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NM23-H1 BLOCKS CELL MOTILITY INDEPENDENTLY OF ITS KNOWN ENZYMATIC ACTIVITIES IN A COHORT OF HUMAN MELANOMA CELLS

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Abstract of Dissertation

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The Graduate School

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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Lexington, KY

Director: Dr. David M. Kaetzel,
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The metastasis suppressor gene NM23-H1 has been shown to possess three enzymatic activities including nucleoside diphosphate kinase, histidine-dependent protein kinase and 3'-5' exonuclease activity. While these properties have been demonstrated *in vitro* using recombinant proteins, the contribution of these activities to suppression of metastatic dissemination is unknown. Site-directed mutagenesis studies were used to identify amino acid residues which are required for proper function of each enzymatic activity associated with H1, providing a platform for studying the importance of each function on an individual basis. To assess the relevance of these activities to melanoma progression, a panel of mutants harboring selective lesions disrupting the enzymatic activities of H1 were overexpressed using stable transfection in two melanoma cell lines, WM793 (isolated from a vertical growth phase human melanoma), and the metastatic derivative cell line 1205LU. *In vitro* correlates of metastasis measuring motility and invasion were used in an attempt to identify the mechanism mediating H1-dependent motility suppression of cancer cells. Surprisingly, all mutants studied retained full motility suppression in this setting, suggesting that the enzymatic functions associated with H1 are not required for inhibiting cell migration. Instead, gene expression analyses conducted on the panel of stable transfectants indicate that differences in steady-state mRNA levels of genes involved in mitogen-activated protein kinase (MAPK) signaling showed significant correlations with H1 expression and motility suppression. RNAi studies have confirmed that H1-dependent modulation of the expression of two genes in particular, *BRAP* and *IQGAP2*, contribute to the observed phenotype, suggesting a novel mechanism used by NM23 to control cellular migration in human melanoma.

KEYWORDS: Melanoma, Metastasis, Motility, Invasion, Metastasis suppressor gene

J. Robert McCorkle

Student's Signature

November 4, 2010

Date

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Table of Contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

Background

Metastasis and Suppressor Genes

The process of tumorigenic transformation and subsequent malignant progression of human cells is driven by genetic and epigenetic alterations that disrupt both intracellular and extracellular homeostasis. This leads to disruption of normal cellular physiology and produces cells which no longer respond appropriately to contextual cues causing uncontrolled proliferation, culminating in the formation of a primary tumor. As the tumor grows and the population of cells rapidly expands, it is thought that a small subset of these cells acquire additional changes which provide a selective advantage by displaying increased plasticity. These cells are then equipped with an enhanced adaptive response that allows them to respond (or fail to respond) to various stimuli outside of the normal repertoire of signaling cues found in the tissue-of-origin, permitting them to thrive in otherwise foreign microenvironments. Although it is still unclear what exactly initiates the conversion of a benign growth to a malignancy, cancer cells that are able to disseminate from the primary tumor, travel and survive in the circulation or lymphatics, then colonize and proliferate at a new site are deemed metastatic. Metastasis is a complex, multi-stepped process (Figure 1.1) paramount to the pathology of cancer as it is responsible for greater than 90% of the deaths of those that succumb to malignant tumors (1). In spite of this, an unbelievably small amount of cancer research funding is dedicated to metastasis research (2). This seems counterintuitive based on the morbidity associated with metastatic disease and the accumulating evidence suggesting tumorigenesis and metastasis are distinct processes controlled by unique mechanisms (3, 4). It is known that disrupting a single step in the metastatic cascade can prevent the formation of secondary tumors (5), enhancing the number of possibilities for unique therapeutic intervention. With greater understanding, the clinical impact from this would appear to be extremely promising as disrupting a process known

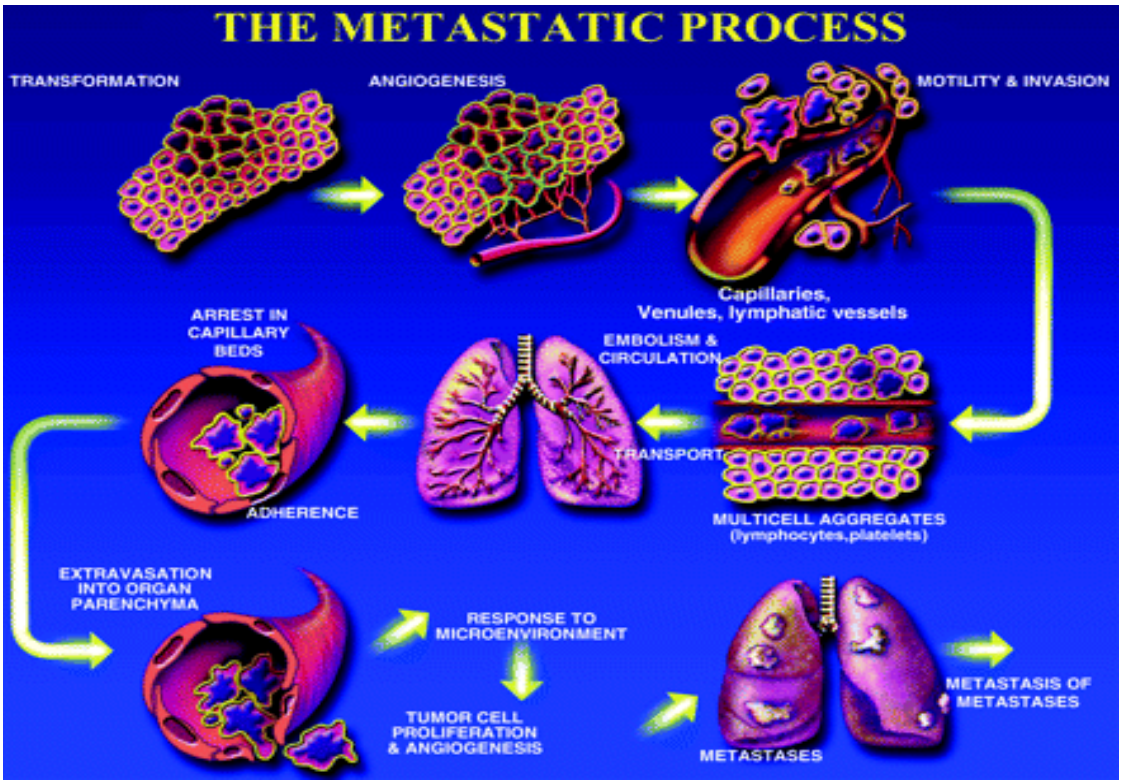

Figure 1.1 Schematic of the Necessary Steps in Metastasis (from (6)). This illustration of the highly dynamic process of metastasis reinforces the idea that a cell must be extremely plastic in order to successfully adapt to the many stresses and rapidly changing microenvironments it will encounter during the transition.

to specifically drive metastatic dissemination almost certainly would have a much greater impact on patient survival than current treatments.

Distinguishing features of metastasis versus tumorigenesis have become more abundant over the past 20 years as more than twenty members of a class of genes known as metastasis suppressors have been identified to date (7). A metastasis suppressor gene (MSG) is defined as an inhibitor of metastatic dissemination that has little or no apparent impact on growth of the primary tumor. Examples of MSG's include KISS1 (8), RhoGDI2 (9), Caspase-8 (10), MKK-4,-6 &-7 (11-13), RKIP (14) and BRMS1 (15). Most data investigating the function of these genes in metastatic contexts suggest that their products have roles in highly conserved eukaryotic signaling cascades and may actually restore signaling programs that mimic that of their normal or benign cellular counterparts (15). The biological processes associated with the MSG's have led them to be separated into 4 classes based on the type of intracellular network that they are associated with: cytoskeletal, mitogenic, stress-activated or survival signaling. These activities commonly impact a cell's motility, morphology, growth and evasion of apoptosis, all traits highly correlated with cancer and metastasis. The wide variety of physiological functions attributed to this class of genes again underscores the disease's complexity, yet highlights a uniquely attractive means of treating metastasis. MSGs have been identified based on consistent patterns of reduced expression in highly metastatic cancers, suggesting negative selective pressure for these genes in the most aggressive cells. Therefore, the exploitation of these genes for use as therapeutics has generated a great deal of interest, as it is thought stimulation of MSG expression by chemical means or via gene therapy may hold promise for preventing advanced disease.

NM23 Gene Family

The first described metastasis suppressor gene was NM23-H1 (H1) which was initially identified in models of murine melanoma and human breast cancer as having significantly reduced expression in highly metastatic clones as compared to the congenic non-metastatic counterparts (16). Since then,

overexpression of H1 has been shown to reduce the metastatic potential of cancer cells derived from melanoma (17-19), breast (20, 21), colon (22), ovarian (23) and oral squamous cell carcinomas (24). In agreement with these studies, *nm23*- knockout mice exhibited more pulmonary metastases in a model of hepatocellular carcinoma while showing no significant impact on the formation of the primary tumor (25). Although it has been more than two decades since the original designation of NM23-H1 as a metastasis suppressor, the mechanism of inhibition has remained elusive in spite of a wealth of experimental data characterizing the molecule in a wide variety of contexts.

The NM23 family of proteins is encoded by 10 genes in humans which are commonly designated –H1 to –H9 (gene symbol: NME1-9), along with the recently added RP2 gene (NME10). The unifying feature of this family is their evolutionarily conserved nucleoside diphosphate kinase (ndpk) protein domain, an archain peptide sequence also found in many prokaryotes. Surprisingly, only isoforms H1-H4, which function as hexamers, & H6 actually exhibit ndpk activity (26). The NM23 gene family is involved in a number of cellular processes such as differentiation, development, apoptosis and metastasis suppression. The H1 isoform along with the highly homologous H2 have been studied most extensively in a variety of contexts and have been shown to consistently block metastasis. Experimental characterization of H1in particular has elucidated *in vitro* biochemical activities in addition to ndpk, including a 3'-5' exonuclease activity and a histidine-dependent protein kinase function. The contribution of each activity to H1-mediated metastasis suppression at this point is unclear.

Nucleoside Diphosphate Kinase

The ndpk function generates nucleoside triphosphates (NTP) from nucleoside diphosphates at the expense of other NTPs, usually adenosine-triphosphates (ATP). This process is catalyzed by the ndpk-dependent transfer of a high-energy γ-phosphate from one nucleotide to the other. This terminal phosphate binds to an ndpk such as NM23, forming a phosphor-enzyme

intermediate through a “ping-pong” type of transfer mechanism (27). A simplified schematic is presented below:

$$N_1DP + N_2TP \xleftrightarrow{\text{ndpk}} N_1TP + N_2DP$$

Biologically, this was initially thought to be a nonessential “housekeeping” function present to promote balance among nucleotide pools in various cellular compartments. However, it has become evident that H1 has diverse influences on cellular development and homeostasis, some of which may indeed rely more on ndpk than originally appreciated. For example, several studies have been conducted to specifically examine the contribution of the ndpk activity to the metastasis suppressor function of H1 (28-31). Although these reports showed ndpk activity to be unnecessary for suppression, full investigation into the contribution of this activity with *in vivo* models of spontaneous metastatic tumor growth were not completed until recently and the resulting data would suggest otherwise (32). Furthermore, evidence from various experimental systems has shown the importance for this function in regulating metabolic processes in both the cytosol and in mitochondria, as well as for the generation of NTPs necessary for synthesis of nucleic acids (33). The contribution of the lattermost function on the efficiency of DNA replication and repair is illustrated by an acquired mutator phenotype observed in *E. coli* lacking *ndk*, the bacterial homolog of NM23 (34). It is thought that this gene family may have similar roles in eukaryotic systems as well (35).

Histidine-dependent Protein Kinase

The description of the histidine-dependent protein kinase activity of H1 first appeared in the literature in 1995 when H1-dependent phosphorylation of a histidine residue in the ATP-citrate lyase gene product was reported (36). A number of other substrates have since been identified including porcine succinyl thiokinase (37), kinase suppressor of ras (KSR) (38) and aldolase C (39). The

mechanism of action is similar to ndpk with the formation of a high-energy phosphorylated intermediate but transfer of the terminal phosphate is directed toward protein substrates instead of nucleoside diphosphates. So far, the best case for an inherent function mediating the metastasis suppressor function of NM23 has been made for the histidine kinase activity as it has been reported to mediate motility suppression and anchorage-dependent growth in a number of cancer cell types (40-42). This effect appears to be the result of H1-dependent phosphorylation of KSR, a scaffolding molecule important for propagation of MAP kinase signals through Raf and MEK. From the large collection of data implicating the MAPK cascade in tumor progression, quite a bit of interest is now focused on H1-mediated suppression of Raf signaling via KSR, and its role in metastasis.

3'-5' Exonuclease

Our lab was the first to describe the third endogenous activity of NM23-H1, a 3'-5' exonuclease function which was shown to preferentially remove overhanging or mismatched bases at the 3' end of deoxyribonucleotide oligomers (43). This finding has generated considerable excitement as 3'-5' exonucleases commonly have roles in DNA repair processes, the disruption of which has been repeatedly linked to tumorigenesis (44, 45). In addition, this activity could play significant roles in metastatic disease by helping preserve genomic integrity. Highly metastatic cells have elevated spontaneous genomic mutation rates when compared to less metastatic counterparts (46), supporting the notion that the exonuclease activity of H1 could be a contributing factor to suppressor function. The ability of the H1 isoform to cleave DNA was not a complete surprise. It had previously been shown that the highly homologous NM23-H2 possessed nuclease activity, targeting nuclease hyper-sensitive elements of the *c-myc* promoter (47) *in vivo* and *in vitro*, in both linear and super-coiled plasmid forms (48). Upon further characterization of the nucleolytic activity of H1, it appeared that the H1-dependent cleavage of DNA exhibited marked differences from that of H2. The H2 molecule consistently generates internal cuts of double stranded

DNA indicative of an endonuclease, while H1 catalyzed the removal of individual nucleotides progressively from the 3' terminus of single-stranded oligodeoxynucleotides in the 5' direction (43, 49). Additional evidence of nuclease activity has been reported for the *E. coli* homolog *ndk* (50), along with a previously undescribed role in genomic maintenance (34), suggesting evolutionary conservation for this function in addition to ndpk, and underscoring the importance of this activity. The contribution of the nuclease function to H1-mediated metastasis suppression is still unclear, however, recent studies have begun to shed light on the necessity for this activity, as well as ndpk, in supporting NM23-dependent genomic integrity and metastasis inhibition (32, 35).

Identification of the multiple biochemical activities of H1 has provided valuable mechanistic insight into this NM23 isoform and its roles within the cell, expanding the realm of possibilities for mechanisms of H1-dependent metastasis suppression. The advent of high-throughput global proteome and gene expression analyses should facilitate discovery, as perhaps the most promise for finally understanding how H1 may mediate metastasis suppression will arise from the identification of gene products with which NM23 demonstrates a physical interaction and/or regulates their expression. Over the last decade, H1 has been shown to bind a variety of proteins, a number of which are components of complex intracellular signaling networks. Some examples are Rad (51), Tiam1 (52), EBNA-3C (53), KSR (38), CDC42 (54), Dbl-1 (55) and Prune (56). The physiological effects of these interactions are variable ranging from regulation of transcription to endocytosis, as well as modulation of MAPK signaling. Should H1 contribute to maintenance of proper signal transduction, the loss of expression could very easily lead to profound biological consequences. Tight regulation of signaling networks is crucial for cellular homeostasis and the disruption of critical protein complexes and networks used to transmit information resulting from genetic mutations, amplifications, etc., can have significant deleterious effects on the cell. In fact, several key genetic components of broad signaling networks are synonymous with cancer and metastasis including Ras,

AKT, and Wnt. The subsequent deregulation of these gene products is commonly found to be the driving force in tumorigenesis and progression.

The MAPK pathway in particular has been a functional target of the H1 molecule according to reports of interactions with gene products Rad, Tiam1 and KSR. MAP kinases have multiple roles in growth, differentiation and survival while also promoting cancer progression in many instances (57-59). Significant contributions are thought to be made by MAPK pathway components to not only proliferation, but also proteolytic degradation of extracellular matrix, invasion, motility and metastasis. Three main branches of MAPK signaling are thought to exist mediated through extracellular signal-regulated kinase 1/2 (ERK 1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 (60). These networks have been extensively characterized and play specific roles within the cell, mediated by chains of different signaling isoforms and scaffolding molecules to direct information-rich phosphorylation of conserved tyrosine and threonine residues. While usually depicted as linear networks, it is known that a large amount of cross-talk occurs within these pathways, increasing complexity but also specificity of signals to generate the desired cellular response (Figure 1.2).

With reports of Ras/MAPK-mediated stimulation of cell motility (61, 62), it should be noted that migration was found to be commonly impacted following the interaction of H1 with a number of these different proteins. This suggests that H1 is incorporated with signaling mechanisms that perhaps underlie the phenotypic response, *e.g.*, suppressing migration and/or invasion. It is understood that these signaling pathways typically induce some level of differential gene expression as the endpoint response of the cascade. As a result, microarray analyses following H1 overexpression in a number of different systems have also been utilized as a tool for investigating how NM23 is able to suppress metastasis, identifying a myriad of genes which may contribute to H1-mediated suppression (63-67). For example, discovery of H1-dependent reductions to transcript levels for the lysophosphatidic acid (LPA) receptor, EDG2, in metastatic breast cancer cells demonstrates yet another level of influence H1 exhibits upon the conduction

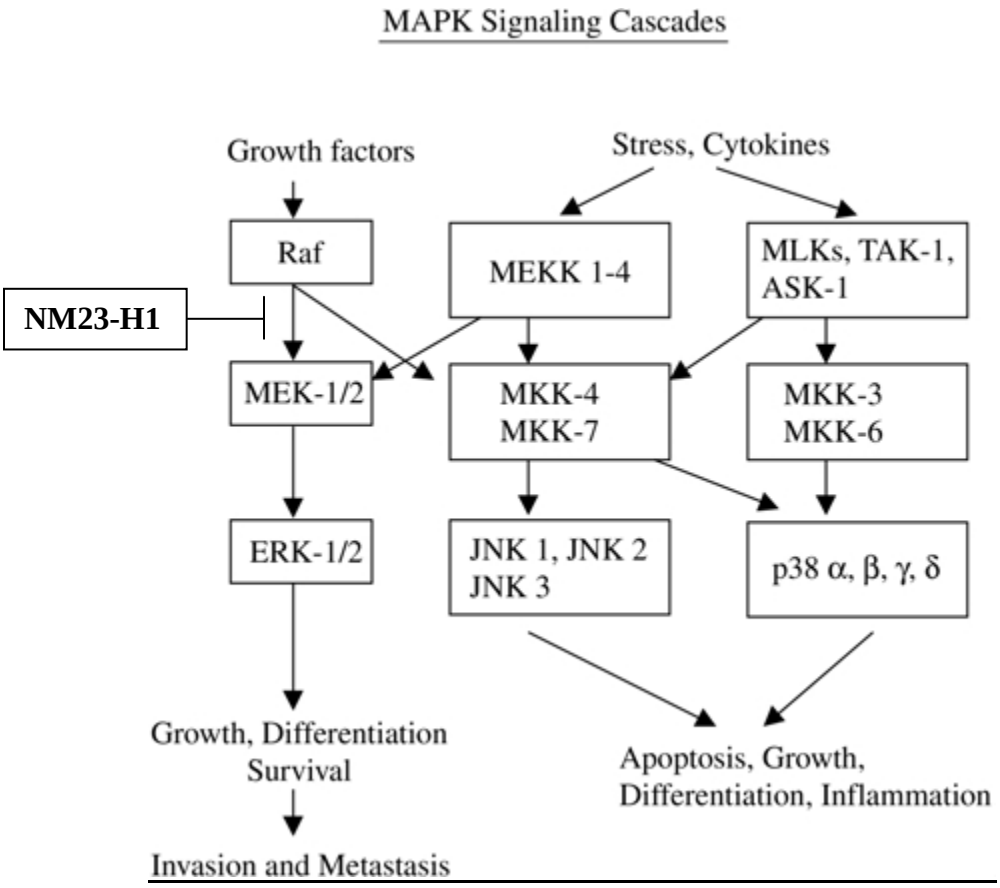

Figure 1.2 Mitogen Activated Protein Kinase (MAPK) Signaling in Mammals (adapted from (60)). The three main branches of the MAPK cascade are depicted and expected phenotypic outcomes displayed. The majority of information has linked H1 to the network propagated through the Raf molecule (far left) and as noted, this arm of the pathway has been implicated in metastasis. Therefore, the Raf-MEK-ERK pathway seems to be an important target for induction of H1-mediated motility and metastasis suppression.

of critical cellular signaling events (64). Collectively, this data would suggest that H1 has a much more global impact on the signaling repertoire than perhaps initially appreciated and this regulation may contribute significantly to the negative regulation of metastasis.

In Vitro Correlates of Metastasis

The clinical promise of targeting metastasis is based on the principle that preventing just one aspect of the cascade can render a cell incapable of successful metastasis, suggesting a multitude of novel approaches could be effective. Achievement has proven difficult however, as treatments aimed specifically at the metastatic process have shown little clinical success (68), encouraging the need for further molecular research. It is generally accepted that *in vivo* models must be used to adequately study metastasis but this approach can be costly, time-consuming, and make it difficult to interpret due to a lack of mechanistic information. However, improvements have been made with recent advancements to *in vivo* imaging techniques, as a recent report has shown the use of laser-scanning microscopy is making it possible to monitor cancer cell dissemination within a live organism (69). While far superior to equipment of the past, access to facilities with such capabilities is just now becoming realistic options for many researchers. Such drawbacks, along with the relative ease in which cell culture assays are conducted, has prompted the use of *in vitro* systems to study the many aspects of metastatic spread. Generally, *in vitro* assays have been designed to recapitulate the individual steps or components of the cascade. For example, common indicators of metastatic potential *in vitro* are motility, invasiveness and ability to colonize in soft agar. Though many techniques are available to assess these traits, there is not an *in vitro* cell culture model that can accurately mimic all of the environmental factors relevant *in vivo* at once. Nonetheless, when used to complement animal studies, these types of assays can be extremely informative but still cannot stand alone as comprehensive.

The use of such culture systems to study the cellular response to H1 has indicated that the enzyme influences a number of aspects of cell fate and behavior, any of which could contribute to the suppression of metastasis. H1-dependent effects on motility/invasion inhibition (70, 71), anchorage-independent growth (29, 31), angiogenesis (72), and promotion of differentiation (73) or cell death (74-76) are common, although for the most part, concise descriptions for mechanisms of action have been lacking. Descriptions of molecular events that lead to a particular phenotype could greatly benefit from the identification of the inherent enzymatic functions necessary for mediating H1-dependent effects. Site-directed mutagenesis experiments have effectively linked the histidine kinase function of H1 to motility suppression and inhibition of metastasis in breast carcinoma cell lines (37, 42), as previously mentioned due to its interaction with the kinase suppressor of ras (KSR). The sites of phosphorylation on KSR were identified as serine residues 392 and 434 which alter the scaffolding properties of KSR and consequently disrupt Ras-initiated MEK signal propagation and cellular motility in MDA-MB-435 and 293T cells (38, 77). Further investigation using site-directed mutagenesis should continue to facilitate the elucidation of the individual activities of H1 and assess the impact of each one in a variety of cellular contexts.

Although mutagenesis has proven useful in dissecting its enzymatic activities, naturally occurring mutations in the H1 gene product are rare. While it is quite common to find genetic or epigenetic mutations of tumor suppressor genes driving oncogenesis (*e.g.*, p53, PTEN, RB, etc.), loss of NM23-H1 generally results from reduced expression and not from inactivating mutations (17). Spontaneous mutations to the NM23-H1 gene in humans have only been found in several cases of high-grade neuroblastoma, where Ser_{120} is replaced with a glycine residue and overexpression is positively correlated with aggressiveness of the disease (78). This mutated gene product is characterized by abnormal structure due to improper folding and fails to confer motility suppression and prevent soft agar colonization in MDA-MB-435 cells *in vitro* when compared to the wild-type molecule (37, 79). Another naturally-occurring

amino acid substitution identified is a serine substitution of the Pro_{97} residue in the *awd* gene, the homolog of NM23 in *Drosophila melanogaster*, was shown to be a conditional dominant mutation known as *Killer of prune* (K^{pn}) (80). Unlike $S_{120}G$, this mutation was not initially linked to cancer and was originally shown to impair development in fruit flies (80-82). Interestingly, both $S_{120}G$ and $P_{96}S$ are reportedly unable to confer metastasis suppression to malignant breast carcinoma cells due to the loss of histidine kinase activity (36, 41). As a result, quite a bit of interest has been shown in these mutants resulting in extensive characterization of their impact on cell biology in a number of models (40, 83-86). Nonetheless, structure-function analyses of a variety of specific mutants have identified individual residues necessary for the multiple activities of the H1 molecule (Table 1.1), allowing for partial mapping of respective active sites and the study of each function in isolation. It has been shown that both kinase activities require the catalytic His_{118} residue (32, 87), with residues Lys_{12}, Tyr_{52}, Arg_{88}, Pro_{96} and Asn_{115} also being reported to facilitate ndpk activity (43, 88, 89). The histidine kinase function is dependent upon Lys_{12} and Pro_{96} as well, along with Ser_{120} (37), while the 3'-5' exonuclease necessitates residues Lys_{12} and Glu_5 (43, 90). A $Q_{17}N$ mutant was also characterized for the H2 isoform where it disrupted the nuclease activity without negatively affecting ndpk (88). While these investigations have uncovered quite a bit about the molecule, complete characterization of all three activities in any mutant prior to the report herein have been lacking.

In addition to *in vitro* characterization of recombinant proteins, forced expression of the wild-type (WT) and mutant forms of NM23 in metastatic cell lines devoid of endogenous H1 expression has provided important evidence into how these functions may relate to H1-mediated metastasis suppression. As mentioned, the strongest case made to date for a mechanism of H1-dependent antimetastatic function is via motility suppression in aggressive cell lines. Without question, the ability of a tumor cell to invade and move through the stroma is a necessity for metastatic spread, specifically for intravasation and extravasation, two rate limiting steps in metastasis. While NM23 is unequivocally linked to

Table 1.1 Amino Acid Residues Reportedly Necessary for Appropriate Enzymatic Function of H1

	Nucleoside diphosphate kinase (ndpk)	Histidine-dependent protein kinase	3'-5' exonuclease
Amino acid residues	K_{12} Y_{52} R_{88} N_{115} H_{118}	K_{12} P_{96} H_{118} S_{120}	E_5 K_{12} F_{33} D_{54} D_{121} E_{129}

motility suppression, various mechanisms have been proposed, from its transcriptional regulation of EDG2 (64), to a direct interaction and regulation of the cytoskeletal machinery (*e.g.* Cdc42) (54) making it difficult to accept a single mechanism as the pathway through which H1 is acting. Forced expression of wild-type and mutant forms of H1 continues to be an attractive model for gathering mechanistic information concerning H1 biology and should help resolve some of the confusion surrounding its actions.

Melanoma Progression and NM23

One of the main goals of this study was to better understand the role of H1 in tumor progression. In solid tumors, this is a daunting task as it is difficult to identify the relatively few aggressive cells within a heterogeneous primary lesion that are programmed for clonal expansion and metastasis. However, in the context of melanoma, development is typically thought to follow a step-wise progression with distinct phenotypic changes associated with increasing levels of aggressiveness (Figure 1.3). Most melanomas arise from a benign nevus following some type of genetic insult (e.g., UV irradiation) that eventually progresses to a radial growth phase (RGP) melanoma. RGP melanoma is classified as a lesion that spreads superficially, confined within the epidermis, outward from the center of the growth and showing no tendency for rapid growth or metastatic ability (91). Events not completely understood then trigger a phenotypic shift which permits the cancer to grow vertically down into the dermis and subcutaneous tissue (vertical growth phase; VGP), creating a route for highly aggressive tumor cells to breach blood or lymphatic vessels and spread throughout the body enabling the progression to metastasis.

In cancer, accumulating genetic or epigenetic mutations to key oncogenes or tumor suppressors drives tumorigenic transformation and a few specific aberrancies have been linked specifically to melanoma. Examples include amplification of genes such as *KIT*, *MITF*, *TBX2* and *MYC* or activating mutations in others as seen with *BRAF*, *NRAS* and β-catenin (92). Mounting evidence is suggesting that many of these anomalies are impacting proliferative, senescence

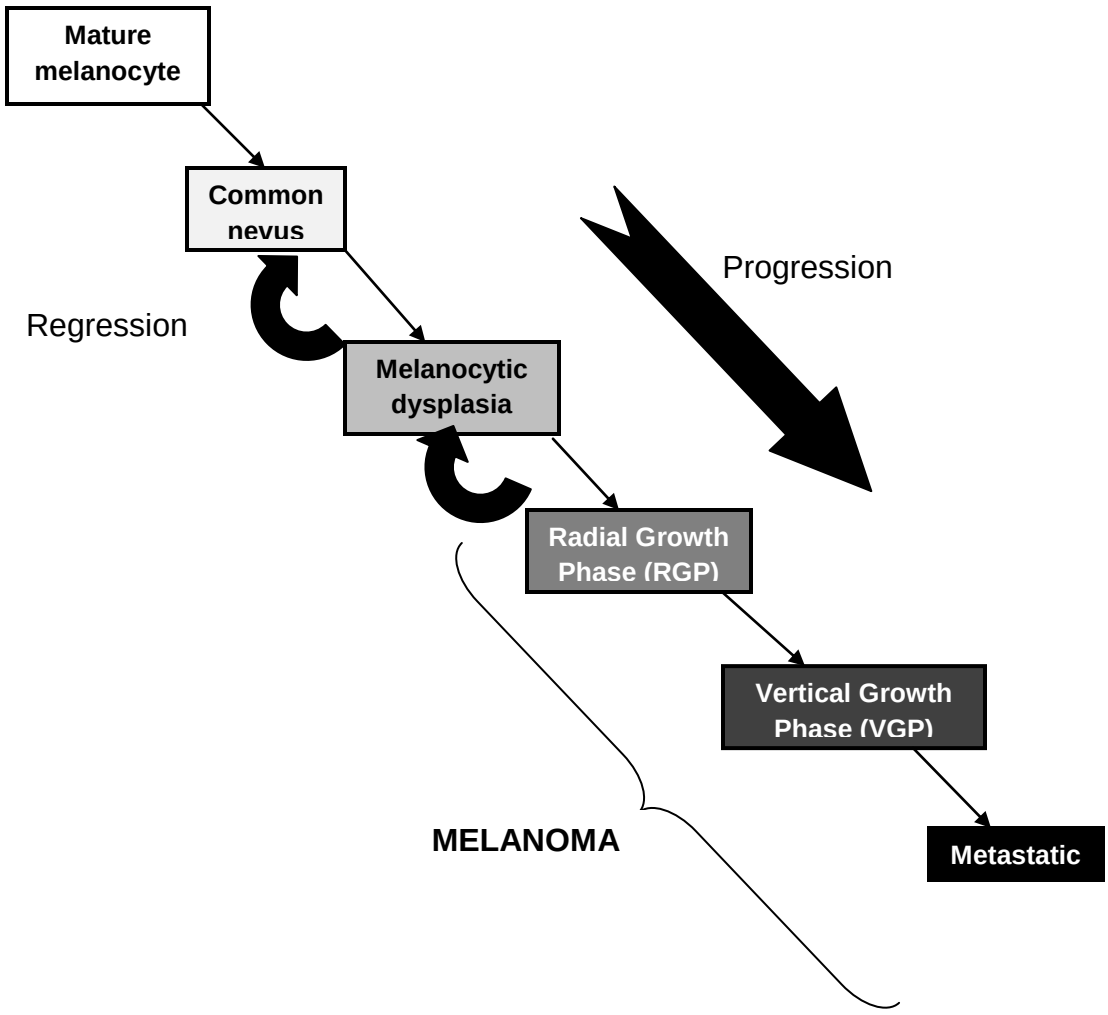

Figure 1.3 Development and Progression of Melanoma. Based on a model proposed by Clark, Elder and Guerry (93, 94), the schematic depicts the sequential steps of melanoma progression. The driving forces behind the transition from one phase to another have not been completely elucidated. Each group is histopathologically distinct with the radial growth phase representing the first recognizable malignant stage. Evidence of spontaneous regression exists for non-tumorigenic lesions albeit through unknown mechanisms.

or cell death pathways in melanoma, conferring survival advantages to the tumor cells and rapidly expanding the likelihood of clonal outgrowth of metastases. It is obvious that certain groups of these genes must be altered independently of one another as many genes are not found to be mutated within the same lesion, while others may be promoting cancer progression. For example, activating mutations to *NRAS* and *BRAF* both play significant roles in the pathogenesis of melanoma however, these lesions have never been observed concurrently in melanoma indicating that this combination has a negative effect on clonal selection (95). While the exact reason for this is unknown, it has been suggested that cells harboring this combination of mutations could be overstimulated, causing catastrophic effects to the cells (92). Other combinations of genetic mutations actually facilitate the disease likely by disabling mechanisms which act as inherent safeguards against oncogenesis, as seen with the matched cohort of melanoma cells used in the studies presented herein. While both VGP WM793 melanoma cells and metastatically derived 1205LU cells harbor an activating mutation to the *BRAF* gene, only the 1205LU cells have acquired inactivating mutations to the tumor suppressor *PTEN*, showing this gene most likely has a role in preventing malignant progression in this system. Additional evidence to support this concept has been shown using normal melanocytes engineered to overexpress *BRAF,* which senesce when grown in culture due to overstimulation of the MAPK cascade (96, 97). Therefore, propagation of a melanoma depends on the selection of cancerous cells that respond to hyperproliferative signals and are able to overcome cell death or senescence, appearing refractory to endogenous safeguards used to maintain normal cellular homeostasis.

Although the NM23-H1 gene has been shown to consistently suppress metastasis in melanoma cells, a clear picture of the way in which it is achieving this effect has yet to emerge. Understanding the events which lead to the formation of a melanoma and ultimately metastasis has helped focus new hypotheses on the most likely areas being impacted by H1. As our interest has shifted to its role within the primary tumor, the biology outlined above would raise the possibility that H1 is somehow helping to reconstruct or maintain a means of

protection within the system that would prevent the expansion of the most aberrant and aggressive cells. However, other specific aspects of suppression have been generally accepted as the significant contributing factor to H1-mediated prevention of metastasis, the most popular of which are reductions in cell motility and anchorage-independent growth. A problem arises though as the extent of interdependence between motility, invasion and contact-inhibited growth with other processes known to prevent melanoma progression, such as genomic instability, has yet to be elucidated. The conundrum then becomes determining how H1 is driving these effects, by individually blocking aspects of motility, invasion or growth in soft agar, or perhaps by controlling a host of factors or even a genetic program that culminates in the observed phenotype. Recent evidence would suggest that the impact of H1 on key signaling pathways controlling a host of biological outcomes is a plausible scenario for mediating these effects. As a result, the conclusions drawn from some models of NM23-dependent metastasis suppression generally accepted within the field may be oversimplified and misleading.

Hypothesis

The multiple enzymatic functions of NM23-H1 individually influence the expression of specific genetic programs culminating in decreased motility, invasion and metastatic potential of melanoma cells.

Project Objective

As we have come to discover the existence of multiple properties inherent to H1, identifying the contributions of each activity in suppressing metastasis has become the focus of a number of studies including many of our own. The focus of my dissertation research has been on the discovery of non-functional mutants of the H1 molecule and characterizing their biological effects in a cohort of melanoma cells. The first part of the study was composed of structure-function analyses of recombinant H1 proteins, identifying amino acid residues necessary for mediating each of the three known enzymatic activities of NM23. This was

followed by investigation of stable cell lines overexpressing mutant and wild-type H1, characterizing their relative capacities for motility and invasion in cell culture systems. Gene expression array analysis was also used in hopes of identifying H1-regulated gene targets responsible for the observed phenotypes. With the identification of novel genes that were regulated by H1 and tracked with motility suppression in this system, the use of RNA interference allowed for the manipulation of target genes in order to assess the contribution of differential gene expression patterns on cell motility. Ultimately, two genes in particular, *BRAP* and *IQGAP2*, were found to mediate the motile phenotype observed in WM793, VGP melanoma cells. These two genes have been linked to the oncogenic Ras signaling cascade, bringing to light a potential novel role for H1 in regulating this pathway. In addition, these findings stongly implicate H1-dependent manipulation of gene expression patterns as a means of conferring motility suppression and, more than likely NM23-mediated metastasis suppression as well.

CHAPTER TWO

NM23-H1-mediated Suppression of Motility and Invasion in Metastatic Melanoma Cells Acts Independently of its Known Enzymatic Activities[1]

Introduction

The identification of the multiple functions of H1 was achieved mainly through the demonstration of these activities *in vitro*. The use of site-directed mutagenesis was a fundamental component of these discoveries as recombinant proteins were expressed and purified from *E. coli* in order to examine the effects of specific amino acid substitutions on normal function. Prior to starting the current study, our initial characterization of mutations disrupting kinase and exonuclease activity (Lys_{12}) originally described for the H2 isoform (89), while confirming the requirement of His_{118} for proper kinase function (43), had been reported (30). The goal was then to identify a selective lesion that hindered exonuclease activity in addition to fully characterizing all mutants of interest in the context of the three known functions, a feat yet to be completed by any other labs to this point.

A report published in 2005 was the first to implicate amino acid residues E_5 and D_{54} as being required for H1-mediated exonuclease activity (90). To validate that these selective lesions disrupt this function exclusively, the E_5A and $D_{54}A$ substitution mutants were tested for their ability to cleave a single-stranded DNA substrate. While recombinant preparations of the H1 mutant $D_{54}A$ showed nuclease activity similar to the wild-type enzyme (data not shown), the E_5A mutant showed >85% reduction in the amount of substrate degraded. Completion of the functional analysis confirmed that the E_5A mutation exhibited the desired phenotype as this particular mutation was disruptive to exonuclease function only while having little impact on the kinase activities. This was a highly sought-after, missing piece of a model which would be used to delineate the

[1] Results published previously: Q Zhang*, **JR McCorkle***, et al. *Int J Can*, 2010
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importance of the individual enzymatic activities of H1 on its ability to suppress metastasis, providing the basis for all subsequent experiments discussed herein.

Testing the biological effects of this collection of specific H1 mutants in a cellular context was achieved using a metastatic melanoma cell line, 1205LU. Stable transfectants of NM23-H1 wild-type (WT) and mutant variants were compared based on *in vitro* motility and invasive capacity as well as proliferation rates. While H1-WT clearly suppresses migration and invasion in this aggressive cell type, an inherent enzymatic function of H1 responsible for mediating this phenotype, including the histidine kinase function, could not be conclusively established. This raises the possibility that H1 is blocking cellular movement through a novel pathway in metastatic melanoma and not via its histidine kinase activity as has been reported (40, 41). Additionally, the kinase functions of H1 were not able to be separated by any of the mutations tested. With His_{118} being the phosphorylated residue in both kinase reactions, it would appear that a mutation impacting this residue should also cause loss of motility suppressive capacity if mediated by a histidine kinase mechanism. However, similar to the observed results in this system using 1205LU cells, $H_{118}F$ conferred full suppression of invasion on cells derived from human prostate carcinoma (31). Further investigation into any link between a histidine kinase function and motility or metastasis suppression requires increased attention in the future to determine if indeed this activity is important for blocking cell motility and/or invasion.

Materials and Methods

Site-directed Mutagenesis and Expression Vectors: Mutant constructs for variants of NM23-H1 were produced as described either through the use of overlap extension modifications of the polymerase chain reaction (98) ($P_{96}S$) or the QuikChange site-directed mutagenesis kit (E_5A) (Stratagene, La Jolla, CA). *E. coli* expression vector pET3c (New England Biolabs, Ipswich, MA) was used for generation of recombinant proteins and were created with the insertion of mutant cDNA molecules between *NdeI* and *BamHI* restriction sites. H1-wild-type and $H_{118}F$ plasmids were kind gifts of Dr. E. Postel (U. of Medicine and Dentistry of New Jersey, New Brunswick, NJ); $K_{12}Q$ construction was previously described (43). Mammalian expression vectors were produced in a similar manner, inserting cDNAs into pCI-EGFP plasmids driven by a cytomegalovirus (CMV)-promoter and containing an internal ribosome entry sequence (IRES) for coexpression of enhanced green fluorescent protein (EGFP). The pSV2*neo* (Clontech, Mountain View, CA) vector facilitated the selection of stable transfectants by conferring resistance to Geneticin (neomycin analog, G418; Clontech).

Size-exclusion HPLC and circular dichroism analyses: Purified wild-type or mutant forms of recombinant NM23-H1 were analyzed using the Shodex gel-filtration KW-800 high-performance liquid chromatography (HPLC) column (Showa Denko, New York, NY), pre-equilibrated in 50 mM Tris-HCl pH 7.5, 0.1 M KCl. Molecular weight estimates were generated using gel filtration molecular weight standard kit ranging from 12 - 200 kDa (Sigma, St. Louis, MO). Circular dichroism analyses were conducted as previously described (43) using a Jasco-810 spectrometer. Secondary structure estimates were derived from the 250-190 nm region of the recorded spectra using CONTILL, SELCON3, and CDSSTR applications (99) found in the CDPro Software package (http://lamar.colostate.edu/~sreeram/CDPro).

Nucleoside diphosphate kinase (ndpk) assays: Ndpk activity was measured as described (27) and adapted for use in a 96-well plate reader (43). Wild-type and mutant recombinant H1 was tested for its ability to generate ADP in the presence of ATP and dTDP in a coupled pyruvate-lactate dehydrogenase assay. Purified H1 was added to the bottom of the wells in 10 μL aliquots (in 20 mM Hepes pH 7.9) followed by the addition of a 140 μL reaction mixture resulting in final concentrations of 0.42 mM β-NADH, 1 mM phosphoenolpyruvate, 5 mM ATP, 2.5 mM dTDP, 6 mM $MgCl_2$, 50 mM KCl, 100 U each of pyruvate kinase and lactate dehydrogenase. Oxidation of NADH was measured at 340 nm every 15 seconds over a 3 minute time period. Specific activity is expressed as units/mg of protein with 1 unit defined as the amount of enzyme necessary to convert 1 μmol of ADP/minute at room temperature ($\varepsilon 340$ of NADH = 6.22 x 10^3 M^{-1} cm^{-1}).

Histidine-dependent protein kinase assays: Histidine kinase activity was measured as originally described (37) with slight modifications (32). [γ-^{32}P] ATP (3000 Ci/mmol; Perkin Elmer, Waltham, MA) was diluted with unlabeled 10 mM ATP lithium salt to a specific activity of 50 Ci/mmol. 20 μg of purified H1 was incubated with 500 μCi [γ-^{32}P] ATP for 15 minutes at room temperature to allow for autophosphorylation. Reactions took place in 100 μl of 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). Samples were cleared with centrifugal filtration at 4^o C to remove unbound ATP. Autophosphorylated H1 (2 x 10^5 cpm) was combined with a 5-fold molar excess of purified recombinant NM23-H2 in a 30 μL reaction mixture of 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. Reactions were stopped using room-temperature SDS sample buffer followed by electrophoresis on 13% SDS-PAGE gels. Gels were dried under vacuum at 80^o C followed by visualization with phosphorimaging using a STORM840 (Amersham Biosciences, Piscataway, NJ). Pixel intensities were measured with ImageQuant software (Amersham Biosciences).

3'-5' exonuclease assays: Activity was measured using a 33-base oligodeoxyribonucleotide substrate derived from a sequence found within the non-coding strand of the 5'SHS silencer region of the platelet-derived growth factor-alpha (PDGF-A) gene (100), as previously described (49). Radiolabeling of the 5'-termini was conducted with [γ-^{32}P]ATP and T4 polynucleotide kinase (Roche, Nutley, NJ) and efficiency of labeling was determined using non-denaturing polyacrylamide gel electrophoresis. Purified radiolabeled substrates (10-20 fmols) were incubated with purified recombinant H1 (0.5 μg) at room temperature in a 15 μl mixture containing 20 mM HEPES buffer (pH 7.9), 2 mM $MgCl_2$, and 100 mM KCl. Samples were resolved on 20% sequencing gels and visualized with phosphorimaging (STORM840, Amersham Biosciences).

Cell lines, culture & stable transfection: The metastatic melanoma cell line, 1205LU was a generous gift of Dr. M. Herlyn (Wistar Institute, Philadelphia, PA). Cells were maintained under 5% CO_2, 37^o C in TU 2% growth medium composed of MCDB153: Leibovitz-15 (4: 1; v: v) (Sigma, St. Louis, MO: Gibco, Carlsbad, CA) with 2 mM $CaCl_2$ (Fisher Scientific, Pittsburgh, PA), 5 μg/mL insulin (Sigma, St. Louis) and 2% fetal bovine serum (FBS) (Gibco). Stable transfectants were generated previously by Dr. Q. Zhang (University of Chicago, Chicago, IL) in our lab as described (32). Briefly, pCI-EGFP based expression vectors along with a pSV2-*neo* plasmid (both kind gifts of Dr. S. Kraner, U. of Kentucky) were co-transfected at a ratio of 1:0.3 μg, delivered using Fugene 6 liposomal reagent (Roche, Nutley, NJ). Forty-eight hours following transfection, cells were exposed to growth media supplemented with 250 μg/mL of G418 for 3 weeks. Cells surviving the selection period were then subjected to fluorescence-activated cell sorting (FACS; FACSCalibur Flow Cytometer, Becton-Dickinson, Mountain View, CA) to obtain a mixed population of stable transfectants.

Cell Proliferation Assays: 1205LU parent and stable transfected cell lines were seeded 1x10^4 cells/well in 96-well plates and grown under normal growth conditions. Relative growth rates were assessed every 24 hours for 5 days using

CellTiter 96 AQ_{ueous} Non-radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI) according to the supplied protocol. Each cell line was measured in at least 5 replicate wells per experiment and results reflect a minimum of 3 independent experiments.

Transwell Cell Motility and Invasion Assays: Cell motility was monitored with Transwell chambers (24-well, 8.0 μm pore) (Corning, Lowell, MA) while cell invasion was determined with BioCoat Matrigel Invasion Chambers (24-well, 8.0 μm pore) (BD Biosciences, San Jose, CA). Rehydration of the membranes for invasion studies was achieved according to protocol in protein-free TU (insulin- and FBS-free) melanoma cell culture media for 2 hours at 5% CO_2, 37^o C. For both motility and invasion assays, 1205LU cells were then seeded 1x10^4 in the upper chamber in TU growth media supplemented with 0.1% FBS and 0.25 μg/mL insulin. The lower chamber was filled with normal TU 2% culture media (2% FBS and 5 μg/mL insulin) and cells were kept for 20-22 hours at 5% CO_2, 37^o C. Cells remaining in the upper compartment were then washed twice with phosphate-buffered saline (PBS, pH 7.4) and non-motile/invading cells were removed using a cotton swab. Cells attached to the underside of the membrane facing the lower chamber were fixed for 10 minutes at room temperature in 100% methanol, followed by three washes in PBS and staining for 1 hour in Gill's hematoxylin #2 (Vector Laboratories, Burlingame, CA). Membranes were extensively washed then allowed to dry overnight at room temperature. Stained cells were counted in 5 non-overlapping, random fields per membrane using light microscopy (10 x 10 magnification). Results are from at least 3 experiments with a minimum of 3 replicate wells each.

Immunoblot analysis: Whole cell lysates were prepared from near-confluent cultures using a modified RIPA lysis buffer (10 mM sodium phosphate, pH 7.2; 150 mM sodium chloride; 0.1% sodium dodecyl sulfate; 1% sodium deoxycholate; 1% Triton X-100; 50 mM sodium fluoride; 2 mM EDTA, pH 8.0; with freshly added sodium vanadate (0.2 mM), and 1 μg/mL each of pepstatin A

and leupeptin). Quantitation of protein concentration was achieved with Bradford microplate assays (Bio-Rad, Richmond, CA) measuring 620 nm absorbance and using γ-globulin for generation of a standard curve. Fifty micrograms of extract was separated on 15% SDS-PAGE gels under reducing conditions, followed by transfer to nitrocellulose membranes (0.4 μm pore size; Bio-Rad) in the presence of 20% methanol. Primary and secondary antibody incubations occurred for 1 hour at room-temperature. Species specific secondary antibodies used were conjugated with horseradish-peroxidase (GE Biosciences, Piscataway, NJ) and signals were generated following incubation for 5 minutes in ECL Plus Western Blot Detection Reagent (GE Biosciences) and detected with X-ray film (Eastman Kodak Co., Rochester, NY). Primary antibodies used included a mouse monoclonal anti-NM23 (BD Biosciences, San Jose, CA) and anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA).

Results

In vitro Characterization of Wild-type and Mutant Variants of H1

As with the previous report describing mutants $H_{118}F$ and $K_{12}Q$ (43), recombinant H1 molecules, in wild-type and mutant form, were bacterially expressed and purified to near homogeneity. Structural defects were then assessed using circular dichroism (CD) spectrometry and size-exclusion HPLC gel filtration, enabling the identification of amino acid residues directly necessary for proper enzymatic activity and ruling out disruption of secondary or oligomeric conformation as cause for the loss of function. CD analysis showed molar ellipticities similar to the wild-type molecule suggesting that the amino acid substitutions E_5A, $Y_{52}A$ and $P_{96}S$ did not significantly alter the secondary structure of H1 (Figure 2.1). On the other hand, the $Q_{17}N$ mutant did in fact exhibit some significantly different secondary structures, specifically, a reduction in α-helix content. In addition, proper oligomerization was confirmed using gel filtration HPLC showing molecular weight estimates of mutants and wild-type H1 were not statistically different from one another, except for the $Y_{52}A$ variant (Table 2.1). The anomalies in structural properties of the $Y_{52}A$ and $Q_{17}N$ mutants prompted the discontinuation of their use in further downstream studies to investigate their effects in cancer cells based on the inability to conclusively link function to amino acid residues. Nonetheless, these results confirmed successful targeting of amino acid residues by site-directed mutagenesis which do not significantly disrupt protein stability and are possibly mediating the observed physiology of the molecule.

While $P_{96}S$ and E_5A had already been shown to impair histidine kinase and 3'-5' exonuclease function respectively (37, 90), the complete characterization of these two mutants, as well as $H_{118}F$ and $K_{12}Q$, in respect to all three activities had yet to be demonstrated. Assessment of histidine kinase function revealed a necessity for residues H_{118}, K_{12}, Y_{52} and to a lesser extent P_{96}, with the E_5A and $Q_{17}N$ mutants displaying relatively normal activity (Figure 2.2). Interestingly, a very similar result was observed for the ndpk functions and the impact of the amino acid substitutions disrupting this function mirrored that of

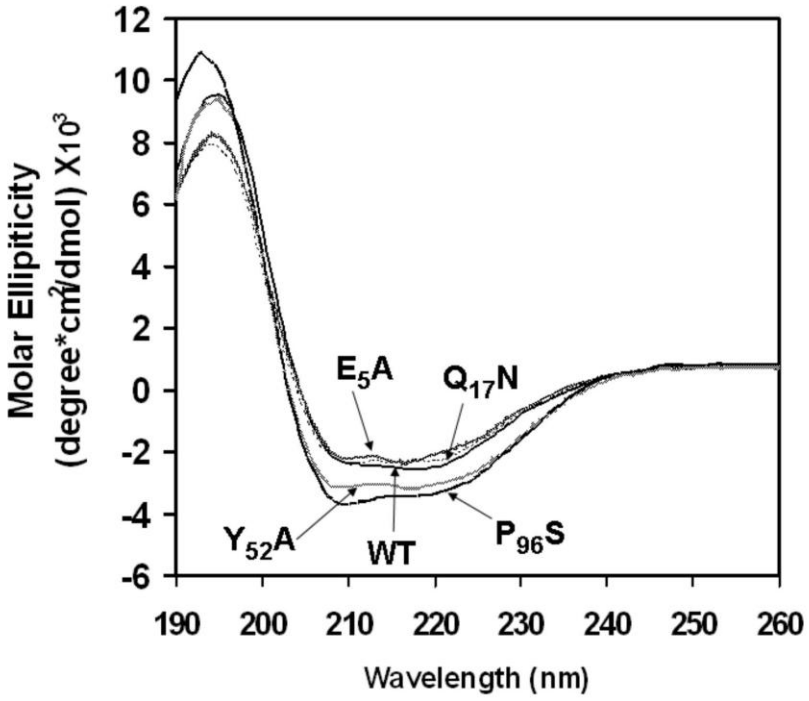


Figure 2.1 Circular Dichroism Spectrum for NM23-H1 Variants. Circular dichroism spectrometry was used to compare secondary structure of wild-type and mutated recombinant H1. Bacterially expressed (*E. coli*) recombinant preparations of wild-type, E_5A, $Q_{17}N$, $Y_{52}A$ and $P_{96}S$ were purified to homogeneity prior to analysis. Mutants $K_{12}Q$, $R_{34}A$ and $H_{118}F$ were tested and described in a previous study (43).

Table 2.1 Molecular Weight and Secondary Structure Estimates of NM23-H1 Variants[1]

Protein	Molecular Weight (kDa)[2]	CD spectrometry[3]			
		α-helix	β-sheet	Turns	Random
WT	87.7 ± 2.1	14.2 ± 2.8	32.6 ± 5.3	20.0 ± 0.1	32.6 ± 3.8
E_5A	85.1 ± 7.1	10.7 ± 2.2	35.0 ± 2.5	19.4 ± 0.6	32.9 ± 1.8
$Q_{17}N$	86.3 ± 1.5	8.8 ± 2.6	40.2 ± 4.3	18.7 ± 2.0	32.3 ± 0.7
$Y_{52}A$	78.4 ± 3.0*	11.4 ± 4.1	35.9 ± 1.9	19.4 ± 1.4	30.7 ± 1.1
$P_{96}S$	84.7 ± 0.4	13.7 ± 1.7	33.0 ± 1.2	19.8 ± 1.3	32.7 ± 2.0

[1]Estimates for mutants $K_{12}Q$ and $H_{118}F$ published previously (43). [2]Results are expressed as means ± standard deviation from 3 replicate measurements on 3 independent protein preparations. [3]Secondary structures are expressed as a percentage of the total structure content (Mean ± standard deviation). *Mean value is significantly different (One-way ANOVA, $p \leq 0.05$).

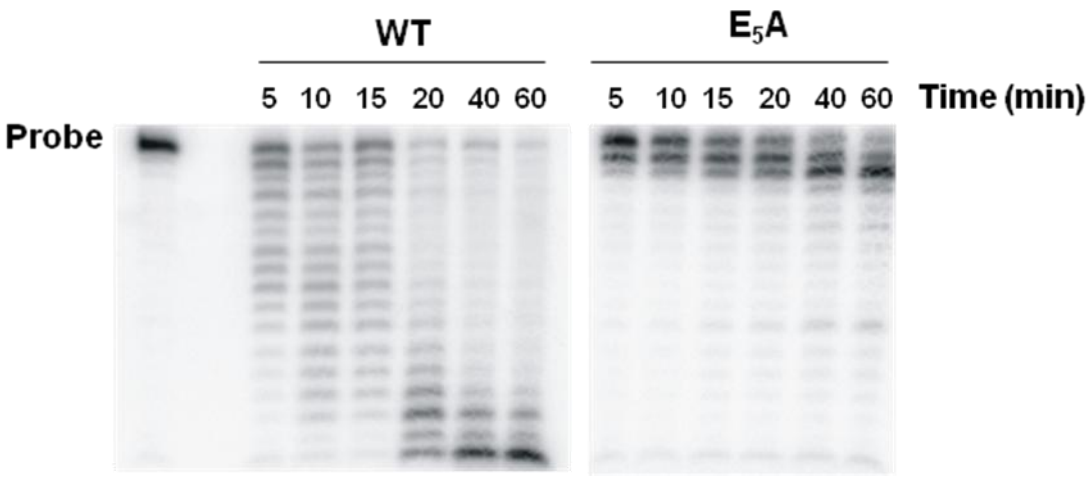


Figure 2.2 Characterization of Exonuclease Activity for the E_5A Mutant. Purified preparations of recombinant NM23-H1 (500 ng) were incubated at room temperature with 5'-radiolabeled single-stranded DNA oligonucleotide substrate (10 fmol) for the indicated times. Cleavage products were resolved on 20% polyacrylamide sequencing gels, dried under vacuum and visualized by phosphorimaging. Results for mutants $K_{12}Q$, $H_{118}F$ and $P_{96}S$ were published previously (32, 43).

the histidine kinase activity (Table 2.2). With respect to the exonuclease, loss of function was observed with the E_5A and $K_{12}Q$ mutants while $P_{96}S$ and $H_{118}F$ displayed no statistical difference from wild-type in regards to nucleolytic activity (Figure 2.3b; Table 2.2). This allowed us to separate the mutants into groups based on enzymatic aberrancies. These include mutants selectively deficient in exonuclease activity (E_5A), tandemly-deficient in both kinase activities ($P_{96}S$, $H_{118}F$) and completely devoid of all three functions ($K_{12}Q$). This panel of mutants permits the examination of the contribution(s) of the components of the enzymatic repertoire of H1, individually and collectively, on metastasis-relevant cellular physiology and ultimately, suppression of disease progression.

NM23-H1 Overexpression in 1205LU Metastatic Melanoma Cells

Stable transfectants of the human melanoma cell line 1205LU were generated for the wild-type form of H1, along with mutants E_5A, $K_{12}Q$, $P_{96}S$ and $H_{118}F$. These cells are metastatic in athymic nude mice (101) and display low endogenous expression of NM23-H1 and H2. Stable transfectants overexpressing NM23-H1 were genereated and subjected to antibiotic selection and flow-cytometry to isolate co-expressing EGFP-positive cells. Transfectants were then pooled to generate the working populations of stable cell lines used for downstream analyses.

As expected with a metastasis suppressor protein, elevated levels of H1 had no bearing on the *in vitro* proliferation rates of these cells (Figure 2.4), an observation that was also seen when grown as tumor explants in athymic nude mice using the same cell lines (32). Wild-type H1 overexpression has consistently reduced motility and invasion in a number of metastatic cell lines. While not surprising, the data presented here show 1205LU cells also exhibit similar reductions upon forced expression of H1, a novel finding for this particular line (Figure 2.5). The impact of the mutations of interest in blocking motility and invasion in these cells however was completely unexpected as all mutant cell lines showed full suppression in this system. This is in conflict with previous reports suggesting that the $P_{96}S$ mutant lacks the ability to impede motility and invasion in cell lines derived from metastatic breast carcinoma and large cell lung

Table 2.2 Molecular Functions of NM23-H1 variants

Protein	NDPK[1]	Histidine kinase[2]	3'-5' Exonuclease[3]
Wild-type	627 ± 36[a] (100)	9.9 (100)	28.4 ± 4.0 (100)
E_5A	438 ± 31[b] (70)	8.2 (83)	3.4 ± 1.5* (12)
$K_{12}Q$	14 ± 0.9[c] (2)	0.9 (9)	5.2 ± 3.5* (18)
$Q_{17}N$	516 ±10[b] (82)	6.5 (66)	31.4 ± 6.0 (110)
$Y_{52}A$	B.D.	0.4 (4)	25.3 ± 3.9 (89)
$P_{96}S$	127 ± 5[d] (20)	1.1 (11)	24.9 ± 5.1 (88)
$H_{118}F$	B.D.	B.D.	33.0 ± 9.0 (116)

B.D.: below detection (< 5 units/mg). [1]NDPK activity is expressed as units/mg (mean ± standard error) derived from at least 3 replicate measurements from 3 independent protein preparations. Percentage of wild-type activity is shown in parentheses. [2]Histidine kinase activity is expressed as the percent conversion of a NM23-H2 substrate to phosphorylated form per minute. [3]Exonuclease activity is shown as fmol of nucleotides removed from a ^{32}P-radiolabeled substrate per 5 minutes (mean ± s.e.) as determined from replicate measurements from at least 3 independent protein preparations. *Means within a column are significantly different ($p \leq 0.05$) as determined by student's t-test. [a-e]Means not bearing a common superscript are significantly different ($p \leq 0.01$) as determined with the student's t-test.

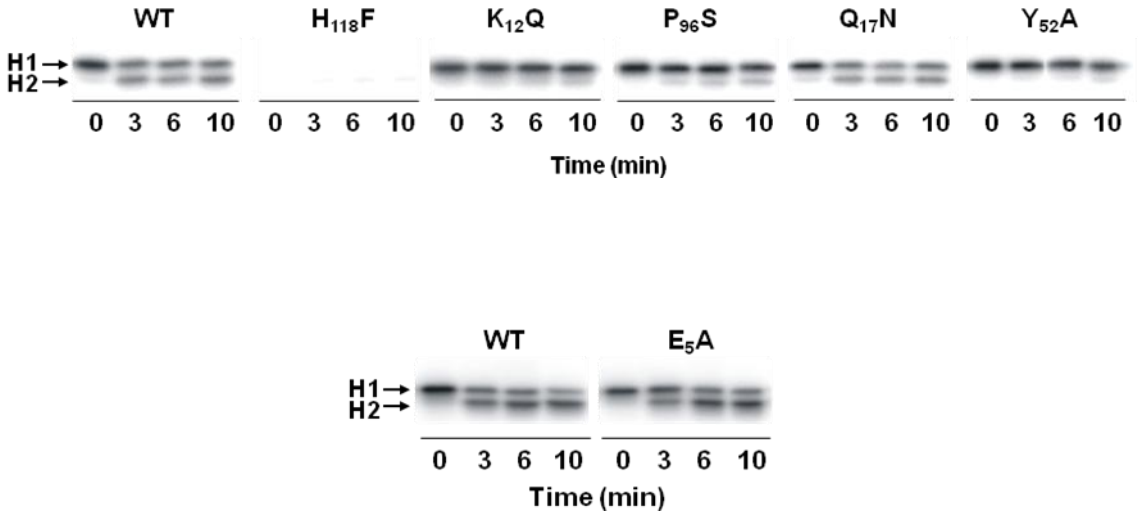


Figure 2.3 Analysis of Histidine Kinase Activity Among H1 Variants. Recombinant NM23-H1 proteins were autoradiolabeled with [γ-^{32}P] ATP, followed by removal of unbound ATP using centrifugal filtration. Labeled H1 was incubated in the presence of 5-fold molar excess of unlabeled recombinant NM23-H2 for the times indicated. Reaction products were resolved using SDS-PAGE and images acquired with autoradiography.

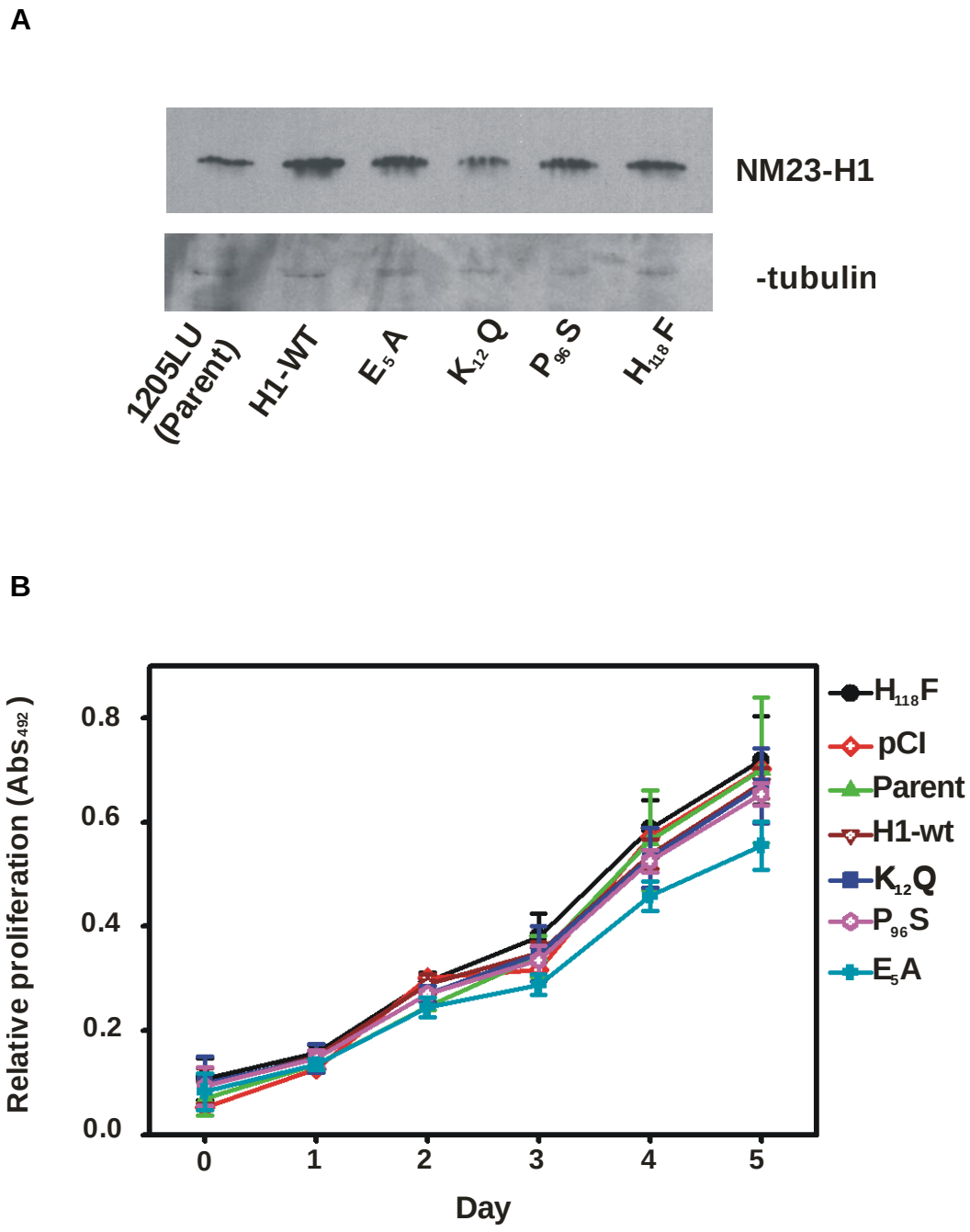


Figure 2.4 Cell Proliferation Rates of 1205LU Stable Transfectants *in vitro*. (A) 1205LU cells transfected with NM23-H1 expression vectors maintain elevated protein levels relative to the parent cell as determined by western blot. (B) Cells were grown under normal conditions and proliferation was assessed using MTS reagent. Data showed no significant difference in growth rate among stable transfectants and control cells.

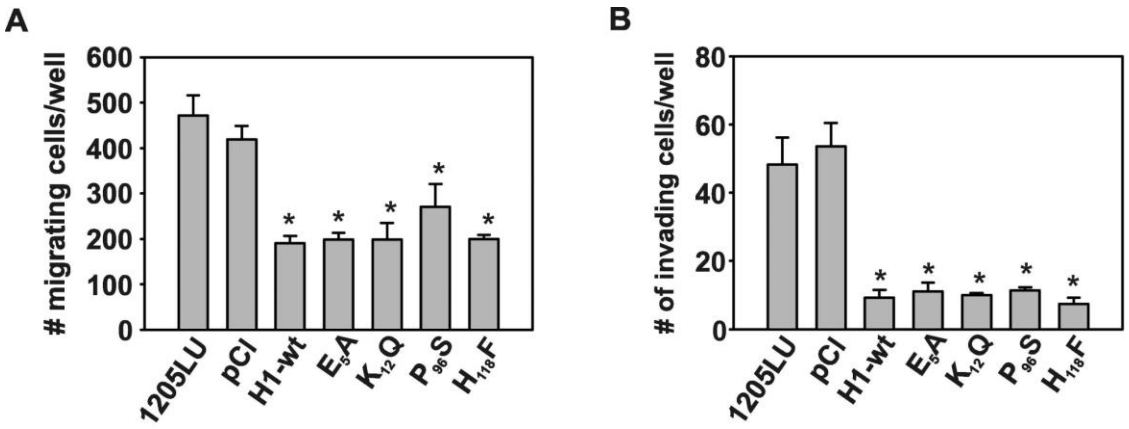


Figure 2.5 NM23-H1 Mediated Inhibition of Motility and Invasion in 1205LU Cells Acts Independently of Its Three Known Enzymatic Functions. Evaluation of the (A) motility and (B) invasive capacities of the 1205LU panel of cell lines in response to FBS using Boyden chambers. Asterisks indicate statistical significance ($p < 0.05$) among treatments based on their respective mean values as determined by one-way ANOVA ($p < 0.001$) and the Holm-Sidak method for all pairwise comparisons.

cancer. Therefore, these results contradict the notion that histidine kinase activity mediates H1-dependent motility suppression, possibly representing an undiscovered mechanism in melanoma by which H1 reduces the locomotor activity of cancer cells. If indeed the enzymatic functions of H1 are not needed for motility suppression in this setting as this data would suggest, it strongly implicates an alternate explanation such as kinase-independent physical interactions with other proteins and/or transcriptional regulation of motility-associated genes as the means for achieving the observed phenotype.

Conclusions

The results presented here show for the first time the complete characterization of a number of non-functional mutants of recombinant NM23-H1. The identification of this panel of mutant variants has provided a means of classifying each in terms of their impact on kinase or nuclease functions, furthering our understanding of the biology of the H1 molecule. While our results would predict that each amino acid residue targeted for substitution is the source of the associated enzymatic activity, the reports concerning instability of $P_{96}S$ cannot be ignored (79, 80, 102). As a result, it is possible that our methods were not sensitive enough to observe a difference in protein structure, leading to an erroneous conclusion concerning $P_{96}S$, which could lead to impairment of H1 function strictly due to anomalies in secondary structure. However, the studies referenced tested stability of the molecules following treatment with denaturants before examining oligomerization. This could explain the failure to observe any differences in $P_{96}S$ structure here as experiments were conducted so as to maintain recombinant H1 in its proper orientation.

While the structure-function analysis was extremely informative, perhaps the most important finding from this study came with the comparison of the motility suppression data to results from *in vivo* experimental and spontaneous metastasis assays conducted in nude mice with the same panel of transfectant cells. Carried out in the lab by colleagues Marian Novak and Qingbei Zhang, the analysis of the metastatic potential of these cells showed the enzymatic activities

of H1 to be necessary for blocking spontaneous metastasis, with exonuclease-deficient mutant cell lines (E_5A & $K_{12}Q$) showing complete loss of suppression and the $H_{118}F$ mutant having an intermediate phenotype (32). This is in stark contrast to the full motility suppression observed in 1205LU cells for all mutants tested when compared to the wild-type molecule. The lack of correlation between migration and metastasis suppression suggests that *in vitro* motility and invasion assays are not the best indicators of metastatic potential, as H1-dependent motility suppression almost certainly contributes to its metastasis suppressor function. Additional underappreciated roles for H1 within the cell influencing metastatic potential, such as maintenance of genomic stability via 3'-5' exonuclease activity, may still exist. More investigation is needed to better understand what contribution H1-dependent motility suppression has on metastasis as it is commonly accepted that impeding cell migration is likely the sole mechanism for generating the observed phenotype. In this particular 1205LU model system, however, metastasis suppression lacks clear correlation with *in vitro* motility and invasion assays, emphasizing the inadequacy of such *in vitro* systems when comprehensively studying the metastatic process.

CHAPTER THREE

Identification of Novel Genes Mediating NM23-H1 Dependent Motility Suppression in WM793 Melanoma Cells

Introduction

Our recent report concerning the effects of increasing H1 expression in the melanoma cell line 1205LU has shed new light on the necessity of the individual functions of H1 in mediating metastasis suppression (32). Specifically, this study showed that H1 was unable to block lung colonization when transfectant cell lines overexpressing the NM23 isoform were introduced directly into the tail-vein of nude mice. Observations in the lab did show however that H1 was able to suppress metastatic spread in nude mouse models of spontaneous metastasis. Spontaneous metastasis assays require the formation of a primary tumor and intravasation instead of direct introduction into the circulation, encapsulating even more of the metastatic process and as such, are a more accurate model for studying the multiple steps necessary for malignant spread. These results would suggest that H1 is acting on cells within the primary tumor, preventing the outgrowth and dissemination of a population of malignant cells. This makes sense in the context of the 1205LU *in vitro* motility and invasion studies as metastatic cells would need some motile capacity to metastasize. Thus, high levels of H1 expression would obviously put these cells at a serious disadvantage for successful spread. The problem is that the data showed a lack of correlation between the *in vitro* and *in vivo* studies suggesting that H1-dependent suppression of motility and invasion was not sufficient for blocking metastasis. So if there is more to the story than just blocking cellular invasion and motility, what else is H1 doing to prevent metastasis? Numerous possibilities exist however analyzing every one directly would not be feasible. Knowing that most physiological processes will impact or are controlled by gene expression programs, the most comprehensive and logical approach to such a broad question is a high-throughput screen such as a gene expression microarray.

This rationale led to the development of a gene chip analysis assessing the impact of elevating otherwise low nm23-H1 expression levels in cells derived from a vertical-growth phase (VGP) human melanoma, in hopes of gaining insight into the types of processes H1 uses to mediate metastasis suppression.

The cell line that was chosen for the differential expression analysis was WM793, a VGP melanoma cell type from which the 1205LU cells were derived (103). These cells are tumorigenic and have exhibited the ability to colonize the lungs of nude mice following tail-vein injection, a process that was blocked by H1 overexpression (personal communication, M. Novak). Melanoma cells derived from the vertical growth phase are thought to possess the capability to metastasize and have a high degree of similarity to cells of corresponding distant metastases (93). Furthermore, with 1205LU cells being established directly from WM793 cells and nearly identical patterns of DNA fingerprinting being reported (104), it was thought that many of the genes altered by H1-overexpression in these VGP cells were likely to be impacted in a similar manner in the metastatic derivative cell lines. Therefore, the aim of this particular set of experiments was to identify genes differentially expressed between the WM793 control cells and H1-transfectants, then, look for common H1-dependent expression patterns that are present in the 1205LU panel which may be influencing metastatic potency.

Materials and Methods

Cell lines and culture: Vertical-growth phase (VGP) WM793 and metastatic WM1158 melanoma cell lines were a generous gift of Dr. M. Herlyn (Wistar Institute, Philadelphia, PA). WRO82 thyroid adenocarcinoma cells were kindly provided by the late Dr. Stephen G. Zimmer (U. of Kentucky, Lexington, KY). The melanoma cell lines were maintained at 5% CO_2, 37^o C in TU 2% growth medium composed of MCDB153: Leibovitz-15 (4:1; v:v) (Sigma, St. Louis, MO: Gibco, Carlsbad, CA) with 2 mM $CaCl_2$ (Fisher Scientific, Pittsburgh, PA), 5 μg/mL insulin (Sigma, St. Louis) and 2% fetal bovine serum (FBS) (Gibco). WRO82 cells were grown at 10% CO_2, 37^o C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1 mM non-essential amino acids (Lonza, Allendale, NJ), 1 mM L-glutamine (Gibco), 100 units/mL penicillin and 100 μg/mL streptomycin (Lonza). Stable transfection of the WM793 cells was achieved in the same manner and using the same constructs as described for 1205LU cells in the previous chapter.

Wound Healing Assays: "Scratch" assays were conducted in 6-well tissue culture plates. 3x10^5 WM793 cells were seeded per well and allowed to attach for ~16 hours under normal growth conditions. The next morning, TU 2% growth medium was changed to protein-free culture medium and the cells were "starved" for 4 hours. A scratch was then generated in the monolayer of cells with a 200 μL pipette tip and 2 washes with media were used to remove any loosely attached cells. The cells were then grown for 48 hours, in the absence of exogenous growth factors to reduce proliferation, at 37^o C, 5% CO_2. For analyses, multiple images were captured (~4 per well) from the same areas of the scratch in 24 hour intervals using light microscopy (4X) and a Nuance imaging system (CRi, Woburn, MA). Motile cells were counted and measured using ImageJ software (U.S. National Institute of Health, Bethesda, MD) (105).

RNA isolation: WM793 cells were initially seeded 1.5 x 10^5 in 100 mm culture dishes and grown for 3 days in TU 2% melanoma media to a confluence of

~80%. Five replicate dishes of each mixed population of stable transfectants or parent cell lines were included and kept as separate and individual samples. Total cellular RNA was harvested using RNEasy RNA Extraction Kit (Qiagen) according to the supplied protocol. Purified RNA samples were diluted to ~ 1 mg/mL with RNAse-free deionized water and transferred to the University of Kentucky Microarray Core facility for cDNA synthesis, hybridization and scanning, using equipment and procedures described here: (http://www.mc.uky.edu/ukmicroarray/affymetrix.htm).

Data analysis: Raw intensity values were imported into Partek Genomics Suite for normalization and analysis. Signals were subjected to background correction based on G-C content, sketch normalization (50000 points) and $\log_2$ transformation. Resulting intensities below 2 were removed as background noise and median values were assembled for collections of probes assigned to each gene to generate signal estimates (RMA method) corresponding to each treatment group. Factors used for the multi-factorial analysis of variance employed by the software were both experimental (cell lines) and batch grouping (scan date). Statistical analysis was performed across the entire experiment for overall ANOVA and pairwise comparisons were conducted between control cells and H1-transductants as well as between H1wt and mutant variants. The same parameters were used for both gene-level and exon-level analyses, however different workflows provided by the software were necessary for separation of the two data sets.

Validation of expression changes with semi-quantitative polymerase chain reaction: Isolated RNA (1 μg) was converted to cDNA using MultiScribe reverse transcriptase and random hexamer primers in 50 μl reactions (Applied Biosystems, Foster City, CA). Semi-quantitative polymerase chain reaction (PCR) was performed with TaqMan Universal PCR MasterMix without UNG and pre-designed TaqMan Gene Expression Assays (Applied Biosystems) using 100-200 ng of cDNA. The NM23-H1 gene was assessed with primers (5'-3'; forward-

CAGAGAAGGAGATCGGCTTGT; reverse-GCACAGCTCGTGTAATCTACCA) and a FAM dye fluorescent probe (TTCCTCAGGGTGAAACC). A list of additional primers pre-designed and commercially available (Applied Biosystems) are described in Table 3.1. Signals were quantified from FAM reporter dyes found on an MGB probe incorporated with a given primer set, and detected with an ABI Prism 7700 (Applied Biosystems). Target gene expression levels were normalized to endogenous beta-2-microglobulin mRNA (Human B2M Endogenous Control, Applied Biosystems).

Table 3.1 Primers Used for Semi-quantitative Polymerase Chain Reaction (PCR) Validation of Microarray Results

Gene[#] (Assay ID*)	**RefSeq**	**Exon junction**	**Length of amplicon (bp)**
EREG (Hs00154995_m1)	NM_001432.2	1-2	118
MMP3 (Hs00968305_m1)	NM_002422.3	6-7	126
NETO2 (Hs00983152_m1)	NM_018092.3	7-8	66
MAGEA1 (Hs00607097_m1)	NM_004988.4	1-2	124
LRP1B-5' (Hs01069129_m1)	NM_018557.2	3-4	125
LRP1B-3' (Hs00218582_m1)	NM_018557.2	89-90	91

Official gene symbol according to HUGO Gene Nomenclature Committee
* Product number for pre-designed primer sets specific for a given gene available from Applied Biosystems: (https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=ABGEKeywordSearch&catID=601267). All probes are labeled with FAM fluorescent dye.

Results

H1 Overexpression Leads to Reductions in WM793 Cell Motility

Empty-vector, wild-type and mutant (E_5A, $K_{12}Q$, $H_{118}F$, and $P_{96}S$) NM23 constructs were introduced by stable transfection into WM793, VGP melanoma cells. Although these cells are evolutionarily similar to 1205LU cells, motility and invasion studies conducted in the same manner as before in Boyden chamber systems failed to show any reduction in cell migration of WM793 cells when overexpressing wild-type or mutant H1. This was unexpected as these cells were predicted to behave like the 1205LU cells and exhibit H1-dependent reductions in motility. In order to eliminate the possibility that this was the result of technical limitations of the assay, an alternate method was used to verify that no phenotypic variation existed.

Wound healing, or "scratch", assays are an extremely simple method commonly used in cell culture systems to assess the ability of a monolayer of cells to repopulate a freshly denuded area in a culture dish. Although these assays limit the ability to stimulate a cell's directional chemotaxis with exogenous attractants, they can be very informative when looking at random motility within a population of cells. In this study, there was no H1-dependent difference in the collective movement of the monolayer of cells as determined by closure of the "wound" over time (Figure 3.1). However, a few striking differences were observed between the control cell lines and the NM23 transfectants. First, the total number of individual cells which were breaking away from the monolayer and migrating into the center of the scratch was significantly higher in the control cells than the H1-overexpressing melanoma cells (Figure 3.2(A)). In addition, the majority of these relatively motile control cells that would gather in the wounded area had stark differences in morphology, acquiring a neuronal-like shape and extending a pair of extremely long, thin dendrites in opposite directions from the cell body (Figure 3.2(C)). This was quantified using imaging software and is presented as average cell length of the motile cells within the "scratch" at 48 hours (Figure 3.2(B)). Mean cell lengths of migrating cells showed a significant difference from controls, yet no variation among any of the H1-transfectants.

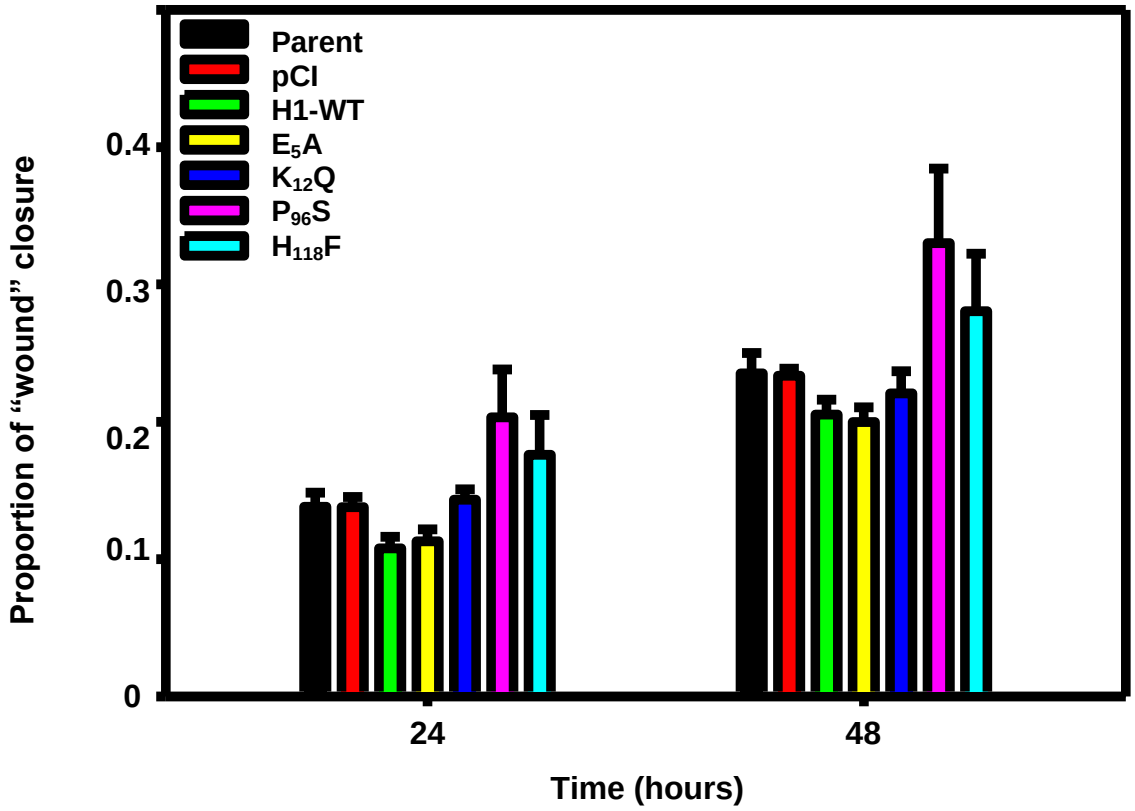
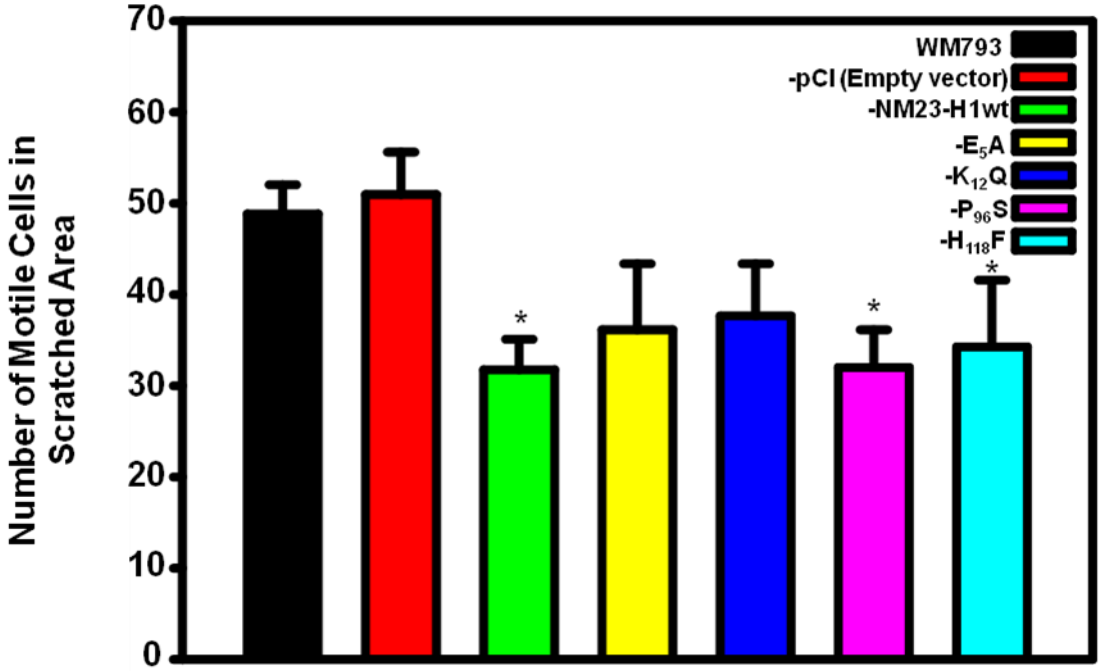
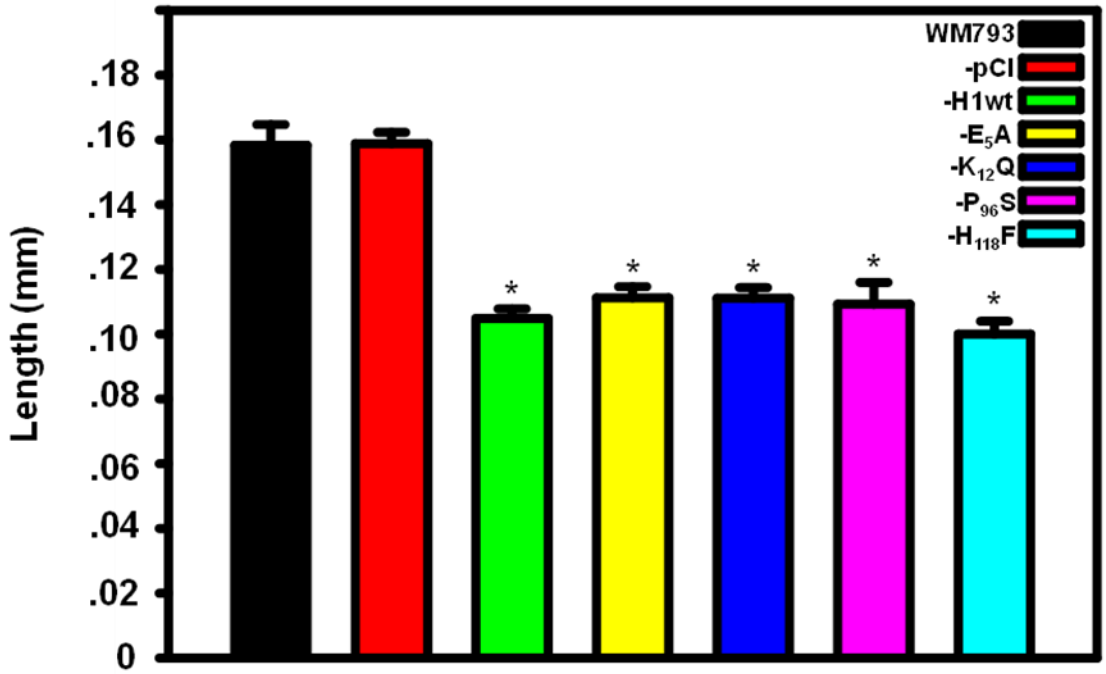


Figure 3.1 WM793 Transfectants Show No Differences in Rates of Wound Closure. Confluent cell monolayers were scratched with a 200μL pipette tip and cultured for 48 hours in protein-free growth media. Images were acquired at 4X magnification every 24 hours, including at initiation of the experiment for reference. Area of denuded space between opposite monolayer fronts was quantified from these images using ImageJ software (NIH) and compared to one another to determine the proportion of the wound that was closed. Statistical analysis using the Kruskal-Wallis one-way ANOVA on ranks showed no statistical significance per time point.

A.


Number of Motile Cells in Scratched Area
70
60
50
40
30
20
10
0
WM793
-pCI (Empty vector)
-NM23-H1wt
-E5A
-K12Q
-P96S
-H118F
*
*
*

B.


Length (mm)
.18
.16
.14
.12
.10
.08
.06
.04
.02
0
WM793
-pCI
-H1wt
-E5A
-K12Q
-P96S
-H118F
*
*
*
*
*

C.

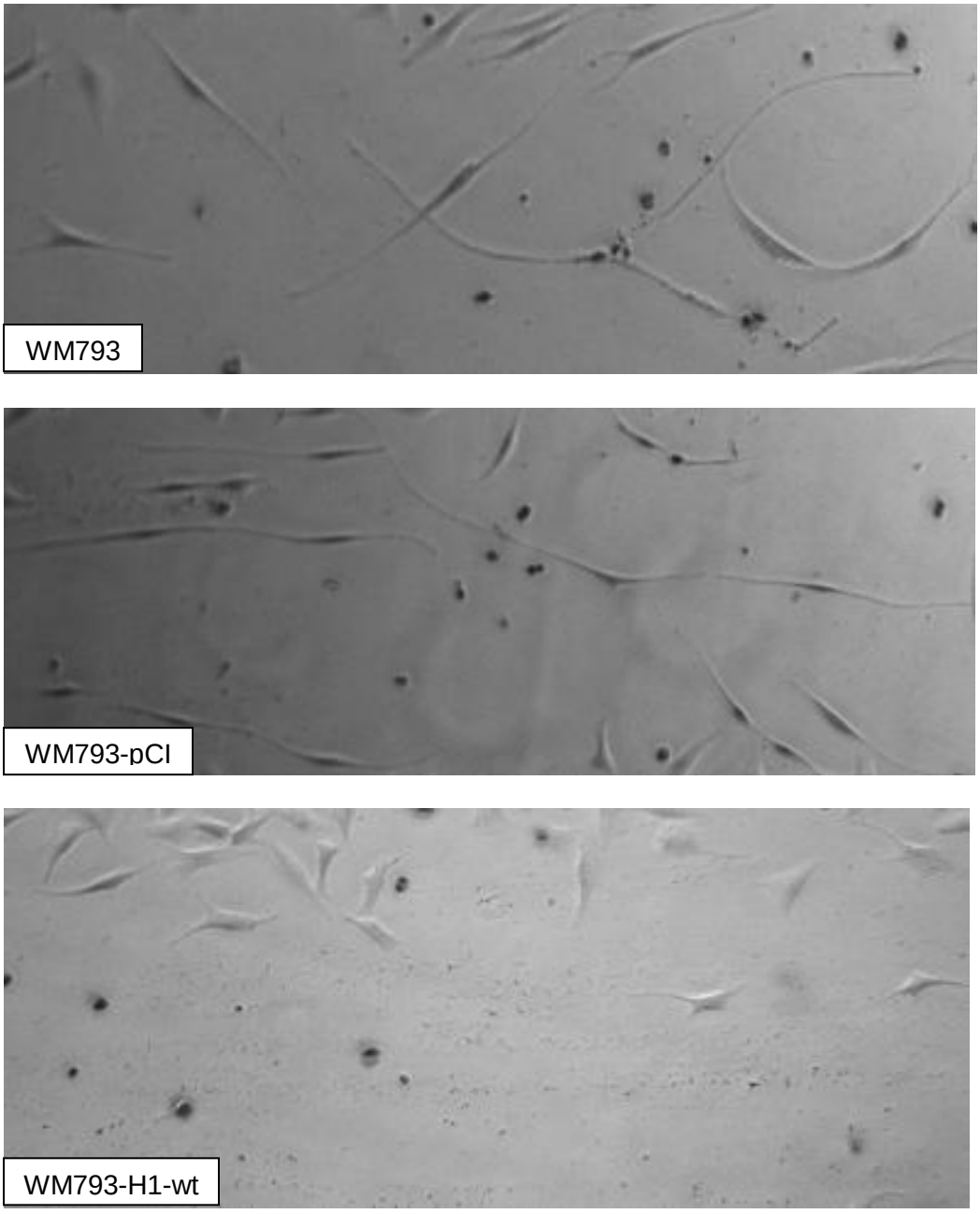


Figure 3.2 Forced Expression of H1 Reduces Single Cell Motility and Prevents a Correlated Morphological Change in WM793 Cells. WM793 cells were seeded at confluence and allowed to attach for ~18 hours before serum-starving the cells for 4 hours. Cells were then removed along a "scratch" and closure of the denuded area was monitored for 48 hours. The motility (A) and length (B) of single cells within the scratched area at 48 hours were determined from images captured at 4X magnification and quantified using ImageJ analysis software. (* = Statistical significance according to 1-way ANOVA and Holm-Sidak method of pairwise comparisons ($p < .05$) for single cell motility (A). Kruskal-Wallis 1-way ANOVA on ranks and Dunn's method were used for comparisons of cell length in (B)). (C) Typical cellular morphology seen among motile cells from the control and H1 lines under light microscopy (4X).

While the H1-dependent reduction in cell motility for these populations of cells is modest when compared to what was seen in the 1205LU model, the correlation between the observed morphological change and migratory ability strongly suggests some type of physiological relevance, although this connection is not understood at this point. These data do provide evidence that H1 is able to suppress random migration, albeit in what appears to be a fairly small subpopulation of the non-metastatic WM793 melanoma cells, a novel finding in this particular cell line.

In accordance with the results in 1205LU cells, mutations disrupting the kinase activities alone ($H_{118}F$ and $P_{96}S$) had no bearing on motility suppression conferred to the WM793 cells showing agreement between the two studies despite utilization of different techniques. Two other mutant lines, E_5A and $K_{12}Q$, showed a similar trend to wild-type H1 in total number of motile cells yet failed to reach statistical significance.

Identification of Genes Regulated by H1 Using Microarray Analysis

In an effort to determine mediators of H1-dependent suppression of cell migration, differential gene expression analysis was conducted on the WM793 stable transfectants and control cells. Prior to the current study, microarray analysis had been conducted to examine the effects of H1-overexpression in two aggressive cell lines lacking endogenous H1 expression, a metastatic melanoma cell line WM1158, and WRO82, cells derived from a metastatic human thyroid carcinoma. In these two lines, H1 levels were elevated through the use of adenoviral delivery and RNA was collected 48 hours later and hybridized to U133 human genome arrays (Affymetrix). A *lacz* containing adenovirus was included as a control for the infection itself. Once the data was analyzed, it became apparent that the transduction of these cells itself was resulting in global gene expression changes which complicated downstream analysis by increasing the likelihood of false positives and masking true effects of H1-WT on the transcriptome. Therefore, microarray analysis using the WM793 panel of stable transfectants was initiated in order to clarify results from the previous studies

while also providing a means of investigating the effect of specific H1 mutations on the expression profiles.

A recent report implicating NM23 in splicing of nascent mRNA (106) led to the decision to use an alternate gene-chip platform to study the WM793 panel. The human Exon 1.0 ST array (Affymetrix) was chosen allowing for the detection of multiple levels of genetic variation by examining the entire transcript. This type of analysis measures changes in both abundance (gene-level) and alternative splicing (exon-level) of gene transcripts. Following data normalization and statistical comparisons, 143 gene-level and/or alternatively spliced targets were found to be significantly different following overexpression of H1 in WM793 cells. This list of genes was used for grouping based on functional ontology; however, the lack of thorough characterization of the vast majority of splice variants in the human genome makes down-stream consequences and validation of a great number of these species extremely difficult. Therefore, most of the genes that were chosen for further investigation were based on the gene-level lists only. Realizing that inherent differences between the chip platforms chosen would confound variations, the previously generated expression data was reanalyzed with the same methods used for the exon array. Raw signal intensity files were normalized and compared statistically using Partek® Genomics Suite (GS) software. Partek employs a mixed model analysis of variance and uses the method of moments estimation (107) as a means of estimating variation attributed to random effects. Initially, the resultant profiles of differentially expressed genes for WM793 control cells were compared to stable transfectant cell lines forced to express NM23-H1 wild-type or mutant variants. A final list of differentially expressed, H1-wt regulated genes was created using criteria consisting of p-values ≤ 0.01 and relative fold changes of at least 1.5 in either direction. This subsequent gene profile consists of 126 genes with 107 being upregulated and 19 downregulated. While there were an unexpectedly low number of genes showing fold-changes of two or more, pushing the detection limits of most routine quantitative techniques used to assess transcript levels, confirmation of results by RT-PCR was still conducted on select targets with

greater relative differences in expression and relevance to cancer and/or NM23-H1 (Table 3.2). These include the ErbB receptors ligand epiregulin (EREG), melanoma antigen A1 (MAGEA1), matrix metalloproteinase 3 (MMP3) and NETO2. Forced expression of H1 was confirmed as well with stable transcript levels being 10-15-fold higher than in the control cells.

Examination of the exon-level data identified one gene in particular that displayed extremely significant differences in alternative-splicing patterns in response to H1-wt overexpression. LRP1B, a member of the low-density lipoprotein receptor family, was represented by at least two species of transcripts in the analysis, with one full-length and one truncated message consisting of just the first fourteen exons of the intact gene product detected. From visualization of the splicing patterns using Partek GS, it would appear that in the WM793 control cells this short form of the transcript is expressed, identified by ENSEMBL as a novel protein coding transcript (ENST00000434794). Overexpression of H1 induced a shift from the spliced to the full length mRNA including all 91 exons, however, it did not appear to stimulate increased transcription. Regions interrogated at the 5' end (spanning exons 3 and 4) of the message using RT-PCR showed no expression differences between H1 and control cells whereas targeting areas corresponding to exons 89 and 90 at the 3' end showed a mean H1-dependent increase of almost 16-fold. Although the biological meaning of the different splice variants is unknown, it should be noted that this gene is a putative tumor suppressor. Expression aberrancies and loss have been noticed in a number of cases of non-small cell lung cancers (NSCLC), earning the nickname, LRP-deleted in tumors (LRP-DIT) (108). It is possible that the absence of transcripts in NSCLC is due to failure to detect this shortened transcript, raising the possibility that the truncated form of this transcript may be tumorigenic, while the full-length message is found in normal cells.

RT-PCR analysis of these same genes in the 1205LU panel showed no expression differences among the lines tested, with two targets dropping below the detection limits (Table 3.3). Failure to see correlation between the profiles for the two transfected cell panels, at least for these genes, suggested that the

Table 3.2 Validation of Genes Identified with Expression Analysis Using RT-PCR[#]

Target genes	WM793 Cell Lines				
	-H1wt	-E_5A	-K_{12}Q	-P_{96}S	-H_{118}F
NM23-H1	9.4 +/- 0.8	11.8 +/- 1.1	11.0 +/- 0.8	11.5 +/- 0.2	15.5 +/- 0.4
LRP1B[ς] (exon 3-4)	1.1	1.1	2.0	1.3	1.2
(89-90)	15.7 +/- 3.2	2.0 +/- 0.6	1.7 +/- 0.4	1.4 +/- 0.3	1.2 +/- 0.4
EREG	2.6 +/- 0.2	5.8 +/- 0.05	2.2 +/- 0.3	3.2 +/- 0.04	1.4 +/- 0.2
NETO2	2.9 +/- 0.2	7.7 +/- 2.0	1.2 +/- 0.3	1.2 +/- 0.02	1.3 +/- 0.1
MAGEA1	4.7 +/- 0.3	2.9 +/- 0.6	1.5 +/- 0.1	1.8 +/- 0.2	2.4 +/- 0.2
MMP3	1.5 +/- 0.2	2.2 +/- 0.5	2.0 +/- 0.4	1.3 +/- 0.02	1.5 +/- 0.1

Up-regulated genes; **Down-regulated.** [#]Genes examined are listed vertically with individual cell lines listed across the top. Numbers represent relative differences when compared to the WM793 parent cell line with semi-quantitative RT-PCR. [ς]According to the analysis, the LRP1B gene was found to be alternatively spliced in the H1-wt cell line. Two different regions of the same transcript (listed in parentheses) were interrogated to verify this result. The boundary between exons 3 and 4 were compared to that of exons 89-90.

Table 3.3 Genes Differentially Expressed in WM793 Cells are Unaltered in 1205LU Cells Following H1-Overexpression

Cell Line	Target Genes				
	EREG	NETO2	MAGEA1	LRP1B (*exon 3-4*)	LRP1B (*exon 89-90*)
WM793 (Parent)	1	1	1	1	1
NM23-H1wt	2.6	2.9	4.7	1.1	15.7
-E_5A	5.8	7.7	2.9	1.1	2.0
-K_{12}Q	2.2	1.2	1.5	2.0	1.7
-P_{96}S	3.2	1.2	1.8	1.3	1.4
-H_{118}F	1.4	1.3	2.4	1.2	1.2
1205LU (Parent)	No amplification (NA)	1	NA	1	1
NM23-H1wt	NA	1.5	NA	1.0	1.0
-E_5A	NA	1.1	NA	1.1	1.2
-K_{12}Q	NA	1.1	NA	1.5	1.6
-P_{96}S	NA	1.1	NA	1.1	1.4
-H_{118}F	NA	1.0	NA	1.6	1.6

Up-regulated; **Down-regulated** relative to parent cell line as determined with semi- quantitative RT-PCR.

ability to translate the findings interchangeably between lines may not be as easy as originally anticipated. It is possible that the metastatic derivative cells have undergone significant changes making them fundamentally different from WM793 cells. Alternatively, it could simply be that the genes interrogated here are not the key factors mediating the H1 phenotype and other members of the expression profile, not yet validated in 1205LU, are essential for suppression.

To better understand the greater biological meaning of the gene expression profiles for the cell lines, grouping based on ontology using the DAVID database (NCBI) was performed on the 143 genes showing H1-dependent differences. A selection of genes of interest are grouped by cellular function and presented here (Table 3.4). From the classifications, the processes that were deemed to be significantly enriched according to the statistical analysis using DAVID ($p < .05$) were translation and cell growth. Other groupings listed are for classification purposes and do not represent biological enrichment per se.

The gene lists from the two metastatic lines proved to be much larger, again, more than likely due to off-target effects of the adenoviral infection. Analysis of the WM1158 transcriptome revealed 1849 genes were regulated by H1 while 1003 genes were differentially expressed in the WRO82 cell line. These lists were created by filtering the data to include only genes showing statistical significance ($p < .01$) between the H1 transductants and the two controls (uninfected & *lacz* infected). The subsequent expression profiles were then used to create lists of functionally associated genes enriched by H1 overexpression in the metastatic cell lines, WRO82 and WM1158, as determined by Ingenuity Pathway Analysis (IPA). A selection of significant genes identified within each ontology group is presented (Table 3.5 [WM1158]; Table 3.6 [WRO82]).

Genes Tracking with the Motility Suppression Profile Modulate Cell Signaling

To more specifically investigate how H1 is inhibiting motility in these cell lines, the microarray data was filtered in a manner that fit the observed

Table 3.4 Grouping Based on Associated Biological Process of NM23-H1-Dependent Genes Differentially Expressed in WM793 Cells[1]

	Induced by H1[3]	Repressed by H1	Alternatively-spliced
Translation/ RNA processing (9.1%)[2]	MRPL16 MRPS6 MRPS11 PTRH1 RPL6 RPL17 RPL35 RPS27L EXOSC8 PRPF18 RBM17 TRMT5 ZCRB1		
Cell Death (7.0%)	BCL2A1 BNIP3 ALDOC* ATXN3 CASP1 CASP4 DYNLL1 SRGN SIAH1		SYNE1
Cell Movement/ Adhesion (7.7%)	CD9 SCG2 SIAH1	SEMA3B FREM2 LGALS3BP SIRPA SDC3	MET NRCAM SLIT3
Cell-cell Signaling (5.6%)	PARK7 ATXN3 CXCL11 EREG* GDF15	APOE SEMA3B	AMH
Cell Cycle/ Growth/ Proliferation (11.9%)	N6AMT1* ESM1* IGFBP3	APOE	NRCAM LZTS1 MET

Table 3.4 (Continued)

Cell Cycle/ Growth/ Proliferation (11.9%)	IGFBP4*		
	MPHOSPH8		
	NDC80*		
	OIP5		
	RAD51		
	CDT1		
	EREG*		
	SIAH1		
	EMP1		
	ZNF259		

[1]Biological annotation assigned from DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). [2]Percentages represent proportion of total H1-regulated genes assigned to each biological process. [3]Genes are listed by their official gene symbol as denoted by HUGO Gene Nomenclature Committee. *Genes which are found to be alternatively-spliced as well as induced transcriptionally by H1.

Table 3.5 Genes Altered by H1-wt Overexpression in WM1158 Cells

	Induced by H1-wt		Repressed by H1-wt	
	Gene*	p-value	Gene	p-value
Cell Death	GADD45A	9.19E-06	TRIM2	7.81E-05
	DUSP5	1.11E-05	CASP6	0.000259
	AKAP12	4.66E-05	ANP32A	0.000396
	PPIF	0.000209	MBD4	0.000867
	NPTX1	0.000212	TRIB2	0.000884
	KLF6	0.000252		
	G0S2	0.001297		
Cellular Growth/ Proliferation	GEM	6.16E-07	PADI4	7.35E-07
	DUSP1	1.88E-06	CALM1	3.62E-05
	EGR1	6.91E-05	EDF1	0.000229
	ATF3	0.000211	CAPRIN1	0.000994
	PPP1R15A	0.000262	PCNA	0.001054
	MAP2K7	0.000366	TOB1	0.001106
	MAFF	0.000381	CCNG2	0.001508
	SGK1	0.001155	ETFDH	0.003576
	RBM9	0.002231		
Cellular Movement	STC1	4.82E-07	CD24	8.59E-06
	MMP3	1.35E-05	TPM1	7.33E-05
	HSP90AA1	1.38E-05	CAPZB	0.000191
	MMP1	1.72E-05	THBS1	0.002831
	AJAP1	6.80E-05	CAPZA2	0.002978
	ITGB3	7.19E-05		
	NT5E	0.000111		
	SERPINB2	0.000206		
	CD55	0.000244		
	TFPI2	0.001135		
Cell Signaling	IL8	9.20E-06	NAT1	0.000901
	IL11	3.30E-06	SS18	0.00378
	EIF5	7.58E-05		
	IL1A	0.00015		
	CALR	0.000602		

Table 3.5
(Continued)

Development	ANGPT2	4.65E-08	RAB4A	3.81E-05
	PTHLH	9.02E-06	TTC3	0.000209
	MYC	1.32E-05	SRI	0.000279
	AREG	2.56E-05	ADH5	0.000312
	HBEGF	6.19E-05	BMP4	0.000536
	IFRD1	6.41E-05	BBS4	0.001483
	JUN	0.000142	DLG1	0.001534
	NID1	0.000213	NUP133	0.001879
	TFPI	0.000551	VEZF1	0.006567
	FGF5	0.000605		
	SYNE1	0.001985		
	INHBA	0.002474		

*Genes are listed by their official gene symbol as denoted by HUGO Gene Nomenclature Committee.

Table 3.6 Genes Altered By H1-wt Overexpression in WRO82 Cells

	Induced by H1-wt		Repressed by H1-wt	
	Gene*	p-value	Gene	p-value
Cellular Movement	CSF2RB	3.84E-07	ZYX	0.000498
	ADAM21	6.68E-05		
	TIMP4	0.00026		
	CD3E	0.000288		
	COL4A3	0.000505		
	F9	0.000806		
	SEMA6D	0.001344		
	LGALS8	0.001191		
	ELMO1	0.002452		
Cellular Growth/ Proliferation	SSX1	5.46E-05	GATA3	0.000462
	ESR1	6.78E-05	FIP1L1	0.000929
	CYP2C9	0.000142	PRC1	0.000995
	RASGRF1	0.000365	UTP6	0.00134
	STAT5A	0.000583	CDK7	0.001453
	ST6GAL1	0.000615	STAT1	0.002397
	GNAO1	0.001036	NUP98	0.002837
			ZFP36L2	0.004026
			SERTAD2	0.004269
			CCNA2	0.004279
Cell Death	SERPINB9	1.10E-05	RPS3	5.10E-05
	RAD51L3	1.48E-05	ATP5S	7.79E-05
	KIF5A	0.000191	CCT6A	0.000199
	SERPINB4	0.000711	PQBP1	0.000323
	PRDM2	0.000817	PSMB1	0.001074
	SFRP1	0.00096	ABCE1	0.00134
	PAK6	0.001913	ASNS	0.001577
			CDC42EP3	0.001804
			ITGB3BP	0.001859
			BCL2L1	0.002547
			PDCD5	0.002846
			HSPA4	0.003247
			PRDX6	0.00393

Table 3.6
(Continued)

	NFKB2	1.61E-05	MAEA	0.000559
	ELAVL2	0.000133	FAS	0.00059
	TYRP1	0.000154	GRSF1	0.000731
	SRPX2	0.000254	IRF7	0.001005
Development	SHROOM2	0.000482	XBP1	0.003044
	UTS2	0.000645	EDF1	0.003306
	EFNB3	0.001143		
	TRIB2	0.001148		
	REL	0.001845		
	ID2	0.004968		
	MS4A1	3.26E-05	PPID	9.95E-05
	HGF	3.86E-05	CAP1	0.002622
	PDE3B	6.44E-05		
Cell	HRH3	9.50E-05		
Signaling	CX3CR1	0.000118		
	CXCL10	0.000121		
	CCR1	0.000222		
	TGFA	0.000374		
	IL4	0.000596		

*Genes are listed by their official gene symbol as denoted by HUGO Gene Nomenclature Committee.

phenotype. In other words, the genes that were shown to be differentially expressed in the H1-wt cell line were further enriched for coordinately regulated genes in the $P_{96}S$ and $H_{118}F$ mutant transfectants, thus tracking with the motility data. The criteria for enrichment did not consider the E_5A- and $K_{12}Q$-regulated genes as these cells did not quite reach statistical significance in the scratch assays, showing an intermediate phenotype not different from either control or wild-type transfectants. The final gene list containing 59 genes facilitated the downstream investigation necessary for characterizing any proposed mechanism of action by pinpointing the genes most likely to be mediating the H1-dependent effects on migration (Table 3.7). Further enrichment was achieved by sorting the gene lists from all three studies into a Venn diagram in order to look for regions of similarity and showing that only nine genes are coordinately altered by H1-wt in these lines (Figure 3.3). These include seven up-regulated (*BRAP*, *DIDO1*, *EXOSC8*, *NLGN4X*, *PABPC3*, *POP5*, *SRPX2*) and two down-regulated genes (*LRP3* and *SEMA4F*). The only gene from this list that also fit the motility profile was *BRAP*, an ubiquitin ligase molecule required for proper conduction of MAPK signaling through Raf and MEK, suggesting yet another important role for H1 in this pathway.

Conclusions

Although still statistically significant, the relative reduction to motility in response to H1 overexpression in the WM793 cell line is unexpectedly small, which is quite interesting considering that these cells have lower metastatic potential but are no less tumorigenic than the 1205LU cells. As classically defined metastasis suppressors mediate their effects exclusively on cells which are metastatically competent, one might expect a more modest impact on a cell derived from a VGP cell line. Therefore, it is possible a quantitative difference may only be observed in the most aggressive cells within a population of VGP melanoma cells. Consequently, many WM793 cells may be unaffected by H1 overexpression simply due to the stage of progression or level of

Table 3.7 List of Genes Regulated by H1 Which Fit the Motility Profile Observed in the WM793 Panel of Transfectants[ξ]

Induced by H1-overexpression		Repressed by H1-overexpression	
Gene Name*	**Gene Symbol**[Ψ]	**Gene Name**	**Gene Symbol**
major histocompatibility complex, class II, DP beta 1	**HLA-DPB1**	steroid-5-alpha-reductase, alpha polypeptide 1	**SRD5A1**
growth differentiation factor 15	**GDF15**	carnitine O-octanoyltransferase	**CROT**
zinc finger protein 195	**ZNF195**	Sad1 and UNC84 domain containing 2	**SUN2**
WD repeat domain 54	**WDR54**	SUMO1 pseudogene 3	**SUMO1P3**
ribosomal protein S27-like	**RPS27L**	Rho guanine nucleotide exchange factor (GEF) 19	**ARHGEF19**
LYR motif containing 5	**LYRM5**	solute carrier family 22 (organic cation/carnitine transporter), member 5	**SLC22A5**
APBB1-interacting protein 1	**APBB1IP**	zygote arrest 1	**ZAR1**
peptidyl-tRNA hydrolase 1 homolog	**PTRH1**	retbindin	**RTBDN**
BCL2/adenovirus E1B 19kDa interacting protein 3	**BNIP3**	IQ motif containing GTPase activating protein 2	**IQGAP2**
small nuclear RNA activating complex, polypeptide 3	**SNAPC3**	cytochrome P450 4V2	**CYP4V2**
NDC80 homolog, kinetochore complex component	**NDC80**	zinc finger, FYVE domain containing 28	**ZFYVE28**
RAD51 homolog	**RAD51**	mesenchyme homeobox 1	**MEOX1**
kelch domain containing 2	**KLHDC2**	solute carrier family 6 (neurotransmitter transporter, L-proline), member 7	**SLC6A7**
ankyrin repeat domain 37	**ANKRD37**	probable tRNA (uracil-O(2)-)-methyltransferase	**C4orf23**
minor histocompatibility antigen HA-8	**KIAA0020**	maltase-glucoamylase (alpha-glucosidase)	**MGAM**
M-phase phosphoprotein 8	**MPHOSPH8**	lymphocyte-activation gene 3	**LAG3**
RNA binding motif protein 17	**RBM17**	metallothionein 1J (pseudogene)	**MT1JP**

Table 3.7 (Continued)

lysosomal-associated membrane protein 1	**LAMP1**		SEC14-like 5	**SEC14L5**
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7	**NDUFA7**			
neuritin 1	**NRN1**			
BRCA1 associated protein	***BRAP***			
enolase 2	**ENO2**			
DNA replication factor Cdt1	**CDT1**			
methyl-CpG binding domain protein 6	**MBD6**			
zinc finger, MIZ-type containing 1	**ZMIZ1**			
centromere protein C 1	**CENPC1**			
sushi-repeat-containing protein, X-linked	**SRPX**			
very low density lipoprotein receptor	**VLDLR**			
ZW10, kinetochore associated, homolog	**ZW10**			
eukaryotic translation initiation factor 3, subunit M	**EIF3M**			
TANK-binding kinase 1	**TBK1**			
non imprinted in Prader-Willi/Angelman syndrome 2	**NIPA2**			
zinc finger protein 267	**ZNF267**			
Emerin	**EMD**			
THAP domain containing 8	**THAP8**			
TIMP metallopeptidase inhibitor 1	**TIMP1**			
cytoskeleton associated protein 2	**CKAP2**			
retinoblastoma binding protein 7	**RBBP7**			
DnaJ (Hsp40) homolog, subfamily B, member 1	**DNAJB1**			
ubiquitin-conjugating enzyme E2Z	**UBE2Z**			

Table 3.7 (Continued)

phosphoseryl-tRNA kinase	**PSTK**	[redacted]	

[ξ]Genes are listed in descending order from highest to lowest relative fold-change (Parent vs. H1-wt; $p \leq 0.01$). Genes included also exhibited $p \leq 0.075$ in $H_{118}F$ and $P_{96}S$ cell lines vs. parent cells with expression changes similar to H1-wt cells.

[*]Annotation from NetAffx (https://www.affymetrix.com)

[Ψ]Official gene symbol according to HUGO Gene Nomenclature Committee

BRAP is highlighted indicating the only gene in this list also induced in WM1158 and WRO82 cells following H1 overexpression

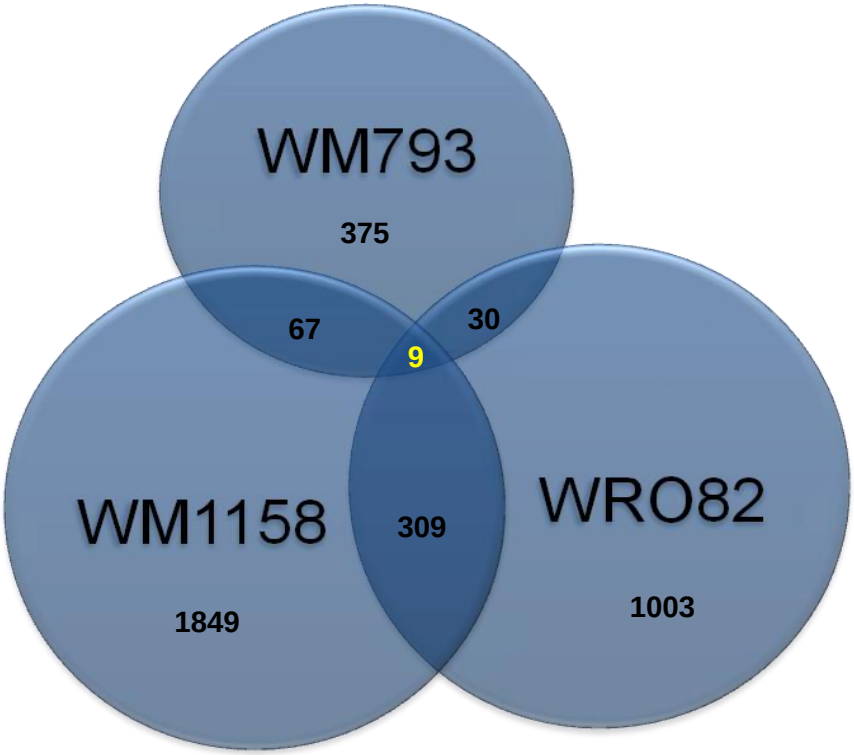


Figure 3.3 Venn Diagram Showing Relative Numbers of H1-Dependent Genes in Each Cell Type. Statistically significant genes ($p \leq .05$) were identified with microarray analysis in the three cell lines shown (WM793, WM1158, WRO82) following H1-overexpression. Numbers of genes in each group are indicated for each relationship. A total of nine genes were found to be altered in all three lines.

aggressiveness. If as expected, only an extremely small proportion (≤ .01%) of cells shed from a primary tumor are able to successfully metastasize (109), the disseminated cells that thrive possess advantageous traits that allow for clonal expansion at the distant site, characteristics that the vast majority of cells found at the point of origin do not have. This establishes a population of cells much more homogeneous than the highly heterogeneous cells in the primary tumor, which probably possess specific attributes acted upon by an MSG such as NM23-H1. Therefore, an abundance of less aggressive cells present in VGP melanoma may explain why the overall reduction in motility is to a much smaller degree than what was observed in 1205LU cells.

A technical difference in the two motility assays used could also contribute to the difference in results. In the scratch assays, cells were seeded at confluence, inducing the formation of cell-cell contacts prior to generating the wound. This negatively impacts the single cell motility through contact inhibition and forces the cells to disassemble intercellular junctions before migrating into the free space. When WM793 cells were plated more sparsely, no differences in motility between H1-transfectants and control cells were observed using this assay (data not shown), suggesting that the role of H1 may be important for maintaining cell-cell adhesion, possibly ensuring tissue integrity.

The morphological differences observed between the H1 panel and control cells in the WM793 line are extremely intriguing. The thin, neurite-like projections extending bilaterally from the control cells seem exacerbated with time in protein-free media, but are positively correlated with enhanced motility as well. And while many H1 transfectants do maintain a spindle-shaped phenotype, the presence of these long dendrites is lacking, with most H1-overexpressing motile cells retaining a shorter spindle or rounded epithelial morphology. An attempt to explain this phenomenon at this point would be speculative however, similar observations in HT-1080 fibrosarcoma and MDA-MB-231 carcinoma cells, deemed a “mesenchymal to amoeboid transition”, correlated invasiveness with such a change in cell shape (110). In these cells, highly elongated spindle morphologies also corresponded to enhanced cell motility and invasion whereas

rounded, “amoeboid” shape changes were associated with relative immobility. The enhanced movement in the referenced study was linked to increased pericellular proteolysis, alterations to Rho activity and changes to structural and adhesion molecules such as F-actin and β1-integrin.

Interestingly, the ERK-MAPK pathway has also been associated with a similar transition in BE colon carcinoma cells (111). As reported, signaling dynamics down-regulated Rho activity while enhancing Rac through ERK-dependent Fra-1 expression, increasing motility through the generation of bipolar protrusions, as well as reductions to numbers of stress fibers and focal adhesions. Investigating the impact of these pathways on migration in other systems is increasing and the link between a “mesenchymal” morphology and enhanced invasive potential has been strengthened as supporting evidence mounts (112). With the Raf-ERK signaling axis being implicated in the H1-dependent motility profile through the identification of *BRAP*, and a corresponding fibroblastoid morphology seen in the more migratory cells, the expression profile was revisited. In addition to *BRAP*, *IQGAP2* and *LCN2* were also differentially expressed and have been linked to the MAPK cascade. As a result, these genes were chosen for further investigation to assess their contribution to H1-mediated reduction in aggressiveness of melanoma cells.

CHAPTER FOUR

NM23-H1-Dependent Expression Changes of the *BRAP* and *IQGAP2* Genes Facilitate Motility Suppression

Introduction

The discovery of metastasis suppressor genes and their subsequent characterization have identified commonality among associated cellular functions, most importantly a consistent impact on signal transduction pathways. While in many cases it is unclear exactly how modulation of these cascades is occurring, familiar components including MAP kinases, small GTPases Rho and Rac, and receptors linked to tyrosine kinase and G-protein coupled signaling have all been targeted by metastasis suppressors (113). In the case of NM23, several lines of evidence exist suggesting that the H1 isoform is able to affect ERK-MAPK signaling through KSR phosphorylation (38), regulation of oncogenic Ras (114), Rad (51), and Rac1 (52). ERK activity has been shown relevant to metastasis in studies of motility and invasion (115, 116), epithelial-mesenchymal transition (EMT) (117), lymphatic spread (118, 119) and *in vivo* models of metastatic disease (120, 121). Examination of the H1-dependent gene expression data has bolstered interest in this pathway as it appears that H1 is controlling expression of several genes whose products are effectors or modulators of MAP kinase signaling.

BRCA1 associated protein (BRAP), also known as IMP for “impedes mitogenic signal propagation”, is induced by H1 in two melanoma cell lines, WM1158 and WM793. Importantly, both of these cell lines harbor $V_{600}E$ activating mutations to B-Raf. The gene product of *BRAP* is an E3 ubiquitin ligase and Ras effector protein that acts as a negative modulator of MEK and ERK activation through preventing the assembly of Raf-MEK complexes (122). Coincidentally, BRAP blocks signal transduction by inactivating KSR and disrupting its ability to link Raf with MEK, inhibiting downstream phosphorylation in the absence of mitogenic stimulation. This effect is very similar to descriptions

of the interaction between H1 and KSR representing a novel, but possibly redundant mechanism by which NM23 is impeding MAP kinase signal transduction. Furthermore, inhibition by BRAP is specific for negative regulation of the amplitude of ERK activation, with no impact on the timing or duration of the response, however, other common MAPK signaling molecules (e.g., JNK and Akt) are unaffected (122, 123). This reduction in ERK activity is again likened to the effect of H1 overexpression described in MDA-MB-435 cells (113).

NM23 induced the expression of the lipocalin-2 (LCN2) gene in the WM793 cell line as well. Although the expression of LCN2 increases following malignant transformation of epithelial cells from the breast, lung, colon and pancreas, suggesting an oncogenic function (124, 125), it also demonstrates the ability to restore expression of E-cadherin and cell polarization, characteristics of normal epithelium (126). The gene product exerts unique effects on Ras-transformed epithelial cells that have undergone EMT, a characteristic common among many metastatic cancer cells which is thought in many cases to drive metastatic progression. LCN2 has exhibited the ability to reverse many characteristics of EMT in Ras-transformed cells, leading to inhibition of tumor growth and cancer metastasis (127). Specifically, LCN2 overexpression was shown to promote E-cadherin stabilization which is opposed by activated MEK, through uncoupling signal transduction between Ras and Raf in 4T1 murine mammary carcinoma cells transfected with constitutively active H-ras. Although the actions of LCN2 appear to act immediately upstream of Raf, demonstrated in the presence of oncogenic Ras, the impact on cells containing constitutively activate B-raf has not been described. Therefore, the ability of LCN2 to reverse any negative effects of hyperactivated Raf signaling is unknown but induction of expression by H1 implicated this molecule as a potential player in NM23-dependent modulation of MAP kinase responsiveness in certain contexts.

A third gene thought to influence mitogen-activated signaling identified by microarray analysis was *IQGAP2*. Surprisingly, this gene is a putative tumor suppressor (128), although its expression was reduced in response to elevated H1 levels in WM793 cells and observations of IQGAP2 induction have been

noted in pancreatic and colorectal cancer studies (129, 130). In addition, the oncogenic isoform IQGAP1 was down-regulated by H1 in the WM1158 expression profile suggesting this gene family may be a common target of NM23. IQGAPs are a highly conserved class of proteins which alter cytoskeletal structure and associated signaling events, exerting influence on CDC42, Rac, E-Cadherin, and β-Catenin, among others (131-134). Functionally these proteins have shown dependence on Rac1 binding for their activity which is important for cell-cell adhesion and motility through regulation of F-actin dynamics. (135) Modulation of signaling through Ras and PI3 kinase pathways has also been shown for IQGAPs, controlling aspects of chemotaxis possibly through differential regulation of these cascades (135).

These three genes, *IQGAP2*, *LCN2*, and *BRAP*, have all demonstrated importance for a variety of mitogenic signaling events. The fact that they all feed in to many of the same branches of the MAP kinase cascade suggested potential importance of this pathway in mediating H1-dependent motility suppression. The role of these genes in the WM793 melanoma model was addressed using lentivirus-based shRNA-mediated gene silencing. RNA interference is a rapid and effective means of assessing relevant functions associated with any given gene. Lentiviral delivery systems are commonly used based on their ability to infect both cycling and non-cycling cells, stable integration into the host cell genome and proving to be less prone to silencing during development allowing for use in embryonic stem cell studies (136). Transduced cells exhibiting antibiotic resistance and loss of the targeted transcript were subjected to wound healing assays and relative motility was measured. Of all the genes tested in these experiments, it appeared that the up-regulation of BRAP as well as the suppression of IQGAP2 contributed the most to the observed H1-dependent phenotypes. The coordinated yet opposite regulation of these two genes would indicate H1 is controlling a set of genetic events which may be ultimately modulating propagation of specific yet unidentified signals, potentially underlying the impedance of metastatic progression.

Materials and Methods

Cell lines and culture: 293T cells were a kind gift of Dr. M. Kilgore (University of Kentucky, Lexington, KY). WM793 melanoma cell lines were maintained at 5% CO_2, 37^o C in TU 2% growth medium composed of MCDB153: Leibovitz-15 (4:1; v:v) (Sigma, St. Louis, MO:Gibco, Carlsbad, CA) with 2 mM $CaCl_2$ (Fisher Scientific, Pittsburgh, PA), 5 μg/mL insulin (Sigma, St. Louis) and 2% fetal bovine serum (FBS) (Gibco). 293T cells were grown at 10% CO_2, 37^o C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1 mM non-essential amino acids (Lonza, Allendale, NJ), 1 mM L-glutamine (Gibco), 100 units/mL penicillin and 100 μg/mL streptomycin (Lonza).

Lentivirus Expression Vectors, Virus Production and Infection: Packaging plasmids pRev, pVSV-G, and pMDL.go.RRE (137, 138) were kind gifts of Dr. Louis Hersh (U. of Kentucky). shRNA-encoding Lentiviral expression vectors (pLKO.1-puro) were purchased from the Sigma-Aldrich MISSION line (St. Louis, MO). For generating recombinant lentivirus, 2 x 10^6 293T human embryonic kidney cells, an efficient lentivirus packaging cell type, were seeded in 100 mm dishes, and transfected using the calcium phosphate method 24 hours later. Transfection media was changed following overnight incubation and lentiviral containing media was harvested ~40 hours later, centrifuged at 1500 x g for 5 minutes, then flash frozen in 0.5 mL aliquots and stored at -80^o C. Viral titer was determined using QuickTiter™ Lentivirus Titer Kit (Lentivirus-Associated HIV p24) (Cell Biolabs, San Diego, CA) and concentration of viral particles for each preparation were normalized to one another based on presence of p24 viral coat protein.

Lentiviral Infection: Cells were seeded 1 x 10^5 in 100mm dishes and infected 24 hours later at 10 MOI then incubated overnight. Viral containing media was then removed and fresh TU 2% was added for 72 hours. Cells were then subjected to 2 μg/mL puromycin for a minimum of 14 days, changing media every 3-4 days. Selected clones were pooled and tested for target gene expression.

RNA isolation: WM793 cells were initially seeded 1.5 x 10^5 in 100 mm culture dishes and grown for 3 days in TU 2% melanoma media to a confluence of ~80%. Total cellular RNA was harvested using RNEasy RNA Extraction Kit (Qiagen) according to the supplied protocol. Concentrations were determined using a NanoDrop® ND-1000 (Invitrogen, Carlsbad, CA).

PCR Analysis: Total cellular RNA (1 μg) was converted to cDNA using MultiScribe reverse transcriptase and random hexamer primers in 50 μl reactions (Applied Biosystems, Foster City, CA). 50-200 ng of cDNA was used for PCR amplification in the presence of sequence specific, exon-spanning primers (Table 4.1). PCR products were separated on 1.5% agarose gels and visualized with ethidium bromide.

Wound Healing Assays: "Scratch" assays were conducted in 6-well tissue culture plates. 3x10^5 WM793 cells were seeded per well and allowed to attach for ~16 hours under normal growth conditions. TU 2% growth medium was then changed to protein-free culture medium and the cell's were "starved" for 4 hours. A scratch was generated in the monolayer of cells with a 200 μL pipette tip and 2 washes with media were used to remove any loosely attached cells. The cells were then grown for 48-72 hours in the absence of exogenous growth factors to reduce proliferation, at 37^o C, 5% CO_2. For analyses, multiple images were captured (~4 per well) from the same area of the scratch in 24 hour intervals using light microscopy (4X) and a Nuance imaging system (CRi, Woburn, MA). Motile cells were counted and measured using ImageJ software (U.S. National Institute of Health, Bethesda, MD) (105).

Table 4.1 Primers Used For shRNA Validation

Name	Seq (5'-3')	Tm (^{o}C)	GC content (%)	Product length (bp)
H1-(F)	CATTGCGATCAAACCAGATG	52.1	45	373
H1-(R)	CAAGCCGATCTCCTTCTCTG	55	55	
LCN2(F)	AGGCCTGGCAGGGAATGCAA	62.4	60	424
LCN2(R)	ACACTGGTCGATTGGGACAGGG	61.2	59	
BRAP(F)	GCGCAGTGCCATGCTGTGTA	61.1	60	460
BRAP(R)	TGGCTCGGGCGTTTGACAGT	62.1	60	
IQGAP2(F)	TTGCTGGGGTCGCTGGGAGA	63.8	65	312
IQGAP2(R)	TGCACGGCTCACCATGTCCG	62.9	65	

(F)-Forward primer
(R)-Reverse primer

Results

Gene Targets Are Effectively Knocked-down Using shRNA Lentivirus Constructs

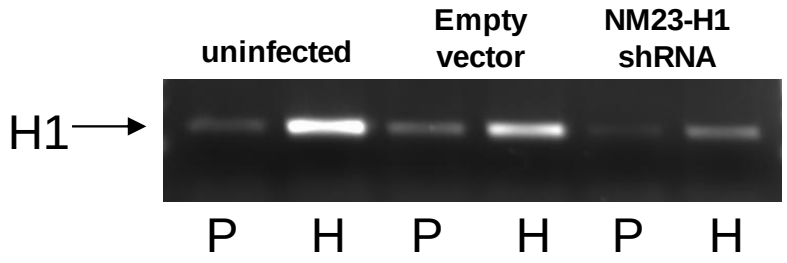
Gene targets of interest were identified from exon array analysis of H1 overexpression in melanoma cells and selected for further analysis based upon their differential expression tracking with H1-dependent motility suppression. In order to examine the function of these genes in WM793 cells, lentiviral delivery of shRNA molecules was used. Groups of 4 to 5 lentivirus-based shRNA constructs targeting each individual gene were tested, and those showing the greatest efficiency of knock-down were chosen for continued study. Stable transductants were generated in both the WM793-pCI and –H1-WT cell lines with silenced expression of NM23-H1, LCN2, BRAP, IQGAP2, as well as empty-vector controls. An additional set of cells co-infected with BRAP and LCN2 shRNA to counteract H1-dependent simultaneous induction to both genes were also created. Examples of typical reductions to expression of NM23-H1 are assessed with PCR analysis or immunoblotting as indicated (Figure 4.1).

Once the effectiveness of lentiviral infection was confirmed, cell lines were monitored in culture for presence of toxicity or gross morphological changes. Interestingly, reducing expression of BRAP, either on its own or in combination with LCN2, increased the presence of extremely long, bipolar dendrites from the WM793 cells in both pCI and H1wt cells, in agreement with what would be predicted in this system. Also, a shift in the morphology of the H1-transfectants that received the NM23-H1 shRNA was observed. Silencing H1 caused cells to acquire a similar morphology to that of the more aggressive pCI control cells, confirming this effect is mediated by the H1 gene and was not due to a non-specific off-target effect (Figure 4.2).

Silencing genes fitting the motility profile recapitulates the observed phenotype

The gene profile tracking with H1-dependent motility suppression implicated BRAP as a potential mediator. From here, additional gene targets

A.



B.

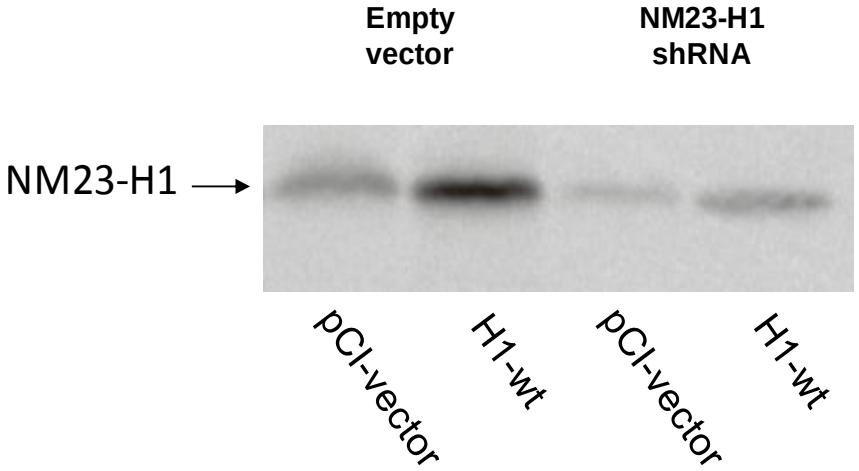


Figure 4.1 Validation of H1 Silencing in WM793 Transfectants. Effectiveness and persistence of H1 knockdown was confirmed following lentiviral infection and puromycin selection. Stable integration and expression of the shRNA construct was confirmed using (A) RT-PCR and (B) immunoblotting analysis. P = pCI; H = H1

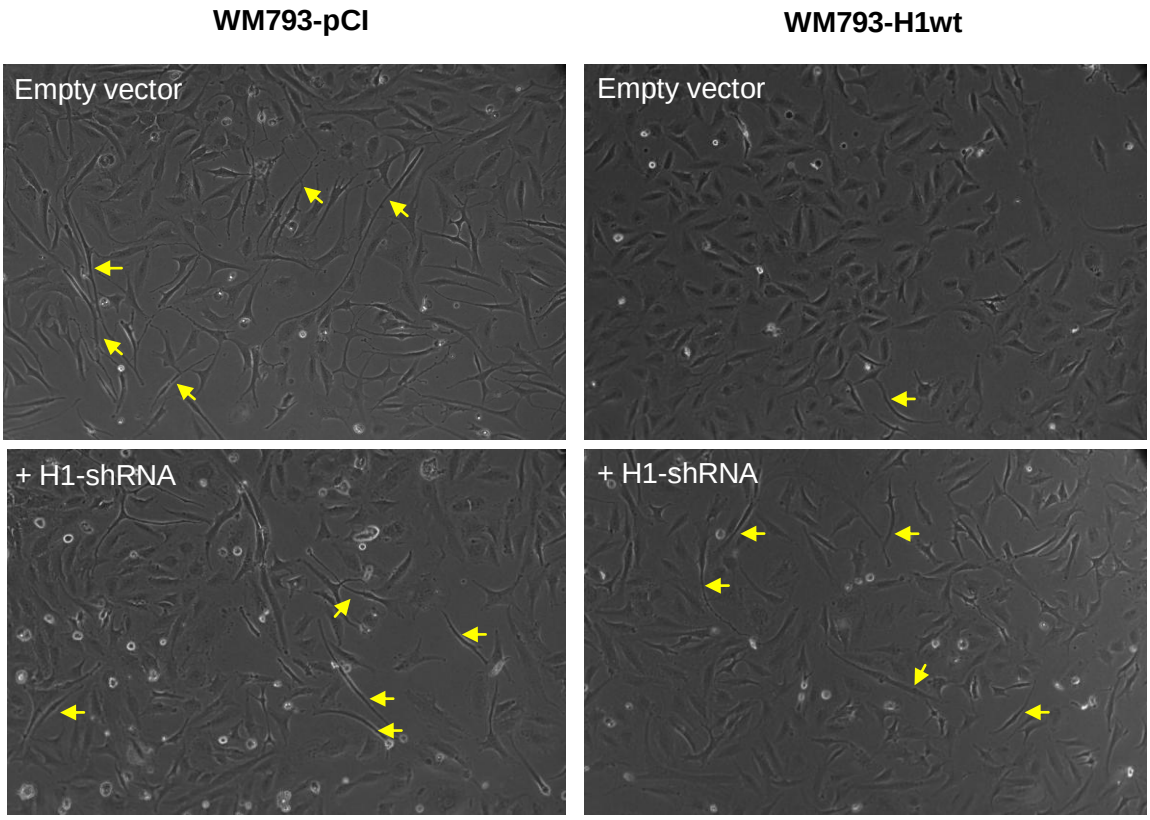


Figure 4.2 H1-expression Alters the Morphology of WM793 Cells. As indicated by the two pictures at the top of the panel, control cells (top left) lacking H1 expression exhibit dendritic processes and much more elongated morphologies than those overexpressing H1wt (top right). Targeted silencing of H1 in the stable transfectants (bottom right) causes the reversion to the morphologies found among pCI control cells.

were selected based on functional similarity to BRAP in hopes of uncovering networks of genes which may coordinate the effects of H1. As a result, stable cell lines exhibiting silenced expression of H1, LCN2, IQGAP2, and BRAP (Figure 4.3) were generated and characterized. Cell lines were cultured and compared based on relative motility in wound healing assays. (Figure 4.4) Manipulation of two of these genes in particular, *IQGAP2* and *BRAP*, strongly suggests their functional involvement in facilitating the actions of H1.

As mentioned, BRAP and LCN2 showed elevated transcript levels in response to H1 overexpression suggesting potential roles in promotion of H1-dependent effects. Indeed, BRAP down-regulation induced the migration of both pCI and H1-WT transfectants while also exacerbating the presence and length of filipodial processes extending from the motile cells. Silencing LCN2 did not appear to induce the same response as the BRAP shRNA. These LCN2-shRNA cells had the same degree of motility and similar morphologies as the control cells. Additionally, the response of these melanoma cells when both BRAP and LCN2 were silenced simultaneously did not appear to be exacerbated beyond BRAP knock-down alone, further indicating that the LCN2 gene product is not a significant factor in this context.

On the other hand, there was one gene tested that was repressed by H1 up-regulation and fit the motility profile, *IQGAP2*. Blocking expression of this gene was expected to suppress migration as it was inversely correlated with NM23. The results supported this prediction as IQGAP2 appeared to be an extremely important mediator of motility in this cell line, with profound reductions to numbers of migrating cells in the absence of this gene. In fact, inhibiting the IQGAP2 gene had a similar effect on the WM793 cells as H1wt overexpression suggesting that H1-dependent reductions to IQGAP2 levels within the cell may play a critical role in suppressing melanoma cell motility.

The results of the RNA interference studies have suggested additional novel roles for H1 in altering expression of molecules previously linked to modified signal transduction in mediating reductions in cancer cell motility. Evidence of functional contributions from BRAP on motility suppression has once

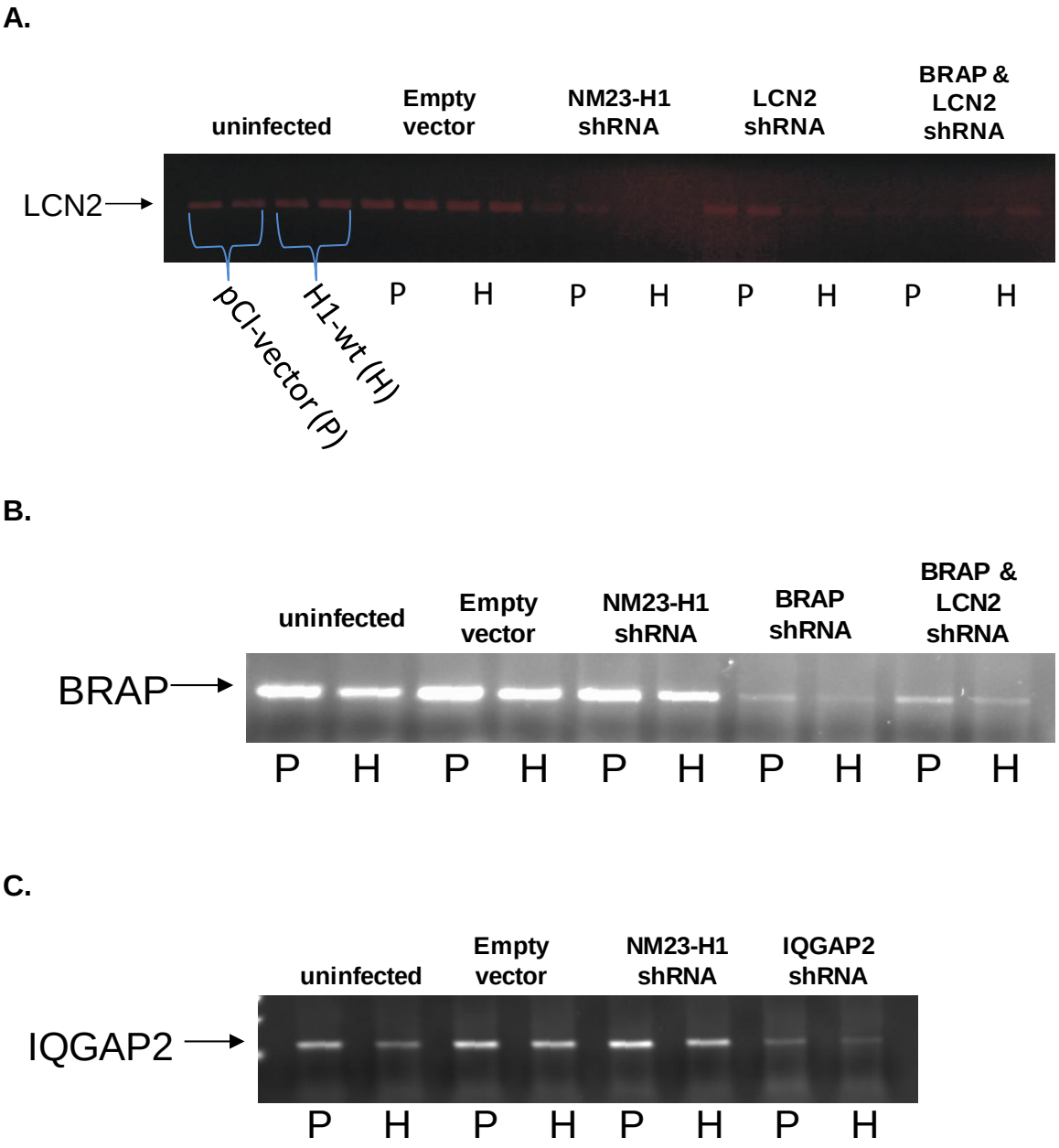


Figure 4.3 Silencing of *IQGAP2*, *BRAP* and *LCN2*. WM793 transfectants were tested for stable integration of shRNA constructs following antibiotic selection. RT-PCR and agarose gel electrophoresis was used to confirm efficacy of the lentiviral infection directed to H1 gene targets (A) LCN2, (B) BRAP, and (C) IQGAP2.

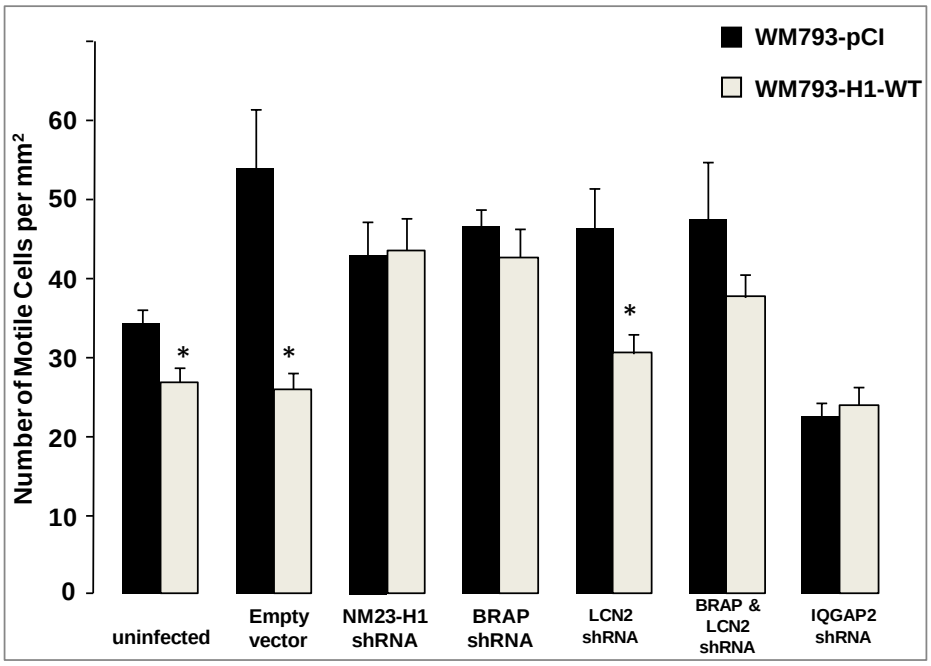


Figure 4.4 BRAP and IQGAP2 Are Mediators of H1-Dependent Motility Suppression. WM793 transfectants were infected with lentivirus containing shRNA expression vectors targeting the genes listed along the x-axis and subjected to wound healing analysis. A scratch was generated along the cell monolayer and cultures were grown 48 hours in serum-free medium. Phase-contrast images were gathered at 4X magnification every 24 hours. ImageJ software was used to determine scale and count individual motile cells. *H1wt dependent effects that exhibit statistical significance vs. pCI as determined by nested 1-way ANOVA and Dunn's test.

again implicated the KSR molecule as a functional target of H1 in some cancers. A simplified model is proposed, illustrating the multiple levels of H1-dependent regulation of a KSR containing branch of the MAPK signaling cascade, and small GTPases including Rac, Rho and Cdc42, incorporating what has been presented previously by others with the novel findings herein (Figure 4.5).

Conclusions

The results of the current round of experiments indicate a novel role for NM23-H1 in altering the expression of key molecules previously shown to regulate intracellular signal transduction in other systems. Modulation of genes BRAP and IQGAP2 facilitates, at least in part, H1-dependent motility suppression and may be found to be of further importance in prevention of melanoma metastasis. The fact that LCN2 did not appear to increase cell motility in the absence of BRAP would suggest that LCN2 is either acting upstream of the point of deregulation, or is not mechanistically involved with this phenotype at all. Supposing hyperactive signaling through the B-Raf mutation $V_{600}E$ is a driving force in the biology of this particular cancer model, it makes sense that LCN2 regulation would be innocuous to the cell as it is upstream of B-Raf, whereas BRAP directly inhibits Raf-MEK propagation. Furthermore, with separate descriptions of physical interactions with H1 and BRAP independently for KSR, it is possible that H1 and BRAP are both found in a protein complex with KSR and perhaps even functionally interact with one another, although there is no known description of this phenomenon.

The position of IQGAP2 in this model is not quite so clear, but again, its profound effect on the behavior of WM793 cells would suggest that, if via MAPK modulation, it may be acting at or below B-Raf signaling in the current schematic, or perhaps through a parallel route. The high degree of cross-talk that exists among many of these oncogenic transduction pathways makes it difficult to accurately depict all of the relevant interactions occurring within a tumor cell.

It is becoming increasingly evident that the Raf-MEK pathway, as well as the small GTPases Rac, Cdc42 and Rho, contributes quite extensively to the

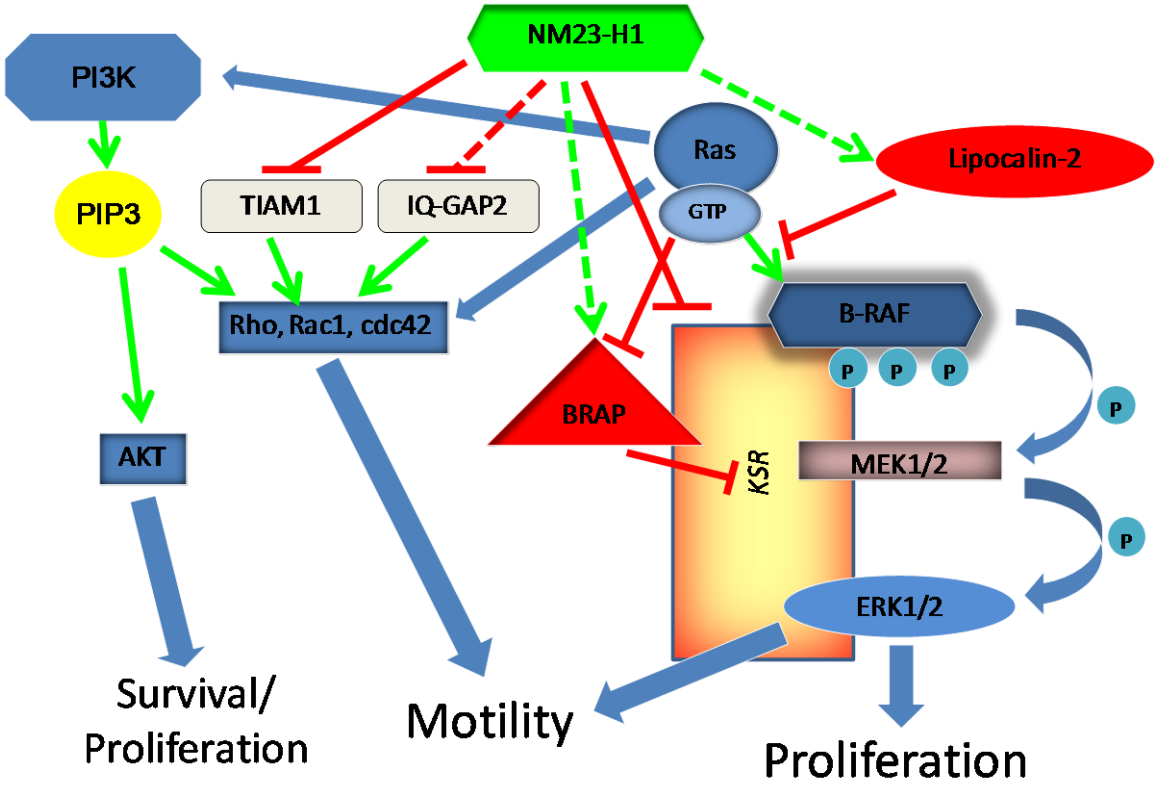


Figure 4.5 Proposed Model for H1-mediated Regulation of a Ras Signaling Pathway. NM23-H1 regulates a number of components of the Ras cascade including TIAM1, IQGAP2, BRAP, KSR and LCN2. The manner in which H1 affects these molecules indicates negative regulation of signal propagation. Activation or induction is indicated with green or blue arrows while repression is illustrated with red blocked lines. The three novel genes identified in this study as being transcriptionally regulated by H1 are designated by dashed lines.

progression of many melanomas. A wealth of experimental data has proven that activation of not only Raf signaling, but also abnormal function of N-Ras, PTEN, and Akt, strongly correlate with melanomagenesis (139). In fact, studies by Dr. M. Herlyn have shown dependence on MEK and Akt signaling pathways in melanoma cells of both VGP and metastatic origin and that inhibition of oncogenic activity with pharmacological compounds targeting MEK and PI3K were able to completely abolish tumors (140). Clinical studies with treatments directed towards mutated B-Raf have also shown promise in advanced melanomas (141). As this pathway seems to be so important for sustaining melanoma tumor growth, it is quite intriguing to see it potentially targeted on so many different levels by a single metastasis suppressor gene. Perhaps reactivation of H1 gene expression could mimic the downstream effects of pharmacological B-Raf inhibitors and could be exploited as a novel therapeutic for the treatment of melanomas harboring the $V_{600}E$ mutation. Moreover, considering the broad range of H1 targets in this pathway, it is quite possible elevating NM23 expression could be just as successful in preventing metastasis of Ras or Raf driven melanomas.

CHAPTER FIVE

Discussion

Recent progress in clinical outcomes using targeted therapies for treating melanoma has produced renewed confidence in the ability to design highly effective pharmacological small molecules, making the advent of personalized medicine a much more likely and logical approach. While quite a bit is unknown, extensive characterization of the development and progression of melanoma has revealed a great deal of information about the disease. For example, findings from a number of studies have identified activating mutations to BRAF or NRAS driving tumorigenesis, providing the basis for a number of clinical trials targeting the associated oncogenic signaling of these gene products. However, in spite of advancements made, melanoma incidence continues to rise. While only accounting for about 4% of newly diagnosed skin cancers, it remains the most deadly, causing 79% of skin cancer deaths (142), with a 5-year survival rate under 5% (143). The elevated mortality associated with melanoma is based on its ability to readily metastasize. As with most aggressive cancers, metastatic melanoma displays inherently high degrees of plasticity, exhibiting multipotency, a trait commonly seen in human embryonic stem cells (ESC). This observation has led to a great deal of interest on stem cell-like properties of many metastatic cancers. The existence and consequences of distinct populations of cancer stem cells on metastatic progression is currently an area of intense investigation, however, common aspects of signaling pathways mediating multipotency of both tumor cells and ESCs has been described (144).

While the genetic and epigenetic mechanisms governing this increased plasticity are incompletely understood, it is highly likely that the same mechanism used by metastatic melanoma cells to rapidly adapt to various microenvironmental cues probably underlies greater therapeutic resistance associated with melanoma. Thus, continued investigation into the basic biology of melanoma, as well as normal melanocytes, is crucial for finally achieving

successful pharmacological intervention for lethal metastasis, as it appears so close to becoming a reality for melanoma. As previously mentioned, the discovery of deregulated MAPK signaling in the majority of melanomas has produced an onslaught of clinical trials. Initial studies using a broad spectrum Raf inhibitor sorafenib (Bayer) on its own were disappointing, however, the next generation of BRAF inhibitors, both specific or more general targeting of multiple kinases including BRAF, are currently being studied clinically. Preliminary results of phase 2 trials for the selective inhibitors PLX4032 (Plexxikon) and GSK2118436 (Glaxo-Smith-Kline) as single use agents in patients with mutated BRAF have been positive, however, as with many prior melanoma treatments, a number of those who initially responded have since become resistant (145). In light of these studies, it is clear that BRAF is an important therapeutic target for melanoma yet targeting it only represents part of the solution. It is becoming apparent that the most aggressive melanoma cells are able to circumvent signaling pathways disrupted pharmacologically, possibly by using parallel, convergent routes however, combining agents which can disrupt multiple networks may prove much more promising for overcoming resistance. (146) Indeed, current reports have indicated that mutated BRAF cooperates with PTEN loss to produce metastatic melanoma, suggesting that targeting Raf-MEK signaling along with PI3K activity may be necessary for eradicating certain types of melanoma (147).

The ramifications from these findings extend to the melanoma model used for the NM23 studies presented herein. It had been previously reported that the WM793 and 1205LU cell lines both harbored the $V_{600}E$ activating mutation to the *BRAF* gene (148). An additional acquired anomaly thought to contribute to the metastatic competency of the 1205LU cells is the loss of the tumor suppressor PTEN through inactivating mutation, suggesting a genetic basis for the differences in aggressiveness between the two lines (149). This is extremely informative as studies using these cell lines in three-dimensional cell culture models showed metastatic 1205LU melanoma cells exhibited resistance to MEK inhibitors (U0126 and PD 98059) and a PI3K inhibitor (LY294002) when

administered individually (140). Interestingly, the same experiments found VGP WM793 cells exhibited toxicity and cell death following 72 hours of U0126 treatment at doses that had no effect on survival of 1205LU cells (1 and 10 μM). Similar results were seen using the PI3K inhibitor alone, however, co-administration of the two inhibitors was effective at synergistically blocking viability and invasiveness of the 1205LU metastatic melanoma cells (140). These results strongly indicate a cooperative function and dependence on multiple signaling pathways for promotion of melanoma metastasis while exposing the short-comings of similar single-agent therapeutics in treating malignant cancers.

The fact that aggressive melanoma cells are able to circumvent disruption of critical signaling events used to promote survival and expansion makes the class of metastasis suppressor genes that much more remarkable. If these data accurately reflect the metastatic process in humans, suppressors must be able to negatively impact progression on multiple levels, as the most aggressive cells will possess the ability to adapt and respond to selective pressure much more rapidly than normal cells. Highly plastic cancers, in theory, would overcome a single barrier implemented by a metastasis suppressor gene with relative ease. Therefore, it seems likely that the scope of functions of a MSG would have to reach well beyond an individual target if successful suppression is to be achieved. On the other hand, the unique feature of metastasis suppressors is their inability to affect primary tumor growth suggesting profound differences between metastatic clones and most cells in the primary tumor. The processes underlying the disparity in aggressiveness are the most likely targets of suppressor action and as a result, understanding this class of genes helps to identify various signaling events important to metastasis. Further identification and characterization of relevant signaling pathways is required in order to elucidate genes and transcription factors activated at the endpoint of a cascade which may play specific, yet more global roles in metastasis, as some have suggested is the case for the transcription factor NFκB (150-152).

It is no surprise that the data here would indicate a role for H1 in modulating cell signaling as this feature is common among MSGs (113). Additional evidence further implicates H1 in multiple pathways with reports of physical interactions with Rad, Tiam1 and KSR. The findings presented here that H1 can also modify expression of other affiliated genes, specifically BRAP and IQGAP2, solidifies the notion that NM23 may be exerting influence upon multiple levels of signaling. Whether these particular interactions are responsible for H1-dependent metastasis suppression is unclear but is sure to be an area of continued investigation.

The microarray studies and subsequent pathway analyses illuminated broader mechanisms through which NM23 may be impacting tumor progression. The high proportion of ribosomal and RNA binding proteins found to be regulated by H1 (~ 9% of significant genes) implicates translational control as another possible means for suppressing metastasis. This is in addition to previously described roles in RNA processing (106) and functional interactions with 40S ribosomal protein S3 (RPS3) (153). Of particular interest to this study, RPS3 was shown to cooperate with H1 to influence ERK signaling in HT1080 cells while appearing to have an additional role in NFκB complexes (154). Years of research have uncovered extensive interdependence between cell signaling and translational control. Both Ras and PI3K mediated signal transduction induce translational machinery and activate the mTOR complex 1 as well as a variety of eukaryotic initiation factors (eIF), resulting in translation of genes involved in cell growth, proliferation and tumor progression (155-157). While the extensive interactions between ribosomal proteins, initiation and elongation factors, and mRNA are reviewed in detail elsewhere (158), the favorable outcomes from clinical trials testing inhibitors of specific eIFs and mTOR underscore the contributions of translational aberrancies in tumor initiation and maintenance (159-162). These studies also lend credence to the idea that H1 regulation of genes controlling translation, via expression changes, direct physical interaction or signal alterations, may play important roles in mediating the H1-dependent phenotype.

Cancer research has been greatly influenced by the concept of cancer stem cells in recent years as characteristics of many aggressive malignant cancers resembled those of embryonic stem cells in having a high propensity for self-renewal and proliferation (163). In the context of this theory, H1and other metastasis suppressors may be exerting effects on a stem cell-like subpopulation of cancer cells within a tumor which would be responsible for generation of metastatically competent progeny. The highly dedifferentiated stem-like cells are thought to give rise to slightly more committed progenitor cells that are still capable of becoming more specialized cell types, indicating that even the progeny are highly undifferentiated. As differentiation is considered to be an irreversible process, the cancer stem cells and their offspring have not yet transitioned to a designated cell fate and are equipped with substantial plasticity, much like an immature cell during development. Therefore, these dedifferentiated cells should easily adapt and thrive in a number of tissue microenvironments, much like the behavior of embryonic and metastatic cells.

This concept may help explain some of the data presented here. The motility associated morphology observed in scratch assays with WM793 cells is reminiscent of an immature melanocyte (164). Melanocytes of both adults and newborns are typically spindle shaped however early melanocytes are typically unpigmented and bipolar whereas differentiated melanocytes produce melanin and are polydendritic (165). The high degree of similarity between the morphology of immature melanocytes and the aggressive WM793 cells is intriguing in light of the cancer stem cell theory. Motile cells displaying bipolar dendrites are perhaps arising from a stem cell compartment and are being observed at a similar point in the primordial lineage as the early melanocyte. If indeed this is the case, these cells would be expected to possess the associated increased plasticity as well. The absence of this sub-population of cells in the H1-transfectants indicates a suppressive effect on this minority, possibly representing metastatically competent clones which would be selectively targeted by a metastasis suppressor. It is plausible that H1 is impeding these cells through the proposed model of intracellular signal modulation, as shRNA studies

would indicate that IQGAP2 and BRAP both play important roles in the observed phenotype. Multiple actions of NM23 may be leading to more tightly regulated, highly specific transduction which perhaps drives these cells towards a more differentiated cell fate, quenching the multipotency and plasticity of these cells and in turn, suppressing their metastatic capability.

In order to ever realize any clinical benefit from metastasis suppressor genes like NM23, a feasible method for reintroduction of the gene must be discovered. The most likely scenarios involve the identification of pharmacological compounds that can induce expression or the development of safe and effective gene therapy systems, as preliminary studies would suggest is possible (166-168). However, it becomes a question of when administration of such a treatment would actually be effective. If metastatic disease is already present, it is possible that a MSG would be innocuous, other than to possibly prevent further dissemination from the metastatic lesions themselves. This is because some MSGs, in particular H1, may affect key aspects of tumor spread independently of tumor growth. Therefore, an already established metastatic lesion may not regress upon forced expression of a MSG. Early detection of a primary tumor and administration of treatment prior to successful secondary site colonization would be required for a positive response, luxuries rarely afforded in clinical settings. It is possible that H1 could still impact dormant micrometastases at a site such as the pulmonary capillary beds that have yet to fully establish themselves, as these cells most likely must undergo further adaptation in order to colonize and would require a greater degree of plasticity than perhaps those of a full blown metastasis. Nonetheless, screening of pharmacological libraries is sure to identify novel compounds that can initiate *de novo* transcription of MSGs and will provide some therapeutic benefit, at least for a specific subset of patients.

An alternate approach was presented here with the identification of genes differentially expressed in an H1-dependent manner. The gene profile was used as a discovery tool for novel accessory proteins which facilitate the activity of H1. Characterization of factors mediating H1-dependent metastasis suppression

could lead to the development of novel therapeutics independent of H1 expression. Indeed, the success of B-Raf inhibitors (e.g., PLX4032) in clinical trials is of no surprise in light of the microarray data as the activity of this serine/threonine protein kinase seems to be consistently targeted by H1, indicating proof of principle. Further investigation into the complete mechanism used by H1 to modulate expression and signaling could stimulate new ideas for improving current treatment designs, preventing acquired resistance and enhancing the effectiveness against metastatic disease.

Future Directions

Before confidently accepting the role of H1 in modulation of MAPK signal transduction, a number of additional studies are in order. First, thorough characterization of altered signaling dynamics mediated by H1 is desired. Identification of specific patterns of phosphorylation changes resulting from H1 overexpression on effector molecules through the use of phosphor-proteome mass spectrometry analyses or simple phosphor-specific immunoblotting could greatly benefit our understanding of these processes. Additional studies using pharmacological inhibitors of MEK, PI3K, B-Raf among others in the panel of NM23 transfectants would shed light on any synergistic effects H1 may have on oncogenic signaling.

H1-dependent motility suppression is mediated at least in part by IQGAP2 and BRAP expression levels in this model of melanoma, however, the role of these genes in metastasis is not yet known. Animal models of tumor progression should be used to assess the contribution of these molecules to H1-mediated suppression of metastasis. Stable cell lines harboring RNAi molecules could facilitate such studies by investigating the dependence on these gene targets for acquisition of H1-dependent phenotypes. While it would be expected that these genes would indeed play important roles in metastasis due to the motility data, it is possible that the correlation between *in vitro* motility suppression and metastasis prevention in nude mice does not exist, as was realized with studies in 1205LU cells described in chapter 2.

A final area of investigation that should be pursued in light of this data is the possible negative influence of H1 on the cancer stem cell compartment. Analysis of effects levied against a stem-like cell by H1 could cause a paradigm shift concerning how we view a metastasis suppressor like H1. Assuming the presence of such a niche exists in tumors, it is plausible for metastasis suppressors to act specifically on this tumor-initiating sub-population, perhaps by applying selective pressure and preventing the outgrowth of highly aggressive progeny cells or through elimination of the metastatically competent cancer stem cells themselves. Isolation and purification of stem-like cells in order to assess basal levels of MSG expression would be an appropriate starting point, with the expectation that many genes in this class should exhibit low endogenous expression in this compartment. If the prediction is true, the development of therapeutic strategies designed to induce H1 expression may hold a great deal of promise for clinical blockade of tumor progression and metastasis.

Appendix

MSG: metastasis suppressor gene

NM23-H1 (H1): nucleoside diphosphate kinase A/ non-metastatic cells 1

WT: wild-type

Amino acid residue abbreviations:

A: alanine

D: aspartic acid

E: glutamic acid

F: phenylalanine

G: glycine

H: histidine

K: lysine

N: asparagine

P: proline

Q: glutamine

S: serine

V: valine

Y: tyrosine

DNA/RNA: deoxyribonucleic acid/ ribonucleic acid

VGP: vertical growth phase

RGP: radial growth phase

CD: circular dichroism

HPLC: high-pressure liquid chromatography

RT-PCR: reverse-transcriptase polymerase chain reaction

SDS: sodium dodecyl sulfate

UV: ultra-violet

ATP: adenosine triphosphate

NTP/NDP: nucleoside triphosphate/ nucleoside diphosphate

EMT: epithelial to mesenchymal transition

ESC: embryonic stem cell

MAPK: mitogen activated protein kinase

MEK: mitogen activated protein kinase kinase

ERK: extracellular signal-regulated protein kinase

B-Raf: v-raf murine sarcoma viral oncogene homolog B1

Ras: Ras oncogene homolog

PTEN: phosphatase and tensin homolog

CDC42: cell division cycle 42

Rac: ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)

Rho: ras homolog gene family

KSR: kinase suppressor of Ras

Tiam1: T-cell lymphoma invasion and metastasis 1

Rad: Ras-related associated with diabetes

NFkB: nuclear factor kappa B

PI3K: phosphoinositide-3 kinase

BRAP: BRCA1 associated protein

IQGAP2: IQ motif containing GTPase activating protein 2

LCN2: lipocalin 2

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