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Grainyhead-like 2 Reverses the Metabolic Changes Induced by the Oncogenic Epithelialmesenchymal Transition: Effects on Anoikis

Joshua C. Farris

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**Grainyhead-like 2 Reverses the Metabolic Changes Induced by the Oncogenic Epithelial-
mesenchymal Transition: Effects on Anoikis**

Joshua C. Farris

**Dissertation submitted to the School of Medicine at West Virginia University in partial
fulfillment of the requirements for the degree of
Doctor of Philosophy in Cancer Cell Biology**

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**Keywords: EMT, grainy head like 2, GLUD1, metabolism, cancer stem cell, reactive
oxygen species, anoikis**

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Abstract

Grainyhead-like 2 Reverses the Metabolic Changes Induced by the Oncogenic Epithelial-mesenchymal Transition: Effects on Anoikis

Joshua C. Farris

When deprived of connections to their extracellular matrix, normal epithelial cells trigger a process of programmed cell death referred to as anoikis. The transcriptional reprogramming event known as Epithelial-Mesenchymal transition (EMT) confers anoikis resistance and ultimately, increased metastatic potential. Distant metastatic disease contributes to the majority of deaths occurring in many cancer types, especially those originating in breast tissue. The ability to metastasize depends critically upon a cancer cell's ability to evade anoikis. Heterogeneity of tumors containing anoikis, therapy resistant populations likely explains the tendency of disease to reappear in distant locations after a period of apparent remission. The wound healing transcription factor, Grainyhead-like 2 (GRHL2), has been previously demonstrated to play an indispensable role in the maintenance of the epithelial phenotype through both suppression of EMT and promotion of the reciprocal event, Mesenchymal-Epithelial transition (MET), accompanied by suppression of the cancer stem cell (CSC) phenotype and re-sensitization to anoikis. Loss of GRHL2 expression is associated with aggressive, metastatic breast tumor types such as claudin low and basal B subclasses of tumor cell lines. Specifically, GRHL2 expression is lost in the cancer stem cell-like compartment of tumors characterized by their enrichment in the CD44^{hi}/CD24^{low} cell population and an EMT phenotype. Constitutive expression of the epithelial enforcer GRHL2 results in increased anoikis sensitivity as well as reduction in tumor initiating frequency. Here, the effects of GRHL2 upon intracellular metabolism in the context of reversion of the EMT/CSC phenotype, with a view toward understanding how these effects promote anoikis sensitivity were investigated. EMT resulted in enhanced mitochondrial oxidative metabolism. While this was accompanied by higher accumulation of superoxide, the overall level of Reactive Oxygen Species (ROS) declined, due to decreased hydrogen peroxide. Glutamate Dehydrogenase 1 (GLUD1) expression increased in EMT, and this increase, via the product α -ketoglutarate (α -KG), was important for suppressing

hydrogen peroxide and protecting against anoikis. GRHL2 suppressed GLUD1 gene expression, decreased α -KG, increased ROS and sensitized cells to anoikis. These results demonstrate a mechanistic role for GRHL2 in promoting anoikis through metabolic alterations.

Dedication

This dissertation is dedicated to my loving wife Breanne Farris, who has consistently supported me through my undergraduate education, medical school, and graduate school. I would also like to dedicate this work to my parents Gena' and Mickey Farris, as well as my brother and sister-in-law Michael and Sun Hee Park. I would like to thank my mother and father-in-law, Richard and Susan Yingling, my sister-in-law Jerica Yingling, Collette, Eric, Aida, and Savannah Sites, as well as the rest of my family and friends. They have supported me through my frustrations, and celebrated my successes. Without their support through my doctoral training, I would have surely failed along with the many failed experiments. Lastly, I would like to dedicate this dissertation to my grandmother, Virgie Pauline Sprouse, whose struggle with cancer inspired me to embark on this path.

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Chapter 1

Introduction and Literature Review

Epithelial-to-Mesenchymal Transition

Epithelial vs. Mesenchymal Cell Adhesion/Polarity

The major role of epithelial tissues is to function as barriers to physiologically separate compartments. Epithelial cells display a unique gene expression profile relative to mesenchymal cells which endows them with the ability to achieve the two major characteristics of epithelial tissues: cell-cell adhesion and cell polarity. Phenotypically, epithelial cells have a tendency to exist as a monolayer in a classically described cobblestone pattern appearance often seen in two-dimensional tissue culture. These epithelial monolayers are maintained due to the expression of specific epithelial proteins including E-cadherin, occludins, desmogleins, claudins, and desmocollins located at cellular junctions (1-3). *In vivo*, however, epithelial cells generally polarize resulting in one side of cells attaching to extracellular matrix (ECM) with its basal side attached to the basement membrane, while the apical surface is oriented toward the lumen of a vessel or duct. Epithelial cells rely on basolateral membrane interactions in order to survive (see section on anoikis below). Cells maintain polarity through organization of tight junctions via claudins and occludins, which restriction the diffusion of integral membrane proteins from apical to basolateral membranes (4). Rab25 and Ankyrin G are epithelial-specific proteins found in the cytosolic compartment of cells which act to ensure proper localization of junctional proteins (5, 6). A major example of how the epithelial architecture maintained by cell-cell adhesion molecules contributes to the gene expression profile of epithelial cells is that of Epithelial-cadherin, or E-cadherin. The extracellular domain of E-cadherin functions to interact homophilically with the extracellular region of adjacent cell E-cadherin proteins. The intracellular domain acts

to orchestrate cortical actin assembly, cell polarity, and influences gene expression through sequestration of β -catenin, all contributing to the epithelial phenotype (7, 8).

Conversely, mesenchymal cells either do not express, or express very low levels of many of the previously mentioned junctional proteins. In fact, a microarray comparison of epithelial vs mesenchymal gene expression profiles reveals that these two phenotypic cell variants differ dramatically (9). The inability to produce tight junctions, adhere with neighboring cells, and thus establish apical-basolateral polarity results in failure to maintain barriers like their epithelial counterparts. Mesenchymal cells tend to migrate as individual cells, and they are generally significantly more invasive (10). Due to these features, mesenchymal cells lack the same dependence on their extracellular matrix for survival as do epithelial cells, resulting in their resistance to cell death when detached from their matrix (see anoikis section below) (11-15). In addition to the loss of cell adhesion and polarity associated proteins, mesenchymal cells gain expression of other mesenchymal markers such as N-cadherin, vimentin, fibronectin, and upregulation of transcription factors associated with repression of the epithelial phenotype (more on this in a later section).

Whereas epithelial cells tend to form linings of organs or ducts due to their ability to establish strong barrier compartments, normal mesenchymal cells in adults tend to be involved in establishment of the extracellular matrix, as is the case with normal adult fibroblasts, or they give rise to other tissue types altogether, as is the case with Mesenchymal stem cells (MSCs). For example, MSCs can give rise to adipocytes, osteoblasts, and chondrocytes.

Epithelial-to-Mesenchymal Transition: Role in Normal Physiology

The epithelial-mesenchymal transition (EMT), a process implicated in cancer progression as well as other pathogenic processes such as fibrosis, describes a major shift which endows epithelial cells with characteristics of mesenchymal cells including disruption of cell-cell adhesion, acquisition of increased motility and invasiveness, chemotherapeutic resistance, and resistance to anoikis. While cellular plasticity between these two states can drive the progression of disease, in the context of normal physiological function, EMT and its converse process termed mesenchymal-to-epithelial transition (MET), are indispensable to events such as embryological development and wound healing (16). Three separate categories of EMT have been described which vary based on the tissue involved as well as the resulting changes (17). Type 1 EMT refers to implantation, embryo formation, and organ development. For example, both Snail1 and 2, well established EMT associated transcription factors, are required in early embryogenesis, as it has been demonstrated that embryos depleted of these factors fail to gastrulate as a result of unyielding epithelial junctions of mesodermal cells, preventing formation of the primitive streak (18, 19). The importance of EMT plasticity is also displayed soon after primitive streak formation, where strong epithelial junctions are required for neural tube closure, which is followed immediately by an EMT event which is required during neural crest delamination (20-22). These EMT'd cells then migrate away from the neural tube only to MET in order to develop into structures such as the somites and the notochord (23).

In adults, wound healing is a dynamic process that requires plasticity between epithelial and mesenchymal states. Type 2 EMT refers to the cellular transitions that occur during wound healing, tissue regeneration, and organ fibrosis. Epidermal wound healing

requires the activity of myofibroblasts both for wound contractility as well as restructuring of the extracellular matrix. Keratinocytes at the wound border recapitulate the process of EMT by exhibiting a partial EMT or “metastable” phenotype in which they are able to move, advancing an attached epithelial sheet rather than invading as individual cells (23, 24). TGF- β induced EMT of epithelial cells also occurs within an organ in some pathogenic states, and this can contribute to the tissue fibrosis wherein fibroblasts generate excessive levels of collagen (25).

EMT and Oncogenesis

Aberrant regulation of normal cellular processes such as cellular proliferation and growth are common features to many malignancies. EMT is another example of a normal process that is often employed inappropriately in the context of cancer progression. Type 3 EMT refers to plasticity between epithelial and mesenchymal states that results in metastatic progression of malignant disease. A multitude of (epi)genetic changes can alter oncogene and tumor suppressor expression resulting in neoplastic growth. This growth is typically characterized by increased proliferation and angiogenesis. Many cues in the microenvironmental milieu can influence cell fate and result in induction of EMT such as nutrient or oxygen starvation (26, 27). It is well established that oncogenes are known to induce senescence in some contexts. There is evidence to suggest that cancer cell EMT events may play a role in the escape of oncogene-induced senescence, thereby leading to the acquisition of many deleterious traits by cancer cells (28-30). These traits will be detailed below.

Migration, Invasion, and Chemotherapeutic Resistance

While aberrant epithelial cell proliferation and angiogenesis are indeed early hallmarks in the initiation of epithelial cancers, the major event which signals the onset of metastatic dissemination of these carcinomas is invasion, often conferred by EMT, leading to cancer cells passing through the basement membrane (17, 31). A widely studied feature that EMT induces in carcinomas is the gain of migratory and invasive properties, as this is considered the final stages in the cascade of oncogenesis leading to metastatic dissemination of disease. The association of EMT with invasion has been observed in many *in vivo* and *in vitro* experimental models in which carcinoma cells acquire mesenchymal traits and upregulate mesenchymal markers such as α -SMA, FSP1, vimentin, and desmin at the invasive front of primary tumors – the ultimate result being the activation of the metastatic cascade including intravasation, circulatory transport to a secondary site, extravasation and formation of micrometastases (17). Generally speaking, one of the major alterations that occurs as a result of EMT that influences this invasion is the loss of the cell-cell adhesion intermembrane protein E-cadherin. Experimentally, enforced expression of E-cadherin abrogates invasive properties in cancer cells, and clinically, expression of E-cadherin is associated with lower risk of tumor invasion (32-35). E-cadherin may block these invasive traits either physically, a result of homophilic interactions with the extracellular domain of other E-cadherin molecules favored more highly than interactions with extracellular matrix. This invasive phenotype is also influenced through other pathways that E-cadherin interacts with such as polarity complex interactions, sequestration of pro-survival factors such as NRAGE, β -catenin, YAP/TAZ (signaling molecules involved in HIPPO signaling), and Smad3 (critical for TGF- β signaling). These pathways will be discussed further in discussions of

anoikis below. In addition to the association with metastatic phenotype, EMT also confers upon cancer cells the pro-survival effects of chemotherapeutic resistance to cytotoxic chemical agents and radiation which have been extensively documented (36-39).

EMT confers Cancer Stem Cell Properties

It was long ago hypothesized in pioneering work, originally in the context of hematological malignancies, that only a small subpopulation of the leukemic cancer cell lines has the intrinsic ability to recapitulate the original disease (40-43). This notion has held true in solid tumors as well, specifically in the context of breast and brain cancers (44, 45). This subpopulation is known as cancer stem cells (CSC) or tumor initiating cells (TICs). There are several explanations for the origins of these populations in tumors. For example, it was demonstrated by Eric Landers group that phenotypic equilibrium between non-CSC and CSC populations are the result of “stochastic state transitions” which is predictable given a particular mathematic algorithm (46). It has also been reported that EMT results in the acquisition of stem cell markers, an increased ability to form mammospheres as well as increase in the ability to seed tumors in animal hosts, both well-established characteristics of stem cells (47). Subsequent work has demonstrated that it is possible to physically segregate two populations from a single tumor sample which differ in their cell-surface antigen marker profiles (eg. CD24 and CD44 cell surface markers in the case of breast cancer). Of critical importance is the observation that when re-implanted in vivo, these CSC populations then produce tumors which are pre-dominantly not CSCs, demonstrating their stem cell-like self-renewal properties. These observations present a unique challenge to therapeutic intervention in that if any CSCs are not eradicated in the treatment of the

primary malignancy, they contain the ability to regenerate the entire disease in the form of local recurrence or even drive the emergence of distant disease at metastatic nodes.

The field of CSCs/TICs is quite controversial, both in terms of their origin as well as what percentage of the total malignancy they truly comprise (44). A common CSC surface profile used in the case of breast cancer is the CD44^{HIGH}/CD24^{LOW} phenotype which has been used to isolate the tumorigenic from the non-tumorigenic population (45). It was shown by Mani et al. that normal immortalized human mammary epithelial (HMLE) cells display a phenotypic conversion toward the CD44^{HIGH}/CD24^{LOW} phenotype following induction of the EMT program (47). It has also been demonstrated that a distinct group of EMT'd cells exists within the epithelial HMLE cell line termed MSP or mesenchymal-subpopulation which is dependent on paracrine and autocrine signaling pathways including TGF- β , canonical as well as noncanonical Wnt activation, as well as BMP pathway inhibition (10). A recent publication by Scheel et al. however, demonstrated that transient Twist1 activation permanently alters cell state and primes epithelial cells for stem cell-like properties which did not require permanent acquisition of the EMT phenotype (48). This finding informs the intimidating reality that any tumor cell subjected to the proper conditions in the tumor microenvironment may undergo EMT and thus potentially become a cancer stem cell highlighting the need for targeted therapy to eradicate this subpopulation.

The extent of involvement of EMT/MET in the area of metastasis is an area of controversy in the field currently. Reversion of mesenchymal to epithelial cells in some contexts has been shown to enhance metastatic colonization (17). This has led to the establishment of a theory opposing the prevailing dogma that instead of EMT as a culprit for metastasis, MET is the critical event which ultimately leads to the establishment of

secondary tumors and eventual death of cancer patients. For example, it has been demonstrated by Nieto and colleagues that downregulation of the EMT inducing homeobox transcription factor Prrx1 is required for cancer cells to seed metastatic locations *in vivo*; however, it was noted in this study that Prrx1 overexpression does not induce cancer stem cell-like traits as does ZEB1, Twist, or Snail mediated EMT events as previously described (47, 49-51). In fact, breast cancer metastases are typically epithelial and express E-cadherin (52). However, it is undeniable that EMT invokes many traits in cancer cells associated with aggressiveness, invasion, anoikis resistance, therapeutic resistance, and tumor recurrence. A potential reconciliation of these views are that EMT/MET plasticity is in fact the major force to endow cancer cells with their malignant properties. An extension of this interpretation would imply that either EMT or MET could be viewed as tumor suppressive or oncogenic events depending on the stage of disease progression. This observation that metastatic tumors often do not resemble the mesenchymal cells which gave rise to them is likely a consequence of differing local microenvironmental stimuli encountered following extravasation into distant organs, where the stress signals initially invoking EMT in the primary tumor are no longer present (31, 53, 54). It is clear that a better understanding of the major regulators of EMT and MET is critical in order to prevent the emergence of treatment resistance metastases and recurrence.

Regulation of EMT

EMT Induction – Transcription Factors

Many factors are known to induce EMT. These factors far outweigh the number of EMT inhibitory elements. A multitude of extracellular signals originating from the stromal tissue associated with the primary tumor are known to stimulate the EMT program including

HGF, EGF, PDGF, and TGF- β . These growth factors activate a horde of receptors to ultimately result in the induction of many proteins including major EMT inducing transcription factors such as Snail, Slug, Twist, Goosecoid, FOXC2, and ZEB1. Many of these factors when overexpressed alone, can lead to the orchestration of the EMT program, while the mechanism varies. Snail and ZEB1, well established E-box binding transcription factors, function as direct repressors of epithelial genes such as E-cadherin (55-58). Twist, Goosecoid, and FOXC2 are inducers of EMT which coordinate through more indirect means, typically through Snail or ZEB1 upregulation (9, 23). Supporting the view of the role of oncogenic EMT, overexpression of these transcription factors leads to progression of malignancy in multiple models.

Very well characterized signaling networks including PI3K, Akt, ERK, MAPK, Ras, RhoB, β -catenin, LEF, c-FOS, and Smad are critical components of the implementation of the EMT program (17). Cell surface integrins also play a role in the activation of EMT, enabled by the disruption of cell-cell and cell-matrix adhesions (30, 47, 59). As previously mentioned, YAP and TAZ, critical components of the Hippo signaling pathway, are examples of pro-survival transcription factors that are also sequestered by cell-cell and epithelial cell polarity complexes (9, 60). Homeoproteins such as HOXA5, LBX1 and SIX1 are further examples of well-established TFs involved in developmental EMT which also play a role in the oncogenic EMT, through direct transactivation of the ZEB1 promoter (61-63).

TGF- β superfamily ligands bind to TGF- β R1/R2 heterodimeric receptors resulting in the serine phosphorylation of Smad2/3 (often referred to as R-Smads or receptor regulated Smads), which then can bind Smad4, resulting in nuclear translocation and upregulation of many genes ranging from growth inhibitory to EMT activating genes such as ZEB1 (64, 65).

It is in large part this enormous range of regulated genes that gives the TGF- β -Smad2/3 axis its context dependent, and seemingly paradoxical effects of either oncogenesis or tumor suppression (66, 67). Recently published work by our lab shines some light on what determines which response a cell line will have to TGF- β signals (ie. Growth arrest vs EMT). This will be discussed further below in the section on Grainyhead-like 2.

Inhibition of EMT

As is evident above, many factors contribute to the induction of the EMT process; however, there are relatively fewer inhibitors of oncogenic EMT. Included in this list are BMPs, the mir200 family, ESRP1/2, and the human homologue of drosophila Grainyhead, Grainyhead-like 2 (GRHL2). BMPs or Bone Morphogenetic Proteins, named for their ability to induce formation of bone and cartilage, belong to the TGF- β superfamily (68). BMPs represent a major class of EMT inhibitors which induce MET through inhibition of TGF- β signaling. Overexpression of BMP family members has been shown to enforce the epithelial phenotype, and pharmacologic studies have verified that TGF- β receptor inhibition results in effects mimicking that of BMPs (10, 68-70). Further, the BMP functional inhibitors chordin and gremlin are known to enhance TGF- β induced EMT (71). This signaling is accomplished via BMP ligand binding to BMPRII/R1B heterodimeric complexes resulting in R-Smad1/5 phosphorylation, which complete with R-Smad2/3 for binding to Smad4 (70).

EMT is further controlled by microRNAs, specifically those of the miR-200 family. These small noncoding endogenous regulators of gene expression have been shown to be downregulated during EMT, and their enforced expression inhibits TGF- β induced EMT and is sufficient to initiate MET in mesenchymal populations (72). It has been well established that the primary mechanism by which the miR-200 family acts to enforce the epithelial

phenotype is through post-transcriptional repression of ZEB1/2, and in fact, a negative feedback loop has been demonstrated such that ZEB transcription factors transcriptionally repress miR-200 expression (64, 72-76).

Yet an additional example of post-transcriptional regulation of the EMT phenotype is that of the mRNA splicing factors ESRP1 and ESRP2. These splicing regulatory proteins are downregulated during EMT. Their depletion by shRNA results in EMT associated with loss of cell polarity and characteristic upregulation of fibronectin, a mesenchymal associated extracellular matrix glycoprotein, and the intermediate filament vimentin. Interestingly, it has been reported that loss of ESRP1 and 2 contributes to the acquisition of EMT traits by independent mechanisms. Stable knockdown of ESRP1 has been shown to result in change in cell motility via induction of Rac1b, whereas loss of ESRP2 results in abrogated cell-cell adhesion through induction of EMT associated transcription factors (77).

Finally, another major negative regulator of EMT is one of the three mammalian homologues of drosophila Grainyhead, known as Grainyhead-like 2 (GRHL2 - also known as TFCP2L3 or BOM). GRHL2 represses the oncogenic EMT through multiple mechanisms including direct repression of the ZEB1 promoter, upregulation of miR-200b/c, upregulation of BMP2, and inhibition of TGF- β -Smad2/3 mediated transcription (78, 79). These and other findings have established GRHL2 as a master regulator of the epithelial phenotype and repressor of EMT plasticity. A more detailed review of the functions of Grainyhead-like 2 transcription factor in the context of cancer will be presented in a later section.

Anoikis

Physiological Role of Anoikis

Normal epithelial cells undergo a program of apoptosis when detached from their ECM or when attached to an inappropriate matrix termed “anoikis”(11). Anoikis, the Greek word meaning “homelessness,” is a normal apoptotic process which ensures epithelial cells, which are often shed at a high frequency, are eradicated, such as in the case of skin or colonic epithelium (15). The process of anoikis ensures that cells are unable to take up residence in inappropriate locations. What factor(s) establish sensitivity vs resistance to anoikis has been the major question in the field since its discovery.

This is of significant importance in the case of oncogenic transformation of cells. Anoikis prevents the translocation of cells which have (epi)genetic changes from attaching elsewhere and exploiting their growth/survival advantages at these secondary locations. Metastasis of cancer cells requires the suppression of anoikis (80-83). The apoptotic mechanism induced by detachment is not specific to this context, and is regulated by normal death-effector machinery common to other forms of cell death signaling pathways (84). Therefore, regardless of the exact mechanism of activation, these signals typically culminate with mitochondrial outer membrane permeabilization (MOMP), resulting in the release of cytochrome c generating a caspase-9 containing apoptosome, ultimately activating effector caspases. Bcl-2 family proteins play anti/pro-apoptotic roles upstream of mitochondrial membrane permeabilization, cytochrome c release, and apoptosome formation as in other apoptotic settings. In this vein, BH3-only family genes (including Bim, Bad, Bmf, Noxa, and Puma) are exquisitely sensitive to integrin-assisted signal transduction, and these pathways have been widely characterized in the setting of anoikis (85). For example, Bim, a BH3-only family protein, has been shown to act as a mediator of anoikis dependent on β 1-integrin engagement, EGFR downregulation, and inhibition of the Erk pathway in detached basal

mammary epithelial cell line MCF10a cells (86). While the results do not appear generalizable to all cell lines or contexts, death receptor signaling has been reported to be involved in anoikis signaling such that the FAS-associated death domain protein (FADD) has been shown to contribute to anoikis sensitivity, though the precise mechanisms are unclear (87).

The influence of other inputs upstream of the apoptotic machinery which influence anoikis such as reactive oxygen species and metabolism have been topics of significant recent research. The contribution of these variables to differences in anoikis sensitivity endowed by the epithelial-mesenchymal transition is poorly understood however, and will be discussed further below.

The Oncogenic Epithelial-to-Mesenchymal Transition Confers Resistance to Anoikis

Among their many deleterious traits, metastatic tumor cells have a compromised anoikis response (14, 88). As discussed previously, EMT is generally viewed as a major contributory program driving metastasis. In addition to increasing migratory and invasive properties, it has become well established that EMT also induces anoikis resistance in cancer cells (14, 89, 90). As was alluded to earlier, many of the core cellular signaling pathways involved in the implementation of the EMT program such as PI3K/Akt and MAPK activation also contribute to cell survival in general, anoikis being no exception (91).

E-Cadherin, Ankyrin-G, Wnt/ β -catenin and TGF- β , and HIPPO Contribute to Anoikis

In the seminal work in which anoikis was first identified, epithelial cells, specifically Madin-Darby canine kidney epithelial (MDCK) cells, were noted to be exquisitely sensitive

to this mode of apoptosis, especially when grown to confluence, an observation also reported elsewhere (11, 92). While the mechanism contributing to this phenomenon is not fully explained, several points of evidence point to a major role of the cell-cell adhesion protein E-cadherin to explain these findings. In a p53-dominant negative tumor model, depletion of E-cadherin in a conditional knockout significantly increased tumor metastasis *in vivo*; moreover, the resulting cell lines produced from this study were anoikis resistant compared to their E-cadherin positive counterparts (93). These findings have also been further generalized to include the Human Mammary Epithelial Cell line (HMLE) cells (94). Interestingly, the major characterized pathways upstream of anoikis resistance observed in mesenchymal cells have a common node emanating from the cell-cell junctional protein E-cadherin. These pathways include the Ankyrin-G/NRAGE/p14ARF, Wnt/ β -catenin, and the HIPPO/YAP/TAZ pathways. While these pathways contribute in individual ways to both EMT, and its conferred anoikis resistance, they are highly interrelated, resulting in significant cross talk between each pathway (95). Each of these pathways and their contribution to anoikis resistance in the context of EMT will be outlined below.

Our lab previously reported the role in anoikis of the E-cadherin associated gene, Ankyrin G (Ank-3), an epithelial cytoskeletal linker protein responsible for sequestration of the transcription factor NRAGE. NRAGE was found to be sequestered by Ankyrin-G, preventing its nuclear translocation where it typically represses the pro-apoptotic gene p14ARF; however, in the context of EMT, E-cadherin and Ankyrin-G downregulation result in unhindered NRAGE to undergo nuclear translocation, repress p14ARF and contribute to anoikis resistance (12). E-cadherin further influences anoikis sensitivity by way of the Wnt/ β -catenin signaling axis. In the context of E-cadherin depletion, which by its

intercellular domain acts to sequester β -catenin, HMLE cells exhibited an increase in β -catenin phosphorylation, stability, and nuclear translocation, resulting in transactivation of target genes and anoikis resistance (94).

Apical/baso-lateral polarity was described above as a major trait of epithelial cells - one that is lost during EMT, either by downregulation of these components or failure of proper localization (96-98). Crumbs, Par and Scribble are polarity complexes in normal epithelial cells which contribute to apical, apical/basolateral border, and basolateral side polarity, respectively (99). The HIPPO kinase pathway has previously been well established as a critical regulator of organ size during development (100). Cell-cell contacts promote the formation of the polarity complexes Crumbs, Par, and Scribble; interactions of the HIPPO signaling pathway with these complexes results in phosphorylation and cytoplasmic sequestration of the pro-survival factors YAP/TAZ (95, 101, 102). When in their unphosphorylated forms, YAP/TAZ are localized to the nuclear compartment, where they interact with Smad proteins (TGF- β signal transducers discussed previously) to activate a large variety of target genes. Loss of cellular polarity is thought to result in resistance to anoikis, at least in part, through deficient HIPPO kinase cascade activation where cytoplasmic retention of YAP/TAZ transcriptional co-activators is lost, resulting in nuclear translocation and repression of pro-apoptotic protein Bim. Further evidence that cell polarity established through HIPPO kinase cascade is linked to anoikis was shown where luminal clearance, a process in which anoikis is required, was shown to be reliant on the cell polarity complex Scribble assessed by MCF10a mammary morphogenesis assays (103).

Of note, the apoptosis stimulating protein of p53 effector protein, ASPP1, acts as an activator of YAP/TAZ transcriptional activity through inhibition of kinases LATS1 or LATS2,

which normally modify these survival co-activators such that they are tagged for nuclear export and degradation. It has been reported that ASPP1 mediated downregulation of Bim mediates cell survival, enhanced cellular migration, and anoikis resistance, indicating an oncogenic role of this HIPPO associated protein, although the exact mechanism of Bim downregulation is unclear at present (104). Further, EMT induced mislocalization of scribble resulting in TAZ activation and increased anoikis resistant mammospheres (102). Hippo signaling may explain the initial observations of increased sensitivity to anoikis of confluent MDCK cells, as it has been reported that the Crumbs complex acts as a mode of sensing cellular density, and these signals are relayed through YAP/TAZ interaction with the TGF- β pathway (105).

Alternative to inhibition of direct transactivation of pro-survival target genes of YAP and TAZ, the HIPPO pathway also functions to inhibit the Wnt and TGF- β pathways, both of which contribute to EMT and anoikis resistance (described above). This is accomplished through direct protein-protein interactions of phosphorylated forms of YAP/TAZ with the Crumbs polarity complex and Smads, resulting in inhibition of both pathways. Therefore, cellular polarity is able to regulate TGF- β signaling beyond E-cadherin direct sequestration of β -catenin. This is accomplished through YAP/TAZ binding to Smad3, which relies on receptor mediated phosphorylation through TGF- β R1/R2. LATS1/2 phosphorylation of YAP/TAZ results in sequestration of these factors to the cytoplasm, effectively binding Smad3 to this compartment as well (95, 106). Therefore, upon loss of cell polarity, Smad sequestration by YAP/TAZ is inhibited, allowing for further reinforcement of EMT through TGF- β signaling. It has also been demonstrated that phosphorylation of YAP is regulated through an integrin-matrix interaction involving actin-tubulin rearrangement where cell

detachment activates LATS1 and LATS2 resulting in YAP sequestration (107). Cross talk of the HIPPO pathway with Wnt activation is also present, as it has been demonstrated that through Casein Kinase I (CKI), YAP and TAZ interact with the disheveled homologue Dvl, inhibiting its activation, resulting in canonical Wnt inactivation (108). Thus, these findings demonstrate the multiple levels of regulation on the HIPPO pathway and cross-talk between other critical pathways for control of anoikis and demonstrates how these are influenced by the EMT phenotype.

Interestingly, there are cellular polarity complexes whose role in contributing to anoikis pathways seems limited or context dependent. Loss of scribble in keratinocyte models have revealed alterations in cell invasion with very little effect on anoikis; however, depletion of Dlg1 (disc large homologue 1) results in maintenance of cell polarity but significantly suppresses anoikis (109).

Direct Influence of EMT regulatory Transcription Factors on Anoikis

Above, an indepth review of major transcription factor regulation of the process of EMT itself was given. Here, the modification by these EMT associated transcription factors to the core apoptotic machinery will be described. As was mentioned earlier, the major E-box binding transcription factors Snail, Slug, Twist, and ZEB1/2 act predominantly as transcriptional repressors of the major adhesion genes such as E-cadherin, and those components contributing to the formation of tight junctions and desmosomes. Given the known critical function of maintenance of cell polarity on preserving anoikis sensitivity, it follows that these EMT factors which disrupt cell polarity will indirectly result in suppression of anoikis due to dysregulation of the pathways described above.

These transcription factors also contribute to the acquisition of anoikis resistance through regulation of cell-survival factors as well. ZEB1 represses the pro-apoptotic gene *Trp73* through direct E-box binding to intron 1 (110). Snail has been described to contribute to cell survival pathways through upregulation of MAPK and PI3K signaling pathways (111). Both, Snail and Slug have been reported to promote resistance to cell death through direct transcriptional repression of pro-apoptotic genes such as BID and caspase-6 (112). Snail also has been shown to suppress genes associated with p53-mediated apoptosis, promoting cell survival (113). The pro-apoptotic gene p14ARF was described above as the major target of the Ankyrin-G sequestered transcription factor NRAGE, which is released following EMT (12). Myc induced apoptosis has been shown to rely on formation of the pro-apoptotic Myc-ARF complex (114). Twist mediates direct repression of p14ARF, preventing apoptosis by interfering with the ability of Myc to form this complex, thereby collaborating with Myc to result in a transformed phenotype rather than inducing cell death (115). The transcriptional co-repressor CTBP is generally required for EMT induction and attenuation of anoikis (55). Mechanistically, this may occur through ZEB1 co-repression of E-cadherin (15), but it has also been demonstrated that CTBP can repress Bik (Bcl-2-interacting killer) resulting in further repression of the anoikis response (116).

In some contexts, NF- κ B is a critical component of EMT initiation in response to inflammatory pathway activation (117, 118). This signaling pathway has also shown to be critical for anoikis regulation in both the context of intestinal epithelial cells, as well as in basal breast mammary epithelial cells where DBC1 was reported to stimulate NF- κ B pathway activation through direct interaction with IKK- β , stimulating its kinase activity, which increased NF- κ B transcriptional upregulation of c-FLIP and bcl-xl survival factors

(119, 120). Other pro-survival target genes of this pathway include several members of the IAP (inhibitor of apoptosis) family, bcl-2, CFLAR, survivin, and XIAP (15). Survivin specifically has been shown to mediate anoikis resistance via complex with XIAP which activates transcriptional activity of NF- κ B to result in fibronectin increase which maintains clustering and β 1-integrin signaling to rescue from anoikis (121).

FAK and ILK signaling pathways– Anoikis and EMT

Activation of FAK and ILK pathways results in suppression of anoikis (92, 122). This is generally by promoting the epithelial-mesenchymal transition resulting in the repression of epithelial adhesion genes such as E-cadherin through multiple pathways; however FAK has been reported to influence expression of multiple E-box binding transcription factors such as Snail, Twist, and ZEB1/2 (123). Co-localization of these proteins with integrins links activation of these pathways with integrin-ligand cell-ECM interactions (124-126). FAK and ILK are activated by attachment to matrix components such as collagen and influence EMT inducing machinery as well as are activated by growth factor induced EMT (mediated by TGF- β , EGF, HGF, etc.) (127-130).

While EMT linked anoikis resistance appeared to be an insurmountable obstacle to cancer researchers for many years, the emergence of master regulators of the epithelial phenotype (e.g. GRHL2) give hope to the possibility that EMT suppression and enhancement of anoikis sensitivity in cancer cells might be a therapeutic possibility through determination of pathways which suppress these important transcription factors (of significant interest are the Wnt, TGF- β , and BMP pathways). This would inform treatment protocols and may greatly reduce metastasis and disease recurrence usually associated with the EMT

phenotype. The mechanisms of GRHL2 induced MET and anoikis sensitivity will be discussed in great detail in later sections. First, alternative contributors to the induction of anoikis will be highlighted.

Reactive Oxygen Species

Reactive Oxygen Species and Intratumoral Cellular Metabolic Needs

It is not fully clear why cancer cells initially engage a program of phenotypic and (epi)genetic alterations, such as EMT, that ultimately culminate with leaving their primary site. This has pointed to the idea that a problem exists in their primary microenvironment. Oncogenic mutations in tumors generally lead to properties that are advantageous to the tumor in the short term such as increased proliferation; however, the primary site in which they are located typically lacks the vasculature to support this activity, leading to challenges such as lack of carbon source nutrients, hypoxia, and resulting reactive oxygen species accumulation.

In some cases, oncogenic alterations may in fact be harmful and threaten cell survival. As was mentioned earlier, c-Myc forms a complex with p14ARF, which, if not accompanied by defects in the basic apoptotic machinery, will lead to devastating consequences. In the case of oncogenic EMT, E-cadherin and the associated cytoskeletal linker protein, Ankyrin-G, are downregulated, resulting in NRAGE nuclear translocation and suppression of p14ARF, which is also directly suppressed by Twist activation (12). Prior to an increase in angiogenesis, the rapidly proliferating tumor cells can be exposed to hypoxic conditions. This results in the need for tumor cells to increase their anabolic processes in the face of anaerobic conditions.

There is evidence that these challenges to the tumor microenvironment may be the very driving force to invoke adaptive changes such as the EMT transcriptional program. This is demonstrated by observations that in hypoxic conditions, HIF1 α stabilization occurs, resulting in the induction of a myriad of genes to combat the hostile environment. Hypoxia, paradoxically, results in the generation and accumulate of reactive oxygen species (80). Stabilization of HIF1 α , usually targeted for ubiquitination by Von-Hippel-Lindau protein (VHL) (131), results in the induction of multiple antioxidant genes (80) as well as EMT inducing transcription factors including Twist, Snail, and ZEB1 (27, 132).

While high ROS levels are known to induce various types of cell damage, tumor cells have been reported to have higher ROS relative to “normal” tissues, and are thought to upregulate antioxidant enzymes in order to keep ROS within a “non-lethal” threshold limit. Of importance, the observation that tumors generally have elevated ROS levels (133), comes with the caveat that these comparisons normally compare bulk, predominantly epithelial tumor samples to “normal” tissue which in the case of many cancer types, especially breast cancer, is often stromal tissue containing an entirely different gene expression profile. Therefore, comparisons of these tissue types which claim ROS as a survival factor in some contexts, should be considered with caution. The true role of ROS is likely as not as simple as either cell survival protagonist or antagonist. The maintenance of ROS within a crucial range may aid in some cellular processes acting as a signaling molecule of sorts (134), while rising beyond that range can be catastrophic, requiring safeguards such as antioxidant gene expression. In fact, when totally quenched by pharmacologic intervention, ROS levels plummet resulting in cell cycle arrest or even apoptosis (135). In light of these observations, the evolving model posits that tumor cells react to changes in their microenvironmental

signals by adopting particular metabolic adaptations which may include, but are not limited to, changes in EMT status, migration, invasion, and importantly for survival distal to the primary site, anoikis resistance (136).

Cellular Sources of Reactive Oxygen Species

There are a multitude of cellular sources of ROS which vary in their triggers, species of ROS they produce, and their implications to biological processes. Classically, ROS generation has been considered to come from two chief sources including oxidative phosphorylation in the mitochondria, and the NADPH oxidase (NOX) complex at the cell membrane, both of which predominantly generate the superoxide species (137). While these processes are important to consider as ROS sources, other major contributors and/or regulators are emerging as critical players in the control of ROS-related anoikis induction. Studies to show what fraction of ROS these pathways contribute to the overall pool are yet to be carried out in most cases.

During normal oxidative phosphorylation, single electrons passed between components of the mitochondrial electron transport chain (ETC) are shunted instead directly to molecular oxygen at a fairly substantial frequency (0.1-2%). This superoxide ($O_2^{\cdot-}$) leak is generally quickly converted to hydrogen peroxide (H_2O_2) by the mitochondrial isoform of superoxide dismutase (SOD2), which can be further detoxified by catalase or glutathione peroxidase (Gpx) to produce harmless water. Individual components of the electron transport chain have differing contributions to ROS generated by this mechanisms however. The majority of ROS contributed by the ETC comes from complexes I and III (137). Experimental depletion of core components of the mitochondrial respiratory complex I

(NDUFS-3 and GRIM-19) by siRNA transduction resulted in ROS reduction generated through the NADH ubiquinone oxidoreductase complex (138). Alternatively, metformin has been demonstrated to abrogate mitochondrial activity through complex I inhibition, resulting in increased ROS level, sensitizing to radiotherapeutic approaches (139). These findings demonstrate the complexity of ROS generation from the mitochondrial electron transport chain as a source, signifying both positive and negative implications toward ROS production.

Mitochondrial uncoupling proteins family members (UCP1-3) generally act to protect cells from processes favoring ROS generation through uncoupling respiratory chain input of substrates for oxidation from ADP phosphorylation output, with implications toward longevity (140). The consequence of these uncouplers on ROS production toward regulation of apoptosis is unclear as ANT (adenine nucleotide translocase) proteins, another major uncoupling source resulting in proton leak in the mitochondrial membrane, have been associated with transient opening of the MPTP (mitochondrial permeability transition pore), but it is unclear at present whether this is a normal part of mitochondrial metabolism or associated exclusively with apoptosis (134). The ROS generating activities of the NOX complex, its regulation, and roles in cellular processes will be discussed in greater detail in a later chapter.

Reactive Oxygen Species and their Reactivity

Oxyradicals are harmful when produced at high levels due to their propensity to interact non-selectively with biomolecules disrupting their function, and this reactivity has been implicated in a number of damaging processes including DNA strand breaks, direct

oxidative damage to DNA bases, and can lead to peroxidation of lipid bilayer components, altering membrane permeability (134, 137). This disruption has a place in normal physiological processes, such as in lysosomal or phagocytic ROS bursts intended to remove unwanted cellular components or microbicidal killing. Overproduction of ROS however, can result in the oxidation of critical cysteine residues, often in the active site of enzymes, modulating or eliminating their activity seen in protein tyrosine phosphatases, lipid phosphatases (e.g. PTEN), MAPK phosphatases, and can also result in disruption of ubiquitin regulatory proteins altering proper protein degradation pathways (141-143). As was alluded to earlier, maintenance of the proper cellular range of ROS is critical for many cellular process, given that complete quenching of ROS results in global hyperactivation PTPs, resulting in broad inhibition of signaling pathways requiring tyrosine phosphorylation (135).

Mitochondria exists as the major source of ROS due to their reliance on aerobic processes utilizing O_2 as the ultimate electron acceptor. Since O_2 is only capable of accepting one electron at a given time, the reduction of O_2 to H_2O can be somewhat muddled, producing reactive intermediates including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and hydroxyl radicals ($OH^{\cdot}$), which vary from least to most reactive, respectively. ROS is a very broad term intending to encompass each of these species, including singlets, doublets, and nonradicals such as hydrogen peroxide (144). While these species are all encompassed by the ROS umbrella, they differ in properties such permeability to cellular compartments as well as the targets with which they have a propensity to interact. For example, superoxide is membrane impermeant; the results of which is restriction to the compartment in which it is produced until it is converted to another, permeable form. Superoxide can be converted

to H_2O_2 , or can react with H_2O_2 via iron sulfur centers to generate hydroxyl radicals via the Fenton reaction. Hydrogen peroxide, relative to superoxide, is less reactive, more membrane permeant, and tends to oxidize proteins with low- pK_a cysteine residues, peroxidases, and unsaturated lipids (145). Hydroxyl radicals on the other hand are highly reactive, and tend to produce secondary radical species. Taken together, it is clear that a variety of reactive oxygen species contribute to the damage of critical cellular components. How these effects are believed to contribute to anoikis will be discussed below.

Reactive Oxygen Species Induce Anoikis

Detachment from ECM has been demonstrated to result in diversion of glucose transporters from the plasma membrane, resulting in increased ROS which inhibits fatty acid oxidation (FAO), resulting in loss of ATP (146). It is unclear at present, however, whether low ATP induces anoikis, or whether production of a ROS burst somewhere in this process is the crucial factor. Antioxidant enzymes such as superoxide dismutase and catalase have been shown to prevent ATP loss following detachment (147). Compounds which act to scavenge reactive oxygen such as α -lipoic acid and reduced glutathione have been shown to inhibit caspase activation in suspension (148). Consistent with these findings, overexpression of antioxidant enzymes (e.g. SOD, catalase) protects from detachment induced cell death while depletion of these enzymes both increases cellular ROS as well as death (147).

Furthermore, ROS has also been shown to contribute to apoptosis directly through mechanisms such as direct mitochondrial peroxidation of cardiolipin, a lipid which sequesters cytochrome c, as well as direct inactivation of the anti-apoptotic Bcl-2 (149).

Recently, ROS and metabolism have been under investigation highlighting their importance in anoikis (80, 146, 147). From a particular isoform derived from an alternative promoter of the SHC1 gene, p66Shc protein is produced in the mitochondria. Here it acts to form a complex with cytochrome c and generates superoxide radicals resulting in mitochondrial dysfunction (150). While cytoplasmically located p66Shc appears to have antioxidant functions, mitochondrial p66Shc has a clear positive link with ROS induction. It has been demonstrated that depletion of p66Shc results in protection from anoikis, although this effect appears to be independent of the cytochrome c interaction domain, but instead relies on activation of RhoA (151). Interestingly, RhoA activation is implicated in ROS production via activation of the NOX complex (152) (which will be described further in a later chapter).

Further, the anti-apoptotic protein Bcl-2, in addition to its roles in MOMP suppression, acts to influence cellular redox balance via mitochondrial membrane proteins where it interacts with complex IV and the small GTPase-Rac1 (153). The critical role of maintaining a low ROS environment in cancer stem-like cells for evading cell death induced by radiation and chemotherapy has been well documented (154-156). The role of ROS suppression in cancer stem-like cells in inhibiting anoikis is hypothetically also a critical feature of this subclass of cells – a feature that appears to be intrinsically related to their metabolic phenotype.

ROS can be produced in response to a number of extracellular stimuli including growth factors, cytokines, and activation of G-coupled protein receptors (157). Further, it has been reported that ROS perturbs a number of cell signaling pathways which (as discussed above) are critical for maintaining anoikis resistance, especially in the context of EMT including ERK, JNK, NF- κ B, FAK, Ras, Rac, and Akt transduction pathways (158).

There are multiple factors which further alter the production of ROS in tumor cells. The importance of glucose and glutamine have been shown (through separate mechanisms which will be discussed in more detail later) to dramatically affect not only cellular metabolic pathways, but also influence the intracellular redox potential, principally through alteration of ROS scavenging pathways. Enzymes which are involved in the processing of these carbon sources thus greatly impact their utilization and shunting to different pathways. It has been shown that in a non-small cell lung carcinoma model, EMT causes upregulation of the neural isoform glucose transporter, GLUT3, via ZEB1 (159). The exact roles of these processes in ROS generation will be detailed below.

Previous work indicates that glucose uptake in suspension declines due to relocation of glucose transporters from the plasma membrane, and that glucose metabolism is integral to maintaining an antioxidant environment through shunting to the pentose phosphate pathway to produce NADPH (146). The result is a sharp increase in cellular ROS level. Reduced glutathione (GSH) and thioredoxin pools (maintained by other processes) play a significant role in neutralizing ROS and are critical for the maintenance of a reduced state requiring the cofactor NADPH (160, 161). Along with the production of antioxidant enzymes such as superoxide dismutase and catalase (discussed above), these are additional pathways in which cells maintain a low ROS environment.

The ROS induced cell death described previously was reported to be caspase-independent, thus non-apoptotic in nature, and was due to inhibition of FAO and ATP decline (146). Here, the authors proposed that this cell death was a consequence of ATP loss resulting in low energy charge; however, it is unclear at present whether their findings are causative or the result of cell death as they evaluate late stage time points in suspended

culture after which caspase activation may have led to ROS pathway activation (e.g. caspase cleavage of PKC resulting in activation of the NOX complex).

In light of these, as well as other studies showing ROS scavenging methods protect from anoikis (148), it is clear that detachment induced ROS burst have clear role in induction of cell death in suspended conditions. ROS can have even more upstream implications in cell survival properties. It has been shown that AMPK activation occurs after cells detach from their matrix (via activation through LKB1 kinase), which results in anoikis resistance through mTORC1 suppression as well as inhibition of acetyl CoA carboxylase, resulting in maintenance of NADPH (162). Also of note, pyruvate kinase isoform M2 (PKM2) tends to be overexpressed in tumor cells. Interestingly, PKM2 is inhibited by high ROS level resulting in a buildup of metabolites prior to the branch step converting pyruvate to acetyl CoA, resulting in abundance of glycolytic intermediates supplying the PPP, increasing NADPH production (163).

Various proliferation inducing oncogenes such as K-Ras, B-Raf, and Myc, described in previous sections to lend survival traits to tumors including anoikis resistance, also result in increased in Nrf2 gene expression. Nrf2 is a master regulator of the ROS cellular milieu when released from its cytoplasmic negative regulatory sequestration partner Keap1. When redox sensitive cysteine residues in Keap1 are oxidized due to oxidative stress, conformational changes result in release of Nrf2, which undergoes nuclear translocation and dimerizing with Maf to transactivate the antioxidant response element (ARE) (164). Through this mechanism, Nrf2 induces numerous antioxidant enzymes, including major targets such as HO-1 and NQO1, the latter reported to have specific roles in ROS detoxification in NSCLC models such that its depletion sensitizes cells to anoikis (165). In addition to antioxidant

enzymes, Nrf2 also transactivates expression of genes related to the pentose phosphate pathway, thereby aiding in maintenance of NADPH. In summary, evidence of the role of ROS suppression in tumorigenesis and anoikis resistance is abundant.

Reactive Oxygen Species and the Epithelial-Mesenchymal Transition

While it appears that ROS and EMT share a paradoxical relationship in that ROS is purported to result in the activation of the EMT program (e.g. hypoxia induced HIF1 α stabilization or NF κ B activation) (166, 167), few studies have been done to examine the eventual ROS environment in cells once they have undergone this transition. Rac1b, an alternatively spliced Rac isoform, is reportedly activated by MMP-associated ectodomain cleavage of E-cadherin, resulting in activation of NOX complexes (168). However, considering this as a source of ROS only takes superoxide species into account. Also, it is unclear from the current reports if ROS induces the EMT phenotype, or rather, selects for EMT subpopulations which are resistant to the harmful effects of ROS. A more global description of ROS in mesenchymal vs epithelial cells is required. How EMT contributes to anoikis through suppression of ROS and altered metabolic function will be discussed in detail in Chapter 2.

Metabolism: Glycolysis, Oxidative Phosphorylation, and Glutaminolysis – Impact on Anoikis

Metabolism has been an area of intensive focus in the study of malignancy since Otto Heinrich Warburg first described the concept of aerobic glycolysis, in which he defined cancer cells as relying extensively on glycolytic metabolism regardless of the presence of abundant oxygen which could be utilized to drive oxidative phosphorylation, a much more

energy efficient means of carbon utilization (169). Through this process, cancer cells not only act to fulfill their energy requirements, they also produce vital biosynthetic precursors for anabolic processes such as fatty acid synthesis, ribose production, amino acid production, and protein glycosylation (170). While these observations were groundbreaking in their time, and brought attention to an extraordinarily important area of research in cancer biology, his original idea has been dramatically oversimplified however, and are insufficient to explain the complex metabolomics that occurs in cancer cells. Warburg's original observations have been widely misinterpreted to mean that cancer cells in fact suppress their mitochondrial oxidative pathways in favor of glycolysis or that their mitochondria are somehow defective. It is now understood that cancer cells even within a single tumors vary dramatically from one portion to another in their reliance on these two primary means of energy utilization (171, 172). Given this observed variety, it is no surprise that there is also significant metabolic diversity when comparing primary tumor, circulating tumor, and metastatic cells (173). The metabolic profile of tumors is likely far more dependent on the regional oxygen/nutrient constraints, soluble factors from the tumor microenvironment heavily influenced by nearby tumor associated stromal fibroblast cells, as well as the infiltration of the tumor with immune cells. Given the great increase in efficiency of EMT plasticity on the ability to migrate away from the primary site, survive in suspension, and colonize a secondary site, it is most likely that the cellular metabolic needs of successful tumor cells are also plastic, resulting in adaptation to their surroundings (136). In connection with this idea, the concept of "metabolic coupling" has emerged, and has been reviewed eloquently by the Lisanti group (174), which has shown that in fact, the majority of glycolysis occurs in the tumor stromal compartment. This is due to influence from

secreted factors from tumor cells, resulting in the production of significant amounts of pyruvate and lactate. It has even been postulated that tumor cells produce oxidative stress to extract nutrients from surrounding stromal cells, resulting in induction of mitophagic processes producing energy rich nutrients that “feed” the cancer cells (174). Tumor cells have abundant monocarboxylate transporters (MCT) family proteins often overexpressed on the cell surfaces of aggressive tumors. These glycolytic end products can then be imported into the tumor cells where they are converted into carbon fuel sources for oxidative metabolism.

In breast cancer cell line models, cancer stem-like cells have been reported to display a shift in metabolism favoring oxidative phosphorylation as a means of ATP production, and interestingly, more metastatic basal and claudin low cell lines show a more significant increase in this population when compared to luminal subtypes (156). Given the metabolic differences seen in these CSC subpopulation, it follows that EMT, which endows cancer cells with properties of CSCs, would influence the oxidative phenotype. It has been reported EMT in breast cancer cells supports increased oxidative phosphorylation, and a greater reliance on alternative fuel sources to carbohydrate catabolism such as fatty acid oxidation is needed to overcome the inefficient process of metastasis (170, 175).

Multiple changes in suspended culture can possibly result in alterations in fatty acid oxidation. Pyruvate dehydrogenase kinase (PDK4), an enzyme which inhibits pyruvate dehydrogenase, has been reported to increase in expression in suspension resulting in a reliance on FAO and alternative fuels for oxidative phosphorylation; however ROS can inhibit these alternate processes (146, 176, 177). The importance of EMT in regulation of these critical metabolic pathways is evident as E-cadherin depletion *in vivo*, either by ZEB1

overexpression or shRNA targeting E-cadherin, altered the metabolic profile of cancer cells which favored oxidative phosphorylation over glycolysis (178).

Cancer cells have been reported to favor glutaminolysis as a carbon source in some contexts, and even display a “glutamine addiction” in many cancer types (179, 180). In fact, even though glutamine is a non-essential amino acid, most mammalian cells cannot proliferate without exogenously supplied glutamine. Increased glutamine consumption is essential for many cancer types during periods of excessive proliferation (181), and has even been linked to aberrant regulation of oncogenes and tumor suppressors (182, 183). Glutaminolysis is the processes by which cells metabolize the amino acid glutamine via its conversion to α -ketoglutarate (α -KG), which proceeds through the tricarboxylic acid (TCA) cycle to produce reducing equivalents for ATP production by means of the ETC. In highly proliferative cancer cells, citrate (an earlier intermediate in the Krebs cycle) is shunted to the cytoplasm where it is involved in the production of NADPH and fatty acids; however, glutaminolysis replenishes this loss with α -KG, a process referred to as anaplerosis (184, 185). Glutaminolysis has been ascribed roles in various cellular processes aside from anaplerosis, the implications of which in anoikis were previously unclear. In addition to replenishing the TCA cycle, glutaminolysis has implications in autophagy and regulation of ROS, the implications of which will be discussed below.

Through the process of glutaminolysis, glutamine undergoes deamidation through an irreversible reaction catalyzed by glutaminase (GLS). Mitochondrial Glutamate Dehydrogenase I (GLUD1 or GDH1) is then responsible for the deamination of glutamate to α -KG (186, 187). GLUD1 will be discussed in more detail as it relates to the induction of anoikis resistance in the following chapter. Regulation of this process is complex and

involves a series of positive and negative feedback regulation of these enzymes. The enzyme glutaminase is inhibited by its product, glutamate by a direct negative feedback regulation. Leucine on the other hand, is a potent allosteric activator of GLUD1. Activation of GLUD1 increases the glutamate to α -KG conversion, decreasing glutamate and removing GLS inhibition. The glutamine bidirection antiporter SLC7A5-SLC3A2 extrudes glutamine and imports leucine into the cell (188). Glutamine modulation of cellular leucine levels therefore directly activates GLUD1 and increases the conversion of glutamate to α KG, further removing GLS inhibition to signal the process forward.

Glutamine is a known regulator of autophagy, a process used by cells to degrade proteins and organelles, replenishing energy charge (189). Activation of autophagy would protect from anoikis by replenishing energy charge, a process inhibited by activation of MTORC1, mammalian target of rapamycin complex I, which is a central regulator of cell growth, translation of mRNA, and metabolic pathways (190-192). The exact mechanisms regarding glutamine inhibition of MTORC, influences over autophagy, and exactly how this impacts anoikis are unclear (193, 194). Pharmacologic inhibition of the major regulators and antiporters involved in this process and their effects on cellular processes such as autophagy, anapleurosis, and anoikis merits further study.

It has been reported that mouse embryonic stem cells, when grown in conditions which maintain their naïve pluripotency, proliferate in the absence of exogenous glutamine; however, when replaced, they utilize higher levels of glutamine and use this to maintain high level of level of intracellular α KG (195). In this study, the authors proposed that embryonic stem cells sustain an elevated α KG: succinate ratio, which maintains their pluripotency, promoting histone/DNA demethylation (195). Conditions that favor elevated α KG:

succinate ratio (e.g. elevated rates of glutaminolysis), thus favor epigenetic changes supporting the stem cell phenotype. As was discussed in detail in previous sections, EMT induces the acquisition of stem cell features and flexible epigenetic state. Given the other major characteristics innate to mesenchymal cells, such as anoikis resistance, the involvement of the process of glutaminolysis and the role of α KG in its regulation in the context of EMT is of critical importance.

The implications of these metabolic alterations on anoikis and EMT are described here. Tumor cell coupling with associated stromal fibroblasts become reliant on these carbon sources for energy production, meaning less flow through glycolytic pathways, and thus less input into the PPP, hampering the production of NADPH. This could initially increase ROS in these tumor cells causing EMT through mechanisms described above (HIF1 α stabilization, NF κ B induction, etc). During the EMT process, in addition to other deleterious features, cells upregulate defenses against this oxidative environment, through Nrf2 and HIF1 α transactivation of ARE, antioxidant defense systems are engaged, resulting in low ROS. Tumor cells that are now reliant on oxidative phosphorylation as an energy production source are now poorly suited to remain in the hypoxic primary tumor environment. Considering these stimuli, cells are encouraged to undergo transient EMT processes, increase invasiveness and anoikis resistance, and leave their primary site. In fact, breast cancer cells recovered from metastatic sites have been observed to contain elevated mitochondrial number and increased mitochondrial membrane potential (136, 196). These findings all support the role of EMT in modulating ROS, metabolism and anoikis.

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Grainyhead-like transcription factor family: Roles in Cancer

The three mammalian homologues of *Drosophila* Grainyhead, termed GRHL1, 2, and 3 have each been demonstrated to play significant roles in carcinogenesis. These homologues share some common targets; however, like their distinct chronological induction during embryogenesis and varied expression patterns in adult tissues, they have unique target genes and effects on cellular processes. In the context of cancer, GRHL1 has been demonstrated to act as a tumor suppressor in both squamous cell carcinoma (SCC) and neuroblastoma (1). In the context of SCC, the tumor suppressive role of GRHL1 was demonstrated in *Grhl1*^{-/-} mice, which developed a higher number of malignant lesions following treatment with topical carcinogen, DMBA as compared to wild type. In this study, Mlacki et al., demonstrated that mechanistically, loss of GRHL1 resulted in a chronic elevation of inflammatory factors as well as a faulty keratinocyte terminal differentiation program (2). Interestingly, the closely related family member, GRHL3, has also been demonstrated to have tumor suppressive functions in SCC via maintenance of its target gene PTEN, where the expression of GRHL3 was demonstrated to be repressed by the proto-oncogenic network mediated through miR-21 (3). The role of GRHL2 in oncogenesis remains contested, and appears to play a role which at the very least might be context and/or tissue specific in its tumor promoting or suppressing behavior. Highlighting the most critical findings in regards to GRHL2 will be the focus of this review.

Suppression of the Oncogenic Epithelial-to-Mesenchymal Transition by Grainyhead-like 2

The epithelial-mesenchymal transition (EMT), has been strongly implicated in the malignant progression of cancer, endowing subpopulations of tumors with enriched metastatic potential as well as stem cell-like properties (4). This cellular event represents a major shift within these epithelial subpopulations, resulting in characteristics reminiscent of mesenchymal cells including disruption of cell-cell adhesion (5), increased motility and invasiveness(6), chemo/radiotherapeutic resistance (7-10), and resistance to matrix-detachment induced cell death, a process referred to as anoikis (11-14). These features represent the manifestations of cellular phenotypic plasticity conferred by passage between these two distinct states. In the context of normal physiological function, EMT and its converse process termed mesenchymal-epithelial transition (MET), are indispensable to events such as embryological development and wound healing (5, 15). However, these normal processes, when dysregulated, can have catastrophic consequences. Perturbations of normal cellular functions such as proliferation and growth are extensively characterized in the context of cancer progression, and an extensive body of evidence has now established EMT/MET plasticity as a normal process employed inappropriately in the context of malignancy.

In breast cancer specifically, loss of GRHL2 expression is seen specifically in the aggressive claudin-low and basal B subtypes, which are known to exhibit a frank EMT phenotype, and are often associated with resistance to therapeutic intervention and poor prognosis (16-18). While a multitude of factors have been shown to induce EMT, these factors far outweigh those which inhibit this event. GRHL2 has emerged as a master regulator of the epithelial phenotype, the mechanisms of which are largely established (18-20). Overexpression of GRHL2 is associated with MET resulting in downregulation of the

mesenchymal markers vimentin, fibronectin, N-cadherin, and CD44S as well as E-cadherin upregulation (18). Snail and ZEB1, well established E-box binding transcription factors, function to directly repress epithelial genes such as E-cadherin (21-24), directly contributing to loss of cell-cell junctional networks of epithelial cells, and further enhancing transactivation of survival genes associated with Wnt signaling through loss of β -catenin sequestration (25). A critical reciprocal feedback loop has been discovered between GRHL2 and ZEB1 such that in ovarian cancer, colorectal carcinoma, and breast cancer cell lines, GRHL2 and ZEB1 expression levels have been found to inversely correlate (20, 26, 27). This relationship is mediated at least partially through direct GRHL2 repression of the ZEB1 promoter as demonstrated through ChIP assay (18), and furthermore, it has also been demonstrated that ZEB1 represses the GRHL2 promoter through direct binding as well (19). Of note, in an superb study by Chung et al., the reciprocal feedback loop between GRHL2 and ZEB1 was confirmed in epithelial ovarian cancer (27); however, they were unable support previous findings (18) that demonstrated direct GRHL2 binding to the ZEB1 promoter. This discrepancy is most likely the result of relative gain difference comparing ChIP-seq technique to direct ChIP assay in which the sensitivity of interaction detected can be significantly modified through DNA fragment input alteration. Further, support for direct GRHL2 binding to the ZEB1 promoter was confirmed by repression of a luciferase tagged ZEB1 promoter construct co-transfected with GRHL2 in a dose-dependent manner as well as enhanced ZEB1 promoter activity in cells expressing stable GRHL2 knockdown (18). Additionally, GRHL2 has been demonstrated to interfere with ZEB1 expression through other mechanisms. The homeoprotein SIX1 is a well-established transcription factor involved in developmental EMT which also plays a role in the oncogenic EMT, through direct

transactivation of the ZEB1 promoter (28, 29). Interestingly, GRHL2 further disrupts ZEB1 expression by altering the Six1-DNA complex resulting in further abrogated transcription at this site (19).

In addition to suppressing ZEB1 transcriptional activity, GRHL2 further supports the epithelial phenotype through suppression of TGF- β /Smad mediated transcription. TGF- β has been extensively described to confer either tumor suppressive (through induction of CDK inhibitor proteins) or tumor promoting (through transcriptional support of EMT) properties dependent on tumor stage. In fact, TGF- β signaling, along with canonical/noncanonical Wnt signaling is upregulated and through autocrine signaling pathways maintain the mesenchymal subpopulation present in Human mammary epithelial lines (HMLE) (30). GRHL2 inhibits Smad mediated transcription, and depletion of GRHL2 is permissive of EMT induction in HMLE cells (18), which are otherwise resistant to this stimulus (due to high endogenous GRHL2 levels) (30). Further, it was confirmed that depletion of GRHL2 in HMLE cells expressing low level K-Ras (HMLER) results in EMT, a phenomenon shown to be dependent on autocrine TGF- β signaling (18). Additionally, GRHL2 was shown to prevent TGF- β induced EMT in MCF10a cells constitutively expressing GRHL2 (20). Notably, GRHL2 was reported to upregulate BMP-2, a member of the bone morphogenetic protein family, belonging to the TGF- β superfamily, which are known to inhibit TGF- β receptor signaling through competition with Smad2/3 for Smad4 binding and nuclear translocation (18, 31).

It has been well established that the primary mechanism by which the miR-200 family acts to enforce the epithelial phenotype is through post-transcriptional repression of ZEB1/2, and in fact a negative feedback loop has been demonstrated such that ZEB

transcription factors also transcriptionally repress miR-200 expression (11, 32-36). It is now well established that GRHL2 positively regulates the transcription of this family of microRNAs. Stable knockdown of GRHL2 resulted in a significant reduction in both miR-200b/c transcript levels (18). Further, GRHL2 was shown to bind directly to the promoter region regulating the polycistronic primary transcript of miR-200b, miR-200a, and miR-429 (27), inducing their expression. Interestingly, it was shown here that stable overexpression of either miR-200b or miR-200a was able to restore E-cadherin expression in OVCA429 ovarian cancer cells expressing stable knockdown of GRHL2, indicating a so-called “double negative” regulatory feedback loop with the miR-200 family, GRHL2, and ZEB1. The positive regulatory effect of GRHL2 on the miR-200 family has also been confirmed in other models including oral squamous cell carcinoma (37, 38). The transcription factor p63 is a multi-isoform relative of p53 critical for proper epidermal development (39). A recent report showed a critical role in the maintenance of the epithelial phenotype for p63 and demonstrates a positive regulatory loop between p63 and GRHL2. It was demonstrated here that depletion of p63 results in loss of GRHL2 and miR-200 family gene expression as well as other epithelial specific markers and resulted in an EMT phenotype (38).

Several of the aforementioned pathways have regulatory control of endogenous GRHL2 expression as well. As previously stated, TGF- β is only able to induce EMT in particular contexts or stages of tumor progression. For example, in the setting of HMLE cells, EMT is only induced by TGF- β in conjunction with either stable GRHL2 knockdown (18) or activation of Wnt signaling (30). It is now clear that activation of the Wnt and TGF- β pathways work in concert to downregulate endogenous GRHL2 expression. Neither activation of the Wnt pathway via BIO compound (GSK3 inhibitor) or stably expressed S33Y

β -catenin nor long term TGF- β treatment resulted in significant downregulation of GRHL2 alone; however, in combination, these pathways downregulated GRHL2 considerably (19). BMP2, which was significantly upregulated in GRHL2 expressing cells (18), acts as an inhibitor of TGF- β signaling. Treatment of cells with BMP2 prevented downregulation of GRHL2 and ZEB1 upregulation in response to Twist mediated EMT (19). As will be demonstrated below, it is clear that a better understanding of how to inhibit these pathways successfully *in vivo* could be a crucial step in inhibition of EMT plasticity and preventing mortality from chemotherapeutic resistance, recurrence, and metastasis.

Anoikis, Cancer Stem Cell Phenotype, Recurrence and Metastasis: Reversal by GRHL2

Among the various deleterious traits of metastatic tumor cells, one especially important for their ability to survive beyond their primary site is a compromised anoikis response (14, 40). Along with increasing migratory and invasive characteristics, it is well established that EMT induces anoikis resistance in cancer cells (14, 41, 42). Many of the core cellular signaling pathways involved in the implementation of the EMT program such as PI3K/Akt and MAPK activation also contribute to cell survival in general, anoikis being no exception (43). Ectopic GRHL2 expression was shown to restore sensitivity to anoikis in MDA-MB-231 breast cancer cells as well as the mesenchymal subpopulation of HMLE cells (18). Accordingly, depletion of GRHL2 by shRNA in HMLE+Twist-ER and MCF10a neoT cells also decreased sensitivity to anoikis (44). While many signaling pathways are aberrantly activated during EMT (e.g. FAK, ILK, TrkB; refer to (13) for an indepth review), and contribute to anoikis resistance, our lab recently showed GRHL2 at least partially enforces

anoikis sensitivity through downregulation of the mitochondrial enzyme glutamate dehydrogenase I (GLUD1) (44). GLUD1, an enzyme shown to have pro-tumorigenic roles in orthotopic models, has been demonstrated to influence the cellular oxidative milieu via production of α -ketoglutarate (α -KG), increasing fumarate production in the citric acid cycle, stabilizing the antioxidant enzyme glutathione peroxidase 1. Further, in this study, Jin et al. demonstrated by IHC that GLUD1 expression correlates with progressive stages of both breast and lung carcinomas (45). Our lab found that overexpression of GRHL2 resulted in a decline in α -ketoglutarate levels, elevating reactive oxygen species (ROS), and impacting mitochondrial metabolism; further, stable knockdown of GLUD1 increased ROS and anoikis sensitivity, while replacement of α -KG in these cells resulted in decreased ROS and enhanced resistance to anoikis (44). Luciferase assays showed that the GLUD1 promoter was significantly repressed by GRHL2 in cotransfection experiments. Here, it was demonstrated that GRHL2 acts to repress the GLUD1 promoter as well as the EMT program in HMLE cells and further suppresses tubulogenesis in MDCK cells via inhibition of the histone acetyltransferase co-activator, p300 (Pifer et al., 2016; *in review*).

Resistance to anoikis is a pre-requisite for tumor metastasis. In order for cancer cells to escape their primary microenvironment, they must be able to shed their connections to the surrounding extracellular matrix in their primary environment and survive intravasation into the vasculature or lymphatic system. Experimentally, enforced expression of E-cadherin abrogates invasive properties in cancer cells, and clinically, expression of E-cadherin is associated with lower risk of tumor invasion (46-49), highlighting the importance of the EMT program in these early stages of metastasis. EMT thus endows subpopulations of tumor cells with anoikis resistance allowing them to survive

dissemination after invading out of their primary niche. In a p53-dominant negative tumor model, depletion of E-cadherin in a conditional knockout mouse transgenic line significantly increased tumor metastasis *in vivo*; moreover, the resulting cell lines produced from this study were anoikis resistant compared to their E-cadherin positive counterparts (50). However, despite these findings, the role of EMT/MET in metastasis is an area of controversy.

Reversion of mesenchymal to epithelial cells in some contexts has been shown to enhance metastatic colonization (5). In fact, breast cancer metastases are often, but not always, epithelial and express E-cadherin (51). As such, it was demonstrated by Nieto and colleagues that depletion of the EMT inducing homeobox transcription factor Prrx1 is required for cancer cells to seed metastatic locations *in vivo*; however, it was noted in this study that Prrx1 overexpression does not induce cancer stem cell-like traits as does ZEB1, Twist, or Snail mediated EMT events as previously described(52-55). This contradicts findings showing that tumor metastases, at least in some cases, are mesenchymal or contain a mixture of mesenchymal and epithelial cells, indicating that the EMT phenotype likely still exists in metastases, at least in early stages of metastatic colonization. This observation that metastatic tumors often do not resemble the mesenchymal cells which gave rise to them is likely a consequence of differing local microenvironmental stimuli encountered following extravasation into distant organs, where the stress signals initially invoking EMT in the primary tumor are no longer present (6, 56, 57). Some studies have posited that following Twist activation and EMT induction, in order for circulating tumor cells to colonize distant sites, it must be silenced (58). While it may be true that MET confers proliferative advantages in metastatic settings, a major flaw with this reasoning is evidenced by the

inhibition of proliferation which occurs due to activation of exogenous Twist in these settings, which may result in misinterpretation of these results.

A potential reconciliation of these views is that EMT/MET plasticity is in fact the major force to endow cancer cells with their malignant properties. An extension of this interpretation would imply that either EMT or MET could be viewed as tumor suppressive or oncogenic events depending on the stage of disease progression. A recent publication by Scheel et al. however, demonstrated that transient Twist1 activation permanently alters cell state and primes epithelial cells for stem cell-like properties which did not require permanent acquisition of the EMT phenotype (58). This finding informs the intimidating reality that any tumor cell subjected to the proper conditions in the tumor microenvironment may undergo EMT even transiently, and thus potentially attaining cancer stem cell-like properties highlighting the need for targeted therapy to eradicate this subpopulation, or rather, prevent its emergence. It is clear that a better understanding of the major regulators of EMT and MET is critical in order to prevent the emergence of treatment resistant metastases and recurrence. Regardless of the seemingly contradictory evidences, it is undeniable that EMT invokes many traits in cancer cells associated with aggressive malignancies, invasion, and anoikis resistance, and that these features are ameliorated by expression of the epithelial enforcer GRHL2. Of critical importance, it was demonstrated through a comprehensive IHC analysis of primary breast tumors that a striking loss of GRHL2 expression occurs at the invasive front of primary tumors, and a significant inverse correlation exists between GRHL2 expression and the presence of lymph node metastases (20). Also, it was noted by the authors in this study that while correlative evidence, basal cell carcinomas, which nearly never metastasize, as well as non-invasive

urinary bladder cancer both showed incredibly strong expression of GRHL2. Conversely, other reports have associated GRHL2 expression with increased risk of metastasis and poor relapse free survival (59, 60). However, these studies do account for that fact that tumors of epithelial phenotype enrich for genes such as GRHL2 or E-cadherin relative to “normal” tissue (which is often predominantly fibroblastic stromal tissue). This provides the implication that the more rapidly an epithelial tumor grows, the more “epithelial” it will appear to techniques such as RNA-seq due to expansion of this compartment. In addition to this, as mentioned previously, the most relevant sub-compartment of the tumor to driving therapy resistance, recurrence and metastasis is a very small proportion of the overall tumor which downregulates GRHL2, therefore this population is masked by techniques that consider the entire tumor.

Disease recurrence several years following initially successful treatment of primary disease is a frequent reality, and is the most common cause of death from cancer. It has been extensively demonstrated that in these settings, plasticity conferred by EMT benefits small populations of cells, bestowing CSC-like and chemoresistant properties to these primary tumor cells, which persist, only to resume growth after a period of dormancy (41, 61-66). In breast cancer, these populations are often identified by a shift in surface expression toward a CD44^{HIGH}/CD24^{LOW} phenotype. In culture, treatment of HMLE cells with taxol derivatives selects for this CSC-like population; conversely, stable expression of GRHL2 significantly sensitizes these, as well as MDA-MB-231 cells, to this therapy. Most importantly, ectopically expressed GRHL2 prevents the chemotherapy-induced emergence of this population capable of tumor initiation (19). In a study by Singh et al, it was demonstrated that epithelial SUM149 cells subjected to metabolic stress such as glutamine deprivation resulted in the emergence

of a “metabolically adaptable,” glutamine independent population, termed SUM149-MA or MA2. It was demonstrated here that in addition to having undergone EMT, these cells now displayed chemotherapeutic resistance to taxol, Erlotinib, doxorubicin, and a number of other both targeted and cytotoxic agents. Upon global gene expression analysis, it was discovered that MA2 cells had significantly downregulated GRHL2 and upregulated ZEB1. The authors noted a significant decrease in histone activation marks H3K4 trimethylation and H3K14 acetylation in the MA population previously reported to be associated with the drug resistant state (67), which when reversed using HDAC inhibitor pretreatment, significantly sensitized cells to chemotherapy (68). Interestingly, it has also been reported that while H3K4me3 marks remain largely unchanged following GRHL2 depletion with shRNA, repressive mark H3K27me3 significantly increased in the promoter regions of genes positively regulated by GRHL2 including *CDH1*, *MIR-200B/200A* and *429*(27). These findings strongly point to a potential role for GRHL2 in epigenetic modification as a mechanism of transcriptional regulation. Observations such as these support a role in both sensitization to chemotherapy by GRHL2, as well as a critical role in abrogating CSC emergence crucial for the initiation of recurrent disease.

Mouse models recapitulating recurrence following regression of primary tumors driven by doxycycline inducible neuNT, Wnt1, or *c-myc* have shown that in comparison to the primary tumors, recurrent disease that emerges following withdrawal of the oncogenic driver have undergone EMT (64, 69, 70). Analysis of tetO-neuNT and tetO-Wnt1 primary vs recurrent RNA expression revealed a striking decrease in GRHL2 expression in recurrent tumors (19). These findings strongly support the hypothesis that GRHL2 is a tumor suppressor, is specifically silenced in the tumor initiating subpopulation of the primary

tumor, and is a major suppressor of disease recurrence. Further evaluation is needed in order to more clearly define the precise role of GRHL2 in preventing recurrence *in vivo*.

GRHL2: Tumor Suppressor or Oncogene?

Despite the extensive evidence reporting GRHL2 as a tumor suppressor and inhibitor of the EMT phenotype, there are contradictory reports in both breast and other malignancies supporting a role of GRHL2 in oncogenesis (1). It is clear that the expression pattern of GRHL2 in adult tissues is immensely complex. GRHL2 is found almost exclusively in tissues of the body that are predominantly epithelial (71), correlating with expression of junctional proteins such as E-cadherin and CLDN4 (72); however, its expression is diversely regulated, showing up and downregulation often within the same tissue type, highlighting its potential for tumor suppressive or oncogenic roles, depending on the context .

It was reported that in oral squamous cell carcinoma, GRHL2 interacted directly with the promoter of hTERT (human telomerase reverse transcriptase) as assessed by promoter magnetic IP (PMP assay). In this model, telomerase, which is required to maintain the malignant phenotype, had higher activity in tumor lines expressing high GRHL2 expression, and depletion of GRHL2 reduced telomerase activity and cell viability, as well as resulted in hypermethylation of the hTERT promoter (73, 74). The precise rationale for this GRHL2-mediated regulation of cellular immortalization in this context is unclear at present. In agreement with these findings, Chen et al demonstrated that depletion of GRHL2 abrogated tumorigenesis and was necessary for maintenance of stemness in a model of oral squamous cell carcinoma (37). Another study reported that the 8q22 gene cluster was involved in death receptor-mediated apoptosis. Here, GRHL2 was identified in a whole-genome RNAi

screen which indicated that it suppressed death receptor signaling (75); however, unlike other target genes identified in this screen, the authors were unable to rescue the RNAi associated phenotype resulting from GRHL2 depletion, therefore an off-target effect could not be eliminated. Also noteworthy, the shRNA pool used in the study was conducted in HT1080 fibrosarcoma lines which have minimal expression of endogenous GRHL2.

Many of these findings can be rationally explained, such as studies which knocked down GRHL2 expression in already mesenchymal HT1080 fibrosarcoma cells (75), or overexpressing GRHL2 in NIH3T3 fibroblast lines (20), whose cancer relevance, as noted by the authors, is questionable. Still other studies contain discrepancies not fully understood at present. For example, while it was reported by Xiang et al that highly invasive 4T1 cells orthotopically injected into the mammary fat pad would metastasize in a period of about 4 weeks, and that the resulting metastases recovered from the lungs displayed an EMT phenotype, they observed a counterintuitive increase in metastasis when injecting 4T1 cells overexpressing GRHL2 (59). Further, GRHL2 was confirmed in this study to enforce the epithelial phenotype. These results are in direct opposition to those showing a direct role of EMT in promoting metastases, rather than inhibiting it (12, 76-83). In our hands, we have found that GRHL2 does not retain several critical functions in some murine cell lines such as 4T1 cells (unpublished observations); therefore, it is possible that in some restricted murine settings, GRHL2 fails to inhibit metastasis.

As detailed above, GRHL2 expression is lost in recurrences which often display EMT phenotype (19); however, other studies have shown that gain of GRHL2 (via amplification of *GRHL2* gene at chromosome 8q22.3) was demonstrated to be a marker of early recurrence in hepatocellular carcinomas (84). However, unlike in the context of basal-B breast cancer

cell lines, where GRHL2 was reported to have no effect on proliferation (18, 20), in the HCC lines studied by Tanaka and colleagues, GRHL2 knockdown resulted in decreased cell proliferation, indicating that in this context, either GRHL2 significantly impacts tumor cell growth and proliferation which may contribute to earlier detection of apparent recurrent disease in some tissue types (84). Likewise, colorectal carcinomas, which have been reported express high levels of GRHL2, have been described to show enhanced proliferation, larger tumor size, and more advanced clinical stage in this setting (85). These findings were verified by depletion of GRHL2 in colorectal cell lines HCT116 and HT29 cells (26); conversely, in our hands, HCT116 cell lines were found to express very low levels of endogenous GRHL2 (unpublished observations). A potential explanation of the role of GRHL2 in regulating cellular proliferation may be found in examination of the reported target gene *ErbB3* by GRHL2. *ErbB3*, with known roles in carcinogenesis and proliferation, was identified as a GRHL2 target gene by Werner et al, and furthermore, was found to contain a single conserved GRHL2 DNA binding site (AACCGGTT) upstream of the transcriptional start site. Binding to this site was demonstrated by EMSA and activation was shown through luciferase cotransfection experiments (20). Importantly, this upregulation was originally noted in NIH3T3 cells overexpressing GRHL2, and the authors noted that in the triple negative breast cancer line MDA-MB-231 cells, where GRHL2 has no reported effect on proliferation, there was no upregulation of *ErbB3* (20). It is possible that differential gene regulation in different cell lines may therefore contribute to findings demonstrating alterations in proliferation by GRHL2. Of note, it has been demonstrated that GRHL2 copy number gain, reported in several cancer types including gastric (86) and hepatocellular carcinoma (84) does not necessarily translate to an increased expression of GRHL2 (71).

However, in a directly conflicting report, it was demonstrated by Xiang et al, that gastric cancer cell lines contained significantly downregulated GRHL2 mRNA and protein levels; moreover, expression of exogenous GRHL2 in this setting significantly inhibited proliferation and promoted apoptosis (87). Further, the authors of this study demonstrated that GRHL2 negatively regulated the pro-survival factors c-myc and Bcl2.

In a finding that has since been discredited in multiple models, Yang et al, reported that in wound healing scratch assays, MDCK and MCF7 cells overexpressing GRHL2 displayed increased invasion relative to vector only expressing cells (60). As these results were not confirmed using assays more specific to migration and invasion such as Boyden chamber assays, the most likely interpretation of these data is that in this cell lines, overexpressing of GRHL2 increased proliferation as it has been reported to do in other models rather than enhanced migration as discussed previously (26, 85). Opposed to this view, it was reported that overexpression of GRHL2 in both MDA-MB-231 and BT549 cells result in MET and significantly suppressed migration in Boyden chamber assays (20). Further, it was demonstrated that depletion of GRHL2 in ovarian cancer cells enhanced migration, invasion, and motility using a matrigel embedded gap invasion assay, a more specific approach for analysis of invasion (27). In fact, this report demonstrated that shGRHL2 cells migrated as individual cells rather than a collective front, and further verified that differences were not due to proliferation differences.

The discrepancies in the reported roles of GRHL2 in tumor progression could resemble that of the TGF- β pathway which has reported roles in both processes. This theory is certainly is conceivable given the well-established interaction of GRHL2 in the inhibition of the TGF- β pathway. Given the critical role of EMT in metastasis and establishment of CSC-

like populations, a better understanding of pathways regulating expression of master regulatory transcription factors such as GRHL2 is crucial for improving patient survival, both in terms of sensitizing to initial chemotherapy as well as preventing the emergence of treatment resistant secondary disease.

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Chapter 2

Grainyhead-like 2 Reverses the Metabolic Changes Induced by the Oncogenic Epithelial-mesenchymal Transition: Effects on Anoikis

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Grainyhead-like 2 Reverses the Metabolic Changes Induced by the Oncogenic Epithelial-
mesenchymal Transition: Effects on Anoikis

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Abstract

Resistance to anoikis is a pre-requisite for tumor metastasis. The epithelial to mesenchymal transition (EMT) allows tumor cells to evade anoikis. The wound healing regulatory transcription factor Grainyhead-like 2 (GRHL2) suppresses/reverses EMT, accompanied by suppression of the cancer stem cell (CSC) phenotype and by re-sensitization to anoikis. Here, we have investigated the effects of GRHL2 upon intracellular metabolism in the context of reversion of the EMT/CSC phenotype, with a view toward understanding how these effects promote anoikis sensitivity. EMT enhanced mitochondrial oxidative metabolism. While this was accompanied by higher accumulation of superoxide, the overall level of Reactive Oxygen Species (ROS) declined, due to decreased hydrogen peroxide. Glutamate Dehydrogenase 1 (GLUD1) expression increased in EMT, and this increase, via the product α -ketoglutarate, was important for suppressing hydrogen peroxide and protecting against anoikis. GRHL2 suppressed GLUD1 gene expression, decreasing α -ketoglutarate, increasing ROS and sensitizing cells to anoikis. These results demonstrate a novel role of GRHL2 in promoting anoikis through metabolic alterations.

Introduction

When deprived of connections to their extracellular matrix, normal epithelial cells trigger a process of programmed cell death referred to as anoikis (1). The ability to metastasize depends critically upon a cancer cell's ability to evade anoikis (2-4). The transcriptional reprogramming event known as the Epithelial to Mesenchymal transition (EMT) confers anoikis resistance and ultimately, increased metastatic potential (5). The increased phenotypic plasticity underlying transitions between epithelial and mesenchymal states is thought to be epigenetically driven (6-10). One additional manifestation of this increased plasticity, aside from EMT, is that cancer stem cell subpopulations emerge, that, like cells resulting from EMT, are resistant to anoikis; these contribute crucially to both metastasis and disease recurrence.

The wound healing regulatory transcription factor, Grainyhead-like 2 (GRHL2), plays an indispensable role in the maintenance of the epithelial phenotype and branching morphogenesis (11-14). Previously, we reported that GRHL2 suppresses the oncogenic EMT, i.e., promotes MET (15-17). The loss of GRHL2 expression is associated with aggressive, metastatic breast tumor types that contain an unusually large fraction of EMT-like subpopulations, and in the cancer stem cell-like subpopulation of tumors resulting from EMT and/or drug resistance. Interestingly, the constitutive expression of GRHL2 invariably led also to anoikis-sensitivity (15, 16). The mechanism underlying this effect was unclear, however.

Reactive oxygen species (ROS) are ubiquitously important in apoptosis through mechanisms such as direct mitochondrial peroxidation of cardiolipin, a lipid which sequesters cytochrome c, as well as direct inactivation of the anti-apoptotic Bcl-2 (18).

Accordingly, ROS contributes to anoikis as well as the non-apoptotic cell death of detached cells accompanying ATP loss (19, 20). It is unclear at present, however, whether changes in ROS levels occur in EMT or cancer stem cell transitions, and, if so, what drives these changes.

The HMLE cell line is an immortalized mammary epithelial cell line that contains a CD44^{hi}/CD24^{low} subpopulation referred to as the Mesenchymal Sub-Population (MSP) that co-expresses EMT and cancer stem cell phenotypes (21, 22). Previously, we reported that MSP cells have low GRHL2 expression, and are resistant to anoikis (15, 16). Forced expression of GRHL2 in MSP suppressed their cancer stem cell-like as well as EMT phenotypes and sensitized them to anoikis. The possibility that GRHL2 achieves the latter effect by altering intracellular metabolism has not been explored.

Glutaminolysis is a critical metabolic pathway on which tumors rely as a major alternate carbon source to glucose, with significant ramifications for cell proliferation, metabolic adaptability and cell survival (23-25). Following deamination of glutamine to produce glutamate, glutamate dehydrogenase-1 (GLUD1 or GDH1) generates the Krebs cycle intermediate α -ketoglutarate (α -KG), which is converted by the Krebs cycle to fumarate, an important cofactor for glutathione peroxidase enzymes. Accordingly, α -KG is an important protective factor against oxidative stress, and GLUD1 is over-expressed in breast and lung carcinomas (26, 27).

In this paper, we report that, in reversing EMT, GRHL2 suppresses GLUD1 expression, elevating H₂O₂ ROS levels and promoting anoikis-sensitivity. GRHL2 also reversed the cancer stem cell-like shift to oxidative phosphorylation-based ATP production and cell survival. These results inform a novel connection between EMT, metabolic pathways, ROS and anoikis-sensitivity, regulated by GRHL2.

Materials and Methods

Cell lines

HMLE, and HMLE+Twist-ER cells and generously provided by R. Weinberg (The Whitehead Institute, Cambridge, MA); MCF10A neoT cells were provided by F. Miller (Karmanos Cancer Center). HMLE and HMLE+Twist-ER cells were maintained in Advanced Dulbecco's Modified Eagle's Medium (DMEM): Ham's F-12 (Gibco) + 5% horse serum + 1X penicillin-streptomycin-glutamine (PSG) + 10 µg/mL insulin, 10 ng/mL EGF, 0.5 µg/mL hydrocortisone. MCF10A neoT cells were maintained in the same media as HMLE cells with the addition of 0.1 µg/mL cholera toxin. If indicated, HMLE+Twist-ER cells were growth in the presence of 4-hydroxytamoxifen (10 ng/mL) in order to activate the ER inducible Twist construct. MSP cells were obtained from HMLE cells by sorting for CD44-APC high population/CD24-Cy5.5 LOW. HMLE cells were sorted according to CD24-Cy5.5 high and CD44-APC low.

Generation of stable cell lines by retroviral transduction

The template for Human GRHL2 was purchased from Open Biosystems (MHS4426-99625903). GRHL2 was subcloned by standard molecular biology protocols into the pMXS-IRES-puro retroviral vector in frame with the C-terminal 3X-FLAG tag (vector contribution of Russ Carstens, University of Pennsylvania). Retroviral packaging and amplification was done in GP2+293T cells by transfection of 4.5 µg of retroviral plasmid and 2.5 µg of pCMV-VSV-G on collagen coated 60 mm² dishes that were pre-coated for >1 hr with 50 µg/mL

collagen using Lipofectamine 2000 (Invitrogen). Plates were refed 4-6 hours following transfection and viral supernatants were harvested approximately 36 hours following refeed. Viral stocks were filtered through 0.45 μm filters (Whatman) and 1 mL of supernatant was used to infect 1 well of target cells. This was followed by 1,400 RPM centrifugation for 1 hour at room temperature followed by 6 hour or overnight incubation. Infected cells were passaged to 100 mm dishes, and incubated for 48 hours following infection. Cells were then selected for puromycin (2 $\mu\text{g}/\text{mL}$ for HMLE or MCF10A neoT cells).

Generation of stable cell knockdown cell lines by lentiviral transduction

Lentiviral GLUD1 short hairpin RNA (shRNA) was purchased from Open Biosystems in the pLKO vector. ShRNA #1 was Open Biosystems catalogue number RHS3979-201758959 and shRNA#2 was RHS3979-201758962. pLKO scramble control vector was generously contributed by the laboratory of Dr. Scott Weed (WVU). Lentiviral constructs were packaged and amplified in 293T cells by transfection of 3.5 μg shRNA vector, 2.3 μg sPAX2, and 1.2 μg CMV-VSV-G on 60 mm^2 dishes that were pre-coated for >1 hr with 50 $\mu\text{g}/\text{mL}$ collagen using Lipofectamine 2000. GRHL2 shRNA was described previously and was shown to duplicate the biologic effects of transfected siRNAs (15).

Western Blotting

SDS-PAGE was conducted using 4-20% gradient Tris-Glycine gels (Invitrogen). Proteins were electrophoretically transferred to polyvinylidene difluoride filters (Immobilon) in 5% methanol Tris-Glycine transfer buffer. PBS + 0.1% Tween-20 + 5% nonfat milk were used for blocking filters, primary antibodies were incubated in PBS+0.1% Tween-

20 + 5% nonfat milk. Primary antibodies were typically incubated between 2 hours at room temperature or overnight at 4 degrees C. Primaries used were: E-cadherin, ms (BD Biosciences), CD44 (HCAM) ms [Santa Cruz Biotech (SCBT)]; GRHL2, rb (Sigma), Akt or pAkt, rb (Cell Signaling); GLUD1, rb (Abcam 168352), α -tubulin, Rb (Cell Signaling), Fibronectin, ms (BD Biosciences), GAPDH, ms (Origene), β -actin, ms (Thermo-pierce), SOD2, Rb (Cell Signaling), HIF1 α , ms (BD Biosciences), p110, ms (BD Biosciences), TOMM20, ms (BD Biosciences), VDAC1, Rb (Cell Signaling). Secondary antibodies for chemiluminescence were either anti-mouse or anti-rabbit, conjugated to horseradish peroxidase (HRP) enzyme (Bio-Rad). Secondary antibodies (Biorad) were used at 1:3000 dilution and filters were incubated for approximately 1 hour at room temperature. Western blots were developed via ECL Super Signal West Pico (Thermo-Pierce).

Anoikis Assays

For anoikis assays, caspase activation was measured using the Caspase-Glo 3/7 assay kit (Promega). Cells were dissociated using TrypLE Express (Invitrogen) and a fixed number of cells (1.5×10^5 cells) were placed per 6 well poly-(2-hydroxyethyl methacrylate) (poly-HEMA) coated low attachment plates in 2.0 ml of normal growth medium + 0.5% methylcellulose for the indicated time. Aliquots of cells were mixed 1:1 with caspase glo reagent at indicated time points and assayed for luminescence utilizing a Wallac Envision Perkin Elmer plate reader according to manufacturer's instructions. Where indicated, time-zero cell death values were subtracted from the data presented to normalize for small loading variation.

ROS Assays

For ROS assay, cells were seeded as above and attached for 48 hours. Cells were detached by trypsinization, and equal numbers of cells were placed in a 15 mL centrifuge tube. Cells were assayed immediately by flow cytometry after staining for 15 min at 37 degrees with 1 uM CM-H₂DCFDA (Invitrogen) protected from light. If indicated, 24 hr ROS was examined after plating cells in low attachment polyHEMA (2 mg/mL) coated 100 mm dishes in 5 mL of 0.5% methylcellulose to prevent excess cell clumping. Cells were harvested by diluting well with 10 mL DME/F12 + 10% Horse Serum containing media. Cells were centrifuged at 2000 RPM for 2 minutes. Pellets were washed 2X with HBSS and stained in 1 uM CM-H₂DCFDA as indicated previously. In some cases, Amplex Red (Invitrogen) assays were used to measure ROS in order to compare attached and suspended conditions and were used according to manufacturer's instructions. For measurement of general cellular superoxide, cells were processed as above and stained with 5 uM dihydroethidium (DHE) (Invitrogen) for 15 minutes at 37 degrees C protected from light followed by flow cytometric analysis at 605 nm fluorescence. Mitochondrial specific superoxide was measured using mitoSOX (Invitrogen) dye by staining cells with 5 uM mitoSOX solution for 10 minutes at 37 degrees C protected from light. Samples were analyzed by flow cytometry at 580 nm fluorescence using a BD LSRFortessa Cell analyzer.

ATP Assays

For the measurement of ATP in attached cells, Cell Titer Glo (Promega) reagent was added to 96 well plates. Luminescence was read after 10 minute incubation at room temperature. Values were normalized based on BCA protein assay (Pierce). To measure ATP in detached conditions, cells were detached and placed in 6 well poly-HEMA dishes. Aliquots

were mixed 1:1 with Cell Titer Glo reagent. Values were normalized based on BCA protein assay (Pierce).

JC1 Assay

Cells were seeded on poly-d-lysine coated MatTek dishes. Cells were incubated in 2 ug/mL JC1 (5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Invitrogen) at 37 degrees for 30 minutes protected from light. After incubation, dye was washed off of cells and replaced with normal growth medium. Cells were analyzed using an Axiovert 100M LSM510 Zeiss Confocal microscope. Cells were excited with an argon-ion laser source at 488 nm. JC1 monomer emission was observed at 529 nm and J-aggregate emission was observed at 590 nm. Images were acquired using 20X/0.75 Plan-Neofluar objective. Software used was Zeiss AIM software, version 3.2.

Oxygen Consumption and Extracellular Acidification Rate

The oxygen consumption rates (OCR) and extracellular acidification rate (ECAR) of adherent cells were measured utilizing the Seahorse XF Extracellular Flux Analyzer (Seahorse Biosciences Inc., North Billerica, MA). This method of analysis allows for real time monitoring of extracellular acidification rates and oxygen consumption based on two fluorimetric probes inserted into the small volume well. Equal numbers of cells (7500 cells per well) were seeded in a defined small volume in Seahorse XF-96 well plates. The following day, wells were refed with fresh HEPES buffered media approximately 1 hour prior to assay. In short, the sensor cartridge was loaded with metabolic inhibitors to target specific components of the electron transport chain at specific times, while monitoring pH change as

well as oxygen consumption. The metabolic inhibitors used are oligomycin (ATP synthase inhibitor), followed by the protonophore FCCP (an uncoupler of oxidative phosphorylation), and finally a combination of rotenone (complex I inhibitor) and antimycin A (complex III inhibitor). Basal OCR and ECAR measurement during time course treatment with the inhibitors listed above allow for calculations of basal OCR, maximum mitochondrial respiratory capacity, OCR due to proton leak, ATP production, spare respiratory capacity, and non-mitochondrial derived OCR. Fatty acid oxidation study was done by treating cells overnight prior to assay with substrate limited media (DME/F12 media + 1% horse serum, 0.5 mM carnitine hydrochloride [pH adjusted], 1X PSG, 0.5 mM D-(+)-Glucose, 5 mM HEPES buffer). On day of assay, substrate limited media was replaced with FAO medium 45 minutes prior to the start of the assay (Homemade Krebs Henseleit Buffer modified with CaCl_2 : To 500 mL sterile water the following was added: 111 mM NaCl, 5.7 mM KCl, 1.25 mM CaCl_2 , 2.0 mM MgSO_4 , 1.2 mM Na_2HPO_4 ; This buffer was supplemented with 2.5 mM glucose, 0.5 mM carnitine, 5 mM HEPES, pH adjusted to 7.4 with NaOH). 15 minutes prior to start of assay, 40 μM Etomoxir was added to the appropriate wells. At the beginning of the assay, either palmitate:BSA or BSA control was added to appropriate wells. Mitostress test was the conducted according to manufacturer's instructions. For glycolytic stress test, cells are deprived of glucose for approximately 1 hour prior to the beginning of the assay. ECAR is measured and glucose is re-introduced to the media. The increase in ECAR is attributed to glucose conversion to pyruvate through glycolysis. Oligomycin is then added to shunt all metabolic function to glycolysis, which results in a large increase in ECAR indicating the glycolytic reserve. Following addition of 2-deoxy-glucose (competitive inhibitor of hexokinase), the non-glucose derived OCR can be calculated.

Flow Cytometry Sorting

Trypsinized cells (1×10^6) were stained with APC tagged mouse anti-human CD44 (BD Biosciences 560890), and PerCP-Cy5.5 tagged mouse anti-human CD24 (BD Biosciences 561647) in 100 μ L of flow sorting buffer (1X PBS, 2.5 mmol/L EDTA, 10 mmol/L HEPES, and 2% horse serum) on ice protected from light for 30 minutes. Following staining, cells were washed with 1X HBSS and resuspended in flow sorting buffer. Cells were analyzed and sorted using a FACS-Aria.

Cell Culture Antioxidant Treatments

For suppression of reactive oxygen species, the following antioxidants were used. Where indicated, HMLE cells were treated with N-acetyl cysteine (NAC) at the concentration indicated for 1 hr prior to assay. Reduced glutathione (GSH) was added to HMLE cells after pH correction to 7.4 and incubated for approximately 1-2 hours depending on experiment at the indicated concentration. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was added to cells at the indicated concentration for overnight pretreatment of HMLE or HMLE+shGLUD1 cells. HMLE or HMLE+shGLUD1 cells were treated with cell permeable di-methyl- α -ketoglutarate (Sigma) which was pH corrected to 7.4. Cells were treated at the indicated concentration between overnight and 2 hour pre-treatment.

RNA Sequencing

RNA was isolated in triplicate from both MSP cells expressing vector alone or stably expressing GRHL2. For RNA isolation, the RNeasy Plus Kit (Qiagen) was used and was

quantified using Nanodrop (Fisher Scientific). The RNA quality check was performed on Bioanalyzer (Agilent). RNA quality check and libraries builds were done by the WVU genomics core. RNA samples were then sent to the University of Illinois Core Facility for 100BP single end data RNA sequencing. FastQC was used to visualize quality of sequencing. After it was determined that no filtering was needed, the reads were mapped to hg38 using STAR. The data was then quantified with featureCounts with the following flag set: —ignore-dup—primary. This count data was used as input into DEseq2 to produce a list of differentially expressed genes with some visualizations also included. The list of genes with FPKM was also acquired and was produced from the count data with a simple custom script.

Kinetic α -KG assay

HMLE, MSP, or MSP+GRHL2 cells were seeded in 6 well dishes in duplicate wells and allowed to attach for 24 hours. Wells were washed 2X with PBS, followed by lysis in 300 μ L RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton-X100, 1% Sodium Deoxycholate, 0.1% sodium dodecyl sulfate, + pierce miniprotease inhibitor table per 10 mL lysis buffer). Lysates were scraped into microfuge tubes and cleared in cold centrifuge at 4°C at full speed for 10 minutes. Supernatants were saved and assayed in 100 μ L reaction buffer (100 mM KPO₄ pH 7.2, 10 mM NH₄Cl, 5 mM MgCl₂, and 0.15 mM NADH added immediately prior to assay). 10 μ L of sample were added to 100 μ L reaction buffer. Immediately prior to the beginning of the assay, 0.7 μ L (0.5 units) of L-Glutamate Dehydrogenase enzyme (bovine liver, sigma G2626) was added to each of the wells using a multi-channel pipettor. Positive control samples were done to ensure linear range of α -KG concentration in samples. OD was read kinetically at 37 °C at 340 nm reading the sample every 10-20 seconds depending on

the number of samples in the assay. Depending on the level of α -KG in the sample, the reaction would proceed more quickly or more slowly. The slope of the line created by monitoring the decline in NADH absorbance as it was used up in the reaction was calculated and indicated the relative concentration of α -KG. Calculated slopes were normalized to protein in the samples as determined quantitatively by BCA assay (Pierce).

Extraction of Cells for LC-MS Analysis

All solvents used were high quality HPLC gradient grade and sterile milliQ grade water was used. Cells were plated in triplicate per condition in 6 well dishes. Cells were seeded for at least 24 hours prior to extraction. Wells were washed 3X with PBS. Extract solution was made up of 50% methanol, 30% acetonitrile, and 20% milliQ water. Extraction buffer was pre-chilled in a dry ice ethanol bath. Eppendorf tubes were pre-chilled in a dry ice/ethanol bath placed in a metal heating block for maximum cooling. Intracellular extracts were made by adding 500 μ L of pre-chilled extraction buffer to wells and placing plate on a shaker in the cold room for 10 minutes. The solution was then transferred into pre-chilled Eppendorf tubes. Samples were centrifuged at 0 $^{\circ}$ C at full speed for 10 minutes. Supernatants were transferred to HPLC vials and stored at -80 $^{\circ}$ C. For LC-MS method, Column was the sequant Zic-pHilic (150 mm $\times$ 2.1 mm i.d. 5 μ m) with the guard column (20 mm $\times$ 2.1 mm i.d. 5 μ m) from HiChrom, Reading, UK. Mobile phase A: 20 mM ammonium carbonate plus 0.1% ammonia hydroxide in water. Mobile phase B: acetonitrile. The flow rate was kept at 100 μ L min $^{-1}$ and gradient as follow: 0 min 80% of B, 30 min 20% of B, 31 min 80% of B, 45 min 80% of B. The mass spectrometer (Thermo Exactive Orbitrap) was operated in a polarity switching mode.

Immunofluorescence

Cells were plated on chamber slides (LabTek154526) using ~0.5 ml medium per chamber. Wells were pre-coated with sterile 0.5 mg/ml poly-lysine or 20 ug/ml collagen for ~1 hour, and washed off prior to plating. Cells were washed in HBSS or D-PBS, and fixed in EM grade 4% formaldehyde in 1X PBS for 20 minutes at room temperature. Cells were washed 2X with PBS. Samples were then permeabilized in 0.5% TX100 in PBS at 4 degrees for 10 minutes. Permeabilization solution was washed 2X with PBS. Cells were incubated in 100mM glycine in PBS for 10 minutes to quench the formaldehyde. Cells were blocked for one hour in PBS+10% goat serum+0.1% Tween-20+0.1%BSA for 1 hour. Primary antibody was diluted in blocking solution, and incubated 1 hr/RT or overnight/4 degrees (TOMM20, ms; BD Biosciences). Primary antibody was typically diluted 1:250. After probing, cells were washed with PBS+0.1% Tween-20, 3X. Secondary antibody (Anti-mouse Alex Fluor 555; Invitrogen) was diluted in blocking solution, and incubated 1-2 hr/RT (typically 1:1000 dilution). Cells were washed again with PBS+0.1% Tween-20, 3X. Samples mounted in ProLong Gold+DAPI.

Statistical Analysis

Error bars in graphs represent SDs. P-values were calculated using a Student's two-tailed t-test.

Results

EMT shifts metabolism from glycolysis to oxidative phosphorylation, which is reversed by GRHL2.

MSP and TGF- β -induced MCF10aneot cells result from EMT that requires GRHL2 down-regulation (15, 16, 28), accompanied by a CD44^{high}CD24^{low} marker profile (figure 1A). Re-expression of GRHL2 sensitized these cell lines to anoikis significantly, as assessed by a caspase activation assay (figure 1B, 1C, figure S1A). Conversely, the stable knockdown of GRHL2 using shRNA conferred anoikis-resistance in HMLE+Twist-ER cells as well as MCF10A neoT cells (figure 1D, S1B). We first endeavored to characterize the effect of EMT and, conversely, the effect of GRHL2-induced MET on metabolism in our system. We found that the ATP level in attached MSP cells was elevated significantly compared to HMLE cells. When cells were suspended, ATP level declined significantly in HMLE cells, but was maintained in MSP cells, even at twenty-four hours of suspension (Figure 2A). GRHL2 reduced the level of ATP in both suspended and attached MSP cells (figure 2B). In light of the higher efficiency of mitochondrial oxidative phosphorylation compared to glycolysis in the production of ATP, these results suggested that EMT perhaps caused a shift to the former. MSP cells had a higher mitochondrial membrane potential ($\Delta\Psi$), reflecting increased oxidative phosphorylation. Conversely, GRHL2 overexpression resulted in decreased $\Delta\Psi$ in MSP cells (Figure 2C). These results, and the marked ATP differences, indicated that EMT shifted cells toward an increased utilization of mitochondrial oxidative phosphorylation to increase basal ATP levels and sustain ATP even in suspended cells.

In order to test the effect of EMT or GRHL2-induced MET on regulation of oxidative metabolism specifically, we compared oxygen consumption rate in HMLE, MSP, and

MSP+GRHL2 cells using the Seahorse mitochondrial stress test. The Seahorse XF assay system measures extracellular acidification rate (ECAR) as well as oxygen consumption rate (OCR) in order to determine fluxes in metabolic parameters that occur upon addition of various inhibitors and stimulators of the electron transport chain. Our results indicated that MSP cells had greater ATP, maximum respiration, as well as spare oxidative capacity relative to HMLE cells, and re-expression of GRHL2 reversed these effects (figures 3A,B). The increased mitochondrial activity of MSP and reversal by GRHL2 were not limited to cells utilizing glucose and glutamine as carbon sources, as similar effects were seen with the fatty acid carnitine as the sole carbon source (figures S4A,S4B). Moreover, HMLE cells had higher glycolytic activity than MSP cells (figure S2A). Stably depleting GRHL2 in both HMLE+Twist-ER and MCF10A neoT cells partially reversed the mesenchymal metabolic phenotype, increasing ATP and maximum respiration (figure S2B, S2C, S2D).

To determine whether the increased OCR observed was due to elevated mitochondrial number, cell lines were stained for a mitochondrial structural protein, TOMM20. Surprisingly, we found that MSP cells had lower mitochondrial staining than HMLE cells (figure S2E, S2F). This may indicate that HMLE cells partially compensate for low mitochondrial activity by up-regulating mitochondrial biogenesis. Taken together, these results confirmed that mesenchymal cells enhanced oxidative phosphorylation, and partially suppressed glycolysis, both of which are counteracted by GRHL2. Confirming this observation, we found that treatment with 2-deoxyglucose (2-DG) resulted in greater decrease in ATP level in HMLE cells (58% of no treatment control) and MSP+GRHL2 cells (59.1% of no treatment control) than in MSP cells (72.1% of no treatment control) (figure

S3A). GLUD1 depletion did not directly affect ATP levels, as reported (29). The loss of GLUD1 did, however, result in a decrease in mitochondrial membrane potential (figure S3B).

EMT suppresses the accumulation of ROS; reversal by GRHL2

To identify genes regulated by GRHL2 underlying its metabolic effects, we conducted RNA-seq analysis. Surprisingly, we did not find that major ETC components or Krebs cycle metabolic enzymes were significantly affected by GRHL2 expression, with the possible exception of pyruvate dehydrogenase kinase 4 (PDK4), but its levels were highly sensitive to the initial conditions of each individual experiment, precluding definitive conclusions as to its regulation (data not shown). GRHL2 did, however, regulate several genes that participate in reactive oxygen species (ROS) regulation (Table 1). Interestingly, GRHL2 expression did not significantly affect expression of the major ROS regulatory enzymes superoxide dismutase or catalase (Table 1).

We compared endogenous ROS levels in HMLE, MSP, and MSP+GRHL2 cells. We found that the MSP cells display a significant reduction in ROS by fluorogenic reporter assay (CM-H₂DCFDA) in both 0 hour and 24 hour suspended conditions, and that GRHL2 increased ROS to the approximate level detected in HMLE cells (figure 4A). Inducing EMT in the basal mammary epithelial cell line, MCF10A neoT, with TGF- β treatment resulted in decreased ROS; further, overexpression of GRHL2 elevated ROS level (figure S5A). Conversely, shRNA depletion of GRHL2 suppressed ROS levels both in HMLE+Twist-ER cells and MCF10A neoT cells (figures 4C, S5D).

To identify the species of ROS present, we analyzed cells with a variety of fluorometric dyes with varying species selectivities. CM-H₂DCFDA staining is a general ROS indicator that responds well to hydrogen peroxide (H₂O₂). In order to verify that this was the predominant species, we stained cells with MitoSOX red, dihydroethidium (DHE), and Amplex red-hydrogen peroxide reporter. Amplex Red, which reports hydrogen peroxide specifically, indicated ROS trends similar to those obtained by CM-H₂DCFDA staining in both attached and suspended conditions (figure 4B). MitoSOX red and DHE staining report mitochondrial and generalized superoxide, respectively. These probes showed that HMLE cells had lower mitochondrial and general superoxide (O₂^{•-}) levels than MSP cells and that overexpression of GRHL2 resulted in a decreased O₂^{•-} level (figure S5B, S5C). Mitochondrial electron transport is the major source of cellular superoxide (30). In this light, our superoxide data correlated well with our data on mitochondrial oxidative phosphorylation. These data indicated clearly that mitochondria are not the major source of higher ROS in normal epithelial cells or GRHL2-reverted epithelial cells, as this ROS was hydrogen peroxide, not superoxide, and mitochondria were more active in mesenchymal cells, which had lower overall ROS.

We further sought to examine the role played by ROS in contributing to anoikis sensitivity. HMLE cells were treated with two common antioxidant compounds including reduced glutathione and the vitamin E derivative, Trolox. We found that these compounds reduced ROS level in cells and significantly reduced both ROS and sensitivity to anoikis (Figure 4D). In figure S5A, we show that MCF10a neoT cells experience a large ROS burst between 0 and 24 hours. Speculatively, this burst may occur much earlier in HMLE cells, resulting in little difference between 0 and 24 hours. It is also plausible that in HMLE cells

at 24 hours, enough cell death has occurred to obscure the ROS burst. These findings support the crucial role of ROS in triggering anoikis.

We have previously reported that CD44S, which is upregulated in MSP cells, contributes to anoikis resistance (31). CD44 was shown elsewhere to decrease ROS by shifting metabolism from oxidative phosphorylation toward glycolysis and pentose phosphate pathways, thereby increasing reduced glutathione levels (32). Consistent with this, the stable knockdown of CD44 resulted in increased ROS, and increased sensitivity to anoikis (figure S7A, S7B). Our metabolic findings were inconsistent with the mechanism proposed in (32) because we observed a shift toward oxidative phosphorylation in MSP cells (figure 3) and because reduced glutathione levels in HMLE and MSP cells were similar (figure S8).

Interestingly, neutralizing cellular ROS in HMLE cells with the scavenger N-acetyl cysteine (NAC) resulted in a significant increase in $\Delta\Psi$ (Figure 4E). Neutralizing ROS in MSP+GRHL2 cells replicated this effect (figure S6A), indicating that the low-ROS cellular environment could play a role in sustaining the elevated mitochondrial function that we observed in mesenchymal cells (figure 2).

EMT up-regulates glutamate dehydrogenase-1; reversal by GRHL2

Our RNA-seq data indicated that GRHL2 down-regulated several genes that have well established effects on ROS in other systems (Table 1). Manipulation of the levels of NCF2/p67PHOX, ALOX15, FAT4 and NQO1, however, produced no significant change in ROS in our cells (data not shown). GRHL2 also down-regulated Glutamate Dehydrogenase 1 (GDH1 or GLUD1), an enzyme implicated in the suppression of ROS (27). By western

blotting, we found that GLUD1 protein was upregulated in MSP cells relative to HMLE cells, and downregulated in MSP by GRHL2 expression, in agreement with our RNA-seq data (figure 5A). Similarly, EMT that was generated by transient activation of Twist-ER protein also up-regulated GLUD1, which was prevented by GRHL2 (figure 5B); similar effects were found in TGF- β -induced MCF10a neoT cells (Figure S8). Conversely, GLUD1 expression increased following depletion of GRHL2 in both HMLE+Twist-ER and MCF10A neoT cells (figure 5C), indicating that GRHL2 is an important repressor of GLUD1.

Depletion of GLUD1 was previously shown to reduce α -ketoglutarate (α -KG) levels, indicating that GLUD1, through glutaminolysis, is a major source of α -KG (27). Interestingly, we found that MSP cells have an elevated α -KG:succinate ratio relative to HMLE cells and that GRHL2 decreased α -KG, using both enzymatic and liquid chromatography/mass spectrometric assays, consistent with the expression levels of GLUD1 (Figure 5D, 5E, 5F).

GLUD1 suppresses ROS and anoikis

Next, we tested the effects of altered GLUD1 and α -KG levels on ROS and anoikis. The stable knockdown of GLUD1 enzyme using shRNA transduction resulted in both an increase in ROS level as well as increased sensitivity to anoikis (figure 6A). A similar effect of GLUD1 knockdown was observed in HMLE+Twist-ER cells (figure S8C).

To confirm that the GLUD1 effect on ROS was mediated by α -KG, we tested the effect of α -KG supplementation using the cell-permeable di-methyl- α -ketoglutarate derivative in GLUD1-depleted cells. This resulted in a dose dependent protection from anoikis as well as a reduction in ROS in HMLE cells (figure 6B). Moreover, the excess anoikis observed in HMLE

cells as well as MSP+GRHL2 cells was suppressed by α -KG replacement, demonstrating that the loss of GLUD1 promoted anoikis through ROS (figure 6C, 6D, S10). Neutralizing ROS with antioxidants or α -KG partially protected HMLE+shGLUD1 cells against anoikis as well (figure 6E, 6F).

These data indicate the critical role of GLUD1 up-regulation during EMT, resulting in protection from anoikis by suppressing ROS, and the converse role of GRHL2 in promoting anoikis through GLUD1 down-regulation (summarized in figure 7).

Discussion

The role and mechanism of the Warburg-like shift to aerobic glycolysis in tumor cells has received extensive investigation, but this effect is now known to be highly context dependent. In fact, recent studies indicate that cancer stem cells (related, in some instances, to EMT) develop an enhanced capacity for and dependence on oxidative phosphorylation ((33), see (34) for an excellent review). Moreover, oxidative phosphorylation can contribute to tumor metastasis (35, 36). This is thought, counter-intuitively, to have advantages for slow-cycling cancer stem cells, such as better maintenance of ATP in nutrient-poor tumor environments due to the higher efficiency of the TCA/ETC pathway (using fatty acids or glutamine as carbon sources); moreover, the tumor microenvironment is rarely, if ever, hypoxic enough to inhibit oxidative phosphorylation (34). Our metabolic results comparing MSP vs. HMLE cells are highly consistent with this new understanding, and they show that GRHL2 reverts MSP cells back to an epithelial type metabolism (higher glycolysis, lower oxidative phosphorylation), accompanying re-sensitization to anoikis.

Metabolic changes due to oncogenic transformation, EMT or transition to cancer stem cells have been reported extensively, with unique effects in each system (35, 37-40). In this paper, we provide a mechanism by which EMT/cancer stem cell transition lowers ROS so as to protect cells against anoikis. Under this model, GRHL2 reverses EMT, suppressing mitochondrial oxidative function by repression of the critical enzyme regulating glutaminolysis, GLUD1, resulting in loss of glutamine utilization, increased ROS level, ultimately shifting metabolism resulting in anoikis sensitivity (Figure 6).

There may be multiple mechanisms of ROS neutralization downstream of α -ketoglutarate. First, it can act synergistically with other compounds (e.g., ascorbic acid) as a co-antioxidant (41). Secondly, the reverse GLUD1 reaction yields glutamate, contributing to glutathione synthesis. Thirdly, α -ketoglutarate is a co-factor for both histone demethylase and DNA demethylase enzymes. Changes in the level of this co-factor could affect gene expression epigenetically, as observed in other settings (42), with effects on metabolism and ROS. Finally, a recent report shows that up-regulated GLUD1 in tumor cells led to increases in α -ketoglutarate, increasing fumarate, in turn, through Krebs cycle enzymes. This contributed to the neutralization of cellular ROS, because fumarate activated glutathione peroxidase activity. GLUD1 up-regulation was, accordingly, shown to be pro-tumorigenic, and this effect was mediated by decreased ROS levels (27). In renal cell carcinoma, fumarate hydratase (FH) deficiency was increased fumarate to millimolar levels, contributing to oxidative stress rather than alleviating it (43). However, the major ROS in that study was superoxide rather than hydrogen peroxide, and GRHL2 did not affect FH level in our cells (data not shown). We speculate that the contradictory effects of

fumarate in FH-deficient vs. GLUD1-over-expressing tumor cells may be reconciled by these considerations.

Interestingly, autophagy, which may play positive or negative roles in anoikis and non-apoptotic cell death after detachment, is regulated by glutaminolysis as well, through mTORC complexes (25). Speculatively, GLUD1/ α -ketoglutarate alterations due to EMT or GRHL2 may affect cell survival through these additional pathways.

The upregulation of GLUD1 would have multiple advantages for EMT/CSC cells, including increased use of glutamine or glutamate as alternative carbon sources in glucose-poor environments, protection against ROS, and the increased ETC/oxidative phosphorylation capability afforded by less ROS-induced mitochondrial damage. Interestingly, the aspartate/glutamate transporter SLC1A6 was down-regulated dramatically by EMT, and, conversely, up-regulated by GRHL2 (table 1). Recently, SLC1A6 was found to act primarily as a glutamate exporter (44). EMT/CSC cells are predicted, therefore, to have increased accumulation of intracellular glutamate, contributing to the pathway that we have emphasized here.

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Table 1. Effect of GRHL2 on expression of ROS-regulatory genes

Gene ID	Fold Change (MSP+GRHL2/ MSP+vec control)	Significance in ROS or Metabolism
ALOX15	+236.0	Can reportedly generate lipid peroxide radicals from arachidonic acid substrates
ALOXE3	+21.0	
ALOX12P2	+18.3	
SLC1A6	+165.7	Glutamate/Aspartate transporter which may alter α -KGDH activity resulting in H_2O_2 radicals and reduce its TCA activity
NCF2 (p67-PHOX)	+23.4	Cytoplasmic subunit of NADPH-Oxidase (NOX) Complex
FAT4	-19.6	Human homologue of Drosophila Fat. Potentially contains Ft _{mito} fragment which binds complex I and stabilizes it to reduce ROS/increase mitochondrial activity
SOD1	-1.4	Soluble superoxide dismutase enzyme involved in cytoplasmic $O_2^{\cdot-}$ detoxification. Not significantly by GRHL2 overexpression
SOD2	+1.2	Mitochondrial isoform of superoxide dismutase involved in conversion of mitochondrial $O_2^{\cdot-}$ into diffusible H_2O_2 . Not significantly altered by GRHL2 overexpression
SOD3	NA	Extracellular superoxide dismutase

Cat	-1.3	Catalase enzyme involved in detoxification of H ₂ O ₂ ROS species. Appears to be slightly regulated by EMT, but not significantly altered by GRHL2 overexpression
NQO1	-1.9	ROS detoxifying enzyme which reduces quinines to hydroquinones – major Nrf2 target gene
GLUD1	-2.5	Reported be the primary source of cellular regulation of α -KG which feeds to TCA cycle to increase Fumarate and stabilize GPx1

Figure Legends

Figure 1. MSP cells are an anoikis resistant subpopulation of HMLE cells which have undergone EMT and lost GRHL2 expression. A. (upper panel): HMLE cells were sorted for CD44 and CD24 by FACS (insert shows up-regulated CD44S in MSP cells); B: Resulting subpopulations were assayed for anoikis sensitivity by caspase 3/7 activation assay; C. MSP cells in which GRHL2 was re-expressed were assayed for anoikis compared to vector control (24 hour time point with zero time subtracted). (lower panel): confirmation of GRHL2 expression by western blotting. D. HMLE+Twist-ER cells depleted of GRHL2 by shRNA transduction were assayed for anoikis; the confirmation of GRHL2 knockdown is shown as part of figure 5C.

Figure 2. ATP levels are elevated in mesenchymal relative to epithelial cells due to increased mitochondrial membrane potential ($\Delta\Psi$) A. HMLE and MSP cells were analyzed for ATP level under attached or suspended (16 h, 24 h) conditions. (The line indicates that the attached level cannot be directly compared to the suspended levels due to proliferation in attached condition). B. GRHL2 suppresses ATP level in MSP cells in both attached and suspended conditions. C. $\Delta\Psi$ is elevated in MSP cells, which is reduced by GRHL2 overexpression (JC1 fluorescent probe; green=monomers to indicate free JC1, orange=aggregates of JC1 proportional to $\Delta\Psi$); (right panel): Image J quantification and ratio calculation of raw intensity pixel density for single phase red and green images.

Figure 3. Shift from glycolysis to oxidative phosphorylation accompanies EMT; reversal by GRHL2 A. MSP cells have higher ATP production, maximum respiration, and higher spare capacity for oxidative phosphorylation than HMLE; B. Reversal by GRHL2

Figure 4. Epithelial cells have higher ROS than mesenchymal cells, and the excess ROS promotes anoikis. A. HMLE cells have higher ROS compared to MSP cells, which is elevated by GRHL2 expression in MSP cells: CM-H₂DCFDA fluorescence at the indicated times of suspension. B. Hydrogen peroxide is the major regulated ROS type (Amplex Red assay); C. shRNA depletion of GRHL2 decreases ROS in HMLE+Twist-ER cells. D. Antioxidant compounds protect against anoikis. MSP+GRHL2 cells were pre-treated overnight with reduced glutathione (GSH, 5 mM) and Trolox (100 μ M) followed by ROS and anoikis assay; E. Suppression of ROS resulted in increased JC1 aggregate formation and elevated $\Delta\Psi$. HMLE cells were treated with N-acetyl cysteine (NAC) at 0.25 and 0.5 mM for 1 hr before staining with JC-1 fluorimetric dye. (right panel): Image J quantification and ratio calculation of raw intensity pixel density of single phase red and green images.

FIGURE 5. GRHL2 regulates Glutamate dehydrogenase-1 and α -ketoglutarate levels.

A, GLUD1 is downregulated by GRHL2 in MSP cells (upper panel: RNA-seq data lower panel: western blot); B. GLUD1 expression is upregulated during EMT and is downregulated by GRHL2 in HMLE-Twist cells; C. shRNA depletion of GRHL2 results in increased GLUD1 protein. D. α -KG is higher in MSP cells relative to HMLE cells, and GRHL2 overexpression decreases it (enzymatic assay measuring loss of NADH absorbance); E. α -KG:succinate ratio is higher in MSP than in HMLE or MSP+GRHL2 cells (mass spectrometric assay). F. α -KG:succinate ratio is decreased by GRHL2 in HMLE cells induced to undergo EMT by Twist-ER activation (mass spectrometric assay).

FIGURE 6. GLUD1 and α -KG regulate anoikis through ROS. A. (upper panel): Western blot analysis of HMLE+shGLUD1 cells. (lower panel): GLUD1 knockdown elevates ROS level; (B) GLUD1 knockdown promotes anoikis as assessed by CM-H₂DCFDA assay and caspase 3/7 activation assay (respectively); C. Dimethyl- α -KG lowers ROS; (D) Dimethyl-

α -KG protects HMLE cells against anoikis; E. DM- α KG and Trolox lower ROS level; (F) DM- α KG and Trolox protect GLUD1 knockdown cells against anoikis.

FIGURE 7. GRHL2 suppresses EMT and results in the loss of GLUD1. This decreases α -KG, suppressing oxidative metabolism, increasing ROS, and sensitizing cells to anoikis.

Figures

Figure 1

Figure 2

Figure 3

Figure 5

Figure 6

Figure 7

Supplemental Figure Legends

Figure S1. GRHL2 sensitizes MCF10A neoT cells to anoikis. A. Caspase 3/7 activation assay on 24 hour anoikis assay (zero hour subtracted). B. shRNA depletion of GRHL2 decreases anoikis sensitivity in MCF10A neoT cells.

Figure S2. EMT causes a metabolic shift from glycolysis to oxidative phosphorylation, that is partially reversed by GRHL2. A. Elevated glycolysis in HMLE cells relative to MSP cells (Seahorse XF-96 glycolysis stress test); (B) shRNA depletion of GRHL2 increases ATP and maximum respiration (Seahorse XF-96 mitostress test) in HMLE+Twist-ER cells; (C) shRNA depletion of GRHL2 increases ATP and maximum respiration in MCF10A neoT cells. D. Graphical representations of Seahorse assays showing OCR measurements prior to and following addition of stimulators and inhibitors of oxidative phosphorylation. E. MSP cells (derived from HMLE+Twist-ER cells, not treated with 4-OHT) have decreased mitochondrial structural protein TOMM20 when compared to HMLE+Twist-ER (not treated with 4-OHT) epithelial subpopulation. F. MSP cells have lower expression of the mitochondrial structural proteins TOMM20 and VDAC1 by western blotting.

Figure S3. MSP cells are able to maintain ATP in spite of inhibition of glucose utilization with 2-deoxyglucose; reversal by GRHL2. A. Cells were treated with 5 and 10 mM 2-DG for 24 hours and evaluated for ATP level (Cell Titer Glo). Error bars are SD of mean. B. Knockdown of GLUD1 in HMLE cells decreases mitochondrial membrane potential (JC1 aggregate formation) (lower panel): Image J quantification and ratio calculation of raw intensity pixel density of red and green images.

Figure S4. Multiple carbon sources, including fatty acids, contribute to the elevated mitochondrial function in MSP cells. A. Elevated FAO in MSP cells relative to HMLE cells (Seahorse XF-96 FAO assay test; Eto = Etomoxir fatty acid oxidation inhibitor); B. GRHL2 re-expression decreases ATP production and maximum respiration in response to a fatty acid substrate (carnitine).

Figure S5. EMT decreases overall ROS and results in anoikis resistance, but elevates superoxide levels. A. (left and middle panels): Treatment of MCF10A neoT cells with TGF- β (5 ng/mL) induced irreversible EMT resulting in decrease in ROS and anoikis; (right panel): Overexpression of GRHL2 increased ROS in MCF10A neoT cells; B. Mitochondrial superoxide was increased by EMT and diminished by GRHL2 overexpression (MitoSOX); C. GRHL2 overexpression also decreased general superoxide level (DHE). D. Depletion of GRHL2 in MCF10A neoT cells decreases ROS by CM-H₂DCFDA staining.

Figure S6. Treatment of MSP+GRHL2 cells with antioxidant compounds results in elevated $\Delta\Psi$. (left panel): Representative images of treated cells. (right panel): Image J quantification and ratio calculation of raw intensity pixel density of single phase red and green images.

Figure S7. Depletion of CD44 with stable shRNA transduction increases anoikis and ROS. A,B. shCD44 cells are more sensitive to anoikis (caspase 3/7 activation assay) and have increased ROS level (CM-H₂DCFDA fluorescence).

Figure S8. HMLE and MSP cells have similar levels of reduced glutathione.

Figure S9. GRHL2 downregulates GLUD1 in MCF10A neoT cells and depletion of GLUD1 from HMLE+Twist-ER cells increases ROS and anoikis. A. 4OHT was added to HMLE+Twist-ER cells for 9 days, causing EMT; 4-OHT was then removed (+/-), causing cells expressing exogenous GRHL2 to revert to an epithelial state, while the control cells remained mesenchymal (15). Lysates were made 9 days after 4OHT removal and analyzed for the indicated proteins by western blotting. B. GRHL2 down-regulates GLUD1 in MCF10AneoT cells induced with TGF- β . C. Stable knockdown of GLUD1 by shRNA transduction increases ROS (left panel) and anoikis (right panel) in HMLE+Twist-ER cells to a similar level as in HMLE cells.

Figure S10. Treatment of MSP+GRHL2 cells with DM- α KG (10 mM) results in decreased ROS and protection from anoikis.

Figure S1

Figure S2

C

Figure S2 cont'd

D

E.

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Figure S2 cont'd

Figure S3

Figure S4

Figure S5

Figure S5 cont'd

Figure S6

Figure S7

Figure S8

Figure S9

Figure S9

Figure S10

Chapter 3

The Effect of GRHL2 on Tumor Recurrence following Chemotherapy or Oncogene Withdrawal

*The following represents unpublished data. Select portions of this work are ongoing projects
in the Frisch Lab.*

Project Overview

Grainyhead genes play an important role in embryogenesis in the context of neural tube closure, wound healing, and carcinogenesis. For a more thorough discuss on the role of Grainyhead-like 2 in cancer, chemotherapy resistance, and tumor recurrence, refer to Chapter 1, part 2 of this dissertation. The major aims of this project were to investigate the effect of GRHL2 overexpression on prevention of either chemotherapy induced recurrence in an orthotopic injection model or oncogene independent recurrence in a transgenic mouse model. These will be detailed below.

Introduction/Project Design

Disease recurrence several years following successful treatment of primary tumors is a frequent reality, and is the most common cause of mortality from cancer. It has been extensively demonstrated that in these settings, plasticity conferred by EMT benefits small populations of cells, bestowing CSC-like and chemoresistant properties to these primary tumor cells, which persist, only to resume growth after a period of dormancy (1-7). In breast cancer, these populations are often identified by a shift in surface expression toward a CD44^{HIGH}/CD24^{LOW} phenotype. In culture, treatment of HMLE cells with taxol derivatives selects for this CSC-like population; conversely, stable expression of GRHL2 significantly sensitizes these, as well as MDA-MB-231 cells, to this therapy. Most importantly, ectopically expressed GRHL2 prevents the chemotherapy-induced emergence of this population capable of tumor initiation (8). It is well accepted that EMT is generally associated with a heightened propensity for metastasis as well as a decreased sensitivity to conventional cytotoxic chemotherapeutics. Further, it has been demonstrated by

Chakrabarty et al. that treatment of cancer cells with the anti-cancer chemotherapeutic agent paclitaxel enriches for CD44 and ALDH expression as well as CD24 downregulation, all features associated with cancer stem cells (9).

In order to elucidate the role of GRHL2 in prevention of chemoresistant CSC-like subpopulations *in vivo*, we designed a doxycycline inducible ectopic GRHL2 construct. This GRHL2 inducible vector has prevented EMT in HMLE-Twist-ER cells when subjected to 4-hydroxytamoxifen induction, further supporting the importance of GRHL2 in suppression of EMT. Work shown here proposed injection of tumor lines containing GRHL2 inducible constructs into syngeneic mice followed by treatment with chemotherapy and subsequent analysis of recurrent populations. In order to determine the role of GRHL2 in protection from EMT/chemotherapy induced CSC enrichment, we planned to develop stable mouse tumor cell line expressing a doxycycline inducible GRHL2 construct, inject these tumor cells orthotopically into the mammary fat pad of syngeneic mice allowing tumors to form to sizes of greater than 5 mm. We would then treat with chemotherapy (approved therapies include Cisplatin, Cyclophosphamide, Doxorubicin, and Paclitaxel), dividing therapy groups further into +/- DOX groups in order to induce GRHL2. We would observe the effect on GRHL2 tumor sensitivity, tumor recurrence, and metastasis. To examine the role of EMT in the generation of the CSC fraction *in vivo*, a murine breast cancer cell line referred to as Py2T was evaluated for potential use in murine syngeneic orthotopic injection experiments. This cell line, derived from a polyoma middle-T antigen mouse transgenic model, has been previously described to undergo EMT either by addition of TGF- β *in vitro* or spontaneously by orthotopic injection *in vivo* (10). Considered together with previous studies, our data indicate a significant role for GRHL2 in the context of prevention of recurrence post-

therapeutic intervention. This section will highlight the technical difficulties experienced in this project and future aims.

An additional focus of this project is to determine the effect of constitutive Grhl2 expression in a murine model of recurrence. Mouse models recapitulating recurrence following regression of primary tumors driven by doxycycline inducible neuNT, Wnt1, or *c-myc* have shown that in comparison to the primary tumors, recurrent disease that emerges following withdrawal of the oncogenic driver have undergone EMT (4, 11, 12). Analysis of tetO-neuNT and tetO-Wnt1 primary vs recurrent RNA expression revealed a striking decrease in GRHL2 expression in recurrent tumors (8). These findings strongly support the hypothesis that GRHL2 is a tumor suppressor, is specifically silenced in the tumor initiating subpopulation of the primary tumor, and that it is major suppressor of disease recurrence. Further evaluation is needed in order to more clearly define the precise role of GRHL2 in preventing recurrence *in vivo*. We hypothesized here that constitutive GRHL2 expression abrogates the emergence of the CSC population enriched for by chemotherapy or EMT, thereby preventing therapy resistant recurrence *in vivo*. The search for a tumor suppressive type transcription factor that might alter this transition from chemosensitive “normal” tumor cell to the chemoresistant CSC could prove critical in producing improved outcomes for patients with breast cancer.

Methods and Materials

Cell Lines

Py2T cell lines were generously donated from the lab of G. Christofori (Department of Biomedicine, University of Basel – Switzerland). Py2T cells were grown in Dulbecco's

Modified Eagle's Medium (DMEM) + 10% Fetal Bovine Serum (FBS) + 1X Penicillin-Streptomycin-Glutamine (PSG). Clonal isolation of the mixed population (epithelial + mesenchymal cells) was done in order to generate a purely epithelial colony of cells. These cells were titled "Py2T C7" (clone 7). HMLE+Twist-ER cells were a generous contribution from R. Weinberg (The Whitehead Institute, Cambridge, MA). HMLE+Twist-ER cells were grown in Dulbecco's Modified Eagle's F12 Media (DME/F12) + 5% horse serum + 1X penicillin-streptomycin-glutamine (PSG) + 10 ug/mL insulin + 10 ng/mL EGF + 0.5 ug/mL hydrocortisone. 4T1 mouse mammary carcinoma cells were ordered from ATCC and maintained in RPMI 1640 Complete media supplemented with 10% FBS + 1X PSG. Where indicated, 4-hydroxytamoxifen (10 ng/mL) was added to the HMLE+Twist-ER cells to activate the Twist-ER protein. Where indicated, Transforming Growth Factor- β (TGF- β) was added to the HMLE+Twist-ER cells (5 ng/mL).

Generation of Stable Cell Lines by Retroviral Transduction

Mouse GRHL2 was amplified from a template purchased from Open Biosystems (MHS4426-99625903) and subcloned via standard molecular biological methodology into pcDNA3.1+-3XF expression vector in order to generate a flag tagged mGRHL2 plasmid (cloned into Sall site using Xho1 compatible enzyme). It was further subcloned into the doxycycline inducible expression vector pLUT (cloned into Not1 and NheI sites). Separately, GRHL2 was subcloned previously into MSCV-IRES-puro retroviral vector (Cieply et al., 2012). Also cloned human GRHL2 into pLUT vector for verification of findings in human cell lines as well as mouse lines. Lentiviruses of mGRHL2-pLUT plasmids were packaged and amplified into 293T cells by transfection of 3.3 ug mGRHL2-pLUT, 2.2 ug psPAX, and 1.2 ug of pCMV-

VSV-G (plasmids obtained from Addgene) with Mirus TransitLT transfection reagent onto 60 mm² dishes. GRHL2-MSCV-IRES-puro retroviral was packaged and amplified into GP2 + 293T cells by transfection of 4.5 ug of retroviral vector, 2.5 ug of pCMV-VSV-G per 60 mm² dish of cells, with Mirus TransitLT transfection reagent. Cells were refed with Dulbecco's Modified Eagle's Medium (DMEM) + 10% Fetal Bovine Serum (FBS) + 1X Penicillin-Streptomycin-Glutamine (PSG) media after incubating with DNA/transfection medium overnight. Viral supernatants were harvested 48 hrs later, filtered through 0.45 micron filters (Whatman), and stored at -80°C. In subsequent infections, 0.6 mL of viral stock was used to transduce cells with plasmid per 1 well of a 6 well dish, and dish was subjected to centrifugation at ~1,400 rpm for 1 hr followed by 6 hours to overnight incubation with viral supernatant. Infected cells were subsequently refed and passaged to larger dishes for selection by puromycin. Following puromycin selection, cells were examined for protein content by Western Blot analysis probing for GRHL2 protein in order to verify protein overexpression in lentivirally transduced lines.

Animal Models

Animal work involving tumor injection and subsequent chemotherapy has all been approved by the West Virginia University Animal Care and Use Committee (ACUC # 11-0706.4, approved 10-31-12). We generated MMTV-GRHL2 transgenic mice by embryo transfer of a GRHL2-flag-MMTV fragment with the help of the WVU animal transgenics core facility. Founders were found to express GRHL2 constitutively in the mammary gland and developed normally (data not shown), as described previously. We crossed these transgenic mice with a double transgenic tetO-neuNT/MMTV-rtTA mouse line in a FVB

background provided by the laboratory of L. Chodosh. At six weeks of age, oncogene expressing transgenic mice either containing the GRHL2 transgene, or GRHL2 negative littermates were induced using 2 mg/mL doxycycline 5% sucrose water. Within 6-7 weeks, primary tumors emerged in both groups and were allowed to grow to approximately 15x15 mm². Tumors were monitored using caliper measurements weekly.

Immunofluorescence

For further evaluation as to the percentage positivity of GRHL2 within Py2T C7 cell populations expressing lentivirally transduced mGRHL2-pLUT, immunofluorescence was carried out in order to confirm ~100% cellular expression. Collagen coated chamber well microscope slides (20 ug/mL) were used to plate cells for IF experiments. 40,000 cells were plated at least 24 hrs prior to fixation. Cells were fixed with 4% paraformaldehyde (PFA) in sterile PBS. PFA was quenched with 100 mmol/L glycine in PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS at 4 degrees for 10 minutes, followed by PBS washing twice. Were blocked for 1 hr in PBS + 10% goat serum + 0.1% Tween-20 + 0.1% BSA. The primary antibodies were used as follows: GRHL2, rb (Sigma) 1:250; Flag, ms (Sigma) 1:250. The secondary antibody was rb or ms Alexaflour 555 (red), 1:1000. Mounting media: Prolong Gold w/ DAPI (Invitrogen). Coverslips were mounted in Prolong Gold. Images were produced with the Axiovert 200M microscope, AxioCam MRM camera, and Axio Vision 4.3.1 software (Zeiss).

Western Blotting

SDS-PAGE was performed with pre-cast 4-20% gradient Tris-Glycine gels (Invitrogen). Proteins were immobilized by electrophoretic transfer to polyvinylidene difluoride filters (Immobilon) in 5% MeOH containing Tris-Glycine transfer buffer. Transfer was done running at either 80V for 3 hours or 50V for 14 hour overnight transfer. Filters were blocked in TBS + 0.1% Tween-20 + 5% nonfat milk. Primary antibodies were incubated in blocking solution either overnight at 4°C or for two hours at room temperature. Secondary antibodies were incubated in blocking solution for 1 hour at room temperature. Primary antibodies were used as follows: E-cadherin, ms (BD Biosciences) 1:1000; Fibronectin, rb (BD Biosciences) 1:1000; GRHL2, rb (Sigma) 1:1000; β -actin, ms (Millipore) 1:1000; Secondary antibodies for ECL chemiluminescence were either anti-mouse or anti-rabbit, conjugated to horse-raddish peroxidase (Bio-Rad) and were used at a 1:3000 dilution. Western blot membranes were developed using (ECL-West Pico, Pierce).

Statistics

GRHL2 expression data was obtained via NCBI Gene Expression Omnibus (accession number GSE10885; ref 3) to compare log expression of GRHL2 among tumor types including normal, luminal, Her2, claudin, and basal in order to develop an appropriate model system to evaluate. Previous work by our lab showed by Tukey HSD tests which compared these tumor lines for statistical differences in GRHL2 expression (Cieply et al., 2012). Any statistical analyses were conducted by R version 2.13.1 (www.r-project.org). Data for gene expression not shown.

Results and Discussion

The Py2T cell line was originally generated by the Christofori laboratory as a model cell line for examining the effects of EMT due to its ability to undergo EMT *in vitro* upon addition of TGF- β , and spontaneously *in vivo* upon orthotopic injection into the mammary fat pad (10). This murine breast cancer line thus seemed a model platform for examining the effects of GRHL2 on EMT in the context of CSC marker enrichment *in vitro* as well as sensitivity to chemotherapy, recurrence, and metastasis *in vivo*. TGF- β induction resulted in EMT in Py2T cells (figure 1). This EMT was accompanied by a striking reduction in GRHL2. Analysis of common markers of EMT (E-cadherin and fibronectin) revealed expected regulation showing downregulation of the epithelial cell-cell junctional marker E-cadherin as well as upregulation of the mesenchymal marker Fibronectin.

Next, we attempted to overexpress human-GRHL2 in multiple murine cell lines (figure 2a, b). These results showed that human-GRHL2 protein was not overexpressed by lenti- or retroviral transduction in mouse cell lines. mRNA analysis by qPCR showed that human GRHL2 was significantly elevated in 4T1 cells expressing h-GRHL2-MSCV-IRES-puro, indicating a defect in protein translation (figure 2c). Based on these findings, it was determined that mouse-Grhl2 would be needed to overexpress Grhl2 protein in murine models.

Murine Grhl2 was successfully overexpressed in the Py2T C7 mouse breast cancer cell line (figure 3) in a doxycycline inducible lentiviral vector (pLUT). Overexpression was confirmed by western blot analysis (figure 3a). However, as was evident by IF analysis, the resulting population had significant heterogeneity in Grhl2 expression showing strong nuclear expression in a majority of cells, while others either lacked nuclear localization of

Grhl2 or lacked even cytoplasmic expression (data not shown). It has been previously observed in our laboratory that injection of heterogenous clones results in failure of GRHL2 to prevent tumorigenesis due to tumor initiation from the GRHL2-neg subpopulation. Analysis of tumors that result shows that cells are devoid of GRHL2 expression indicating selection for GRHL2 negative cells (data not shown). Based on this, we attempted to isolate more homogenous expressers of Grhl2 by cloning out individual cell populations. We isolated a cell population that contained a higher number of Grhl2 positive cells than the parent population (figure 3b). However, upon treatment with TGF- β , these cells failed to undergo EMT, regardless of the presence of Grhl2 (figure 4). It was concluded that the lack of effect of TGF- β was due to overpassaging of the cells or selection against the TGF- β responsive population during passaging. The cells did form protrusions at the leading edge of colonies, but this occurred in both + and – GRHL2 cells. In 4T1 murine breast cancer cells, Grhl2 was insufficient to prevent TGF- β induced EMT (data now shown). In order to verify that the DOX inducible GRHL2 construct resulted in sufficient GRHL2 induction to prevent EMT in our previously characterized models, human-GRHL2 pLUT was transduced into HMLE+Twist-ER cells. The resulting line was confirmed by immunofluorescence to contain a homogenous, nuclear localized GRHL2 positive population. Simultaneous GRHL2 induction and treatment with 4-hydroxytamoxifen confirmed that GRHL2 overexpression protects against EMT in the context of activation of the Twist-ER fusion gene (figure 5b). These results indicate that there may be restricted murine cell line settings in which perhaps Grhl2 lacks appropriate co-factors required for enforcing the epithelial phenotype. It was determined that experiments utilizing Grhl2 overexpression in murine models in order to achieve syngeneic implantation of primary tumors would not be practical.

Elucidating the effect of chemotherapy on emergence of recurrent disease in orthotopic injection of human tumors in immunocompromised NSG mice will be an area of future research.

Primary mammary tumors induced by doxycycline driven by a tetO-neuNT gene regress upon doxycycline withdrawal, but recur at a high rate, forming tumors that have an EMT signature which are no longer reliant on the initiating oncogene (4, 11, 13). While the primary tumors are epithelial and express high levels of GRHL2, recurrent tumors have significantly lower expression of this transcription factor and are morphologically mesenchymal. This is a highly clinical relevant model of disease recurrence in HER2-positive breast cancer populations whose tumors contain EMT-like subpopulations conferring resistance to targeted therapies such as trastuzumab (5), resulting in emergence of treatment resistance recurrent disease. We tested the supposition that enforced Grhl2 expression in this murine model would prevent recurrence of oncogene-independent EMT-like tumors. GRHL2 positivity in GRHL2-transgenic mice was confirmed by genotyping using Taqman expression array designed to amplify exogenous GRHL2-flag (figure 6a). GRHL2-MMTV expression was confirmed in mammary tissue of founder pups by qPCR using trans-exonic primers to identify exogenous GRHL2 only (figure 6b). We induced 6 week old transgenic mice (tetO-neuNT/tetO-rtTA with GRHL2 transgene or littermate negative controls) to drive the development of the primary tumor. Following withdrawal of doxycycline, tumors regressed rapidly within around 17 days. The tumors recurred at varying times such that 5 out of 17 control mice (29.4%) developed recurrences while 0 out of 13 Grhl2-positive mice developed recurrent tumors (0%) (figure 7). These tumors were verified to be EMT-like and have lost GRHL2 expression in the control tumor (figure

8). While the recurrence frequency of control mice with primary HER2 driven tumors was significantly lower than previously reported (14), recent findings suggest that primary tumors must reach a larger size ($>25 \times 25 \text{ mm}^2$) before discontinuing oncogene activation. An area of future research will be to induce a larger number of GRHL2-positive and control mice, allowing primary tumors to grow to greater primary size in an attempt to achieve a greater frequency of recurrence so that greater statistical significance can be reached.

The findings presented here preliminarily support the conclusion that GRHL2 contributes to the prevention of CSC enrichment in EMT subpopulations or following chemotherapeutic intervention. These findings highlight the potential clinical relevance of determination of pathways which might influence endogenous GRHL2 expression and prevent the emergence of GRHL2^{negative} subpopulations enriched for CSC-like characteristics. Further study is needed to better characterize the role of GRHL2 in preventing these recurrences *in vivo*.

Figure Legends

Figure 1: The Effect of TGF- β on EMT Markers in Py2T Cell Line. Py2T cells (mixed population) treated for 11 days with TGF- β (5 ng/mL). Lysates were made on day 11 of TGF- β induction, and were analyzed by western blot analysis. Samples were probed with antibodies for GRHL2, E-cadherin (E-cad), Fibronectin, and β -actin as a loading control.

Figure 2: Ectopic human-GRHL2 expression in mouse mammary cancer cell lines. A) Inducible human GRHL2-pLUT was transduced into mouse mammary carcinoma cell line Py2T had no effect on GRHL2 protein level. B) Attempt to express an alternate GRHL2 form

in MSCV-IRES-puro vector in mouse mammary carcinoma cell line 4T1. No increase in GRHL2 expression was observed. C) mRNA expression human-GRHL2 in 4T1 mouse mammary carcinoma cell lines by RT-qPCR.

Figure 3: Expression of mouse-GRHL2-pLUT in Py2T C7 cell line. A) mGRHL2-pLUT expression induced by doxycycline (western immunoblot analysis). B) Immunofluorescence data of Py2T C7+mGRHL2-pLUT +/- DOX probed with Flag antibody.

Figure 4: TGF- β failed to induce EMT in Py2T clone 7 cells. After isolation of higher GRHL2 expressing colonies from Py2T + mGRHL2 heterogenous populations, control cells were now resistant to TGF- β induced EMT.

Figure 5: HMLE+Twist-ER + hGRHL2-pLUT +/- doxycycline. A) Immunofluorescence data of HMLE+Twist-ER+hGRHL2-pLUT cells either induced with DOX or control cells probed with Flag antibody. B) Cells were simultaneously treated with 4OHT (10 ng/mL) and doxycycline (1 ug/mL) and were passaged for 14 days with continued 4OHT/DOX induction.

Figure 6. Confirmation of MMTV-GRHL2 transgenic. (a) represents qPCR amplification plot using trans-exonic GRHL2 primers and DNA from GRHL2-MMTV founder vs. control mouse; (b) represents qRT-PCR using GRHL2-FLAG specific primers using RNA from F1 mouse mammary tissue derived from founder (6G) or control mice.

Figure 7: GRHL2 suppresses recurrence of neuNT-driven tumors in the MMTV-GRHL2 Tg; MMTV-rtTA Tg; tetO-neuNT model.

Figure 8: GRHL2 is lost in recurrent tumors of MTB/TAN current mice. Right panel: Confirmation of GRHL2 expression from primary tumor of GRHL2-MMTV+ transgenic mouse (probed for flag).

FIGURES

Figure 4

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

GRHL2(-)/MTB(+)/TAN(+) mice

MTB(+)/TAN(+)
mice primary tumor
samples

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Chapter 4

EMT ROS Mechanisms: A brief description of the NOX complex and FAT cadherins

The following represents unpublished data.

Overview and Preliminary Findings

As discussed extensively in Chapter 2, GRHL2 overexpression alters the ROS, ATP, and mitochondrial membrane potential in MSP cells. In order to characterize the specific mechanism by which GRHL2 promotes anoikis and generates ROS in our model system, we analyzed the expression of specific genes involved in ROS scavenging, mitochondrial metabolism, NADPH oxidase (NOX) complex genes, as well as FAT atypical cadherin proteins. ROS generation is known to paradoxically accompany hypoxia, a factor commonly resulting from and influencing the poorly vascularized portions of tumors, which stabilizes HIF1 α (1). HIF1 α is thought to contribute to the induction of EMT through upregulation of several EMT associated transcription factors (e.g., ZEB1, Twist, and Snail) (2, 3). HIF1 α transcriptional activity can serve to ameliorate ROS through upregulation of antioxidant response genes (1).

The NADPH oxidase complex is made up of multiple isoforms which are differentially expressed in a variety of tissues. The catalytic subunit, NOX, found in the plasma membrane, binds to its dimeric partner p22^{phox}, resulting in the assembly of several cytoplasmic regulatory factors such as p67^{phox} and p47^{phox}, which are responsible for generation of a ROS burst, commonly occurring in the setting of immune function (4, 5). The potential contribution of the NADPH oxidase complex to tumorigenesis has even been linked to a specific role in EMT regulation of the NOX complex through Rac1b (5).

Another emerging mechanism of ROS production and metabolic control relates to the FAT atypical cadherin protein family. FAT cadherins are enormous cell adhesion molecules (>500 kDa) which have been demonstrated to play a critical role in regulation of planar cell polarity (PCP) as well as Hippo pathways for tissue organization and organ size regulation respectively (6, 7). It was recently shown by Sing et al that in *Drosophila*, the mitochondrial

electron transport chain relies critically on an imported intracellular cleaved peptide originating from FAT protein (termed Ft^{Mito}) which localizes to the mitochondria to stabilize the holoenzyme NADPH dehydrogenase ubiquinone flavoprotein (Ndufv2), a core component of complex I, which results in increased mitochondrial function, and interestingly, decreases production of reactive oxygen species (8). FAT4 is the most closely related human homologue of drosophila FAT protein (9). Recent evidence has indicated that FAT4 protein expression is significantly upregulated in human triple-negative breast cancer (9), specifically in the claudin low subclass in which GRHL2 expression is lost (10). The role of antioxidant gene expression, NOX complex activation, and FAT protein on ROS as well as the involvement of GRHL2 in regulating these pathways was explored here.

Preliminary evidence indicates that differential antioxidant gene expression, the NOX complex, and/or FAT atypical cadherins may play specific roles in the resistance to anoikis seen in MSP cells. Suppression of reactive oxygen species using pharmacological ROS scavengers protects cells from anoikis (refer to Chapter 2 published manuscript). Before discovering the regulation GLUD1 by GRHL2, we first reasoned that if EMT or GRHL2 expression leads to altered antioxidant gene expression, as is known to accompany EMT, this may explain why ROS is significantly different between HMLE and MSP cells. While we confirmed that overexpression of SOD2 (mitochondrial isoform of superoxide dismutase) did somewhat decrease sensitivity to anoikis in HMLE cells, we found that this effect was very minor (figure 1). Also, we were also able to confirm that several antioxidant genes are upregulated during EMT including catalase, SOD2, and NQO1, which were upregulated in MSP cells relative to HMLE cells (Figure 2). We further confirmed protein upregulation in MSP cells of both catalase, SOD2, and HIF1 α by western blot analysis (figure 2). However,

these proteins were not downregulated following GRHL2 re-expression (data not shown). This indicated that while EMT may upregulate antioxidant response elements to ameliorate ROS accumulation which may occur in their microenvironments, this was likely not the mechanism by which GRHL2 increases ROS.

Through analysis of publicly available microarray data (11), we have discovered that multiple important components of both the NADPH oxidase as well as the atypical cadherin FAT protein family are differentially expressed in MSP relative to HMLE cells. Overexpression of GRHL2 significantly upregulates a major cytoplasmic binding partner of NOX involved in producing ROS bursts known as p67^{phox} (figure 3), but does not appear to significantly regulate p22^{phox}, or any of the catalytic NOX isoforms analyzed (data not shown). Further, stable knockdown of GRHL2 resulted in decreased expression of the cytoplasmic regulator of the NOX complex. We reasoned that if alternate regulation of one of the major components of the NOX complex in cells which have undergone EMT or GRHL2 induced MET contributes to ROS bursts in suspended culture, this might elucidate the mechanism of GRHL2 induced ROS accumulation. However, we found that transient depletion of neither p22^{phox} nor p67^{phox} by siRNA transfection had any significant protective effect on anoikis (figure 4) and did not alter the ROS observed in our system (data not shown). The primary assay used in ROS determination was CM-H₂DCFDA which is a fluorescent probe for general ROS, being most sensitive to cellular hydrogen peroxide. We later conducted assays to determine the precise ROS species being overproduced in our model which we determined to be predominantly hydrogen peroxide (H₂O₂) (see Chapter 2). Since ROS produced via the NADPH oxidase system is predominantly that of superoxide (O₂^{•-}), which was in fact elevated in MSP cells relative to HMLE cells (reflective of their

heightened mitochondrial activity), we reasoned that the NOX complex was likely not the source of ROS contributing to the GRHL2 mediated ROS burst observed in our system.

The FAT cadherin family of proteins have been shown recently to alter mitochondrial activity through intracellular cleavage (8). Despite the tumor suppressor role described of FAT proteins (Ft protein) in drosophila (8, 12), FAT protein homologues have been shown to potentially play roles in tumor suppression as well as oncogenesis in several cancer types (9). We planned to examine the effect of FAT4 atypical cadherin expression on regulation of metabolism, ROS, and anoikis (JC1, Seahorse, ATP Cell Titer assays, Caspase 3/7 activation assays) after targeted shRNA knockdown of FAT4 protein. FAT4 is significantly upregulated in MSP cells (figure 5a). This could increase Ft^{Mito} protein, and consequently, lead to an increase in mitochondrial activity seen in MSP cells. GRHL2 expression in MSP cells downregulated FAT4 mRNA expression (figure 5b). Given the extraordinarily large size of FAT cadherin proteins, this precluded detection by western blot analysis. We attempted shRNA stable knockdowns in MSP cells; however, given the inability to analyze protein expression by western blotting, we were unable to verify these knockdowns by western blot. Further, depletion of FAT4 had no effect on ROS or anoikis in our model.

Analysis of our RNA-seq data revealed regulation of a mitochondrial enzyme referred to as glutamate dehydrogenase 1 (GLUD1 or GDH1) which upon further pursuit proved to decrease hydrogen peroxide level as well as anoikis – a mechanism dependent on the production of α -ketoglutarate as described in more detail in Chapter 2 (13). The mechanisms described in this chapter were promising molecular pathways regulated by GRHL2, but proved to have no effect on ROS or anoikis in our model.

Figure 1

Figure 2

Figure 3

Regulation of p67-PHOX by GRHL2

Figure 4

Figure 5a

FAT Atypical Cadherins

Figure 5b

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Chapter 5

The Role of Tubulins in Regulation of Anoikis through Mitochondria

The following represents unpublished data which reflected our views of ROS mechanisms and contributions to anoikis at the time. Since these observations were made, our views on the links between ROS, metabolism, and anoikis have been updated by more recent findings (see Chapter 2).

Introduction

Detachment from extracellular matrix induces an apoptotic program or non-apoptotic cell death process collectively called “anoikis” (1). For a more complete review of this topic, refer to Chapter 1. The anoikis response is compromised in cells that undergo the oncogenic Epithelial-Mesenchymal Transition (EMT), facilitating tumor metastasis. Tumor metastasis is critically dependent upon mechanisms that allow tumor cells to evade anoikis, such as EMT (2-5). The basic mechanisms of anoikis regulation are critical to our understanding and therapeutic development, and while the core machinery influencing anoikis is identical to that of apoptosis, much is unknown regarding the aberrant activation of pathways allowing mesenchymal cells to escape this process.

Many signaling mechanisms are engaged in the activation of the anoikis response of normal epithelial cells, a subset of which are clearly defective in tumors, a result of epigenetic, genetic or other modifications. These findings have been demonstrated in tissue culture as well as animal models of metastatic tumor progression (3-5). Other matrix-dependent cell death events likely closely related to anoikis, but non-apoptotic (caspase-independent) in nature, have been demonstrated as well (“metabolic cell death”) (6, 7).

Both caspase-dependent and caspase-independent forms of anoikis are critically regulated through cellular metabolic pathways (6, 7). These effects may be the result of ATP depletion and/or excessive generation of reactive oxygen species (ROS). For example, the three-dimensional acinar morphogenesis of MCF10a cells in

matrigel, imitating terminal end bud clearance *in vivo*, relies on both caspase-dependent and caspase-independent processes. The latter is likely the result of inhibition of glucose transport due to sequestration of transporters, and partly because fatty acid oxidation (FAO), which generally acts as a backup for ATP maintenance when carbohydrate sources are lacking—is inhibited by ROS. In this context, ROS accumulates to high levels due to lack of pentose phosphate pathway input, which then fails to produce NADPH reducing equivalents. ErbB2 specifically, has been reported to rescue these phenotypes, protecting from anoikis (8). The early events connecting cytoskeletal changes to these downstream effects are not yet understood, however.

For an indepth discussion of ROS, its cellular sources, reactivity, and consequences, refer to the Reactive Oxygen Species section of Chapter 1. Under normal conditions, ROS is generated in the process of normal mitochondrial function as electrons are shunted directly to oxygen from components of the electron transport chain during oxidative phosphorylation. ROS levels have been shown to increase dramatically in detached cells (8). This suggests that suppression of ROS in detached cells could be a means of anoikis evasion for tumor cells. For example, in detached cells, pyruvate dehydrogenase kinase 4 (PDK4) is upregulated, inactivating pyruvate dehydrogenase (PDH) and attenuating mitochondrial oxidative phosphorylation; further, experimental depletion of PDK4 stimulates ox-phos, ROS production, and promotes anoikis (9). Many tumor cells have been observed to over-express antioxidant enzymes, resulting in ROS neutralization in detached cells,

rescuing these cells from anoikis (10). These observations powerfully demonstrate the pro-anoikis effect of ROS.

Integrin engagement results in basal stimulation of ROS at least in attached cells, permitting anoikis resistance, through the ubiquitous increase of tyrosine phosphorylation (including Src) due to tyrosine phosphatase's exquisite sensitivity to inactivation by ROS (11). In summary, mitochondrial oxidative phosphorylation affects anoikis, both of caspase-dependent and -independent types, through both ATP (protective) and ROS (protective or promoting, depending upon context). The precise mechanisms by which cells generate ROS and maintain energy charge is unclear and requires further examination.

Voltage Dependent Anion Channels (VDAC1-3) transport ADP, ATP, NAD(H) and various metabolic intermediates across the outer mitochondrial membrane (MOM), thereby regulating metabolic flux of the mitochondria, earning them the title of "governators" of metabolism (12). VDACs are the most abundant channel of the mitochondrial outer membrane. At low transmembrane voltage, VDACs are fully open to anion transport. They are closed to anions/selective for cations at higher voltages. VDAC greatly affects $\Delta\psi$ (mitochondrial outer membrane potential) (13) which affects ATP, ROS generation, and may even be part of the MPT (mitochondrial permeability transition) pore, although this idea is somewhat controversial and evidence for this idea is not uniformly expressed in the literature (14).

Importantly, VDACs are regulated by several signaling inputs, including protein kinase A (PKA) or GSK-3 β (which phosphorylate VDACs), or via direct interactions with Bcl-2 protein, hexokinases, or free tubulin dimers (12, 15, 16).

Phosphorylation of the VDAC channel itself occurs on the “cis” (cytosolic) side of the channel by kinases such as GSK3 β and PKA, activities of which have been reported to be altered during suspension (17, 18). This phosphorylation greatly (by orders of magnitude) affects the sensitivity of VDAC to tubulin dimers, which affects their propensity to close. Given their dramatic effects on mitochondrial metabolism, which may affect ATP and ROS level, VDACs affect cell survival vs. apoptosis cell fate decisions (19).

In fact, VDACs control cell survival in a number of settings. Persistent closure of VDAC has been shown to compromise the integrity of the outer mitochondrial membrane, leading to cytochrome c release and apoptosome-mediated apoptosis (20, 21). There are multiple methods of VDAC modulation to simulate physiological opening or closing of VDAC channels. A VDAC-specific compound, erastin, induces non-apoptotic cell death specifically in Ras-transformed cell lines via VDAC-2 and VDAC-3 inhibition (22). Conversely, a bacterial pillus protein, FimA, enters host cells, where it binds and stabilizes the fully open, hexokinase-interacting conformation of VDACs, resulting in protection of host cells against apoptosis (23). Opening and closure of VDAC clearly is more complicated in its regulation of cell survival as in other contexts, opening of VDACs can trigger apoptosis (24).

Microtubules make up the internal structure of both cilia and flagella, form the framework for secretory vesicles, and organelles. They play a major role in mitosis during cytokinesis. Microtubules shift back and forth between soluble and insoluble pools rapidly, free dimer and microtubule polymers respectively. α and β tubulin dimers, but not microtubules, interact with the VDACs, stabilizing the closed

conformation of the membrane channel. This interaction occurs through the C-terminal tail of tubulin, which is extensively modified (15). Heterodimers combine to form rows of protofilaments, which then make up microtubules. These microtubules are the basis of cell shape and movement. Under stable conditions, heterodimers attach to both ends of microtubules, thereby increasing microtubule length. Destabilization of microtubules causes a greater loss of tubulin dimers from the microtubules, leading to a higher concentration of free dimers.

In vivo, pharmacologic agents which act by stabilizing microtubules (e.g., paclitaxel) decrease free tubulin levels, which increases mitochondrial membrane potential. Conversely, drugs which destabilize microtubules (e.g., nocodazole), increase free tubulin dimers, decreasing mitochondrial membrane potential (25). The free to polymerized tubulin ratio may act as a metabolic programmer of mitochondrial function through regulation of VDAC channels (12). The role of tubulin may be rationalized because non-polymerized free tubulin, which tends to be elevated in cancer cells (e.g., potentially due to the overexpression of stathmin/Op18 in some contexts, a microtubule-destabilizing factor), may aid in the maintenance of the glycolytic phenotype, maintaining the Warburg effect. Mechanistically, Op18 binds two molecules of $\alpha\beta$ dimers into a T2S complex which it sequesters away from the polymerized pool of tubulin, thus decreasing microtubules and increasing free tubulin (26). Physiologically, Op18 protein aids in rapid dissociation of microtubules to allow better control of microtubule-tubulin dynamics during processes like chromosome segregation in mitosis. Patient data suggests that Op18 (which is

expressed in various subtypes of breast cancer and several other malignancies) negatively impacts response to microtubule targeting drugs.

Most studies have focused on cell death in the context of matrix detachment related to alteration of signaling pathways regulated through cell-cell adhesion molecules and integrins that are transduced by the actin cytoskeleton. An area of increasing research involves metabolic alterations which occur after cells are placed in suspension, and how those changes contribute to cell death. The connection between cytoskeleton networks and metabolism is presently unknown.

In this project, we hypothesized that cell-matrix detachment and/or EMT alters the microtubule cytoskeleton. This alters the pool of free tubulin dimers available for interaction with and regulation of the conductivity of the Voltage Dependent Anion Channels (VDACs) on mitochondria. VDACs regulate cellular metabolism, in numerous ways, including ATP levels and reactive oxygen levels, which together determine cell survival vs. cell death. A better understanding of this pathway may reveal the importance of microtubule cytoskeleton in anoikis for the first time, integrating cytoskeletal and metabolic mechanisms of cell death, and will indicate the novel ramifications of the use of microtubule drugs in the treatment of cancer.

Methods

Microtubule Stability Assays

To prepare the lysates, aliquots of D-PBS and MST buffer were equilibrated to 37°C in a water bath. MST buffer consisted by 85 mM PIPES, pH corrected to 6.9, 1 mM EGTA, 1 mM MgCl₂, 2M Glycerol, 0.5% TX-100. Microcentrifuge tubes were

labeled and pre-warmed to 37°C. Cells were plated in 6 well dishes and allowed to attach for 36 hours. Cells were trypsinized, spun at 1200 RPM at room temperature, followed by placement 1.5mL of 1% methylcellulose containing MCF10a medium in low attachment polyHEMA dishes (2 mg/mL). At the indicated time of suspension, cells were transferred to a pre-warmed microfuge tube and spun down for 15 seconds at 7,000rpm. The supernatant was discarded, washing of the pellet in 1mL of 37° D-PBS. After spinning, the supernatant was discarded and cells were resuspended in 300uL of 37° MST (microtubule stabilization buffer) buffer containing 4uM taxol and Roche protease inhibitor. Samples were centrifuged at full speed for 10 minutes. Supernatant was saved in pre-warmed microfuge tube and mixed with an equal volume of 2X SDS sample buffer. After careful removal of the remaining supernatant, the pellet was resuspended in 500uL of 1X SDS sample buffer. Samples were boiled at 95° C for 3 minutes. To prepare attached samples, cells were washed twice with approximately 1mL of 37° D-PBS. The cells were then scraped into 300uL of 37° MST buffer containing 4uM taxol and Roche protease inhibitor. Lysates were centrifuged, and supernatant and pellet were retained as described previously.

Western Blotting

SDS-PAGE was conducted using 4-20% gradient Tris-Glycine gels (Invitrogen). Proteins were electrophoretically transferred to polyvinylidene difluoride filters (Immobilon) in 5% methanol Tris-Glycine transfer buffer containing 5% methanol. PBS + 0.1% Tween-20 + 5% nonfat milk were used for blocking filters, primary antibodies were incubated in PBS+0.1% Tween-20 + 5% nonfat milk. Primary

antibodies were typically incubated between 2 hours at room temperature or overnight at 4 degrees C. Primaries used were: β -tubulin, Rb (Cell Signaling), VDAC1, Rb (Cell Signaling); . Secondary antibodies for chemiluminescence were either anti-mouse or anti-rabbit, conjugated to horseradish peroxidase (HRP) enzyme (Bio-Rad). Secondary antibodies (Biorad) were used at 1:3000 dilution and filters were incubated for approximately 1 hour at room temperature. Western blots were developed via ECL Super Signal West Pico (Thermo-Pierce).

Anoikis Assays

For anoikis assays, caspase activation was measured using the Caspase-Glo 3/7 assay kit (Promega). Cells were dissociated using TrypLE Express (Invitrogen) and a fixed number of cells (1.5×10^5 cells) were placed per 6 well poly-(2-hydroxyethyl methacrylate) (poly-HEMA) coated low attachment plates in 2.0 ml of normal growth medium + 0.5% methylcellulose for the indicated time. Aliquots of cells were mixed 1:1 with caspase glo reagent at indicated time points and assayed for luminescence utilizing a Wallac Envision Perkin Elmer plate reader according to manufacturer's instructions. Where indicated, time-zero cell death values were subtracted from the data presented to normalize for small loading variation.

ROS Assays

For ROS assay, cells were seeded as above and attached for 48 hours. Cells were detached by trypsinization, and equal numbers of cells were placed in a 15 mL centrifuge tube. Cells were assayed immediately by flow cytometry after staining for

15 min at 37 degrees with 1 uM CM-H₂DCFDA (Invitrogen) protected from light. If indicated, 24 hr ROS was examined after plating cells in low attachment polyHEMA (2 mg/mL) coated 100 mm dishes in 5 mL of 0.5% methylcellulose to prevent excess cell clumping. Cells were harvested by diluting well with 10 mL DME/F12 + 10% Horse Serum containing media. Cells were centrifuged at 2000 RPM for 2 minutes. Pellets were washed 2X with HBSS and stained in 1 uM CM-H₂DCFDA as indicated previously. In some cases, Amplex Red (Invitrogen) assays were used to measure ROS in order to compare attached and suspended conditions and were used according to manufacturer's instructions. For measurement of general cellular superoxide, cells were processed as above and stained with 5 uM dihydroethidium (DHE) (Invitrogen) for 15 minutes at 37 degrees C protected from light followed by flow cytometric analysis at 605 nm fluorescence. Mitochondrial specific superoxide was measured using mitoSOX (Invitrogen) dye by staining cells with 5 uM mitoSOX solution for 10 minutes at 37 degrees C protected from light. Samples were analyzed by flow cytometry at 580 nm fluorescence using a BD LSRFortessa Cell analyzer.

JC1 Assay

Cells were seeded on poly-d-lysine coated MatTek dishes. Cells were incubated in 2 ug/mL JC1 (5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Invitrogen) at 37 degrees for 30 minutes protected from light. After incubation, dye was washed off of cells and replaced with normal growth medium. JC1 monomer emission was observed at 529 nm and J-aggregate emission was

observed at 590 nm. Samples were analyzed by flow cytometry using a BD LSRFortessa Cell analyzer.

siRNA transfections

MCF10a PG2 cells were grown to ~70% confluence and transfected with siRNA (5 nM) targeting either non-target control siNT (Ambion, Austin TX), VDAC1, VDAC2, or VDAC3. siNT was Silencer Select Negative control #1 siRNA (Cat #4390844). siRNAs targeting VDACs were Silencer select siRNAs: VDAC1 (Cat # 4390824; ID:s14769), VDAC2 (Cat # 4392420; ID:s14771), VDAC3 (Cat # 4392420; ID: 230730). siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Quantitative reverse transcription PCR analysis

MCF10a PG2 cells were plated on collagen coated 60mm² dishes and treated as indicated. Cells were harvested using RNAeasy Plus mini kit (Qiagen), analyzed using Nanodrop, and converted to cDNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) with oligo dT primers and 1ug of RNA. cDNA was analyzed

using SYBR Green PCR Master Mix on an Applied Biosystems 7500 Real Time PCR System. Primers were as follows: VDAC1 (F: GGGTGCTCTGGTGCTAGGT; R: GACAGCGGTCTCCAACCTTCT), VDAC2 (F: CCAAATCAAAGCTGACAAGGA, R: TTTAGCTGCAATGCCAAAAC) VDAC3 (F: TTGACACAGCCAAATCCAAA, R: TGTTACTCCCAGCTGTCCAA). CBX1-F (AAGTATTGCAAGGCCACCCA), CBX-

R(TTTTCCCAATCAGGCCCCAA). Values reported were determined using the $\Delta\Delta CT$ VDAC primer sequences were amplified as follows using CBX1 as an internal control

Results

The findings presented in this chapter were preliminary data of my previous work. This project proposed to determine the effect of cell-matrix adhesion on the tubulin/VDAC/mitochondrial axis in normal epithelial cells or in cells resulting from epithelial-mesenchymal transition (EMT). To this end, we evaluated the effects of EMT on free vs polymerized tubulins, VDACs, and mitochondrial function. We also planned to test the function of components of the tubulin/VDAC/mitochondrial axis on anoikis in normal epithelial cells or in cells resulting from EMT, including the effects of altered VDACs, and the effects of altered tubulins/microtubules. Here, our preliminary findings that led us to these hypotheses will be discussed, and how the findings contributed to our current views of metabolism, ROS, and anoikis (which was previously discussed in Chapter 2). Ultimately our findings were that manipulation of VDACs had minimal impact on metabolic function, ROS, and anoikis.

Polymerized tubulin and free tubulin levels were determined through lysis in microtubule stabilizing buffer (containing paclitaxel) followed by western blotting of soluble vs. insoluble fractions, as described previously (25). We found that free tubulin shifts to polymerized microtubules rapidly following cell-matrix detachment of the normal epithelial cell line MCF10a (figure 1a); however, the microtubule stability assays were highly inconsistent and given the start conditions, in some experiments we were unable to see this effect (figure 1b). We next assayed MCF10a

neoT +/- TGF- β , which induces a stable EMT event in this context, in order to determine the effect of EMT on the response of tubulin polymerization in detached culture. While it was expected that EMT would result in stabilization of the free tubulin dimer pool (closing VDAC, decreasing ROS, and protecting from anoikis), we were unable to show this expected phenomenon in our cells. We decided to shift our focus to that of the microtubule associated proteins Op18 and Tau.

Free tubulin dimers have been reported to interact with VDACs, causing closure of the anion channel activity (12, 15). Overexpression of the microtubule destabilizing protein Oncoprotein 18 (Op18; otherwise known as Stathmin) increased free tubulin dimers levels (Figure 2a). We expected that an increase in free tubulin dimers would close VDAC channels, decreasing mitochondrial activity, decreasing ROS and protection from anoikis. However, we found that overexpression of Op18 in MCF10a PG2 cells resulted in increased cell death in suspended culture (figure 2b). Our original assumption was that this increase in the free pool of tubulin would result in increased VDAC binding, resulting in their closure, but this may not be the case. It is quite possibly that binding of tubulin dimers in the T2S quaternary complex with Op18 prevents their binding to VDAC channels, effectively competitively inhibiting free tubulin binding, resulting in a paradoxical opening of VDAC channels. Alternatively, if free tubulin dimer binding and closing VDAC channels results in decreased ATP, this may negatively influence cell survival in suspension, resulting in increased anoikis as shown here.

Further manipulation of tubulin dynamics was accomplished via overexpression of the Alzheimer associated neural protein, Tau (also known as MAP4

or Microtubule associated protein 4). Tau primarily tends to bind to microtubules and stabilize them, leading to a decrease in the total tubulin dimer pool, hypothetically resulting in increased mitochondrial activity through lack of tubulin induced VDAC closure. We found that increasing the polymerized pool results in significantly lower colony formation by the same assay (figure 2c). We hypothesized that decreased tubulin would result in removal of VDAC suppression opening the channel to increase ROS and lead to oxidative stress potentially causing cell death. We extended these findings to the MDCK cell line (Madin-Darby canine kidney). When Tau was overexpressed in this model, we found that MDCK cells were more sensitive to anoikis at 4, 8, and 24 hours, assessed by caspase 3/7 activation assay (figure 2d). However, this effect was not reinforced when cell death was assessed more generally by colony formation assay (figure 2e).

Given that modification to proteins involved in microtubule polymerization could have multiple, even paradoxical effects, we next reasoned that if tubulin binding to VDAC results in VDAC closure, removal of VDAC by siRNA transfection would be equivalent to closing the channel. VDAC knockdown was confirmed by western blot analysis and RT-qPCR (figure 3a, b; VDAC3 antibody was not specific for this isoform but rather detected Pan-VDAC – data not shown). Utilizing isoform specific siRNA transfection targeting VDAC1, 2, and 3 individually, we found that cells depleted of VDAC1 protected somewhat against anoikis; however, this effect was very small (figure 3c). Conversely, depletion of VDAC2 appeared to further sensitize cells to anoikis (figure 3c). VDAC3 depletion did not have a consistent effect on anoikis as average fold change (relative to siNT control) showed that depletion of this isoform

had no significant effect on caspase activation when averaged between two independent experiments (figure 3d).

In order to determine the role of VDAC channels in ROS regulation in our system, ROS bursts were measured by CM-H₂DFCDA fluorescence by flow cytometry. Independent depletion of VDAC1, 2, and 3 all ameliorated ROS generated by incubation in suspension relative to non-target control cells (figure 3e). While this result is consistent with the view that VDAC closure decreases mitochondrial activity, hampering ROS generated by oxidative phosphorylation, VDAC knockdown did not correlate with loss of mitochondrial activity in excess of that seen in the control samples however as assessed by JC1 fluorimetric assay (figure 4). If ROS were in fact coming from loss of mitochondrial oxidative phosphorylation activity, we would have expected to see a greater decrease in JC1 signal in suspended VDAC knockdown samples. Further, we found no significant effect of either microtubule stabilization alteration (through Op18 or Tau) on ATP level or basal JC1 signal (data not shown). Given the results presented here, we concluded that the Tubulin/VDAC/ROS axis was not playing a significant role in the induction of anoikis in our model.

Discussion

The results of this study were largely inconclusive or counter to our original hypothesis. We attempted to show whether the open or closed state of VDACs is more favorable for anoikis, and, whether the primary outcome is a reprogramming effect that occurs prior to detachment vs. a more significant effect during the detachment/suspension phase. Given the difficulty of showing consistent effects of

microtubule alterations on anoikis (see Op18 and Tau anoikis results), we concluded that any effect on anoikis was likely due to alterations of tubulin dynamic homeostasis. We were unable to find a link between regulation of tubulin dynamics and the Epithelial-Mesenchymal transition.

A determination regarding the effect of higher or lower ratio of free: polymerized tubulin and which condition was more favorable to anoikis was unable to be determined due to difficulty isolating free vs polymerized tubulin forms given its incredible instability at room temperature. It was anticipated that the direction of the tubulin effect would be consistent with the direction of the VDAC effect (more free tubulin=closed VDAC=metabolic effects arising from closed VDAC= cell death). Given the range of phenotypes that are correlated here, any consistency in these models proved very difficult to achieve.

While we were able to show a very robust effect of VDAC knockdown on ROS, this phenotype likely was due to an unconsidered alternative source, as the ROS decrease was not associated with an accompanying decrease in JC1 or ATP decline in detached conditions beyond that which occurred the control cells.

In an attempt to evaluate experimentally manipulated tubulin levels effects on anoikis, we overexpressed both microtubule associated proteins Op18 and Tau. As discussed previously, our original assumption was that alterations in the free pool of tubulin via Op18 induced destabilization of microtubules would close VDACs, due to direct tubulin binding, however, this may not be the case. It is possible that binding of tubulin dimers in the T2S quaternary complex with Op18 prevents their binding to VDAC channels, effectively competitively inhibiting free tubulin binding, resulting in

a paradoxical opening of VDAC channels. Additionally, Op18 overexpression could potentially result in cell death even if it does cause tubulin-VDAC binding, because any dysregulation of this tightly controlled program in tubulin dynamics could be problematic for cells. Op18 may also bind to the tips of protofilaments and directly destabilize growing microtubules. A final alternative is that Op18 induces accumulation of free tubulin which is able to close VDACs, and that the effect on anoikis seen is due to a loss of mitochondrial activity, causing metabolic catastrophe (see Chapter 2).

VDAC1 is typically the most abundant isoform expressed in cells (27). VDAC1/2 are most sensitive to tubulin induced blockade, while VDAC-3 is expressed in significantly lower abundance, and is far less affected by tubulin dynamics (28). Isoform 3 is thought to contribute most to typical mitochondrial membrane potential in steady state while VDAC1/2 are generally kept closed by endogenous free tubulin. In suspension, VDAC1/2 may open due to a decrease in free tubulin which would result in oxidative injury. Effects of VDAC knockdown (~VDAC closure) on anoikis were largely insignificant and/or inconsistent. We found that knockdown of VDAC1 resulted in small protection from anoikis, whereas VDAC3 depletion had no effect on anoikis. Interestingly, depletion of VDAC2 sensitized cells significantly. It was previously observed that the pro-apoptotic protein, Bak, is sequestered through an interaction with VDAC2; furthermore, it was demonstrated here that overexpression of VDAC2 inhibited apoptosis through Bak sequestration (29). Disruption of this interaction releases Bak through VDAC knockdown likely releases Bak from the mitochondria inducing apoptosis in detachment, potentially representing an

interesting area of future research. Given the results presented here, we concluded that the Tubulin/VDAC/ROS axis was not playing a significant role in the induction control of mitochondrial function contributing to anoikis in our model.

Figure Legends

Figure 1. a) Western blot showing effect of cell-matrix detachment on tubulin: microtubule ratios. MCF10aneoT cells were lysed in mitochondrial stabilization buffer in the attached state or following suspension for the indicated periods of time, fractionated into free/supernatant (F) or microtubule/pellet (M) fractions and analyzed by western blotting for β -tubulin. b) Effect of EMT on tubulin: microtubule ratios in attached vs detachment conditions. MCF10aneoT cells shown here either before or after treatment with TGF- β (resulting in irreversible EMT). p = pellet (insoluble microtubule fraction); s = supernatant (soluble tubulin dimer fraction)

Figure 2. a) Western blot showing effect of Op18 overexpression in MCF10a PG2 cells on tubulin: microtubule ratios. Op18 elevated free tubulin: microtubule ratio by destabilization of polymerized microtubules. b) Op18 constitutive expression was associated with increased anoikis, assessed by colony formation assay conducted after suspension in 0.5% methylcellulose containing media in polyHEMA low

attachment wells for indicated times. c) Tau (MAP4) overexpression in MCF10a PG2 cells resulted in significant sensitization to detachment induced cell death by colony formation assay as described above. d) Tau overexpression in MDCK cells sensitized to anoikis (caspase 3/7 activation assay; Promega Caspase-Glo kit). e) Colony formation assay following suspension in MDCK cells +/- Tau overexpression as described above.

Figure 3. a) Confirmation of siRNA depletion of specific VDAC isoforms 1 and 2. Lysates made following single VDAC isoform knockdown (lysates made 72 hrs after transfection). b) RT-qPCR amplification of 3'UTR of individual VDAC mRNA transcripts. c) Caspase-3/7 activation assay (Promega) showed increase in anoikis for VDAC2 knockdown, but VDAC1 knockdown protected from anoikis. d) Relative caspase activation fold change compared to nontargeting control siRNA for siVDAC isoforms for two independent experiments. e) Loss of VDAC1, 2, and 3 results in decrease in ROS burst following detachment (24 hr suspension) as assessed by CM-H₂DCFDA staining followed by flow cytometric analysis. f) Relative fold change in ROS burst (vs siNT control) average values from 2 independent experiments (assessed by flow cytometry following CM-H₂DCFDA staining).

Figure 4. a) JC1 (Orange J-aggregate/Green monomer) ratio in MCF10a PG2 cells depleted of VDAC1 assessed by flow cytometry showing mitochondrial membrane potential in MCF10A PG2 +/- siVDAC isoforms.

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

a.

Figure 5

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Chapter 6

Overall Discussion and Future Directions

The EMT has been demonstrated extensively to confer resistance to anoikis. Here, it was demonstrated that EMT resulted in increased mitochondrial oxidative metabolism resulting in maintenance of ATP during suspension. Increased oxidative phosphorylation in mesenchymal cells was accompanied by increased production of mitochondrial superoxide; however, general ROS was suppressed due to decreased production of hydrogen peroxide (H_2O_2). We established here that glutamate dehydrogenase 1 (GLUD1) was upregulated during EMT, resulting in increased production of α -ketoglutarate which was critical for suppression of H_2O_2 and protection from anoikis. The work presented here demonstrated that the wound healing transcription factor GRHL2, through suppression of EMT, sensitizes cells to anoikis, at least in part through downregulation of GLUD1 resulting in depletion of α -ketoglutarate and increased production of ROS. Therefore, this thesis has established a novel pathway for control of anoikis through metabolic alteration and ROS suppression occurring during the oncogenic EMT, and demonstrated an additional mechanism by which GRHL2 sensitizes to anoikis.

Warburg metabolism has been extensively studied in the context of tumor cells; however, these findings are largely context dependent (1, 2). Recent work has demonstrated that the CSC phenotype is in fact dependent on oxidative phosphorylation (3, 4). Moreover, oxidative metabolism has been shown to be enhanced in distant metastases, highlighting the critical importance of a better understanding of how tumor metabolism influences these metastatic, chemoresistant subpopulations *in vivo* (5, 6). As discussed previously, the precise cues inducing cellular migration from the primary tumor are incompletely understood. What is clear at present is that EMT is a major mechanism by which cells acquire the (epi)genetic alterations to aid invasion from their primary site, and further, evade anoikis

during tumor dissemination. It has been postulated that oncogenic mutations in subpopulations of tumors may lead to the acquisition of properties that are advantageous to the growth of tumors in the short term such as those that increase proliferation; however, these changes are ultimately detrimental in the primary site which may lack the proper vasculature to support this activity. This can lead to challenges including lack of carbon source nutrients, hypoxia, and results in reactive oxygen species accumulation. Metabolic alterations due to oncogenic transformation, EMT or transition to cancer stem cells have been reported extensively, with unique effects in each system (5, 7-10).

Paradoxically, oxygen deprivation results in the generation and accumulation of reactive oxygen species (11). HIF1 α stabilization due to hypoxic conditions reduces mitochondrial biogenesis (12), results in the induction of multiple antioxidant genes (11), and signals EMT via induction of transcription factors including Twist, Snail, and ZEB1 (13, 14). In fact, in our own hands, MSP cells were found to have stabilized HIF1 α compared to parental HMLE cells (unpublished data; refer to Chapter 4). This could potentially, at least in part, explain the decreased mitochondrial load present in MSP cells (Chapter 2), which are paradoxically more efficient than those in their epithelial counterparts. Alternatively, HMLE cells may upregulate production of mitochondria due to their lack of functional activity compared to mesenchymal cells. The reactive oxygen generated from hypoxic conditions in some cases leading to the induction of the EMT program is thus the likely culprit of gene expression changes that ameliorate these toxicities, resulting in a relatively low ROS environment compared to epithelial cells.

As noted earlier, tumor tissues are known to have high ROS levels relative to “normal” tissues, and are thought to upregulate antioxidant enzymes in order to keep these harmful

oxidants within a “non-lethal” threshold limit. The maintenance of ROS within a crucial range may aid in some cellular processes, acting as a signaling molecule of sorts (15), while rising beyond that range can be catastrophic. This requires the presence of safeguards such as robust expression of antioxidant genes. However, it has also been shown that if driven to critically low levels the result is cell cycle arrest or even apoptosis (16). In fact, ANGPTL4 was reported to protect from anoikis by stimulating ROS of the superoxide variety from the NOX complex stimulating Src leading to increased activity of PI3K and ERK pro-survival signaling (17). Note that in our hands (as has been reported elsewhere), ROS suppression, assessed by CM-H₂DCFDA detecting H₂O₂ primarily, protects from anoikis. This discrepancy may be due to a species or source specific effect or may lie in the fact that short term caspase activation assays may only show effects of increased caspase activity due to alleviation of ROS suppressing cysteine active sites of caspases, which is overcome at later time points. In fact, our findings support that anoikis resistant MSP cells have higher superoxide level relative to HMLE or MSP+GRHL2 cells (Chapter 2). We interpret this result as a readout of mitochondrial oxidative phosphorylation generating ROS from complex’s I and III – but perhaps these cells have some yet unexplored mechanism by which they induced c-SRC activation through increased superoxide (17). Based on RNA-seq, we found that GRHL2 upregulates ANGPTL4 significantly (3.8 fold) which would support a role in ROS generation, however in our model, GRHL2 overexpression decreased superoxide level, both from general sources as well as specifically from mitochondria and increased H₂O₂, indicating lack of generalizability of these previously reported findings to our system.

Another yet unexplored pathway through which GRHL2 may exert regulatory control over anoikis is through that of the atypical FAT cadherin family of proteins. GRHL2 greatly

downregulates expression of FAT4 specifically, which is highly upregulated in mesenchymal cells (Chapter 4). FAT4, the mammalian homologue most closely related to *Drosophila fat* (18), has been described to play a role in both regulation of planar cell polarity (PCP) as well as HIPPO signaling. As extensively described in Chapter 1, YAP and TAZ are pro-survival factors that mediate Hippo pathway signaling which are critical pro-survival factors with described roles in anoikis (19, 20). Regulation of the FAT cadherin family members may be yet another means by which GRHL2 enforces epithelial cell sensitivity to anoikis through regulation of Hippo signaling, a pathway which merits further study.

Our lab has previously demonstrated that reversal of EMT by GRHL2 suppresses the oncogenic EMT, enforces the default epithelial cell state, and resulted in reversal of the cancer stem cell-like state (21, 22). It has been extensively demonstrated that in these settings, plasticity conferred by EMT benefits small populations of cells, bestowing CSC-like and chemoresistant properties to these subpopulations that resume growth after a period of dormancy (23-29). In culture, treatment of HMLE cells with taxol derivatives enriches for this CSC-like population; conversely, stable expression of GRHL2 significantly sensitizes these, as well as MDA-MB-231 cells, to this therapy. Critically, ectopically expressed GRHL2 prevents the chemotherapy-induced emergence of this CSC-like population (22). Further, SUM149 cells subjected to metabolic stress such as glutamine deprivation largely die, but the rare resistant populations which result are described as a “metabolically adaptable,” glutamine independent population (30). In addition to having undergone EMT, these cells now display chemotherapeutic resistance to a multitude of chemotherapeutic agents including taxol, Erlotinib, doxorubicin, and other targeted and cytotoxic agents. These cells are reported to have significantly downregulated GRHL2 and upregulated ZEB1 (a finding

confirmed by our laboratory). Preliminary evidence suggests that re-expression of GRHL2 in this model significantly sensitizes MA cells to chemotherapeutic intervention. Additionally, significant changes in histone activation marks H3K4 trimethylation and H3K14 acetylation have been reported to be associated with the drug resistant state (31), which when reversed using HDAC inhibitor pretreatment, significantly sensitized cells to chemotherapy (30). This highlights the critical need for a better understanding of how GRHL2 regulates epigenetic modification of genes to sensitize to chemotherapies. Prevention of the emergence of CSC-like populations will be a critical step to abrogate chemo/radiotherapy resistant recurrence or metastases developing after initially successful treatment of primary disease. As established previously by our lab (22), Wnt and TGF- β signaling collaborate to coordinately downregulate GRHL2, while inhibition of these pathways maintains endogenous GRHL2. It was established here that BMP2 was able to inhibit TGF- β signaling to maintain GRHL2 protein levels. Combining BMP pathway activation and Wnt inhibitors with conventional cytotoxic or targeted chemotherapy may establish a means by which chemotherapy resistant subpopulations are sensitized, preventing recurrence of disease. A better understanding of the pathways which regulate endogenous GRHL2 levels is critical to establish a practical means by which to translate this knowledge to patient care.

The findings presented here establish a role of GRHL2 in anoikis beyond that of previously described pathways by which EMT influences anoikis. Here, we provided a mechanism by which EMT decreases ROS as to protect cells against anoikis. Under this model, GRHL2 reverses EMT, suppressing mitochondrial oxidative function and α -ketoglutarate level through the repression of the critical enzyme regulating glutaminolysis,

GLUD1, resulting in loss of glutamine utilization, increased ROS level, ultimately shifting metabolism resulting in anoikis sensitivity.

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EDUCATION

Ph.D.	M.D.-Ph.D. Dual degree scholar: 07/2010-05/2018 Anticipated degree date: Ph.D.: 05/2018 Time in Ph.D.: 07/2012-06/2016	West Virginia University, Morgantown, WV Department of Biochemistry, Program in Cancer Cell Biology, School of Medicine Dissertation Title: Grainyhead-like 2 Reverses the Metabolic Changes Induced by the Oncogenic Epithelial-Mesenchymal Transition: Effects on Anoikis Dissertation Advisor: Steven M. Frisch Ph.D. Dissertation Defense Date: 06/03/2016
M.D.	M.D.-Ph.D. Dual degree scholar: 07/2010-05/2018 Anticipated degree date: M.D.: 05/2018	West Virginia University, Morgantown, WV School of Medicine
B.S. (Summa Cum Laude)	Chemistry: 08/2007-05/2010 Thesis: 08/2009-05/2010	Lenoir-Rhyne University, Hickory, NC Department of Chemistry Thesis Title: Antioxidant Effects of Phenolic Compounds on Oxidative Stress to Low Density Lipoproteins Thesis Advisor: Joshua Ring Ph.D.
B.S. (Summa Cum Laude)	Biology: 08/2007-05/2010 Honors Thesis: 08/2008-05/2010	Lenoir-Rhyne University, Hickory, NC Department of Biology Honors Thesis: The Effect of Meditative Breathing on Pulse Transit Time: An Analysis in the Frequency Domain Honors Thesis Advisor: Stephen Scott M.D.
A.A.	Associates of Art 08/2005-05/2007	McDowell Technical Community College, Marion, NC

Research Experience

MD/PhD Trainee, Dept. of Biochemistry, WVU Advisor: Steven M. Frisch, PhD. Dissertation Title: "Grainyhead-like 2 Reverses the Metabolic Changes Induced by the Oncogenic Epithelial-Mesenchymal Transition: Effects on Anoikis" 2012-2016

Research Rotation - Laboratory of J. Michael Ruppert, PhD

Summer 2011

JC. FARRIS | 1

Honors Biology Research, Lenoir-Rhyne University. Advisor: Stephen Scott MD. “The Effect of Meditative Breathing on Pulse Transit Time: An Analysis in the Frequency Domain” **2008-2010**

Publications

Farris JC, Pifer PM, Zheng L, Gottlieb E, Denvir J, Frisch SM. Grainyhead-like 2 Reverses the Metabolic Changes Induced by the Oncogenic Epithelial-mesenchymal Transition: Effects on Anoikis. *Molecular Cancer Research*. 2016. Apr 15. pii: molcanres.0050.2016

Pifer PM, **Farris JC**, Thomas AL, Stoilov P, Denvir J, Smith DM, et al. Grainyhead-like-2 inhibits the coactivator p300, suppressing tubulogenesis and the epithelial-mesenchymal transition. *Molecular Biology of the Cell*. 2016

Cieply B, **Farris JC**, Denvir J, Ford HL, Frisch SM. Epithelial-mesenchymal transition and tumor suppression are controlled by a reciprocal feedback loop between ZEB1 and grainyhead-like-2. *Cancer Res*. 2013 10/15; 73(20): 6299-6309.

Manuscript(s) Under Review

Cifarelli C, Cifarelli D, **Farris JC**, Pifer PM, Frisch SM. Modulation of Radiation Sensitivity in High Grade Glioma via Cadherin-Mediated Cell-Cell Interaction. *Under Review*

Techniques

Cell and Molecular Biology: Tissue Cell Culture with sterile technique, PCR Primer Design, Molecular Cloning Techniques (Including sub-cloning, site directed mutagenesis, and plasmid preparation), RNA isolation (from primary tissues and cells), DNA purification, qRT-PCR, RNA seq sample preparation, immunofluorescent staining, ingenuity pathway examination, 3D culture mammosphere assays, cell transfection, mammalian cell retroviral and lentiviral transduction, luciferase reporter assays, immunoblotting (western blot), immunoprecipitation, chromatin immunoprecipitation, flow cytometry based assays including cell surface antigen staining, assays for reactive oxygen species (CM-H₂DCFDA, mitoSOX, mitotracker green FM, and DHE staining), Anoikis assays (caspase 3/7 activation assays, cell death elisa, cell viability clonogenic assays), soft agar assays, generation of transgenic murine lines, working with murine models of mammary cancer, mouse genotyping, mouse colony maintenance, confocal microscopy based JC1 mitochondrial assays.

HONORS & AWARDS

Class Honors (Top 15%), West Virginia University School of Medicine 2010 - 2012

- Awarded in Human Function, Physical Diagnosis & Clinical Integration (Spring 2011), Neurobiology, Behavioral Science/Psychopathology, Health Care Ethics, Pathology, Microbiology, Pharmacology, and Physical Diagnosis & Clinical Integration 2 (Fall 2011 and Spring 2012)

Special Commendation for Academic Excellence – WVU School of Medicine 2011 and 2012

First Honor Graduate Spring 2010

Steeleman Science Scholarship	Spring 2010
North Carolina Academy of Science – 2nd Place Award	Spring 2010
Lenoir-Rhyne Sponsor-a-Bear Scholarship	2009-2010
Biology Honors Program	2008-2010
Lenoir-Rhyne University President’s List – Six Semesters	2007-2010
CRC – Chemistry Achievement Award	Spring 2008
Phi Theta Kappa Honors Society	2006-2007
Honors Deans List – McDowell Technical Community College	2005-2007

Presentations

Farris JC, Frisch SM. Regulation of Anoikis through Metabolic Reprogramming during EMT or GRHL2-mediated MET. Department of Biochemistry – Cancer Cell Biology Dissertation Defense. June 3rd, 2016

Farris JC, Pifer PM. The MD/PhD Career Track. West Virginia Cancer Institute Summer Undergraduate Research Program. May 2016

Farris JC, Frisch SM. Regulation of Anoikis through Metabolic Reprogramming during EMT or GRHL2-mediated MET. Department of Biochemistry – Cancer Cell Biology Seminar. November 4th, 2015.

Farris JC, Frisch SM. Regulation of Anoikis through Metabolic Reprogramming during EMT or GRHL2-mediated MET. Biochemistry Departmental Seminar. October 29th, 2015.

Farris JC, Frisch SM. Regulation of Anoikis through Metabolic Reprogramming during EMT or GRHL2-mediated MET. Department of Biochemistry – Cancer Cell Biology Proposal Defense. March 11th, 2015.

Farris JC, Frisch SM. Exploring the Role of Tubulins in Regulating Anoikis through Mitochondria. Department of Biochemistry – Cancer Cell Biology Seminar. March 19, 2014.

Farris JC, Frisch SM. The Role of Grainyhead-like factors in Tumor Recurrence and Metastasis after Chemotherapy. Department of Biochemistry – Cancer Cell Biology Seminar. February 27, 2013.

COMMITTEES and LEADERSHIP EXPERIENCE

MD/PhD Student Admission Committee	2015
<ul style="list-style-type: none"> • Interviewed perspective incoming MD/PhD candidates 	
President of Chi Beta Phi Academic Honors Society	2009 – 2010

TEACHING EXPERIENCE

Physics Teaching Assistant	2008 - 2009
<ul style="list-style-type: none">Assisted in both Physics 101 and 102 lab teaching exercises and graded exams/lab work	
Geology Teaching Assistant	2008 - 2009
<ul style="list-style-type: none">Assisted in Geology lab teaching exercises and graded exams/lab work	
Farris JC. Common Anokis Assay Techniques and Methodology. BMS 706 Cellular Methods Course. October 2015	
Mentorship of undergraduate researchers	2012-2016

Collegiate Activities

Alumnus of Lenoir-Rhyne University (2007-2010)	
Circle K Volunteer Society Member	2008 - 2010
Health Career Internship	2009-2010

WORK EXPERIENCE

Frisch Laboratory Graduate Researcher	2012-2016
<ul style="list-style-type: none">West Virginia University - Department of Biochemistry	
Physics and Geology Laboratory Teaching Assistant	2008-2009
<ul style="list-style-type: none">Lenoir-Rhyne University - Physics Department	
Saw Mill Worker	2003-2007
<ul style="list-style-type: none">Peeled and processed poplar logs into home siding	

VOLUNTEER ACTIVITIES

Community Service*

Homeless Care Packages	January 2016
<ul style="list-style-type: none">Raised funds and helped make care packages which contained cold weather items, and distributed to homeless individuals in Morgantown	
Graduate Student Organization Mad Scientist Children's Library Learning Event	May 2016
WV State Science Bowl Competition - Science Judge	February 2016
Graduate Student Organization Relay for Life Mad Scientist Event Volunteer	June 2015

WV State Science Bowl Competition – Science Judge	February 2015
Assisted in Graduate Student Orientation Activities at Wes Vaco-WVU forest	August 2014
Health Science & Technology Academy Judge	May 2014
AAPS Science Experiment Demonstrations at Boys and Girls Club of America	May 2014
Children’s Home Society of WV Valentine Bowling Activity for Foster Children	February 2012
Burke United Christian Ministries Soup Kitchen and Food Pantry Volunteer	2008-2010
Medical Mission trip to Guatemala (Surgical Mission Trip)	June 2009

**Over 100 hours logged in undergraduate; Over 60 hours in medical school*

PERSONAL INTERESTS & HOBBIES

Traveling, hiking, camping, reading, playing guitar

Appendix AI

Cieply B., **Farris JC.**, et al.: Epithelial-Mesenchymal Transition and Tumor Suppression Are Controlled by a Reciprocal Feedback Loop Between ZEB and Grainyhead-like 2. *Cancer Research* 2013 73(20); 1-11

Cancer Research

Epithelial–Mesenchymal Transition and Tumor Suppression Are Controlled by a Reciprocal Feedback Loop between ZEB1 and Grainyhead-like-2

Benjamin Cieply, Joshua Farris, James Denvir, et al.

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Epithelial–Mesenchymal Transition and Tumor Suppression Are Controlled by a Reciprocal Feedback Loop between ZEB1 and Grainyhead-like-2

Benjamin Cieply¹, Joshua Farris¹, James Denvir³, Heide L. Ford⁴, and Steven M. Frisch^{1,2}

Abstract

Epithelial–mesenchymal transition (EMT) in carcinoma cells enhances malignant progression by promoting invasion and survival. EMT is induced by microenvironmental factors, including TGF- β and Wnt agonists, and by the E-box-binding transcription factors Twist, Snail, and ZEB. Grainyhead-like-2 (GRHL2), a member of the mammalian Grainyhead family of wound-healing regulatory transcription factors, suppresses EMT and restores sensitivity to anoikis by repressing ZEB1 expression and inhibiting TGF- β signaling. In this study, we elucidate the functional relationship between GRHL2 and ZEB1 in EMT/MET and tumor biology. At least three homeodomain proteins, Six1, LBX1, and HoxA5, transactivated the ZEB1 promoter, in the case of Six1, through direct protein–promoter interaction. GRHL2 altered the Six1–DNA complex, inhibiting this transactivation. Correspondingly, GRHL2 expression prevented tumor initiation in xenograft assays, sensitized breast cancer cells to paclitaxel, and suppressed the emergence of CD44^{high}CD24^{low} cells (defining the cancer stem cell phenotype in the cell type studied). GRHL2 was downregulated in recurrent mouse tumors that had evolved to an oncogene-independent, EMT-like state, supporting a role for GRHL2 downregulation in this phenotypic transition, modeling disease recurrence. The combination of TGF- β and Wnt activation repressed GRHL2 expression by direct interaction of ZEB1 with the GRHL2 promoter, inducing EMT. Together, our observations indicate that a reciprocal feedback loop between GRHL2 and ZEB1 controls epithelial versus mesenchymal phenotypes and EMT-driven tumor progression. *Cancer Res*; 73(20); 1–11. ©2013 AACR.

Introduction

The oncogenic epithelial–mesenchymal transition (EMT) contributes to tumor progression by enhancing tumor cell invasiveness and anoikis-resistance, which may enhance the early steps of metastasis (1–5). Chemo- and radioresistance also accompany EMT, confounding stable patient responses to treatment, especially in the claudin-low subclass of breast cancer, where a frank EMT-like pattern of genes is expressed (6–9). In certain contexts, EMT also promotes the transition of tumor cells to cancer stem cell (CSC)/tumor-initiating cells, although CSCs can arise independently of EMT (10–14).

The transcription factors ZEB1 and ZEB2 (SIP1) are pivotal activators of EMT. ZEB1 and, subsequently, ZEB2 were iden-

tified as the first repressors of the mammalian E-cadherin promoter (15, 16). They are now known to regulate cytoskeletal, cell polarity, cell adhesion, and apoptosis-regulatory genes that collectively suffice for EMT induction (17, 18). ZEB1 is induced by transcription factors that include NF- κ B, Twist/Snail (acting in concert), TCF4, and LBX1 (19–22). Conversely, ZEB1 expression is translationally attenuated by mir-200b/c, whose expression is, in turn, repressed by TGF- β signaling, explaining (in part) the engagement of stable autocrine TGF- β signaling in the maintenance of EMT (23).

Grainyhead-like-2 (GRHL2) is a transcription factor that regulates the EMT/MET-related processes of wound healing, epidermal junction assembly, and neural tube closure (24–27). Previously, we reported that GRHL2 is a generalized suppressor of oncogenic EMT, through at least two mechanisms – direct repression of ZEB1 expression and inhibition of the TGF- β pathway (28). Here, we show that GRHL2 inhibited transactivation of the ZEB1 promoter mediated by the homeodomain proteins Six1, LBX1, and HoxA5. GRHL2 acted as a tumor suppressor gene, by the criterion of suppressed tumor-initiation frequency. In addition, GRHL2 sensitized tumor cells to paclitaxel and prevented the emergence of CD44^{high}CD24^{low} mammary epithelial cells. The combination of Wnt and TGF- β pathway activation upregulated ZEB1 expression. ZEB1 reciprocally repressed GRHL2 expression through a direct interaction with the GRHL2 promoter. These observations reveal a novel GRHL2–ZEB1 reciprocal feedback loop

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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that drives EMT versus MET in response to extracellular signals.

Materials and Methods

Cell lines

HMLE and HMLE+twist-ER were kindly provided by R. Weinberg (Whitehead Institute, Cambridge, MA) and the MDA-MB-231LN by E. Pugacheva (West Virginia University, Morgantown, WV). Cells were cultured and stable cell lines were generated via retroviral transduction as described previously (28). GRHL2 was expressed using either MSCV-IRES-puro or MSCV-IRES-GFP (pMIG, Addgene) and mixed populations or multiple clonal lines were used as indicated. The β -catenin S33Y mutant (provided by S.P.S. Monga, University of Pittsburgh, Pittsburgh, PA) was subcloned into the pMXS-IRES-PURO retroviral vector [contributed by Russ Carstens, University of Pennsylvania (Philadelphia, PA) in-frame with a C-terminal FLAG tag]. The human Six1 sequence was also subcloned into the pMXS-IRES-PURO vector. ZEB1 shRNA (V3LHS_356186, Open Biosystems) and ZEB1 cDNA were expressed via the pTRIPZ and pLUT lentiviral vectors, respectively, as described previously (28). Human H-rasV12 in MSCV-IRES-Blast (Addgene) was used to express activated Ras in CD44^{low} (flow-sorted) HMLE cells for tumor assays. HMLE+Ras cells were subjected to stable GRHL2 knockdown (Open Biosystems RHS4430-99291384) using pGIPZ, followed by selection for puromycin resistance and sorting for GFP.

Xenograft assays

MDA-MB-231LN stably expressing either empty-MSCV-IRES-Puro or GRHL2-IRES-Puro were trypsinized and resuspended in growth media, and the indicated cell numbers in 0.1 mL were injected into the fourth inguinal mammary fat pad of female BALB/c nude mice (Charles River) at approximately four weeks of age. HMLE+Ras cells with GRHL2 or control short-hairpin RNA (shRNA; see earlier) were trypsinized and suspended in growth medium; the indicated cell numbers were injected in 0.1 mL into the fourth inguinal mammary fat pad of female nonobese diabetic/severe combined immunodeficient (NOD/SCID; Charles River). Assays were carried out for 10 weeks (HMLER) or for indicated times. Tumor volume was determined using the formula $\pi(L1 \times L2^2)/6$, where $L1$ is the long axis and $L2$ is the short axis. Tumor burden was monitored according to the WVU ACUC Tumor Burden Policy. Animal studies were approved by the West Virginia University Animal Care and Use Committee.

Western blotting

Electrophoresis, transfer, immunoblotting, and antibodies used were described (28). Other antibodies used here were: rabbit anti-vimentin (Cell Signaling Technology), mouse anti-FLAG (M2; Sigma), and mouse anti-GAPDH (Thermo Scientific).

Chemosensitivity assays

MDA-MB-231 cells with stable GRHL2-MSCV-IRES-puro or empty MSCV-IRES-puro were plated in 96-well dishes at a

density of 5,000 cells per well and assayed in biological triplicate for cell viability after three days of incubation, with the indicated concentration of paclitaxel, using Presto-Blue (Invitrogen) according to the manufacturer's protocol. For the chemo-induced marker analysis, the protocol was modeled after the study by Gupta and colleagues (38). HMLE cells expressing empty-MSCV-IRES-GFP or GRHL2-MSCV-IRES-GFP were treated with paclitaxel (10 nmol/L) for four days, followed by seven days of recovery and flow analysis for CD44 using a CD44-PE antibody (BD Biosciences). HMLE induced to undergo EMT by ectopic twist were used as a positive control for the CD44^{high} phenotype.

Quantitative reverse transcription PCR analysis of primary vs. recurrent mouse tumors

RNAs from primary and recurrent tetO-neuNT ($n = 5$ for each group; ref. 29) or tetO-Wnt1 ($n = 4$ for each group; ref. 30) were provided by L. Chodosh (University of Pennsylvania) or E. Gunther (Penn State University, Hershey, PA) and analyzed for GRHL2 expression using a β 2-microglobulin internal control as described previously (28), using the primers: mGRHL2-f: caaggacgaccagcgcagca; mGRHL2-r: ggccctccccctgtctctga; mB2M-f: tggctttctggtgcttctgc; mB2M-r: gggtggaactgtgttacgtacgtag.

BIO, TGF- β , and BMP2 treatments

HMLE cells were treated with 5 μ M 6-bromo-3-[(3E)-1,3-dihydro-3-(hydroxyimino)-2H-indol-2-ylidene]-1,3-dihydro-(3Z)-2H-indol-2-one (BIO; ref. 31) and/or TGF- β 1 (5 ng/mL) for four days. BIO was removed and the TGF- β was continued for another six days; samples were then analyzed by Western blot. HMLE+twist-ER were treated with either 50 nmol/L 4-hydroxytamoxifen (4OHT), 0.5 μ g/mL BMP-2 (R&D Biosystems), or both for 7 to 10 days and then analyzed by Western blot.

Reporter assays

Cotransfections, luciferase, and β -galactosidase assays as well as the GRHL2 expression vector and ZEB1-WT- promoter reporter were described previously (28); all values represent the average relative luciferase activity/ β -galactosidase activity of biological duplicates; error bars represent SD from the mean. The transcription factors used in the cotransfection/reporter assays were obtained and cloned as follows: hLBX1 was obtained from D. Haber (Dana-Farber Cancer Center) in pBABE and cloned into pCDNA3.1. The hHOXA5 was purchased from Addgene and subcloned from pET28B into pCDNA-3.1. mTwist (pBABE-mTWIST-ER) was purchased from Addgene and the twist cDNA was subcloned into pCDNA-3.1 (GC rich Phusion buffer). HA-Snail-pCDNA-3.1 was purchased from Addgene. The GRHL2 genomic sequence from -625 to +335 (relative to the transcription start site of the standard GRHL2 transcript) was subcloned into pGL4.14 using the following primers: Gra-prom-f3: CTCACCTGTTT-GAAAATCTG and Gra-prom-r12: TGTTTGATCCAATGAA-CTTGC, using the Bacterial Artificial Chromosome clone 2058A8 (Invitrogen) as a template; these coordinates were based on the GRHL2 transcript NM_024915. The band resulting from this PCR was reamplified with similar primers

containing NheI (forward) and BglII (reverse) restriction sites, and subcloned into pGL4.14 (Promega).

The ZEB1 promoters containing a mutant downstream homeobox site or deleted upstream homeobox site or both were generated as follows. The downstream site was mutated by using the mutagenic primers Mut-f3: cgtgcctcctctccccacagtcgtaggaaaacttttccc Mut-r3: gggaaaagtcttctacggactgggtgggagagggaggcagc in the Quickchange-II XL kit (Agilent) with the ZEB1 promoter/pGL3basic clone (provided by Antonio Garcia de Herreros, University of Barcelona) as a template. The deletion of the upstream site was generated by amplifying the same template in two separate PCR reactions, using Ant-f: TAATTTACGCGTCCTTAAGGTCCTGCACGGCG, Ant-r: TTTAAAAAGCTTCCGCCATGATCCTCTCGC (reaction 1) and Ant-f2: tatataGCGGCCGAAGGGAAGGGAAGGGAGTCC, Ant-r2: TTTAAAGCGGCCGCTTCAATGAGATTGAACCTCA. Following restriction digestion and isolation from an agarose gel, fragments were three-way ligated into pGL3basic.

Chromatin immunoprecipitation assays

The protocol for cross-linking, immunoprecipitation, de-cross-linking, and quantitative PCR (qPCR) analysis was as described previously (28). For Six1 protein interaction with the ZEB1 promoter, five dishes of HMLE+ empty vector (pMXS-IRES-puro) or FLAG-Six1 were immunoprecipitated with mouse anti-FLAG-M2-agarose or mouse IgG (Santa Cruz BioTechnology)-agarose in conjunction with DNA/protein-blocked protein A/G agarose (Pierce/Thermo). Primers to amplify the ZEB1 promoter were: f: gcccgagcctccaacttt; r: tgctaggagccggcggttt. For interaction of ZEB1 protein with the GRHL2 promoter, five dishes of HMLE expressing ZEB1 (in pLUT vector) were processed. To pull down ZEB1, 1 µg of the following antibodies (3 µg in total) were used: ZEB1, rb, Sigma HPA004820; ZEB1, rb, Cell Signaling Technology 3396S; and ZEB1, rb, Santa Cruz Biotechnology SC-25388. The control antibody was rabbit immunoglobulin G (IgG, 3 µg; Santa Cruz Biotechnology). Primers to analyze the ZEB1 chromatin immunoprecipitation (ChIP) were: positive control: Ecad-ZEB1-f: ggcggcgagtgaaacctca; Ecad-ZEB1-r: gggctggagtctgaactga; GRHL2 promoter set 1: f: cgccgctccactcggtgaaa; r: tgcgcgatgattggctgggg; GRHL2 promoter set2: f: tcagctctcagcagctgccg; r: gaggcgcgctcagtgtaaggc. The negative control primer set for all ChIP assays was from an intergenic region upstream of the GAPDH locus: GAPDH-f: atgggtgccactggcgatct; GAPDH-r: tgccaaagcctagggaaga.

Electrophoretic mobility shift assay

Purified recombinant Six1 protein was generated as described previously (32). Purified recombinant GRHL2 protein was obtained by expressing the GRHL2 coding sequence in *Escherichia coli* (E. coli) BL21 using the pGEX-6P3 vector and purifying the fusion protein as described previously (15). After binding to the glutathione Sepharose resin (GE Healthcare), the protein was cleaved free of GST sequence using Pre-Scission Protease, which was then dialyzed against electrophoretic mobility shift assay (EMSA) buffer (see further) using Slide-A-Lyzer Mini-dialysis Unit (Thermo Scientific). 2× EMSA buffer was: 30 mmol/L HEPES pH 7.6, 20% glycerol, 1 mmol/L

EDTA, 200 mmol/L KCL, 0.5% NP40, 5 mg/mL BSA (added fresh), 10 mmol/L DTT (added fresh). For 20 µL binding reactions: 10 µL 2× EMSA buffer+1 µL poly dIdC (0.5 µg/µL)+2 µL annealed 100 nmol/L oligonucleotide, and recombinant protein (as indicated). Oligonucleotides for EMSA were synthesized with Cy5 groups on both 5' ends (Operon) and annealed at 10 mmol/L Tris/1 mmol/L EDTA/100 mmol/L NaCl. Binding reactions were incubated at 4°C for 20 minutes, 15 minutes at room temperature, and analyzed on 6% polyacrylamide/TBE gels in 0.25× TBE buffer (pre-run for 1 hour) at 170V. The fluorescent signals were imaged using the LiCor Odyssey imaging system.

Oligo sequences (Forward strands) ZEB1-WT-Promoter: (Cy5)-GGAAGGTGATGTCGTAAAGCCGGGAGTGTCTGTAACCCAGGTGCGGTGGG

ZEB1-MT-Promoter: (Cy5)-GGAAGGTGATGTCGTCCCCGC-CGGGAGTGTCTGTCCCCAGGTGCGGTGGG

GRHL2-Pos-Con: (Cy5)-AACTATAAAACCGGTTTATCTAGTTGG

Six1-Pos-Con: (Cy5)-GGGGGCTCAGGTTTCTGTGGC

Negative control (from E-cadherin promoter): (Cy5)-TGGCCGGCAGGTGAACCCTCAGCCAATCAGCGCTACGGGGGGCGGTGCTCCGGGGCTCACCTGGCTGCAG

Flow cytometry

Trypsinized cells (1×10^5) were stained with APC mouse anti-human CD44 (BD 560890) and PerCP-Cy5.5 mouse anti-human CD24 (BD 561647) in 100 µL of PBS containing 2.5 mmol/L EDTA, 10 mmol/L HEPES, and 2% horse serum, followed by washing and analysis using a FACS-Aria.

Statistical methods

Error bars in graphs represent SDs, using a two-tailed *t* test. *P* values for tumor assays were calculated using the two-tailed Fisher exact test. Tumors were considered present when the tumor volume was reported to be greater than 100 mm³. ORs and *P* values for tumor incidence were calculated using the Fisher exact Test as implemented in R v2.15.1 (www.r-project.org).

Results

GRHL2 inhibits the activation of ZEB1 expression by homeodomain proteins

Previously, we reported that GRHL2 binds and represses the ZEB1 promoter so as to suppress EMT (28). To characterize this transcriptional regulation further, we analyzed the ZEB1 promoter using a transcription factor-binding prediction program (Genomatix) and identified two tandem homeodomain consensus binding sites, overlapping with and just upstream of the putative GRHL2 site. Alignment of this sequence between several species revealed that both the putative GRHL2 binding site as well as these homeobox sites were highly conserved (Fig. 1A). In this light, we tested the effect of two homeodomain factors that were previously shown to be involved in EMT induction in breast cancer cells – LBX1 and Six1 – on the activity of the ZEB1 promoter (22, 33). These factors activated transcription of the ZEB1 promoter significantly; mutation

Figure 1. Homeodomain proteins LBX1 and Six1 activate ZEB1 expression and are inhibited by GRHL2 protein. A, the region of interest from the ZEB1 promoter is conserved between various species: Ms, *Mus musculus*; Hs, *Homo sapiens*; Bp, *Bos primigenius*; Dr, *Danio rerio*. Bold, homeobox consensus sites; italics, grainyhead consensus sites. B, LBX1 and Six1 activate transcription from the ZEB1 promoter in a GRHL2-sensitive manner. The ZEB1 promoter-luciferase reporter with wild-type sequence (WT), the 3' homeobox site mutated (MUT), or both the mutation of the latter site plus deletion of the 5' homeobox binding site (DD) were cotransfected into MSP cells together with the indicated transcription factors; values represent the average of biologic duplicates from one representative of two experiments. C, Six1 induces the endogenous ZEB1 gene and EMT in unfractionated HMLE cells. HMLE cells expressing vector or FLAG-Six1 were analyzed for the indicated EMT markers (top) or morphologic changes (bottom).

of the 3' homeodomain binding site substantially diminished transactivation by these factors. The double deletion totally eliminated it, and, interestingly, GRHL2 cotransfection inhibited the transactivation by both homeodomain factors (Fig. 1B).

We then tested the effect of Six1 on endogenous ZEB1 expression. HMLE cells are a mixed population containing 5% to 10% mesenchymal cells in equilibrium with epithelial cells (11). HMLE cells were infected with Six1 retroviral expression constructs. Interestingly, Six1 by itself upregulated ZEB1, downregulated GRHL2, and induced EMT (Fig. 1c). Upon removal of the CD44^{high} subpopulation by flow sorting before infection with Six1 retrovirus, however, induction of ZEB1 by Six1 required the concomitant knockdown of GRHL2 through an shRNA (previously shown to have identical effects with other shRNAs targeting GRHL2), consistent with the antagonistic effect of GRHL2 on EMT that we observed previously (Supplementary Fig. S1; ref. 28). Knock-down of the individual homeodomain proteins examined failed to block ZEB1 induction during EMT, indicating that multiple redundant factors, likely to include additional, unidentified factors, activate ZEB1 (data not shown). In this connection, HoxA5, another factor that was predicted to bind these two sites, also showed robust, GRHL2-sensitive ZEB1 activation as well as the ability

to induce mammosphere formation in HMLE cells, although frank EMT was not evident (Supplementary Figs. S2 and S3; data not shown).

We tested Six1 for selective, direct binding to an oligonucleotide corresponding to the conserved sequence of the ZEB1 promoter. Six1 protein interacted preferentially with the wild-type, but not the doubly mutated (transcriptionally inactive), sequence as did the GRHL2 protein (Fig. 2a and b). The addition of stoichiometric amounts of GRHL2 protein diminished the Six1-DNA complex dramatically, indicating either a competition for DNA binding or an inhibitory Six1-GRHL2 interaction, which the data do not allow us to distinguish (Fig. 2C). ChIP analysis revealed that Six1 was recruited to the endogenous ZEB1 promoter, indicating a direct Six1 protein-ZEB1 promoter interaction (Fig. 2D).

The combined data support a model under which GRHL2 is a general inhibitor of homeodomain protein-induced ZEB1 expression and EMT.

GRHL2 suppresses primary tumor growth and sensitizes tumor cells to chemotherapy-induced cytotoxicity

Previously, we reported that GRHL2 suppressed or reversed the oncogenic EMT, in part, through the repression of the *ZEB1* gene (28). EMT can increase the frequency of

Figure 2. GRHL2 and Six1 bind specifically to sequences in the ZEB1 promoter, and GRHL2 alters this complex. Wild-type (wt) or mutant (mut) oligonucleotides corresponding to the conserved sequence of interest from the ZEB1 promoter were used to analyze and demonstrate the specificity of GRHL2 protein (A) or Six1 (B) protein interactions. C, GRHL2 positive control oligo (GRHL2PC), Six1 positive control oligo (Six1PC), oligo from the E-cadherin promoter, which neither protein is predicted to bind (NC), and WT ZEB1 promoter oligo were used in an EMSA with the indicated combinations of GRHL2 and Six1 proteins. D, Six1 protein interacts with the ZEB1 promoter *in vivo*. Chromatin from HMLE expressing either vector or FLAG-Six1 were used in a ChIP assay and analyzed by qRT-PCR using ZEB1 promoter primers or GAPDH control primers. Values are the average of biologic duplicates from one representative of two experiments.

breast CSCs in certain cell lines such as HMLE (12), and consistent with this, GRHL2 suppressed mammosphere generation (28). Here, we tested the effects of GRHL2 on tumor-initiation frequency, a measure of CSC frequency. HMLER cells are immortalized mammary epithelial cells that express H-rasV12 ectopically and have not undergone EMT, but are induced to do so by stable GRHL2 knockdown using multiple, independently targeting shRNAs (28, 34). As reported previously, HMLER cells possessed a low tumor-initiation frequency, but this frequency was increased significantly by the knockdown of the *GRHL2* gene (Fig. 3A). Correspondingly, the knockdown of GRHL2 increased the

frequency of CD44^{high}CD24^{low} cells (Supplementary Fig. S4), which have been correlated with the CSC phenotype in this cell line previously (12, 28). To extend this to another cell line, and use the converse approach, the triple-negative breast cancer line, MDA-MB-231LN was stably transduced with a GRHL2-expressing retrovirus or with or empty vector (28) and injected. GRHL2 suppressed the frequency of tumors significantly (Fig. 3b). These data were consistent with the ability of GRHL2 to suppress the tumor initiation-promoting effect of EMT (11, 12, 35). In conjunction with the functional assays and clinical correlations – downregulation of GRHL2 expression in EMT-like tumors – that we

Figure 3. GRHL2 suppresses tumor-initiation capacity and sensitizes breast cancer cells to chemotherapy-induced cytotoxicity. **A**, GRHL2 suppresses tumor initiation (knockdown approach). Immunodeficient mice were injected orthotopically with HMLER cells expressing control shRNA versus GRHL2 shRNA (one of two GRHL2 shRNAs shown previously to suppress EMT in this cell line; ref. 22); individual tumor volumes at 10 weeks are shown. **B**, GRHL2 suppresses tumor initiation (overexpression approach). MDA-MB-231LN cells expressing GRHL2 (three individual clones, labeled G1, G2, and G3) or MDA-MB-231LN cells with empty vector (vec) were assayed at the indicated time points. (Mice bearing vector control tumors were sacrificed between 4.5 and 7 weeks due to signs of tumor burden-related illness.) ORs for tumor initiation were 0.0108 and 0.0558 for the early and late time-point data, respectively. **C**, GRHL2 is downregulated during the transition from primary to recurrent phenotype in tetO-Wnt1 and tetO-neuNT mouse models; qRT-PCR on RNAs from independent tumors, normalized against β 2-microglobulin, conducted in technical duplicate.

published previously (28), these results identify *GRHL2* as a tumor suppressor gene.

In doxycycline-inducible mouse mammary tumor models, the expression of neuNT, Wnt1, or *c-myc* promotes primary tumors that regress upon the removal of doxycycline. The tumors recur frequently, and the recurrent tumor cells have undergone EMT, providing a model for evolution from oncogene/proliferation rate-driven tumors to oncogene-independent, EMT-driven recurrences (29, 30, 36). We analyzed RNA from the tetO-neuNT and tetO-Wnt1 mouse models for GRHL2 expression by quantitative reverse transcription (qRT)-PCR. In both cases, GRHL2 expression was greatly reduced in the recurrent tumor relative to primary (Fig. 3C). These findings indicate that GRHL2 is downregulated during tumor evolution to an EMT phenotype, further supporting the tumor suppressor function of GRHL2, in a mouse model for disease recurrence.

EMT is associated with chemoresistance (37, 38). We reasoned that the mesenchymal-epithelial transition induced by GRHL2 would be associated with enhanced sensitivity to the microtubule-targeting chemotherapy drug, paclitaxel. Ectopic GRHL2 expression induced dose-dependent cell death in the otherwise refractory MDA-MB231LN tumor cell line (Supple-

mentary Fig. S5). HMLE cells contain a subpopulation of CD44^{high}CD24^{low} cells that is enriched by paclitaxel treatment; this subpopulation has enhanced tumor-initiation frequency (11, 38). We confirmed previous reports that, following paclitaxel treatment and recovery, this subpopulation predominated (Supplementary Fig. S5). The paclitaxel-selected cells also expressed lower levels of GRHL2 than the original population, consistent with their previously reported EMT phenotype. Ectopic GRHL2 expression prevented the chemotherapy-induced emergence of CD44^{high}CD24^{low} cells, indicating that GRHL2-mediated suppression of this phenotype was resistant to the effects of challenge with a chemotherapy drug (Supplementary Fig. S5; ref. 28). The current data did not, however, allow us to exclude the possibility that paclitaxel generated a second, as yet uncharacterized, CSC subpopulation from HMLE that was not suppressed by GRHL2.

Based on the extensive data supporting an oncogenic role for EMT, the suppression of EMT by GRHL2, the effects of GRHL2 on tumorigenicity and chemosensitivity, and the downregulation of GRHL2 during EMT in cell culture, patient, and mouse models, we set out to identify pathways that downregulate GRHL2 expression.

Figure 4. Wnt and TGF- β cooperate to downregulate GRHL2. HMLE cells treated with the canonical Wnt pathway agonist BIO (A) or stably expressing β -catenin S33Y (B) were assayed for GRHL2 downregulation in response to TGF- β treatment by Western blotting; each is one representative of two duplicate experiments. C, HMLE cells that stably expressed Twist-ER protein were treated with or without 4-OHT (to activate Twist-ER) in the presence or absence of the TGF- β pathway antagonist BMP2, and cell lysates were assayed for GRHL2 and other indicated protein expression.

GRHL2 is downregulated by Wnt and TGF- β

TGF- β is able to induce EMT in only restricted cell contexts (39). In HMLE cells, TGF- β induced EMT only in conjunction with Wnt pathway agonists or in cells where GRHL2 had been knocked down, providing insight into this restriction (11, 28).

Activation of the Wnt signaling pathway by transient treatment with the GSK-3 inhibitor, 6-bromo-3-[(3E)-1,3-dihydro-3-(hydroxyimino)-2H-indol-2-ylidene]-1,3-dihydro-(3Z)-2H-indol-2-one (BIO; ref. 31) or, by stable expression of the degradation-resistant β -catenin S33Y mutant (40), downregulated GRHL2 expression only modestly, as did extended treatment with TGF- β . Coactivation of both pathways, however, caused a dramatic downregulation of GRHL2 expression (Fig. 4A and B), accompanied by upregulation of the GRHL2-repressed target gene, *ZEB1*. BMP2, a functional antagonist of TGF- β signaling and Twist-induced EMT (11, 41), alleviated both GRHL2 downregulation and *ZEB1* induction in response to the activation of Twist-ER protein by 4-hydroxytamoxifen (Fig. 4C).

These observations signified that the Wnt and TGF- β pathways collaborated to downregulate GRHL2 as an intermediate step in EMT, correlating with increased *ZEB1* expression, which prompted us to investigate the potential role of *ZEB1* in repressing GRHL2.

ZEB1 represses the GRHL2 promoter directly

To test the role of *ZEB1* in the downregulation of GRHL2, HMLE cells with an estrogen-inducible Twist fusion protein (HMLE+Twist-ER) were induced to undergo EMT by the addition of 4-hydroxytamoxifen. As we reported previously, this upregulated *ZEB1* and downregulated GRHL2 (28). Suppression of *ZEB1* with siRNA, however, largely prevented the GRHL2 downregulation (Fig. 5A). To test this in a different

context using a different knockdown reagent, HMLE cells with doxycycline-inducible *ZEB1* shRNA expression or control cells were induced with the combination of BIO and TGF- β . Depletion of *ZEB1* by *ZEB1* shRNA alleviated the downregulation of GRHL2, indicating that the Wnt+TGF- β -mediated downregulation of GRHL2 is dependent on *ZEB1* expression (Fig. 5B). Conversely, we expressed *ZEB1* ectopically in HMLE cells using a doxycycline-inducible lentiviral vector. Induction of *ZEB1* downregulated GRHL2 expression as well as the previously characterized *ZEB1* target, E-cadherin (Fig. 5C; ref. 15). These observations indicated that *ZEB1* repressed GRHL2 expression.

We then explored the possibility that the *ZEB1* protein might repress the *GRHL2* gene through direct interaction with promoter sequences. As an initial test, we subcloned a genomic fragment containing the sequences from -625 to +335 (relative to the transcription start site of the standard GRHL2 transcript) into a luciferase reporter vector and assayed this for transcriptional activity. The transcriptional activity of the GRHL2 promoter clone was substantially greater in HMLE cells than in the mesenchymal subpopulation cells (MSP) derived from HMLE, consistent with the low expression of endogenous GRHL2 in the latter, validating this promoter clone (Fig. 5D; ref. 28). Cotransfected *ZEB1* repressed the promoter, nearly to background level. By contrast, two other E-box binding EMT-related transcription factors, Snail and Twist, only modestly repressed the promoter. Conversely, the promoter activity assayed in MSP cells was stimulated by transfection of *ZEB1* siRNA (Fig. 5E).

Inspection of published *ZEB1* ChIP-seq data indicated binding sites positioned near the transcription start site of GRHL2 (Fig. 6A). By ChIP analysis using an anti-*ZEB1* antibody, we detected an interaction of *ZEB1* protein with GRHL2 sequences near the transcription start site, with efficiency

Figure 5. ZEB1 downregulates the GRHL2 promoter. **A** and **B**, si/shRNA-mediated knockdown of ZEB1 partially alleviated GRHL2 downregulation caused by 4-OHT-activated Twist-ER protein (**A**) or BIO plus TGF- β treatment (**B**). **C**, HMLE cells stably expressing a doxycycline-inducible ZEB1 expression construct were induced with doxycycline and lysates were analyzed for ZEB1, GRHL2, E-cadherin, and Akt by Western blotting. **D**, the GRHL2 promoter in a luciferase reporter vector was cotransfected with Snail, Twist, or ZEB1 expression vectors in HMLE cells or the promoter was transfected by itself into MSP cells; normalized luciferase activity values are shown. **E**, HMLE-MSP transfected with control or ZEB1 siRNA were transfected with the GRHL2 promoter-luciferase construct; normalized luciferase activity values are shown. Values are the average of biologic duplicates from one representative of two experiments.

comparable with that of the E-cadherin promoter and significantly stronger than a negative control sequence (the intergenic region upstream of the *GAPDH* gene; Fig. 6B). When combined, these data indicated that the ZEB1 protein represses the GRHL2 promoter directly, establishing that a mutual antagonism between expression of ZEB1 and GRHL2 directs tumor cells toward epithelial or mesenchymal phenotypes.

Discussion

Our observations inform a model of reciprocal antagonism between ZEB1 and GRHL2 as a pivotal mechanism underlying EMT (Fig. 7). Under this model, GRHL2 and ZEB1 – each regulated by microenvironmental factors – repress each other's transcription. GRHL2 represses ZEB1 by inhibiting at least three activators of the ZEB1 promoter: LBX1, Six1, and HoxA5.

We focused on these three transcription factors in light of the functional importance of homeodomain consensus sites for activity of the ZEB1 promoter (Fig. 1). Although numerous homeodomain proteins may have partially redundant functions, confounding efforts to show an effect of knockdown/knockout of an individual factor, their overall significance in diverse cancer types is established (reviewed in ref. 42). In particular, previous reports have implicated LBX1 and Six1 in breast-cancer-related EMT (22, 33, 43). Although Six1 was shown previously to induce ZEB1 expres-

sion, this was proposed to be posttranscriptional in colorectal cancer cell lines (44), in contrast with our evidence indicating a direct interaction of Six1 with the ZEB1 promoter.

Importantly, GRHL2 protein, the first direct transcriptional repressor of the *ZEB1* gene to be reported (28), interfered with the transcriptional activation of the ZEB1 promoter by all three of these factors. Although the GRHL2 protein effectively abolished the Six1 protein–DNA complex on the ZEB1 promoter oligonucleotide that contained two tandem Six1 binding sites, there was no effect on Six1 protein binding to a positive control sequence containing only one binding site (data not shown), suggesting that GRHL2 selectively affects two Six1 protein molecules on DNA versus a single Six1 molecule. We were unable to detect an interaction of GRHL2 with Six1 protein in cotransfection assays (data not shown), suggesting that they interact only on DNA.

The antagonistic effects of GRHL2 versus ZEB1, mediated, in part, by homeodomain activators of ZEB1, may relate conceptually to previously reported developmental roles of these genes. GRHL2 is required for neural tube closure, the stage of development directly preceding delamination. Subsequently, anterior HOX genes (*HoxA1* and *HoxB1*) induce EMT during delamination of the neural crest, through activation of Snail and *Msx1/2* transcription (45). Speculatively, the homeodomain factors may also activate EMT factors such as ZEB1, thereby repressing GRHL2 expression, which would indicate, analogously, functional opposition of

Figure 6. ZEB1 protein interacts directly with the GRHL2 promoter. A, ChIP-seq data (Encode project; ref. 55) for ZEB1 reveal a potential binding site near the transcription start site. B, confirmation of ChIP-seq. Chromatin from HMLE expressing tet-ON ZEB1 was subjected to ChIP using ZEB1 antibody and analyzed by qRT-PCR using the indicated primers. The average of biologic duplicates from one representative of two experiments is shown.

these factors during development that mirrors their respective functions in regulating the oncogenic EMT.

We have now shown the other half of the reciprocal feedback loop (Fig. 7) as well: ZEB1 is also a direct repressor

of GRHL2 (Figs. 4 and 5). When combined, this represents a switch that dictates cellular phenotype between epithelial and mesenchymal states, formally analogous to the relationship between ZEB1 and mir200 (46). Thus, one effect of

Figure 7. A reciprocal feedback loop between Grainyhead-like-2 and ZEB1 controls EMT and tumor suppression. Details are explained in the text.

inducing ZEB1 expression by microenvironmental factors is to repress GRHL2 expression, stabilizing ZEB1 expression. In this connection, we found that neither TGF- β nor the canonical Wnt pathway stimulation was sufficient to induce ZEB1/downregulate GRHL2; however, the two in conjunction tipped the balance in favor of ZEB1. This finding is precisely consistent with those showing that multiple microenvironmental signals are needed to regulate EMT or MET (10, 47) and may prove to be the underlying mechanism. Other combinations of factors induce ZEB1 and EMT, at least in specific cell contexts, including TGF- β + FGF2, TGF- β + TNF- α , TGF- β +EGF(R), TGF- β +mechanotransduction from extracellular matrix, or factors in the "senescent cell secretory phenotype" (SASP; refs. 19, 48–51). It is also notable that Six1 expression activates autocrine TGF- β and Wnt signaling loops, suggesting that it activates ZEB1 both directly and indirectly (33).

Our results indicate that these combinations of factors may downregulate GRHL2 as an intermediate step leading to EMT. Moreover, stable GRHL2 expression suppressed EMT, decreased tumor frequency, and sensitized tumor cells to the effects of chemotherapeutic drugs. Thus, factors that downregulate GRHL2 are potential drug targets for novel therapies acting to enhance chemotherapeutic responses and prevent disease recurrence. In this connection, it is interesting to note that the related gene, *GRHL3*, is a tumor suppressor in squamous cell carcinoma that upregulates PTEN (52, 53). During development, GRHL2 and GRHL3 regulate some unique and other common genes (54), suggesting that the biologic functions of GRHL2 should be investigated in the context of other Grainyhead family members for optimal translational benefit.

Cellular commitment to undergo an oncogenic EMT is formally analogous to cell-cycle control in that both involve a transition from dependence on extracellular signals to an autonomous, stable state. For example, the cell cycle becomes autonomous beyond the mitogen-driven restriction point, activation of cyclinD-CDK4/6 complexes, and maintains that

state by a number of mechanisms, including stable activation of cyclin E expression by free E2F. Similarly, oncogenic EMT is initiated by microenvironmental factors, for example, TGF- β and Wnt agonists. We propose that the transition to an autonomous state is defined, in part, by downregulation of GRHL2, stabilizing autocrine signaling through ZEB1, TGF- β , Wnt, and other factors. Thus, GRHL2 downregulation is an "oncogenic EMT restriction point."

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: B. Cieply, S.M. Frisch

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Cieply, J. Farris, S.M. Frisch

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Cieply, J. Farris, J. Denvir, H.L. Ford, S.M. Frisch

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Cieply, H.L. Ford, S.M. Frisch

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Appendix All

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Grainyhead-like-2 inhibits the co-activator p300, suppressing
tubulogenesis and the epithelial-mesenchymal transition

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Abstract

Developmental morphogenesis and tumor progression require a transient or stable breakdown of epithelial junctional complexes to permit programmed migration, invasion and anoikis-resistance, characteristics endowed by the Epithelial-Mesenchymal Transition. The epithelial master-regulatory transcription factor Grainyhead-like 2 (*GRHL2*) suppresses and reverses EMT, causing a mesenchymal-epithelial transition to the default epithelial phenotype. Here, we investigated the role of *GRHL2* in tubulogenesis of Madin-Darby Canine Kidney (MDCK) cells, a process requiring transient, partial EMT. *GRHL2* was required for cystogenesis, but it suppressed tubulogenesis in response to Hepatocyte Growth Factor (HGF). Surprisingly, *GRHL2* suppressed this process by inhibiting the histone acetyltransferase co-activator, p300, preventing the induction of matrix metalloproteases and other p300-dependent genes required for tubulogenesis. A thirteen amino acid region of *GRHL2* was necessary and sufficient for the inhibition of p300, the suppression of tubulogenesis and the interference with EMT. The results demonstrate that p300 is required for the partial or complete EMT occurring in tubulogenesis or tumor progression, and that *GRHL2* suppresses EMT in both contexts, through inhibition of p300.

INTRODUCTION

p300 is a co-activator of transcription that interacts with at least four hundred DNA-factors to activate transcription from thousands of enhancers/promoters, mostly contingent upon its intrinsic histone acetyltransferase (HAT) activity. In this context, it is viewed as an “integrator” factor that serves as a nexus among multiple signaling pathways and programs of gene expression (Yao *et al.*; Kamei *et al.*, 1996; Vo and Goodman, 2001; Bedford *et al.*, 2010). Accordingly, p300/CBP null mice die at E9.5-11.5 with massive deficits of mesenchymal cells and erythroid cells, as well as defects in neurulation and heart development (Yao *et al.*; Oike *et al.*, 1999). p300 is essential for the differentiation of muscle, erythroid lineages, osteoblast, oligodendrocytes, and stem cells (Puri *et al.*, 1997; Oike *et al.*, 1999; Blobel, 2000; Polesskaya *et al.*, 2001; Narayanan *et al.*, 2004; Teo and Kahn, 2010; Zhang *et al.*, 2016). A recent study has demonstrated p300 is a marker for super-enhancers, positioning it to control cell state transitions (Witte *et al.*, 2015).

Grainyhead-like 2 (GRHL2) is one of the three genes comprising a family of mammalian transcription factors related to *Drosophila* Grainyhead, the first zygotically encoded transcription factor expressed during the maternal to zygotic transition (the MZT) (Harrison *et al.*). *GRHL2* is important for epithelial barrier assembly in, for example, the morphogenesis of kidney collecting ducts, placenta, lung alveoli and the mammary gland ductal system (through *OVOL2*, a *GRHL2* target gene) (Gao *et al.*, 2013; Watanabe *et al.*, 2014; Aue *et al.*, 2015; Walentin *et al.*, 2015). Grainyhead factors are also critical for diverse developmental events that involve the formation of epithelial barriers, including epidermal assembly, neural crest formation, and, in the adult, wound-healing (Wilanowski *et al.*, 2002; Gustavsson *et al.*, 2008; Wang and Samakovlis, 2012; Mlacki *et al.*, 2015). *GRHL2* activates the expression of multiple genes encoding epithelial cell

adhesion molecules (e.g., E-cadherin, desmosomal proteins, tight junction proteins), and due to its widespread expression, may be generally important for enforcing an epithelial phenotype (Rifat *et al.*; Senga *et al.*; Wilanowski *et al.*, 2002; Ting *et al.*, 2003; Ting *et al.*, 2005; Auden *et al.*, 2006; Werth *et al.*, 2010; Boglev *et al.*, 2011; Cieply *et al.*, 2012; Tanimizu and Mitaka, 2013; Aue *et al.*, 2015). Correspondingly, the GRHL2 expression plays an important role in suppressing the oncogenic EMT, thereby functioning as a tumor suppressor that enhances anoikis-sensitivity (Cieply *et al.*, 2012; Cieply *et al.*, 2013; Mlacki *et al.*, 2015); in fact, the gene most tightly correlated with E-cadherin expression in human tumors was GRHL2 (Kohn *et al.*, 2014).

The opposing roles of GRHL2 in establishing the epithelial state vs. p300 in differentiation - including EMT -- motivated us to investigate a potential role for GRHL2 in inhibiting p300 function. Kidney tubulogenesis is contingent upon temporal and spatial regulation of epithelial cells, in which GRHL2, which is expressed in the ureteric buds of the developing kidney plays a critical organizing role (Schmidt-Ott *et al.*, 2005; Aue *et al.*, 2015; Walentin *et al.*, 2015). Madin-Darby Canine Kidney (MDCK) tubulogenesis in response to Hepatocyte Growth Factor (HGF) models some aspects of kidney collecting duct tubulogenesis *in vivo* and requires a transient, partial EMT (pEMT) (Pollack *et al.*, 1998; O'Brien *et al.*, 2002; Pollack *et al.*, 2004; Leroy and Mostov, 2007; Hellman *et al.*, 2008; Jung *et al.*, 2012; Zhang *et al.*, 2014). HGF causes MDCK cells to undergo cell scattering in two-dimensional culture, and induces tubulogenesis of MDCK cysts that form in a three-dimensional collagen gel (Wang *et al.*, 1990,1991 #4686; Pollack *et al.*, 2004). Induction of MMP1 and MMP13 by HGF through AP1/p300 complexes is a required for tubulogenesis in the MDCK model (Benbow and Brinckerhoff, 1997; Clark *et al.*, 2008; Hellman *et al.*, 2008; Chacon-Heszele *et al.*, 2014).

Targeted knockout studies indicate a role for HGF/Met in kidney development, and, relatedly, HGF/Met signaling is a potent stimulator of Wnt signaling, a crucial signaling pathway in this process, validating the *in vivo* relevance of this approach (Monga *et al.*, 2002; Bridgewater *et al.*, 2008; Ishibe *et al.*, 2009).

Herein, we report that *GRHL2* promoted cystogenesis but suppressed tubulogenesis. GRHL2 protein interacted functionally with p300, inhibiting its HAT activity and transcriptional activation of target genes, including matrix metalloproteases. A small (thirteen amino acid) sequence of GRHL2 was important for inhibition of p300, suppression of tubulogenesis and the reversal of EMT. These results mechanistically position GRHL2, an enforcer of the epithelial default phenotype, as an antagonist of p300, a co-activator of differentiation-specific genes, with important ramifications for developmental and tumor biology.

RESULTS

GRHL2 suppresses cell scattering and tubulogenesis

In light of the epithelial programming role of *GRHL2*, we tested the effect of HGF on GRHL2 levels in MDCK cells. Interestingly, we found that HGF down-regulated the expression of endogenous GRHL2 protein levels at early time points (figure 1A). When constitutively expressed in MDCK cells, *GRHL2* significantly inhibited HGF-induced cell scattering compared to vector control cells (figure 1B). Conversely, MDCK cells with GRHL2 shRNA knockdown demonstrated enhanced HGF-induced cell scattering; retention of E-cadherin expression showed that EMT did not occur in response to the knockdown of GRHL2 alone (figure 1C and see video

in supplemental figure S1). GRHL2 had only modest effects on the phosphorylation status of two established HGF/Met signaling mediators, Erk and Akt (figure S2).

We then examined the effects of *GRHL2* on HGF-induced tubulogenesis in the three-dimensional collagen model. *GRHL2* expression caused the formation of larger cysts compared to vector control cells, consistent with previous reports in hepatic bile duct cells (figure 1D) (Tanimizu and Mitaka, 2013). *GRHL2* significantly suppressed tubulogenesis/branching morphogenesis following HGF treatment (figure 1D); a similar effect of murine *Grhl2* was observed in mouse inner medullary collecting duct cell line (figure S3A), indicating the important role of GRHL2 down-regulation in tubulogenesis. Conversely, the stable knockdown of GRHL2 prevented cystogenesis (figure S3B), presumably by down-regulating epithelial adhesion molecules (Senga *et al.*; Werth *et al.*, 2010). Transient knockdown of GRHL2 following cyst formation using a doxycycline-induced shRNA vector, however, clearly indicated that the loss of GRHL2 promoted tubulogenesis (figure S4A). There was no effect of the GRHL2 shRNA on cell proliferation (figure S4B). These results indicated that GRHL2 suppressed HGF-activated cell scattering and tubulogenesis.

GRHL2 suppresses the expression of matrix metalloproteases

To identify *GRHL2* target genes responsible for the attenuation of cellular responses to HGF/Met signaling, RNA-Seq was used to compare MDCK cells with constitutive GRHL2 expression vs. GRHL2 depletion, either with no treatment or with HGF induction (24 hours). This experimental scheme allowed for two distinct comparisons: genes regulated by HGF in the absence vs. presence of GRHL2, and genes regulated by GRHL2 with vs. without HGF induction. GRHL2 down-regulated several known matrix metalloproteases (MMP) family members (Table 1, confirmed by qPCR in figure 2A). In light of the established importance of

specific MMPs for tubulogenesis in the MDCK and mIMCD-3 cell culture models (Hotary *et al.*, 2000; Jorda *et al.*, 2005; Hellman *et al.*, 2008; Chacon-Heszele *et al.*, 2014), we verified their importance for branching tubulogenesis in embryonic kidneys cultured ex-vivo using the generalized MMP inhibitor, batimastat (figure S5). The down-regulation of MMPs by GRHL2 could contribute significantly to the tubulogenesis-suppressing effect of constitutive GRHL2 expression, although additional contributions from EMT/MET-related GRHL2 genes are likely.

To investigate the transcriptional mechanisms of MMP gene regulation by GRHL2, MMP1 and MMP14 promoters were assayed as luciferase reporter constructs by cotransfection in HT1080 fibrosarcoma cells, in which the endogenous MMPs are expressed constitutively (Tang and Hemler, 2004; Nonaka *et al.*, 2005). GRHL2 was found to suppress *MMP1* and *MMP14* promoters using adenovirus E1a protein, a known repressor of *MMP* genes, as a positive control (figure 2B). These results demonstrated that GRHL2 repressed *MMP1* and *MMP14* promoters; however, examination of the promoter sequences utilized in our reporters constructs did not indicate the presence of GRHL2 DNA binding consensus sites (Wilanowski *et al.*, 2002; Gao *et al.*, 2013; Aue *et al.*, 2015; Walentin *et al.*, 2015), suggesting a more subtle mechanism for repression not involving direct DNA binding.

GRHL2 inhibits p300 function

The AP-1 transcription factor family is important in the regulation of most *MMP* genes (Clark *et al.*, 2008). GRHL2 significantly suppressed AP-1 activity on minimal reporter constructs in HT1080 and 293 cells (figure 3A). GRHL2 did not appear to affect the expression of *FOS* or *JUN* family members following HGF induction (figure S6).

In light of the functional similarities between adenovirus E1a and GRHL2 (see Discussion), we compared a published list of genes that E1a down-regulated through p300 interaction (Ferrari *et al.*, 2014) against our list of GRHL2-downregulated genes; this latter list was derived from the RNA-seq analysis that we performed on GRHL2-expressing human mesenchymal subpopulation cells (MSP), reported in (Farris *et al.*, 2016) (as comparison of canine vs. human gene lists proved uninformative for technical reasons). About 43.5% of the genes that E1a repressed through p300 were also repressed by GRHL2 (figure 3B). Downstream Effector Analysis/Ingenuity Pathway analysis revealed that the expression of GRHL2 suppressed the regulation of numerous p300-associated genes that were up-regulated or down-regulated by HGF in the absence of GRHL2 expression (figure 3C and figure S7). These results suggested that GRHL2 could potentially inhibit p300 function.

To investigate this, the effect of GRHL2 upon the activity of a GAL4-DNA binding domain-p300 fusion protein was assayed by transient transfection using a GAL4-responsive luciferase promoter, an established method for testing p300 coactivator function without confounding effects from primary DNA binding activator proteins (Snowden *et al.*, 2000). Adenovirus E1a protein repressed the AP-1 and GAL4/GAL4-p300 reporter activities, but a p300-non-binding mutant of E1a (Ferrari *et al.*, 2014) failed to do so (figure S8), validating the p300-dependence of transcription in these assays. GRHL2 inhibited the co-activator function of p300 in this assay (figure 3D). As additional negative controls, GRHL2 did not affect the ability of a p300-independent GAL4-activator fusion protein (VP16-GAL4; (Kutluay *et al.*, 2009)) to activate the same reporter; another transcription factor unrelated to GRHL2, c-Myc, failed to inhibit GAL4-p300 function in this assay (figure S9). These results indicated that GRHL2 was a

potent suppressor of p300 function. The highly related GRHL1 and GRHL3 proteins were found to have similar effects in the reporter assay (figure S10).

GRHL2 inhibits the histone acetyltransferase (HAT) activity of p300

The HAT domain of p300 acetylates numerous substrates, including lysines 18 and 27 in the N-terminal tail of histone 3 (H3) (Ogryzko *et al.*, 1996; Kasper *et al.*, 2010; Jin *et al.*, 2011). Using recombinant proteins, GRHL2 inhibited acetylation of H3K27 by p300 in a dose dependent manner in vitro (figure 4A). Similar results were obtained using the p300 HAT domain alone (amino acids 1283-1673) rather than full-length p300 (figure S11). Recombinant GRHL1 and GRHL3 proteins also inhibited the HAT activity of p300 (figure S12).

To confirm these in vitro effects in cells, we analyzed histone acetylation by CHIP-qPCR using the human cell line, HT1080 (as Encode reference data reporting histone acetylation are available for the human genome.) Consistent with the in vitro results, the acetylation of H3K27 was inhibited in HT1080 cells expressing GRHL2 constitutively, specifically on promoters that GRHL2 repressed, including *MMP1*, *MMP14*, *MMP2*, and *ZEB1*; acetylation of GRHL2-upregulated gene promoter-associated H3K27 was either not affected (*CDH1*, *ESRP1*) or up-regulated (*RAB25*) (figure 4B). Unlike the effect of E1a on p300, there was neither an effect of GRHL2 on global H3 acetylation nor global chromatin condensation, assayed by Western blotting (figure 4B) or by immunofluorescent localization of a LacI-mCherry-GRHL2 protein (Ferrari *et al.*, 2014), respectively (figure S13).

GRHL2 interacted with p300 by the criteria of co-immunoprecipitation of transiently co-transfected genes, or of endogenous proteins (figure 4C). The recombinant proteins interacted

only weakly, however (figure S14) suggesting that the interaction is transient or requires post-translational modifications absent from our recombinant proteins.

GRHL2 inhibits the C-terminal transactivation domain of P300

Multiple domains of p300 can co-activate transcription independently. One of these is the C-terminal domain (1665aa-2414aa) which interacts with the p160 family of co-activators (Stiehl *et al.*, 2007). Previous reports suggested that the activity of the C-terminal domain is stimulated by auto-acetylation, catalyzed by the p300 HAT domain (Stiehl *et al.*, 2007). In light of the HAT-inhibitory effect of GRHL2 described above, we hypothesized that GRHL2 inhibited the C-terminal domain through this mechanism. We confirmed that transcriptional activation by the C-terminal domain, assayed as a GAL4-C-term fusion protein, was stimulated by a co-transfected p300 HAT domain, and that the latter domain had no detectable ability to activate transcription independently (figure 5A). GRHL2 inhibited the enhanced transactivation by the p300 C-terminal domain in the presence of co-transfected p300 HAT (figure 5B). Embedded within the C-terminal domain is a small sub-domain called the IRF-3 Binding Domain (IBID; amino acids 2050-2096) that mediates the interaction with the p160 co-activator family, including the family member Steroid Receptor Coactivator-1 (SRC-1) (Sheppard *et al.*, 2001). While neither the IBID domain of p300 nor SRC-1 alone activated transcription efficiently, the combination of both activated transcription, as reported previously (figure 5C) (Sheppard *et al.*, 2001). GRHL2 potently inhibited transcription that was driven by the complex of p300-IBID and SRC-1 (figure 5D). Interestingly, mutation of the two lysines (K2086 and K2091) of the IBID domain that are acetylated *in vivo* abrogated the ability of the p300-IBID/SRC1 complex to activate transcription (figure 5E). These results indicate that the transcriptional activation by the p300 C-terminus -- which is supported by SRC-1 and abrogated by GRHL2 -- depends upon

acetylation of at least these (and probably other) lysines by p300 HAT, or perhaps other GRHL2-sensitive HATs that remain to be determined.

A small region of GRHL2 inhibits p300 and is critical for suppressing tubulogenesis and EMT

To identify the region of GRHL2 responsible for p300 inhibition, whole domains of GRHL2 were deleted initially (figure 6A) and assayed for inhibition of HAT activity and transcriptional activation by GAL4-p300. In light of the lack of GRHL2 binding sites in most GRHL2-repressed promoters (data not shown), the surprising result was that the DNA binding domain (amino acids 245-494) was critical for inhibition (figure S15, S16). GST-GRHL2 protein sub-fragments derived from the DNA binding domain were assayed for HAT inhibition (figure 6B). The region 325-475aa, and within that, the thirteen amino acid sequence 425-437aa, inhibited HAT activity potently (figure 6C), and a synthetic peptide (aa420-442) was also able to inhibit p300 HAT activity (figure S15C). The 425-437 fragment (IRDEERKQNRKKG) is an α -helix conserved amongst GRHL1, GRHL2, and GRHL3 (Kokoszynska *et al.*, 2008). A GRHL2 Δ 425-437 construct (with a synthetic nuclear localization signal) was unable to repress an AP-1-promoter, GAL4-P300 transactivation (assayed on a GAL4-Luc reporter), the *MMP1* promoter or the *MMP14* promoter (figure 6D). We recently reported that GRHL2 repressed the expression of the glutamate dehydrogenase-1 gene (*GLUD1*), with important consequences for metabolism and anoikis (Farris *et al.*, 2016). Interestingly, GRHL2 also repressed this promoter in a manner that required amino acids 425 to 437 (figure S17). GRHL2 Δ 425-437 was still able to interact with p300 in a co-transfection assay, however (figure S18).

We hypothesized that the inhibition of p300 function could provide a mechanism for some aspects of transcriptional reprogramming by GRHL2. Consistent with the inability of the

GRHL2 Δ 425-437 to repress the AP1-dependent promoters *MMP1* and *MMP14* (figure 6C), this mutant was unable to suppress HGF-induced tubulogenesis in MDCK cells (figure 7A). Moreover, wild-type GRHL2 reverses EMT in Mesenchymal Subpopulation (MSP) cells, a cancer stem cell-like subpopulation derived from the mammary epithelial cell line HMLE (Cieply *et al.*, 2012; Cieply *et al.*, 2013). The GRHL2 Δ 425-437 mutant failed to reverse EMT, by the criteria of cell morphology as well as EMT marker protein and mRNA expression (figure 7B). These results indicated that the inhibition of p300 by GRHL2 contributes significantly to transcriptional reprogramming involved in both tubulogenesis and EMT (figure 8).

DISCUSSION

Previously, we proposed that the epithelial cell is the default phenotype from both developmental and cancer biologic points of view. Under this hypothesis, the epithelial phenotype is adopted automatically by a cell in the absence of genomic/epigenetic alterations or inductive differentiation signaling (Frisch, 1997). Consistent with this idea, E-cadherin-expressing cells are the first to emerge at the late two cell stage; these eventually give rise to all differentiated cell types of the adult organism (Hyafil *et al.*, 1980; Vestweber and Kemler, 1985; Fleming *et al.*, 1989; Larue *et al.*, 1994). The adenovirus-5 E1a protein, which interacts with and negatively modulates p300, enforces an epithelial phenotype, suppresses EMT, enhances anoikis, suppresses MMP expression, and exerts a ubiquitously acting tumor suppression effect in human tumor cells (Frisch and Mymryk, 2002).

The default idea has been invoked in recent studies from other laboratories, to explain, for example, the mesenchymal-epithelial transition (MET) occurring at metastatic sites (Thiery and Sleeman, 2006; Polyak and Weinberg, 2009; Tam and Weinberg, 2013). The default hypothesis could be explained molecularly if critical co-activators such as p300 were required

for differentiation-specific but not epithelial-specific gene expression (Frisch, 1997).

Accordingly, we reported previously that GRHL2, shown here to antagonize the co-activator p300, inhibits and reverses the oncogenic EMT, suppressing tumor initiation, drug-resistance and anoikis-resistance (Cieply *et al.*, 2012; Cieply *et al.*, 2013).

The novel observation that GRHL2 antagonizes p300-dependent pathways in the context of tubulogenesis provides a potentially unifying mechanism for both the developmental and oncologic effects of GRHL2. GRHL2 probably regulates tubulogenesis by additional, p300-independent mechanisms. For example, cells which fail to down-regulate GRHL2 appropriately may fail to down-regulate cell adhesion molecules in response to morphogenic factors (Werth *et al.*, 2010; Senga *et al.*, 2012). For example, E-cadherin relocates from cell junctions to diffuse membrane pattern in protrusive cell extensions during tubulogenesis; this relocation might be envisioned to require GRHL2 down-regulation and subsequent loss of junctional complex components in these specific cells (Pollack *et al.*, 1998).

p300 contributes to tubulogenesis and cancer-related EMT in multiple important ways providing a mechanism for GRHL2, via inhibition of p300, to suppress these processes. With regard to cancer, transcription factors that utilize p300/CBP and additional coactivators that interact with p300/CBP have been implicated in the oncogenic EMT, tumor drug-resistance and tumor recurrence (Matthews *et al.*, 2007; Qin *et al.*, 2009; Santer *et al.*, 2011; Zhou *et al.*, 2012; Delvecchio *et al.*, 2013; Ringel and Wolberger, 2013; Yang *et al.*, 2013; Xu *et al.*, 2014; Cho *et al.*, 2015). The upregulation of matrix metalloproteases (MMPs) associated with the oncogenic EMT requires AP-1, Ets and other p300-dependent factors (Westermarck and KÄHÄRi, 1999; Sun *et al.*, 2004; Chou *et al.*, 2006; Clark *et al.*, 2008; Lee and Partridge, 2010; Santer *et al.*, 2011).

With regard to tubulogenesis, the p300-dependent Ets family transcription factors Etv4 and Etv5, are key transcriptional regulators of this developmental process (Yang *et al.*, 1998; Jayaraman *et al.*, 1999; Goel and Janknecht, 2003; Foulds *et al.*, 2004; Lu *et al.*, 2009; Oh *et al.*, 2012; Wollenick *et al.*, 2012). In addition, the family of p300-dependent TCF/ β -catenin transcription complexes is a central hub for Wnt signaling, crucial for nephron induction (Li *et al.*, 2007; Bridgewater *et al.*, 2008; Miller and McCrea, 2010; Ma *et al.*, 2012).

p300 activates transcription through an array of mechanisms; acetylation of histones, acetylation of transcription factors, the binding of the p300 bromodomain to acetylated histones to promote transcriptional activity, and recruitment of histone methyltransferases (e.g., Set1b complex) (Mizzen and Allis; Ogryzko *et al.*, 1996; Gu and Roeder, 1997; Ito *et al.*, 2001; Wolf *et al.*, 2002; Stiehl *et al.*, 2007; Chen *et al.*, 2010; Tang *et al.*, 2013). By inhibiting the p300 HAT activity, GRHL2 could enforce an inactive state on p300 in at least two ways. First, p300 HAT activity is substantially increased by auto-acetylation of an autoinhibitory loop on the HAT domain, which GRHL2 would suppress, stably inhibiting the enzyme (Thompson *et al.*, 2004). Secondly, the p300 HAT domain potentiates p300 C-terminal domain (1665aa-2414aa) transactivation (Stiehl *et al.*, 2007), which GRHL2 blocked. The minimal domain for p300 C-terminal transactivation is amino acids 2000-2180 which include the IRF-3 binding domain (IBID) (Lin *et al.*, 2001; Matsuda *et al.*, 2004). The p300 IBID domain cooperates with SRC1, a p160/steroid receptor coactivator family member, to activate transcription (Sheppard *et al.*, 2001). By inhibiting the HAT activity of p300, GRHL2 suppressed co-activation by the p300-IBID complex, which was highly dependent upon the two known acetylation sites, K2086 and K2091 for activity. Interestingly, SRC1-p300 has been shown previously to induce TWIST expression and EMT in breast cancer (Qin *et al.*, 2009), and GRHL2 is a potent EMT suppressor.

Although more than four hundred DNA binding proteins interact with p300 and utilize it as a co-activator, the inhibitory effect of GRHL2 on p300 is nearly unique, shared by only one other family of proteins called the E1a-like inhibitor of differentiation (EID) proteins. EID1 inhibits p300 HAT activity (MacLellan *et al.*, 2000) and EID3 blocks the SRC-1/CBP interaction, while the mechanism of EID2 is controversial (Ji *et al.*, 2003; Miyake *et al.*, 2003; Båvner *et al.*, 2005). The effects of EID proteins upon EMT or tubulogenesis have not yet been investigated to our knowledge.

GRHL2 regulates intracellular metabolism and this could indirectly affect p300 by altering the levels of the co-factors for p300 HAT, acetyl-CoA or crotonyl-CoA (Li and Li, 2015; Farris *et al.*, 2016). This may provide an additional, more indirect mechanism for GRHL2 to affect p300 function.

In summary, p300 and related co-activators are crucial for cells to differentiate. Under the default-phenotype hypothesis, this can be extended to critical roles in EMT, in contexts such as tubulogenesis and oncogenesis. The inhibition of p300 by GRHL2 provides a mechanism for GRHL2 to enforce the default epithelial phenotype.

MATERIALS AND METHODS

Cell lines

293 HEK cells without T antigen (Doug Black, UCLA, Los Angeles), MDCK cells (Clone M8 described previously in Frisch *et al.* (Frisch and Francis, 1994), and HT1080 cells (ATCC) were cultured in advanced Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) with 10% fetal bovine serum (Hyclone) and 1x penicillin-streptomycin-glutamine (PSG) (Invitrogen). miMCD-

3 cells (ATCC) cultured in advanced DMEM:Ham's F12 (Gibco) with 10% fetal bovine serum and 1x PSG. HMLE and HMLE MSP cells were described previously (Cieply *et al.*, 2013).

Generation of stable cell lines by retroviral transduction and lentiviral transduction

Full-length GRHL2/pMXS-IRES-Puro and retroviral packaging protocol were described previously (Farris *et al.*, 2016). GRHL2 Δ 425-437 was subcloned into pMXS-IRES-Puro from GRHL2 Δ 425-437-NLS-3xFLAG/pCDNA3.1+. GRHL2 shRNA/pGipZ and scramble control shRNA/pGipz were obtained from Open Biosystems and packaged as previously described (Cieply *et al.*, 2012). The shRNA construct in pTRIPZ was made by subcloning a MluI-XbaI fragment from pGIPZ.

Cell Scattering Assays

MDCK cells were plated on collagen coated 6 well dishes to give 25% cell confluency on HGF induction. Cells were treated with 60ng/ml recombinant human HGF (RD Systems) in DMEM media or fresh DMEM media for the time periods indicated. The cells were imaged and then harvested for protein or RNA analysis. Images were obtained using a Zeiss Axiovert 200M, AxioCam MRM camera, Phase 20x/0.55, RT, Axiovision Rel. 4.8 software. Cell scattering time lapse movies were taken every 15 minutes over a 24 hour period. Time lapse images were obtained using a Nikon Eclipse TE2000-E with Photometrics CoolSNAP HQ2 Monochrome CCD with Phase 40x/0.75 objective using Biopetechs Delta T4 Culture Dish Heater, Prior ProScan II Encoded Motorized Stage, and OKO LABS Digital Stage Top Incubator (37 degrees and 5% CO₂). For each data point, four 40x images were stitched together using 10% overlap with background correction in NIS-Elements AR Nikon.

Western Blotting

Protein lysates were incubated at 95 degrees for 5 minutes in 1X SDS lysis buffer (125mM Tris-HCl, pH 6.8, 0.04% SDS, 0.024% bromophenol blue, 5% β -mercapatoethanol, and 20% glycerol). SDS-Page was run in 4%-20% gradient Novex Tris-Glycine gel (Invitrogen). Proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon) in Tris-Glycine buffer with 5% methanol. Membranes were blocked for 1 hour in 1xTBS+ 5% nonfat milk. Primary and secondary antibodies were incubated in 1xTBS+5% nonfat milk+ 0.1% Tween-20. Primary antibodies were incubated for 2hour/RT or overnight/4 degrees at 1:1000 dilution. Primary antibodies used were: GRHL2, rb (Sigma); β -actin, ms (Thermo-pierce); Fibronectin, ms (BD Biosciences); Vimentin, ms ((Santa Cruz Bio-tech [SCBT]); β -actin, ms (Sigma); E-cadherin, ms (BD Biosciences); β -catenin, ms (BD Biosciences); GAPDH, ms (Origene); acetyl-lysine, rb (Millipore); H3K18AC, rb (Cell Signaling [CS]); H3K27AC, rb (CS); total H3, rb (CS); GST, ms (Thermo-pierce); p300,rb (SCBT sc-584); p300, rb (SCBT sc-585); FLAG, ms (Sigma); GAL4, rb (SCBT). Goat anti-rabbit or anti-mouse horseradish peroxidase conjugated secondary antibodies (Bio-rad) were used at 1:3000 dilution and incubated for 1hour/RT. Western blot membranes were developed using ECL-West Pico (Pierce) and analyzed using standard chemiluminescence techniques.

MDCK Tubulogenesis assay

MDCK Tubulogenesis assay was performed as previously described (Elia and Lippincott-Schwartz, 2001). Briefly, collagen matrix was made by mixing 10x MEM (Invitrogen), 100x PSG, 23.5g/L NaHCO_3 (Invitrogen), sterile water, and HEPES (Invitrogen) to working concentrations. Vitrogen Bovine Collagen (Advanced Biomatrix 5005-b) was added to a final concentration of 2mg/mL. NaHCO_3 was used to bring the solution to pH 7.3 via pH strip indicator (Elia and Lippincott-Schwartz, 2001). In a 24-well plate, 100 μ l of working collagen solution was placed in

a 1.0 micron pore cell culture insert (Falcon) and allowed to solidify for 3 hours in tissue culture incubator. Sub-confluent MDCK cells were trypsinized and counted using Countess Cell counting machine (Invitrogen). Cells were pipetted into the working collagen solution to give a final concentration of 5,000 cells/ml. Then, 250 μ l of cell/collagen solution was pipetted onto the pre-solidified collagen matrix in the cell culture insert. The cell-collagen matrix was allowed to solidify for 2 hours at 37 degrees and 5% CO₂ and then 250 μ l and 500 μ l of media were place on cell-collagen matrix cell culture insert and in 24 well the, respectively. Media were changed every 3 days. After 6 days, 60 ng/ml recombinant human HGF was added to indicated wells, and branching morphogenesis was monitored by microscopy. MDCK cyst staining was performed as previously described (Elia and Lippincott-Schwartz, 2001), with the only modification being the substitution of 7mg/mL fish skin gelatin for Triton X-100 in permeabilization buffer. Cysts were stained with Alexa Fluor 488 Phalloidin (Molecular Probes) and Hoescht 33258 (Molecular Probes). Representative 2 day post-HGF images were obtained using a Zeiss 710 Confocal with Airyscan, Apochromat 40x/1.2 W Autocorr M27, RT, AxioObserver.Z1 software, and Diode 405 and Argon 488 lasers. Images were processed in Adobe Photoshop. Tubulogenesis was observed by day 2 post-HGF addition and quantified on day 5 post-HGF addition.

RNA-Seq Assay

MDCK+GRHL2/pMXS and MDCK+shGRHL2/Gipz cells were plated in collagen coated 60mm² dishes to obtained 25% confluency upon HGF treatment. Cells were treated with either 60ng/ml HGF in DMEM complete or DMEM complete media alone for 24 hours; RNA was isolated from all four treatment conditions in triplicate using the RNeasy Plus Kit (Qiagen) and quantified using Nandrop (Fischer). Raw data were aligned to the CanFam3 reference genome using TopHat2 (Kim *et al.*, 2013). For each sample and each gene, the number of reads

generated from that sample and mapping to that gene were counted using RSamtools. Genes were as defined in the ensembl database v82 (Cunningham *et al.*, 2015). The resulting count table was analyzed using DESeq (Anders and Huber, 2010), performing two independent comparisons: samples with HGF compared to those without, with GRHL2 overexpressed, and, samples with HGF compared to those without, both expressing GRHL2 shRNA. Genes were considered differentially expressed if they showed a false discovery rate of 0.05 or less and a fold change of 1.5 or higher in either direction. Genes found to be regulated by HGF with shRNA against GRHL2, but not found to be regulated by HGF with GRHL2 overexpressed were uploaded to Ingenuity Pathway Analysis (Qiagen), where a Core Analysis was performed using default settings.

Quantitative reverse transcription PCR analysis

MDCK parent, GRHL2/pMXS, and shGRHL2/pGipZ cells were plated on collagen coated 60mm² dishes and treated as indicated. Cells were harvested using RNAeasy Plus mini kit (Qiagen), analyzed using Nanodrop, and converted to cDNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) with oligo dT primers and 1ug of RNA. cDNA was analyzed using SYBR Green PCR Master Mix on an Applied Biosystems 7500 Real Time PCR System. Values reported were determined using the delta delta CT method using canine CBX1 as internal control. Primers were as follows: CBX1-F (AAGTATTGCAAGGCCACCCA), CBX-R (TTTTCCTCAATCAGGCCCCAA), GRHL2-F (TTCCTCCCCAGGAGAGATGG), GRHL2-R (GGCCCAACACACGGATAAGA), MMP1-F (TGACTGGAAAGGTCGACACG), MMP1-R (GACCTTGGTGAAGGTCAGGG), MMP3-F (CTGACCCTAGCGCTCTGATG), MMP3-R (GTCCTGAGGGATTTTCGCCA), MMP13-F (GGACTTCCCAGGGATTGGTG), MMP13-R (ATGACACGGACAATCCGCTT), MMP14-F (CCCAGAGGAGGGCATGTTTT), MMP14-R

(GACTCCCCACCCTTCCAATG), FOS-F (GGGAGGACCTTATCTGTGCG), FOS-R (ACACACTCCATGCGTTTTGC), FOSL1-F (ACACCCTCCCTGACTCCTTT), FOSL1-R (CTGAAGAGGGCATGGGATTA), FOSB-F (GGAGAAGAGAAGGGTTCGCC), FOSB-R (CACAACTCCAGACGCTCCT), JUN-F (GACCTTCTACGACGATGCCC), JUN-R (TTCTTGGGGCACAGGAAGT), JUNB-F (GTGAAGACACTCAAGGCCGA), and JUNB-R (AGACTGGGGGAATGTCCGTA).

Luciferase Reporter Assays

HT1080 or 293 NOT cells were transiently transfected using Lipofectamine 2000 (Invitrogen) at a 1µg DNA: 3µl Lipofectamine ratio. After 20 minute incubation in 300µl of Opti-MEM (Invitrogen), a total of 1.0µg of DNA was transfected into a 12 well with DMEM media and refed 4 hours later with DMEM media. After 28-36 hours post-transfection, the cells were washed twice with cold PBS, lysed in 200µl of 1x Cell Culture Lysis Buffer (Promega), frozen for at least 1 hour at -80 degrees, thawed on ice, and centrifuged at 13,200rpm. The supernatants were assayed for luciferase activity (Promega) and β-galactosidase activity as internal control (2x β-galactosidase assay reagent, 200mmol/l sodium phosphate, pH 7.3, 2mmol/L MgCl₂, 100mmol/L 2-mercaptoethanol, and 1.33mg/mL o-nitrophenylgalactoside). MMP1 promoter (-1200 to +60) and MMP14 promoter (-1114 to -242) luciferase reporter plasmids were generated by subcloning the promoter from genomic DNA using PCR into the pGL3-basic luciferase reporter plasmid. Primer sequences were: MMP1prom-F (TTATTACTCGAGCCCTCCCTCTGATGCCTCTGA), MMP1prom-R (TATATAAAGCTTCTCCAATATCCCAGCTAGGAA), MMP14prom-f (TTATTACTCGAGCCCTTTCTCACGGTCAGCTT), MMP14prom-R (TATATAAAGCTTTCTCGCTCTCTCCTCTGGTC). GRHL2/pcDNA3.1, TK-LacZ and

E1a/pCDNA3.1 plasmids were described previously (Grooteclaes and Frisch, 2000; Cieply *et al.*, 2012). E1A p300 binding mutant was a generous gift from Arnold Berk (UCLA, Los Angeles, California) subcloned into pCDNA3.1+. GRHL2 Δ 425-437 was generated by cloning GRHL2 1-424aa and GRHL2 438-625aa fragments and ligating together with BglII and then ligating into pCDNA3.1+ using BamHI and SalI. Primers used were GRHL2-1-424-F (TATATAGGATCCATGTCACAAGAGTCGGACAA), GRHL2-1-424-R (ATATAAAGATCTTTTTCTTTCTGCTCCTTTGT), GRHL2-438-625-F (TAAATTAGATCTAAAGGCCAGGCCTCCCAAAC), GRHL2-438-625-R (TTATATGTCGACCTAGATTTCCATGAGCGTGA). pGL4.44[luc2P/AP1 RE/Hygro] (Promega) was used to assay for AP-1 responsive promoter activity and control plasmid was pGL4.27 (Promega). Phorhol 12-myristate 13-acetate (PMA) induction (10ng/mL) (ThermoFisher) was used for assaying AP-1 activity in transfected 293 cells. Full length p300-GAL4/VR1012 and EV-GAL4/VR1012 were generous gifts from Neil Perkins (Newcastle University, UK); they were assayed on the GAL4 responsive reporter pG5-Luc (Promega). GAL4- p300 HAT (1283-1674aa), GAL4- p300C-terminus (1665-2414aa), and GAL4- p300 IBID (2050-2096) were generated by subcloning PCR fragments (MluI-NotI) into pBIND (Promega). Primer sequences were: p300 c-terminal-F (TATATAACGCGTATCGCTTTGTCTACACCTGCAAT), p300 c-terminal-R (TTTAAAGCGGCCCGCCTAGTGTATGTCTAGTGTAC), p300 HAT-F (TATATAACGCGTATAGGAAAGAAAATAAGTTTTCT), p300 HAT-R (TTTAAAGCGGCCCGCCTAGTCCTGGCTCTGCGTGTGCAG), p300 IBID-F (TATATAACGCGTATGCCTTACAAAACCTTTTGCG), and p300 IBID-R (TTTAAAGCGGCCCGCCTAATTAGAGTTGGCATACTTGG). To generate the p300-IBID

Mutant (K2086A and K2091A), the p300 IBID lysine nucleotide sequences were mutated to alanine and flanking Mlu1 and Not1 restriction enzyme were added to the 5' and 3' ends, respectively. The sequence was then generated as a gBlock gene fragment (IDT). The p300 mutant sequence was 5' CACAACCAGG ATTGGGCCAG GTAGGTATCA GCCCACTCGC ACCAGGCACT GTGTCTCAAC AAGCCTTACA AAACCTTTTG CGGACTCTCA GGTCTCCCAG CTCTCCCCTG CAGCAGCAAC AGGTGCTTAG TATCCTTCAC GCCAACCCCC AGCTGTTGGC TGCATTCATC GCGCAGCGGG CTGCCGCGTA TGCCAACTCT AATCCACAAC 3". The IBID mutant fragment was digested and ligated into pBIND vector. HA-SRC1/pACTAG was a generous gift from Timothy Beischlag (Simon Fraser University, Canada).

Immunoprecipitation

GRHL2-STAP contained full-length human GRHL2 fused to a C-terminal myc-Streptavidin Binding Peptide-S-Tag sequence derived by ligating a synthetic oligonucleotide containing the S-tag to a PCR product containing the myc-SBP tags from the pCeMM-CTAP vector described (Burckstummer *et al.*, 2006). 293 NOT cells were plated on collagen coated 6 well dishes and transfected using 1µg of empty vector/pCDNA3.1+ or GRHL2-S-TAP/pCDNA3.1+ and 1ug of empty vector/VR1012 or p300/VR1012) with 6ul of lipofectamine (Invitrogen) in 300ul of Opti-MEM (Invitrogen). Cells were incubated for 4-6 hours with DMEM media, and then refed with DMEM media. After 28 hours post-transfection, cells were lysed in 600µl of cold p300 Lysis Buffer (25mM Tris-HCl pH8, 75mM NaCl, 0.5mM EDTA, 10% glycerol, 0.1% NP40, and protease inhibitor tablet (Roche)). Sample lysates were passed through 1ml syringe with 27g needle 3 times and precleared in 4 degree microcentrifuge at 13,200rpm for 10 minutes. Total lysate samples were obtained, mixed 1:1 with 2X SDS Lysis buffer (250mM Tris-HCl, pH 6.8,

0.08% SDS, 0.048% bromophenol blue, 10% β -mercapatoethanol, 40% glycerol) and heated for 5 minutes at 95 degrees. Lysates were precipitated for 2 hours with 40ul of a 50% of S protein-agarose (EMD Millipore) which had been pre-equilibrated with p300 lysis buffer containing 10mg/ml BSA. Immunoprecipitation lysates were washed three times with p300 lysis buffer, diluted with 2X SDS lysis buffer, and heated for 5 minutes at 95 degrees. Lysates were processed using previously described western blotting methods. For Endogenous p300 and Ectopic GRHL2 co-immunoprecipitation, MSP cells with empty vector/pMXS or GRHL2/pMXS were used in the experiment. For endogenous p300 and endogenous GRHL2 co-immunoprecipitation, parental HMLE cells were used in the experiment. The co-immunoprecipitation method was identical between the different cell lines. For each cell line, 2 100mm dishes were grown to 80% confluency, washed twice with cold PBS, lysed in 1mL of p300 lysis Buffer with protease inhibitors, and passed through 27g needle three times. After lysates were precleared at 13,200rp, 10 μ g of p300 antibody (SCBT, SC-584) or rabbit IgG (Jackson) was added to lysates and incubated overnight on 4 degree wheel. Pre-equilibrated Protein A-Sepharose (45 μ l) (GE Healthcare) was added and incubated for 90 minutes. Samples were washed 3 times in p300 lysis buffer, added 45ul of 2X SDS lysis buffer, and heated at 95 degrees for 5 minutes. Lysates were processed using previously described western blotting methods.

Histone Acetylation Assays

Histone acetylation reactions contained indicated amounts of recombinant baculovirus flag-tagged p300 (37.5ng) (Active Motif) or baculovirus p300 HAT (1283-1673) (Sigma), 250ng of H3 (Cayman), 100 μ M acetyl-CoA (MP Biomedical), and indicated amounts of prescission cleaved GRHL2, GST-GRHL2 or GST alone in 30 μ l of p300 acetylation buffer (50mM Tris-

HCl pH 8.0, 1mM DTT, 0.1mM EDTA, 10% glycerol and protease inhibitor). Acetylation reactions were incubated for 15 minutes at 30 degrees on heat block. Reactions were diluted with 2x SDS Lysis buffer and incubated for 5 minutes at 95 degrees. Lysates were processed using previously described western blotting methods.

Recombinant protein purification

Full length GRHL2 was subcloned into pGEX-6P-3 vector (GE Healthcare) and transformed in to BL21 bacteria (Invitrogen). GRHL2 fragments were subcloned into pGEX-6p-3 using BamH1 and Sal1. A colony was selected and grown in 20mL of LB+amp overnight at 37 degrees. The following day, 10 mL of culture was inoculated into 500 mL of LB+amp and incubated for approximately 2 hours at 37 degrees with shaking until OD~ 0.6 was obtained. IPTG was added to final concentration of 0.1mM and incubated overnight on shaker at room temperature. The bacteria were spun in chilled centrifuge for 10 minutes at 5000rpm. Bacterial pellet was resuspended in 10 mL of BL21 lysis buffer (1xPBS, 1% Triton-100, 1mM DTT, 0.4% lysozyme and protease inhibitor tablet), incubated for 10 minutes, and sonicated twice for 30 seconds. The suspension was cleared at 13,000 rpm for 10 minutes. To the supernatant, 150µL of 50% glutathione-sepharose beads (GE Healthcare) was added and incubated for 2 hours with rotation at 4 degrees. Glutathione-sepharose beads were washed three times with glutathione washing buffer (1xPBS, 1mM DTT and 0.1% Triton-X) and eluted for 30 minutes with glutathione elution buffer (100mM Tris pH 8, 150mM NaCl, 20mM Glutathione, and protease inhibitors). Alternatively, glutathione-sepharose beads with GRHL2 protein attached were washed 3 times in PreScission cleavage buffer (50mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM DTT, pH 7.0). The GST-GRHL2 on glutathione-sepharose beads was cleaved by adding 80ul of Prescission Protease (GE Healthcare) per mL of PreScission cleavage buffer at 5 degrees

overnight. The sample was spun at 2,000rpm and the supernatant with the GST cleaved GRHL2 was obtained. Proteins were dialyzed against p300 acetylation buffer overnight at 4 degrees using 20,000 MWCO Slide-A-Lyzer MINI Dialysis Unit (Thermo-Scientific). Protein was analyzed on SDS-PAGE by Coomassie Blue staining and quantified by comparison of Coomassie staining to a BSA standard and/or a BCA assay. Synthetic peptides were purchased from ThermoFischer Scientific with n-terminus acetyl group and c-terminus amidation: GRHL2 420-442aa (GAERKIRDEERKQNRKKGKGQAS) and scramble (IDEKNKGRERQRK)

Chromatin Immunoprecipitation (ChIP)

For ChIP assay, HT1080 cell lines expressing empty vector/pMXS or GRHL2/pMXS cells were used, and chromatin generated as described previously with the following modifications (Kumar *et al.*): (i) Immunoprecipitation was performed with 3ug of H3K27-Ac antibody (Cell Signaling) or rabbit IgG (Jackson) to 3.3×10^6 cell-equivalents in ChIP RIPA buffer. (ii) Protein A-sepharose was used instead of FLAG-agarose beads. (iii) qPCRs were performed using 2X SYBR green master mix (Applied Biosystems; 10 uL), 0.08 uL of 100 micromolar primer, 1 uL ChIP DNA in a 20 uL reaction. Primer sequences were as follows: MMP1-1-F

(AACCTCAGAGAACCCCGAAG), MMP1-1-R (TACTAACACTGCGCACCTGA) MMP1-2-F (CAGAGTGGGGCATGAGTAGG), MMP1-2-R (TGTCTTCGGTACTGGTGACC), MMP14-F (AAGTAAGTGAGCTTCCCGGC), MMP14-R (GGAGTTCGCCCCAGTTGTAA), MMP2-F (TCGCCCATCATCAAGTTCCC), MMP2-R (CCCCCAAGCTGTTTACCGAA), ZEB1-1-F (GCGAGGCGTGGGACTGATGG), ZEB1-1-R (AAAGTTGGAGGCTCGGCGGC), ZEB1-2-F (CTGCACGGCGATGACCGCT), ZEB1-2-R (TTCCGCTTGCCAGCAGCCTC), CDH1-F (ACTCCAGGCTAGAGGGTCACC), CHD1-R (CCGCAAGCTCACAGCTTTGCAGTTCC), ESRP1-F (GGGAGCTTGGTCAAGTCAAC),

ESRP1-R (TCTTAAATCGGGCCACGCAG), RAB25-F (AGGTCCTGTCCCTTTTTCGC), RAB25-R (TTGGGGGTAAGGGGACTTCT), GAPDH-F (ATGGTTGCCACTGGGGATCT), and GAPDH-R (TGCCAAAGCCTAGGGGAAGA). Results were normalized to GAPDH values.

Ex Vivo Kidney Culture

All experiments involving mice were approved by WVU IACUC. *Hoxb7creEGFP* transgenic mice and ex vivo kidney culture method were described previously (Zhao *et al.*, 2004). We removed embryonic kidney at E13.5 and the kidneys were cultured in 1uM batimastat (Tocris) or vector in culture media for 48 hours. Images were taken of GFP ureteric buds on Olympus MVX10 Macro Zoom, ORCA-Flash 4.0 Monochrome camera, Objective 2x, Zoom 4x, FITC, and using CellSen Imaging Software. Ureteric buds were counted for quantification.

Confocal microscopy of transfected RRE.1 cells

RRE.1 cells, wt e1a-NLS-LacI-mCherry, and empty vector NLS-LacI-mCherry were generous gift from Arnold Berk (UCLA, Los Angeles) and experiment was performed as previously described (Verschure *et al.*, 2005; Ferrari *et al.*, 2014). GRHL2 was subcloned into empty vector NLS-LacI-mCherry using Sal1. Images were taken on Zeiss Axiolmager Z1 microscope LSM 510 Confocal, 63x/0.75 LDPlan-neofluar, DAPI and Rhodamine, and Zeiss Zen Software. Images were quantified using mean area on Image J.

Statistical Analysis

Error bars in graphs represent standard deviation. P-values were calculated using a Student's two-tailed t-test.

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Table 1.

Genes regulated by GRHL2		
Gene ID	Fold Change (shGRHL2+HGF/GRHL2+HGF)	Significance
MMP-1	31.7	Interstitial collagenase, necessary for MDCK tubulogenesis, p300-dependent expression
MMP-9	5.8	Gelatinase B, p300-dependent cancer invasiveness, upregulated in EMT
MMP-13	15.1	Collagenase 3, Necessary for MDCK tubulogenesis, p300-dependent expression
MMP-14	5.82	Membrane-type matrix metalloprotease, involved in EMT regulation and invasiveness phenotype
MMP-19	3.5	Increases invasion in multiple cancers
MMP-24	9.6	

Table 1- HGF-induced MMPs expression in absence of GRHL2 compared to the presence of GRHL2

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Figure Legends

Figure 1. GRHL2 suppresses HGF-induced cell scattering and tubulogenesis, and is down-regulated by HGF.

A. HGF induction downregulates endogenous GRHL2 protein. Western blot and densitometric quantitation of HGF treatment time course in MDCK cells are shown. HGF induction downregulates endogenous GRHL2 mRNA in MDCK cells; qPCR results expressed as ratios compared to vector control. B. Constitutive GRHL2 expression in MDCK cells suppresses HGF-induced cell scattering; images represent representative morphologies of cells treated for 42 hours. C. GRHL2 knockdown in enhanced HGF-induced cell scattering (16 hours), but MDCK shGRHL2 cells did not undergo an EMT phenotypic change when EMT markers were examined via western blotting. D. Constitutive GRHL2 expression in MDCK cells prevents tubulogenesis (blue-nuclei and green-actin). Scale bar equals 20 microns. Graph represents quantification of percentage of cysts that demonstrated tubulogenesis.

Figure 2. GRHL2 suppresses the induction of MMP genes and MMP promoters by HGF.

A. GRHL2 suppresses the induction of MMP genes (qPCR analysis; EN: endogenous GRHL2, CE: constitutively expressed GRHL2, KD: GRHL2 shRNA knockdown). B. GRHL2 suppresses the induction of MMP promoters by HGF. HT1080 cells were cotransfected with either MMP1 or MMP14 promoter-luciferase reporter constructs and GRHL2, E1A, or empty expression vectors. Values represent relative luciferase activity normalized to TK- β -galactosidase control.

Figure 3. GRHL2 suppresses AP-1 and p300 function.

A. GRHL2 suppresses AP1 function. HT1080 and 293T cells were cotransfected with AP-1 response element- luciferase reporter construct and GRHL2 expression vector or empty vector. Phorhol 12-myristate 13-acetate (PMA) was used to induce AP-1 signaling in 293T cells. Values represent relative luciferase activity normalized to TK- β -galactosidase control. B. Venn diagram comparing GRHL2-

repressed genes (Farris *et al.*, 2016) vs. p300 target genes identified via E1a (Ferrari *et al.*, 2014).

C. GRHL2 suppresses the p300 pathway. p300 effector and target genes induced by HGF in MDCK cells with GRHL2 shRNA vs. cells with constitutive GRHL2 determined by IPA analysis quantitation of the number of unregulated, HGF up-regulated or HGF down-regulated genes for both cell lines is shown in the graph. The p300 interactome diagram on which this is based is shown in figure S7. D. GRHL2 suppresses p300 function. GAL4-minimal promoter-luciferase activation by a co-transfected GAL4-p300 was assayed in HT1080 cells in the presence or absence of cotransfected GRHL2 expression vector; values represent relative luciferase activity normalized to TK- β -galactosidase control.

Figure 4. GRHL2 suppresses the HAT activity of p300. A. In vitro HAT assays with recombinant H3, p300 and GRHL2 proteins. Coomassie stains to assess the quality of recombinant proteins used throughout this study are shown in figure S19. B. GRHL2 inhibits the acetylation of H3 on GRHL2-repressed but not GRHL2-induced promoters in vivo (ChIP assay). Crosslinked chromatin from HT1080+vector HT-1080+GRHL2 cells immunoprecipitated with H3K27-Ac or rabbit IgG; the indicated promoters were assayed for H3K27Ac by qPCR using the primers in Materials and Methods. * indicates p values of < 0.05. GRHL2 does not affect global histone H3K18-Ac or H3K27-Ac (western blotting of total histones). C. GRHL2 interacts with p300. (*left panel*): co-transfection of indicated expression constructs, followed by co-immunoprecipitation/western blotting; (*middle panel*): co-immunoprecipitation of retrovirally expressed S-tagged GRHL2 with endogenous p300; (*right panel*): co-immunoprecipitation of endogenous GRHL2 and endogenous P300.

Figure 5. GRHL2 inhibits the C-terminal transactivation domain of p300. A. The p300 HAT domain stimulates transactivation by the p300 C-terminus (co-transfection in HT1080 cells). B. GRHL2 inhibits transactivation by the p300 C-terminus. HT1080 cells were cotransfected with GAL4 response element luciferase reporter in the presence of the indicated expression constructs. C. The IBID domain of p300 and the co-activator SRC-1 synergize to activate transcription. HT1080 cells were cotransfected with GAL4 response element luciferase reporter I in the presence of the indicated expression constructs. D. GRHL2 inhibits transactivation by the IBID-SRC-1 complex. HT1080 cells were cotransfected with GAL4 response element luciferase reporter in the presence of the indicated expression constructs. E. Transactivation by the IBID-SRC-1 complex is contingent upon lysines 2086 and 2091. HT1080 cells were cotransfected with GAL4 response element luciferase reporter in the presence of the indicated expression constructs. (a-e): Values represent relative luciferase activity normalized to TK- β -galactosidase control. F. Schematic of p300 domains. KIX- CREB-binding domain, Bd- bromodomain, HAT- histone acetyltransferase domain, and IBID- IRF-3 binding domain.

Figure 6. A small region within the DNA binding domain of GRHL2, amino acids 425-437, inhibits p300. A. Schematic of GRHL2 domains. TAD- transactivation domain , DBD- DNA binding domain, and DD- dimerization domain. B. HAT assays using the indicated GRHL2 fragments, assayed as GST-fusion proteins C. GRHL2 aa425-437 inhibits p300 HAT activity. The indicated fragments derived from GRHL2 were assayed as GST-fusions (left panels) or as recombinant peptides (right panel) for inhibition of HAT activity. D. GRHL2 amino acids 425-437 are required for inhibition of an AP-1 reporter, GAL4 reporter in conjunction with GAL4-

p300, MMP1 reporter or MMP14 reporter. Values represent relative luciferase activity normalized to TK- β -galactosidase control.

Figure 7. The p300-inhibitory domain of GRHL2 is important for the suppression of tubulogenesis and reversion of EMT. A MDCK cells expressing GRHL2 wild-type or GRHL2 Δ 425-437 were assayed for tubulogenesis (blue-nuclei and green-actin). Scale bar equals 20 microns.; quantitation is shown in the middle panel and Western blot confirmation of protein levels in the right panel. B. MSP cells expressing GRHL2 wild-type or GRHL2 Δ 425-437 were assayed for reversion of EMT, by western blotting for EMT markers (left panel), cell morphology (middle panel), or qPCR (right panel).

Figure 8. GRHL2 suppresses tubulogenesis and EMT by inhibition of p300.

Figure 1

A.

B.

Figure 2

A.

B.

C.

D.

Figure 3

A.

B.

C.

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Supplemental Figure Legends

Figure S1. GRHL2 shRNA accelerates HGF-induced cell scattering (videomicroscopy).

Cell scattering time lapse movies were taken every 15 minutes over a 24 hour period. Time lapse images were obtained using a Nikon Eclipse TE2000-E with Photometrics CoolSNAP HQ2 Monochrome CCD with Phase 40x/0.75 objective. (left panel): MDCK + vector; (right panel): MDCK+GRHL2 shRNA

Figure S2. GRHL2 did not affect Erk or Akt signaling downstream of HGF/Met. Lysates from HGF induction time course using the indicated cell lines was analyzed by western blotting using the indicated antibodies. Densitometry data represent ratios of phospho-Akt or phospho-ERK to the corresponding total protein levels.

Figure S3. Effect of GRHL2 on cyst formation. A. Constitutive GRHL2 expression in mIMCD-3 cells prevents tubulogenesis. B. Knockdown of GRHL2 prevents cyst formation in MDCK. (left panel) GRHL2 knockdown by western blotting. (middle panel) Quantification of cysts per well. (right panel) Representative images from MDCK collagen cysts

Figure S4. Knockdown of GRHL2 following cyst formation (inducible shRNA) promotes tubulogenesis. A. MDCK cells with doxycycline inducible GRHL2 shRNA were induced with HGF in the presence or absence of doxycycline to induce GRHL2 shRNA. Cyst extensions were quantified 24 hours after HGF induction. Quantification indicates the number of cysts that did not have extensions at 24 hours. B. GRHL2 does not alter proliferation in MDCK cells. The indicated cell lines were plated at equal density and counted in triplicate wells at three days post-plating.

Figure S5. MMPs are important for kidney tubulogenesis. E13.5 ex vivo mouse kidney cultures were grown for 48 hour with batimastat or solvent control and imaged. Ureteric buds were counted for quantification.

Figure S6. GRHL2 did not affect *FOS* or *JUN* family members. *FOS*, *FOSL1*, *FOSB*, *JUN*, and *JUNB* mRNA expression levels were determined in the indicated cell lines at one or four hours post-HGF induction via qPCR.

Figure S7. GRHL2 suppresses the p300 pathway. p300 effector and target genes induced by HGF in MDCK cells with GRHL2 shRNA vs. cells with constitutive GRHL2 determined by IPA analysis quantitation of the number of unregulated, HGF up-regulated or HGF down-regulated (red=up-regulated, green=down-regulated, and grey=unregulated)

Figure S8. Validation of p300-dependence of reporter assays using WT vs. p300-non-binding mutant of E1A. HT1080 cells were cotransfected with the indicated reporters and expression constructs. Values represent relative luciferase activity normalized to TK- β -galactosidase control.

Figure S9. Further validation of reporter assays: A. c-Myc did not inhibit GAL4-p300 pG5 assay. B. GRHL2 did not inhibit GAL4-VP16 activation of GAL4- responsive element. HT1080 cells were transfected with the indicated reporters and expression constructs. Values represent relative luciferase activity with GRHL2 present compared to empty vector alone normalized to TK- β -galactosidase control.

Figure S10. GRHL1 and GRHL3 inhibit transactivation by p300. HT1080 cells were cotransfected with the indicated reporters and expression constructs. Values represent relative luciferase activity normalized to TK- β -galactosidase control.

Figure S11. GRHL2 suppresses activity of the HAT domain (alone) of p300. Recombinant GRHL2 inhibits recombinant p300 HAT (1283-1673) acetylation of H3K27 in a dose dependent manner in the H3 acetylation assay. Coomassie stains to assess the quality of recombinant proteins are shown in figure S19.

Figure S12. GRHL1 and GRHL3 inhibit p300 HAT activity. Recombinant GRHL1 and GRHL3 inhibit p300's acetylation of H3K27 in H3 acetylation assay. Coomassie stains to assess the quality of recombinant proteins are shown in figure S19.

Figure S13. GRHL2 did not cause global changes in heterochromatin condensation like E1A. (left panel) GRHL2-lacI-mCherry, E1a-lacI-mCherry or lacI-mCherry expression constructs were transfected into RRE cells and confocal microscopic images of chromatin condensation were quantitated.

Figure S14. Recombinant GRHL2 and p300 pulldown interaction. Recombinant GRHL2 and p300 were assayed for interaction by p300 IP/GRHL2 western blot.

Figure S15. Mapping of GRHL2 domains involved in inhibiting HAT activity in vitro. A. Recombinant GST-GRHL2 fragments were generated without the GRHL2 transactivation domain (136-625), DNA binding domain (Δ 245-494), or dimerization domain (1-520) and assayed for inhibition of p300 HAT activity. B. Recombinant GST-GRHL2 fragments were generated within the GRHL2 transactivation and assayed for inhibition of p300 HAT activity. Coomassie stains to assess the quality of recombinant proteins are shown in figure S19. C.

Synthetic peptide (aa420-442) and scramble peptide were assayed for inhibition of p300 HAT activity. (right panel) Quantification of H3K27-Ac levels compared to no peptide control.

Figure S16. Mapping of GRHL2 domains involved in inhibiting transactivation by GAL4-p300 in reporter assays. HT1080 cells were cotransfected with the indicated reporters and expression constructs. Values represent relative luciferase activity normalized to TK- β -galactosidase control.

Figure S17. GRHL2 aa 425-437 important for repressing GLUD1 promoter through p300. HT1080 cells were cotransfected with the indicated reporters and expression constructs. Values represent relative luciferase activity normalized to TK- β -galactosidase control.

Figure S18. GRHL2 Δ 425-437 interacts with p300 in a co-transfection assay. p300 and wt GRHL2 or GRHL2 Δ 425-437 expression vectors were transfected into 293 cells. Lysates were immunoprecipitated with p300 antibody and probed for GRHL2 interaction.

Figure S19. Confirmation of recombinant proteins (Coomassie blue staining). A. Prescission cleaved GRHL2 for figure 4A H3 acetylation assay. B. GST-GRHL1 and GST-GRHL3 for figure S12 H3 acetylation assay. C and D. GST-GRHL2 fragments for figure S15 H3 acetylation assay. E. GST-GRHL2 fragments for figure 6A H3 acetylation assay. F. GST-GRHL2 fragments for figure 6B H3 acetylation assay.

Figure S2

A.

B.

Figure S3

A.

B.

Figure S4

Figure S5

Figure S6 FOS expression

FOSL1 expression

FOSB expression

JUN expression

JUNB expression

Figure S6

Figure S7

Figure S8

Figure S9

Figure S10

Figure S11

Figure S12

Figure S13

Figure S14

Figure S15

Figure S16

Figure S17

Figure S18

Figure S19