analyses of GAPDH (GAPDH and IB:GAPDH) are loading controls. **(C)** Cellular levels of mRNA of endogenous p68 (En-p68) and exogenous p68 (HA-p68) in sublines of SW620 by RT-PCR using the primer pairs indicated in bottom panel. Endogenous p68 was knocked down and HA-p68s, wild-type (WT) and Y593F mutant (Y593F) were exogenously expressed. Vec means the cells were infected with virus carries empty vector. The Clone# represents the selected sublines. RT-PCR analyses of mRNA of GAPDH (GAPDH) are loading control.

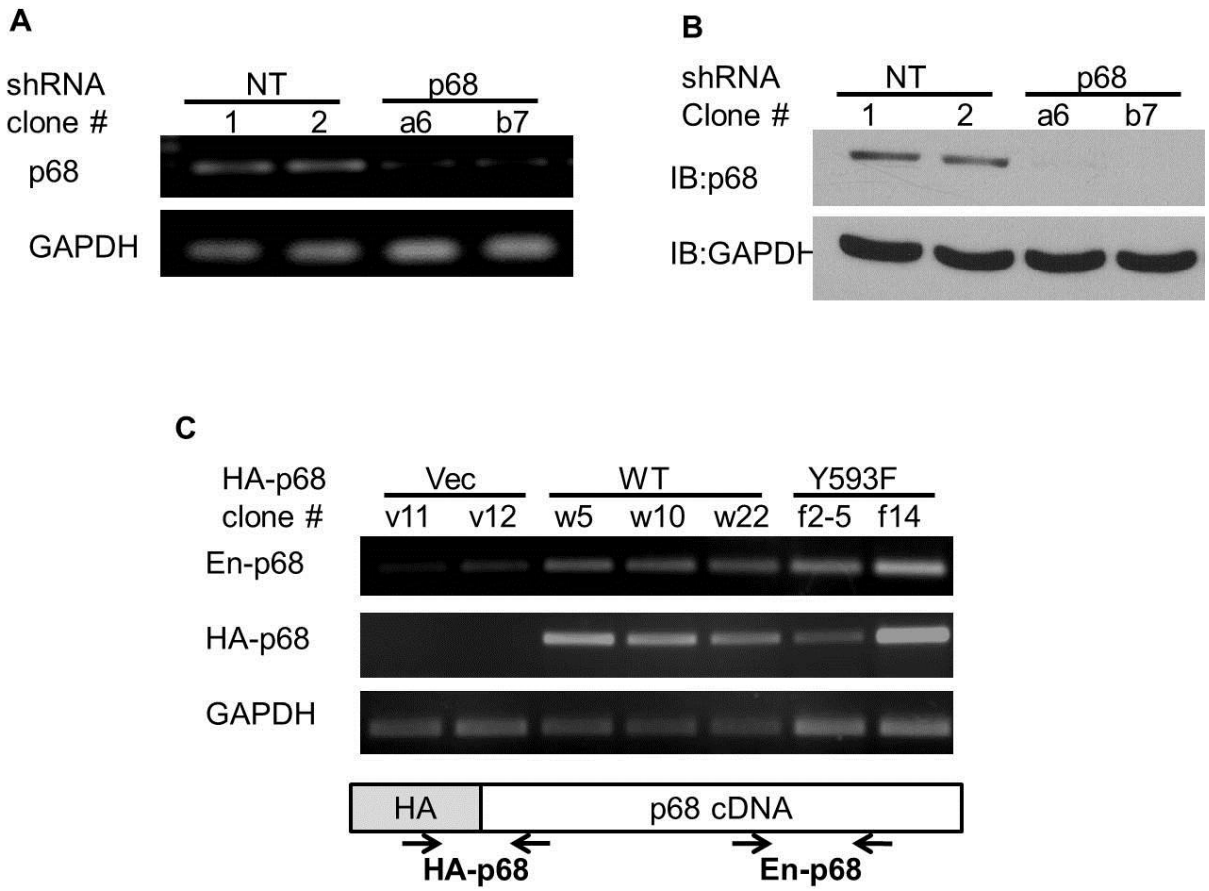
A
shRNA
clone #
NT
1
2
p68
a6
b7
p68
GAPDH
B
shRNA
Clone #
NT
1
2
p68
a6
b7
IB:p68
IB:GAPDH
C
HA-p68
clone #
Vec
v11
v12
WT
w5
w10
w22
Y593F
f2-5
f14
En-p68
HA-p68
GAPDH
HA
p68 cDNA
HA-p68
En-p68

Figure 3.1

Figure 3.2 Growth conditions of established SW620 sublines

**(A)** & **(B)** Growths of SW620 cells in which p68 was knocked down by shRNA at indicated times were analyzed by MTT assay. The cell growth were presented as relative cell proliferation by defining the growth of cells that were treated with non-target shRNA (NT) as 1 in **(A)** or the cell growth rate at day one as 1 in **(B)**. **(C)** & **(D)** Growths of SW620 cells in which p68 was knocked down by shRNA and HA-p68s, wild-type (WT) or Y593F mutant (Y593F), were expressed in the p68 knockdown cells at indicated times were analyzed by MTT assay. The cell growth were presented as relative cell proliferation by defining the growth of cells that were infected with virus carries empty vector (Vec) as 1 in **(C)** or the cell growth rate at day one as 1 in **(D)**. Error bars represent standard deviations of three experiments.

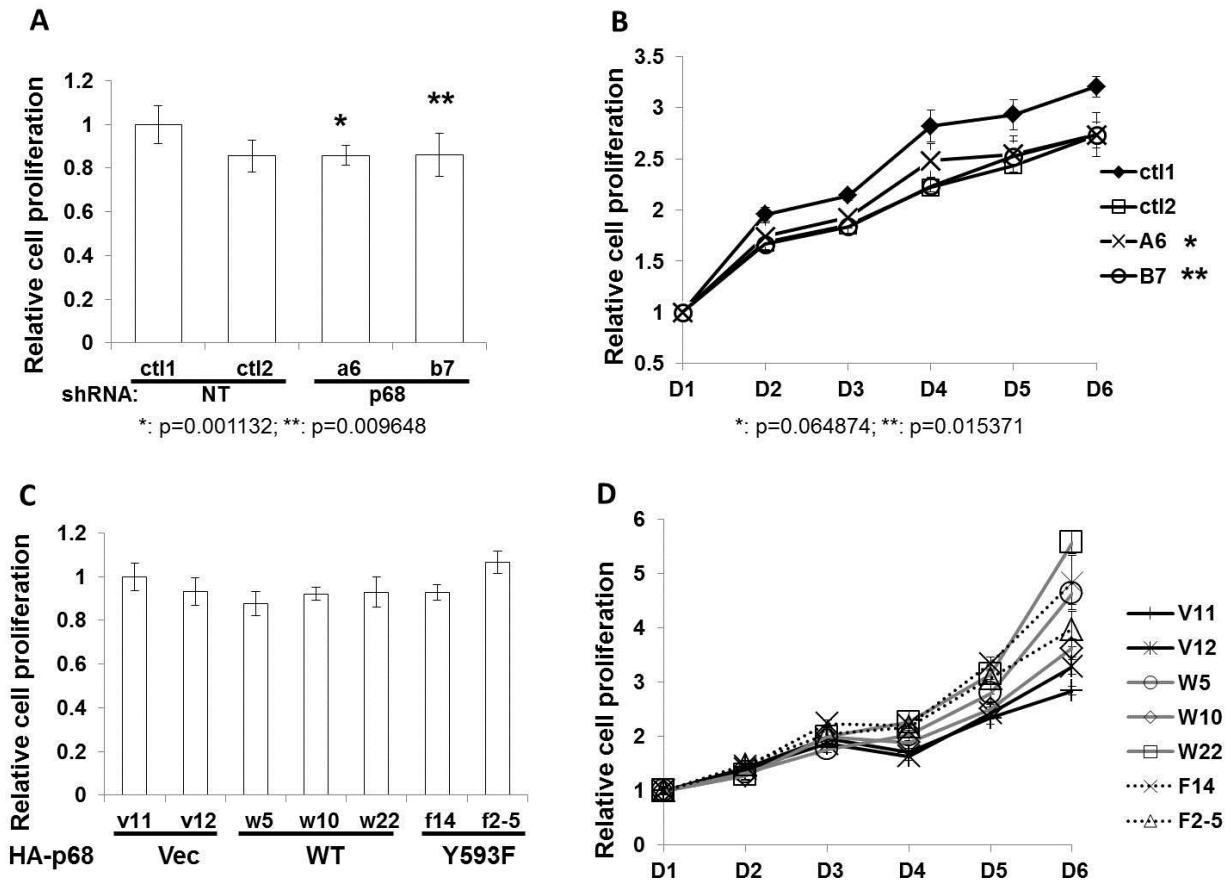
A
Relative cell proliferation
1.2
1
0.8
0.6
0.4
0.2
0
*
**
ctl1
ctl2
a6
b7
shRNA:
NT
p68
*: p=0.001132; **: p=0.009648
B
Relative cell proliferation
3.5
3
2.5
2
1.5
1
0.5
D1
D2
D3
D4
D5
D6
ctl1
ctl2
A6 *
B7 **
*: p=0.064874; **: p=0.015371
C
Relative cell proliferation
1.2
1
0.8
0.6
0.4
0.2
0
v11
v12
w5
w10
w22
f14
f2-5
HA-p68
Vec
WT
Y593F
D
Relative cell proliferation
6
5
4
3
2
1
0
D1
D2
D3
D4
D5
D6
V11
V12
W5
W10
W22
F14
F2-5

Figure 3.2

Figure 3.3 Cell migration and epithelial/mesenchymal marker characterization in SW620 sublines

**(A)** & **(B)** Cell migration of SW620 cells in which p68 was knocked down by shRNA **(A)** and HA-p68s, wild-type (WT) or Y593F mutant (Y593F), were expressed in the p68 knockdown cells **(B)** was measured by Boyden chamber assay. The cell migrations were presented as relative cell migration by defining the migration rate of the cells that were treated with non-target shRNA (NT) as 1 in **(A)** or of the cells that were infected with virus that carries empty vectors after p68 knockdown (Vec) as 1 in **(B)**. Error bars represent standard deviations of three experiments. **(C)** & **(D)** Cellular mRNA levels of E-cadherin (E-cadherin), Snail, and Vimentin (Vimentin) in SW620 cells in which p68 was knocked down by shRNA **(C)** and HA-p68s, wild-type (WT) or Y593F mutant (Y593F), were expressed in the p68 knockdown cells **(D)** were measured by RT-PCR. NT in **(C)** means the cells were treated with non-target shRNA. Vec in (D) means the cells were infected with virus that carries empty vectors after p68 knockdown. Analyses of mRNA levels of GAPDH are loading controls.

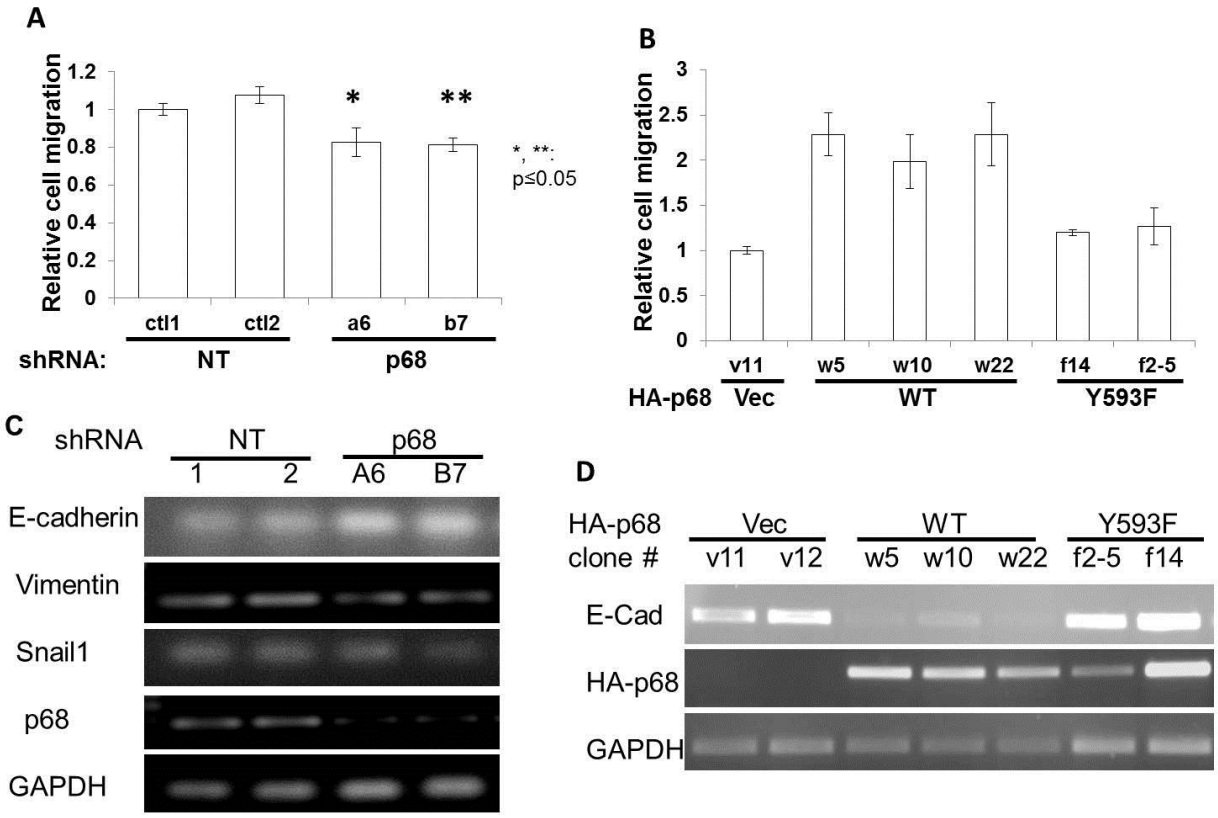
A
Relative cell migration
1.2
1
0.8
0.6
0.4
0.2
0
*
**
*, **:
p≤0.05
ctl1
ctl2
a6
b7
shRNA:
NT
p68
B
Relative cell migration
3
2.5
2
1.5
1
0.5
0
v11
w5
w10
w22
f14
f2-5
HA-p68
Vec
WT
Y593F
C
shRNA
NT
p68
1
2
A6
B7
E-cadherin
Vimentin
Snail1
p68
GAPDH
D
HA-p68
Vec
WT
Y593F
clone #
v11
v12
w5
w10
w22
f2-5
f14
E-Cad
HA-p68
GAPDH

Figure 3.3

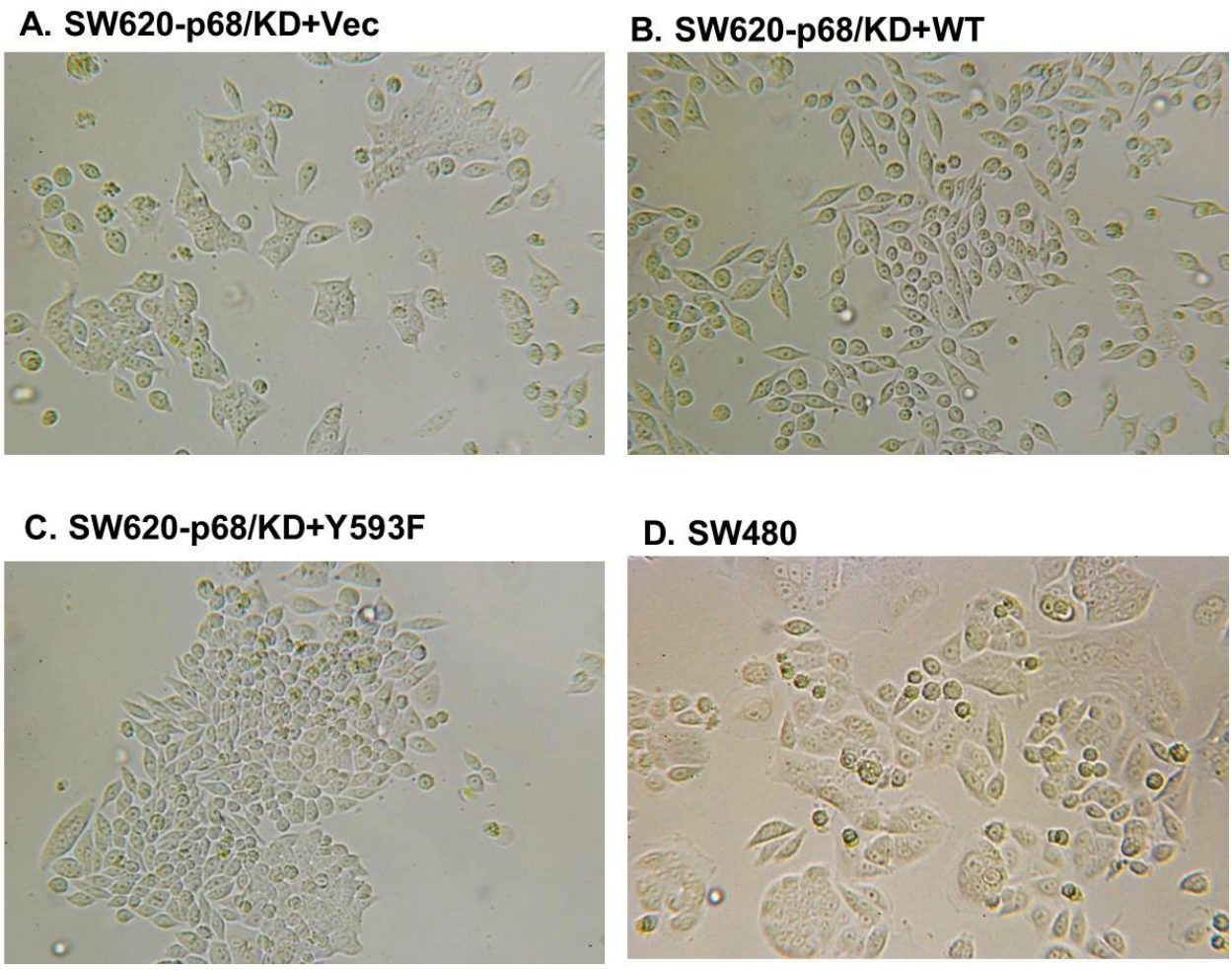


Figure 3.4 Morphology of SW620 sublines and SW480 cells

Representative phase contrast microscopic images of indicated SW620 cells and its derived sublines, and SW480 cells

Figure 3.5 The effects of p68 mutations on metastasis of xenograft of SW620 tumors.

**(A)** Tumor growth was monitored by measuring tumor volumes every three days and the tumor volumes were calculated by formula; Tumor volume = 1/2 x (width) 2 x length. **(B)** The end point weight of harvested tumors of SW620 and its derived sublines after 28 days growth. **(C)** The mRNA levels of human cytokarotine-18 (hCK-18) in the spleen extracts of tumor-bearing mice that carry tumors of SW620 or its derived sublines (indicated) were analyzed by RT-PCR. The mRNA levels of mouse β-actin (mActin) in the spleen extracts are loading controls. Error bars in **(A)** & **(B)** represent standard deviations of three experiments.

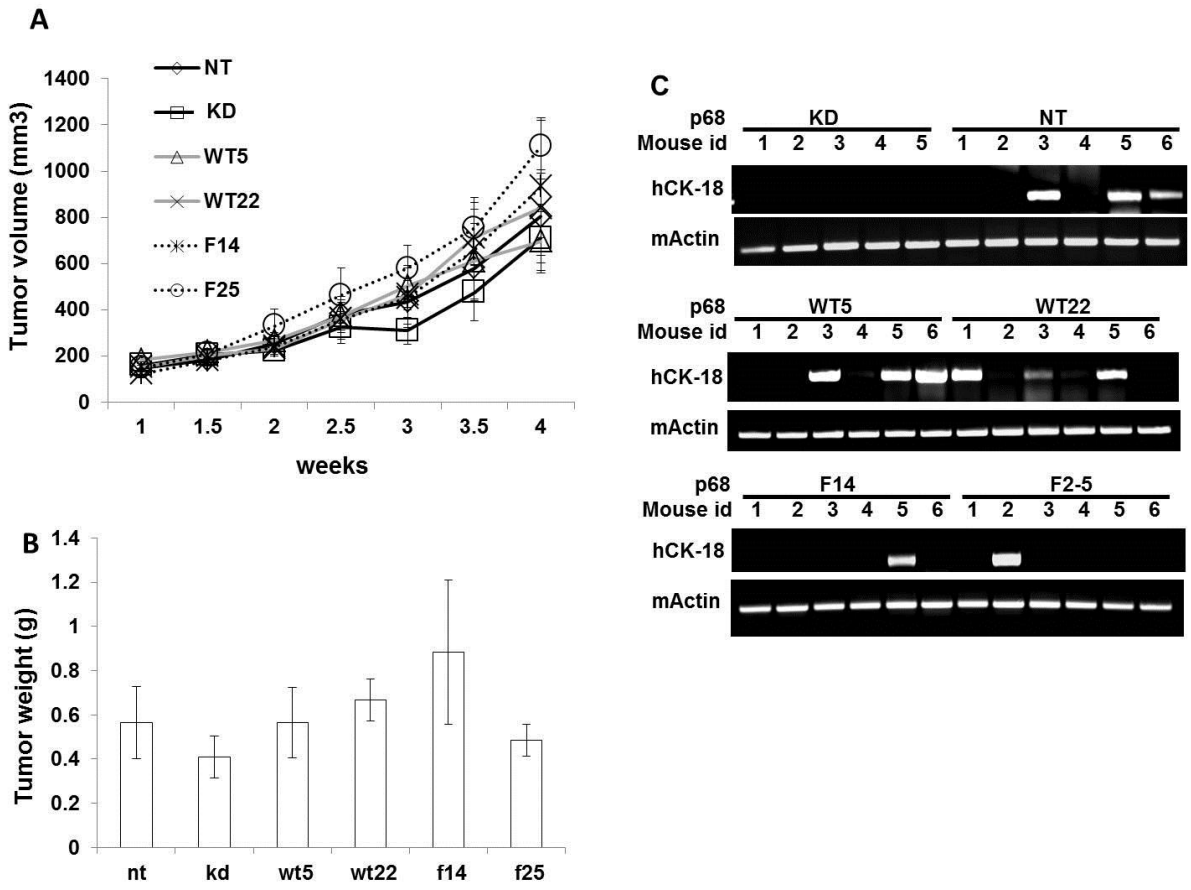
A
NT
KD
WT5
WT22
F14
F25
Tumor volume (mm3)
1400
1200
1000
800
600
400
200
0
1
1.5
2
2.5
3
3.5
4
weeks
B
Tumor weight (g)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
nt
kd
wt5
wt22
f14
f25
C
p68
Mouse id
KD
NT
1 2 3 4 5 1 2 3 4 5 6
hCK-18
mActin
WT5
WT22
F14
F2-5

Figure 3.5

Figure 3.6 mRNA levels of p68 in SW620 tumors

The mRNA levels of endogenous and exogenous p68 in the implanted tumors of SW620 and its derived sublines were examined by RT-PCR using primer pairs to cover different regions of ORF of the p68 **(illustrated in Upper penal).** RT-PCR examination of mRNA levels of human cytokarotine-18 (hCK-18) and human β-actin (hActin) in the tumor tissue extracts are loading controls.

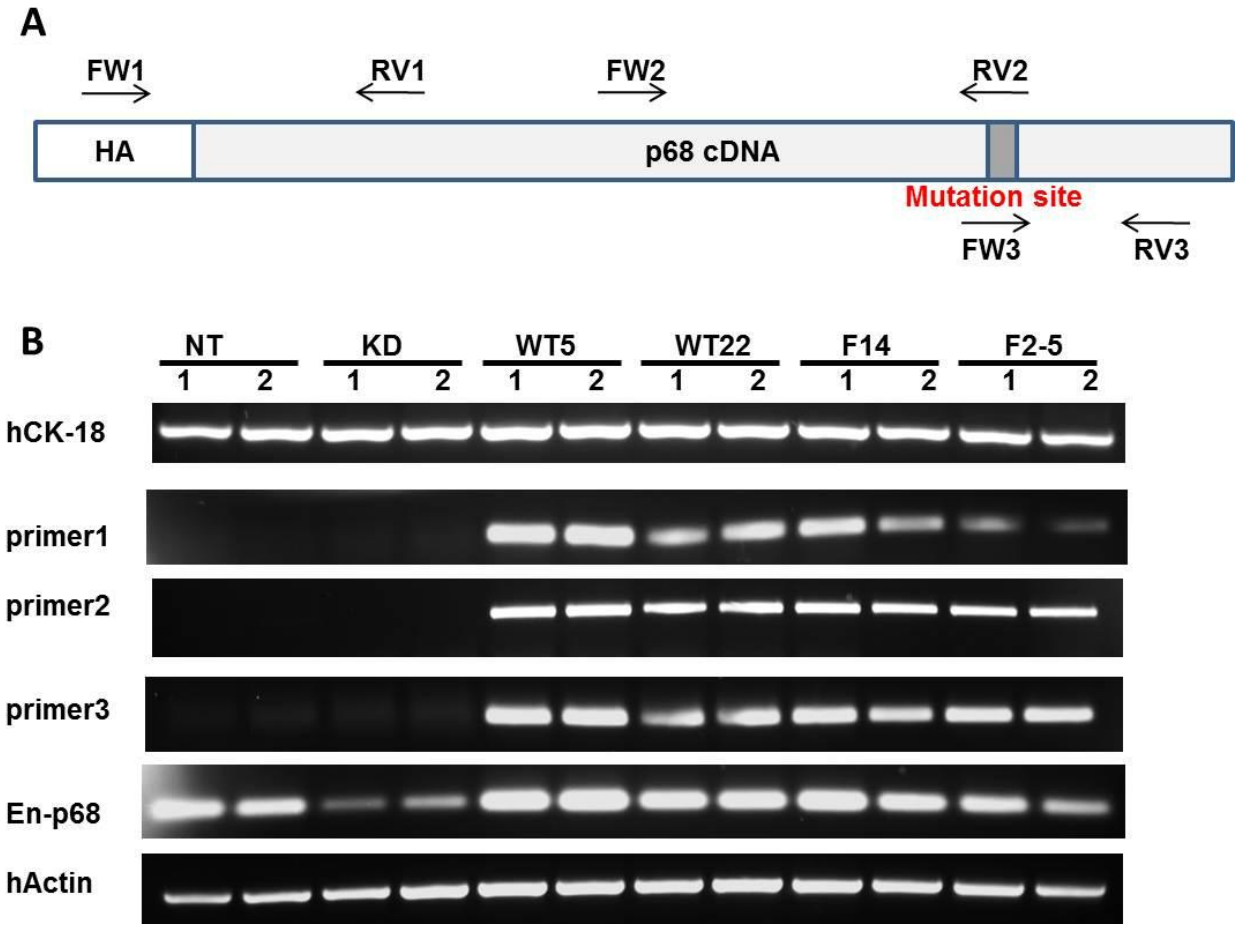
A
FW1
RV1
FW2
RV2
HA
p68 cDNA
Mutation site
FW3
RV3
B
NT
KD
WT5
WT22
F14
F2-5
1 2 1 2 1 2 1 2 1 2 1 2
hCK-18
primer1
primer2
primer3
En-p68
hActin

Figure 3.6

Figure 3.7 Phosphorylation level of p68 at Y593 in colon cancer tissues

**(A)** & **(B)** Tests of specificity of antibody raised against Y593 phosphorylated p68 **(A)** Immunoblot analyses of bacterially expressed recombinant p68 that was treated/untreated by c-Abl (+/-) and YOP (+/-) using different antibodies: Antibody against the Y593 phosphorylated p68 (IB:p-p68), antibody against tyrosine phosphor-protein (IB:pY20), and antibody against p68 (IB:p68). **(B)** Immunoblot analyses of exogenous HA-p68s (WT and indicated mutant) that were immunoprecipitated by anti-HA antibody (IP:HA) using antibody against the Y593 phosphorylated p68 (IB:p-p68). Immunoblot of total p68 in the IPs indicate amount of IPed HA-p68. The input (IB:HA and IB:GAPDH) are the immunoblots of cell extracts without IP and are loading controls. **(C)** Immunoblot analyses of p68 in colon cancer and corresponding normal tissue samples (indicated by the patent ID numbers) using antibodies against the Y593 phosphorylated p68 (IB:p-p68) and p68 (IB:p68). Immunoblot of GAPDH (IB:GAPDH) is a loading control. **(D)** Quantitation of the immunoblots in **(C)** by normalizing the IB signals of Y593 phosphorylated p68 to the total p68 IB signals (relative intensity of Y-593 phosphor-p68). Error bars represent standard deviations of three experiments.

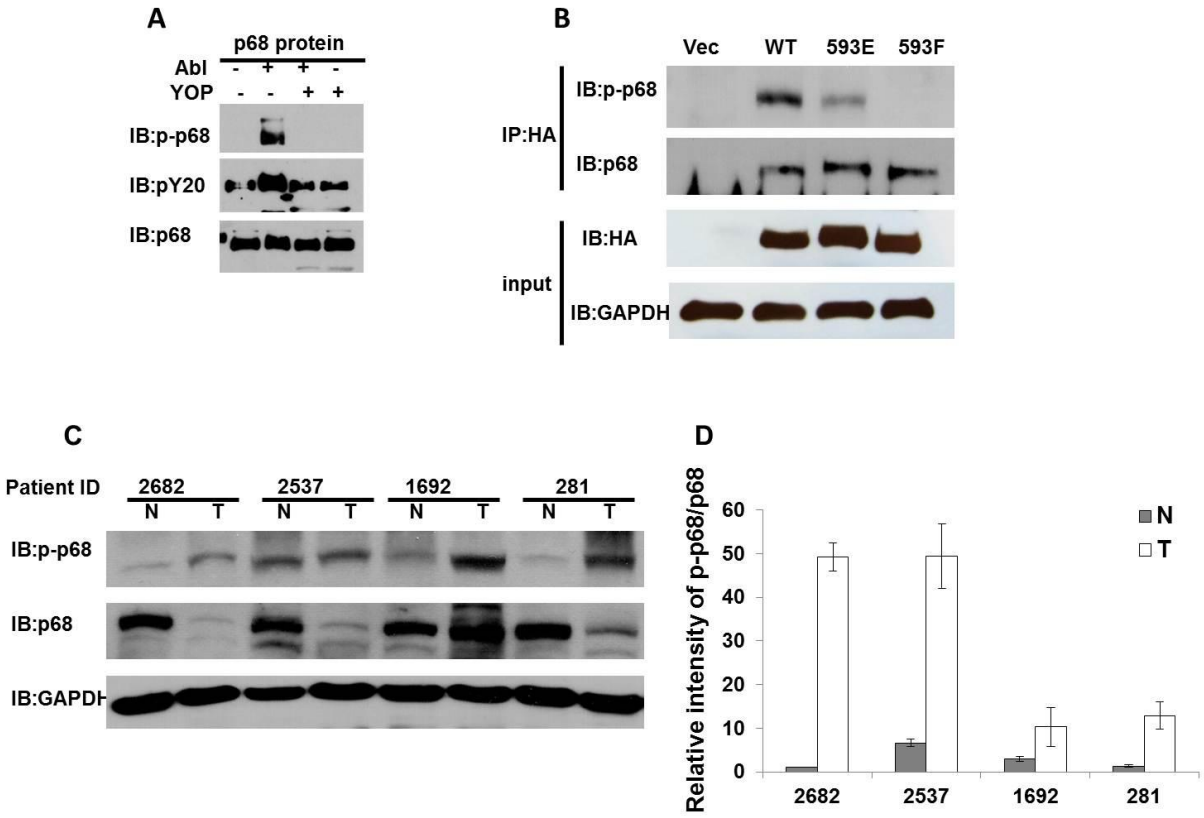
A
p68 protein
Abl - + + -
YOP - - + +
IB:p-p68
IB:pY20
IB:p68
B
Vec WT 593E 593F
IP:HA
IB:p-p68
IB:p68
input
IB:HA
IB:GAPDH
C
Patient ID 2682 2537 1692 281
N T N T N T N T
IB:p-p68
IB:p68
IB:GAPDH
D
Relative intensity of p-p68/p68
60
50
40
30
20
10
0
N
T
2682 2537 1692 281

Figure 3.7

Figure 3.8 Comparison of immunoblot of p68 between tissue extracts and cultured cell extracts.

Immunoblot analyses of p68 in the tissue extracts from colon cancer tissue samples (T) and corresponding normal tissue samples (N) using antibody against the Y593 phosphorylated p68 (IB:p-p68, Upper panel) and antibody against p68 (IB:p68, middle panel). M means the tissue extracts prepared from metastatic tumors in small bowl. The panels on the right are the same immunoblots using extracts of SW620, HCT116, and HT29 cultured colon cancer cells. The numbers on the left are the molecular weight markers. Immunoblot of GAPDH (IB:GAPDH) is a loading control.

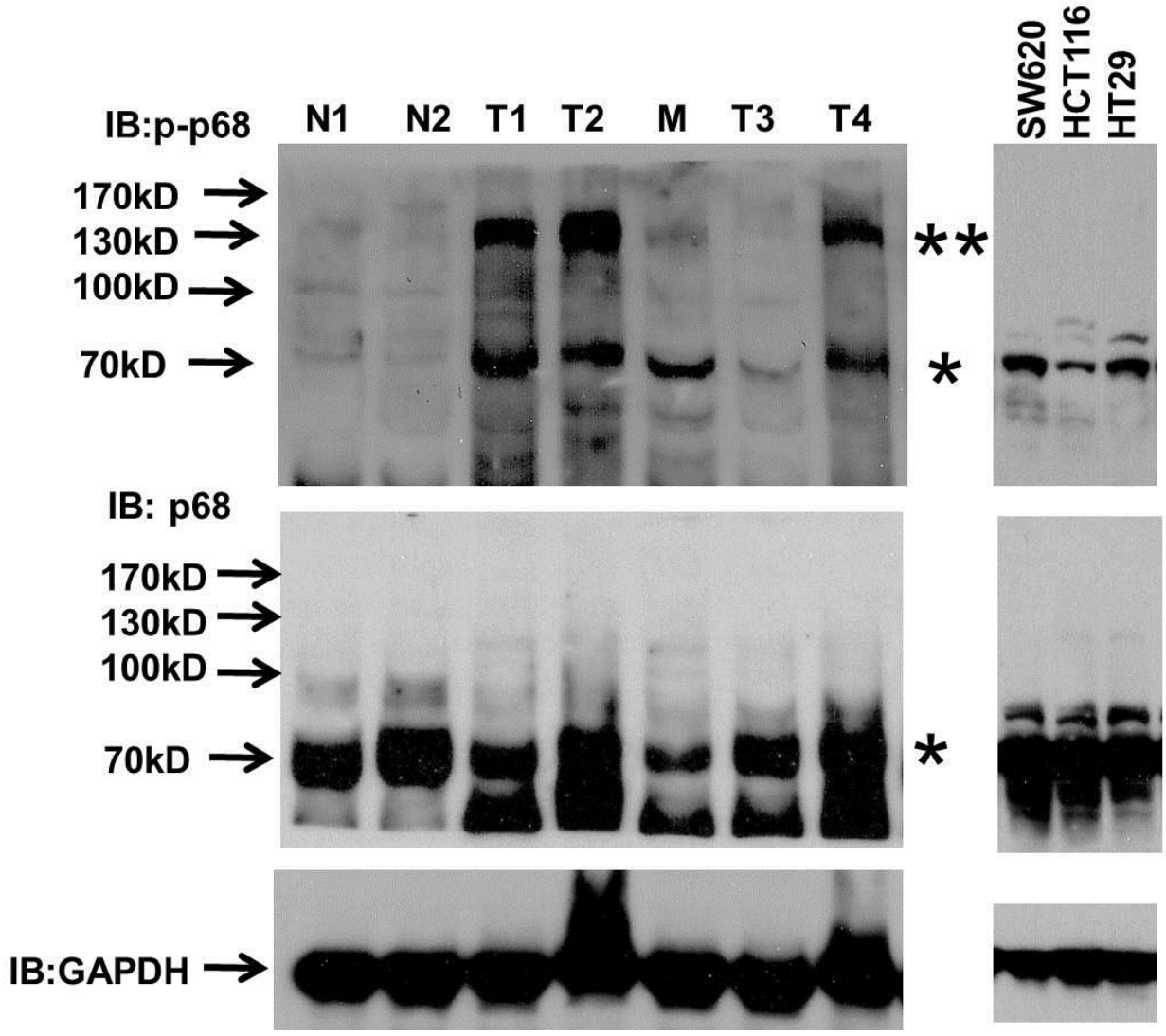
IB:p-p68
N1
N2
T1
T2
M
T3
T4
SW620
HCT116
HT29
170kD
130kD
100kD
70kD
**
*
IB: p68
170kD
130kD
100kD
70kD
*
IB:GAPDH

Figure 3.8

Table 1 Summary of the tumor xenograft experiment

Tumor Metastasis Rate versus Tumor Growth

| **Mouse group** | **NT** | **p68KD** | **KD+Wt-p68 Wt-5** | **KD+Wt-p68 Wt-22** | **KD+Y593F F14** | **KD+Y593F F2-5** |
|---|---|---|---|---|---|---|
| Mets rate | 50% | 0% | 50% | 50% | 17% | 17% |
| tumor weight Ave. (g)±SE | 0.5662 ± 0.163 | 0.4106 ±0.094 | 0.5646 ±0.160 | 0.6668 ±0.096 | 0.883 ±0.327 | 0.4854 ±0.074 |
| tumor volume Ave.($mm^3$) | 803.97 ±201.29 | 708.70 ±149.00 | 697.20 ±128.16 | 839.25 ±127.27 | 933.14 ±298.04 | 1106.51 ±113.40 |

# CHAPTER 4: MATERIALS AND METHODS

## 4.1 Materials

### 4.1.1 Chemicals

| | |
|---|---|
| Alcohol | VWR international, Radnor, PA |
| Acetic Acid | VWR international, Radnor, PA |
| Acrylamide | VWR international, Radnor, PA |
| Adenosine Triphosphate solution | Thermo Scientific, Rockford, IL |
| Agar | Sigma Aldrich, St. Louis, MO |
| Agarose | Sigma Aldrich, St. Louis, MO |
| Ammonium Persulfate | Sigma Aldrich, St. Louis, MO |
| Ampicillin | Sigma Aldrich, St. Louis, MO |
| Autoradiography film | Fisher Scientific, Pittsburgh, PA |
| Bacto Yeast Extract | BD Biosciences, Franklin Lakes, NJ |
| Bacto Tryptone | BD Biosciences, Franklin Lakes, NJ |
| β-Glycerophosphate Disodium | Sigma Aldrich, St. Louis, MO |
| Bis-Acrylamide | VWR international, Radnor, PA |
| β-Mercaptoethanol | Sigma Aldrich, St. Louis, MO |
| Bromophenol Blue | EMD, Gibbstown, NJ |
| Cell culture media | Cellgro, Manassas, VA |
| Coomassie blue | Sigma Aldrich, St. Louis, MO |
| Dithiothreitol | Sigma Aldrich, St. Louis, MO |
| Dimethyl Sulfoxide | Sigma Aldrich, St. Louis, MO |
| ECL Western Blot substrate | Thermo Scientific, Rockford, IL |
| Ethanol, 200 Proof | VWR International, Radnor, PA |
| Ethidium Bromide | Sigma Aldrich, St. Louis, MO |
| Ethylenediaminetetraacetic Acid | Sigma Aldrich, St. Louis, MO |
| Fast SYBR Green Master Mix | Life Technologies, Grand Island, NY |
| Formaldehyde | Sigma Aldrich, St. Louis, MO |
| Formalin, 10% solution | Sigma Aldrich, St. Louis, MO |
| G418 Disulfate Salt | Sigma Aldrich, St. Louis, MO |
| Glycerin | VWR international, Radnor, PA |
| Glycine | VWR international, Radnor, PA |
| Glycol ether diamine tetraacetic acid (EGTA) | Sigma Aldrich, St. Louis, MO |
| HEPES | Sigma Aldrich, St. Louis, MO |
| Hydrochloric acid | VWR international, Radnor, PA |
| Igepal (NP-40) | Sigma Aldrich, St. Louis, MO |
| Imidazole | Sigma Aldrich, St. Louis, MO |
| Isopropyl Alcohol | VWR international, Radnor, PA |
| Isopropyl ß-D-1-thiogalactopyranoside | Sigma Aldrich, St. Louis, MO |
| Kanamycin | Sigma Aldrich, St. Louis, MO |

| | |
|---|---|
| Lipofectamine 2000 Transfection Reagent | Life Technologies, Grand Island, NY |
| Magnesium Chloride | Sigma Aldrich, St. Louis, MO |
| Methanol | VWR International, Radnor, PA |
| Ni-NTA agarose | Qiagen Inc., Valencia, CA |
| PCR Master Mix | Promega, Madison, WI |
| Penicillin-Streptomycin solution | Cellgro, Manassas, VA |
| Phenylmethylsulfonyl Fluoride | Sigma Aldrich, St. Louis, MO |
| Ponceau solution | Sigma Aldrich, St. Louis, MO |
| Protein A agarose | Thermo Scientific, Rockford, IL |
| Protein G agarose | Millipore, Billerica, MA |
| Protein inhibitor cocktail | Sigma Aldrich, St. Louis, MO |
| Phosphatase inhibitor cocktail I (Ser/Thr) | Sigma Aldrich, St. Louis, MO |
| Phosphatase inhibitor cocktail II (Tyr) | Sigma Aldrich, St. Louis, MO |
| Puromycin hydrochloride | Invivogen, San Diego, CA |
| Sodium Acetate | Sigma Aldrich, St. Louis, MO |
| Sodium Chloride | VWR International, Radnor, PA |
| Sodium Bicarbonate | Sigma Aldrich, St. Louis, MO |
| Sodium Dodecyl Sulfate | Sigma Aldrich, St. Louis, MO |
| Sodium Fluoride | Sigma Aldrich, St. Louis, MO |
| Sodium Hydroxide | Sigma Aldrich, St. Louis, MO |
| Sodium Orthovanadate | Sigma Aldrich, St. Louis, MO |
| Sodium Pyruvate solution | Cellgro, Manassas, VA |
| N,N,N’,N’-TetramethylEthylenediamine | Sigma Aldrich, St. Louis, MO |
| Thiazolyl Blue Tetrazolium Bromide (MTT) | Sigma Aldrich, St. Louis, MO |
| Trichostatin A | Sigma Aldrich, St. Louis, MO |
| Tris base | Fisher Scientific, Pittsburgh, PA |
| Triton X-100 | Sigma Aldrich, St. Louis, MO |
| Trypan Blue solution | Sigma Aldrich, St. Louis, MO |
| 0.25% Trypsin-EDTA | Cellgro, Manassas, VA |
| Tween-20 | Sigma Aldrich, St. Louis, MO |
| Western Blot Stripping Buffer | Thermo Scientific, Rockford, IL |

### 4.1.2 Kits

| | |
|---|---|
| Bio-Rad Protein Assay | Bio-Rad Laboratories, Hercules, CA |
| Biocoat Cell Migration System | BD Bioscience, Franklin Lakes, NJ |
| Colorimetric HDAC Activity Assay Kit | BioVision, Mountain View, CA |
| Dual-Luciferase Reporter Assay | Promega, Madison, WI |
| GoScript Reverse Transcription System | Promega, Madison, WI |
| Nuclear Extraction Kit | Active Motif, Carlsbad, CA |
| pSIREN-RetroQ shRNA kit | Clontech, Mountain View, CA |
| RNeasy Mini Kit | Qiagen Inc., Valencia, CA |
| QIAprep Spin Miniprep Kit | Qiagen Inc., Valencia, CA |
| QIAquick Gel Extraction Kit | Qiagen Inc., Valencia, CA |
| QuikChange II Site-Directed Mutagenesis Kit | Stratagene, La Jolla, CA |

| | |
|---|---|
| QuikCHIP Kit | IMGENEX, San Diego, CA |
| Wizard® plus DNA purification System | Promega, Madison, WI |
| Virapower Lentiviral Expression System | Life Technologies, Grand Island, NY |

### 4.1.3 Laboratory Equipment

| | |
|---|---|
| 7500 Fast Real-Time PCR System | Life Technologies, Grand Island, NY |
| Allegra 6R Refrigerated Benchtop Centrifuge | Beckman Coulter, Brea, CA |
| C25 Incubated Floor Shaker | New Brunswick Scientific, Enfield, CT |
| Class II Biosafety Cabinet | Labconco, Kansas City, MO |
| EC3 BioImaging System | UVP, Upland, CA |
| Mastercycler Gradient | Eppendorf, Hauppauge, NY |
| UV-1700 Spectrophotometer | Shimadzu North America, Columbia, MD |
| Victor3 Multilable Plate Reader | Perkin Elmer, Waltham, MA |

### 4.1.4 Enzymes and Recombinant Proteins

| | |
|---|---|
| Alkaline phosphatase, Shrimp | Promega, Madison, WI |
| C-Abl protein tyrosine kinase | New England BioLabs, Ipswich, MA |
| DNase | Promega, Madison, WI |
| HDAC1 | Affymetrix, Santa Clara, CA |
| MBD3 | BioClone, San Diego, CA |
| *Pfu*-DNA polymerase | Promega, Madison, WI |
| Proteinase K | Sigma Aldrich, St. Louis, MO |
| PDGF-AA | Pepro Tech, Rocky Hill, NJ |
| PDGF-BB | Pepro Tech, Rocky Hill, NJ |
| RNase A | Thermo Scientific, Rockford, IL |
| Restriction enzymes | Thermo Scientific, Rockford, IL |
| T4 Polynucleotide Kinase | Promega, Madison, WI |
| T4-DNA ligase | Thermo Scientific, Rockford, IL |
| YOP protein tyrosine phosphatase | New England BioLabs, Ipswich, MA |

### 4.1.5 Antibodies

| | |
|---|---|
| β-Actin antibody (mouse monoclonal) | Santa Cruz Biotechnology, Santa Cruz, CA |
| E-cadherin (mouse, monoclonal) | BD Biosciences, Franklin Lakes, NJ |
| GAPDH antibody (mouse monoclonal) | Thermo Scientific, Rockford, IL |
| HA antibody (mouse monoclonal) | Roche Applied Science, Indianapolis, IN |
| HA antibody (rabbit polyclonal) | Lab generated |
| 6x-His antibody (rabbit polyclonal) | Abcam, Cambridge, MA |
| Histone 2A (rabbit polyclonal) | Cell Signaling Technology, Danvers, MA |
| HDAC1 antibody (rabbit polyclonal) | Abcam, Cambridge, MA |
| HDAC1 antibody (mouse monoclonal) | Santa Cruz Biotechnology, Santa Cruz, CA |
| KAT3A/CBP antibody (rabbit polyclonal) | Abcam, Cambridge, MA |

| | |
|---|---|
| MBD3 antibody (rabbit polyclonal) | ABGENT, San Diego, CA |
| MBD3 antibody (mouse monoclonal) | IMGENEX, San Diego, CA |
| Mi-2 (CHD4) antibody (rabbit polyclonal) | Abcam, Cambridge, MA |
| Mi-2 antibody (goat polyclonal) | Santa Cruz Biotechnology, Santa Cruz, CA |
| Myc antibody (mouse polyclonal) | Santa Cruz Biotechnology, Santa Cruz, CA |
| p68 (C-terminal) antibody (rabbit polyclonal) | Life Technologies, Grand Island, NY |
| p68-rgg antibody (mouse monoclonal) | Auburn University Hybridoma Facility |
| SNAIL 1 antibody (rabbit polyclonal) | Abcam, Cambridge, MA |
| P-Tyr-100 antibody (mouse monoclonal) | Cell Signaling Technology, Danvers, MA |
| P-Tyr (pY20) antibody (mouse monoclonal) | Santa Cruz Biotechnology, Santa Cruz, CA |
| Vimentin antibody (mouse monoclonal) | BD Biosciences, Franklin Lakes, NJ |

### 4.1.6 Vectors and siRNA/shRNA Sequence

| | |
|---|---|
| pHM6 mammalian expression vector | Roche Applied Science, Indianapolis, IN |
| pLenti6/V5-D-TOPO | Life Technologies, Grand Island, NY |
| pLenti6/V5-DEST | Life Technologies, Grand Island, NY |
| Snail-pGL2 | Fujita, et al. (2003) |
| Human DDX5 NM_004396, GCAAGUAGCUGCUGAAUAUUU | Thermo Scientific, Rockford, IL |
| Human MBD3 NM_003926, SMART pool | Thermo Scientific, Rockford, IL |
| Human SNAI1 NM_005985, SMART pool | Thermo Scientific, Rockford, IL |
| Human p68 shRNA, GAACTGCTCGCAGTACCAA | (Shin et al 2007) |

### 4.1.7 Bacteria Stains

| | |
|---|---|
| BL21-CodonPlus (DE3)-RIL | Stratagene, La Jolla, CA |
| JM109 | Promega, Madison, WI |
| One shot Stbl3 | Life Technologies, Grand Island, NY |
| XL1-Blue supercompetent cells | Stratagene, La Jolla, CA |

### 4.1.8 The Mammalian Cell Lines

| Cell Line | ATCC No. | Medium | Source |
|---|---|---|---|
| HEK293 | CRL-1573 | DMEM/10% FBS | human embryonic kidney |
| HT-29 | HTB-38 | Mccoy's 5A/10% FBS | Human colon cancer |
| HCT-116 | CRL-247 | Mccoy's 5A/10% FBS | Human colon cancer |
| SW480 | CCL-228 | L-15/10% FBS | Human colon cancer |
| SW620 | CCL-227 | L-15/10% FBS | Human colon cancer |
| WM115 | CRL-1675 | MEM/10% FBS | Human melanoma |
| WM266 | CRL-1676 | MEM/10% FBS | Human melanoma |
| H460 | HTB-177 | RPMI 1640/10% FBS | Human lung cancer |
| H146 | HTB-173 | RPMI 1640/10% FBS | Human lung cancer |

### 4.1.9 Buffers

| | |
|---|---|
| Coomassie Blue Stain Buffer | 0.025% Coomassie Blue, 50% Methanol, 10% Acetic Acid |
| Destain Buffer | 7% Acetic Acid, 5% Methanol |
| PBS-buffer 10X | 1.5M NaCl, 30mM KCl, 15mM $KH_2PO_4$, 60mM $Na_2HPO_4$ |
| RIPA buffer, 10X | Millipore, Billerica, MA |
| SDS Running Buffer | 25mM Tris, 200mM Glycine, 0.1% SDS |
| TBE-buffer 10X | 900mM Tris-HCl, 440 mM Boric Acid, 20mM EDTA |
| TBS-buffer 10X | 200mM Tris-HCl (pH 7.5), 1.37M NaCl, |
| TE Buffer | 10mM Tris-HCl (pH 8.0), 1mM EDTA |
| Transfer Buffer | 25mM Tris, 200mM Glycine, 20% Methanol |
| MTT stock solution 10X | 5mg MTT in 1ml medium without phenol red, store in dark |
| MTT Solubilization Solution | 10% Triton X-100, 0.1N HCl in anhydrous isopropanol |

## 4.2 General Techniques

### 4.2.1 Bacterial Culture

All *E. coli* strains were used for amplification of plasmid DNA or expression of recombinant protein. Bacteria were grown in liquid LB (Lauria-Bertani) medium (1% bacto-tryptone w/v, 0.5% bacto-yeast-extract w/v and 1% NaCl w/v). For selection, the media contained ampicillin (50µg/ml) or kanamycin (50µg/ml). Agar-plates were made with LB-ampicillin or - kanamycin medium supplemented with agar (15 g/L). For long-term storage, the transformed bacteria were mixed with sterile glycerol (30% (v/v)) and stored at –80°C.

Preparation of Frozen Stocks of Competent Cells

This protocol is based on the method described by Mike Scott (Department of Neurology, UCSF). The desired strain was streaked on a LB plate and then incubated overnight at 37°C. Next day, a single colony was inoculated into 20 ml of TYM broth (2% Bacto-Tryptone, 0.5% Yeast Extract, 0.1M NaCl, 10mM $MgSO_4 \cdot 7H_2O$) in a 250 ml flask. Cells were grown to mid-log phase ($OD_{600}$ ~ 0.2-0.8) and transferred to 80ml of TYM in a 2 L flask with vigorous agitation

until reaching 0.5-0.9 $OD_{600}$. After which the culture was further diluted to 500 ml in the same vessel. When the cells were grown to $OD_{600}$ 0.6, the flask was cooled in ice-water with gentle shaking and centrifuged at 4,000 rpm for 15 minutes at 4°C. Cell pellet was then suspended in 100ml of cold TfB Ⅰ (30mM KOAc, 50mM $MnCl_2$, 100mM KCl, 10mM $CaCl_2$, 15% (v/v) glycerol) and re-spun at 4,000 rpm, 8 minutes at 4°C. Pellet was resuspended in 20ml of cold TfB Ⅱ (10mM Na•MOPS, pH7.0, 75mM $CaCl_2$, 10mM KCl, 15% glycerol) by gentle shaking on ice. Aliquot 0.1-0.5ml aliquots in pre-chilled microfuge tubes, store at -70°C.

### 4.2.2 Transformation

An aliquot of competent cells was removed from -70°C and thawed at room temperature until just melting. The cells were placed on ice, added with desired DNA and stood on ice for 30 minutes. After which, cells were incubated at 37°C for 5 minutes, diluted 1:10 in LB, and then grown for 90 minutes at 37°C. The culture was spread on a LB-agar plate with appropriate antibiotic(s) selection.

### 4.2.3 Deoxyribonucleic Acid Techniques

Preparation of Plasmid DNA

Small scale purification of DNA plasmid was prepared with QIAprep Spin Miniprep Kit by following manufacturer's instruction. Generally, 2 ml of medium supplemented with appropriate antibiotic(s) were inoculated with a single bacterial colony from a selective agar plate and then incubated for 16-18 hours by vigorous shaking at 37ºC. The cell suspension was centrifuged for 5 min at 2,500 rpm and the supernatant was removed by aspiration. The bacterial pellets were suspended completely in 250 µl of suspension buffer supplemented with RNase A. The bacterial cells were lysed by adding 250 µl of alkaline lysis buffer and the lysate was then

neutralized and adjusted to high-salt binding conditions. The protein precipitate in the lysate was removed by centrifugation and the DNA-containing sample was applied to the QIAprep column. Endonuclease and salts were efficiently removed by several washing steps. The plasmid DNA was then eluted from the column with 50µl of elution buffer or water.

Large scale purification of plasmid DNA (up to 100 µg) was prepared using the Wizard-Plus DNA isolation System. A small amount (100µl) of bacterial culture were amplified to 100ml with appropriate antibiotic(s) and incubated for 16-18 hours by vigorous shaking at 37ºC. The culture was centrifuged at 5,000 rpm for 10 min at 4ºC and the pellet was proceeded by following the manufacturer's instruction.

Quantification of Nucleic Acid Concentration

Concentration of nucleic acids was determined by a spectrophotometer in the wavelength of 260nm. One unit of optical density at 260nm ($OD_{260}$) equates approximately 50µg/ml of double-stranded DNA or 40µg/ml of single-stranded DNA and RNA. The ratio of $OD_{260}$ to $OD_{280}$ provides an estimation of the purity of the purified nucleic acid. Highly pure DNA or RNA is characterized by the ratio of 1.8 to 2.0.

Gel Electrophoresis of Nucleic Acids

Nucleic acids that differ in size were separated and analyzed by electrophoresis in agarose gel. Nucleic acids have negative charges from their phosphate backbone and thus tend to migrate toward the anode in an electric field. DNA fragments from 0.5K to 2K base pairs (bp) are usually well separated in 1% agarose gels (w/v). Smaller DNA fragments (100-500 bp) were separated in 1.5-2% agarose gels. Agarose was dissolved in 1X TBE buffer by heating up to 65°C. Ethidium bromide (10mg/ml) was then added to the gel solution with a final concentration

of 500ng/ml. The DNA fragments separated in the gel were intercalated by ethidium bromide and illuminated under the ultraviolet light. The size of each fragment was estimated using molecular weight markers.

DNA Extraction from Agarose Gel

QIAquick Gel Extraction Kit was used for extraction and purification of DNA fragments from agarose gel after electrophoresis. The silica column in the kit is designed to isolate DNA from unwanted impurities. In principle, QG buffer solubilizes a particular slice of agarose gel and optimizes the condition of DNA to bind to the silica membrane. The ethanol-containing washing buffer is then use to remove salts, agarose and dyes. Elution of DNA is more efficient under a basic and low-salt condition with 30-50µl of EB buffer or water.

Polymerase Chain Reaction (PCR)

PCR are used to characterize, analyze, and synthesize a specific piece of DNA, by exploiting the polymerases. Three major steps are involved in PCR. Firstly, the double helix strands of the target genetic material must be denatured and separated by heating to 90-96°C. Secondly, the primers designed for targeting a specific gene anneal to their complementary bases on the single-stranded DNA. Thirdly, the targeted DNA fragment is amplified by the polymerase. The major components in PCR reaction mixture include polymerase buffer, dNTP (0.2µM), DNA template (0.1-1µM), forward and reverse primers (0.1-1µM), polymerase and water. The ration of the each component was based on different manufacturer's instruction. The reaction was carried out in a thermocycler with repeated 28-32 cycles of three steps (e.g. 30 sec 94ºC, 30 sec 55ºC and 2 min 72ºC) and a final step of 10 min incubation at 72ºC to complete the partly amplified DNA fragments.

Restricted Endonuclease Digestion

Restriction endonucleases are found in bacteria and responsible for protecting host DNA by recognizing and cleaving a specific sequence of the foreign DNA invading into bacterial cells. The nature of sequence specificity of restriction enzymes makes them a potent tool in the recombinant DNA technology. In principle, small scale of DNA digestion was performed at 37ºC for 2 hours of incubation. The products were analyzed by gel electrophoresis or purified by gel extraction. For ligation purpose, large scaled digestion of up to 10 µg DNA was incubated overnight at 37ºC. The formula for the digestion was based on each manufacturer's instruction.

Ligation

Ligation is used to create a covalent bond between the 5´-phosphate and the 3´-OH of two fragments of DNA or two ends in single DNA. Generally, ligation is used to insert a gene into a vector to generate a plasmid expressing that particular gene. The enzyme used in ligation is T4 DNA ligase. This enzyme ligates DNA fragments with overhanging, cohesive ends that are complementary to each other. In the cloning process, the linearized vector was treated with phosphatase to eliminate the 5´-phosphate group in order to avoid its self-ligation. The reaction mixture generally contained 3 folds molar excess of insert to vector DNA. The ligation reaction was incubated at room temperature for 4 hours or 4°C for overnight, followed by transformation or storage at 4°C.

Cloning of pHM6-p68 with Mutations on p68 shRNA Targeting Sequence

The original clone of pHM6-p68 was accomplished by a previous member, Dr. Chunru Lin. In principle, p68 cDNA were subcloned into the mammalian expression vector, pHM6, by *Hind* III site with HA epitope at the N-terminus. The subcloned p68 was subjected to site-

directed mutagenesis on the p68 shRNA targeted site in order to express in the p68 knockdown cells. The sequence of mutagenesis primers is listed in the table along with all primer sequences used in this dissertation.

Site-directed Mutagenesis

QuikChange II site-directed mutagenesis kit was used to create mutations on DNA sequence which in turn to replace, delete or insert single or multiple amino acids. The general procedure utilizes a plasmid with a DNA fragment of interest and two synthesized complementary oligonucleotide primers containing desired mutations. The oligonucleotide primers were extended by *PfuUltra* high-fidelity (HF) DNA polymerase in a PCR reaction. Extension of the oligonucleotide primers generated a mutated plasmid containing staggered nicks. The PCR product was then treated with *Dpn* I endonuclease. *Dpn* I (target sequence: 5´-Gm6ATC-3´) was used to specifically digest the parental DNA template which has methylated modification and to retain the synthesized DNA with desired mutations. (DNA isolated from almost all *E. coli* strains is methylated and therefore susceptible to *Dpn* I digestion.) The DNA products containing the desired mutations were then transformed into XL1-Blue supercompetent cells. The correct mutations on the plasmids were verified by DNA sequencing.

### 4.2.4 RNA Isolation

Total RNA in mammalian cells or tumor and spleen tissues from xenograft tumor model was isolated by using RNeasy Mini kit (Qiagen, Valencia, CA) according to manufacturer's instruction. To isolate RNA, the samples were directly lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. The tissue lysates were passing through the

shredder column for further homogenization and cleared by centrifugation. Both tissue and cell lysates were applied to affinity column for RNA binding. RNAs were selectively bound to a silica-gel-based membrane. Contaminants were efficiently washed away by high-salt buffer. After remove extra ethanol, high-quality RNA is then eluted in 30-50 µl of DEPC-treated water. The quality of the isolated RNAs was determined by examining the 18S and 28S rRNAs via agarose gel electrophoresis. The integrity and 2:1 intensity ratio of the 28S and 18S rRNAs suggested high quality of the isolated RNAs.

### 4.2.5 Protein Techniques

Recombinant p68 Protein Purification

The method used for expression and purification of p68 RNA helicase is described in a previous report (Yang and Liu 2004b). The p68 expressing plasmid (pET-30a-p68) with 6x HIS tag at N-terminus of the open reading frame was transformed to BL21-Codon-Plus (DE3) competent cells. An individual colony was cultured in 2ml of LB media with 50µg/ml of kanamycin at 37°C overnight with vigorous shaking. On the second day, 100µl of bacterial culture was transferred to 2ml of fresh media for further incubation of 1.5hr. The protein expression was induced by adding 1mM of IPTG to culture media followed by incubation for 4 hours at 37°C. A small fraction of bacteria culture before and after IPTG induction was collected and analyzed by SDS-PAGE followed by coomassie blue staining to evaluate the induction ratio of the interested protein.

The colony with best induction ratio was selected for large scale protein purification. The bacteria culture was amplified from 200µl to 100ml of fresh LB media with appropriate antibiotic(s) and incubated overnight at 37°C with vigorous shaking. On the second day, the

bacteria culture was further amplified to 1 liter LB media with antibiotic(s) and continued to incubate until the absorbance at 600nm reached 0.8. The bacterial culture was added with 1mM of IPTG and incubated for another 4hrs. The culture was centrifuged at 5,000 rpm at 4°C for10min. After washing with PBS, bacterial pellet was either stored at -80°C or continued for protein purification.

Bacterial pellet was lysed by 10ml of lysis buffer (50mM Tris-HCl pH 7.5, 300mM NaCl, 10mM imidazole) supplemented with 1mg/ml of lysozyme and incubated for 30 min at 4°C. The lysate was sonicated for 6 x 20 s with 30 s pause to break down DNA. The lysate was centrifuged at 10,000 x g for 30 min at 4°C. The recombinant proteins carrying the 6x His tag were purified under native condition by using a gradient concentration of imidazole. The imidazole ring is part of the structure of histidine in the 6x His tag which binds to the nickel-NTA agarose resin. The clear lysate was applied to the Ni-NTA column which was pre-equilibrated by lysis buffer. The binding of endogenous proteins with histidine residues to Ni-NTA resin was prevented by adding 10-50mM of imidazole in the lysis buffer. The lysate passed through the column by gravity. The column was then washed by 4ml of wash buffer (50mM Tris-HCl pH 7.5, 300mM NaCl, 20mM imidazole) for three times. The 6x His tagged proteins were eluted by 500µl of elution buffer (50mM Tris-HCl pH 7.5, 300mM NaCl, 250mM imidazole) and analyzed by SDS-PAGE.

Quantitation of Protein Concentration

The protein amount in cellular and tissue lysates was quantitated using Bio-Rad protein assay. The solution being measured contains 2µl of the protein lysate, 798µl of distilled water and 200µl of Bio-Rad dye. Different BSA concentrations (2.0-10.0 µg/ml) were used to generate

a standard curve. The absorption at wavelength 595nm was measured using a spectrophotometer. The absorption values were converted to the concentration according to the standard curve.

Preparation of Whole Cell Lysates

Cells under the desired condition were washed twice with ice-cold PBS. The cells were lysed with RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors and scraped off from the culture dishes using a rubber policeman. The lysate was vortexed vigorously and incubated for 1 hour at 4°C. The lysate was then centrifuged at 1,2000rpm for 15 min at 4°C to remove cellular debris. The supernatant was stored at –80°C until use.

Preparation of Tissue Lysates

Frozen patient tissue specimens were obtained from University of Massachusetts Medical School Cancer Center (Worcester, MA). Tissue specimens (200 ~300mg) were weighted and diced into small pieces using a clean razor blade. Collected pieces were immersed in liquid nitrogen and disrupted in a pre-chilled 1.5ml micro-centrifuge tube on ice using an electric pestle. The disrupted tissue was lysed in 500µl of 1X RIPA buffer and further homologized by using the pestle manually for 10 strokes. The lysate was rotated for 60 min at 4°C and then centrifuged at 1,2000rpm for 15 min at 4°C to remove cellular debris. The supernatant was stored at –80°C until use.

Preparation of Nucleic Extract

Nucleic Extraction Kit was used to prepare nucleic extract according to manufacturer's instruction. Cells grown in a 100mm culture dish were collected in ice-cold PBS in the presence of phosphatase inhibitors. Then, the cells were re-suspended and incubated in hypotonic buffer for 15 min on ice to swell the cell membrane and make it fragile. Addition of the Detergent

caused leakage of the cytoplasmic proteins into the supernatant. The cytoplasmic supernatant was collected by centrifuging at 10,000 g for 30 s at 4°C. The nucleus pellet was lysed by Complete Lysis Buffer (added with 1mM DTT, protease inhibitor, and phosphatase inhibitor) and the nuclear proteins were collected and stored at -80°C until use.

SDS-PAGE

The gel apparatus was set up and the separating gel monomer (12% acrylamide, 0.375M Tris-HCl pH8.9, 0.1% SDS, 0.396M sucrose, 0.042% APS, 0.083% TEMED) was prepared. TEMED was added prior to pouring gel. The separating gel was allowed to polymerize before adding stacking gel by overlaying gently with absolute ethanol. After the separating gel has polymerized, the overlay was decanted and the stacking gel monomer (5% acrylamide, 0.125M Tris-HCl pH6.7, 0.1% SDS, 0.07% APS, 0.15% TEMED) was prepared. As the separating gel, the stacking gel was added with TEMED just before pouring. The stacking gel was inserted with comb immediately and allowed to polymerize completely before running. Samples were diluted with 5X sample buffer (175mM Tris-HCl pH7.0, 5mM EDTA, 10% SDS, 20% sucrose, 0.01% bromophenol blue, 28.8mM β-mercaptoethanol) and heated at 95 C for 5 minutes prior to loading. Then samples were loaded in gel and run at constant voltage, 50-100 V, in SDS-PAGE running buffer (50mM Tris, 380mM glycine, 0.1% SDS) for ~3 hours.

Western Blot

Protein samples were loaded and separated in a SDS polyacrylamide gel. The electrophoresed proteins in gel were transferred onto a nitrocellulose membrane (Millipore) and the membrane was incubated in blocking solution (5% skim milk/TBST (20mM Tris-HCl pH7.4, 150mM NaCl, 0.1% Tween-20)) for 1 hour at room temperature or overnight at 2-8°C. The

membrane was washed with washing buffer (TBST) for 15 minutes with 3 changes of buffer at room temperature and then incubated with primary antibodies which were diluted in blocking buffer for 2 hours at room temperature or overnight at 2-8°C. The probed membrane was washed 3 times with washing buffer for 15 minutes, 5 min for each, at room temperature and incubated with an appropriate secondary reagent such as goat anti-mouse IgG-HRP at 1:5000 dilution in blocking buffer for 1 hour at room temperature. The membrane was then washed with washing buffer for 15 minutes with 3 changes of buffer at room temperature and the signal was detected with chemiluminescence reagents.

Immunoprecipitation

Immunoprecipitation (IP) is used in a variety of applications, such as to study protein-protein interactions, to determine specific enzymatic activity, and to analyze protein post-translational modifications. An interested protein or protein complex in the cell or tissue lysate was precipitated in an appropriate IP lysis buffer to form an immune complex, which includes the antigen (protein(s)), primary antibody and Protein A- or G- or a secondary antibody-conjugated agarose beads. In principle, the lysate was diluted by 1x RIPA buffer supplemented with protease and phosphatase inhibitors to 500 µl. 1-2µg of primary antibody were added to capture specific protein. After gentle rotation overnight at 4°C, 20 µl of 50 % agarose slurry was added to the mixture for further incubation of one hour. After extensive wash, proteins captured by agarose were eluted and analyzed by SDS-PAGE and western blot.

Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) is a tool developed to study protein-DNA interactions (Solomon et al 1988Solomon et al 1988). In principle, the living cells are fixed using

formaldehyde, which cross-links and stabilizes protein-DNA interactions. The fixed cells are lysed and the lysate is subjected to sonication or enzymatic digestion to acquire small fragments of DNA mostly ranging from 200-1000bp. Then, the specific protein/DNA complexes in the lysate are immunoprecipitated using an antibody against the protein of interest. The immunoprecipitates are treated with high salt buffer for at least four hours at 65°C to reverse the cross-link. After reversing cross-link, the solution is treated with RNase A and then proteinase K to remove RNA and protein. The precipitated DNA is then amplified by a specific pair of primers to determine whether the interested protein binds to the particular DNA region.

The protocol used in here is mainly based on the manual supplied with the QuikCHIP kit (IMGENEX). SW620 cells with the proper treatment described in chapter 2 were fixed with 3.7% formaldehyde in complete culture media for 10 min at room temperature. The fixation process was stopped by 1x Glycine buffer for 5 min at room temperature. The cells were then washed with ice-cold PBS and collected by scrapping followed by centrifugation for 5 min at 4°C. The cell pellets were either frozen at -80°C or resuspended in 1ml SDS lysis buffer supplemented with 1X protease inhibitor cocktail and 1X PMSF. After incubate on ice for 10 min, the lysate was sonicated on ice at 50% amplitude with 15 cycles of 20 seconds on 30 seconds off. After sonication, the samples were centrifuged and the shearing efficiency was checked as per the manufacturer's instructions. Samples were pre-cleared with 75µl of Protein G/Salmon Sperm DNA for 1 hour. The samples were then immunoprecipitated overnight with 1-5µg of appropriate antibody or Rabbit IgG antibody in 500µl of ChIP dilution buffer (Imgenex). On the following day, 75µl of Protein G/Salmon Sperm DNA was added and the samples were incubated at 4 ºC on a rotating platform for 2 hours. The beads were washed extensively using the wash buffers contained in the kits. The protein/DNA was eluted from the

beads using 1% SDS containing 1M $NaHCO_3$. Next, the samples were treated with RNAse A and decrosslinked at 65 ºC overnight. The following day, the samples were treated with Proteinase K at 45 ºC for 1 hour. Finally, the samples were purified by Phenol/Chloroform extraction/Ethanol Purification. The purified samples were then used in PCR reactions using primers directed toward the DNA sequences of interest and the products were run on a 2% agarose gel.

### 4.2.6 Antibody Generation and Purification

Polyclonal rabbit antibody against Y593 phosphorylated-p68 (pY593-p68) was generated by immunizing rabbits with KLH conjugated peptide CNQQA[pY]AYPATA, corresponding to residues 588-599 of the p68 protein (GenBank accession number NM_004396). Blood sample was taken 2 weeks after the fourth boost, and the anti-serum was tested for specific immune reactivity followed with IgG purification.

The procedures of IgG purification are described as follows. The column was packed with protein G beads (Millipore, Billerica, MA) and washed with 10 column volumes of 100 mM Tris HCl buffer, pH8.0 (0.135M NaCl, 100 mM Tris HCl). The serum was run through the column. The column was washed with 10 column volumes of 100mM Tris HCl buffer, pH8.0, and then 10 column volumes of 10mM Tris HCl, pH 8.0. The antibody was eluted off the protein G beads with 100mM glycine, pH 2.7, and the eluted was collected into eppendorfs containing a 15% final fraction volume of 2M Tris-HCl, pH8.0. The fractions were tested by SDS-PAGE followed by Commassie blue staining. The IgG positive fractions were concentrated by Amicon columns (Millipore, Billerica, MA) and tested by Western Blot for specific immune reactivity.

### 4.2.7 Cell Proliferation Assay

Counting viable cells stained with a vital dye is a traditional method to determine cell growth. Trypan blue staining is used to determine the integrity of cell membrane and thus assume cell proliferation or death. However, this staining method is not sensitive and practical to use for high throughput screening. Radioactive labeling, usually the tritium-labeled thymidine, is accurate but time-consuming and risky of handling radioactive substances. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) method is simple, accurate, and yields reproducible results. Yellow MTT, dissolving in medium or balanced salt solutions, is reduced to insoluble purple formazan in the mitochondria of living cells. The purple crystals are dissolved in acidified isopropanol, which can be quantified by measuring at a certain wavelength (usually between 500 and 600nm) by using a spectrophotometer. This reduction can take place only when the mitochondrial dehydrogenases are active, which therefore is directly proportional to the number of viable cells. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance. The application of the MTT method does have limitations that are affected by: (1) the physiological state of cells and (2) variance in mitochondrial dehydrogenase activity in different cell types. However, it is useful in the measurement of cell growth in response to various stimuli, such as mitogens, growth factors, and cytotoxicity reagents. The procedures used here are based on the manufacturer's protocol.

Cells with the proper treatment were seeded to a 96-well tissue culture plate with an amount of $10^4$ cells per well. On the second day, the culture cells were replaced with 100µl of MTT-containing media (MTT stock (5mg/ml) in an amount equal to 10% of the culture medium volume) without phenol red. The cells were returned to the incubator for 2 hours. After the incubation period, the resulting formazan crystals were dissolved by adding 100µl of MTT

solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) and mixed well by pipetting up and down. The absorbance at a wavelength of 570 nm was measured by using a spectrophotometer. The background absorbance was eliminated by measuring the absorbance at a wavelength of 690 nm and subtracted from the 570 nm measurement. A blank containing complete medium without cells was included. Each group of cells was repeated in 8 wells for every independent experiment. The statistical result was based on three independent experiments.

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# CHAPTER 5: CONCLUSIONS

Over the past decade, p68 RNA helicase has been implicated in multiple cellular functions. It has been shown that p68 is involved in pre-mRNA splicing, alternative splicing, and ribosome biogenesis (Guil et al 2003, Jalal et al 2007, Lin et al 2005). Apart from these vital cellular processes, p68 was shown to function as a transcriptional co-regulator through interacting with various transcription factors, such as p53, HDAC1, RNA Pol II, CBP/p300, and SMAD3 (Fuller-Pace 2006). However, the molecular basis that p68 is involved in the transcriptional regulation remains largely unknown. Recently, it has been suggested that posttranslational modification of p68 by sumoylation switches this protein from a transcriptional coactivator to a corepressor, where the sumoylated p68 favors recruiting HDAC1 to the specific responsive promoter (Fuller-Pace et al 2007). Therefore, it is likely that posttranslational modification plays a key role in determining the cellular function of p68 in different contexts.

A previous study in our laboratory demonstrated that tyrosine phosphorylations of p68 are associated with cancer development and cell proliferation (Yang et al 2005a). Phosphorylation of p68 at Y593 residue was shown to promote PDGF-induced epithelial-mesenchymal transition (EMT) through increasing the stability and nuclear translocation of β-catenin (Yang et al 2006). Given that EMT has been implicated in cancer metastasis (Chaffer and Weinberg 2011) and our observation that tyrosine phosphorylation of p68 is relatively higher in metastatic cancer cell lines than their non-metastatic counterparts, we speculated that Y593-phosphoryalted p68 may promote cancer metastasis. Interestingly, in the ChIP-on-chip assay, my colleague identified that p68 interacted with the Snail1 promoter (Carter 2009). Snail1 has been implicated to induce EMT through transcriptional repression of E-cadherin in both embryonic development and cancer progression (Batlle et al 2000, Cano et al 2000). The expression of

Snail1 is also associated with cancer metastasis (Kudo-Saito et al 2009). Thus, we reasoned that phosphorylation of p68 at Y593 may promote EMT through regulating the expression of Snail1 and the consequent cancer metastasis. In this dissertation, we showed that phosphorylation of p68 at Y593 represses E-cadherin expression through transcriptional upregulation of Snail1. Insight into the molecular basis revealed that Y593-phosphorylated p68 upregulates Snail1 by displacing HDAC1 from the Snail1 promoter. We also observed a positive correlation between tyrosyl-phosphorylated p68 and colon cancer metastasis in a xenograft model. These findings may provide a new route for therapeutic purposes in cancer treatment.

## 5.1 Transcriptional Regulation of Snail1 by p68 RNA Helicase

In our study, we showed that Y593 phosphorylation of p68 RNA helicase repressed E-cadherin expression by upregulating transcription of the Snail1 gene. Transcriptional regulation is considered a major mechanism for controlling Snail1 expression during the embryonic development and tumor progression. Studies in breast cancer cells revealed that activation of estrogen receptor (ER) pathway suppresses Snail1 expression via the involvement of the nucleosome remodeling and histone deacetylation (NuRD) repressor complex. In accordance with this finding, we observed that p68 regulates Snail1 transcription through association with the MBD3:Mi-2/NuRD repressor complex. The phosphor-p68 activates transcription of the Snail1 gene by dissociating HDAC1 from the Snail1 promoter. In contrast, the expression of the non-phosphorylable p68 Y593F mutant leads to more association of HDAC1 to MBD3:Mi-2/NuRD complex on the Snail1 promoter, which results in transcriptional repression. Knockdown of p68 did not affect the assembly of the MBD3:Mi-2/NuRD complex on the Snail1 promoter; whereas, knockdown of MBD3 impaired the recruitment of p68 to the Snail1 promoter, suggesting that p68 is recruited indirectly to the Snai1l promoter by interacting with

MBD3 or other components in the Mi-2/NuRD complex. The p68 RNA helicase has been implicated in transcriptional regulation of many genes (Bates et al 2005, Endoh et al 1999a, Metivier et al 2003). However, the role of the p68 RNA helicase in transcriptional regulation is not fully understood. A recent study revealed that *Drosophila's* p68 functions as a transcriptional repressor by resetting nucleosome structure via unwinding the RNA transcripts from its DNA template (Buszczak and Spradling 2006). Therefore, it is likely that p68 can target different chromatin remodeling and/or modification complexes to fulfill its role as a transcription coactivator or corepressor in a context-dependent manner. Our studies may reveal another model for the functional role of p68 in transcriptional regulation through remodeling or rearranging the protein complex that assembles at a specific gene promoter.

How p68 regulates the dynamic composition of MBD3:Mi-2/NuRD complex on the Snail1 promoter in SW620 cells remains a question. The observation that HDAC1 dissociated from the Snail1 promoter could be due to two possibilities. (1) The unphosphorylated p68 recruited HDAC1 to the promoter. The phosphor-p68 could not function as a recruiter. (2) The phosphor-p68 may 'displace' HDAC1 from the Snail1 promoter. The unphosphorylated p68 could not function as a protein 'displacer'. However, the underlying mechanics is not known. The ATPase activity of p68 may play a role in this regulation. Firstly, previous studies in our laboratory have shown that the phosphor-p68 displays β-catenin binding-dependent ATPase activity, suggesting a possibility that phosphor-p68 may utilize protein-binding as a substrate to drive its ATPase activity and displace HDAC1 from MBD3:Mi-2/NuRD complex. However, more effort is required to verify that the phosphor-p68 can indeed displace HDAC1 from MBD3:Mi-2/NuRD complex. Secondly, p68 may use its ATPase activity to execute a function similar to other nucleosome remodeling complex such as SWI/SNF ATPases to rearrange the

chromatin structure on a specific promoter, which subsequently affects gene transcription. Another possibility is that p68 may directly affect the composition of MBD3:Mi-2/NuRD complex to regulate gene transcription. The association of phosphor- or non-phosphor-p68 may induce a different conformational change in the MBD3:Mi-2/NuRD complex, which affects the affinity of HDAC1 to this complex and leads to two opposite outcomes of gene transcription.

The MTA family, as a component of MBD3:Mi-2/NuRD complex, was shown to play a critical role in regulating the function of this complex (Fujita et al 2003, Xue et al 1998b, Zhang et al 1999a). The expression of MTA proteins is closely correlated with the ER signaling in the development of normal mammary gland and breast cancer (Manavathi et al 2007). MTA1 was originally identified in metastatic breast cancer cells and has a role in promoting invasive phenotypes in cells (Toh et al 1994). On the other hand, MTA3 protein serves as a corepressor in Mi-2/NuRD complex to repress the transcription of Snail1, which in turn suppresses the invasive growth in breast cancer cells (Fujita et al 2003). Studies in transgenic mice demonstrated that MTA3 is implicated in suppression of ductal branching in the mammary gland (Zhang et al 2006), whereas MTA1 exhibits an opposite effect with more branching phenotypes (Bagheri-Yarmand et al 2004). Given that p68 was shown to function as a transcriptional coactivator in the ER signaling (Endoh et al 1999b, Watanabe et al 2001), it is likely that phosphorylated and unphosphorylated p68 may have a role in inducing the expression of different MTA proteins, which leads to distinct effects on the function of Mi-2/NuRD complex. Although the detailed molecular mechanism remains unknown, our data provide evidence that p68 regulates gene expression by modulating the association of components in the transcriptional regulatory complex. It is not clear how p68 is recruited to this complex, yet the action of p68 on regulating

gene transcription may depend on different cellular environment and the context of specific promoters.

Whether p68 is a constitutive member of the MBD3:Mi-2/NuRD complex is unknown. P68 was not identified in the originally isolated NuRD complex (Bowen et al 2004, Tong et al 1998, Xue et al 1998a, Zhang et al 1999b). Our data showed co-immunoprecipitation of p68 with the Mi-2 and MBD3, implicating that p68 is a part of protein components of the NuRD complex. In contrast, knockdown of p68 did not affect the association of the MBD3:Mi-2/NuRD complex with Snail1 promoter, suggesting that p68 is not required for the NuRD complex to be recruited to the Snail1 promoter. This observation seems to disagree with the idea that p68 is a constitutive component of the MBD3:Mi-2/NuRD complex. A possible explanation is that p68 only interacts with a sub-population of MBD3:Mi-2/NuRD complexes. Alternatively, the association of p68 and MBD3:Mi-2NuRD complex may be context-dependent. Recently, p68 was found to interact with HDAC1 in a promoter-specific manner (Wilson et al 2004), which seems to support the concept of context dependency. Another explanation is that phosphorylated p68 RNA helicase may compete with HDAC1 to assemble to the MBD3:Mi-2/NuRD complex. The unphosphorylated p68 and Y593F mutant are unable to compete. Thus, there may be two populations of Mi-2/NuRD complexes in SW620 cells. One contains phosphor-p68 and the other contains HDAC1.

It was originally demonstrated that p68 exhibits RNA-dependent ATPase activity (Iggo and Lane 1989). Whether the ATPase activity of p68 is required for its function as a transcription co-regulator may vary in different cellular context. P68 has been shown to be involved in multiple signaling pathways and act as a transcriptional coactivator (Endoh et al 1999a, Rossow and Janknecht 2003) or corepressor (Bates et al 2005, Wilson et al 2004). A study in estrogen

receptor signaling reported that the ATPase activity of p68 is not required for its function as a transcriptional coactivator (Endoh, Maruyama et al. 1999). It was shown that p68 interacts with transcription coactivators, such as CBP/p300, along with the RNA Pol II to activate gene transcription (Rossow and Janknecht 2003). In the study of skeletal muscle differentiation, reducing the level of p68 impaired the assembly of TBP, RNA Pol II, and the ATPase SWI/SNF chromatin remodeling complex on the promoter region of MyoD gene (Caretti et al 2006). These findings suggest that p68 may serve as an adaptor to facilitate the assembly of the transcription regulatory complex on the specific promoter. In such cases, the ATPase activity of p68 may be dispensable. However, we did not observe that p68 affects the assembly of MBD3:Mi-2/NuRD complex on the Snail1 promoter. Therefore, we cannot rule out a possibility that ATPase activity of p68 plays a role in regulating the composition of MBD3:Mi-2/NuRD on the Snail1 promoter.

It is not clear whether more genes are regulated by p68 through modulating the components of the Mi-2/NuRD complex. The Mi-2/NuRD complex has been shown to play a vital role in embryonic development. It was evident that MBD3 knockout mice die during early embryogenesis due to the malfunction of the Mi-2/NuRD complex (Hendrich et al 2001). Accordingly, p68 was shown to play a vital role in early embryonic development (Stevenson et al 1998). Therefore, it is likely that p68 cooperates with MBD3:Mi-2/NuRD complex to regulate transcription of many genes that are essential for embryonic development. Our findings may extend the functional role of p68 RNA helicase to global gene regulation by altering the protein-protein interactions within a multi-protein complex.

Histone deacetylases (HDACs) are enzymes that modify chromatin structure and subsequently repress gene expression. HDACs are usually associated with a particular promoter region along with other multi-protein complexes, such as NuRD or Sin3 complex (Knoepfler and

Eisenman 1999, Narlikar et al 2002). While most studies concentrated on the mechanism by which the HDAC activity and its associated complex are recruited to a specific gene promoter (Forsberg and Bresnick 2001, Kurdistani and Grunstein 2003, Neely and Workman 2002), our studies provided a possible mechanism by which HDACs can be displaced from their associated complex with the assistance of a DEAD-box RNA helicase. Furthermore, our findings suggested a reversible mechanism that controls gene transcription by recruiting or dissociating HDACs from a specific promoter. Whether this reversible mechanism also contributes to the transcriptional regulation of other genes remains elusive. Given that tyrosine phosphorylations of p68 are closely associated with cancer development (Yang et al 2005b), we speculate that displacement of HDACs by the phosphor-p68 is a dysregulated route for tumor progression. It is likely that phosphor-p68 may regulate the transcription of a set of genes through a similar mechanism to promote cancer development.

In this dissertation, we showed that phosphor- and non-phosphor-p68 affect the association of HDAC1 to the Mi-2/NuRD complex differently on the Snail1 promoter, where phosphor-p68 forces the dissociation of HDAC1 from the Snail1 promoter, and non-phosphor-p68 recruits more HDAC1 to the Snail1 promoter. However, how different phosphorylation status of p68 leads to two opposite outcomes of HDAC1 association to the Mi-2/NuRD complex remains a question. A previous study showed that p68 can associate directly with HDAC1 to function as a transcriptional corepressor in a promoter-specific manner (Wilson et al 2004b). A later study further demonstrated that the SUMO modification of p68 on the lysine 53 (K53) is required for the interaction of p68 and HDAC1 and the subsequent transcriptional repression activity (Jacobs et al 2007). The K53R mutation of p68 (non-SUMO-modified p68) abolishes p68-HDAC1 interaction and the transcriptional repression activity. On the other hand, the

SUMO modification of p68 inhibits its function as a transcriptional coactivator. Therefore, it is evident that the posttranslational modification of p68 is important to determine the function of p68 as either a transcriptional coactivator or corepressor in cells. As we observed that the transcriptional regulation of Snail1 by p68 via the involvement of HDAC1 is p68 tyrosine phosphorylation-dependent, we speculate that tyrosine phosphorylation and sumoylation may have crosstalk to precisely regulate the function of p68 as a transcriptional coregulator. Here we provide two possibilities to explain the molecular mechanisms behind this regulation. First, the tyrosine phosphorylation of p68 may cause a conformational change within the protein structure, which inhibits the SUMO-modification of p68 and its interaction with HDAC1. The phosphor-p68 without the SUMO modification thus dissociates HDAC1 from the Mi-2/NuRD complex, suppressing the transcriptional repression activity of this complex on the Snail1 promoter. Second, the non-phosphorylated p68 may serve as a preferred substrate for SUMO E3 ligase, which generates more SUMO-modified p68. The SUMO-modified p68 associates and recruits more HDAC1 to the Mi-2/NuRD complex, thus enhancing the transcription repression on the Snail1 promoter. However, whether the phosphorylation of p68 affects its interaction with HDAC1 directly and has a role in facilitating other posttranslational modifications remain a question. Given that p68 is involved in the transcriptional regulation of many genes, it is likely that posttranslational modification is critical to determine the function of p68 as a transcriptional coregulator and explains the versatile role of p68 in cells.

## 5.2 The Role of p68 RNA Helicase in Cancer Metastasis

Most cancer related deaths are the consequence of cancer metastasis. Despite intensive studies over decades, the molecular mechanisms that control this death-leading process are still poorly defined. Previous studies in our laboratory reported that phosphorylation of p68 RNA

helicase at Y593 mediates the effects of growth factors in promoting EMT (Yang et al 2006). Also, in the first part of this dissertation, we demonstrated that phosphorylation of p68 at Y593 plays a role in upregulating the transcription of Snail1 gene, which was shown to play a key role in the induction of EMT during both embryonic development and cancer progression. Given by the recently established concept that EMT may be a critical step to initiate metastasis in epithelial carcinoma cells, we reasoned that phosphorylation of p68 at Y593 may play a role in determining the metastatic potential of cancer cells. Here, we showed that phosphorylation of p68 RNA helicase at Y593 promotes cancer metastasis in the nude mice xenograft model of SW620 human colon cancer cells. In our experiments, we found that knockdown of p68 completely abolished the metastatic potential of SW620 cells. Re-expression of wild-type p68 completely recovered the metastatic potential of the cells, whereas the re-expression of the non-phosphorylable Y593F mutant p68 did not have a significant effect on the recovery of metastasis. Furthermore, we observed that p68 became strongly phosphorylated at Y593 in tissue samples of colon cancer patients, while the phosphorylation is not or weakly detected in corresponding normal tissues, indicating that the functional role(s) of the p68 phosphorylation is physiologically relevant to colon cancer development.

In this study, our results showed that replacement of endogenous p68 with the non-phosphorylable Y593F mutant did not result in a significant difference in tumor growth rates. On the other hand, it showed a strong increase of the cell-cell attachments in the cells with the expression of p68 Y593F mutant. Consistent with this observation, we observed a decrease in cell migration and an increased expression of E-cadherin in the p68 Y593F mutant expressing cells. Thus, it is clear that phosphorylation of p68 at Y593 promotes cancer metastasis by affecting cell morphology and migration capability.

Why phosphorylated and unphosphorylated p68 play different functional roles in the cells is an interesting issue. Although we observed that p68 Y593F mutant failed to promote cancer metastasis in the xenograft tumor model, the expression of Y593F mutant successfully recovered the tumor growth compared with that of wild-type expressing tumors. Interestingly, we did not observe a significant difference of tumor growth rate between p68 Y593F mutant and wild-type expressing tumors, suggesting that the regulation of tumor growth is Y593 phosphorylation-independent. One explanation is that unphosphorylated p68 may play a dominant role in maintaining cellular processes, such as pre-mRNA splicing and ribosome biogenesis, which are critical for cell growth. Therefore, overexpression of non-phosphorylable p68 has the ability to recover cell growth. However, upon certain environmental stimulation, the tyrosine phosphorylation of p68 is enhanced and leads the function of this protein in promoting EMT. The consequence is that the phosphorylated p68 promotes cancer metastasis. Another possibility is that phosphorylated and unphosphorylated p68 may have different substrate specificities and enzymatic activities, which may target distinct sets of genes. Therefore, switching between a phosphorylated and unphosphorylated status may be a key to determine a particular role of p68 in the cells.

Immunoblot analyses of p68 and Y593 phosphorylated p68 in cultured cells and tumor tissues revealed very different results. P68 was detected at mobility around 68 kDa in SDS-PAGE by the antibodies against p68 and the phosphorylated p68 in the extracts of cultured colon cancer cells, while the majority of p68 was detected at higher molecular weight (MW) (~150 kDa) in the extracts of the tumor tissues but not the normal tissues. This high MW immunoblot band was strongly detected by using the antibody against Y593 phosphorylated p68. The observation suggests that p68 is extensively modified in tumor tissues, and the modifications are

lost in the cultured cells. One possibility is that the communications between tumor cells and stroma cells do not present in the case of cultured tumor cells. Alternatively, tumor cells in the tumor mass are grown under hypoxia conditions, while the cells are usually cultured under normoxia condition. Therefore, it is possible that the high MW modification of p68 is driven by hypoxia. Since p68 with high MW modification was detected much stronger using antibody against the Y593 phosphorylated p68, it is possible that phosphorylation facilitates the posttranslational modification. The high molecular weight modification(s) was observed earlier by Causevic and co-workers. Based on the detection of the same mobility band by antibody against ubiquitin, it was suspected that p68 is ubiquitinated in cancer tissues (Causevic et al 2001). Since this band is only observed in tumor tissues but not in cultured tumor cells, it is quite challenging to study the functional role of this high MW modification(s) of p68. Therefore, whether this high MW modification(s) of p68 plays a role in colon cancer progression remains an open question.

Cancer metastasis is a complicated process that involves multiple steps. Although it is very challenging to observe the EMT process during tumor metastasis, accumulating evidence supports that EMT is an essential step for carcinoma cells to acquire migration and invasion phenotypes. Combining the previous studies and recent findings in our laboratory, we found that phosphorylation of p68 at Y593 plays a role in mediating colon cancer metastasis. Firstly, the phosphorylation of p68 at Y593 can facilitate the nuclear translocation of β-catenin and thus promote EMT in response to growth factor stimulation (Yang et al 2006). Secondly, our data showed that phosphorylation of p68 at Y593 promotes the transcriptional activation of Snail1 through dissociating HDAC1 from Snail1 promoter. Finally, in the xenograft tumor model of human colon cancer cells, we showed that phosphorylation of p68 at Y593 is essential for caner

metastasis. Furthermore, in the analyses of tumor tissues from colon cancer patients, we observed a positive correlation between Y593 phosphorylated p68 and cancer development. These findings suggest that p68 may be used as a potential marker for diagnosis and prognosis.

In the process of treating cancer patients, several major methods are used in current clinic, including chemotherapy, radiation, and surgery. The treatment of cancer usually combines more than one method to increase the effectiveness. One of the major reasons causing the failure of cancer treatment is the metastasis during cancer development. Therefore, finding a drug that specifically targets the metastasis process will make the cancer treatment more promising. Since it has been shown that treatment of some anti-cancer drugs, including TNFα and TRAIL, reduces the tyrosine phosphorylation of p68 in cancer cells (Yang et al 2005a), it is possible that designing a drug, such as phosphorylated Y593-containing short peptides, to compete the effect of phosphor-p68 may be a way to inhibit cancer metastasis. Therefore, additional efforts are required for testing the application of the tyrosine phosphorylation of p68 as a treatment target.

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