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Role of TMS1/ASC in Breast Epithelial Anoikis and Migration

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Abstract

Cellular detachment from the extracellular matrix (ECM) induces a form of apoptosis, referred to as "anoikis." Most cancer cells are resistant to anoikis and survive independently of ECM interactions. Previously, we identified a pro-apoptotic gene, *TMS1* that is epigenetically silenced in breast cancers. Overexpression of TMS1 is sufficient to induce procaspase-8 cleavage in breast cells in the absence of death receptor-ligand interactions. Additionally, TMS1 aggregates to form 'specks' in some apoptotic cells, although the function of this structure remains unclear. Here we established a role for TMS1 in anoikis and uncovered a nonapoptotic role for TMS1 in cell migration. We found that cellular detachment induces TMS1 expression. In addition, we showed that silencing TMS1 confers resistance to anoikis through a delay in procaspase-8 cleavage and accumulation of BimEL. ERK1/2 signaling negatively regulates BimEL accumulation during detachment. We found that silencing TMS1 resulted in persistent ERK1/2 signaling during anoikis. We conclude that loss of TMS1 promotes resistance to anoikis in part through misregulation of ERK1/2 signaling and suppression of BimEL accumulation. During the course of these studies a nonapoptotic role for procaspase-8 in adhesion and migration was elucidated by many groups. The next phase of our research was aimed towards deciphering a role for TMS1 upstream of procaspase-8 in anoikis. Thus, we next investigated a nonapoptotic role for TMS1 in these processes. We found that adhesion to fibronectin induced the redistribution of TMS1 to barrel-like structures. Silencing TMS1 delayed FAK and ERK1/2 signaling in response to integrin-fibronectin interactions. Phosphorylated FAK and centrosomal markers colocalized with TMS1 at barrel-like structures in a subset of cells. Through functional analyses, we further

demonstrated a role for TMS1 in cell migration. These data suggest that an association of TMS1 with centrosomes results in the formation of a critical signaling center that may regulate migration. In conclusion, our data demonstrate distinct functions for TMS1 in anoikis and migration. Overall, results from these studies have increased our understanding of the intricate functions TMS1 exhibits in epithelial cells and has provided great insight into the consequences of aberrant methylation and silencing of TMS1 observed in many cancers.

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Chapter 1

Introduction

In the US, fifty percent of all men and a third of all women will develop cancer during their lifetimes. In 2010, ~570,000 Americans are predicted to die of cancer. Breast cancer is the second leading cause of cancer deaths among women, exceeded only by lung cancer. Since 1998, death rates from breast cancer have continually decreased due to advancements in the detection and treatment of this disease. In addition, the five year survival rate for women with localized disease or for those with locally invasive breast cancer at the time of diagnosis is 98% or 83%, respectively. However, this number drops to 23% for women with late stage disease at the time of diagnosis [1]. Further advancements regarding the underlying molecular mechanisms of breast cancer progression are greatly needed in order to improve the outcome of this devastating disease.

Cancer Genetics

Cancer in its simplest definition is a disease characterized by uncontrolled cellular growth that spreads to distant sites [1]. Due to the complex network of signaling pathways that control cellular processes such as cell division and death, numerous genetic and epigenetic events are necessary to initiate and maintain such growth in cells. Normal cells continually interact with the surrounding extracellular matrix and respond to resulting cues such as the presence or absence of growth factors. In this manner, tissue environment can regulate cellular growth, division, and death. Cancer cells arise from normal cells that have lost the ability to regulate some or all of these processes and thus exhibit uncontrolled growth [1]. In 2000, Hanahan *et al.* proposed six acquired properties of cells that result in cancer including self-sufficiency in growth signals, resistance to

growth inhibitory signals, apoptotic evasion, limitless cell division, sustained angiogenesis, and tissue invasion and metastasis. Accumulated alterations in two classes of genes are acquired through these traits. These mutations typically result in gain of function alterations in oncogenes and loss of function alterations of tumor suppressor genes (TSGs) [2].

Oncogenes are defined as genes that function by positively impacting the cellular mitogenic pathway. Proto-oncogenes are normal growth genes that give rise to oncogenes when activated by viral insertions, point mutations, chromosomal rearrangements or gene amplification [3]. The discovery of the first oncogene, viral *src* (*v-src*) can be traced to the early 1900s, when Peyton Rous established the idea that cancer could be transmitted through a cell-free extract. At this time, Rous showed that a filtered agent was sufficient to induce tumor formation in chickens [4,5]. Years later, Bishop *et al.* found that *v-src* was the oncogene responsible for the tumor formation Rous originally observed in chickens [6]. Numerous oncogenes have since been identified including *myc* [7] and *ras* [8]. The *myc* gene provides an example of an oncogene that is often amplified or overexpressed in breast cancer. *Myc* encodes a transcription factor whose target genes play roles in growth, transformation, angiogenesis, and cell-cycle control [9]. Thus, amplification of this proto-oncogene promotes cellular growth and division. The human *ras* genes encode the highly related Harvey-Ras (H-Ras), neuroblastoma-Ras (N-Ras), and Kristen-Ras (K-Ras) proteins which are the founding members of the Ras superfamily of GTPases that control a diverse set of signaling pathways involved in cellular proliferation, growth and differentiation [10]. Ras proteins are active when bound to GTP and inactivated upon its hydrolysis to GDP. Point

mutations of Ras that prevent the hydrolysis of a bound GTP to GDP result in its constitutive activation. Specific *ras* mutations are associated with the development of different cancers. K-Ras is preferentially mutated in pancreatic carcinomas, colon carcinomas, and lung carcinomas. N-Ras and H-Ras are more commonly mutated in melanoma and bladder cancer, respectively [11].

In contrast to oncogenes, TSGs typically inhibit cellular growth, differentiation, or senescence and are often mutated, physically lost [3], or epigenetically silenced in cancers [12]. During the 1980s, cell fusion experiments first suggested the existence of TSGs [13]. Further insights regarding tumor suppressors were gained from studying retinoblastoma, a disease characterized by childhood eye tumors. From examinations of retinoblastoma cases and published reports, Dr. Alfred Knudson developed a two-hit hypothesis through which he proposed an explanation for the early onset of retinoblastoma in children with the inherited form of this disease. Knudson suggested that carriers with a germline mutation in a hypothetical retinoblastoma gene were predisposed to tumor formation. He reasoned that for individuals who inherited one mutant allele, the probability of acquiring a somatic mutation that disrupted the remaining wild-type allele was greater than that of a noncarrier acquiring two somatic mutations [14]. Therefore, in direct contrast to the role of oncogenes in tumor formation, both alleles of a tumor suppressor gene are commonly inactivated resulting in tumor development (Figure 1). However, in some cases, inactivation of a single allele of a TSG results in haploinsufficiency wherein a single functional copy of a TSG is unable to produce wild-type levels of the gene product resulting in tumorigenesis [15].

Whereas inherited mutations in TSGs or oncogenes contribute to an increased disposition to cancer, the vast majority of cancers have been shown to be sporadic in nature and lack a clear family history [16]. Sporadic cancers typically arise from spontaneous mutations in proto-oncogenes and TSGs. Mutations are thought to arise from environmental exposure to certain cancer-causing agents including cigarette smoke, radiation, hormones, and some dietary components [16]. For example, acquired mutations in a TSG involved in cell cycle control and cell death, *TP53*, occurs in the development of ~50% of human cancers such that it is one of the most frequently mutated genes in cancer [17].

A single mutation in one allele of a proto-oncogene and inactivation of one or two alleles of a TSG may be sufficient to induce uncontrolled cell growth in some cells. However, in the absence of further mutations it is not adequate to result in cancer. In 1991, Dr. Lawrence Loeb reasoned that the spontaneous mutation rate in cells was sufficient to support a two-hit hypothesis in the initiation of cancer. However, he argued that most cancers exhibit more than just two mutations, yet the cellular spontaneous mutation rate could not account for all of these mutations. He suggested that cancers exhibit a mutator phenotype whereby the accumulations of mutations are not simply resultant of tumor progression but instead contribute to its development [18]. In this regard, an inherent state of genetic instability permits cancer cells to acquire mutations that allow them to overcome barriers to growth including lack of oxygen and low levels of proliferation. These mutations are retained and accumulate as cancer cells continually meet and overcome future barriers to growth and proliferation.

Others have argued against a role for genetic instability in tumor progression. In 2008, Dr. Walter Bodmer reasoned that selective advantage of cells with low mutation rates can contribute to cancer progression [19]. He suggested that while some tumors develop a mutator phenotype during their formation, it is not a necessary contribution to cancer [19]. This hypothesis is based on the idea that not all mutations acquired during tumor development are involved in cancer progression. Mutations are classified as either 'drivers' or 'passengers' to describe their contribution to cancer development. Driver mutations confer growth advantages and as such are clonally selected along the cell lineage resulting in tumor growth [20]. These mutations typically cluster in cancer-specific genes (e.g. *TP53* and *KRAS*). In contrast, passenger mutations do not offer growth advantages and are not selected during cancer progression. A key aspect in the prevention and treatment of cancer includes the identification and specific targeting of genes with mutations that drive cancer initiation and progression [20].

DNA methylation

In addition to point mutations and physical loss of DNA sequence, epigenetic inactivation of TSGs plays a role in the loss of TSGs in cancer. Epigenetic alterations include heritable changes in DNA structure and accessibility rather than changes in DNA sequence [12]. DNA methylation in human cells occurs at cytosines in the dinucleotide 5'-CpG-3'. CpG islands are regions of DNA with an unusually high frequency of CpG sites and are found in the promoter regions of more than 70% of human genes [12,21]. While most CpGs in the human genome are methylated, those found in CpG islands are normally unmethylated. However, in cancer cells, this pattern is disrupted such that CpG

islands become abnormally methylated [22]. In 1994, Herman *et al.* were one of the first to link aberrant methylation of gene promoters to cancer development when they showed that the normally unmethylated CpG island of the *von Hippel Lindau (VHL*) gene became hypermethylated in sporadic clear cell renal cancers [23]. The VHL syndrome is a hereditary form of cancer in which individuals who inherit a mutation in the *VHL* gene and are predisposed to the development of hemangioblastomas of the brain, spine, and retina and renal clear cell carcinomas [24]. In 1995, Gonzalez-Szulueta *et al.* showed that the tumor suppressor, *p16*, is also regulated by promoter methylation and is aberrantly silenced in bladder cancers [25]. Methylation of *p16* has also been observed in breast cancers [26]. A number of TSGs similarly deregulated through aberrant promoter DNA methylation have since been identified including *BRCA1* [27] and the *estrogen receptor* (*ER*) [28,29] in breast cancers and *APC* in colon cancer [27].

The change in methylation of CpG islands in promoter regions of genes is accompanied by local changes in histone modifications including histone H3 and H4 hypoacetylation, hypomethylation of H3K4, and hypermethylation of H3K9. The resulting changed chromatin architecture causes these promoters to become transcriptionally repressed [12,30]. Thus, aberrant methylation of promoter region CpG islands is an epigenetic mechanism of silencing that frequently results in the loss of function of TSGs in many cancers.

Breast Cancer

The normal breast is composed of layers of fatty and connective tissue that surround a branching network of ducts emanating from lobules. Mammary ducts

function to transport milk that is produced by lobules to the nipple where they converge in a radial arrangement [31]. Thus, an important structural aspect of the anatomy of both lobules and ducts is that they contain hollow lumen (Figure 2). The earliest stages of breast cancer include ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) and are characterized by aberrant filling of ducts and lobules with tumor cells that have not yet infiltrated the basement membrane. DCIS is the most common noninvasive form of breast cancer accounting for 80% of all *in situ* breast cancers [16].

The 1980s and 1990s displayed an increase in the occurrence of *in situ* breast cancers among all age groups likely due to advances in detection. Since 1999, incidences of *in situ* breast cancers have continued to increase among women under the age of 50. However, a decrease in mortality rate has also been observed during this time suggesting that early detection may improve overall survival [16]. Unlike the linear progression model Vogelstein *et al.* proposed for the development of colon carcinomas [32], breast cancer is thought to result from a complex series of genetic and epigenetic events promoting divergent pathways to invasive cancers [33]. Within ten years, ~30-50% of locally treated DCIS patients recur with invasive carcinomas indicating that DCIS represents a pre-malignant state that is associated with the development of some invasive cancers [34-36].

Invasive breast cancer is a regional stage disease and represents the most common form of breast cancer of which 80% are infiltrating ductal carcinomas. This disease is characterized by breast cells that have acquired the ability to invade surrounding tissue (Figure 3). The real danger of invading cells is that they have the potential to invade to the lymphatic and circulatory systems allowing them a means to spread and colonize

distant organs in a process termed metastasis [16]. Breast cancers preferentially metastasize to the lung, liver, bone and brain [37] ultimately disrupting the functions of these vital organs. Adjuvant therapy including chemotherapy or endocrine therapy following surgical removal of tumors is typically administered to ensure complete eradication of cancer cells including those that may be present at distant sites. Since it is not currently possible to accurately predict which breast cancer patients will develop subsequent late-stage disease, 80% of women receive adjuvant chemotherapy even though only a fraction of these (40%) will develop recurrent metastatic disease and subsequently die. Thus, women who could successfully be treated by local treatment of disease including surgery and radiation are 'over treated' and needlessly endure the acute, physiologically toxic side effects of chemotherapy [38]. Therefore, further insights regarding the molecular biology of breast cancer development are needed in order to identify biomarkers predictive of progression to advanced disease.

Pathologists assign surgically removed breast tumors a grade that defines the degree of differentiation of the tumor relative to normal breast tissue. A low grade tumor is termed 'well differentiated' and consists of cells that appear relatively normal whereas high grade tumors are 'poorly differentiated' and are composed of cells that lack characteristics of their normal counterparts. High grade tumors are also composed of cells that tend to grow and spread more quickly. Although DCIS is considered a precursor lesion to breast cancer, these lesions are often given a nuclear grade based on the appearance of cells filling ductal lumen. A high number of abnormal cells or evidence of necrosis in DCIS may be associated with the development of a more

aggressive tumor. Additionally, factors such as surgical margin (proximity of cancer cells to edge of specimen) and size are noted [16].

While grade determines the differentiation of a primary tumor, breast cancer stage is used to describe the extent of cancer within the body. A standardized system of staging cancer is used in the diagnosis of breast cancer termed the American Joint Committee on Cancer (AJCC) TNM system. Analyses regarding the size of the tumor (T), the number of involved lymph nodes (N), and spread to sites of metastases (M) following surgical removal allow pathologists and clinicians to assign tumors to a stage group. Noninvasive cancers are assigned stage 0 while the most progressive disease is referred to as stage IV [1].

Breast tumors display great diversity in their genetic makeup and responses to treatment [39]. Following surgical removal of breast tumors, samples are tested for the presence or absence of hormone receptors and HER-2 expression and treated accordingly. Women with tumors that express hormone receptors are more likely to be responsive to hormonal therapy such as treatment with the anti-estrogen, tamoxifen. HER-2-positive tumors show amplification of the proto-oncogene *HER2* and are treated with trastuzumab, a monoclonal antibody that specifically targets HER2 protein [1].

In 2000, Perou *et al.* established a molecular classification system for breast tumors based on gene expression profiles that included the following subtypes: ER-positive, HER-2-positive, basal, and normal breast-like carcinomas [39]. In 2001, Sorlie *et al.* further classified ER-positive tumors into three more subtypes (luminal A, B, and C) [40]. Classification of tumors into ER positive and negative groups supported earlier epidemiologic and biomarker studies that suggested these two categories represented

biologically different diseases [41]. Sorlie *et al.* also showed that the basal and HER-2-positive breast cancer subtypes were associated with poor clinical outcome suggesting that the gene expression profile for a given breast tumor can be predictive of disease progression [40].

Further gene expression analyses have contributed as prognostic indicators of breast cancer and have been reviewed by Morris *et al* [41]. The Amsterdam 70-gene profile or Mammaprint was developed in an effort to distinguish patients likely to recur with late stage disease from those who were not. Tumors were isolated from two distinct patient populations with lymph node negative disease: those who showed recurrence of distant metastases and those that never did. A differential gene expression profile was subsequently generated. This 70-gene signature has been shown to correlate with clinical outcome and is predictive of distant metastases in Stage I-II patients. Another predictive test is the 21-gene Recurrence Score (Oncotype DX) which was developed through a screen of 250 candidate genes gathered from the literature, genomic databases and gene expression profiling. The expression levels of 21 resultant genes are used to generate a Recurrence Score predictive of distant metastases in patients with ER positive, lymph node negative disease. Together the intrinsic subtypes and prognostic indicator tests have contributed to the diagnoses and treatment of breast cancer [41].

Integrin-ECM interactions

Most solid tumors, such as breast cancer, arise from epithelial cells and tissues. Integrins are a class of receptors that mediate the interactions between epithelial cells and the extracellular matrix (ECM) [42]. These αβ heterodimers contain large amino-

terminal extracellular domains, transmembrane domains, and short cytoplasmic tails [42]. In humans, eighteen α and eight β subunits have been identified. Binding of the cytoplasmic tails of αβ subunits to each other maintains integrins in an inactive 'bent' state [42]. Activation of ligands results in either a partially extended or a ligand-bound 'open' state [43].

Integrin signaling may be mediated through 'inside-out' or 'outside-in' mechanisms. During the former, cytoplasmic proteins such as talin bind the C-terminal tails of β integrins promoting integrin clustering and impacting their affinity for ligands including fibronectin, laminin, and collagen. Talin is an actin binding protein and also acts to link integrins to the actin cytoskeleton. Inside-out signaling regulates cell adhesion and migration by modulating the strength of integrin-ECM interactions [43,44].

Conversely, integrins can also transmit signals from the extracellular space to the cell through outside-in signaling. Binding of ligands to integrin receptors results in conformational changes such as separation of αβ cytoplasmic domains allowing for binding of intracellular molecules including the nonreceptor tyrosine kinases focal adhesion kinase (FAK) and Src followed by recruitment of scaffolding and signaling proteins including paxillin and talin [45]. Recruitment of these proteins results in the formation of a focal adhesion complex that activates signaling involved in cell adhesion, migration, and survival including the phosphoinositide 3-OH kinase (PI(3)K), and extracellular signal-related kinase (ERK1/2) pathways [45-47].

FAK was identified in the early 1990s as a protein that was tyrosine phosphorylated in response to integrin engagement [47,48] and independently as a substrate of v-Src [47,49]. FAK consists of N- and C-terminal domains that flank a

kinase domain (Figure 4) [45]. The exact mechanisms leading to FAK activation upon ECM-integrin binding have not been elucidated. However, studies have found that the C-terminal domain of FAK, termed the focal adhesion targeting (FAT) domain is responsible for targeting FAK to the cytoplasmic domain upon integrin-ligand binding [47]. Additionally, the N-terminal FERM domain of FAK regulates the activity of the centrally located kinase domain. In its inactive state the FERM domain of FAK directly binds to the kinase domain preventing access to the activation loop containing the key autophosphorylation site, Y397 (Figure 4). Binding of FAK to integrin receptors displaces the FERM domain resulting in rapid autophosphorylation of FAK at Y397 [47]. This autophosphorylation event results in a binding site for SH2 domain-containing proteins including Src and PI3K. Upon recruitment to focal adhesion complexes, these enzymes act to further phosphorylate FAK at numerous sites [45,47]. For example, Src-mediated phosphorylation of FAK occurs at five residues, Y407, Y576/7, Y861, and Y925 (Figure 4) all of which promote the release of adhesions at the trailing edge of migrating cells [50]. Additionally, phosphorylation of FAK by Src at Y925 directly recruits Grb2 an adaptor protein that promotes Ras activation demonstrating a direct link between integrin engagement and activation of the ERK1/2 pathway [45]. Thus, in addition to mediating adhesion and migration of epithelial cells to the basement membrane, integrins play a significant role in initiating survival pathways of matrix-attached cells.

Mammary epithelial cells primarily bind a laminin-rich ECM through specific αβ integrin receptors [51,52]. Growth of primary mammary epithelial cells on a reconstituted basement membrane matrix (derived from Engelbreth-Holm-Swarm murine

tumor) results in the formation of alveoli-like structures that secrete milk-specific proteins including caseins, lactoferrin, and transferrin [51]. Targeted transgene expression of the matrix metalloproteinase, stromelysin-1 which degrades ECM components including laminin, fibronectin, and collagen in the mouse mammary gland results in shrunken alveoli with reduced milk production in lactating glands [53]. Together these data demonstrate that interactions between the ECM and epithelial cells play key roles in the development and maintenance of mammary gland architecture both *in vitro* and *in vivo*.

Apoptosis

Apoptosis was first described by Kerr *et al.* [54] as a highly organized programmed cell death process. During development, this programmed cell death plays a key role in morphogenesis and tissue sculpting. For example, apoptosis is necessary in the formation of mouse paws which are initially formed as spade-like structures during development. Apoptosis of cells between digits allows for their separation and subsequent development of paws. Additionally, apoptosis functions to rid organisms of structures that become unnecessary during their maturation. An example of this essential role of apoptosis is demonstrated by the removal of tails as tadpoles develop into adult frogs [55]. In adults, apoptosis contributes to tissue homeostasis as a direct complement to cellular proliferation. Additionally, this death mechanism functions in the removal of cells that have incurred DNA damage or pathogen infection [56]. Thus, dysregulation of this process is associated with a wide range of pathologies including cancer, autoimmune diseases, and degenerative diseases. For example, rheumatoid arthritis is thought to

result from an overabundance of inflammatory cells in joints due to misregulation of apoptotic pathways. By avoiding cell death, inflammatory cells are able to persist in these regions where they destroy cartilage and bone [57].

The structural cellular changes associated with apoptosis occur in two distinct stages [54]. This first involves membrane blebbing and nuclear and cytoplasmic condensation. Nuclear and DNA fragmentation subsequently occurs and is followed by separation of cell surface protuberances. These steps result in the formation of membrane bound cellular components termed apoptotic bodies. During the second stage, these apoptotic bodies are phagocytosed by neighboring cells [54]. Thus, apoptosis is a highly organized cell death process that prevents the release of cellular contents into the extracellular space which can trigger a subsequent inflammatory response. This factor of apoptosis distinguishes it from another form of cell death termed necrosis that typically occurs in response to injury resulting from overwhelming physical or chemical cellular stress [56]. In contrast to apoptosis, necrosis is characterized by cell swelling, organelle swelling, and loss of membrane integrity. The end result of this cell death includes the release of intracellular contents resulting in inflammation [56]. Additionally, apoptosis is distinguishable from autophagy, another highly organized cellular death process. During autophagy, cells are not lysed but instead become compartmentalized into vacuoles or 'autophagosomes' that are self-digested through lysosome-mediated proteolysis. While autophagy may also play a role in tissue development and homeostasis, its role in these events is not as well defined as that of apoptosis [56]. In addition to mediating cell death, autophagy can contribute to cell survival by allowing cells to adapt to nutrient limitations

through catabolization of cellular components and by promoting targeted destruction of damaged organelles and misfolded proteins [56].

Caspases (cysteine-dependent aspartate specific proteases) are a family of enzymes that execute programmed cell death [58]. These enzymes exist as zymogens within the cell and are activated by a proteolytic cleavage cascade upon receipt of specific signals [58]. The first caspase gene, *ced-3*, was identified in the model organism *Caenorhabditis elegans* [59] and the apoptotic cascade has since been found to be a highly conserved cellular process [60]. Soon after its identification, the protein encoded by ced-3 was found to be homologous to the newly discovered mammalian protease interleukin-1β converting enzyme (ICE/caspase-1) [61,62] originally identified in monocytes [63]. Eleven human caspases (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14) have since been identified with roles in both apoptosis and inflammation [64] as well as other nonapoptotic cellular processes [60].

Caspases have a highly conserved protease domain consisting of a catalytic cysteine-histidine side chain important in cleaving substrates at aspartate residues [65]. These zymogens consist of three domains including an N-terminal prodomain and the p20 and p10 domains. During activation, caspases are cleaved between the prodomain and p20 domains and between the p20 and p10 domains. Mature caspases form as heterotetramers composed of two p20/p10 heterodimers containing two active sites [66].

Sequence similarities of caspases have been used to classify them into the following subgroups: inflammatory caspases: caspase-1, -4, -5; apoptotic initiator caspases: caspase-2, -8, -9, -10; and apoptotic effector caspases: caspase-3, -6, -7 (Figure 5). Caspase-14 is primarily expressed in the epidermis and may function in

differentiation [67]. Apoptotic caspases are classified as initiator or effector caspases depending on their temporal role in cell death. Initiator caspases are the first to be activated and typically contain protein-protein interaction domains in their N-terminus to mediate homotypic interactions resulting in self-aggregation, cleavage, and activation. Activated initiator caspases cleave their downstream targets, effector caspases. In contrast to initiator caspases, effectors lack homotypic interaction domains and are responsible for the proteolytic cleavage events that result in the morphological changes associated with apoptosis [67].

The death domain (DD) superfamily consists of domains commonly found in proteins that mediate apoptosis and inflammation. These structurally related domains include the DD, death effector domain (DED) and caspase-recruitment domain (CARD) [68]. The pyrin domain (PYD) was later identified as a DD related domain [69-71]. All four of these domains share a characteristic fold and architecture consisting of an antiparallel six-helix bundle. However, the length and orientation of helices differs among each subfamily. These domains have been shown to be involved in primarily homotypic interactions resulting in both cell death and inflammation [70].

Two pathways of apoptosis

Apoptosis can be initiated from two distinct signaling cascades: the intrinsic pathway or the extrinsic pathway (Figure 6). The intrinsic pathway of apoptosis is initiated by intracellular death signals and is mediated by the Bcl-2 family of proteins and the initiator caspase, caspase-9. Members of the Bcl2 family share one or more of four 'Bcl-2 homology' (BH) domains, BH1, BH2, BH3, and BH4. Proapoptotic Bcl2 family

members include the "BH3-only" proteins (e.g. Bim, PUMA, Bad, and Bmf) and the multidomain members consisting of the BH1, BH2, and BH3 domains (Bax and Bak). Antiapoptotic members of the Bcl-2 family including Bcl-2, Bcl-xL, and Mcl-1 contain all four of the BH domains and inhibit apoptosis by binding and functionally inhibiting proapoptotic Bcl2 family members [72].

BH3-only proteins are the primary sensors of apoptosis and can be transcriptionally or post-translationally regulated to mediate apoptosis. For example, p53 responds to DNA damage to induce *PUMA* transcription and Bad is activated when dephosphorylated during growth factor deprivation [73]. Additionally, *Bim* is transcriptionally regulated by both the PI3K [74] and ERK1/2 pathways [75]. Bim isoforms are further regulated by specific phosphorylation events mediated by ERK1/2 and JNK. One consequence of these post-translational modifications is the sequestration of Bim by the microtubule network [76]. Additionally, ERK1/2 mediated phosphorylation of the major isoform of Bim at Ser69 promotes its ubiquitination and proteasomal degradation [77].

The primary function of BH3 only proteins is to bind antiapoptotic family members, releasing the negatively regulated proapoptotic multidomain members, Bax and Bak [78]. A recent model regarding the function of BH3-only proteins proposes that they may function to activate Bax and Bak either as 'direct activators' through putative physical interactions (e.g. Bim, Bid) or as derepressors by binding and sequestering the anti-apoptotic proteins, Bcl-2, Bcl-xL, or Mcl-1 [78]. In this model, antiapoptotic proteins bind direct activators (e.g. Mcl-1 sequesters Bim in nonapoptotic cells) preventing interactions with Bax and Bak. During apoptosis, derepressor BH3-only

proteins bind anti-apoptotic members releasing BH3-only direct activators to activate Bax and Bak. However, direct activation of Bax and Bak is controversial (Figure 6) [78,79].

Bak is constitutively present at mitochondria whereas Bax is primarily cytosolic and translocates to the mitochondria during apoptosis [78]. Both initiate apoptosis by inserting into the mitochondrial membrane to form pores resulting in permeabilization of the outer mitochondrial membrane (OMM). The precise mechanisms in the formation of pores in the OMM and their regulation by Bcl2 family members are not well understood [78]. OMM permeabilization results in the release of proapoptotic proteins that occupy the intermembrane space. One such protein is cytochrome c, which upon release from the mitochondria binds the cytosolic protein, Apaf-1 resulting in the recruitment and oligomerization of procaspase-9 and the formation of the apoptosome complex. Assembly of the apoptosome leads to downstream activation of procaspase-3 and cleavage of cellular substrates resulting in apoptosis (Figure 6) [66,80].

The extrinsic pathway is initiated by extracellular death signals (e.g. fas/CD95 and tumor necrosis factor α (TNFα)) that bind plasma membrane-bound receptors and is primarily mediated by the initiator caspase, caspase-8 (Figure 6) [81]. Death receptors are members of the TNF gene family that share cysteine-rich extracellular domains. Upon ligand binding, these death receptors trimerize, allowing the DDs of the individual receptors to cluster and recruit the adaptor molecule fas-associated death domain (FADD). FADD in turn recruits procaspase-8 through DED-DED interactions, forming a death induced silencing complex (DISC) that promotes self-cleavage of procaspase-8 and activation of downstream caspases such as procaspase-3 [80,81]. Caspase-8 can also

activate the BH3-only protein Bid resulting in cytochrome c release and activation of procaspase-9 (Figure 6).

Additionally, two different cell types have been identified with regard to responses to death receptor/ligand binding [82]. In type I cells, death ligands induce activation of procaspase-8 resulting in direct activation of procaspase-3. Overexpression of Bcl2 in these cells does not block procaspase-8 or -3 cleavage and does not affect the sensitivity of cells to the fas death ligand, suggesting that death is independent of the mitochondria and caspase-9. In contrast, in type II cells procaspase-8 and -3 are activated downstream of the mitochondria and can be blocked by overexpressing Bcl2 [82].

Anoikis

Epithelial cells require both growth factors and adhesion to a basement membrane for survival; the disruption of either results in apoptosis (Figure 7). Dr. Steven Frisch termed this phenomenon "anoikis" meaning "homelessness" in Greek [83]. Cancer cells are resistant to anoikis and survive independent of ECM interactions, suggesting that these cells have acquired the ability to suppress apoptosis in the absence of integrin-mediated survival signaling [84]. Crucial events during carcinogenesis include invasion of the surrounding stroma by epithelial-derived cancer cells and cellular spread to distant sites to form metastases. Both of these events require that cancer cells survive in the absence of matrix-attachment, thus anoikis resistance plays an important role in both the initiation and progression of human cancer [84].

The molecular events that link loss of integrin-ECM contacts to the activation of apoptotic machinery are not well understood. Some evidence has implicated caspase-8 as

a mediator of anoikis whereby expression of a dominant negative form of FADD in suspended epithelial cells resulted in an inhibition of anoikis [85,86]. Moreover, in endothelial cells, attachment to the ECM protected cells from fas-induced apoptosis, while detachment from the matrix sensitized cells to fas-induced cell death [87]. Additional evidence suggested that anoikis in these cells was regulated by the balance between expression of c-Flip, an endogenous antagonist of caspase-8, and caspase-8 itself [87]. In adherent endothelial cells, c-Flip expression was high while caspase-8 levels remained low. Upon detachment, c-Flip levels were reduced relative to caspase-8 suggesting that downregulation of c-Flip was necessary for detachment-induced procaspase-8 activation [87]. Altogether, these results implicate caspase-8 in anoikis. However, the precise mechanism by which procaspase-8 is activated in detached epithelial cells has not been elucidated. In this regard, it is interesting to note that activation of procaspase-8 may occur by a death receptor independent mechanism, as there is evidence that extracellular death receptor antagonists failed to inhibit anoikis [86].

While the above studies suggested that caspase-8 cleavage is an initiating event in anoikis, other studies have also implicated other mediators of apoptosis in detachment-induced cell death. For example, overexpression of the anti-apoptotic protein, Bcl-xL protected ovarian cancer cells from anoikis [88]. Additionally, groups have shown a crucial role for the BH3-only protein Bim in anoikis [75,89,90]. Growth of the human breast epithelial cell line, MCF10A, in suspension resulted in a significant upregulation of the three major isoforms of Bim [Bim-extra long (BimEL), Bim-long (BimL), and Bim-short (BimS)]. This upregulation of Bim was specific, since the levels of several

other BH3-only proteins were not altered by detachment. Moreover, short-interfering RNA (siRNA) knockdown of Bim inhibited anoikis in detached epithelial cells [75]. Interestingly, the *Bim* knockout mouse demonstrated that disruption of Bim prevents apoptosis and delays clearance of luminal cells in terminal end branches of mammary ducts during puberty, highlighting the role for Bim in anoikis as well as the role of anoikis in breast development [91].

Identification of TMS1/ASC

Target of Methylation-Induced Silencing (*TMS1*) was originally identified in a screen for downstream targets of aberrant DNA methylation [92]. This screen was performed using the normal human diploid fibroblast cell line, IMR90 and its immortalized derivative stably overexpressing DNA cytosine-5-methyltransferase-1 (DNMT1) referred to as HMT1E1. *TMS1* was identified as a gene that was expressed in IMR90 cells but silenced in HMT1E1 cells. *TMS1* localizes to chromosome 16 and contains three exons spanning ~1.4kb of genomic DNA. The *TMS1* locus produces two distinct mRNA species resulting from alternative splicing. The more abundant and most widely studied of these gene products results from transcription of all three exons and the less abundant form lacks exon 2 (Figure 8) [92].

The initial study identifying TMS1 showed that it is methylated and silenced in ~40% of breast cancer cell lines. Moreover, TMS1 was shown to be methylated in ~40% of primary human breast tumors [92]. Other studies have shown that TMS1 is methylated in many different cancers. Methylation of TMS1 was observed in 65% of prostate cancers [93]. In this study, TMS1 was also shown to be methylated in 64% of

high grade-prostatic intraepithelial neoplasia (PIN), a precursor prostate cancer lesion and 28% of normal, adjacent tissue samples suggesting that methylation of TMS1 is an early event in prostate cancer progression [93]. Epigenetic silencing of TMS1 has also been observed in human glioblastomas [94], lung cancers [95], and ovarian cancers [96]. Together, these data suggest that methylation and silencing of TMS1 contributes to the development of many cancer types. However, the precise function of TMS1 and how its silencing contributes to cancer progression remain unclear.

Simultaneously, TMS1 was also identified as the apoptosis-associated speck-like protein containing a CARD (ASC) in a screen for proteins that mediate the cytoskeletal changes associated with apoptotic cells [97]. In this analysis, the promyoleukemic cell line, HL-60, were treated with retinoic acid to induce cell death. Monoclonal antibodies (mAb) were then generated against the triton-X-100 insoluble fractions of untreated and retinoic acid treated cells. One mAb recognized a 22kDa protein that was named ASC as it became triton-insoluble upon retinoic acid treatment and formed a distinct spherical structure in a subset of cells that was named a 'speck.' [97]. Moreover, formation of this structure significantly correlated with drug induced apoptosis suggesting a potential apoptotic role for the speck.

TMS1 is a bipartite adaptor protein composed of a PYD linked to a CARD. Both of these domains are members of the death domain superfamily commonly found in proteins that mediate apoptosis and inflammation [98]. Studies from our laboratory and others show that TMS1 functions in apoptosis and other groups have also demonstrated that TMS1 mediates inflammation. Our laboratory has shown that ectopic expression of TMS1 in the kidney epithelial cell line, HEK293, promotes apoptosis in a time-dependent

manner through procaspase-9 activation [99]. Additionally, upon induction of TMS1 expression, the subcellular localization of TMS1 was redistributed from a diffuse cytoplasmic staining to a punctuate perinuclear structure (Figure 9). Interestingly, while the timing of the appearance of these structures coincided with cell death, their formation preceded caspase activation [99] suggesting that caspase activation is not necessary in the formation of this structure. Other evidence indicates TMS1 as a p53 responsive tumor suppressor that mediates DNA-damage-induced apoptosis [100] and suggests that TMS1 mediates cell death in a Bax dependent manner (Figure 10) [100,101].

Additional studies have shown that TMS1 promotes caspase-8 dependent cell death. Forced oligomerization of TMS1 was sufficient to induce caspase-8 mediated apoptosis in HEK293 cells. Additionally, the PYD of TMS1 was shown to coimmunoprecipitate with a catalytically inactive caspase-8 suggesting a potential interaction between these two proteins [102]. More recent work from our laboratory indicates that TMS1 promotes caspase-8-dependent apoptosis in MCF7 breast cancer cells. Death receptor ligand (TNFα and TNF-related apoptosis-inducing ligand (TRAIL)) treatment of MCF7 cells induced TMS1 mRNA and protein levels (Figure 10) [103]. Whereas TMS1 was shown to be dispensable for procaspase-8 activation induced by these ligands, it could promote procaspase-8 cleavage independent of death receptor-ligand interactions [103]. Together these data show a distinct function for TMS1 in apoptosis in many cell types.

TMS1 and inflammation

The best characterized role for TMS1 in inflammation involves its function in inflammasome formation. The inflammasome is a multiprotein molecular complex that forms in response to pathogenic stimuli and promotes the oligomerization and activation of procaspase-1, analogous to procaspase-9 activation by the apoptosome [104]. Procaspase-1 self-activation leads to its proteolytic cleavage into heterodimers consisting of two 10 and 20kDa subunits. These enzymatically active heterodimers cleave pro-interleukin 1β (pro-IL-1β) and pro-IL-18 to allow for the secretion of mature IL-1β and IL-18.

Inflammasome assembly occurs in response to recognition of pathogen-associated molecular patterns (PAMPs) which are microbial specific structures such as lipopolysaccharide (LPS), peptidoglycan, and microbial nucleic acids [105]. The innate immune receptors responsible for sensing PAMPs include the membrane associated receptors, Toll-like receptors (TLRs) and the cytoplasmic receptors, Nod-like receptors (NLRs). NLR family members are characterized by having a tripartite protein structure consisting of a variable N-terminal domain that may consist of a CARD domain or a PYD among other domains, followed by a Nod domain, and a C-terminal leucine rich repeat responsible for detecting PAMPs [105]. Multiple inflammasomes have been identified and named after their respective NLR members (NLRP1/NALP1, NLRP3/NALP3/cryopyrin, and NLRC4/IPAF). TMS1 bridges some NLR members to procaspase-1 by using its PYD to bind PYD-containing NLRs and its CARD to interact with procaspase-1. Some NLRs lack a PYD and are thought to associate with procaspase-1 directly through CARD-CARD interactions. However, even in those cases TMS1 is still required for procaspase-1 activation [105].

TMS1 *knockout*

The essential role for TMS1 in procaspase-1 activation in response to pathogenic infection has been well established by studies involving knockout mice. Mariathasan *et al.* generated a TMS1-deficient knockout and showed that TMS1 was necessary for activation of TLRs leading to procaspase-1 activation in macrophages [106]. Additionally, TMS1 played a key role in procaspase-1 activation in response to the intracellular pathogen *Salmonella typhimurium*. Macrophages null for TMS1 exhibited deficiencies in the cleavage and maturation of pro-IL-Iβ and pro-IL-18. Additionally, cell death involving procaspase-1 was completely abrogated in TMS1 and IPAF deficient cells, indicating an interesting link between the inflammatory and cell death pathways [106].

TMS1 deficient mice were also generated by Yamamoto *et al* [107]. These authors showed that upon LPS infection, TMS1 null macrophages were deficient in procaspase-1, pro-IL-Iβ and pro-IL-18 maturation. However, these TMS1 deficient cells produced other proinflammatory cytokines including TNF-α and IL-6 supporting a specific role for TMS1 in procaspase-1 activation. Previous studies had shown that procaspase-1 deficient mice were resistant to LPS induced acute liver injury. Consistent with a role for TMS1 in procaspase-1 activation, TMS1 null mice escaped LPS-induced acute liver injury similar to procaspase-1 -/- and IL-18 -/- mice [107]. Altogether, these data support an essential role for TMS1 in the processing of pro-IL-18 and -1β in response to procaspase-1 activation and in mediating an inflammatory response to

pathogenic infection. However, studies using TMS1 knockout mice to decipher an *in vivo* tumor suppressor role for TMS1 in apoptosis have not been addressed.

Objective

At the start of this thesis, TMS1 had a well characterized role as a tumor suppressor that is methylated and silenced in many cancers including breast cancer [92-94,98,108-112]. However, the consequences of aberrant methylation and silencing of *TMS1* in breast cells and its subsequent contribution to breast tumorigenesis had not been elucidated. Some studies including work by our laboratory had established a function for TMS1 in apoptosis [100-103]. More specifically, forced oligomerization of TMS1 was sufficient to induce caspase-8 mediated cell death independent of death receptor ligation. Additionally, an *in vitro* interaction between the N-terminus of procaspase-8 and TMS1 was identified [102]. Work in our laboratory generated further evidence elucidating an endogenous role for TMS1 in response to death ligand treatment of breast cancer cells. Overexpression of TMS1 in these cells was sufficient to induce procaspase-8 cleavage independent of death receptor-ligand interactions [103]. However the precise mechanisms by which TMS1 promoted procaspase-8 cleavage was still not well understood.

In 1994, a distinct form of apoptosis was discovered that occurred in response to loss of adhesion and was termed ‘anoikis’ [83]. Early events that occurred in cells undergoing anoikis involved death receptor-independent cleavage of procaspase-8 [86] and upregulation of the BH3 only protein Bim [75,90,113]. Moreover, cells that aberrantly filled mammary ducts were thought to be resistant to anoikis allowing them to

persist in an environment that should otherwise promote cell death. Preliminary data in our laboratory showed specific loss of TMS1 in cells aberrantly filling the ducts of a subset of DCIS lesions. However, a thorough examination of these samples had not yet been addressed.

Other studies aimed to elucidate the immune function of TMS1 in both cell culture systems [114,115] and through the use of *TMS1* knockout mice [106,107]. Results from these analyses showed that TMS1 activates procaspase-1 in response to numerous pathogenic stimuli. Interestingly, overexpression of TMS1 in various cell lines including immune cells resulted in the formation of a distinct speck-like structure thought to be associated with an apoptotic role for TMS1 [92,98]. While this structure could be seen endogenously in immune cells [97], visualization of speck-like structures in epithelial cells only occurred upon overexpression of TMS1. In addition, the precise stimulus, mechanism, and functional consequences of speck formation were not well understood.

Based on the proapoptotic nature of TMS1 and its staining pattern in a subset of DCIS lesions, we hypothesized that aberrant epigenetic silencing of TMS1 allowed breast epithelial cells to bypass anoikis and consequently form mammary ducts with a filled luminal space (Figure 11). The goal of this thesis was to further our understanding of the functional role of TMS1 in breast cancer progression. Our main objectives included determining a role for TMS1 in breast cell anoikis and elucidating the molecular mechanisms by which TMS1 promotes death upon cellular detachment. During the progression of this thesis, a new role for procaspase-8 was established in cellular

adhesion and migration. Thus, in our final objective, we expanded our hypothesis to explore a similar role for TMS1 in breast epithelial adhesion and migration.

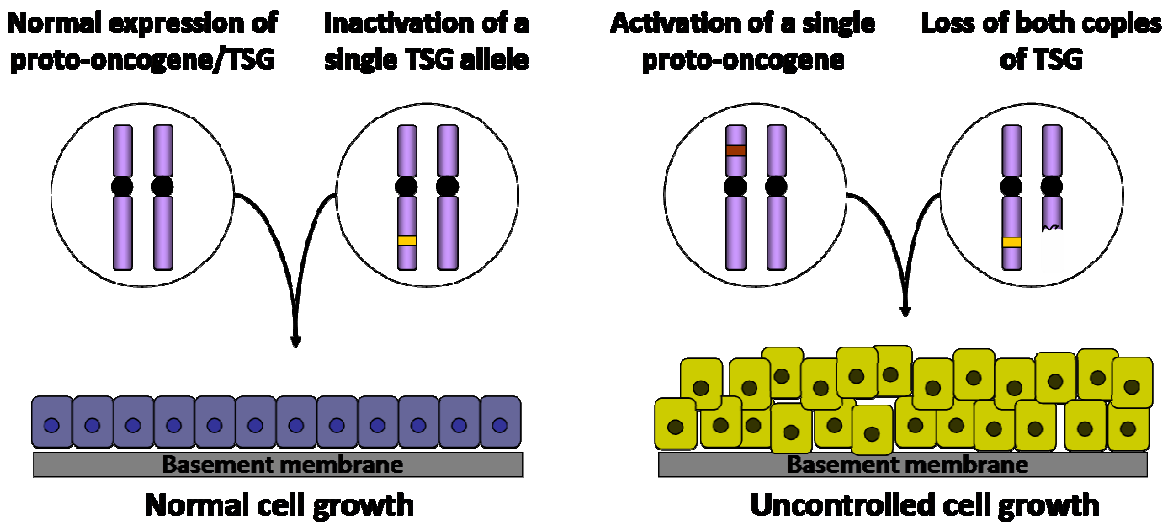

Figure 1: Genetic and epigenetic events that result in uncontrolled cell growth. Normal expression of proto-oncogenes and TSGs promote normal cell growth. An important distinction between oncogenes and TSGs is that activation of a single allele of an oncogene will result in uncontrolled cell growth yet loss of both homologous alleles of a TSG must occur in order to achieve similar consequences.

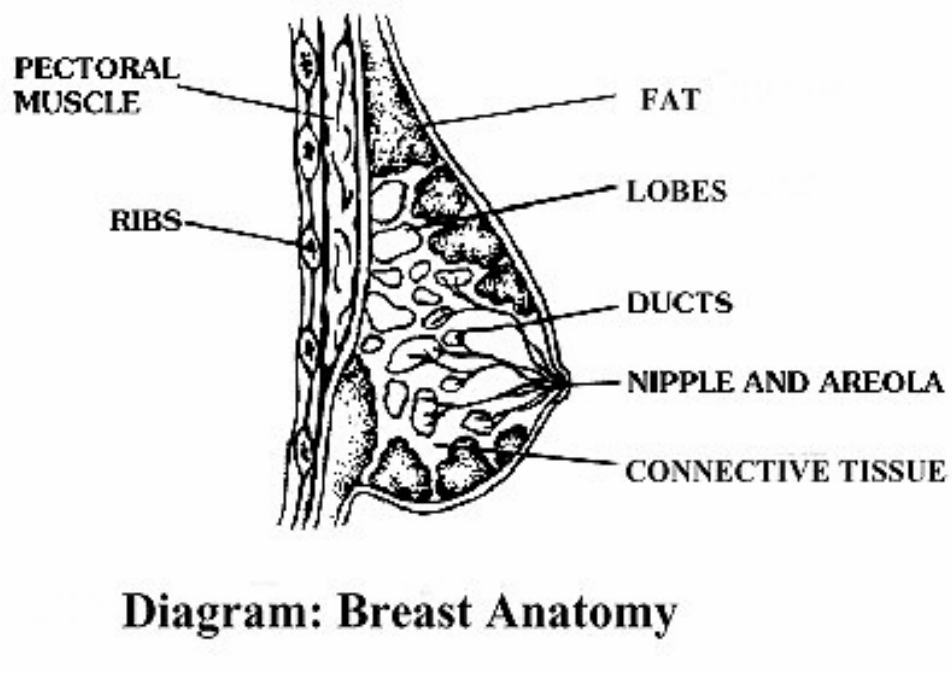

Figure 2: Normal anatomy of the breast. Breasts are composed of a network of ducts and lobules that are surrounded by connective and fatty tissue. Mammary ducts act to transport milk from lobules to the nipple and contain hollow lumen. Reprinted from the National Institute of Health at http://mammary.nih.gov/index.html.

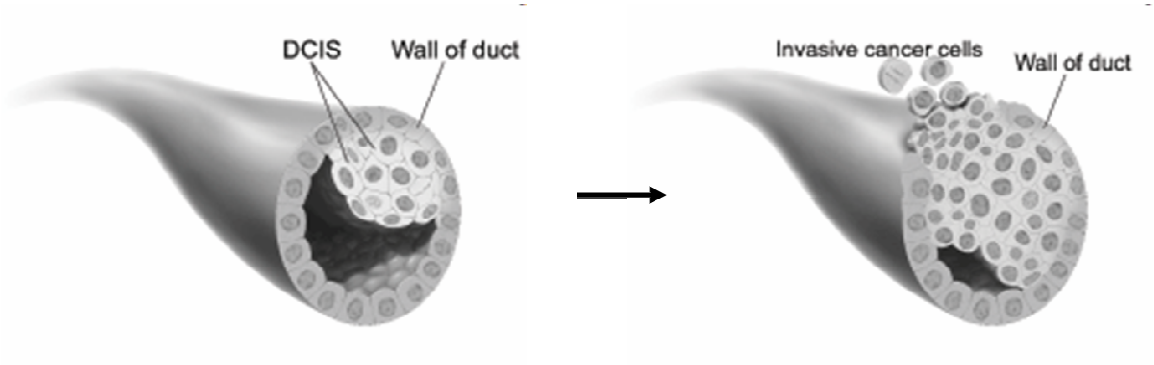

Figure 3: DCIS lesions can progress to invasive ductal carcinomas. DCIS is characterized by breast cells that have acquired resistance to anoikis and thus fill mammary ducts. Invasive ductal cancers arise when these aberrant cells acquire the ability to infiltrate surrounding tissue. Invading cells have the potential to migrate to other sites in the body through entering the lymphatic or blood circulatory systems. Adapted from National Cancer Institute at www.cancer.gov.

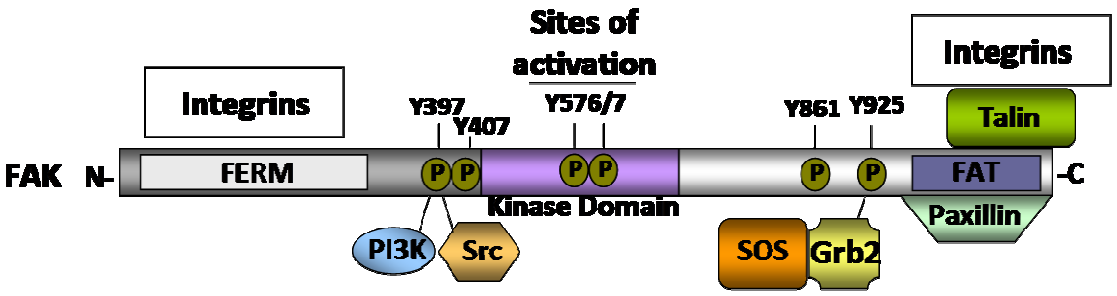

Figure 4: Schematic of the domains and Src-mediated phosphorylation sites of focal adhesion kinase. The N-terminal FERM domain mediates auto-inhibition of FAK by binding to the centrally located kinase domain and preventing access to the autophosphorylation site, Y397. Binding of FAK to integrin receptors displaces the FERM domain resulting in rapid autophosphorylation of FAK at Y397. Phosphorylation at this site serves as a binding site for SH2-containing proteins such as Src and PI3K which further phosphorylate FAK. Src mediates Y407, Y576/7, Y861, and Y925 phosphorylation all of which promote the release of adhesions at the trailing edge of migrating cells. Additionally, phosphorylation of FAK by Src at Y925 directly recruits Grb2 an adaptor protein that promotes ERK1/2 signaling. The C-terminal domain is responsible for targeting FAK to the cytoplasmic domain upon integrin-ligand interactions and binds the adaptor proteins talin and paxillin in the formation of the focal adhesion complex.

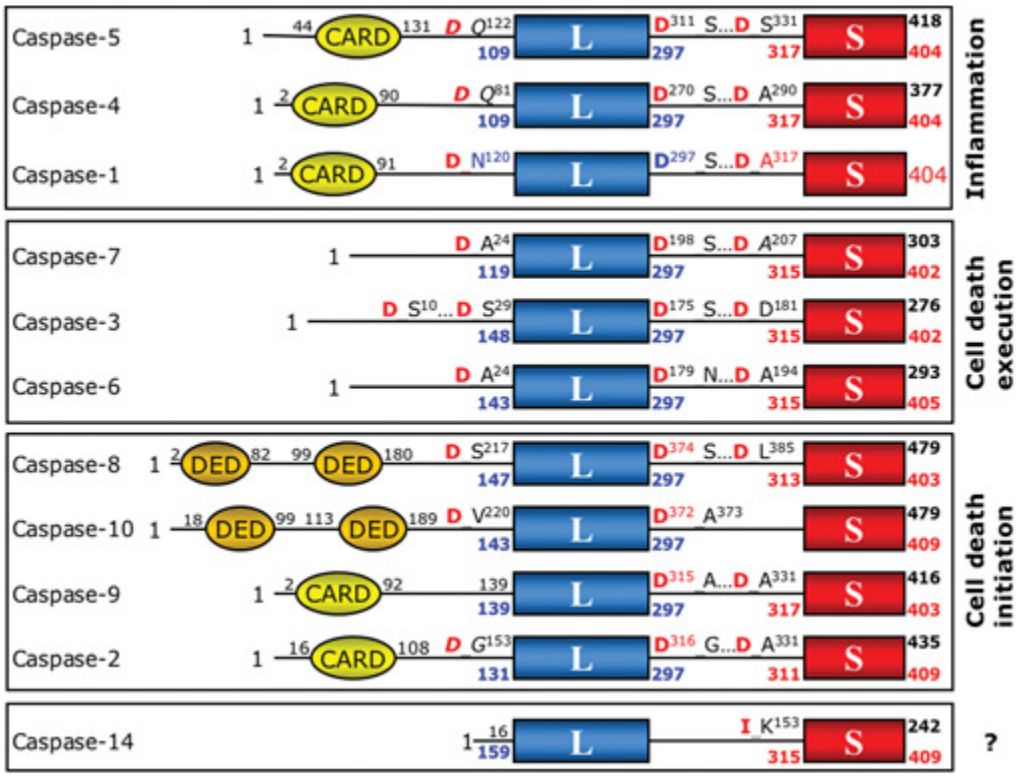

Figure 5: Domain organization of human caspases. Human caspases have been grouped according to their sequence similarities. Notice that sequence identity divides caspases-1 to -10 into three subfamilies, in accordance with the physiological distinction between inflammatory, initiator, and effector caspases. In contrast with the widespread distribution of these family members, caspase-14 is found mainly in the epidermis, may be involved in keratinocyte differentiation, and is not activated *in vivo* at an Asp residue. The positions of maturation cleavage sites are given, with the P1 aspartate residue highlighted in red (in italics in cases where the usage of the site has not been confirmed experimentally). Reprinted from [67].

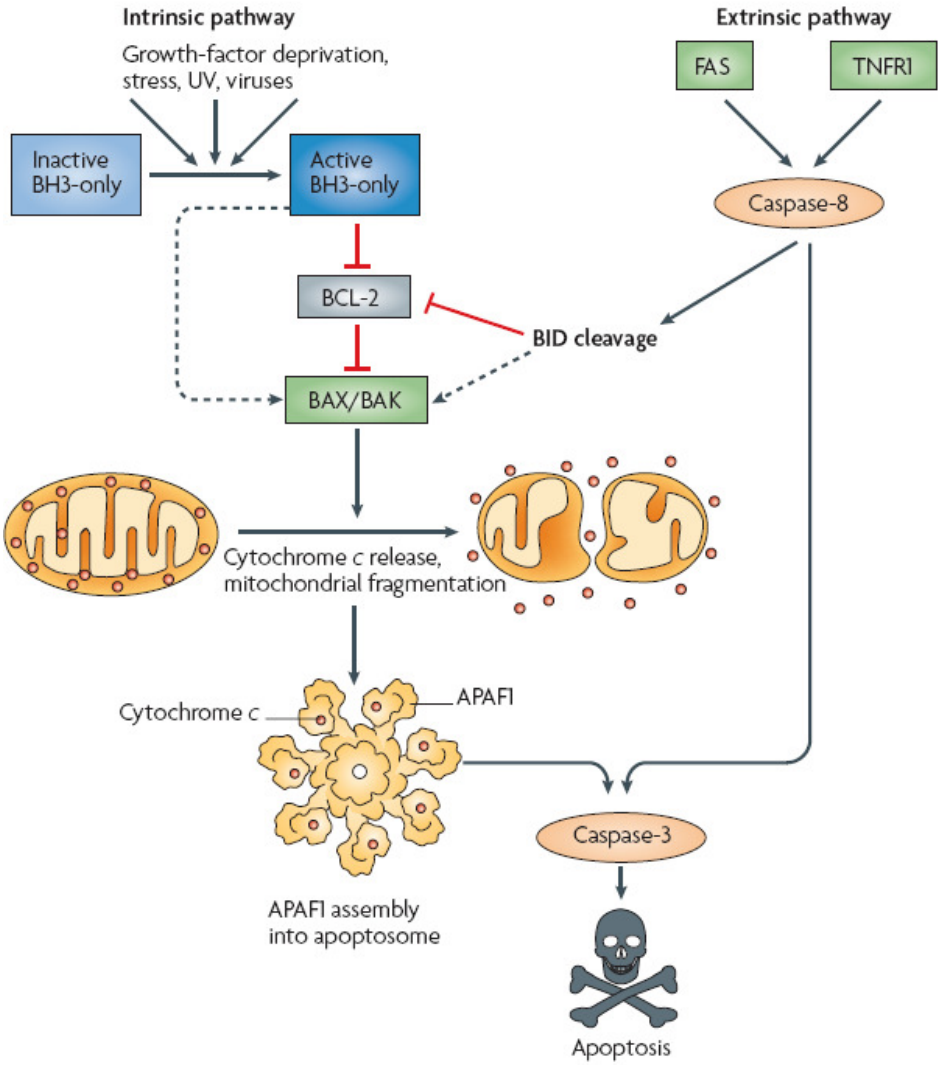

Figure 6: Scheme depicting intrinsic and extrinsic pathways of apoptosis. Apoptosis can be induced by cell surface receptors, such as fas and tumor necrosis factor receptor-1 (TNFR1) (extrinsic pathway, right), or by various genotoxic agents, metabolic insults or transcriptional cues (intrinsic pathway, left). The intrinsic pathway starts with BH3-only protein induction or post-translational activation, which results in the inactivation of some BCL-2 family members. This relieves inhibition of BAX and BAK activation, which in turn promotes apoptosis. Some BH3-only proteins, such as BIM and PUMA, may also be able to activate BAX and/or BAK (as shown by the dotted line). Once activated, BAX and BAK promote cytochrome c release and mitochondrial fission, which leads to the activation of APAF1 into an apoptosome and activates caspase-9 to activate caspase-3. Caspases in turn cleave a series of substrates, activate DNases and orchestrate the demolition of the cell. The extrinsic pathway can bypass the mitochondrial step and activate caspase-8 directly, which leads to caspase-3 activation and cell demolition. The BCL-2 family regulates the intrinsic pathway and can modulate the extrinsic pathway when cleavage of BID communicates between the two pathways. Reprinted with permission from Nature Publishing Group originally published in [78].

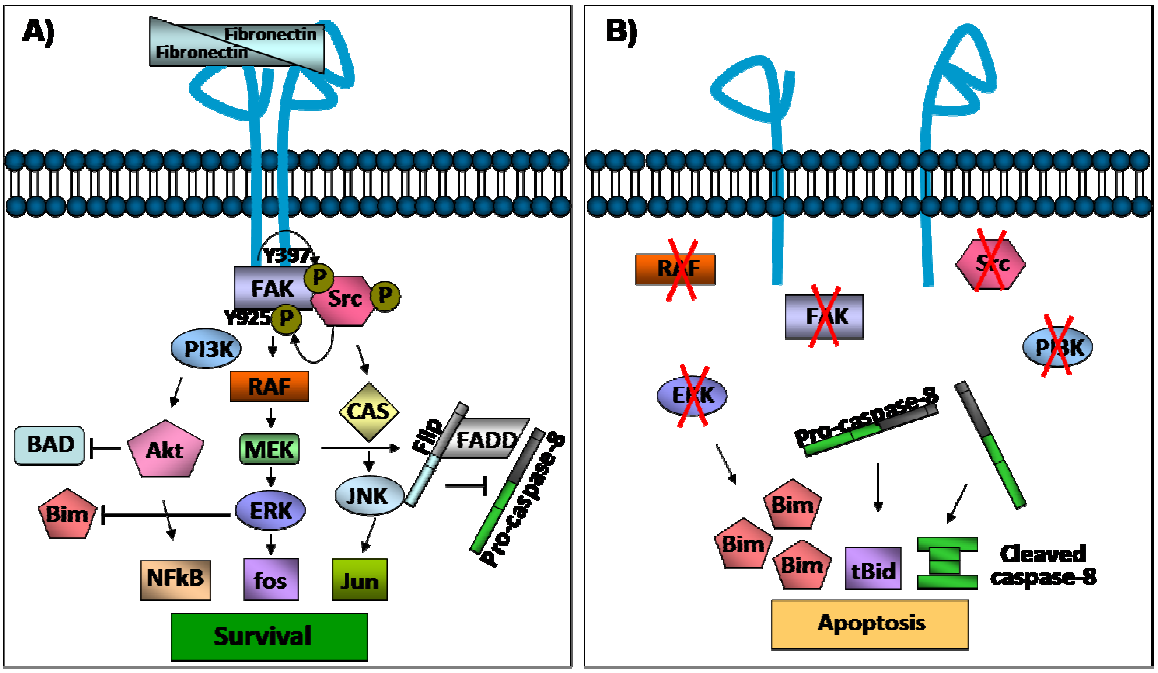

Figure 7: Adhesion promotes survival signaling whereas detachment results in anoikis. A) Integrin-ECM interactions promote the formation of a focal adhesion complex that activates survival signaling. Survival pathways have specific roles in inhibiting the apoptotic machinery in attached cells. B) Loss of integrin-ECM engagement results in downregulation of survival signaling and the onset of apoptosis. While the precise signals resulting in anoikis in detached cells are unclear, upregulation of Bim and death ligand-independent cleavage of procaspase-8 are known to occur upon detachment of epithelial cells.

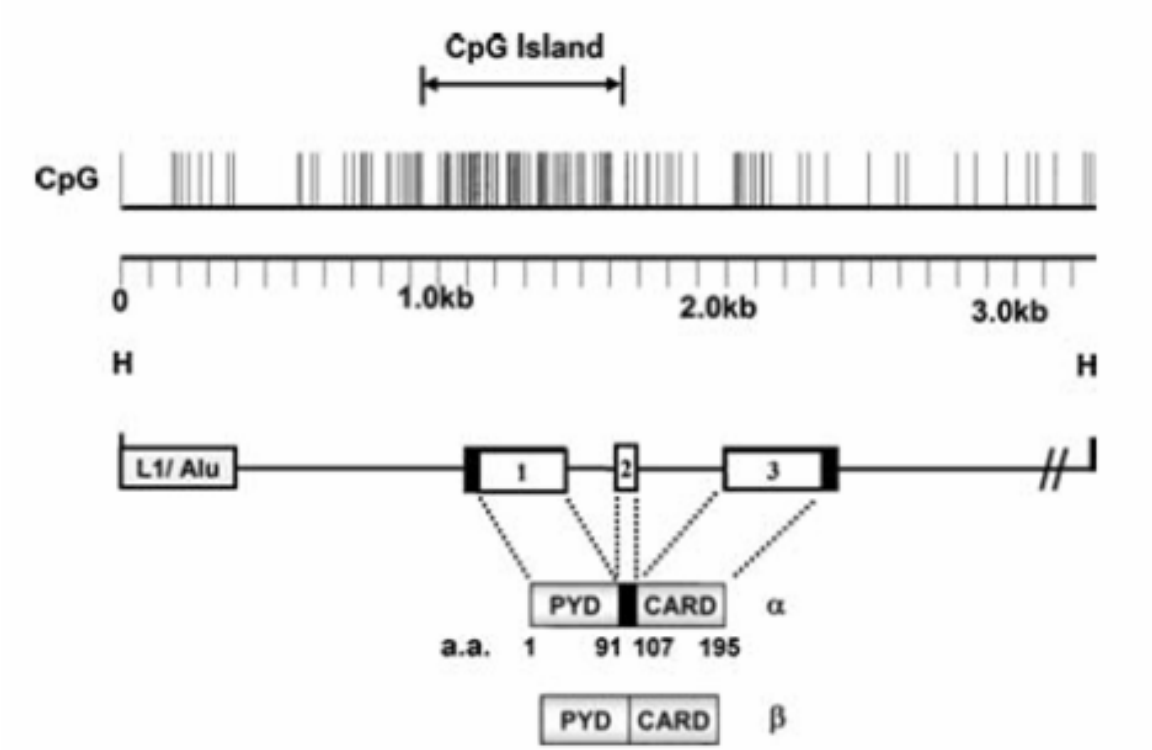

Figure 8: Structure of the TMS1/ASC locus and protein. TMS1/ASC locus on chromosome 16p11.2 encodes a 770 bp mRNA. Open boxes indicate exons. Two splice variants have been detected and are indicated. Expression of the two appears to be concordant in all studies thus far with the α isoform being the predominant message. The frequency of CpG residues and the location of the CpG island is shown. Both splice variants retain the PYD and CARD. Reprinted with kind permission from Springer Science Business Media originally published in [98] copyright 2004 Kluwer Academic Publishers.

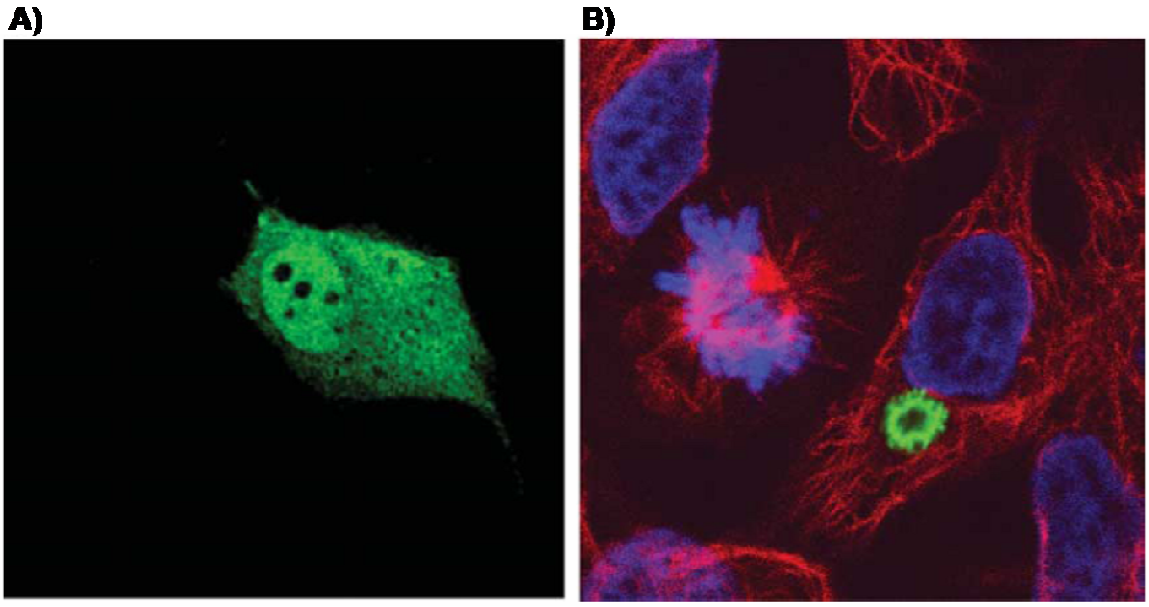

Figure 9: Subcellular localization of TMS1/ASC. HEK293 cells were transiently transfected with TMS1/ASC, fixed at 24 hours and stained for TMS1/ASC using the EU107 rabbit polyclonal antibody (green). A) TMS1/ASC detected throughout the cell. B) TMS1/ASC localized to the ball-like structure. For context, cells were counterstained for β-tubulin (red) and nuclei (blue). Reprinted with kind permission from Springer Science Business Media: originally published in [98] copyright 2004 Kluwer Academic Publishers.

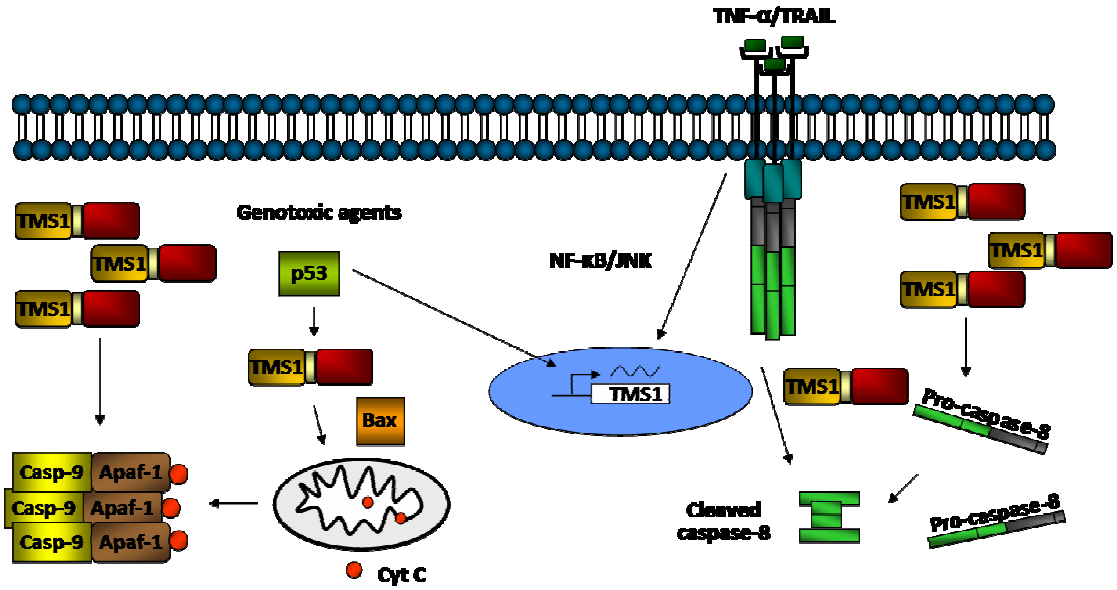

Figure 10: TMS1 and apoptosis. From left to right: Initial studies showed that overexpression of TMS1 induced caspase-9 dependent death in HEK293 cells. Other evidence showed p53 induced TMS1 in response to genotoxic agents resulting in Bax dependent apoptosis. More recent data has shown that the death ligands TNF-α and TRAIL induce TMS1 expression and promote caspase-8 dependent apoptosis in breast cancer cells. Overexpression of TMS1 in these cells is sufficient to induce caspase-8 cleavage in the absence of death receptor - ligand binding.

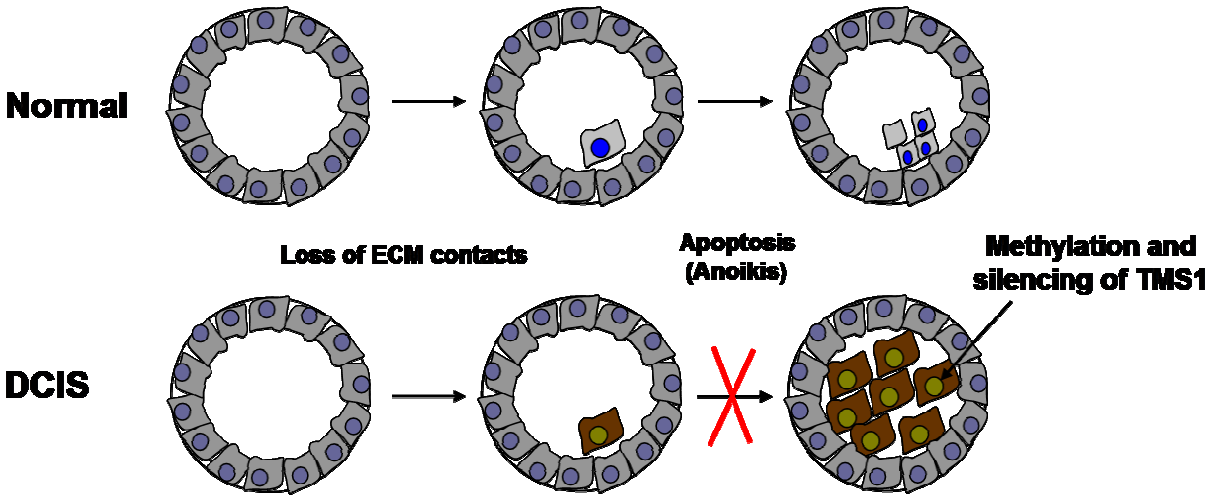

Figure 11: Hypothesis that silencing of TMS1 promotes resistance to anoikis. In normal mammary glands, cells that enter a hollow lumen undergo detachment-induced apoptosis (anoikis) resulting in maintenance of a hollow lumen. During DCIS, cells that have entered the lumen have acquired altered characteristics that allow them to persist. We hypothesize that one of these altered characteristics is the silencing of the proapoptotic gene *TMS1*.

Chapter 2

Silencing of TMS1/ASC promotes resistance to anoikis in breast epithelial cells

Portions of this chapter have been published in Parsons MJ[#], Patel P[#], Brat DJ, Colbert L, Vertino PM. *Cancer Research*. 2009 Mar 1;69(5):1706-11[116]. # These authors contributed equally. Patel P contributed to data shown in Figures 2-8.

Introduction

Epithelial cells require adhesion to the extracellular matrix (ECM) for survival. Adhesion maintains proper tissue architecture by dictating cellular properties such as shape, polarity, and proliferation. Molecularly, these properties are mediated by integrins, which bind components of the ECM and induce downstream signaling through cytosolic adaptor molecules. Detachment from the substratum and loss of integrin-mediated survival signals induce a form of programmed cell death termed ‘‘anoikis’’ [83]. Anoikis is important for organogenesis and tissue homeostasis, and for ensuring that cells remain in their proper cellular context. One of the hallmarks of cancer is the acquired ability to invade basement membranes and neighboring tissues, which requires that cells survive in an anchorage-independent state and develop the means to resist anoikis. Thus, understanding how epithelial cells avoid anoikis is a critical task in understanding, preventing, and treating human cancer.

The molecular events that mediate anoikis are incompletely understood and can be cell type dependent. Both the intrinsic/mitochondrial apoptotic pathway, which is governed by the initiator caspase-9, and the extrinsic/death receptor–mediated pathway of apoptosis, which is governed by the initiator caspase-8, have been implicated. The involvement of the mitochondrial pathway is supported by studies showing that overexpression of the antiapoptotic molecules Bcl-2 or Bcl-xL inhibit anoikis [86]. In addition, the proapoptotic Bcl-2 family members, Bax, Bid, and Bim, translocate to the mitochondrion during anoikis, where they may function to promote cytochrome c release and cell death [75,117].

There is also evidence supporting a role for caspase-8 in anoikis. Extracellular death ligands [e.g., Fas, TRAIL, tumor necrosis factor a (TNFα)], promote caspase-8 activation through a multimeric complex called the death inducing signaling complex [118]. However, recent evidence indicates that anoikis is dependent on caspase-8, independent of death receptor ligation, although the mechanism for its activation remains unknown [86,119]. Upregulation of the caspase-8 inhibitor c-Flip has been observed in many transformed cell lines that are resistant to anoikis [119], and inhibition of c-Flip in these cells restores sensitivity to anoikis [119]. Furthermore, several studies have proposed a nonapoptotic role for caspase-8 as a mediator of cellular adhesion and motility [120-123].

TMS1 (also known as ASC and PYCARD) is a bipartite signaling molecule that participates in apoptosis and inflammation. Although the precise function of TMS1 in apoptosis is unclear, overexpression or forced oligomerization of TMS1 in epithelial cells is sufficient to induce apoptosis via a mechanism that is dependent on caspase-8 [101-103]. There is also evidence to suggest that TMS1-induced apoptosis is dependent on the intrinsic mitochondrial pathway in some cell types [99-101]. Importantly, TMS1 is subject to aberrant DNA methylation and epigenetic silencing in a number of different tumor types [92-94,98,112], suggesting that loss of TMS1 confers a survival advantage to tumor cells. However, the mechanism by which silencing of TMS1 contributes to carcinogenesis remains unclear. Here, we examined the effect of TMS1 expression on anoikis in breast epithelial cells, and discovered a novel role for TMS1 in detachment-induced cell death and breast cancer progression.

Materials and Methods

Cell culture and anoikis assays. MCF10A cells were obtained from the Karmanos Cancer Institute and maintained in DMEM/F12 plus 5% fetal bovine serum, 20 ng/ml epidermal growth factor, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, and 2 mM glutamine. MCF10A/pBabe and MCF10A/Bcl-2 cells were a kind gift from Dr. Joan Brugge (Harvard Medical School, Boston, MA; ref. 21). For anoikis assays, cell culture dishes were coated twice with 20mg/ml poly-HEMA (Sigma) in 95% ethanol and dried overnight. poly-HEMA–coated dishes were washed twice with 1XPBS and seeded with 2.5 X 10^6 MCF10A cells per 10-cm dish in complete medium with 0.5% methylcellulose (Sigma). Methylcellulose containing medium was prepared by adding methylcellulose and a magnetic stir bar to a 250ml glass bottle, which was then autoclaved. Serum-free DMEM-F12 was heated to 65˚C and added to the sterile methylcellulose. The mixture was stirred at room temperature for 30 minutes and then stirred overnight at 4˚C. Components listed above were added to produce complete medium containing 0.5% methylcellulose. Treatments with MEK inhibitor, PD98059 (Calbiochem) were conducted at 40 µM.

Immunohistochemistry. Archived specimens of paraffin-embedded normal breast tissue (n = 4) and infiltrating ductal carcinoma (n = 20) with associated ductal carcinoma *in situ* (DCIS) were obtained from the Avon/Grady Memorial Hospital Breast Tumor Bank. Sections were deparaffinized, subjected to antigen retrieval, and incubated with primary antibody. The TMS1 antibody was an affinity-purified rabbit polyclonal antibody (EU107) raised against a peptide encompassing amino acids 182 to 195 of human TMS1,

and has been described [94]. The E-cadherin antibody is from Invitrogen (#15068). Immunocomplexes were detected by the avidin-biotin complex method, using diaminobenzidine as the chromogen (DAKO). Sections were counterstained with hematoxylin.

Immunoblotting. Cells were pelleted, washed three times in 1 X PBS, and lysed with RIPA buffer (150mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS and 50mM Tris pH 8.0) or 1% NP-40 buffer (150mM NaCl, 1% NP40, and 50mM Tris pH 8.0, 5mM EDTA) containing 1 X protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche, Indianapolis, IN, USA), 2mM Na_3VO_4, 2mM Na-molybdate, and 2mM Na-pyrophosphate (Sigma). Total protein was separated by SDS–PAGE gel, transferred to PVDF or nitrocellulose (BioRad, Hercules, CA, USA), and probed with the indicated primary antibody. Immunocomplexes were detected by incubation with horseradish peroxidase-conjugated secondary antibody and chemiluminescence detection (Pierce, Rockford, IL, USA). The antibodies used were as follows: anti- ASC/TMS1 (MBL or Protein Tech), β-tubulin (Sigma), Bim (Stressgen), caspase-8, p44/42 MAPK/ERK1/2, phospho-p44/42 MAPK/ERK1/2 (Cell Signaling Technologies), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam), nuclear factor-κB (NF-κB) p65, Bcl2 (Santa Cruz), PARP (Alexis Biochemicals), and PARP p85 (Promega).

siRNA transfection. MCF10A cells (5.5 $X10^5$) were seeded in 10-cm dishes and transfected the following day with 200 nM of the indicated siRNA using Oligofectamine (Invitrogen). siRNA duplexes were from Dharmacon and had the following sequences:

TMS1 siRNA#1, 5'CGAGGGUCACAAACGUUGAdTdT3' (sense); TMS1 siRNA#2, 5'GCAAGAUGCGGAAGCUCUUdTdT3'; p65 siRNA, 5'GCCCUAUCCCUUUACGUCAdTdT3'. siControl NonTargeting siRNA#1 (Dharmacon) or siRNA against lamin a/c were used as controls.

Reverse transcription and real-time PCR: Total RNA was isolated from MCF10A cells using the RNeasy Mini kit (Qiagen) and pretreated with DNase I. cDNA was then synthesized using random hexamer primers and MMLV- reverse transcriptase. TMS1, Bim or 18S primers were used to amplify cDNA in triplicate using a reaction mixture that contained 1 µL of the appropriately diluted DNA sample, 0.2 µM primers, and 12.5 µL of IQ SYBR Green Supermix (Bio-Rad). The reaction was subjected to a hot start for 3 min at 95°C and 40 cycles of 95°C, 10 s; 55°C, 30 s. cDNA quantities were determined relative to a standard curve generated using MCF7 cDNA. Primers for real-time PCR analysis were for TMS1: 5'TCCAGCAGCCACTCAACG3'and 5'GCACTTTATAGACCAGCA3'; Bim: 5'TGCAGACATTTTGCTTGTTCAA3' and 5'GAACCGCTGGCTGCATAATAAT3' and 18S: 5'GAGGGAGCCTGAGAAACG G-3' and 5'-GTCGGGAGTGGGTAATTT GC-3'.

Co-immunoprecipitation: MCF10A cells were allowed to grow on tissue-culture treated or polyHEMA coated dishes for 24 hours at which time they were lysed in 1%NP40 buffer (recipe above). Lysates were pre-cleared with BSA-blocked protein-G agarose beads. Precleared lysates were incubated with anti-mouse IgG or anti-TMS1 (MBL) and rocked overnight at 4°C. BSA-blocked protein-G agarose beads were once again added

to each sample which was rocked for an additional 2 hours at 4°C. Immunocomplexes were subsequently washed in lysis buffer 4 X 10 min, boiled in 2X laemmli buffer, separated by electrophoresis on SDS- 10% polyacrylamide gels, and analyzed by western blot.

Results

TMS1 is a proapoptotic protein that is subject to epigenetic silencing in a significant proportion of breast and other cancers [92-94,98,112]. We have previously shown that in normal breast tissue, TMS1 is selectively expressed in the ductal and lobular epithelium, whereas its expression is absent from the underlying myoepithelial and stromal cell compartments [98]. We extended these findings to examine the expression of TMS1 in DCIS lesions and invasive breast carcinomas. In DCIS lesions occurring adjacent to areas of invasive ductal carcinoma, we found that although the ductal epithelial cells in direct contact with the stroma retained TMS1 expression, a subset of the DCIS lesions examined showed reduced TMS1 expression in the majority of the epithelial cells that had filled the breast duct (Figure 1A, i–iii). Furthermore, it seemed that the cells closest to the basement membrane retained TMS1 expression, whereas those in the center of the duct lacked TMS1 expression. This pattern was noted in both solid-form (Figure 1A, i and ii) and cribriform (Figure 1A, iii) DCIS. This did not seem to be a reflection of a generalized loss of epithelial cell characteristics, as the central cells that selectively lose TMS1 expression retain E-cadherin expression (Figure 1B). In addition, although the majority of invasive breast carcinomas examined showed varying degrees of TMS1 expression (Figure 1A, iv and v), ~16% (3 of 19) of invasive ductal

carcinomas had completely lost TMS1 expression (Figure 1A, vi). Note that even in invasive breast carcinomas that lose TMS1 expression (Figure 1A, vi, ca), infiltrating ‘‘normal’’ inflammatory cells retain TMS1 expression (Figure 1A, iv and vi, inf). These data suggest that TMS1 expression is selectively lost in the aberrant epithelial cells filling the breast duct in some DCIS lesions and invasive breast carcinomas. Considering the limited data set, we cannot determine the relationship between TMS1 expression in DCIS and invasive carcinomas (i.e., whether loss of TMS1 in certain DCIS lesions ultimately gives rise to invasive ductal carcinomas lacking TMS1 expression). Nevertheless, the data are suggestive that loss of TMS1 accompanies the transition from DCIS to invasive carcinoma during the progression of breast cancer.

Considering the proapoptotic nature of TMS1 [99,103] and the unusual expression pattern observed in DCIS lesions, we hypothesized that downregulation of TMS1 might play a role in the resistance of breast epithelial cells to anoikis. To test this idea, we examined the effect of detachment on TMS1 expression in the nontransformed breast epithelial cell line MCF10A. Forced suspension of MCF10A cells led to a significant induction of TMS1 expression (Figure 2). This upregulation occurred with similar kinetics to that of Bim, a proapoptotic BH3-only protein known to be upregulated during anoikis (Figure 2). Previous work from our laboratory has shown that TMS1 is upregulated in breast epithelial cells in response to TNFα stimulation in an NF-κB-dependent manner [103]. In contrast, siRNA-mediated knockdown of NF-κB did not impact suspension-induced upregulation of TMS1 (Figure 2C and D), indicating that upregulation of TMS1 occurs independently of this pathway.

We next determined the effect of TMS1 silencing on the apoptotic response to detachment in MCF10A cells. Cells transfected with control siRNA or siRNA directed against TMS1 were seeded onto poly-HEMA–coated tissue culture dishes and analyzed over 48 hours. The forced suspension of control MCF10A cells induced a time-dependent cleavage of procaspase-8 and the downstream caspase target PARP (Figure 3), indicating that the cells are undergoing apoptosis. Strikingly, knockdown of TMS1 led to a significant delay in the apoptotic events associated with anoikis. Neither cleavage of procaspase-8 nor PARP cleavage was observed in TMS1 knockdown cells until 48 hours postdetachment (Figure 3). Consistent with the observed delay in PARP cleavage, knockdown of TMS1 in MCF10A cells conferred a 2- fold protection from cell death after 24hours in suspension (data not shown). This degree of protection is similar to previous reports describing the effect of Bim knockdown on detachment-induced apoptosis in breast epithelial cells [75]. In this regard, we also examined the effect of TMS1 on the regulation of Bim. Induction of Bim was evident by 8 hours postdetachment in control cells (Figure 3). Strikingly, both the magnitude and timing of Bim protein accumulation were significantly inhibited in TMS1 knockdown cells (Figure 3). The effect of TMS1 knockdown was specific, as a similar delay in apoptotic events (caspase-8 cleavage, PARP cleavage) and inhibition of Bim upregulation were not observed when transfected with siRNAs targeting lamin a/c or caspase-1 (data not shown). Taken together, these data illustrate that caspase-8 cleavage, PARP cleavage, and Bim upregulation are severely inhibited in cells lacking TMS1 expression, and that loss of TMS1 confers resistance to anoikis.

Bim is regulated by both transcriptional and posttranslational mechanisms. In the latter case, integrin-mediated survival signaling through the mitogen-activated protein kinase (MAPK) pathway leads to ERK-mediated phosphorlyation of serine 69 on BimEL, the largest isoform of Bim [77]. This phosphorylation stimulates BimEL ubiquitination and subsequent degradation by the proteasome [77]. The cessation of ERK signaling after detachment from the ECM induces both an increase in Bim transcription [75], and the dephosphorylation of BimEL, allowing for stabilization and accumulation of Bim protein [77]. To examine in more detail the effect of TMS1 downregulation on detachment-induced upregulation of Bim, we performed extensive time course experiments. Suspension of MCF10A cells caused a time-dependent increase in TMS1 protein expression, which preceded that of BimEL (Figure 4A). Again, knockdown of TMS1 severely inhibited the induction of BimEL expression (Figure 4A). The effect of TMS1 loss on Bim expression occurred primarily at the level of protein accumulation, as there was little effect of TMS1 knockdown on the detachment-induced upregulation of Bim mRNA (Figure 4B). We further examined the effect of TMS1 knockdown on ERK activation. Interestingly, even under attached, unstimulated conditions, there were increased levels of phospho-ERK in cells lacking TMS1 relative to controls (e.g., 48-hour monolayer cultures; time, 0 hour; Figure 5). Detachment and a shift to fresh serum-containing medium led to a stimulation of ERK phosphorylation that was both greater in magnitude and persisted longer in TMS1 knockdown cells compared with control cells (Figure 5). These data indicate that there is a pool of ERK that is constitutively active in the absence of TMS1 and that the delay in detachment-induced Bim upregulation occurring in the absence of TMS1 may be due to misregulation of ERK signaling. Taken

together, these data suggest that TMS1 acts upstream of Bim and that the resistance to anoikis observed in TMS1 knockdown cells may be, in part, due to the inhibition of Bim induction.

To determine whether the inhibition of detachment-induced Bim upregulation in TMS1 knockdown cells is due directly to TMS1 loss or rather is a consequence of the observed delay in anoikis, we compared the effects of TMS1 knockdown on the induction of anoikis in MCF10A cells constitutively overexpressing Bcl2 [124]. As shown above, knockdown of TMS1 inhibited both the upregulation of BimEL protein and apoptosis after 24 hours in suspension (Figure 6). Overexpression of Bcl2 had no effect on detachment-induced increase in BimEL or TMS1 expression in control cells, nor did it affect the delayed kinetics of Bim accumulation in TMS1 knockdown cells (Figure 6). It did, however, prevent detachment-induced apoptosis, as indicated by the reduced cleavage of PARP (Figure 6). Taken together, these data indicate that the detachment-induced increase in TMS1 protein expression is caspase-independent and not a downstream consequence of anoikis, as has been observed for BimEL induction [77]. These data further indicate that the effect of TMS1 loss on detachment-induced upregulation of BimEL protein expression is direct, and not due to an overall inhibition of anoikis.

As the kinetics of TMS1 induction mirrored that of Bim, we next hypothesized that these two proteins may be regulated by similar pathways during anoikis. To determine if TMS1 is regulated by ERK1/2 signaling, MCF10A cells were grown in attached conditions in the presence or absence of the MEK inhibitor, PD98059, for up to 48 hours and analyzed for TMS1 expression. Treatment of cells with this inhibitor did

not significantly impact TMS1 protein or mRNA upregulation, yet induction of BimEL protein levels were observed (Figure 7A-B). Moreover, inhibition of ERK1/2 signaling during anoikis did not affect detachment-induced upregulation of TMS1 (Figure 7C). Together, these data suggest that the regulatory mechanisms controlling TMS1 expression during anoikis are distinct from some pathways that regulate Bim.

In addition to regulating BimEL accumulation during anoikis, our data suggest that TMS1 is necessary for cleavage of pro-caspase-8 upon cellular detachment (Figure 3). Activation of caspase-8 is an early event in anoikis of certain epithelial cell lines [85,86]. We have shown that overexpression of TMS1 is sufficient to induce cleavage of caspase-8 [103]. Furthermore, Masumoto *et al.* have shown an interaction between tagged caspase-8 and TMS1 in overexpression studies [102]. Therefore, we sought to determine if TMS1 directly engages procaspase-8 in response to matrix detachment thereby promoting its self-cleavage and activation. MCF10A cells were grown on poly-HEMA coated (suspended conditions) and uncoated (attached conditions) tissue-culture dishes. TMS1 was then immunoprecipitated at various time points and blots were probed for caspase-8. While we were able to successfully precipitate TMS1 in attached and suspended cells, we were unable to detect caspase-8. A faint band does appear to run above the heavy chain in both attached and suspended samples precipitated with the TMS1 antibody that is not present in samples precipitated with an IgG antibody. This band may correspond to the upper band of the doublet seen in the input for caspase-8 (Figure 8) although these data are not conclusive.

Discussion

In summary, we find that TMS1 plays an important role in breast epithelial cell anoikis. We provide evidence that loss of TMS1 expression promotes resistance to anoikis, which may be mediated at least in part by the inhibition of detachment-induced upregulation of BimEL. Previous work has shown that anoikis in breast epithelial cells is dependent on Bim, in that factors that inhibit Bim upregulation, such as siRNA against Bim itself, or constitutive activation of the ERK1/2 pathway, also prevent detachment-induced cell death [75,77]. Our data indicate that apoptosis induced by detachment of MCF10A cells is similarly dependent on the induction of TMS1, and furthermore, that TMS1 is necessary for detachment-induced accumulation of BimEL. TMS1 seems to play a role in the suppression of ERK1/2 signaling, as its downregulation leads to ERK1/2 activation. These data suggest that TMS1 acts upstream of Bim, and may act as a link between loss of integrin signaling and the onset of anoikis, such that in the absence of TMS1, survival signaling through the ERK1/2 pathway persists even in the absence of matrix attachments.

In previous work, we and others have shown that TMS1 overexpression or forced oligomerization induces apoptosis, and that TMS1-induced apoptosis is dependent on caspase-8 [101-103]. Anoikis may be another context in which TMS1 regulates caspase-8 activity. However, we were unable to detect an interaction between TMS1 and procaspase-8 in suspended MCF10A cells. One technical issue that we have yet to overcome is that caspase-8 is approximately the same size as the heavy chain of IgG. Thus, detection of the heavy chain has impeded our efforts to determine if caspase-8 is being pulled down with TMS1. Alternatively, regulation of procaspase-8 cleavage by TMS1 may occur independently of a physical interaction. First described by Helfer *et al.*

[120], several recent studies have shown a novel, nonapoptotic role for procaspase-8 in cell adhesion and migration [121-123]. Collectively, these studies have shown that procaspase-8 is required for adhesion to the ECM and Src-dependent activation of the ERK1/2 pathway. This scaffolding function is independent of caspase-8 catalytic activity, and seems to be mediated by an adhesion-stimulated recruitment of procaspase-8 and Src to a complex at the lamella and Src-mediated phosphorylation of procaspase-8 [121,123,125]. Our data suggest that TMS1 is necessary for the efficient cleavage of procapase-8 at the onset of anoikis, which would both limit the pool of procaspase-8 available for adhesion and promote downstream apoptotic signaling.

In conclusion, we report a novel role for TMS1 in anoikis. TMS1 is upregulated after detachment of breast epithelial cells, and siRNA-mediated knockdown of TMS1 causes these cells to be resistant to anoikis. This resistance correlates with a delay in Bim protein upregulation and procaspase-8 cleavage, providing two mechanisms by which TMS1 may contribute to anoikis. Significantly, our results demonstrating the deleterious effects of TMS1 loss on sensitivity to anoikis are supported by *in vivo* data from DCIS lesions, where we see that aberrant epithelial cells persisting in the lumen of the breast duct in DCIS lesions no longer express TMS1. Together, our data illustrate that the epigenetic silencing of TMS1 observed in breast cancer and other tumor types might contribute to the progression of carcinomas by allowing epithelial cells to bypass anoikis.

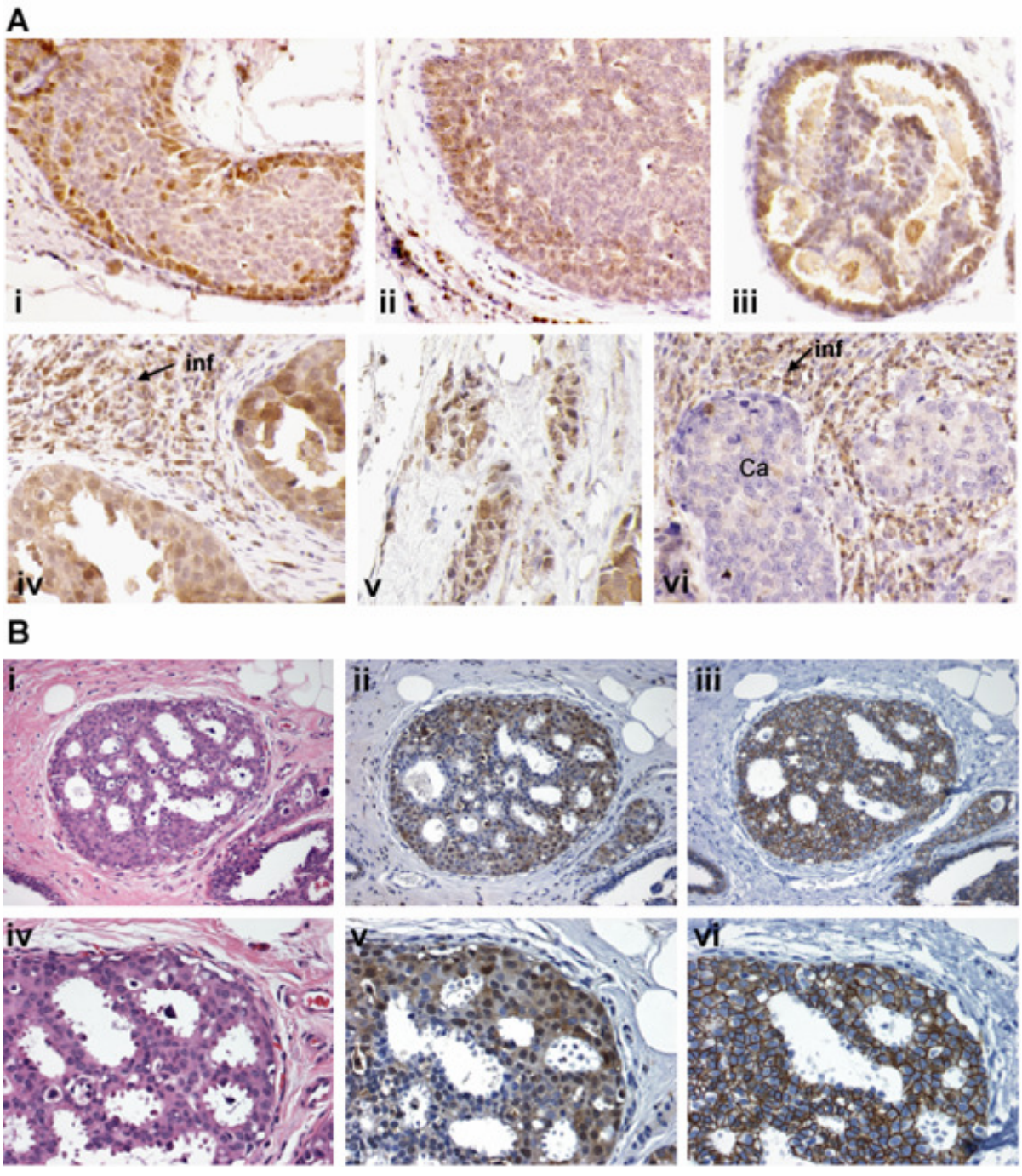
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Figure 1: TMS1 expression in primary DCIS and invasive ductal carcinomas. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded breast tumor tissue samples. The TMS1 antibody was an affinity-purified rabbit polyclonal antibody (EU107) raised against a peptide corresponding to amino acids 182 to 195 of human TMS1, and was used at a dilution of 1:400 as described [94,98]. Immunocomplexes were detected by the avidin-biotin complex method, using diaminobenzidine as the chromogen. Slides were counterstained with hematoxylin (blue). A) i to iii, TMS1 expression in DCIS. Note the unusual staining pattern in DCIS lesions with retention of TMS1 expression in the cells closest to the myoepithelium and basement membrane and loss of expression in the more dysplastic cells filling the luminal space. This pattern was most often observed in solid-form DCIS (i and ii) and cribiform DCIS (iii) lesions. iv to vi, TMS1 expression in invasive ductal carcinomas. Whereas some infiltrating glands retain TMS1 expression (iv and v), 3 of 19 invasive carcinomas showed complete loss of TMS1 expression (vi). Note that even in ductal carcinoma cases that lose TMS1 expression (vi; ca), surrounding normal inflammatory cells (iv and vi; inf) retain TMS1 expression. B) Relationship between TMS1 and E-cadherin expression in primary DCIS. Parallel sections were stained with H&E (i) or analyzed for the expression of TMS1 (ii) or E-cadherin (iii).The TMS1 antibody (EU107) was used at a dilution of 1:400, and the E-cadherin antibody (Invitrogen #15068) was used at a dilution of 1:50. Note the unusual TMS1 staining pattern in DCIS lesions with retention of TMS1 expression in the outermost cells and loss of expression in the more dysplastic cells filling the luminal space. The same cells that lose TMS1 expression retain E-cadherin expression. iv to vi, higher power (600) magnifications of the same lesion shown in i to iii (100). Shown is a representative DCIS lesion from a single individual. Published in Parsons MJ[#], Patel P[#], Brat DJ, Colbert L, Vertino PM. *Cancer Research*. 2009 Mar 1;69(5):1706-11[116].

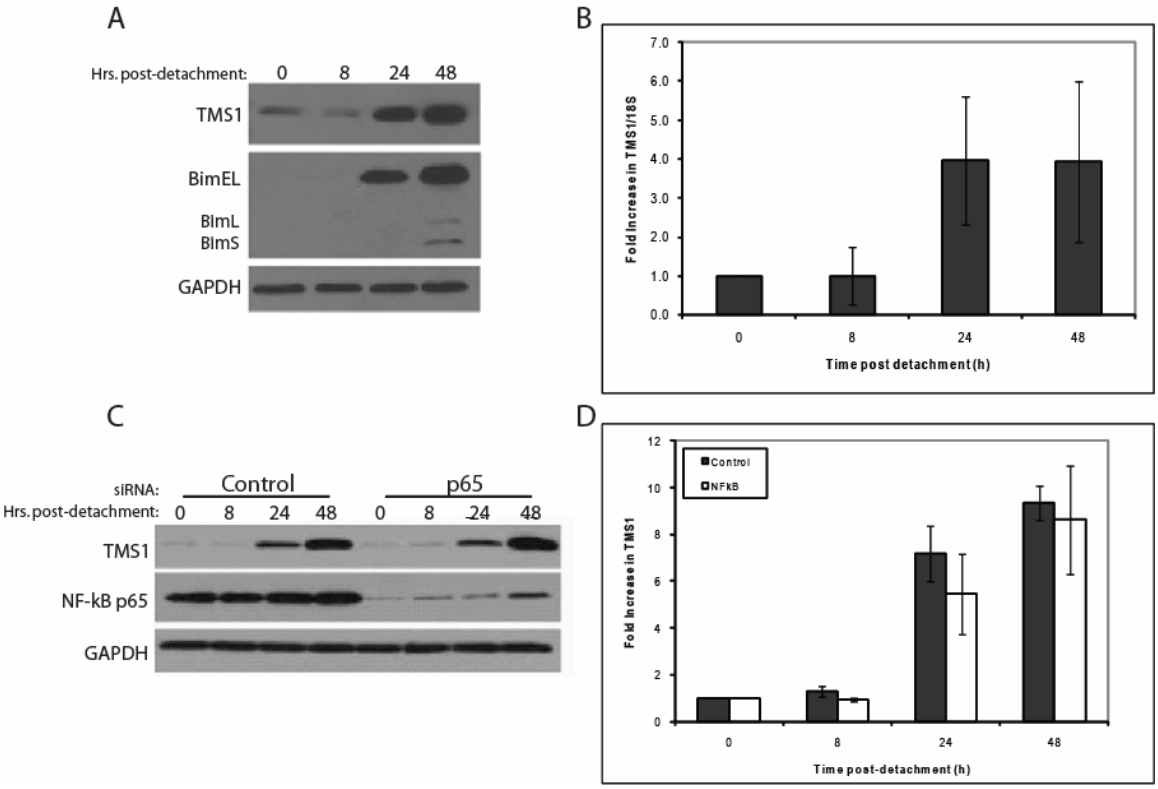


Figure 2: TMS1 protein and mRNA are induced during anoikis. MCF10A cells were plated onto poly-HEMA coated plates and harvested at the indicated time points. A) protein lysates were subjected to western blot analysis using antibodies against TMS1, Bim, and GAPDH. B) total cellular RNA was isolated and TMS1 mRNA expression levels were quantified by reverse transcription and real-time PCR. Relative starting quantities were determined by comparison to MCF7 cDNA standard curve included in each run. Shown is the relative increase in TMS1 mRNA compared with time zero after normalization to an 18s rRNA internal control. Columns, mean of three independent experiments performed in triplicate; bars, SD. C) MCF10A cells were transfected with either 200 nM scrambled siRNA (control) or siRNA targeting the p65 subunit of NF-κB (p65). After 48 h, cells were trypsinized and plated onto poly-HEMA–coated dishes. Lysates were collected and analyzed by Western blot with antibodies to TMS1, the p65 subunit of NF-κB, and GAPDH. D, total cellular RNA was isolated and TMS1 mRNA expression levels were determined and analyzed by real-time PCR as described in B. Shown is the relative expression of TMS1 mRNA compared with time zero after normalization to an 18srRNA internal control. Columns, mean of three independent experiments performed in triplicate; bars, SD. Published in Parsons MJ[#], Patel P[#], Brat DJ, Colbert L, Vertino PM. *Cancer Research*. 2009 Mar 1;69(5):1706-11[116].

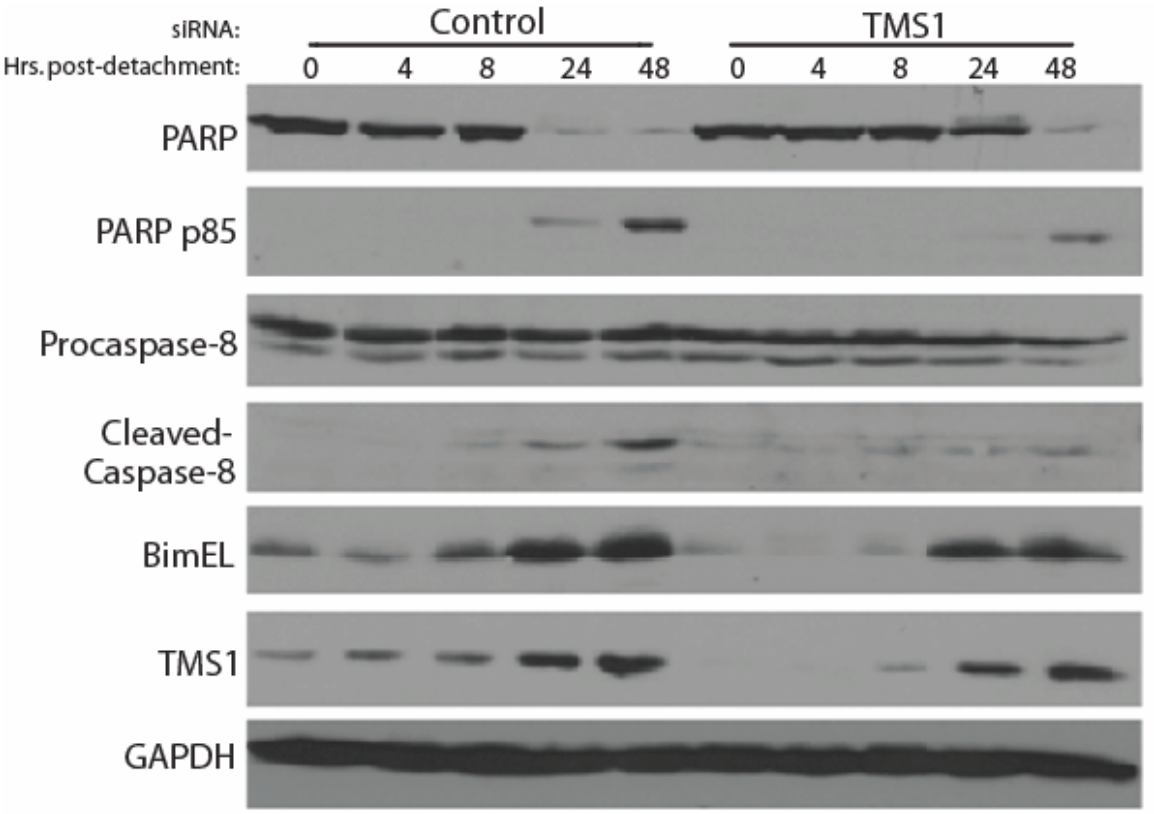


Figure 3: Knockdown of TMS1 expression inhibits anoikis. A, MCF10A cells were transfected with 200 nM of scrambled siRNA (control) or siRNA#2 targeting TMS1 (TMS1). After 48 h, cells were plated on poly-HEMA–coated dishes, and protein was isolated at timed intervals and subjected to western analysis with the indicated antibodies. Shown is a representative of three independent trials. Published in Parsons MJ[#], Patel P[#], Brat DJ, Colbert L, Vertino PM. *Cancer Research*. 2009 Mar 1;69(5):1706-11[116].

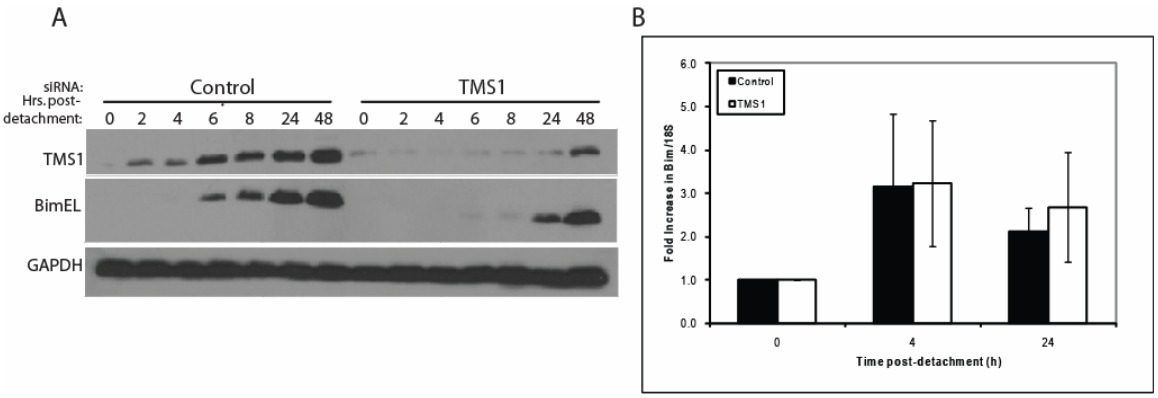


Figure 4: Impact of TMS1 knockdown on Bim upregulation during anoikis. MCF10A cells were transfected with 50nM scrambled siRNA (control) or siRNA#1 targeting TMS1 (TMS1). After 48 h, cells trypsinized and plated onto poly-HEMA–coated plates. A) Lysates were collected at the indicated time points and subjected to Western blot analysis using the indicated antibodies. Shown is a representative of three independent trials. B) Bim mRNA expression levels were quantified by real-time PCR analysis. Relative starting quantities were determined by comparison to MCF7 cDNA standard curve included in each run. Shown is the increase in Bim mRNA relative to time zero after normalization to an 18s rRNA control. Columns, mean of three independent experiments assayed in triplicate; bars, SD. Published in Parsons MJ[#], Patel P[#], Brat DJ, Colbert L, Vertino PM. *Cancer Research*. 2009 Mar 1;69(5):1706-11[116].

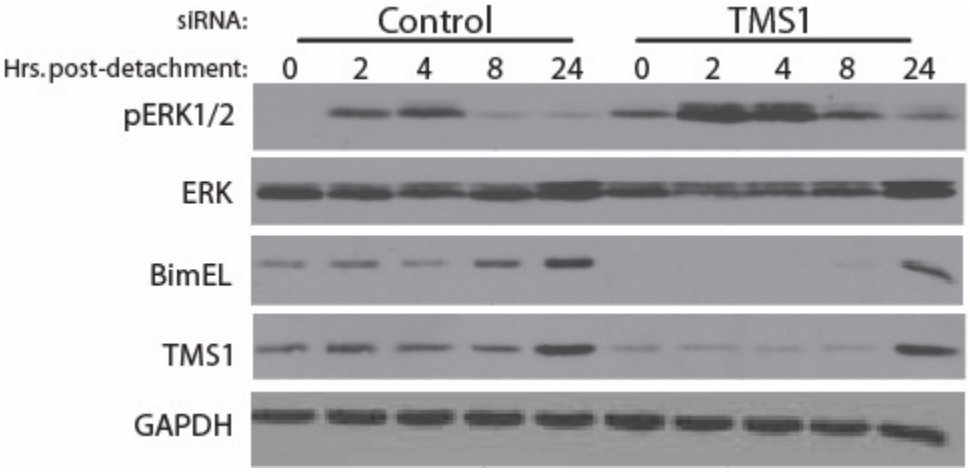


Figure 5: Silencing TMS1 promotes persistent ERK1/2 activation. MCF10A cells were transfected with 50 nM scrambled (control) siRNA or siRNA targeting TMS1. After 48 h, cells were trypsinized and plated onto poly-HEMA–coated plates. Lysates were collected at the indicated time points and subjected to western blot analysis using the indicated antibodies. Shown is a representative of three independent trials. Published in Parsons MJ[#], Patel P[#], Brat DJ, Colbert L, Vertino PM. *Cancer Research*. 2009 Mar 1;69(5):1706-11[116].

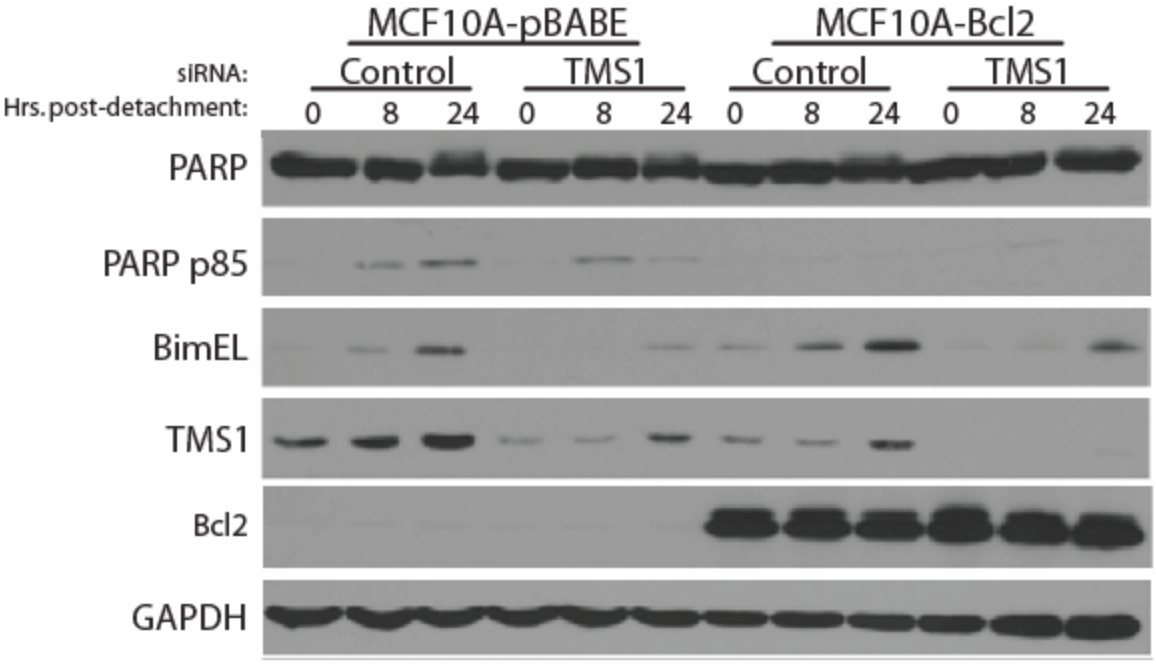


Figure 6: Requirement for TMS1 in detachment-induced Bim accumulation is apoptosis-independent. MCF10A-pBABE and MCF10A-Bcl-2 cells were transfected with 200 nM scrambled (control) or TMS1#1 siRNA. After 48 h, cells were trypsinized and plated onto poly-HEMA–coated dishes. Lysates were collected at the indicated time points and subjected to Western blot analysis with the indicated antibodies. Shown is a representative of three independent trials. Published in Parsons MJ[#], Patel P[#], Brat DJ, Colbert L, Vertino PM. *Cancer Research*. 2009 Mar 1;69(5):1706-11[116].

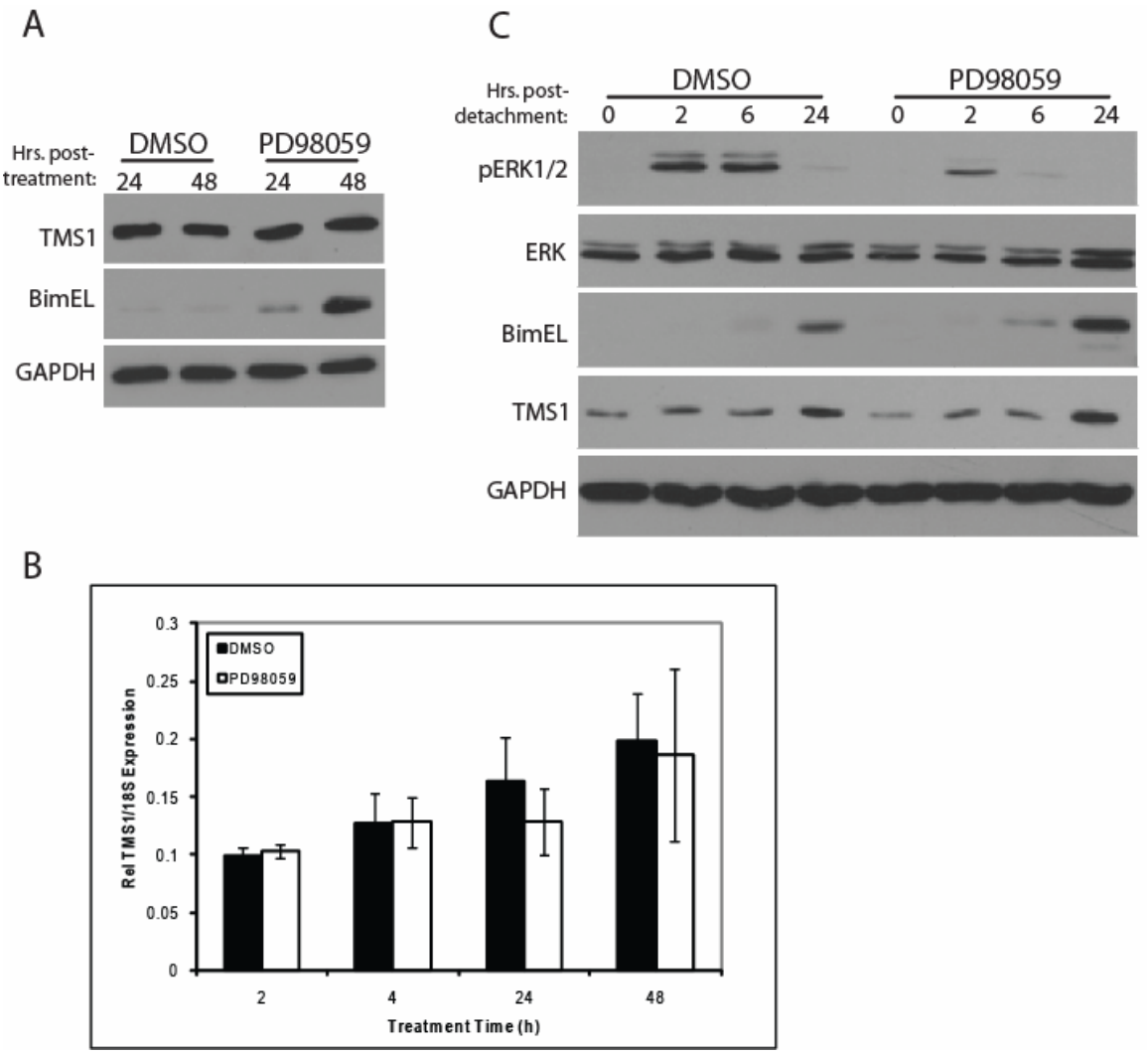


Figure 7: Effect of ERK inhibition on TMS1 expression. MCF10A cells were treated with the MEK inhibitor, PD98059, (40µM) for the indicated time points. A) Lysates were collected and analyzed by western blot. Shown is a representative of three independent trials. B) Total RNA was collected and TMS1 transcript levels were quantified using reverse transcription and real time PCR analyses. Shown is the relative expression of TMS1 mRNA after normalization to an 18srRNA internal control. Columns, mean of three independent experiments performed in triplicate; bars, SD. C) MCF10A cells were plated onto polyHEMA-coated dishes in the presence or absence of the MEK inhibitor, PD98059, (40µM) for the indicated time points. Lysates were collected and analyzed by western blot using the indicated antibodies. Shown is a representative of three independent trials.

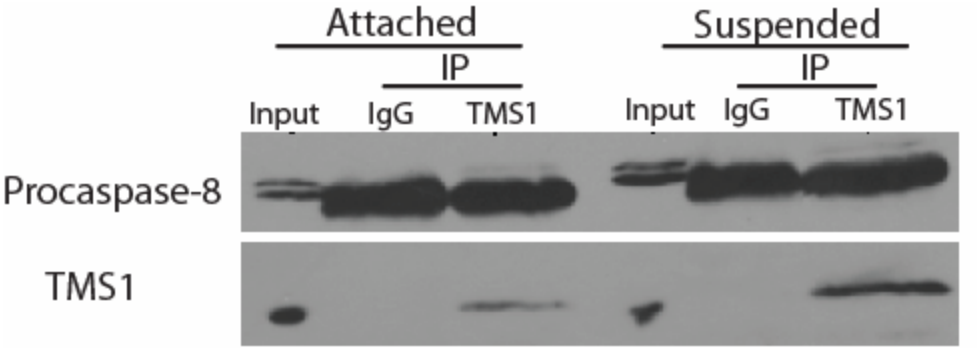


Figure 8: Immunoprecipitation of TMS1 in attached and detached cells. MCF10A cells were grown on uncoated (attached) or polyHEMA coated (suspended) tissue-culture dishes for 24 hours. Lysates were precipitated from these samples with a control antibody (IgG) or a TMS1 specific antibody. The resulting lysates were analyzed by western blot using antibodies against caspase-8 and TMS1. Shown is a representative of two independent trials.

Chapter 3

A nonapoptotic role for TMS1 in breast epithelial cell migration

Patel P completed all work shown in figures in this chapter except Figure 7A-C. For these experiments, Patel P performed siRNA knockdown of TMS1 and Erik R. Kline, Ph.D. (Hematology and Oncology, Winship Cancer Institute) performed subsequent wounding and live cell imaging analyses. Additionally, Adam I. Marcus, Ph.D. (Hematology and Oncology, Winship Cancer Institute) aided in capturing images shown in Figures 1A-C and 4A.

Introduction

Caspases are a family of enzymes that execute programmed cell death. These enzymes exist as inactive zymogens within the cell and become activated by a proteolytic cleavage cascade upon receipt of specific signals [58,67]. Death signals such as DNA damage or death receptor-ligand binding result in the oligomerization and activation of the initiator caspases, procaspase-9 or procaspase-8 that upon activation cleave downstream effector caspases including procaspase-3, -6, and -7. Effector caspases cleave their cellular substrates, resulting in cell death [67].

While the apoptotic functions for caspases are well established, many studies have also suggested nonapoptotic roles for these proteins in other cellular processes including innate immunity, cellular proliferation, and cell migration [60,126]. For example, the first identified caspase, caspase-1, exhibits a nonapoptotic role in inflammation. In response to pathogenic infection, caspase-1 cleaves the pro-inflammatory cytokines pro-interleukin-1β and -18 to generate an inflammatory response [127]. Additionally, caspase-11 exhibits a non-apoptotic function in murine cell migration. Caspase-11 physically and functionally interacts with actin-interacting protein 1 (AIP1), a binding partner of cofilin, to positively regulate actin depolymerization [128]. Most recently, procaspase-8 has been shown to exhibit a nonapoptotic role in cell adhesion and migration [123,125,129,130]. The molecular switch in function may be mediated by an interaction with Src as procaspase-8 is a target of Src-mediated phosphorylation at Y380 [125]. Phosphorylation of procaspase-8 at residue Y380 acts as a toggle such that when phosphorylated it can no longer be cleaved and instead is recruited to the cellular periphery where it interacts with Src [121] and PI3K [123]. Other evidence indicates that

an interaction between procaspase-8 and Src is necessary for cell adhesion and downstream activation of the ERK1/2 pathway [122].

TMS1 (target of methylation-induced silencing 1) was originally identified by our laboratory in a screen for targets of methylation-mediated silencing and simultaneously by an independent group as ASC (apoptosis speck-like protein containing a CARD), a protein that formed speck-like structures in retinoic acid treated leukocytes [97]. Subsequent studies showed that TMS1 is methylated in 40% of human breast tumors [92]. *TMS1* has since been found to be methylated and silenced in a variety of cancers [92,93,98,111,112,131] supporting a tumor suppressor role for this gene. However the precise function of TMS1 remains unclear. The *TMS1* locus encodes a bipartite protein consisting of an N-terminal pyrin domain (PYD) and a C-terminal CARD domain [99,101-103]. Forced oligomerization of TMS1 is sufficient to induce caspase-8 dependent apoptosis. Additionally, there is evidence to suggest that the PYD of TMS1 can physically interact with a catalytically inactive caspase-8 [102]. While TMS1 is dispensable for TNF-α and TRAIL induced procaspase-8 cleavage, overexpression of TMS1 is sufficient to induce cleavage of procaspase-8, independent of death receptor-ligand binding [103].

In addition to its function in apoptosis, TMS1 exhibits a well studied nonapoptotic role in mediating inflammasome formation in macrophages resulting in activation of procaspase-1 [106,107,114]. Inflammasomes are multiprotein complexes that contain TMS1, procaspase-1, and pattern recognition receptors (PRRs) that recognize different pathogenic stimuli [104]. Inflammasomes vary by the PRR that they contain and some of these multi-protein structures depend on TMS1 to link PRRs to procaspase-1 [115].

Aggregation of TMS1 is associated with the formation of this complex [132] and inflammation-induced cell death [133].

Binding of extracellular matrix ligands such as fibronectin, collagen, or laminin to integrin receptors recruits intracellular signaling molecules including the nonreceptor tyrosine kinases focal adhesion kinase (FAK) and Src to the cytoplasmic domain of integrins. Recruitment of these proteins results in the formation of a signaling complex referred to as a focal adhesion complex that consists of proteins such as FAK, paxillin, and talin. Formation of focal adhesion complexes results in the activation of signaling pathways involved in cellular adhesion, migration and survival [45-47].

FAK, Src, and ERK1/2 signaling have well established roles in cell migration [45,46,134]. Illic *et al.* demonstrated that FAK null fibroblasts exhibit a 50% reduction in average speed relative to wild-type cells through time-lapse analyses of randomly migrating cells [135]. Defects in migration of FAK null cells were also observed in transwell migration assays and were attributed to an increased number of focal adhesions in these cells suggesting that FAK plays a key role in the turnover of focal adhesions [135]. FAK/Src signaling is necessary for FAK-mediated migration [134]. Src-mediated phosphorylation of FAK occurs at five residues, Y407, Y576/7, Y861, and Y925 all of which were shown to promote the release of adhesions at the trailing edge of migrating cells [50]. Moreover, phosphorylation at FAKY925 acts as a binding site for Grb2, an adaptor protein that promotes MAPK/ERK1/2 activation. ERK1/2 promotes migration through phosphorylation of substrates such as myosin light chain kinase (MLCK) and calpains. Both events promote focal adhesion turnover in polarized cells [136,137].

Primary cilia have become well recognized as sensory organelles that coordinate pathways controlling cellular processes such as cell migration [138]. Primary cilia are microtubule based organelles that emanate from the basal body and are characterized by microtubule doublets (A plus B tubules) arranged in a 9 + 0 pattern of cytoskeleton similar to the mother centriole from which it originates [139]. Both platelet-derived growth factor receptor-alpha (PDGFR-α) [140] and epidermal growth factor receptor (EGFR) [141] localize to the ciliary membrane. Interestingly, ligation of PDGFR-α in fibroblasts results in activation of the Akt and ERK1/2 pathways and rapid accumulation of pMEK1/2 along the length of the cilium. In the absence of a primary cilium, PDGF signaling through the MAPK pathway is inhibited [142].

Centrosomes consist of a pair of centrioles ('mother' and 'daughter') embedded in the pericentriolar material which is a matrix of proteins including members of the pericentrin family. The mother centriole gives rise to the basal body of cilia. Centriole duplication and cilia formation are tightly linked to the cell cycle. Centrosomal duplication begins at the onset of S phase in which new 'daughter' centrioles are assembled into mother centrioles. These nascent centrioles elongate through the S and G2 phases. At the G2/M transition, the newly duplicated centrosomes migrate away from each other to establish the poles of the mitotic spindle. At the end of mitosis, each daughter cell inherits a pair of centrioles that are disengaged (i.e. no longer display an orthogonal association between mother and daughter centrioles). Cells then proceed to G1 or may enter arrest and form primary cilia [143,144].

Centrosomes organize microtubules into an astral array that functions in cell division, polarity, and intracellular trafficking [144]. Recent studies have also shown a

role for centrosomes in cell migration. pFAKS732 localization to centrosomes was shown to be essential for neuronal migration in development of the brain in the mouse embryo [145]. Additionally, mutations in the centrosomal protein, pericentrin, similarly inhibited neuronal migration in the mouse brain [146].

In Chapter 2, we elucidated a role for TMS1 in anoikis or detachment-induced apoptosis in breast epithelial cells. We found that silencing TMS1 promoted resistance to anoikis by hindering accumulation of the BH3 only protein, BimEL, and cleavage of procaspase-8 upon detachment. In light of recent data showing a role for procaspase-8 in adhesion and migration, we hypothesized that TMS1 may similarly be an important regulator of these cellular processes in breast epithelial cells. Here we explored a nonapoptotic role for TMS1 in adhesion and migration in the immortalized breast epithelial line, MCF10A.

Materials and Methods

Cell culture and antibodies. MCF10A cells were a kind gift from Dr. Joan Brugge (Harvard Medical School, Department of Cell Biology) and maintained in DMEM/F12 plus 5% fetal bovine serum, 20ng/ml epidermal growth factor, 0.5ug/ml hydrocortisone, 100ng/ml cholera toxin, 10ug/ml insulin, and 2mM glutamine (complete media). Cells were starved prior to conducting signaling assays (Figure 2) by rinsing 2X in PBS and incubating in DMEM/F12 for 16 hours. Serum starvation to induce cell growth arrest and cilia formation (Figure 6) was conducted in DMEM/F12 plus 20ng/ml epidermal growth factor, 0.5ug/ml hydrocortisone, 100ng/ml cholera toxin, 10ug/ml insulin, and 2mM glutamine for 24 hours. Fibronectin was purchased from Millipore and used to coat

cell culture surfaces according to manufacturer instructions at a final concentration of 4µg/cm^2. The following antibodies were purchased from Cell Signaling Technology, anti- p44/42 MAPK/ERK1/2, phospho-p44/42 MAPK/ERK1/2, pFAKY925. Other antibodies used were pFAKY397 (Invitrogen), FAK (BD Biosciences), TMS1 for immunofluorescence (MBL) and for western blot (ProteinTech), GAPDH (Abcam) and β-tubulin (Sigma).

siRNA Transfection. MCF10A cells (6.0 x 10^5) were seeded in 10 cm dishes and transfected the following day with 200nM of the indicated siRNA using Oligofectamine (Invitrogen). siRNA duplexes were purchased from Dharmacon (Lafeyette, CO) and had the following sequence: TMS1 siRNA: 5'-CGA GGG UCA CAA ACG UUG A dTdT-3' (sense). Dharmacon siControl NonTargeting siRNA #4 was used as a control.

Western blot analysis. MCF10A cells (1 X 10^6) were plated on nontissue-culture petri dishes that were pre-coated with fibronectin (described above). At each time point, cells were washed in ice-cold PBS and scraped into 1% NP40 lysis buffer (50mM Tris pH 7.5, 2mM EDTA, 100mM NaCl, 1%NP40) containing 1 X protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche, Indianapolis, IN, USA), 2mM Na_3VO_4, 2mM Na-molybdate, and 2mM Na-pyrophosphate (Sigma, St Louis, MO, USA). Lysates were then incubated on ice for at least 10 min, followed by centrifugation at 13,000g for 10 min at 4°C. Cleared lysates were separated by electrophoresis on SDS (10%: FAK, 15%: TMS1 and ERK1/2, GAPDH)-polyacrylamide gels and transferred to a PVDF membrane (BioRad). Blots were blocked in 5% nonfat dry milk in 0.1% Tween-20 in 1X TBS

(TTBS) for 1 hour at room temperature. Incubation with 1° antibody was done overnight at 4°C [diluted in 5% milk (TMS1 1:1000, p44/42 MAPK 1:1000, β-tubulin 1:1000, GAPDH 1:10,000, or 5% BSA (p-FAKY397 1:1000, p-FAKY925 1:1000, FAK 1:1000, phospho-p44/42 MAPK 1:1000) in TTBS], followed by incubation in horseradish peroxidase-conjugated secondary antibodies and detected by chemiluminescence (Thermo Scientific).

Immunofluorescence. Glass coverslips were precoated with fibronectin in 24-well dishes. MCF10A cells (5 X 10^4) were plated onto coverslips for up to 24 hours. Cells were fixed in PHEMO buffer (68mM PIPES, 25mM HEPES, 15mM EGTANa2, 3mm $MgCl_26H_2O$, and 5ml DMSO) containing 3.7% formaldehyde, 0.05% glutaraldehyde, and 0.5% Triton X-100 for 10 min at room temperature (RT). Coverslips were blocked in 10% goat serum in PBS for one hour at RT. Coverslips were incubated with primary antibodies against TMS1 (MBL) 1:250, pFAKY397 1:500, and pFAKY861 1:500 (BD Biosciences) overnight at 4°C. Alexafluor 488 or 555 secondary antibodies incubations were done at 1:500 for 1hour at RT (Invitrogen). All antibodies were diluted in 5% goat serum in PBS. Coverslips were incubated in 300mM 0.4 µmol/L 4‘,6-diamidino-2-phenylindole (DAPI) to stain nuclei. Coverslips were mounted using mounting medium (Electron Microscopy Sciences) and dried overnight in the dark. Imaging analyses were performed using the Zeiss LSM510 Confocal Microscope (Zeiss) with a 63X (numerical aperture, 1.4) Apochromat objective and analyzed by the LSM510 image browser.

Coimmunoprecipitation: MCF10A cells were allowed to grow on fibronectin-coated dishes for 24 hours at which time they were lysed in 1% NP40 buffer (50mM Tris pH 8.0, 5mM EDTA, 150mM NaCl, and 1% NP40). Lysates were pre-cleared with BSA-blocked protein-G agarose beads. Precleared lysates were incubated with anti-mouse IgG or anti-TMS1 (MBL) and rocked overnight at 4°C. BSA-blocked protein-G agarose beads were once again added to each sample which was rocked for an additional 2 hours at 4°C. Immunocomplexes were subsequently washed in lysis buffer 4 X 10 min at 4°C, boiled in 2X laemmli buffer, separated by electrophoresis on SDS-10% polyacrylamide gels, and analyzed by western blot.

Adhesion assay. MCF10A cells (3×10^4) were plated onto uncoated or fibronectin-coated 96-well tissue culture dishes. Cells were allowed to adhere over a two hour time course. At each time point, cells that failed to adhere were removed and each well was washed 1 X with PBS. After washing, regular growth media was added to the well until the last time point was collected at which time cell number was determined using the Cyquant Proliferation Assay (Invitrogen).

Transwell migration. Inserts (8.0µM, BD Biosciences) were placed in 24 well dishes. MCF10A cells were trypsinized, quenched in Resuspension media (DMEM-F12 containing 20% FBS and 2mM L-glutamine) and washed 2X in PBS. Cells (5×10^4) were plated into each insert in complete MCF10A media lacking EGF. Complete MCF10A media (containing 5ng/ml EGF) was added to the bottom chamber of the 24-well dish. Cells were allowed to migrate for eight hours. Following each time point, cells

that failed to migrate to the bottom filter were removed with q-tips and migrated cells were fixed and stained using the HEMA 3 stain set (Fisher Scientific). Filters were mounted onto glass coverslips and dried overnight at room temperature. Cells were counted across ten random fields.

Boyden chamber/Matrigel invasion assay. Matrigel (Trevigen) was diluted to final concentration of 272µg/ml in DMEM-F12 and used to pre-coat 8.0µM inserts (BD Biosciences) for 1 hour at 37°C. MCF10A cells were trypsinized, quenched in Resuspension media (DMEM-F12 containing 20% FBS and 2mM L-glutamine) and washed 2X in PBS. Cells (5×10^4) were plated onto Matrigel-coated inserts in complete MCF10A media lacking EGF. Complete MCF10A media (containing 5ng/ml EGF) was added to the bottom chamber of the 24-well dish. Cells were allowed to invade for 24 hours. Following each time point, cells that failed to invade to the bottom filter were removed with q-tips and invaded cells were fixed and stained using the HEMA 3 stain set (Fisher Scientific). Filters were mounted onto glass coverslips and dried overnight at room temperature. Cells were counted across ten random fields.

Live cell wounding assay. Two-well chamber slides (LabTek) were pre-coated with fibronectin. Subsequently, 4×10^4 cells were plated into each well and allowed to attach overnight. Cells were then transfected with control (Luciferase) siRNA or siRNA targeting TMS1 and allowed to reach confluency. After 48hours, a 1µL or 10µL pipette tip was used to generate a wound in each well. Live-cell wound closure was observed using the temperature- and CO_2-controlled Confocal-Ultraview ERS system, and images

were analyzed using the Volocity software. Four representative DIC images of the wounds were acquired every 10 min for up to 20 hours at 63X magnification using the automatic ASI stage. Wound closure was quantified by measuring the width of the wound at each hour time-point and expressing it as a percent of the original wound width at time-point 0. The wound closure of the four representative fields was averaged for luciferase and TMS1 siRNA groups for each experiment, and the experiments were repeated three times.

Results

We previously showed that TMS1 supports anoikis of breast epithelial cells in part through promoting cleavage of procaspase-8. Moreover, recent studies by independent groups have indicated that procaspase-8 pools at the periphery of attached and migrating neuroblastoma [129] and lung cancer cells [147] and interacts with Src and PI3K to mediate cell adhesion and migration [130]. These data coupled with the knowledge that TMS1 can bind directly to procaspase-8 [102] prompted us to examine an association of TMS1 with focal adhesions of breast epithelial cells where we hypothesized it could physically interact with procaspase-8. Such an association in attached cells would allow TMS1 and procaspase-8 to be in close proximity whereby upon detachment, TMS1 would be poised to mediate rapid and efficient procaspase-8 cleavage and subsequent cell death. To determine the subcellular distribution of TMS1 in attached cells, MCF10A cells were plated on fibronectin and TMS1 localization was examined by immunofluorescence over a 24 hour time course. We found that TMS1 distribution in attached cells was primarily diffuse throughout the cell and TMS1 did not

localize to focal adhesion complexes (Figure 1A). Interestingly, we found that in a subset of cells attached to fibronectin TMS1 localized to a distinct structure (Figure 1A) located most often at the apical surface of cells (Figure 1B). Confocal image analyses of these structures indicated that they were composed of small TMS1 aggregates that clustered to form a hollow, barrel-shaped structure with a width of ~1μM and a height of ~2μM (Figure 1C). Additionally, only one apical structure was typically observed in a given cell. To determine the mechanisms driving TMS1 localization to these structures, MCF10A cells were plated on uncoated or fibronectin-coated coverslips over twenty-four hours and the number of cells exhibiting TMS1 localization to barrel-shaped structures was quantified. As shown in Figure 1D, TMS1 never localized to apical structures in cells plated on uncoated coverslips at the 1 or 6 hour timepoints. However, we found that TMS1 redistributed to these structures in 1% of cells attached to uncoated coverslips following 24 hours of growth. In contrast, pre-coating of coverslips with fibronectin resulted in TMS1 distribution to apical structures at all time points tested (Figure 1D and data not shown). However, even under these conditions, the maximum number of cells with apical structures was observed at the 24 hour time point with ~3% of cells showing localization of TMS1 to these structures. Together these data suggest that adhesion to fibronectin induces TMS1 localization to apical, barrel-like structures in MCF10A cells.

The finding that adhesion to fibronectin induces redistribution of TMS1 to apical structures suggests that TMS1 might play a role in signaling downstream of integrin-fibronectin interactions. Binding of fibronectin to integrin receptors promotes the association of FAK with the cytoplasmic surface of integrins, resulting in autophosphorylation of FAK at tyrosine 397 and the assembly of focal adhesion

complexes that promotes signaling involved in cell adhesion, migration, and survival [45]. To investigate a role for TMS1 in fibronectin-induced signaling, we examined the impact of TMS1 silencing on adhesion-induced phosphorylation of FAK and downstream signaling events. MCF10A cells treated with control siRNA or siRNA targeting TMS1 were plated on fibronectin-coated dishes and adhesion-induced signaling was assessed over 60 minutes. Knockdown of TMS1 did not significantly impact pFAKY397 (Figure 2). Next, we examined the impact of TMS1 silencing on other downstream pathways. Autophosphorylation of FAK at Y397 promotes binding of SH2-containing proteins such as Src which acts to further phosphorylate FAK at additional sites. Src-mediated phosphorylation of FAKY925 directly recruits the adaptor protein, Grb2 and results in activation of the ERK1/2 pathway [45]. In control cells, pFAKY925 began to accumulate 20 minutes after adhesion to fibronectin. Interestingly, we found that knockdown of TMS1 significantly delayed phosphorylation of FAKY925 to 60 minutes post adhesion (Figure 2) as well as downstream activation of ERK1/2 (Figure 2). These data suggest that TMS1 plays a critical role in fibronectin-induced FAK signaling that results in downstream activation of the ERK1/2 pathway.

Our data showing that TMS1 promotes adhesion-induced signaling events in response to integrin-fibronectin binding suggest that TMS1 may be necessary for cell adhesion. Thus, we next sought to determine the functional impact of TMS1 silencing on adhesion. MCF10A cells treated with control siRNA or siRNA targeting TMS1 were allowed to adhere to fibronectin-coated dishes over two hours and the number of cells was quantified using a Cyquant proliferation assay (Invitrogen). These data showed that knockdown of TMS1 did not significantly impact adhesion in these cells (Figure 3).

Together, these data suggest that TMS1 is dispensable for adhesion-induced phosphorylation of FAK at Y397 and cell adhesion but plays a role in downstream FAK and ERK1/2 signaling.

The above studies indicate that adhesion of MCF10A cells to fibronectin induces TMS1 localization to distinct apical structures and that silencing TMS1 disrupts fibronectin-induced signaling. Thereafter, we determined whether FAK or ERK localized to apical structures. Interestingly, pFAKY397 localized with TMS1 to barrel-like structures in a subset of cells. pFAKY397 colocalization with TMS1 occurred at structures that were typically located in the most apical planes of the cell (Figure 4A, third and fourth panels). However, there was little colocalization between TMS1 and pFAKY397 at focal adhesions at the cellular periphery on the most basal planes of the cell. These data suggest that the association of pFAKY397 with TMS1 is specific to the apical structure (Figure 4A, second panel). The observation that pFAKY397 colocalized with TMS1 prompted us to examine a specific interaction between these proteins. However, attempts to co-immunoprecipitate pFAKY397 and TMS1 were inconclusive (Figure 4D).

To determine if FAK could associate with apical structures when phosphorylated at sites other than Y397, we examined the cellular distribution of pFAKY861 in these cells. Src-mediated phosphorylation of FAK at Y861 promotes the release of adhesions at the trailing edge of migrating cells [50]. We observed colocalization of pFAKY861 with TMS1 at apical structures in a subset of cells (Figure 4B). Quantitation of cells showing colocalization between pFAK and TMS1 showed that in 90% of cells where TMS1 was at the apical structure, pFAKY397 was also observed whereas pFAKY861

colocalized with TMS1 only 30% of the time (Figure 4C). Together these data suggest that TMS1 may localize to a critical signaling center in epithelial cells with a potential role in fibronectin-induced signaling through the FAK and ERK1/2 pathways.

Our data suggest that pFAK and TMS1 colocalize to a common structure. Given the barrel-like appearance of this structure and its striking resemblance to the cross section a centriole, we next examined a potential association of TMS1 with centrosomes. MCF10A cells were plated on fibronectin and stained with antibodies to TMS1 and the centrosomal markers, pericentrin and gamma-tubulin. TMS1 colocalized with both pericentrin (Figure 5A) and gamma-tubulin (Figure 5B) in a subset of cells suggesting that fibronectin induces TMS1 redistribution to centrosomes in a subset of cells.

Centrosomal duplication occurs during S phase and migration of duplicated centrosomes occurs in late G2 [143]. In Figure 5A, the two left most cells each contain two centrosomes suggesting that they have separated their centrioles and are either at G1/S or G2 phases of the cell cycle. Of these, the cell on the right has two centrosomes that are present near each other and are visible in a single plane of the cell suggesting that this cell is in the early stages of G2. In contrast, the centrosomes in the cell on the left are visible in two different planes suggesting that they have begun to migrate away from each other and that this cell is in late G2. Colocalization of TMS1 with pericentrin specifically occurred at the centrosome at the apical surface of the cell in late G2. These data suggest that TMS1 association with centrosomes is also linked to the cell cycle and is specific to centrosomes at the apical surface of cells.

Centrioles are precursors of the basal body that gives rise to a primary cilium. An association of TMS1 with centrosomes in the most apical planes of cells suggests that

TMS1 may function in cilia formation. To determine if TMS1 exhibits a role in the formation of primary cilia, we next examined the impact of TMS1 knockdown on cilia formation. siRNA treated MCF10A cells were plated on fibronectin-coated coverslips and serum starved to induce cell-cycle arrest. Cells were then fixed and stained with an antibody against acetylated-alpha tubulin, a commonly used marker of cilia (Figure 6A). Control and TMS1 knockdown cells presenting a primary cilium were counted at random and the percentage of cells with cilia was determined. As shown in Figure 6B, knockdown of TMS1 did not inhibit primary cilia formation.

Disruption of FAK [50,135] and ERK1/2 [136,137] signaling are associated with defects in cell migration. To determine the impact of TMS1 silencing on cell migration, a live cell wounding assay was performed in control and TMS1 knockdown cells. MCF10A cells were transfected with control or TMS1 siRNA and allowed to reach confluency at which time a pipette tip was used to generate a wound across the monolayer. Live cell imaging was then used to track wound closure over 24 hours. Silencing TMS1 resulted in a significant delay in wound closure (Figure 7A). Cell movement during wound healing can include measurements of individual cell velocity and the meandering index (an index of movement pattern). Movement tracks were generated for randomly chosen individual cells in both control and TMS1 knockdown samples to measure these parameters. Together these data showed that while transient silencing of TMS1 delayed overall wound closure, it did not significantly affect individual cell velocity (Figure 7B) or the meandering index (Figure 7C).

We next used the transwell migration assay to further evaluate breast epithelial cell migration. In this assay, a dual-chamber system of growth is used to separate cells

from a chemoattractant by a membrane filter. Cells must migrate through pores in the filter to gain access to the chemoattractant. Previous work has shown that EGF induces MCF10A migration [148]. We found that knockdown of TMS1 resulted in a ~50% inhibition of migration of MCF10A cells towards an EGF stimulus (Figure 8A). Together, our data suggest that TMS1 is necessary for efficient migration of epithelial cells.

The ability of cells to migrate is necessary for cell invasion which is a prerequisite for the development of cancer metastases [149]. To determine the impact of TMS1 silencing on cell invasion we performed a Boyden chamber assay, which is an adapted form of the transwell migration assay. Membrane filters separating cells from a chemoattractant were pre-coated with Matrigel, a commercially available extracellular matrix. In this assay, cells can only gain access to a chemoattractant by invading through a layer of Matrigel prior to migrating through pores. We found that knockdown of TMS1 resulted in a ~50% inhibition in cell invasion through a layer of Matrigel (Figure 8B). Together these data suggest that while TMS1 is dispensable for cell adhesion to fibronectin, it promotes epithelial cell migration and invasion. Altogether these data establish a nonapoptotic role for TMS1 involving its redistribution to barrel-like structures, integrin-mediated cell signaling, and breast cell migration and invasion.

Discussion

Our data illustrate that fibronectin induces the relocalization of TMS1 to an apical, barrel-like structure. In a subset of cells, pFAK and centrosomal markers colocalize with TMS1 in these structures suggesting that these barrel-like structures may

act as a critical signaling center in epithelial cells. We provide evidence that loss of TMS1 prevents integrin-mediated activation of FAK and ERK1/2 signaling and results in a delay in cell migration across a wounded epithelial layer or migration across a transwell chamber towards an EGF stimulus. Moreover, we show that TMS1 functions in breast epithelial invasion through a basement membrane matrix in that silencing TMS1 inhibited cell invasion in a Boyden chamber assay.

TMS1 was originally identified as a protein that localized to distinct structures in retinoic acid treated cells. These structures were shown to be composed of smaller TMS1 aggregates that clustered to form a hollow sphere referred to as a 'speck' [97]. One proposed function of TMS1 aggregation in the formation of a speck-like structure is death. Formation of specks in leukocytes correlated with drug-induced apoptosis [97] and has also been associated with an inflammation-induced form of cell death termed pyroptosis characterized by cell swelling, lysis, and release of cellular contents [133]. Our laboratory showed that HEK293 cells overexpressing TMS1 underwent apoptosis that was associated with redistribution of TMS1 from the cytoplasm to perinuclear spherical structures. However, we also showed that speck formation was independent of apoptosis in that inhibition of apoptosis had no effect on speck formation [92]. Moreover, Richards *et al.* transfected HeLa cells with tagged TMS1 and examined these cells for evidence of apoptosis (visual identification of cell shrinkage, membrane blebbing and nuclear fragmentation) over a 72 hour time course. The percentage of speck-containing cells that showed signs of apoptosis was shown to increase as a function of time post-transfection [150]. Visualization of the speck and subsequent cell death in these studies were dependent on treatment of cells with death-inducing stimuli or ectopic

expression of TMS1. In contrast, we have shown that adhesion to fibronectin induces the relocalization of endogenous TMS1 to a barrel-like structure that is typically observed at the apical surface of cells. Whether this structure is the same as the 'speck' observed in other studies is presently unknown. In addition, we find that attachment to fibronectin and subsequent growth of MCF10A cells in complete media induces TMS1 aggregation and localization to apical structures in these cells. These findings suggest that distribution of TMS1 to these structures in MCF10A cells is dependent on cell adhesion and growth on a natural substratum rather than initiation of a cellular death process.

We found that pFAKY397 and pFAKY861 colocalize with TMS1 at the barrel-like structure in a subset of cells suggesting that this structure may play a role in FAK-dependent signaling in epithelial cells. However, we were unable to detect pFAKY397 coprecipitating with TMS1 in lysates from cells attached to fibronectin. These results may be due to the fact that colocalization of these two proteins was confined to a small subset (~3%) of cells. Additionally, it is possible that the pool of TMS1 that is precipitated from these cells consisted of soluble protein and that TMS1 in the apical structure is not efficiently extracted under the conditions used (e.g. 1% NP40 buffer). Further experiments will be necessary to determine if these proteins interact in MCF10A cells.

Our data suggest that TMS1 may be associated with centrosomes in a subset of cells. We showed that TMS1 and centrosomal markers localized to the barrel-like structures at the apical surface in a subset of cells. We also found that TMS1 and pFAK colocalized at these apical, barrel-like structures. Therefore, it is possible that both TMS1 and pFAK are redistributed to centrosomes where they may function to regulate

cell migration. This hypothesis is consistent with work by Xie *et al.* who demonstrated that pFAKS732 localizes to centrosomes and a centrosome-associated microtubule fork that is necessary for neuronal migration [145]. Phosphorylation of FAK at S732 is mediated by the serine/threonine kinase Cdk5 (cyclin-dependent kinase 5) a known regulator of neuronal migration [145,151]. However, the mechanisms by which an association of pFAKS732 with centrosomes might regulate migration remain unclear [151]. An independent study also demonstrated a key role for centrosomes in cell migration whereby a mutation in pericentrin inhibited neuronal migration in the developing mouse embryo [146]. Thus, localization of TMS1 to centrosomes may result in regulation of fibronectin-induced signaling pathways involved in migration.

Centrosomes consist of centrioles one of which gives rise to the basal body of primary cilia during cell cycle arrest. Our data suggest that the association of TMS1 with centrosomes is also tightly associated with the cell cycle. TMS1 and pericentrin colocalization occurred predominantly in cells that appeared to be in late G2 and was specific to the most apical centrosome. Since primary cilia emanate from mother centrioles that migrate to the apical surface of cells, we also examined the impact of TMS1 silencing on primary cilia formation. Our preliminary data suggest that TMS1 is not essential for primary cilia formation on a population level. However, this analysis does not discount a precursor role for TMS1 in cilia formation such that in the absence of TMS1, cilia formation is delayed but not otherwise hindered. Additionally, efficient knockdown of TMS1 in randomly counted cells was not assessed. Thus, it is also possible that cells that had efficiently silenced TMS1 lacked cilia but were indistinguishable from cells that had inefficiently silenced TMS1 and contained cilia.

Our findings showing specific colocalization of pFAKY397 with TMS1 in apical structures and not at focal adhesions are consistent with our other data showing that TMS1 silencing has little impact on autophosphorylation of FAKY397 in response to integrin engagement or cell adhesion to fibronectin. Together these data suggest that relocalization of TMS1 to barrel-like structures is dispensable for FAKY397 phosphorylation and cell adhesion, yet localization of pFAKY397 to these structures may be crucial in downstream signaling controlling other cellular processes such as cell migration. In this regard, we provide evidence that TMS1 plays a key role in ERK1/2 activation in response to fibronectin-integrin interactions. A downstream consequence of these events could include an impact on cell migration. Studies have demonstrated that ERK1/2 substrates such as m-calpain and MLCK mediate turnover of focal adhesions during cell migration [136,137]. However the precise mechanisms by which ERK1/2 signaling regulates cell migration are not yet well understood [46]. A role for TMS1 in the activation of ERK1/2 signaling may provide one mechanism by which TMS1 could impact cell migration.

Our data show that cells that have silenced TMS1 migrate across a wounded epithelial cell layer less efficiently than control cells. However, TMS1silencing has no significant impact on individual cell velocity or directional movement in live cell wounding assays. Because we used transfection of siRNA as an approach to knockdown TMS1, cells that efficiently knocked down TMS1 could not be accurately distinguished from cells still expressing TMS1 during the collection of cell movement tracings. Alternatively, the mechanisms that dictate directional movement may differ. However, a previous study showing that TMS1 and the proinflammatory protein, pyrin colocalize

with actin at the leading edge of migrating monocytes support a role for TMS1 in directional migration and suggest that this role may be mediated through regulation of cytoskeletal signaling pathways [152].

In conclusion, we report a novel, nonapoptotic role for TMS1 in epithelial cells that includes regulation of FAK and ERK1/2 signaling pathways and a function in cell migration and invasion. We show that fibronectin induces redistribution of TMS1 to a barrel-like structure in breast epithelial cells. Additionally, pFAK and centrosomal markers colocalize with TMS1 at this structure in a subset of cells suggesting that it may represent a critical signaling center in epithelial cells. We show that TMS1 is necessary for fibronectin-induced signaling through the FAK and ERK1/2 pathways. Live cell wounding and transwell migration assays demonstrate that TMS1 promotes cell migration. Moreover, we also find that TMS1 plays a role in cell invasion through an extracellular matrix. Together these data show that in addition to the well established roles for TMS1 in both apoptosis and inflammation, TMS1 exhibits a non-apoptotic role in breast cell migration and invasion.

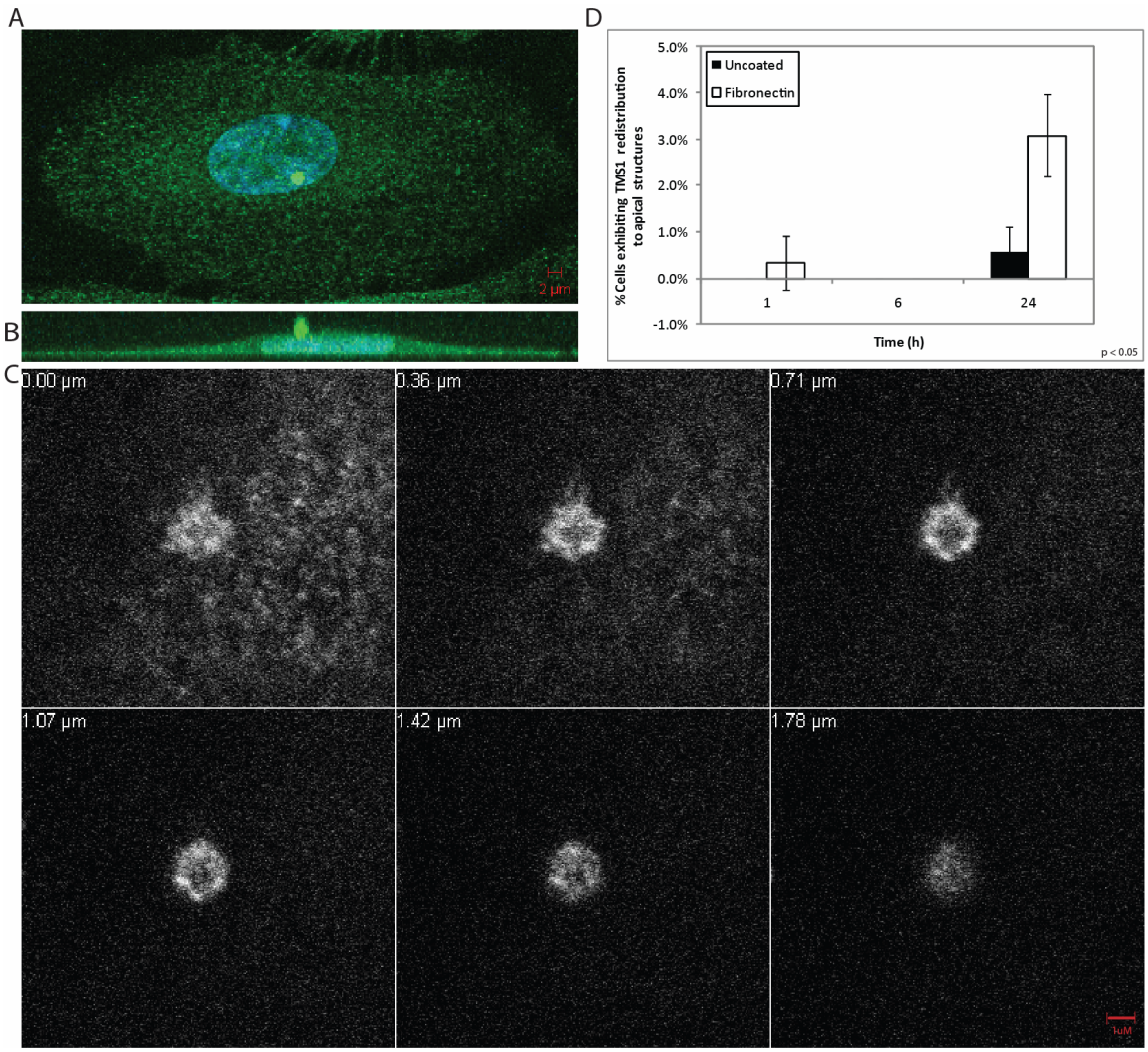


Figure 1: Fibronectin induces redistribution of TMS1 to barrel-like structures. MCF10A cells were allowed to adhere to fibronectin for 24 hours at which time they were fixed and stained with a TMS1 antibody and the nuclear stain, DAPI. A) Shown is a cell exhibiting TMS1 localization to an apical structure. B) A view of the cell depicted in A across the z-axis. C) A series of z-stacks of an apical structure from the basal plane of the structure to the most apical plane of the structure. All images (A-C) are representatives from three independent experiments. D) Quantitation of apical structures in MCF10A cells attached to uncoated or fibronectin-coated coverslips. Shown is the mean of three independent experiments with error bars representing the standard deviation. Statistical significance was determined using the student's *t*-test.

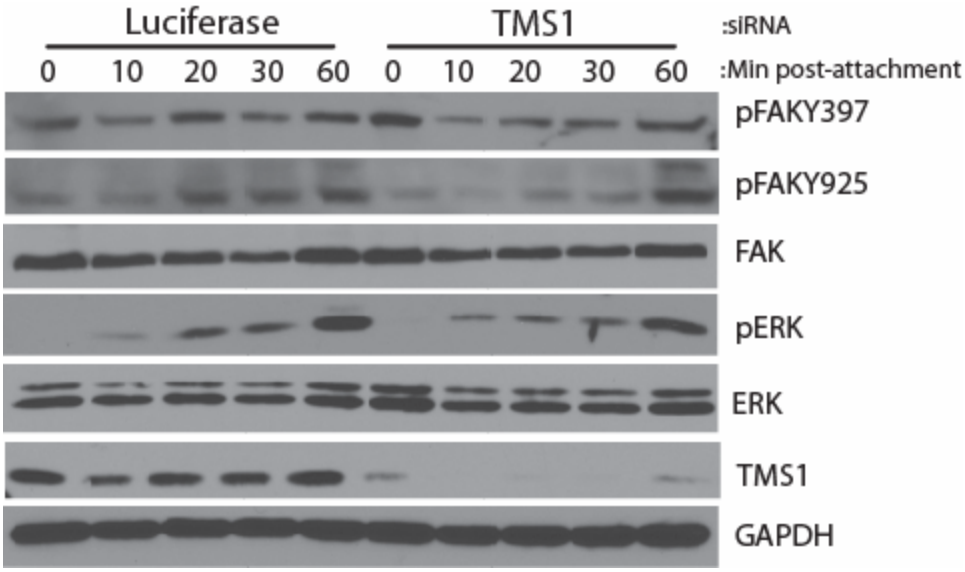


Figure 2: TMS1 is necessary for fibronectin-induced activation of the ERK1/2 pathway. MCF10A cells were treated with siRNA targeting Luciferase (control) or TMS1. After 48 hours, cells were serum starved for an additional 24 hours. Following starvation, cells were trypsinized and plated onto fibronectin-coated dishes. At the indicated time points, cells were harvested and lysates were subjected to western blot analysis with the indicated antibodies. Shown is a representative of three independent experiments.

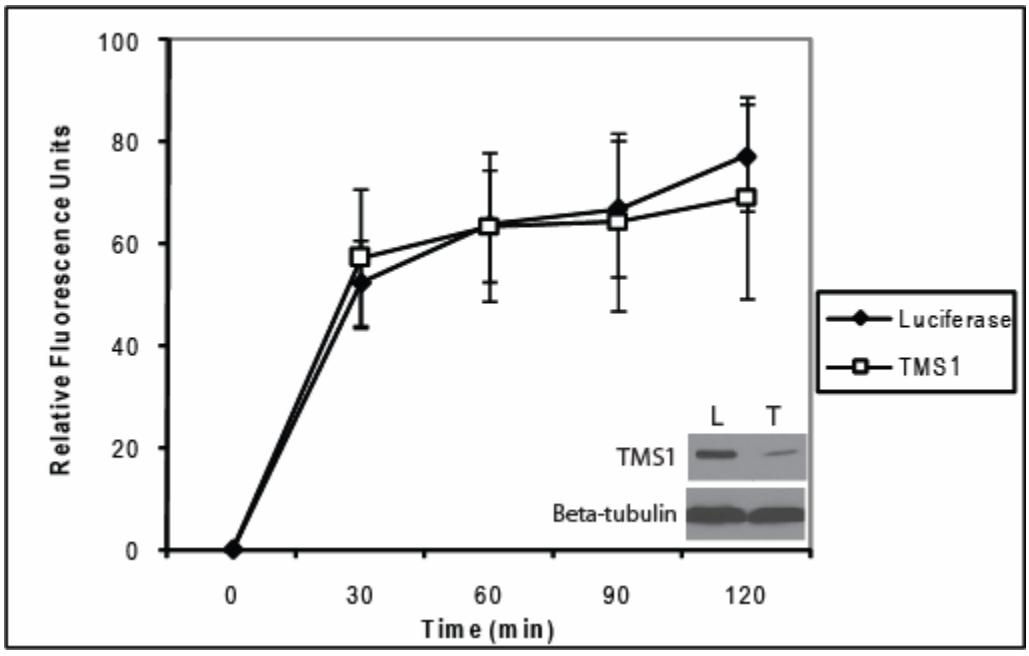
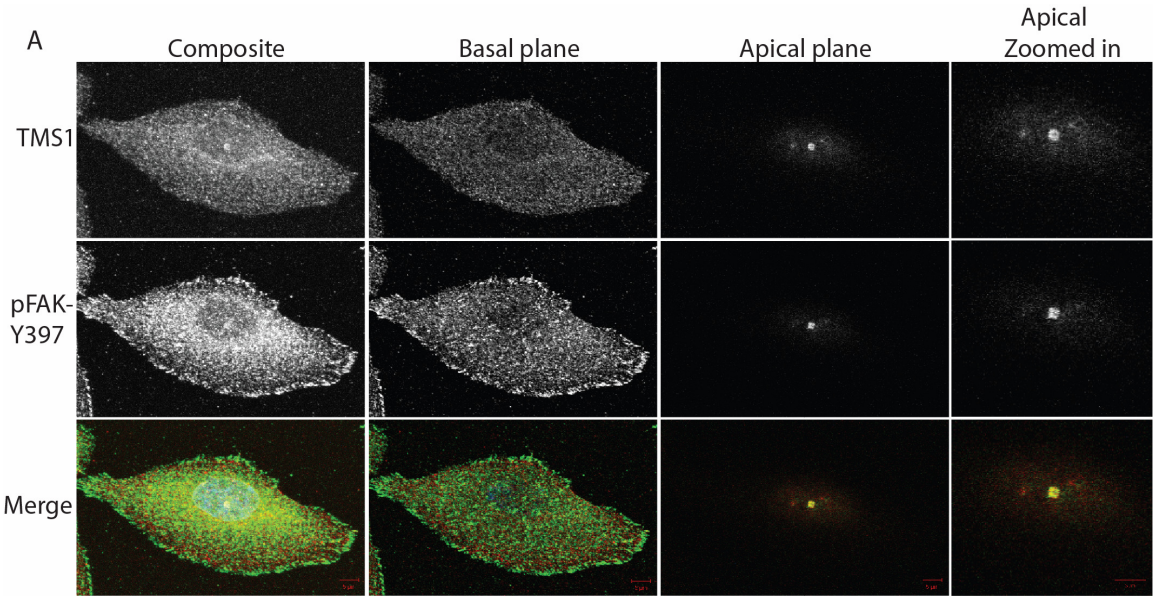


Figure 3: TMS1 is dispensable for cell adhesion to fibronectin. MCF10A cells were treated with siRNA targeting Luciferase (L) or TMS1 (T). After 48 hours, a cell adhesion assay was performed on fibronectin-coated dishes. Adherent cells were quantified using the Cyquant kit. Shown is the mean of three independent trials where errors bars represent the standard deviation. A representative western blot showing knockdown of TMS1 is included in the inset.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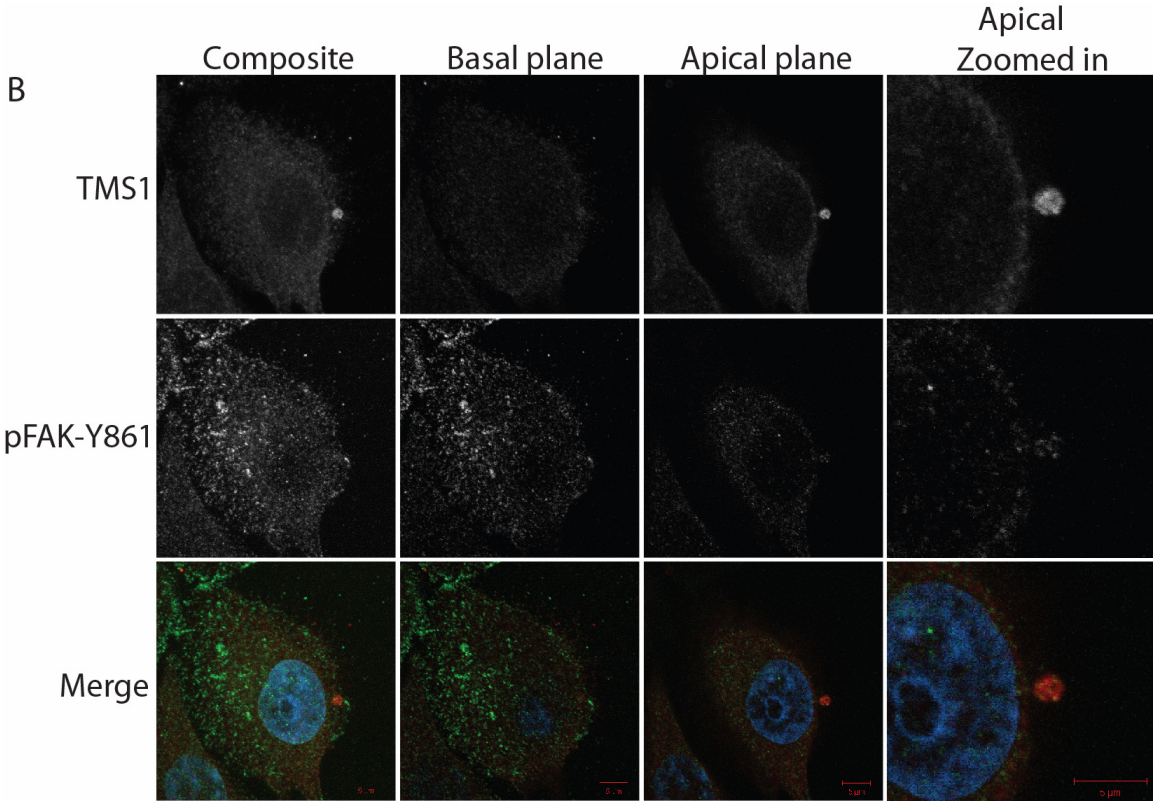
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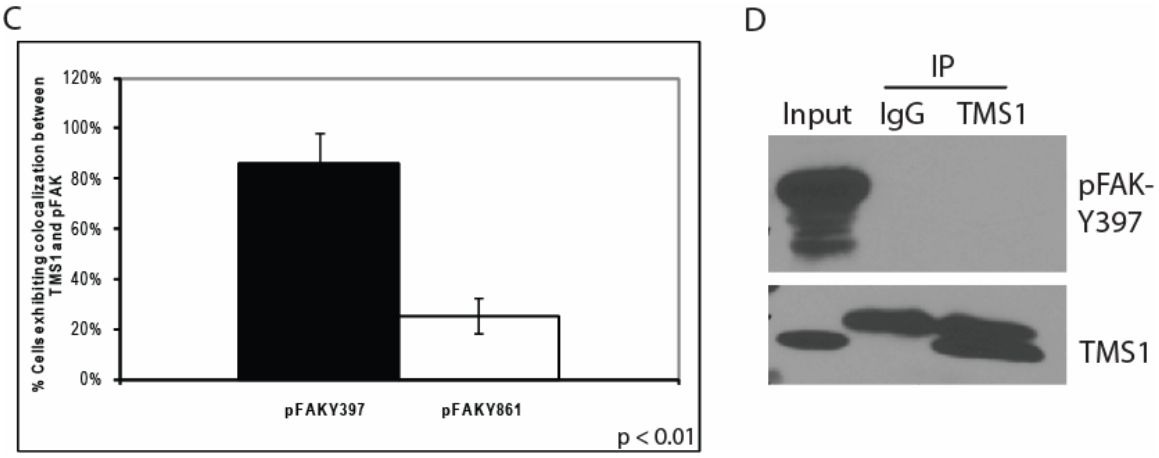
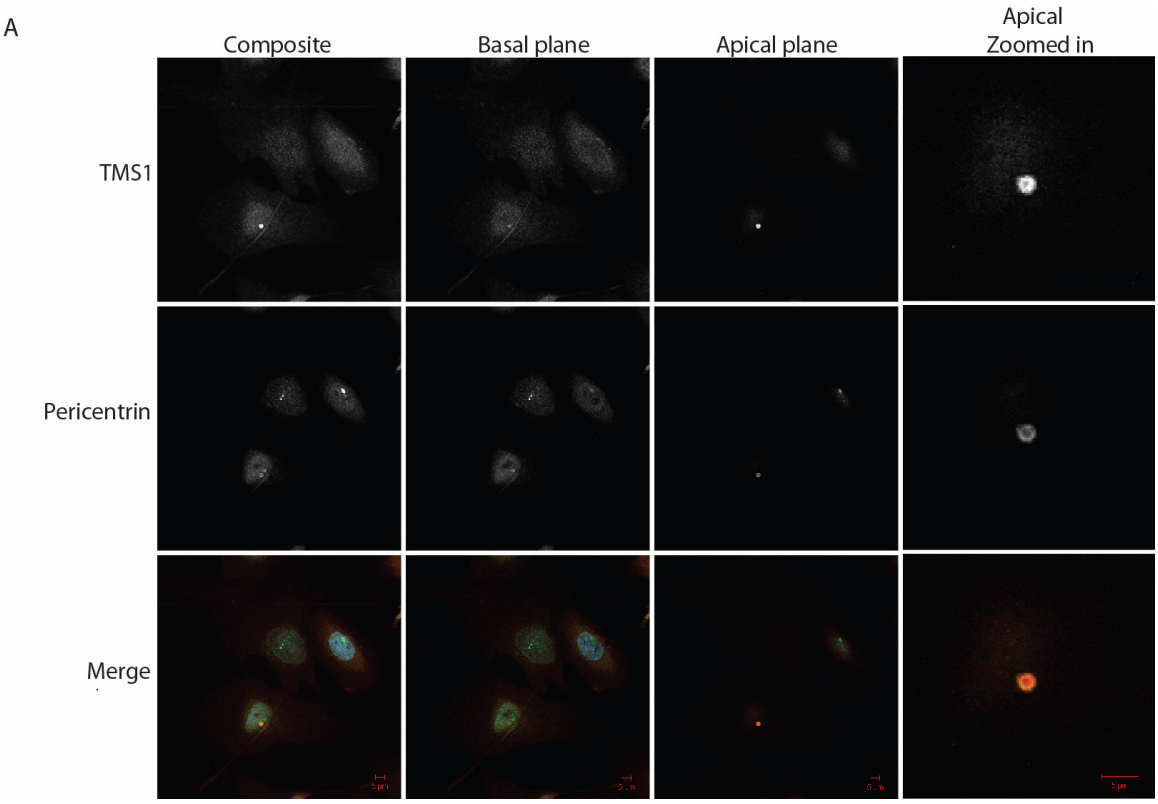


Figure 4: pFAK colocalizes with TMS1 at apical structures in a subset of cells. MCF10A cells were allowed to grow on fibronectin-coated coverslips for 24 hours A-B) Cells were stained with antibodies to TMS1 and pFAKY397 (A) or pFAKY861 (B). Shown are representative cells from three independent experiments exhibiting localization of pFAKY397 or pFAKY861 to an apical structure. The first panel shows a composite image of a series of confocal z-stacks. The second panel shows the most basal plane of the cell. The third panel shows an apical plane of the cells and a zoom magnification of this plane is shown in the fourth panel. Scale bar: 5 µM. C) Quantitation of apical structures in MCF10A cells exhibiting colocalization of pFAKY397 or pFAKY861 with TMS1. Shown is the mean of three independent experiments where error bars represent the standard deviation. Statistical significance was determined using the student's *t*-test. D) Cells were lysed and TMS1 was immunoprecipitated. Resulting samples were probed with antibodies to TMS1 and pFAKY397. Shown is a representative of two independent experiments.


A
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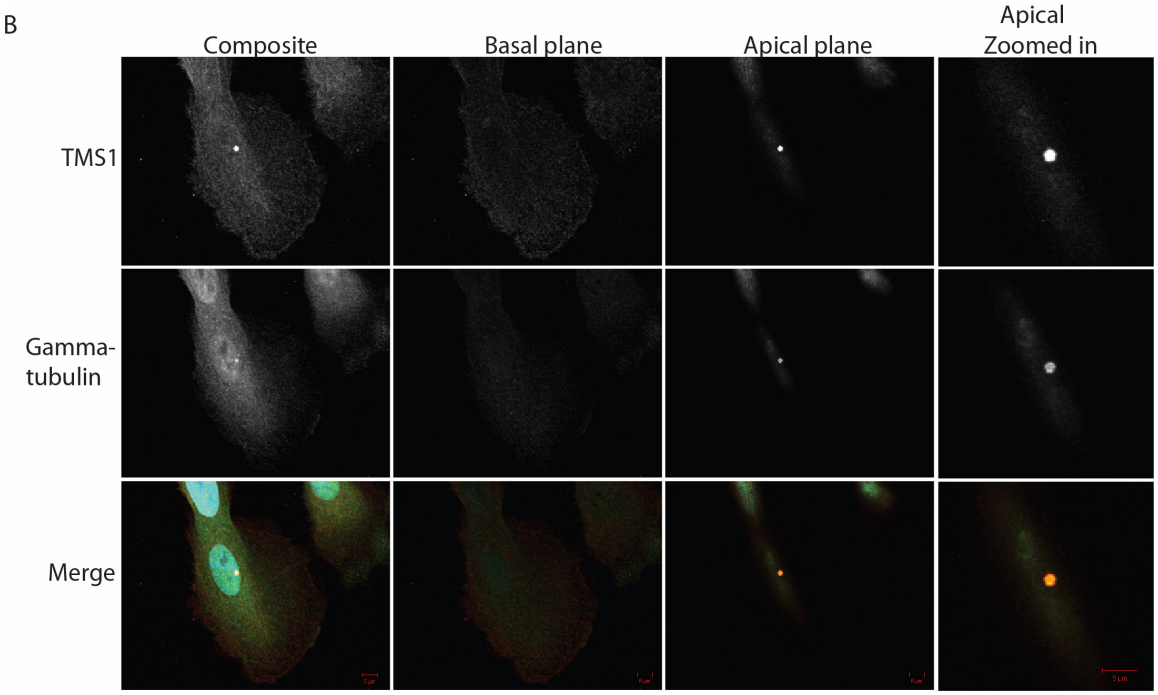
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TMS1
Gamma-
tubulin
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Figure 5: TMS1 localizes to centrosomes. MCF10A cells were allowed to grow on fibronectin-coated coverslips for 24 hours at which time they were fixed and stained with antibodies to TMS1 and pericentrin (A) or gamma-tubulin (B) and DAPI. Shown are representative cells from two independent experiments exhibiting co-localization of TMS1 with pericentrin or gamma-tubulin. The first panel shows a composite image of a series of confocal z-stacks. The second panel shows the most basal plane of the cell. The third panel shows an apical plane of the cells and a zoom magnification of this plane is shown in the fourth panel. Scale bar: 5 μM.

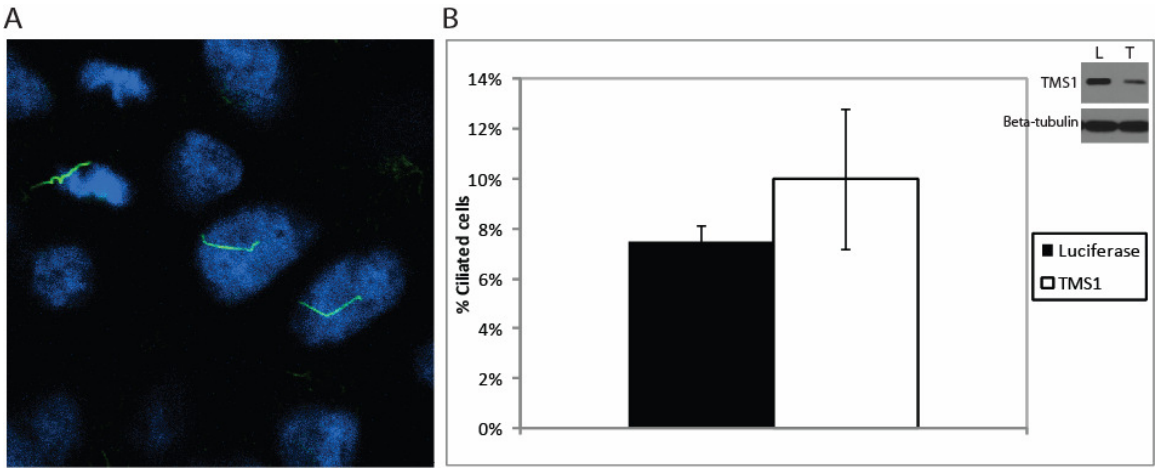


Figure 6: TMS1 is dispensable for cilia formation at a population level. A) MCF10A cells were stimulated to enter G_0 by plating at confluency and allowing growth for 24 hours. Cells were then serum starved for an additional 24 hours at which time they were fixed and stained with an antibody to acetylated-alpha tubulin and DAPI. Shown is a representative field of MCF10A cells exhibiting primary cilia from three independent experiments. B) MCF10A cells were treated with siRNA targeting luciferase or TMS1. After 48 hours cells were plated and stained as described in A. The percentage of cells exhibiting cilia were quantified. Shown is a representative of three independent experiments where error bars show standard deviation of duplicates.

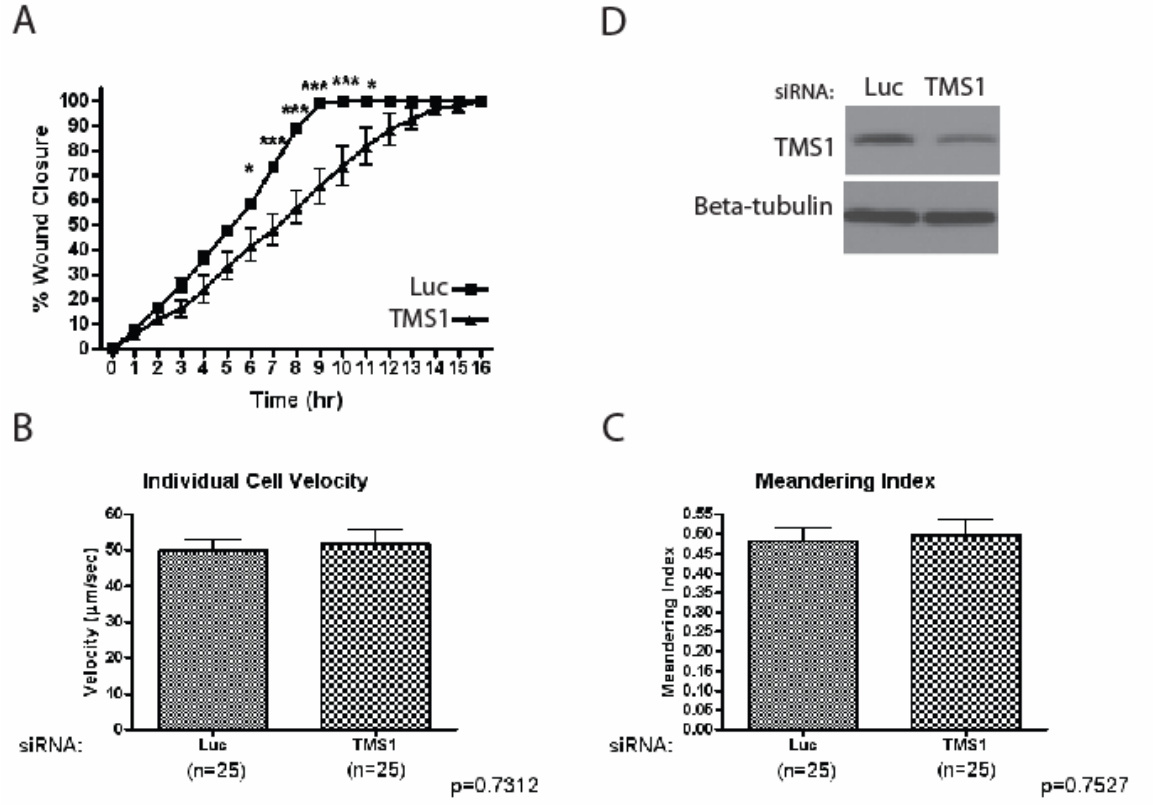


Figure 7: Silencing TMS1 delays wound closure. MCF10A cells were treated with siRNA targeting Luciferase or TMS1. After 48 hours, A) cells were wounded with a pipette tip and live cell imaging was used to track wound closure in four different regions of a given wound. The mean percentage wound closure of quadruplicates is plotted over time where the error bars represent the standard deviation. Shown is a representative of three independent trials performed in quadruplicate. B-C) Individual cell movement tracks were generated for control and TMS1 knockdown cells. Velocity software was then used to determine individual cell velocity (B) and the meandering index (C) for tracked cells (n=25 cells). D) Lysates were collected once wound closure was complete and analyzed by western with the indicated antibodies. Shown are graphs from a representative experiment repeated three times.

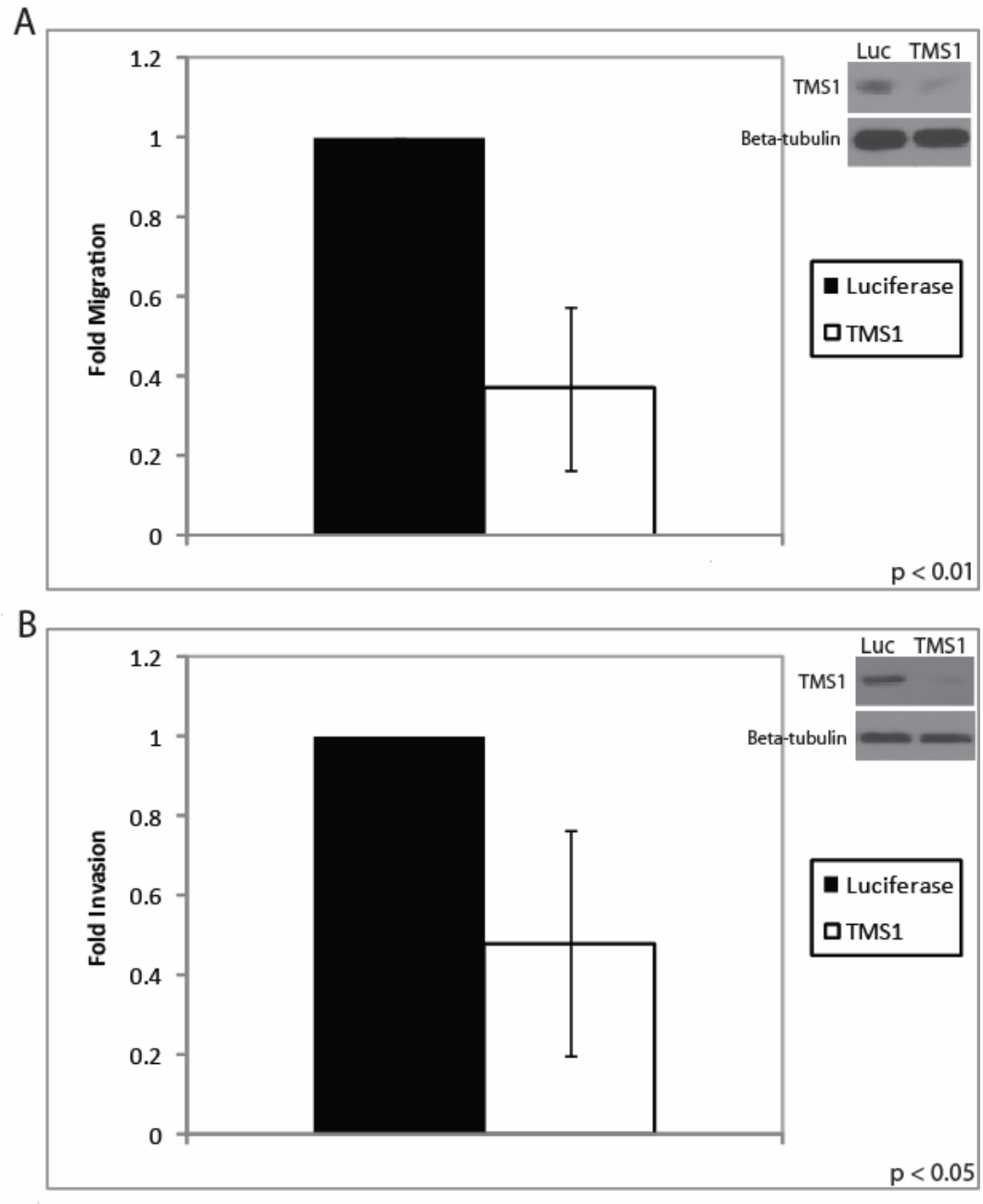


Figure 8: TMS1 is necessary for efficient migration across a transwell chamber and invasion through an extracellular matrix. MCF10A cells were treated with siRNA targeting Luciferase or TMS1. After 48 hours, cells were plated onto transwell chamber inserts and allowed to migrate (E) or invade through a layer of Matrigel (F) towards an EGF stimulus. Following 8 or 24 hours, migrated or invaded cells were fixed and stained. Filters were mounted onto glass coverslips and cells were counted across ten random fields. Shown is the mean of three independent trials performed in triplicate where the errors bars represent the standard deviation. Statistical significance was determined using the student's *t*-test. A representative blot showing TMS1 knockdown is shown in insets.

Chapter 4

Discussion

Summary

This thesis has focused on elucidating the functional roles of the adaptor protein, TMS1 in breast epithelial cells. Overall, our data indicate two distinct roles for TMS1 in both epithelial anoikis and migration. We find that detachment induces TMS1 mRNA and protein levels in breast epithelial cells (Figure 1A). In addition, we show that silencing TMS1 confers resistance to anoikis in that cells treated with siRNA targeting TMS1 were delayed in death as evidenced by a delay in PARP cleavage, procaspase-8 cleavage, and accumulation of BimEL. Furthermore, we establish a specific role for TMS1 in the upregulation of BimEL during detachment that was dependent in part on the regulation of ERK1/2 signaling (Figure 1A).

During the course of this project, a newly discovered nonapoptotic role for procaspase-8 in cell adhesion and migration influenced our studies aiming to determine the function of TMS1 in promoting procaspase-8 cleavage during anoikis. Our resulting efforts have elucidated a distinct nonapoptotic role for TMS1 in breast epithelial migration and invasion (Figure 1B). We have observed that adhesion to fibronectin induces the redistribution of TMS1 into barrel-like structures in breast epithelial cells. Silencing TMS1 inhibits fibronectin-induced activation of the FAK and ERK1/2 pathways. pFAK and centrosomal markers colocalize with TMS1 in barrel-like structures at the apical surface of a subset of cells suggesting that TMS1 may associate with centrosomes to mediate signaling (Figure 1B). We further demonstrate that TMS1 is necessary for efficient epithelial cell migration. Moreover, we show a role for TMS1 in breast cell invasion through a basement membrane matrix. These data suggest that

epigenetic silencing of TMS1 plays an intricate role in breast cancer progression that may be highly context-dependent.

TMS1 is an adaptor protein consisting of two protein-protein interaction domains suggesting that the diverse roles of TMS1 in anoikis and cell migration may be regulated through key protein interactions that occur in specific contexts. In this regard, the binding partners of TMS1, which include itself, may distinguish between the proapoptotic or nonapoptotic functions of TMS1 in a given cell. Anoikis and migration are physiological processes involved in the development of multicellular organisms. Proteins such as TMS1 that are constitutively expressed yet primarily function in relatively infrequent events (e.g. cell death and migration) may exhibit dual roles to allow cells to rapidly and efficiently respond to transient signals that regulate these processes. Overall, the work presented in this dissertation has helped to elucidate how anoikis and cell migration are executed on a molecular level and has greatly contributed to the overall understanding of cell biology.

Roles of TMS1 in anoikis

Upregulation of BimEL and cleavage of procaspase-8 are two events known to occur early upon cellular detachment of epithelial cells. The finding that TMS1 is necessary for both of these events suggests that TMS1 may act to sense loss of integrin-ECM signals to initiate anoikis early upon detachment of breast epithelial cells. Consistent with this hypothesis we found that preventing caspase-mediated death through stable expression of the anti-apoptotic protein, Bcl-2, failed to prevent detachment-induced accumulation of TMS1 protein but nevertheless prevented apoptosis.

Additionally, the impact of TMS1 silencing on BimEL accumulation during anoikis occurred similarly in both control MCF10A cells as well those stably expressing Bcl2 suggesting that the impact of TMS1 on BimEL accumulation also occurred prior to the induction of apoptosis in these cells.

Our data indicate that one way that TMS1 impacts anoikis is through an affect on Bim. TMS1 is dispensable for *Bim* mRNA upregulation, but plays an essential role in upregulation of BimEL protein, the largest and most predominant isoform of Bim. Interestingly, the role for TMS1 in the regulation of Bim was specific to the EL-isoform, as there was little impact of TMS1 silencing on BimL or BimS induction during anoikis (data not shown). BimEL is a target for ERK1/2 mediated phosphorylation that promotes its ubiquitination and degradation. Elevated signaling through the ERK1/2 pathway provided one mechanism by which TMS1 silencing impacted BimEL during anoikis (Figure 2). Consistent with a specific role for TMS1 in the regulation of BimEL, this is the only isoform of Bim that contains an ERK1/2 docking site and is thought to be regulated post-translationally by this pathway [153].

Detachment of MCF10A cells resulted in an upregulation of TMS1 mRNA and protein levels. Previous work in the lab had shown that NF-κB signaling induced TMS1 mRNA and protein expression in response to treatment with TNF-α and TRAIL death ligands [103]. Thus, it seemed likely that this pathway would similarly be important in TMS1 upregulation during detachment-induced apoptosis. However, our data indicate that loss of NF-κB signaling during suspension does not significantly impact the induction of TMS1 protein and mRNA. Additionally, other work in the lab has shown that the JNK pathway, which also stimulated TMS1-induced apoptosis in response to

death ligand treatment [103], is also dispensable for TMS1 upregulation during detachment [116]. Together these data suggest that regulatory pathways that control TMS1 expression in response to detachment differ from those that impact its expression in response to other proapoptotic stimuli.

In addition to a role for TMS1 in BimEL upregulation, we also show that TMS1 is necessary for detachment-induced cleavage of procaspase-8. Previous work has shown that TMS1 directly binds caspase-8 [102]. These authors also demonstrated that caspase-8 is necessary for apoptosis induced by forced oligomerization of TMS1 [102]. TMS1 may promote procaspase-8 cleavage through a physical interaction during anoikis. Suspension of breast epithelial cells resulted in an upregulation of TMS1 protein levels which might directly promote procaspase-8 aggregation and self cleavage through TMS1-mediated homotypic interactions. While this appears to be an attractive hypothesis regarding a mechanism for TMS1 in detachment-induced cleavage of procaspase-8, we were unable to detect a physical interaction between TMS1 and procaspase-8 upon cellular detachment. One technical issue that we have yet to overcome is that procaspase-8 is relatively the same size as the heavy chain of IgG. Thus, detection of the heavy chain has impeded our efforts to determine if procaspase-8 is being pulled down with TMS1 in suspended cell lysates.

Procaspase-8 cleavage could contribute to anoikis by cleaving downstream effector caspases such as caspase-3 resulting in cell death. Two cleavage events are necessary to activate procaspase-8 [154]. The first results in the production of the p43/41 intermediate and the second produces the active p18 fragment [154]. We showed that TMS1 is necessary for cleavage of procaspase-8 into its intermediate form. We were

unable to detect the smaller cleavage product perhaps to due technical issues (e.g. samples were run on a 12% gel, thus this cleavage product may have run off the gel or transferred through the membrane into the buffer) or perhaps because the second cleavage event does not occur during anoikis. In this regard, cleavage and loss of the full length procaspase-8 might alternatively contribute to anoikis by downregulating prosurvival signaling through ERK1/2 rather than directly initiating a cleavage cascade that results in cell death. Evidence suggests that Src-mediated phosphorylation of procaspase-8 at Y380 acts as a toggle such that when phosphorylated at this residue procaspase-8 cannot be cleaved to mediate apoptosis and instead promotes cell adhesion in part through promoting ERK1/2 activation. Thus, TMS1 may promote procaspase-8 cleavage in detached cells by promoting its dephosphorylation at Y380. This event would downregulate survival ERK1/2 signaling which would result in the release of ERK1/2-negatively regulated pro-apoptotic proteins such as BimEL and promote death in the absence of enzymatically active caspase-8.

TMS1/ASC aggregation

The original study identifying TMS1 showed etoposide treatment promoted the formation of a distinct structure containing TMS1 referred to as a 'speck'. Formation of these structures correlated with drug-induced apoptosis in leukocytes [97]. Specks have also been shown to form in response to pathogenic infection of monocytes, [132] and pro-inflammatory proteins have been shown to colocalize with TMS1 at these structures suggesting that it is associated with inflammasome formation [98,155]. Additionally, aggregation of TMS1 in speck-like structures were visualized in cells undergoing

pyroptosis, an inflammation-induced cell death [133]. Together these studies suggest that TMS1 aggregation is closely linked to its apoptotic and non-apoptotic functions. Murine TMS1 has also been shown to self-associate and form aggregates [156]. Thus, aggregation of TMS1 also appears to be a quality inherent to this protein.

There is evidence to suggest that dimerization of both the PYD and CARD is involved in the aggregation of TMS1 whereby independent expression of each domain was sufficient to form aggregates. Additionally, both homophillic and heterophillic interactions between the PYD and CARD were observed by coimmunoprecipitation [157]. These data suggest that TMS1 oligomerization results from aggregation of both homophillic and heterophillic dimers composed of TMS1 PYDs and CARDs.

The three dimensional structure of the PYD of TMS1 has also been elucidated and was shown to be composed of six helices arranged in a classical death domain fold [158]. Moreover, mutational analyses of all charged amino acid residues comprising the PYD of TMS1 as well as those composing a large hydrophobic side chain identified a series of amino acid residues (Lys21, Leu25, Lys26, Pro40, Arg41, Asp48, and Asp51) that were necessary for TMS1 oligomerization [159].

Cheng *et al.* examined the kinetics of speck formation in HeLa epithelial cells ectopically expressing YFP-TMS1. Analyses from time lapse videos of these cells demonstrated that specks occur rapidly, within three minutes after nucleation. Additionally, the process of speck formation was shown to result from diffusion of TMS1 to speck nucleation sites rather than microtubule-dependent active transport. Speck formation was defined as a two step process whereby TMS1 dimers diffuse randomly until they reach a growing speck at which point they bind to the speck surface.

Following binding the remaining cellular concentration of TMS1 rapidly drops, presumably to ensure that a second speck does not form [160]. Together these studies have elucidated some aspects of TMS1 aggregation, yet the precise mechanisms resulting in formation of TMS1 aggregates and their exact functions remain unclear.

In MCF10A cells, we observe the maximum number of cells showing redistribution of TMS1 to barrel-like structures after 24 hours of growth on fibronectin, significantly longer than the 3 minute window of time necessary for speck formation. However, the precise stimulus that initiates redistribution of TMS1 to apical structures in MCF10A cells remains unknown. Perhaps after receiving the right signal, TMS1 aggregation also occurs within 3 minutes in MCF10A cells attached to fibronectin. Interestingly, consistent with the notion that conditions immediately following speck formation act to inhibit formation of a second speck in one cell, we typically only observe TMS1 distribution to a single barrel-like structure per cell. However, we have on a very rare occasion observed a single cell with what appear to be two different apical structures containing TMS1. The significance of TMS1 localization to a second structure in these cells remains unclear.

TMS1 and cell migration

Potential role for TMS1 in regulating FAK signaling during migration

A significant contribution of this thesis includes the findings that TMS1 has nonapoptotic functions in breast epithelial cells. We find that TMS1 is necessary for fibronectin-induced activation of FAK and downstream ERK signaling. Our data indicates that TMS1 promotes cell migration across a wounded epithelial layer and a

transwell chamber towards a chemoattractant. We also show a role for TMS1 in cell invasion through a layer of Matrigel, a commercially available extracellular matrix. In addition, we find that providing cells with a natural substratum and media rich in growth factors results in the redistribution of TMS1 to apical, barrel-like structures.

A previous study investigated the possibility that the TMS1 speck associated with aggresomes, which are large perinuclear aggregates of misfolded proteins that are ultimately targeted to lysosomes for degradation [160]. These authors found that while TMS1 colocalized with two proteasomal components including the 20S subunit of the proteasome and vimentin at specks, these TMS1 aggregates represented distinct structures from aggresomes with a unique process of growth [160]. However this data did not rule out the possibility of a functional association of TMS1 with the proteasome. Based on our data showing an association of pFAK with TMS1, it would be interesting to infer a potential role for TMS1 in the turnover of these proteins (Figure 3).

A second notable observation is that at most ~3% of cells attached to fibronectin exhibited TMS1 redistribution to barrel-like structures. Thus, a remaining question is whether the ~3% of cells exhibiting TMS1 in apical structures is an ever-changing population (e.g. cells that are migrating) or instead represents a distinct class of cells that are inherently capable of forming this structure. If the former is true, it is possible that in migrating cells TMS1 acts to efficiently turnover pFAKY397 at the rear end of the cell where focal adhesions are being disassembled so that it can be incorporated at the front end of the cell where new adhesions are being created (Figure 3). A role for TMS1 in this regard is consistent with our data showing that TMS1 promotes migration but is dispensable for cellular adhesion to fibronectin. Additionally, this structure may act to

sequester soluble TMS1 in migrating cells in order to prevent TMS1-mediated apoptosis from occurring during these conditions. By sequestering TMS1 in an insoluble state during this process, cells may ensure that anoikis is not prematurely initiated as focal adhesions are continually disassembled and reassembled at the rear end and leading edge of migrating cells. Prolonged detachment and loss of integrin-extracellular matrix signals may then result in release of TMS1 into the cytosol where it can act to promote anoikis. Interestingly, this hypothesis might account, in part, for our results showing that TMS1 protein levels were increased prior to induction of *TMS1* transcription in response to detachment (Chapter 2, Figures 2 and 4).

Association of TMS1 with centrosomes may mediate cell migration and primary cilia formation

We find that pericentrin and gamma tubulin colocalized with TMS1 at the apical structure in a subset of cells suggesting that TMS1 may be associated with centrosomes. These data are currently preliminary, yet may provide another mechanism through which TMS1 impacts cell migration. Xie *et al.* previously demonstrated that the association of pFAKS732 with centrosomes in neurons played an essential role in their migration during the development of the neocortex of embryonic mouse brains [145]. Phosphorylation of FAK at S732 is mediated by the serine/threonine kinase Cdk5 [145]. However, the mechanisms by which an association of pFAKS732 with centrosomes might regulate migration remain unclear [151]. Additionally, an independent study reported that siRNA-mediated knockdown of pericentrin in mouse interneuronal cells inhibited their migration from the rostal migratory stream to the olfactory bulb through *in vitro* culture

of mouse brain slices [146]. These data demonstrate a key role for centrosomes in neuronal cell migration. Moreover, evidence in the literature suggests that integrin-ECM interactions can regulate centrosome function [161-163]. Thus, it is possible that TMS1 may act as a link between cellular adhesion to fibronectin and integrin-mediated regulation of centrosome function such that upon integrin-fibronectin interactions TMS1 may redistribute to centrosomes with pFAK to promote cell migration.

An association of TMS1 with centrosomes may additionally be intriguing in that this association suggests a precursor role for TMS1 in primary cilia formation. Almost every vertebrate cell is able to present a single primary cilium that acts as a sensory organelle [164]. These cellular structures have developed unique sensory modalities which allow them to monitor the extracellular environment and transit information to the interior of the cell [138,165]. Growth factors, morphogens, G-protein-coupled receptors, ion channels, and hormone receptors have been localized to the primary ciliary membrane suggesting a role for cilia in the regulation of a variety of signaling pathways that regulate cellular processes such as migration [138]. Both PDGF receptor-α [140] and EGFR [141] have been shown to localize to the ciliary membrane. Interestingly, activation of PDGF receptor-α in fibroblasts results in signaling through the Akt and ERK1/2 pathways and rapid accumulation of pMEK1/2 throughout the cilium. In the absence of a primary cilium, PDGF signaling through the MAPK pathway is inhibited (Figure 4B). These data are especially intriguing in light of our data showing a role for TMS1 in the activation of ERK1/2 during attachment. Thus, a role for TMS1 in cilia formation may represent one mechanism by which TMS1 could regulate ERK1/2 signaling.

Primary cilia assembly is tightly associated with the cell cycle (Figure 4A) and typically form when cells enter growth arrest/G1. These organelles are then shed before cells enter mitosis. Notably, localization of TMS1 to barrel-like structures has never been observed in mitotic cells. However, it appears that a role for TMS1 in this process is dispensable in that knockdown of TMS1 failed to prevent cilia formation in MCF10A cells. However, while these data suggest that TMS1 is not essential for cilia formation, they do not rule out a role for TMS1 in cilia formation that is not readily detected by this assay (i.e. cilia formation may be delayed in the absence of TMS1). Furthermore, these results may also be due to the ineffectiveness of siRNA-mediated knockdown of TMS1 to prevent localization of TMS1 to apical, barrel-like structures. While we have seen that treatment of cells with siRNA targeting TMS1 resulted in a slight decrease in the number of cells showing TMS1 redistribution to barrel-like structures, these structures are still visible in cells treated with TMS1 siRNA. Interestingly, an independent study found that TMS1 'specks' display an extended period of stability up to 72 hours after the cell in which it was formed underwent cell death [166]. Thus, it is possible that TMS1 protein that is present at barrel like structures similarly exhibits an extended half life. If so, siRNA mediated knockdown of TMS1 might prevent the redistribution of TMS1 to apical structures. However, its effects may be masked by stable TMS1 aggregates already present at these structures. Thus, siRNA knockdown of TMS1 may not allow for adequate silencing of TMS1 that would result in complete inhibition of TMS1 localization to apical structures in these cells. In order to fully understand the functional consequences of loss of TMS1, studies may need to be performed in stably silenced cell lines.

Recent evidence supports a role for primary cilia in cancer development. The tumor suppressor von Hippel Lindau (VHL) is implicated in hereditary and sporadic renal cell carcinomas. The best characterized function of VHL is as an E3 ubiquitin ligase that ubiquitinates the α subunit of hypoxia inducible factor (HIFα). Loss of VHL results in an accumulation of HIFα which is presumed to have a causal role in the development of renal cell carcinoma [167]. Recently, VHL was shown to localize to primary cilia [168,169]. Additionally, VHL is necessary for directional growth of microtubules [170] providing one mechanism by which VHL may regulate cilia formation [168]. However, its precise role in ciliary function is unknown. Notably, renal cell carcinoma cell lines that lacked VHL did not present cilia in cell culture and re-expression of VHL rescued their ciliogenesis defect [168]. An independent study showed that renal cell carcinoma-associated mutations in VHL abrogated cilia formation. However, cells expressing a mutated form of VHL not associated with renal cell carcinoma were still able to form cilia. Moreover, the function of VHL in cilia formation was independent of HIFα accumulation suggesting two independent roles for VHL in renal cell carcinoma development [169]. Similarly, a role for TMS1 in ciliary function may provide a mechanism by which loss of TMS1 might promote tumor progression.

Rac and cell migration

The Rho family of GTPases include Rac, Rho, and cdc42 [171]. These proteins are active when bound to GTP and inactivated upon its hydrolysis to GDP. Guanine nucleotide exchange factors (GEFs) activate the Rho family of proteins by promoting binding to GTP. GTPase activating proteins (GAPs) stimulate the intrinsic hydrolytic

activity of GTP-binding proteins to form the inactive GDP-bound state [171]. The Rho family of proteins regulates actin dynamics. Changes in actin cytoskeleton are required for cellular processes such as cytokinesis, intracellular trafficking, and cell migration. Each family member plays a specific role in actin reorganization. For example, Rho is required for stress fiber formation and Rac is necessary for membrane ruffles [171].

In 2004 Raymond *et al.* developed a gain of function screen to identify partners of RotundRacGAP/RacGAP(84C), a GTPase in *Drosophila* that has been shown to share 60% similarity with the human male germcell RacGAP (MgcRacGAP) protein [172]. Through this screen, these authors demonstrated a genetic interaction between *Drosophila* TMS1 (dTMS1) and RacGAP(84C) such that dTMS1 enhanced the rough eye phenotype induced by overexpression of RacGAP(84C) [173]. However, overexpression of TMS1 did not affect eye phenotypes of flies expressing dominant negative forms of Rac, cdc42, or Ras suggesting that the genetic interaction between TMS1 and RacGAP(84C) occurs independently of these proteins [173]. The consequences of an interaction between dTMS1 and RacGAP(84C) is currently unknown but may provide a link by which TMS1 could impact cell migration through regulation of other RacGAP(84C) targets.

TMS1 and ERK1/2 signaling

Interestingly, we show that in detached cells TMS1 inhibits ERK1/2 signaling allowing for the proper accumulation of BimEL and subsequent cell death (Chapter 2). However, our data investigating a nonapoptotic role for TMS1 suggest that during adhesion to fibronectin TMS1 promotes ERK1/2 signaling (Chapter 3). Thus, the role for

TMS1in the regulation of ERK1/2 signaling appears to be intricately complex. We have not yet established how TMS1 impacts the ERK1/2 pathway during attachment or detachment. Recent studies involving the *Drosophila* ortholog of TMS1 have unveiled some potential players. Giot *et al.* conducted a high throughput yeast two-hybrid analysis across the entire proteome of *Drosophila melanogaster* and constructed a genome-scale protein interaction map for this organism. Results from these analyses included generation of a map of 4679 proteins displaying 4780 interactions that were deposited in FlyBase, GRID (General Repository of Interaction Datasets), BIND (Biomolecular Interaction Network Database), and DIP (Database of Interacting Proteins) [174]. A putative interaction between dTMS1 and *Drosophila* sprouty (dSPRY), a negative regulator of ERK1/2 signaling, resulted from this study.

A role for dSPRY in the regulation of ERK1/2 signaling was first described by Casci *et al*. who demonstrated that dSPRY-mediated regulation of ERK1/2 signaling was involved in tracheal development in the fly embryo. These authors went on to show that dSPRY is tightly associated with the plasma membrane and through GST-pull down assays showed that it can physically interact with Drk, a homolog of Grb2, and Ras GAP-1 [175]. Four mammalian isoforms of SPRY exist with SPRY2 showing a similar function in mammalian lung development to that of dSPRY. Sequence analyses of SPRY proteins have failed to identify obvious enzyme motifs or protein-protein interaction motifs [176]. Yet a number of interactions with SPRY proteins have been identified. For example, SPRY1/2 have been shown to bind to Grb2 preventing its interaction with the tyrosine phosphatase, Shp2 or fibroblast-growth-factor receptor substrate 2 (FSR2), both implicated in activation of ERK1/2 signaling [177].

Moreover, SPRY1/4 and 2 have been shown to be phosphorylated on conserved tyrosine residues (Y53 and Y55 respectively). Phosphorylation of SPRY proteins at this conserved tyrosine residue allows them to temporally regulate ERK1/2 signaling whereas mutations at these residues result in dominant negative functions. For example, overexpression of SPRY1 in a neuronal cell line was shown to result in transient ERK1/2 activation. Conversely, expression of SPRY1 bearing the mutation Y53F resulted in sustained ERK1/2 signaling [177]. Interestingly, regulation of ERK1/2 signaling during anoikis is also temporally mediated in that detachment initially results in transient activation of this pathway but long-term growth in suspension results in inhibition of ERK1/2 signaling [178]. Moreover, our results show that knockdown of TMS1 resulted in persistent ERK1/2 signaling during anoikis (Chapter 2, Figure 5). Thus, interactions with SPRY proteins may provide one mechanism by which TMS1 could impact the ERK1/2 pathway during anoikis.

A number of scenarios may account for the regulatory mechanisms observed between TMS1 and ERK1/2 in attached and detached cells. For instance, an interaction between TMS1 and SPRY could positively impact ERK1/2 signaling such that during attachment an interaction between TMS1 and SPRY would sequester SPRY from ERK1/2 activating proteins such as Grb2, preventing inhibition of ERK1/2 signaling. Upon detachment, TMS1 would release SPRY resulting in downregulation of ERK1/2 signaling and accumulation of BimEL (Figure 5A). Conversely, in a second scenario, an interaction between TMS1 and SPRY may enhance its negative regulation of the ERK1/2 pathway. In this instance, binding of TMS1 to SPRY would be necessary for it to bind and sequester Grb2 thereby inhibiting ERK1/2 signaling during anoikis. In contrast,

during attachment, the absence of an interaction between TMS1 and SPRY would prevent it from sequestering Grb2 resulting in ERK1/2 activation (Figure 5B). Both of these scenarios are consistent with our data showing that TMS1 promotes signaling through the ERK1/2 pathway during attachment, yet prevents pERK1/2 accumulation in detached cells.

TMS1 and breast cancer progression

In our initial studies, we showed that a subset of DCIS lesions showed silencing of TMS1 in the aberrant cells filling mammary duct lumen. Additionally, we found that a subset of invasive lesions lost TMS1 expression altogether, suggesting that silencing of TMS1 may contribute to breast cancer progression (Chapter 1). Evidence from Mirza *et al.* supports this hypothesis. These authors determined the methylation status of four genes implicated in breast cancer including *BRCA1*, *TMS1*, *ERα* and *Progesterone Receptor B* (*PRB*) in breast cancer tumors and matched serum derived circulating DNA. Results from these analyses showed that 24% (12/50) of invasive breast cancer lesions and paired sera exhibited hypermethylation of TMS1. Additionally, 10/12 tumors with TMS1 methylation also showed methylation of ERα and *PRB* with 7 showing reduced ER expression. However, a subset of tumors also displayed heterogeneous TMS1 methylation and their paired sera failed to show methylation of TMS1. Overall, these authors concluded that loss of TMS1 was associated with reduced ER expression and advanced tumor stage [179].

A more recent study also included *TMS1* in a panel of genes tested for methylation status in normal breast tissue and paired preinvasive lesions (atypical ductal

hyperplasia and DCIS) and invasive breast tumors [180]. However, these analyses failed to show a link between *TMS1* methylation and breast cancer progression. Three genes were shown to exhibit differential methylation patterns between normal and pathologic samples including *APC*, *CDH1* (which encodes E-cadherin), and *CTNNB1* (which encodes β–catenin). Of these only CDH1 showed a statistically significant difference in methylation status between DCIS and invasive ductal cancers [180]. Thus, the impact of aberrant silencing of *TMS1* in breast cancer progression remains unclear.

DCIS represents a stage of breast cancer that does not necessarily predict the outcome of disease [33]. While some patients diagnosed with DCIS recur with invasive breast cancers, others only show subsequent development of more DCIS lesions. Kerlikowske *et al.* recently identified factors in women diagnosed with DCIS that predicted incidences of subsequent DCIS versus subsequent invasive carcinomas [181]. These authors showed that women with DCIS lesions that were p16, COX-2, and Ki67 positive had increased risk of incidence of recurring invasive cancer relative to women whose lesions did not express these biomarker combinations. Moreover, risk of subsequent invasive cancer was higher for women with DCIS lesions that were diagnosed through palpation versus those diagnosed through mammography [181]. While information resulting from histopathogic analyses including size, nuclear grade, and evidence of necrosis did not predict incidence of invasive cancer, it was predictive of subsequent DCIS whereby lesions with positive or uncertain surgical margins were strongly associated with recurrence of DCIS. Additionally, DCIS lesions in women who developed subsequent DCIS were associated with different profiles of biomarker expression that was characterized as ER-/ERBB2+/Ki67+ or p16+/COX-2-/Ki67+ [181].

Our initial studies demonstrated a key role for TMS1 in breast cell anoikis suggesting that silencing of TMS1 may play a role in lumen filling of some DCIS lesions. We also observed silencing of TMS1 in a subset of invasive breast cancers suggesting that silencing of TMS1 may accompany the progression of some pre-invasive breast cancer lesions to an invasive state. However, our recent data showing a nonapoptotic role for TMS1 in breast cell migration and invasion would suggest that DCIS lesions that retain TMS1 expression and perhaps occur through functional loss of an alternate mediator of anoikis may preferentially progress to an invasive state (Figure 6). Together the data we present in this thesis, suggest that silencing TMS1 might be associated with those lesions that never progress to an invasive state. Thus, TMS1 may prove to be a clinically relevant biomarker that in conjunction with the biomarkers listed above could be predictive of pre-invasive lesions.

Future directions

Regulation of TMS1 during anoikis

In our initial studies we focused our efforts in elucidating a role for TMS1 in breast cell anoikis. Our data and previous work in the laboratory indicate that survival signaling through the ERK1/2 and JNK MAPK pathways is dispensable for TMS1 regulation during anoikis. An unanswered question is how TMS1 is upregulated during anoikis. The *TMS1* promoter region includes part of a ~600bp CpG island that is unmethylated in cells expressing TMS1 and is flanked by 5' and 3' methylation boundaries. Four DNase hypersensitive sites (HS) labeled HS1-4 are present across the *TMS1* locus. The locations of HS1 and HS3 coincide with the CpG island methylation

boundaries while HS2 forms in the center of the CpG island. The fourth HS maps to ~1kb downstream of the end of the coding region [110].

Previous work conducted in the laboratory addressing the role of TNF-α in the transcriptional upregulation of TMS1 showed that *cis*-acting sequences lying outside the promoter region of TMS1 likely play a role in the induction of TMS1 mRNA. In these studies, it was shown that 1000bp of sequence containing the TMS1 promoter failed to be induced by TNF-α treatment in luciferase reporter assays. Yet, treatment of MCF7 cells with this ligand resulted in changes in chromatin architecture including a pronounced distinction of DNase hypersensitive sites. The specific events required to induce *TMS1* transcription in response to death ligand treatment were not elucidated. (Parsons MJ and Vertino PM, Dissertation, 2006).

Since this time, other efforts in the laboratory have been focused on understanding the chromatin architecture of the active TMS1 locus. One key finding from these studies is that active TMS1 contains two distinct H4K16 peaks (active chromatin marks) at HS1 and HS3 [182]. It would be interesting to see if this or other active histone marks are enhanced at the *TMS1* locus during anoikis relative to adhesion. It is possible that in suspended cells, the TMS1 locus would show greater peaks of H4K16Ac at HS1 and HS3. These experiments may shed light on the transcriptional regulation of TMS1 during anoikis.

In addition to JNK and ERK1/2, downregulation of a third major survival pathway, the PI3K/Akt pathway, is implicated in anoikis. We have not yet explored a role for this pathway in the downregulation of TMS1 in attached cells such that upon detachment, downregulation of PI3K survival signaling would result in an upregulation

of TMS1. PI3K signaling has been shown to downregulate proapoptotic genes such as *Bim*, *Puma*, and *Nox* through negative regulation of the forkhead box O (FOXO) [183] and p53 transcriptions factors [184].

FOXO transcription factors can positively or negatively regulate transcription of genes involved in cell cycle progression, survival, metabolism, DNA repair, and other cellular pathways. Humans have four members of this family, FOXO1, FOXO3a, FOXO4, and FOXO6. Negative regulation of FOXO transcription factors by PI3K occurs through phosphorylation-mediated sequestration of FOXO proteins in the cytoplasm [185]. Loss of PI3K signaling releases FOXO proteins allowing them to translocate into the nucleus and activate specific gene expression.

FOXO3a has been shown to positively regulate *Bim* transcription [74,186]. Moreover, FOXO3a has been shown to bind at the sequence 5'AAAAATA3' [187] upstream of some target genes. A search for the sequence 5'AAAAATA3' upstream of the TMS1 promoter found that it was present ~500bp upstream of the TMS1 promoter. In contrast, the FOXO1 specific sequences 5'ATAAATA3' and 5'GTAAACA3' were not found within this searched sequence suggesting that PI3K-mediated downregulation of TMS1 may specifically involve the FOXO3a transcription factor. Additionally, it is possible that other FOXO transcription factors could positively regulate *TMS1* by binding *cis*-acting sequences more distal to the TMS1 promoter region.

An independent study has provided evidence that indicates a role for p53 in the upregulation of TMS1 in Bax-dependent apoptosis in response to genotoxic agents [100]. However, previous work in the laboratory has failed to show a role for p53 in the induction of TMS1 in response to DNA damaging agents [103]. Thus, there exists a

potential for a PI3K-mediated role for p53 in the transcriptional regulation of *TMS1* during anoikis, yet a role for this transcription factor in upregulation of TMS1 during detachment has not been thoroughly explored.

Interestingly, we find that accumulation of TMS1 protein occurs prior to transcriptional induction of TMS1 during detachment. Thus, post-transcriptional and/or post-translational regulations of TMS1 may account for TMS1 accumulation prior to transcriptional induction of *TMS1* during detachment. Post-translational regulatory mechanisms may promote degradation or sequestration of TMS1 protein in attached cells whereby detachment would halt its degradation and/or release TMS1 from its site of sequestration. We have explored a role for TMS1 as a target of ERK1/2 (Chapter 2) phosphorylation that may mediate these regulatory mechanisms. However, results from these analyses suggest that this kinase is not involved in the regulation of TMS1 (Chapter, Figure 7).

Src mediates the phosphorylation event at Y380 of procaspase-8 that promotes the nonapoptotic role for procaspase-8 in adhesion and migration [125]. Analyses of TMS1 protein sequence show putative Src target sites, thus TMS1 may similarly be regulated by this pathway. Initial attempts at determining a role for Src in the regulation of TMS1 protein were conducted in HEK293 cells ectopically expressing TMS1 and a constitutively active Src. However, we were unable to see a difference in TMS1 expression or appearance of alternately migrating bands (indicative of phosphorylation) in these cells relative to controls (data not shown). An alternative approach to determine a role for Src in the regulation of TMS1 could involve treatment of cells with the Src inhibitor, PP2. TMS1 protein and mRNA expression levels could be tested in attached

and suspended cells in the presence or absence of this inhibitor. Additionally, protein lysates could be immunoprecipitated with anti-phosphotyrosine prior to probing for TMS1 to determine if differential expression of tyrosine phosphorylated-TMS1 occurs (or differs) in control and PP2 treated samples. Results from these analyses may determine if Src plays a role in the regulation of TMS1 during attachment or detachment.

TMS1 and primary cilia formation

In our more recent studies, we have shown that TMS1 localizes to apical, barrel-like structures in a subset of epithelial cells allowed to adhere to a natural substratum. Our data suggest that this structure represents a critical signaling center through a possible association with centrosomes. An association of TMS1 with centrosomes may also link TMS1 to a role in the formation of primary cilia. In order to address a role for TMS1 in the assembly of this organelle, GFP-TMS1 and a fluorescently–tagged cilia marker (e.g. YFP-acetylated alpha tubulin to mark the full-length of cilia including its basal body, YFP-gamma tubulin to mark centrosomes) could be ectopically expressed in cells that produce cilia (MCF10A, HEK293, etc). Following transfection, cells could be observed and analyzed by live cell imaging to determine the relationship between accumulation of TMS1 at the apical surface and cilia formation. The kinetics of an association of TMS1 and centrosomes or other primary cilia components that ultimately give rise to a mature primary cilium could also be determined. Moreover, stably knocked down TMS1 cells or TMS1 -/- mouse epithelial cells could be transfected with a fluorescently–tagged cilia marker. Growth of these cells on fibronectin could be

observed by live cell imaging to determine if primary cilia formation is delayed or otherwise hindered in the absence of TMS1.

In vivo *roles of TMS1*

As discussed in Chapter 1, two groups have created TMS1 knockout mice. Analyses involving these mice have focused on elucidating the role for TMS1 in procaspase-1 activation in response to pathogenic stimuli. More recently, Allen *et al.* conducted studies using a line of TMS1 -/- mice generated by Mariathasan *et al.* [106] to show that inflammasome mediators play a protective role against colitis associated cancer (CAC) of the colon [188]. These authors used clinically relevant models [dextran sulfate sodium (DSS) and azoxymethane + DSS] of ulcerative colitis that results in an increased risk of colorectal cancer to examine the impact of loss of key inflammasome components such as TMS1 in colon tumorigenesis. Through these analyses, they demonstrated that loss of TMS1 resulted in increased disease outcome including enhanced tumor burden, polyp formation and morbidity in mice. Interestingly, this study provides the only present link between loss of TMS1 expression and cancer progression in the mouse [188].

Together our data demonstrate an intriguing dual role for TMS1 in epithelial cell anoikis and migration. We find that a subset of DCIS lesions exhibit specific loss of TMS1 expression in cells that aberrantly fill the lumen of mammary ducts. In addition, our results show that attachment to fibronectin induces an unexpected role for this proapoptotic protein in survival signaling through the FAK and ERK1/2 pathways. Thus, in order to fully understand the functional importance of TMS1 in breast tissue, future studies could involve examining mammary duct development in TMS1 -/- mice. Results

from these studies may show that similar to the Bim knockout [91], silencing TMS1 may delay the hollowing of mammary ducts during breast development. Additionally, histological staining of breast tissue with an antibody against TMS1 may allow us to determine if TMS1 redistributes to barrel-like structures in epithelial cells *in vivo*. If tissue architecture plays a critical role in TMS1 redistribution, it is possible that a greater percentage of cells in mammary tissue will show TMS1 localization to these apical structures relative to cells in culture. Examination of the consequences of TMS1 silencing on epithelial architecture may further elucidate the balance by which the diverse functions of TMS1 are regulated *in vivo*. Finally, crossing clinically relevant breast cancer models (e.g. MMTV-PyMT or MMTV-ErbB2/neu mice [189]) with the TMS1 knockout may provide further insights into the functional impact of TMS1 on breast cancer progression. These analyses may shed light on the functional impacts of aberrant methylation and silencing of TMS1 observed in breast cancers.

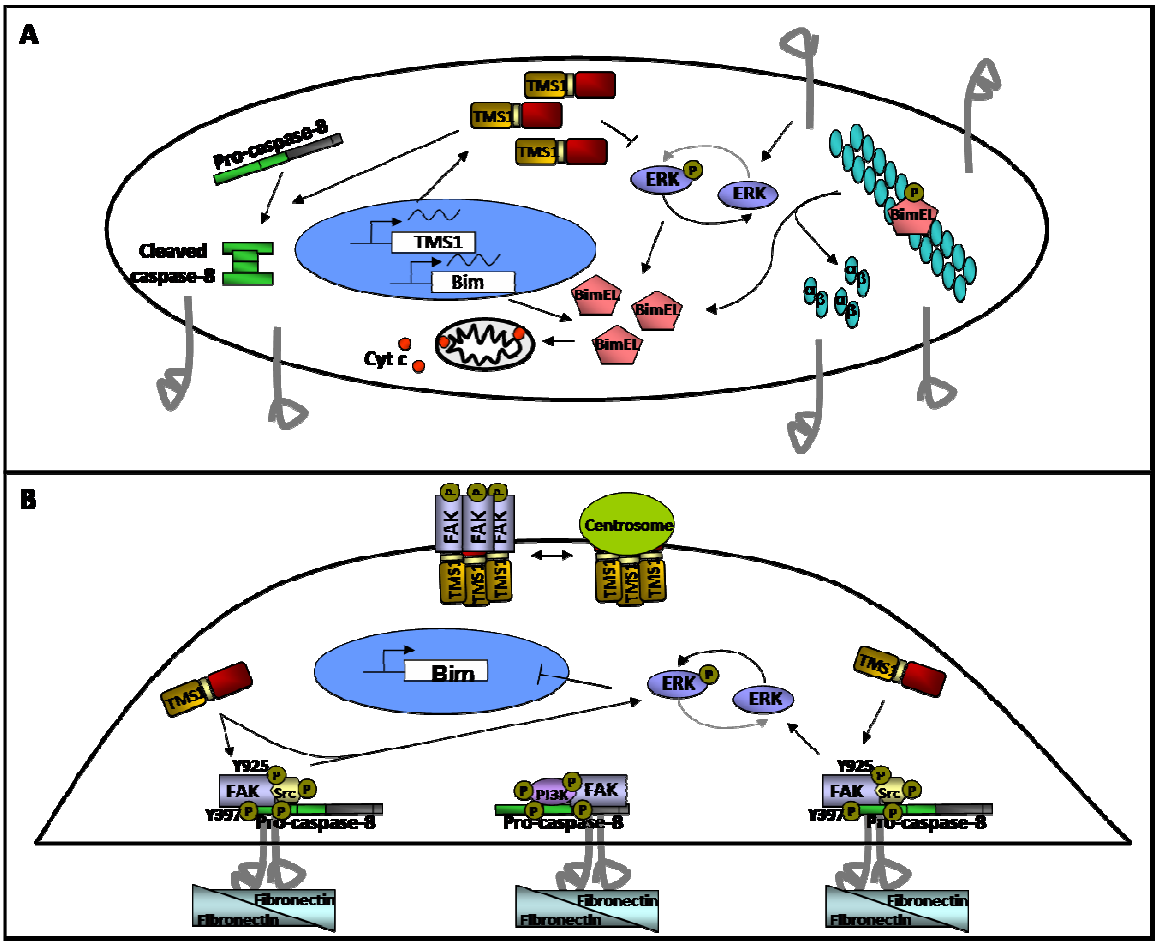


Figure 1: A model depicting the functions of TMS1 elucidated through work addressed in this thesis. A) We found that cellular detachment induces TMS1 mRNA and protein levels. Additionally, TMS1 was necessary for detachment-induced cleavage of procaspase-8 and accumulation of BimEL but dispensable for transcriptional induction of *Bim* during anoikis. ERK1/2 signaling negatively regulates BimEL. We further showed that TMS1 plays an essential role in inhibiting signaling through the ERK1/2 pathway during anoikis providing one mechanism by which TMS1 regulates BimEL accumulation. B) We showed that adhesion of breast epithelial cells to fibronectin results in the redistribution of TMS1 to apical structures. Moreover, we found a role for TMS1 in regulating FAK signaling and activation of ERK1/2. Both pFAK and centrosomal markers were shown to colocalize with TMS1 at apical structures in a subset of cells suggesting a role for the apical, barrel-like structure as a critical signaling center in epithelial cells. Functional consequences of loss of TMS1 included an inhibition in breast cell migration. Moreover, we show that TMS1 is necessary for efficient breast cell invasion through an extracellular matrix.

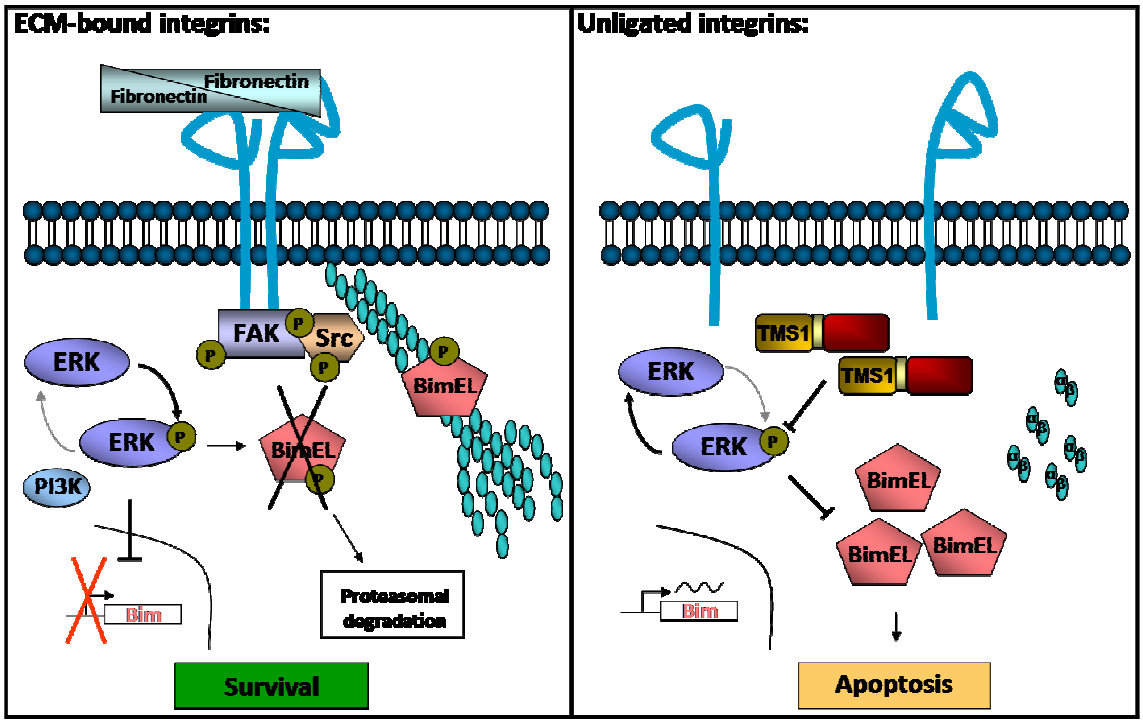


Figure 2: Role of TMS1 in BimEL accumulation during anoikis. In attached cells, survival signaling through the ERK1/2 and PI3K pathways inhibit *Bim* transcription. Additionally, BimEL is a target of ERK1/2 phosphorylation at Ser69 promoting its ubiquitination and proteasomal degradation. Finally, phosphorylation of BimEL at other sites allows it to be sequestered by the cytoskeletal network. These regulatory mechanisms collectively maintain low levels of BimEL in attached cells. Upon loss of integrin-ECM signals, downregulation of ERK1/2 and PI3K signaling results in induction of Bim transcription, prevention of BimEL degradation, and release of BimEL from the cytoskeletal network, resulting in an accumulation of BimEL and activation of the intrinsic pathway of apoptosis. Our work demonstrates that during detachment TMS1 specifically impacts BimEL protein levels by inhibiting ERK1/2 signaling. Thus, in its absence elevated levels of pERK1/2 prevent accumulation of BimEL during detachment.

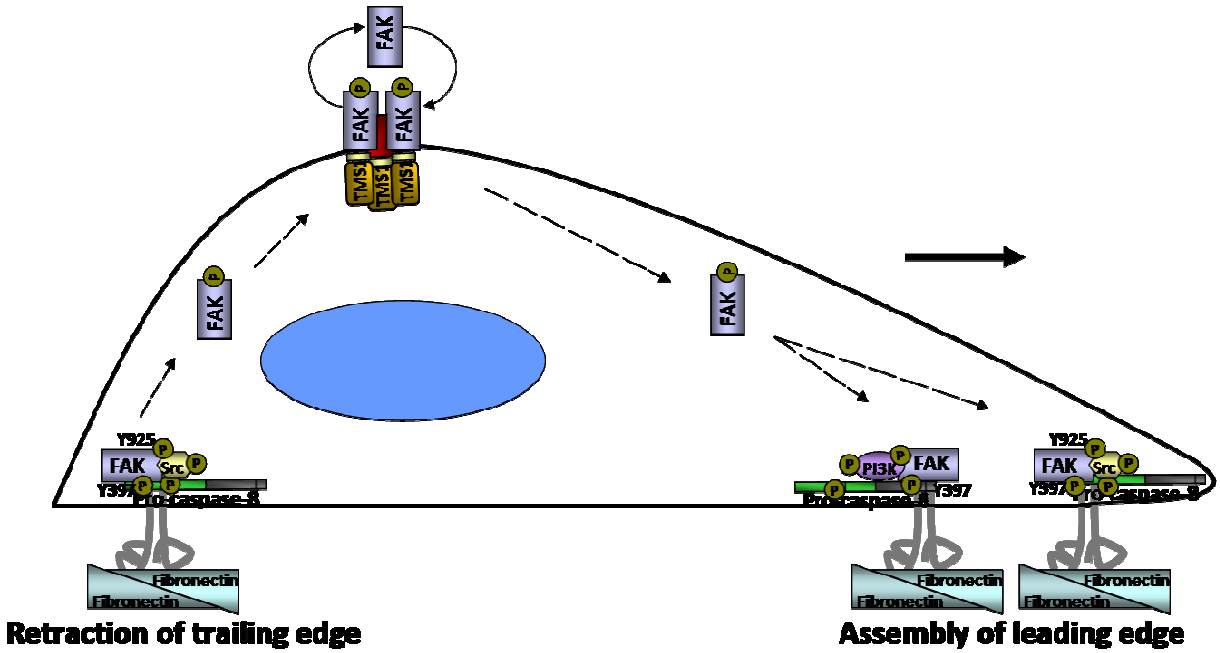


Figure 3: A model depicting a role for TMS1 in migration. During migration focal adhesion complexes are continually disassembled at the rear end of the cell and reassembled at the leading edge. We have shown that pFAK colocalizes with TMS1 at apical structures in a subset of cells. A potential role for TMS1 in migration may include turnover of pFAKs from the trailing edge of the cell so that they may be incorporated in newly assembled focal adhesions at the leading edge. Additionally, the sequestration of TMS1 at the cell surface may ensure that soluble levels of TMS1 remain low during these transient stages of detachment and reattachment ensuring that migrating cells do not prematurely undergo anoikis.

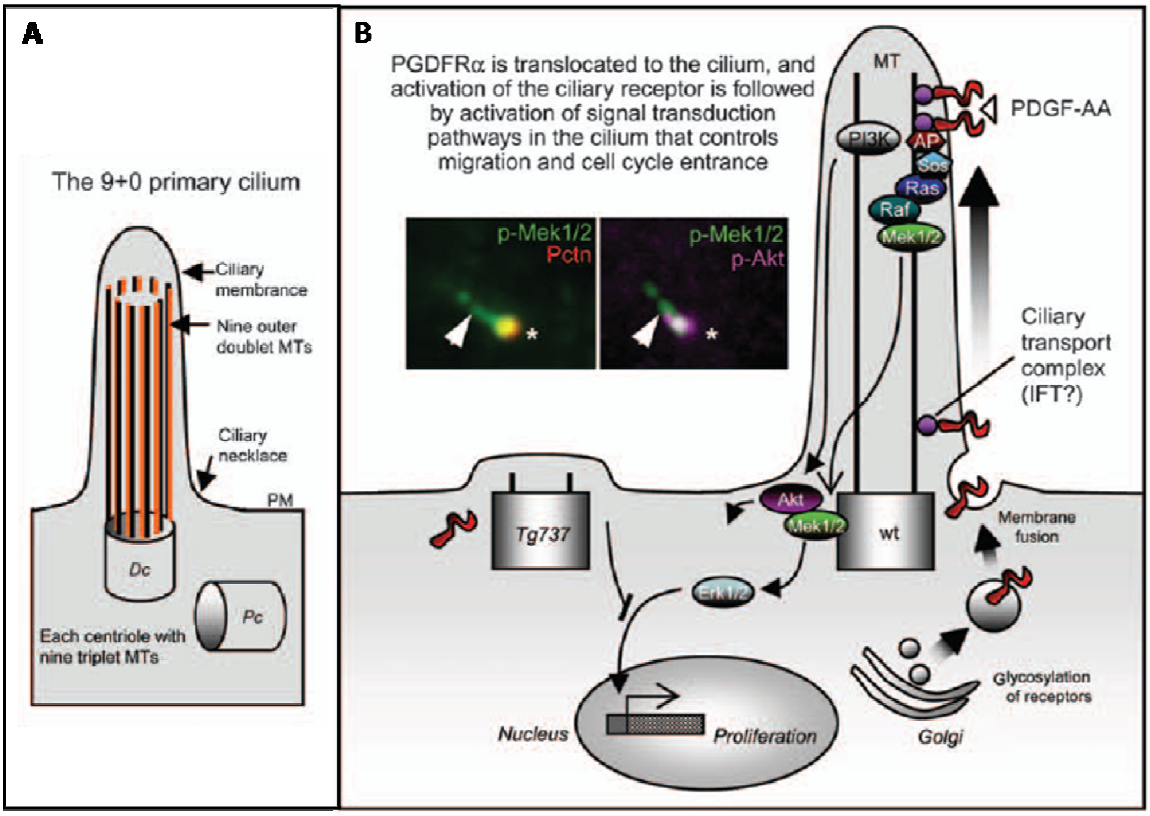
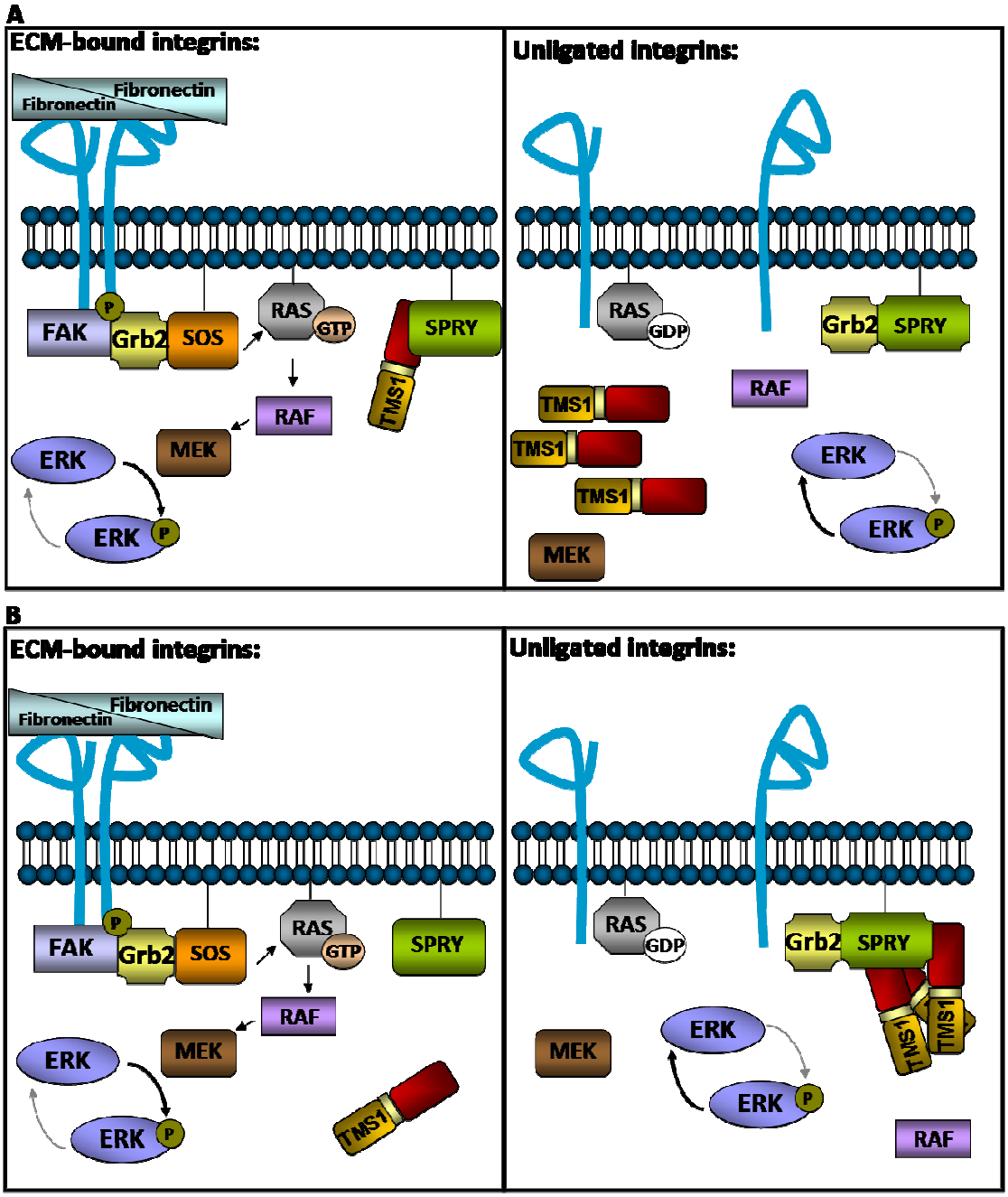


Figure 4: Structure of primary cilia and role in PDFGR/ERK1/2 signaling. A) Schematic view of the 9+0 primary cilium emerging as a solitary organelle from the distal centriole (Dc) of the centrosome (mother centriole; basal body) that is positioned beneath the plasma membrane in growth-arrested cells. B) Upon growth arrest and assembly of the cilium, glycosylated PDGFR-α is targeted to the ciliary necklace and transported into the ciliary membrane by an unknown mechanism. Stimulation with its ligand, PDGF-αα, results in dimerization and activation of PDGFR-α, which sets off the activation of a series of signal transduction cascades in the cilium and/or at the ciliary base including the Mek1/2-Erk1/2 and PI3K/Akt pathways, leading to changes in nuclear target genes that control cell cycle entrance. Activation of PDGFR-α and its signal transduction pathways is blocked Tg737orpk mutant cells that form no or very short primary cilia. Immunofluorescent analysis of the primary cilium in NIH fibroblasts. Left image: localization of PDGFR-α to the primary cilium (red); the nucleus is stained with DAPI (blue) and the cilium is visualized with anti-acetylated alpha-tubulin antibody (tb, green). Upper and lower right images: Upon stimulation with PDGF-αα and activation of ciliary PDGFR-α, Mek1/2 and Akt become activated by phosphorylation (p-Mek/2, green; p-Akt, magenta) in the primary cilium. The ciliary base is visualized with anti-pericentrin antibody (upper right image, red). Arrows mark the cilium and asterisks mark the ciliary base. Reprinted with permission from John Wiley and Sons [139].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RAS
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Figure 5: A model depicting roles for TMS1 in the differential regulation of ERK1/2 signaling in attached and detached cells. A) If an interaction between TMS1 and SPRY resulted in positive regulation of ERK1/2 such an interaction in attached cells would prevent binding of SPRY with a second known binding partner, Grb2. Thus, Grb2 would be able to positively impact ERK1/2 signaling. Upon detachment, TMS1 would release SPRY such that it could bind Grb2 and prevent its activation of the ERK1/2 pathway. B) Conversely, an interaction between TMS1 and SPRY may negatively regulate ERK1/2 signaling. In this scenario, attached cells would fail to show an interaction between TMS1 and SPRY. In the absence of this interaction, SPRY would be unable to bind and sequester Grb2 resulting in ERK1/2 signaling. Upon detachment induction of TMS1 would result in an interaction with SPRY allowing it to bind Grb2 and prevent activation of ERK1/2.

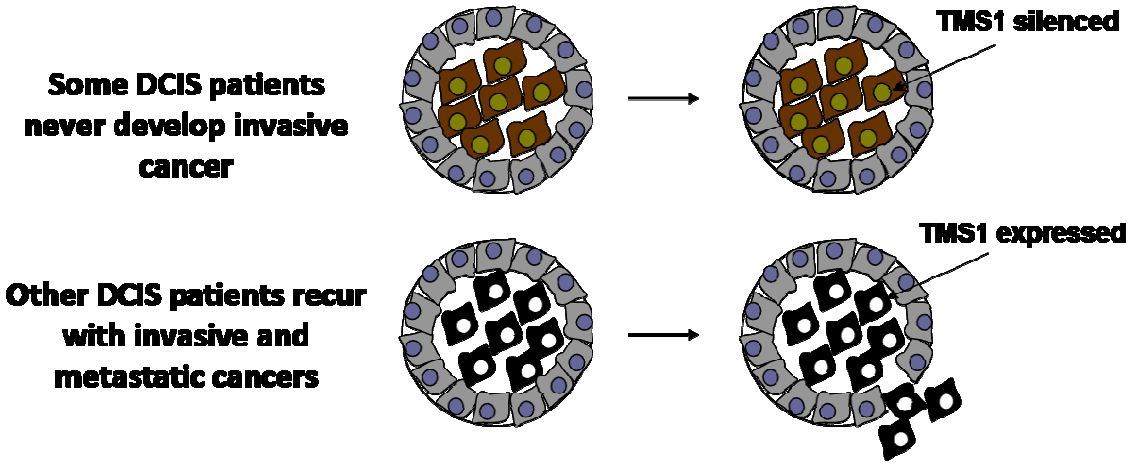

Figure 6: A model depicting a role for TMS1 in the progression of some DCIS lesions. We have shown that TMS1 is silenced in cells aberrantly filling the lumen in a subset of DCIS lesions. Additionally, our data indicate a nonapoptotic role for TMS1 in breast cell migration and invasion. Thus, taking into considerations the diverse functions of TMS1, our data would suggest that those DCIS lesions that exhibit loss of TMS1 may be less likely to become invasive relative to lesions that resulted from functional loss of an alternate mediator of anoikis and retained expression of TMS1.

Appendix

3D Morphogenesis assay

Introduction

The normal mammary gland is composed of a branching network of ductal-lobule units that are characterized by a double-layer of outer myoepithelial and inner luminal epithelial cells surrounding a hollow lumen [190]. Lobules consist of acini or alveoli that perform the secretory functions of the mammary gland. Ducts emanate from lobules to transport their secretions to the nipple. The basement membrane surrounds both mammary ducts and lobules separating them from the stroma which consists of fat, connective tissue, blood vessels, and lymphatics [31]. Together these various components exist in conjunction to form the intricate *in vivo* organization of the mammary gland.

Three dimensional (3D) epithelial culture conditions have been developed that allow mammary epithelial cells to form spherical structures that resemble their *in vivo* organization [191]. Over the course of 14 days, MECs cultured on an extracellular matrix (ECM) undergo a series of morphogenetic events resulting in the formation of spherical structures termed acini composed of a single layer of polarized epithelial cells surrounding a hollow lumen [192]. This technique can be used to study mammary morphogenesis *in vitro*.

Work by Dr. Joan Brugge has shown that MCF10A cells grown on a commercially available ECM, Matrigel, undergo a series of morphogenetic changes that recapitulate the *in vivo* architecture of mammary ducts. MCF10A cells seeded on Matrigel first cluster to form solid spheres composed of an outer layer of polarized epithelial cells. Apicobasal polarization is an early event in MCF10A morphogenesis that persists throughout growth of these cells in a 3D assay. Polarized epithelial cells are

oriented such that their basal surface contacts the basement membrane and their apical poles point towards the lumen. The internal cells undergo selective apoptosis, due to the absence of matrix contacts resulting in the formation of a hollow lumen (Figure 1) [192]. Studies using this assay by Dr. Brugge and others indicate that an imbalance between proliferative signals and apoptotic signals results in the formation of a filled lumen. When MCF10A cells overexpressing oncogenes are grown on an exogenous ECM (Matrigel), they still form spherical structures with a hollow lumen since their excessive proliferation is balanced by an accompanied increase in apoptosis [124]. Thus, in order for tumor cells to populate a lumen, they must be able to overcome apoptosis. Bim, a critical mediator of anoikis has been shown to play a significant role in hollow lumen formation during MCF10A morphogenesis. siRNA knockdown of Bim blocks luminal apoptosis and delays hollow lumen formation of 3D acini [89]. Additionally, hypoxia-mediated ERK1/2 survival signaling promotes luminal filling in part through downregulation of Bim [193]. Similarly, the *Bim* knockout mouse demonstrates that disruption of Bim prevents apoptosis and delays clearance of luminal cells in terminal end branches of mammary ducts during puberty [91]. These results suggest that disruption of proapoptotic proteins that mediate anoikis may significantly impact normal mammary development.

TMS1 is a proapoptotic gene whose expression is frequently lost in breast cancers due to epigenetic silencing [92]. How TMS1 silencing contributes to breast cancer development is not known. Our data show that TMS1 is selectively expressed in the luminal epithelial cells that line normal mammary ducts, and decreased TMS1 expression is observed in the dysplastic cells that fill the lumen of a subset of DCIS lesions.

Additionally, we have shown a key role for TMS1 in anoikis in part through regulation of BimEL. Thus, we aimed to determine the impact of TMS1 silencing on mammary morphogenesis in an MCF10A 3D culture model.

Materials and Methods

Cell culture: MCF10A cells were a kind gift from Dr. Joan Brugge and maintained in DMEM/F12 plus 5% fetal bovine serum, 20ng/ml epidermal growth factor, 0.5ug/ml hydrocortisone, 100ng/ml cholera toxin, 10ug/ml insulin, and 2mM glutamine. Matrigel was purchased from BD Biosciences (Cat# 356230) with preferential protein concentrations of 10-12mg/ml. Matrigel solidifies at room temperature so aliquots were stored at -80°C and thawed on ice overnight at 4°C prior to use.

3D culture of MCF10A cells: This assay was performed according to methods outlined by Debnath *et al.* [192]. Briefly, 40µL of Matrigel was added to each well of an eight-well chamber and incubated at 37˚C for 15 minutes to allow Matrigel to completely solidify. During this time MCF10A cells were trypsinized and quenched with Resuspension media (20% FBS in DMEM/F12). Cells were then counted and diluted in Assay media (lacking EGF) to a concentration of 2.5E4 cells/ml. A second stock of Assay media was prepared containing 4% Matrigel and 10ng/ml. Cells diluted in Assay media were mixed with Assay media containing Matrigel and EGF in a 1:1 ratio. 400µL of the final cell mixture was added to each Matrigel coated well such that each well contained 5E3 cells in media containing 2% Matrigel and 5ng/ml EGF. Cells were refed with Assay media containing 2% Matrigel and 5ng/ml EGF every 4 days.

Immunofluorescence. MCF10A acini were fixed in 2% formalin for 20 minutes at room temperature (RT) followed by permeabilization in 0.5% Triton X-100 for 10 min at 4°C. Spheres were rinsed 3X in PBS-glycine (130 mM NaCl, 7 mM Na2HPO4, 3.5 mM NaH2PO4, 100 mM glycine) for 15 minutes per wash. Cells were blocked in IF buffer (130 mM NaCl, 7 mM Na2HPO4, 3.5 mM NaH2PO4, 7.7 mM NaN3, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween-20) + 10% goat serum for 1–1.5 h at room. Primary antibodies were diluted in blocking buffer at 1:100 and incubated with cells overnight at 4°C. Cells were rinsed in IF buffer 3X for 20 minutes at RT. Alexafluor 488 secondary antibodies incubations were done at 1:500 for 1hour at RT (Invitrogen). The stain 0.4 µmol/L 4‘,6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei. Cells were mounted using mounting medium (Electron Microscopy Sciences) and dried overnight in the dark. Imaging analyses were performed using the Zeiss LSM510 Confocal Microscope (Zeiss) with a 63X (numerical aperture, 1.4) Apochromat objective.

shRNA production and transduction: 7E5 HEK293 cells were plated per 6 cm dish and allowed to grow overnight. The following morning cells were transfected with packaging virus, envelope virus, and shRNA using fugene (Roche). 18 hours post-transfection, media was changed to 30% FBS in DMEM. Following 24 hours media was harvested. A second harvest was collected after an additional 24 hours. 1E5 of MCF10A/MCF7 cells were plated in per well of a 12-well dish and allowed to grow overnight. 100-750µL of

viral harvest was used to transduce cells in the presence of 6µg/ml polybrene. Transduced cells were subsequently selected with 0.75µg/ml puromycin.

Immunoblotting. Cells were pelleted, washed three times in 1 X PBS, and lysed with RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris pH 8.0) containing 1 X protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche, Indianapolis, IN, USA). Total protein was separated by SDS–PAGE gel, transferred to PVDF or nitrocellulose (BioRad, Hercules, CA, USA), and probed with the indicated primary antibody. Immunocomplexes were detected by incubation with horseradish peroxidase-conjugated secondary antibody and chemiluminescence detection (Pierce, Rockford, IL, USA). The antibodies used were as follows: anti-ASC/TMS1 (Protein Tech), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam).

Results

Our first task in addressing this hypothesis was to establish the 3D culture conditions outlined by Debnath *et al.* [192] necessary to generate acini. Previous work by the Brugge laboratory established the time-frame of morphogenetic events during culture of MCF10A cells in Matrigel that result in the formation of hollow acini [194]. These events occur over the course of 14-20 days and include apicobasal polarization, cell growth arrest, and luminal apoptosis [192]. The occurrence of these events can be examined by immunofluorescent staining of acini and visualization using confocal microscopy. We initiated growth of MCF10A cells on Matrigel using the methods outlined in Debnath *et al.* [192] and were able to recapitulate staining and visualization of

these spheres with suggested antibodies (Figure 2). The golgi of polarized cells orient toward the apical surface, and consequently, golgi proteins such as GM130 serve as markers of the apical region of epithelial cells. Immunofluorescent staining of spheres for GM130 showed that cells became polarized as early as 4 days and remained polarized after 14 days of growth on Matrigel (Figure 2A), consistent with published reports. Additionally, only cells in the outer region of spheres maintained contact with the ECM as evidenced by immunostaining for laminin (Figure 2B), a component of the ECM. Finally, staining with the nuclear stain, DAPI, indicated that spheres resulting from 14 days of growth on Matrigel were hollow (Figure 2C). Altogether, these data suggest that conditions outlined by the Brugge laboratory by which to grow MCF10A cells in a 3D morphogenesis assay were successfully recapitulated.

In order to determine if TMS1 silencing delays or otherwise hinders epithelial polarization, survival signaling and/or detachment-induced apoptosis of breast epithelial cells in this 3D model of mammary morphogenesis, we next aimed to generate a stable knockdown of TMS1 in MCF10A cells. In order to address this task, we obtained short-hairpin RNA (shRNA) constructs targeted against TMS1. Additionally, a nontargeting pLKO.1 shRNA vector was used as a negative control. These constructs were co-transfected in HEK293T cells with constructs expressing viral packaging proteins. Viral aliquots were then collected and used to transduce MCF10A cells under selective pressure against puromycin. Protein was collected from viral-treated MCF10A cells and analyzed for TMS1 expression by western blot. All tested TMS1 shRNA constructs showed little impact on TMS1 expression relative to control cells transduced with virus expressing an empty pLKO.1 vector (Figure 3A). Inefficient knockdown of TMS1 by

these shRNA constructs could have resulted from an extremely low viral titer, reduced transduction of MCF10A cells with virus carrying TMS1 shRNA, or a minimal impact of expressed shRNA constructs on *TMS1* message. Therefore we next determined the impact of these shRNA constructs against TMS1 expression in the more readily transfectable breast cancer cell line, MCF7. As shown in Figure 3B, shRNA vector #3 targeting TMS1 silenced TMS1 expression in these cells. These data suggest that at least one shRNA construct is able to effectively inhibit expression of TMS1 in these cells.

Discussion

In summary, we have recapitulated a method described by the Brugge laboratory [192] to generate hollow spheres that demonstrate proper epithelial polarization and cell-specific contact with the ECM. However, we have been unable determine if silencing TMS1 impacts hollow lumen formation in this assay. Our efforts using an shRNA approach to generate a stable line of MCF10A cells silent for TMS1 have been unsuccessful. Interestingly, transduction of virus expressing the control vector, pLKO.1, reduced TMS1 expression in MCF10A cells but not in MCF7 cells (although an untreated MCF7 control was not included) suggesting that this control may have some nonspecific effects in this cell line. Additionally, similar to immortalized mouse embryonic fibroblasts and the NIH3T3 fibroblast cell line, MCF10A cells exhibit low transfection efficiency [195]. This observation in conjunction with our data suggest that some of the technical difficulties we have experienced in creating an MCF10A cell line stably silenced for TMS1 may be intrinsic to this cell line. However, laboratories have successfully developed stable lines of MCF10A cells [89,192,196], suggesting that with

the right conditions these drawbacks should be overcome. In order to establish a stable knockdown of TMS1 in MCF10A cells, it may be beneficial to determine the viral titer of samples and optimize the viral concentration necessary to transduce this cell line.

Reginato *et al.* demonstrated that siRNA knockdown was sufficient to downregulate Bim for up to 15 days of growth in this assay [89]. Thus, siRNA knockdown of TMS1 may similarly be sufficient to investigate the impact of TMS1 silencing on lumen formation of mammary acini. 3D culture of murine mammary epithelial cells has shown that these cells are also able to form acinus-like structures [197]. Thus, an alternative approach to investigate a role for TMS1 in the growth and development of these spheres would be to obtain the TMS1 knockout mouse made by other groups [106,107] from which mammary epithelial cells could be extracted. Comparing growth of these cells to mammary epithelial cells from wild-type mice would allow us to determine if TMS1 silencing delays or otherwise hinders the formation of a hollow lumen.

In addition to examining a role for TMS1 in lumen formation, further studies using this assay may provide a means to study the non-apoptotic role of TMS1 in cell migration and invasion in mammary morphogenesis. In this regard, a recent study by Chen *et al.* demonstrated a role for the MAPK kinase kinase, MLK3, in proliferation, apoptosis, and invasion using this assay. These authors demonstrated that overexpression of MLK3 in MCF10A cells allowed them to both avoid growth arrest and apoptosis to form abnormally large acini. In addition, overexpression of MLK3 in these cells induced expression of the proinvasive proteins, Fra-1 and vimentin in mammary acini in a JNK-dependent manner. The role for MLK3 in cell invasion was supported by Boyden

chamber assays [198]. Having observed a similar role for TMS1 in invasion of MCF10A cells, it would be interesting to generate a line of MCF10A cells stably overexpressing TMS1 and determine if its overexpression similarly accompanies expression of pro-invasive proteins in acini formed during 3D culture. Experiments such as these may allow us to explore our hypothesis regarding the proapoptotic and migratory roles of TMS1 in the progression of DCIS to invasive lesions (Chapter 4, Figure 6).

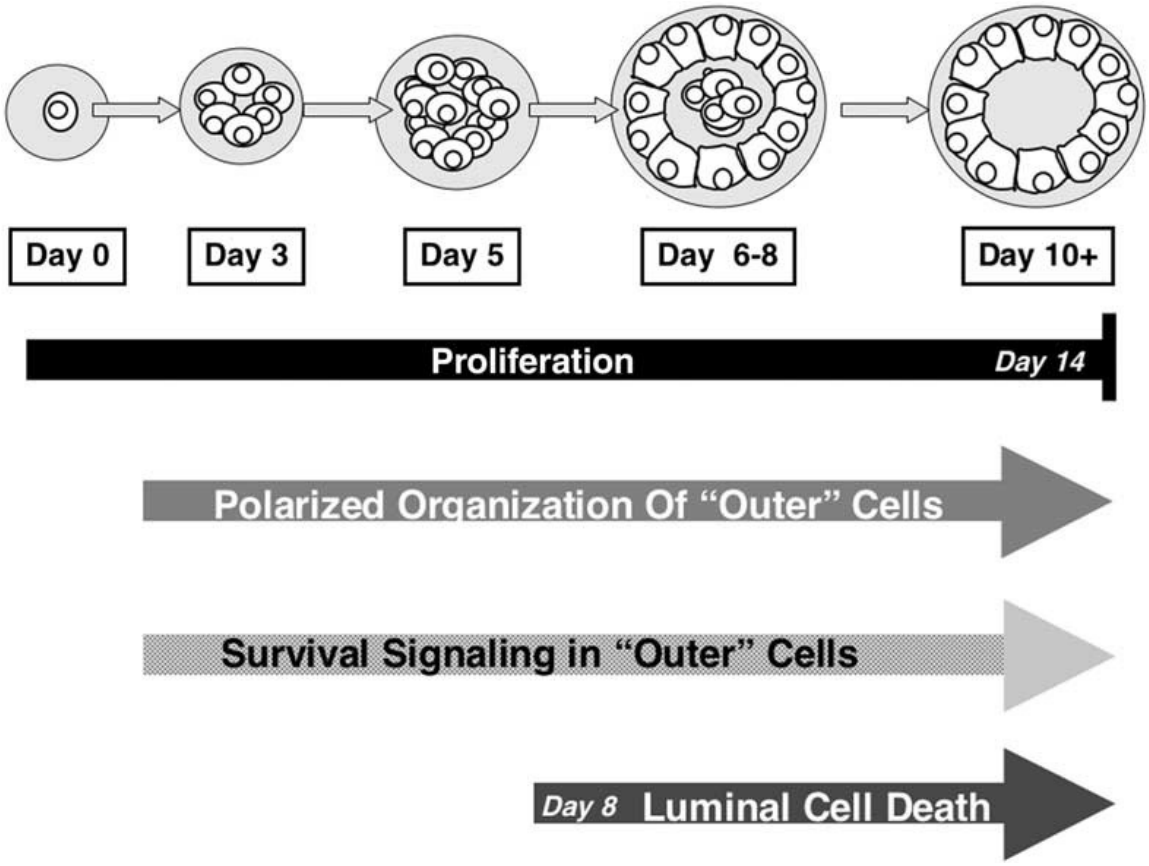

Figure 1: Schematic of biological events during MCF10A acinar morphogenesis. Following 3-4 days of growth on Matrigel, MCF10A cells cluster in small groups. Cells become polarized with their basal surface in contact with the ECM and their apical surface oriented towards the lumen. During days 5-8, two distinct populations arise: highly polarized cells in contact with the ECM that receive survival signaling and an inner subset of cells that are poorly polarized and detached from the matrix. By day 8, the inner cells began to die resulting in the formation of a hollow lumen. Reprinted with permission from Elsevier [192].

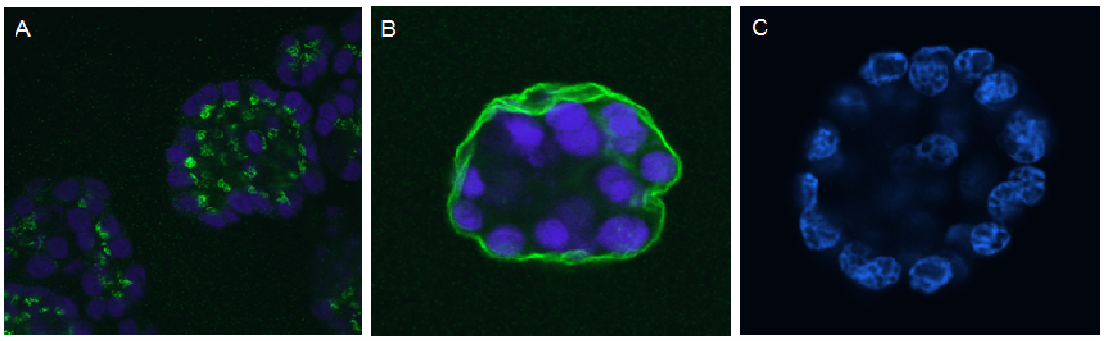

Figure 2: Formation of hollow spheres that mimic mammary gland development. A) MCF10A cells were seeded onto Matrigel and allowed to grow for 14 days. Resultant spheres were stained with an antibody to GM130 and DAPI. The stained spheres show that epithelial cells that line the hollow lumen are polarized with their apical surface oriented toward the lumen. B) Spheres resulting from 20 days of growth were stained with an antibody to laminin and DAPI and show that only cells that line the outer region of the spheres are in contact with the ECM. C) Spheres stained with DAPI showing formation of a hollow lumen following 14 days of growth on Matrigel. Shown are representative images from at least two independent experiments.

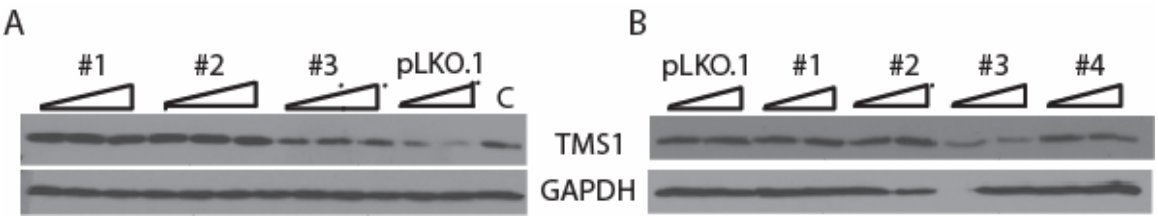

Figure 3: Effect of shRNA targeting TMS1 on its expression. A) MCF10A cells were infected with lentivirus expressing shRNAs against TMS1 (1-3) or an empty vector (pLKO.1). Following infection cells were selected against puromycin for five days at which time protein was collected and analyzed for TMS1 expression. A protein sample from untreated MCF10A cells was also used as a negative control. Shown is a representative image from three independent experiments. B) MCF7 cells were infected as described in A. A fourth shRNA vector targeting TMS1 was included in this analysis. This experiment was performed one time.

References:

1. American Cancer Society, I. (2010) Breast Cancer Facts and Figures 2009-2010. vol. American Cancer Society.
2. Hanahan, D. and Weinberg, R.A. (2000) The hallmarks of cancer. *Cell*, **100**, 57-70.
3. Goldberg, Y.P., Parker, M.I. and Gevers, W. (1991) The genetic basis of cancer. *S Afr Med J*, **80**, 99-104.
4. Rous, P. (1910) A Transmissible Avian Neoplasm. (Sarcoma of the Common Fowl.). *J Exp Med*, **12**, 696-705.
5. Rous, P. (1911) A Sarcoma of the Fowl Transmissible by an Agent Separable from the Tumor Cells. *J Exp Med*, **13**, 397-411.
6. Bishop, J.M., Baker, B., Fujita, D., McCombe, P., Sheiness, D., Smith, K., Spector, D.H., Stehelin, D. and Varmus, H.E. (1978) Genesis of a virus-transforming gene. *Natl Cancer Inst Monogr*, 219-23.
7. Payne, G.S., Bishop, J.M. and Varmus, H.E. (1982) Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. *Nature*, **295**, 209-14.
8. Chang, E.H., Furth, M.E., Scolnick, E.M. and Lowy, D.R. (1982) Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. *Nature*, **297**, 479-83.
9. Chen, Y. and Olopade, O.I. (2008) MYC in breast tumor progression. *Expert Rev Anticancer Ther*, **8**, 1689-98.
10. Mitin, N., Rossman, K.L. and Der, C.J. (2005) Signaling interplay in Ras superfamily function. *Curr Biol*, **15**, R563-74.
11. Schubbert, S., Shannon, K. and Bollag, G. (2007) Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer*, **7**, 295-308.
12. Baylin, S.B. (2005) DNA methylation and gene silencing in cancer. *Nat Clin Pract Oncol*, **2 Suppl 1**, S4-11.
13. Klein, G. (1987) The approaching era of the tumor suppressor genes. *Science*, **238**, 1539-45.
14. Knudson, A.G., Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A*, **68**, 820-3.
15. Santarosa, M. and Ashworth, A. (2004) Haploinsufficiency for tumour suppressor genes: when you don't need to go all the way. *Biochim Biophys Acta*, **1654**, 105-22.
16. American Cancer Society, I. (2010) Cancer Facts and Figures 2010. Atlanta, vol. American Cancer Society.
17. Hainaut, P. and Hollstein, M. (2000) p53 and human cancer: the first ten thousand mutations. *Adv Cancer Res*, **77**, 81-137.
18. Loeb, L.A. (1991) Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res*, **51**, 3075-9.
19. Bodmer, W., Bielas, J.H. and Beckman, R.A. (2008) Genetic instability is not a requirement for tumor development. *Cancer Res*, **68**, 3558-60; discussion 3560-1.
20. Stratton, M.R., Campbell, P.J. and Futreal, P.A. (2009) The cancer genome. *Nature*, **458**, 719-24.

21. Bird, A.P. (1986) CpG-rich islands and the function of DNA methylation. *Nature*, **321**, 209-13.
22. Costello, J.F., Fruhwald, M.C., Smiraglia, D.J., Rush, L.J., Robertson, G.P., Gao, X., Wright, F.A., Feramisco, J.D., Peltomaki, P., Lang, J.C., Schuller, D.E., Yu, L., Bloomfield, C.D., Caligiuri, M.A., Yates, A., Nishikawa, R., Su Huang, H., Petrelli, N.J., Zhang, X., O'Dorisio, M.S., Held, W.A., Cavenee, W.K. and Plass, C. (2000) Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet*, **24**, 132-8.
23. Herman, J.G., Latif, F., Weng, Y., Lerman, M.I., Zbar, B., Liu, S., Samid, D., Duan, D.S., Gnarra, J.R., Linehan, W.M. and et al. (1994) Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A*, **91**, 9700-4.
24. Linehan, W.M., Lerman, M.I. and Zbar, B. (1995) Identification of the von Hippel-Lindau (VHL) gene. Its role in renal cancer. *JAMA*, **273**, 564-70.
25. Gonzalez-Zulueta, M., Bender, C.M., Yang, A.S., Nguyen, T., Beart, R.W., Van Tornout, J.M. and Jones, P.A. (1995) Methylation of the 5' CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. *Cancer Res*, **55**, 4531-5.
26. Tlsty, T.D., Crawford, Y.G., Holst, C.R., Fordyce, C.A., Zhang, J., McDermott, K., Kozakiewicz, K. and Gauthier, M.L. (2004) Genetic and epigenetic changes in mammary epithelial cells may mimic early events in carcinogenesis. *J Mammary Gland Biol Neoplasia*, **9**, 263-74.
27. Esteller, M. (2000) Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes. *Eur J Cancer*, **36**, 2294-300.
28. Lapidus, R.G., Ferguson, A.T., Ottaviano, Y.L., Parl, F.F., Smith, H.S., Weitzman, S.A., Baylin, S.B., Issa, J.P. and Davidson, N.E. (1996) Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. *Clin Cancer Res*, **2**, 805-10.
29. Leader, J.E., Wang, C., Popov, V.M., Fu, M. and Pestell, R.G. (2006) Epigenetics and the estrogen receptor. *Ann N Y Acad Sci*, **1089**, 73-87.
30. Laird, P.W. (2005) Cancer epigenetics. *Hum Mol Genet*, **14 Spec No 1**, R65-76.
31. Weigelt, B. and Bissell, M.J. (2008) Unraveling the microenvironmental influences on the normal mammary gland and breast cancer. *Semin Cancer Biol*, **18**, 311-21.
32. Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M. and Bos, J.L. (1988) Genetic alterations during colorectal-tumor development. *N Engl J Med*, **319**, 525-32.
33. Simpson, P.T., Reis-Filho, J.S., Gale, T. and Lakhani, S.R. (2005) Molecular evolution of breast cancer. *J Pathol*, **205**, 248-54.
34. Frykberg, E.R. and Bland, K.I. (1994) Management of in situ and minimally invasive breast carcinoma. *World J Surg*, **18**, 45-57.
35. Frykberg, E.R. and Bland, K.I. (1994) Overview of the biology and management of ductal carcinoma in situ of the breast. *Cancer*, **74**, 350-61.

36. Erbas, B., Provenzano, E., Armes, J. and Gertig, D. (2006) The natural history of ductal carcinoma in situ of the breast: a review. *Breast Cancer Res Treat*, **97**, 135-44.
37. Palmieri, D., Smith, Q.R., Lockman, P.R., Bronder, J., Gril, B., Chambers, A.F., Weil, R.J. and Steeg, P.S. (2006) Brain metastases of breast cancer. *Breast Dis*, **26**, 139-47.
38. Weigelt, B., Peterse, J.L. and van 't Veer, L.J. (2005) Breast cancer metastasis: markers and models. *Nat Rev Cancer*, **5**, 591-602.
39. Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S.X., Lonning, P.E., Borresen-Dale, A.L., Brown, P.O. and Botstein, D. (2000) Molecular portraits of human breast tumours. *Nature*, **406**, 747-52.
40. Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Eystein Lonning, P. and Borresen-Dale, A.L. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*, **98**, 10869-74.
41. Morris, S.R. and Carey, L.A. (2007) Molecular profiling in breast cancer. *Rev Endocr Metab Disord*, **8**, 185-98.
42. Takada, Y., Ye, X. and Simon, S. (2007) The integrins. *Genome Biol*, **8**, 215.
43. Shattil, S.J., Kim, C. and Ginsberg, M.H. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol*, **11**, 288-300.
44. Liddington, R.C. and Ginsberg, M.H. (2002) Integrin activation takes shape. *J Cell Biol*, **158**, 833-9.
45. Schlaepfer, D.D., Hauck, C.R. and Sieg, D.J. (1999) Signaling through focal adhesion kinase. *Prog Biophys Mol Biol*, **71**, 435-78.
46. Huang, C., Jacobson, K. and Schaller, M.D. (2004) MAP kinases and cell migration. *J Cell Sci*, **117**, 4619-28.
47. Zhao, J. and Guan, J.L. (2009) Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev*, **28**, 35-49.
48. Guan, J.L., Trevithick, J.E. and Hynes, R.O. (1991) Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Regul*, **2**, 951-64.
49. Kanner, S.B., Reynolds, A.B., Vines, R.R. and Parsons, J.T. (1990) Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc Natl Acad Sci U S A*, **87**, 3328-32.
50. Brunton, V.G., Avizienyte, E., Fincham, V.J., Serrels, B., Metcalf, C.A., 3rd, Sawyer, T.K. and Frame, M.C. (2005) Identification of Src-specific phosphorylation site on focal adhesion kinase: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior. *Cancer Res*, **65**, 1335-42.
51. Barcellos-Hoff, M.H., Aggeler, J., Ram, T.G. and Bissell, M.J. (1989) Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development*, **105**, 223-35.

52. Shaw, L.M. (1999) Integrin function in breast carcinoma progression. *J Mammary Gland Biol Neoplasia*, **4**, 367-76.
53. Sympson, C.J., Talhouk, R.S., Alexander, C.M., Chin, J.R., Clift, S.M., Bissell, M.J. and Werb, Z. (1994) Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J Cell Biol*, **125**, 681-93.
54. Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, **26**, 239-57.
55. Alberts B, J.A., Lewis J, Raff M, Roberts K, Walter P (2002) *Molecular Biology of the Cell, 4th edition*. Garland Science, New York.
56. Nelson, D.A. and White, E. (2004) Exploiting different ways to die. *Genes Dev*, **18**, 1223-6.
57. Hutcheson, J. and Perlman, H. (2008) BH3-only proteins in rheumatoid arthritis: potential targets for therapeutic intervention. *Oncogene*, **27 Suppl 1**, S168-75.
58. Salvesen, G.S. and Dixit, V.M. (1997) Caspases: intracellular signaling by proteolysis. *Cell*, **91**, 443-6.
59. Ellis, H.M. and Horvitz, H.R. (1986) Genetic control of programmed cell death in the nematode C. elegans. *Cell*, **44**, 817-29.
60. Yi, C.H. and Yuan, J. (2009) The Jekyll and Hyde functions of caspases. *Dev Cell*, **16**, 21-34.
61. Black, R.A., Kronheim, S.R. and Sleath, P.R. (1989) Activation of interleukin-1 beta by a co-induced protease. *FEBS Lett*, **247**, 386-90.
62. Kostura, M.J., Tocci, M.J., Limjuco, G., Chin, J., Cameron, P., Hillman, A.G., Chartrain, N.A. and Schmidt, J.A. (1989) Identification of a monocyte specific pre-interleukin 1 beta convertase activity. *Proc Natl Acad Sci U S A*, **86**, 5227-31.
63. Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and Horvitz, H.R. (1993) The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell*, **75**, 641-52.
64. Lamkanfi, M., Declercq, W., Kalai, M., Saelens, X. and Vandenabeele, P. (2002) Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ*, **9**, 358-61.
65. Stennicke, H.R. and Salvesen, G.S. (1999) Catalytic properties of the caspases. *Cell Death Differ*, **6**, 1054-9.
66. Hengartner, M.O. (2000) The biochemistry of apoptosis. *Nature*, **407**, 770-6.
67. Fuentes-Prior, P. and Salvesen, G.S. (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J*, **384**, 201-32.
68. Weber, C.H. and Vincenz, C. (2001) The death domain superfamily: a tale of two interfaces? *Trends Biochem Sci*, **26**, 475-81.
69. Martinon, F., Hofmann, K. and Tschopp, J. (2001) The pyrin domain: a possible member of the death domain-fold family implicated in apoptosis and inflammation. *Curr Biol*, **11**, R118-20.
70. Kohl, A. and Grutter, M.G. (2004) Fire and death: the pyrin domain joins the death-domain superfamily. *C R Biol*, **327**, 1077-86.

71. Bertin, J. and DiStefano, P.S. (2000) The PYRIN domain: a novel motif found in apoptosis and inflammation proteins. *Cell Death Differ*, **7**, 1273-4.
72. Martin, S.S. and Vuori, K. (2004) Regulation of Bcl-2 proteins during anoikis and amorphosis. *Biochim Biophys Acta*, **1692**, 145-57.
73. Willis, S.N. and Adams, J.M. (2005) Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol*, **17**, 617-25.
74. Dijkers, P.F., Medema, R.H., Lammers, J.W., Koenderman, L. and Coffer, P.J. (2000) Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol*, **10**, 1201-4.
75. Reginato, M.J., Mills, K.R., Paulus, J.K., Lynch, D.K., Sgroi, D.C., Debnath, J., Muthuswamy, S.K. and Brugge, J.S. (2003) Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol*, **5**, 733-40.
76. Puthalakath, H., Huang, D.C., O'Reilly, L.A., King, S.M. and Strasser, A. (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell*, **3**, 287-96.
77. Marani, M., Hancock, D., Lopes, R., Tenev, T., Downward, J. and Lemoine, N.R. (2004) Role of Bim in the survival pathway induced by Raf in epithelial cells. *Oncogene*, **23**, 2431-41.
78. Youle, R.J. and Strasser, A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*, **9**, 47-59.
79. Chipuk, J.E., Bouchier-Hayes, L. and Green, D.R. (2006) Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ*, **13**, 1396-402.
80. Fulda, S. and Debatin, K.M. (2006) Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*, **25**, 4798-811.
81. Krammer, P.H. (2000) CD95's deadly mission in the immune system. *Nature*, **407**, 789-95.
82. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H. and Peter, M.E. (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*, **17**, 1675-87.
83. Frisch, S.M. and Francis, H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol*, **124**, 619-26.
84. Simpson, C.D., Anyiwe, K. and Schimmer, A.D. (2008) Anoikis resistance and tumor metastasis. *Cancer Lett*, **272**, 177-85.
85. Frisch, S.M. (1999) Evidence for a function of death-receptor-related, death-domain-containing proteins in anoikis. *Curr Biol*, **9**, 1047-9.
86. Rytomaa, M., Martins, L.M. and Downward, J. (1999) Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. *Curr Biol*, **9**, 1043-6.
87. Aoudjit, F. and Vuori, K. (2001) Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. *J Cell Biol*, **152**, 633-43.
88. Frankel, A., Rosen, K., Filmus, J. and Kerbel, R.S. (2001) Induction of anoikis and suppression of human ovarian tumor growth in vivo by downregulation of Bcl-X(L). *Cancer Res*, **61**, 4837-41.

89. Reginato, M.J., Mills, K.R., Becker, E.B., Lynch, D.K., Bonni, A., Muthuswamy, S.K. and Brugge, J.S. (2005) Bim regulation of lumen formation in cultured mammary epithelial acini is targeted by oncogenes. *Mol Cell Biol*, **25**, 4591-601.
90. Woods, N.T., Yamaguchi, H., Lee, F.Y., Bhalla, K.N. and Wang, H.G. (2007) Anoikis, initiated by Mcl-1 degradation and Bim induction, is deregulated during oncogenesis. *Cancer Res*, **67**, 10744-52.
91. Mailleux, A.A., Overholtzer, M., Schmelzle, T., Bouillet, P., Strasser, A. and Brugge, J.S. (2007) BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. *Dev Cell*, **12**, 221-34.
92. Conway, K.E., McConnell, B.B., Bowring, C.E., Donald, C.D., Warren, S.T. and Vertino, P.M. (2000) TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. *Cancer Res*, **60**, 6236-42.
93. Collard, R.L., Harya, N.S., Monzon, F.A., Maier, C.E. and O'Keefe, D.S. (2006) Methylation of the ASC gene promoter is associated with aggressive prostate cancer. *Prostate*, **66**, 687-95.
94. Stone, A.R., Bobo, W., Brat, D.J., Devi, N.S., Van Meir, E.G. and Vertino, P.M. (2004) Aberrant methylation and downregulation of TMS1/ASC in human glioblastoma. *Am J Pathol*, **165**, 1151-61.
95. Machida, E.O., Brock, M.V., Hooker, C.M., Nakayama, J., Ishida, A., Amano, J., Picchi, M.A., Belinsky, S.A., Herman, J.G., Taniguchi, S. and Baylin, S.B. (2006) Hypermethylation of ASC/TMS1 is a sputum marker for late-stage lung cancer. *Cancer Res*, **66**, 6210-8.
96. Terasawa, K., Sagae, S., Toyota, M., Tsukada, K., Ogi, K., Satoh, A., Mita, H., Imai, K., Tokino, T. and Kudo, R. (2004) Epigenetic inactivation of TMS1/ASC in ovarian cancer. *Clin Cancer Res*, **10**, 2000-6.
97. Masumoto, J., Taniguchi, S., Ayukawa, K., Sarvotham, H., Kishino, T., Niikawa, N., Hidaka, E., Katsuyama, T., Higuchi, T. and Sagara, J. (1999) ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. *J Biol Chem*, **274**, 33835-8.
98. McConnell, B.B. and Vertino, P.M. (2004) TMS1/ASC: the cancer connection. *Apoptosis*, **9**, 5-18.
99. McConnell, B.B. and Vertino, P.M. (2000) Activation of a caspase-9-mediated apoptotic pathway by subcellular redistribution of the novel caspase recruitment domain protein TMS1. *Cancer Res*, **60**, 6243-7.
100. Ohtsuka, T., Ryu, H., Minamishima, Y.A., Macip, S., Sagara, J., Nakayama, K.I., Aaronson, S.A. and Lee, S.W. (2004) ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway. *Nat Cell Biol*, **6**, 121-8.
101. Hasegawa, M., Kawase, K., Inohara, N., Imamura, R., Yeh, W.C., Kinoshita, T. and Suda, T. (2007) Mechanism of ASC-mediated apoptosis: bid-dependent apoptosis in type II cells. *Oncogene*, **26**, 1748-56.
102. Masumoto, J., Dowds, T.A., Schaner, P., Chen, F.F., Ogura, Y., Li, M., Zhu, L., Katsuyama, T., Sagara, J., Taniguchi, S., Gumucio, D.L., Nunez, G. and Inohara, N. (2003) ASC is an activating adaptor for NF-kappa B and caspase-8-dependent apoptosis. *Biochem Biophys Res Commun*, **303**, 69-73.

103. Parsons, M.J. and Vertino, P.M. (2006) Dual role of TMS1/ASC in death receptor signaling. *Oncogene*, **25**, 6948-58.
104. Martinon, F., Burns, K. and Tschopp, J. (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell*, **10**, 417-26.
105. Franchi, L., Eigenbrod, T., Munoz-Planillo, R. and Nunez, G. (2009) The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol*, **10**, 241-7.
106. Mariathasan, S., Newton, K., Monack, D.M., Vucic, D., French, D.M., Lee, W.P., Roose-Girma, M., Erickson, S. and Dixit, V.M. (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature*, **430**, 213-8.
107. Yamamoto, M., Yaginuma, K., Tsutsui, H., Sagara, J., Guan, X., Seki, E., Yasuda, K., Yamamoto, M., Akira, S., Nakanishi, K., Noda, T. and Taniguchi, S. (2004) ASC is essential for LPS-induced activation of procaspase-1 independently of TLR-associated signal adaptor molecules. *Genes Cells*, **9**, 1055-67.
108. Levine, J.J., Stimson-Crider, K.M. and Vertino, P.M. (2003) Effects of methylation on expression of TMS1/ASC in human breast cancer cells. *Oncogene*, **22**, 3475-88.
109. Martinez, R., Setien, F., Voelter, C., Casado, S., Quesada, M.P., Schackert, G. and Esteller, M. (2007) CpG island promoter hypermethylation of the pro-apoptotic gene caspase-8 is a common hallmark of relapsed glioblastoma multiforme. *Carcinogenesis*, **28**, 1264-8.
110. Stimson, K.M. and Vertino, P.M. (2002) Methylation-mediated silencing of TMS1/ASC is accompanied by histone hypoacetylation and CpG island-localized changes in chromatin architecture. *J Biol Chem*, **277**, 4951-8.
111. Virmani, A., Rathi, A., Sugio, K., Sathyanarayana, U.G., Toyooka, S., Kischel, F.C., Tonk, V., Padar, A., Takahashi, T., Roth, J.A., Euhus, D.M., Minna, J.D. and Gazdar, A.F. (2003) Aberrant methylation of TMS1 in small cell, non small cell lung cancer and breast cancer. *Int J Cancer*, **106**, 198-204.
112. Yokoyama, T., Sagara, J., Guan, X., Masumoto, J., Takeoka, M., Komiyama, Y., Miyata, K., Higuchi, K. and Taniguchi, S. (2003) Methylation of ASC/TMS1, a proapoptotic gene responsible for activating procaspase-1, in human colorectal cancer. *Cancer Lett*, **202**, 101-8.
113. Quadros, M.R., Connelly, S., Kari, C., Abrams, M.T., Wickstrom, E. and Rodeck, U. (2006) EGFR-dependent downregulation of Bim in epithelial cells requires MAPK and PKC-delta activities. *Cancer Biol Ther*, **5**, 498-504.
114. Srinivasula, S.M., Poyet, J.L., Razmara, M., Datta, P., Zhang, Z. and Alnemri, E.S. (2002) The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. *J Biol Chem*, **277**, 21119-22.
115. Taniguchi, S. and Sagara, J. (2007) Regulatory molecules involved in inflammasome formation with special reference to a key mediator protein, ASC. *Semin Immunopathol*, **29**, 231-8.
116. Parsons, M.J., Patel, P., Brat, D.J., Colbert, L. and Vertino, P.M. (2009) Silencing of TMS1/ASC promotes resistance to anoikis in breast epithelial cells. *Cancer Res*, **69**, 1706-11.

117. Valentijn, A.J. and Gilmore, A.P. (2004) Translocation of full-length Bid to mitochondria during anoikis. *J Biol Chem*, **279**, 32848-57.
118. Ashkenazi, A. and Dixit, V.M. (1998) Death receptors: signaling and modulation. *Science*, **281**, 1305-8.
119. Mawji, I.A., Simpson, C.D., Hurren, R., Gronda, M., Williams, M.A., Filmus, J., Jonkman, J., Da Costa, R.S., Wilson, B.C., Thomas, M.P., Reed, J.C., Glinsky, G.V. and Schimmer, A.D. (2007) Critical role for Fas-associated death domain-like interleukin-1-converting enzyme-like inhibitory protein in anoikis resistance and distant tumor formation. *J Natl Cancer Inst*, **99**, 811-22.
120. Helfer, B., Boswell, B.C., Finlay, D., Cipres, A., Vuori, K., Bong Kang, T., Wallach, D., Dorfleutner, A., Lahti, J.M., Flynn, D.C. and Frisch, S.M. (2006) Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions. *Cancer Res*, **66**, 4273-8.
121. Barbero, S., Barila, D., Mielgo, A., Stagni, V., Clair, K. and Stupack, D. (2008) Identification of a critical tyrosine residue in caspase 8 that promotes cell migration. *J Biol Chem*, **283**, 13031-4.
122. Finlay, D. and Vuori, K. (2007) Novel noncatalytic role for caspase-8 in promoting SRC-mediated adhesion and Erk signaling in neuroblastoma cells. *Cancer Res*, **67**, 11704-11.
123. Senft, J., Helfer, B. and Frisch, S.M. (2007) Caspase-8 interacts with the p85 subunit of phosphatidylinositol 3-kinase to regulate cell adhesion and motility. *Cancer Res*, **67**, 11505-9.
124. Debnath, J., Mills, K.R., Collins, N.L., Reginato, M.J., Muthuswamy, S.K. and Brugge, J.S. (2002) The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. *Cell*, **111**, 29-40.
125. Cursi, S., Rufini, A., Stagni, V., Condo, I., Matafora, V., Bachi, A., Bonifazi, A.P., Coppola, L., Superti-Furga, G., Testi, R. and Barila, D. (2006) Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *Embo J*, **25**, 1895-905.
126. Park, S.M., Schickel, R. and Peter, M.E. (2005) Nonapoptotic functions of FADD-binding death receptors and their signaling molecules. *Curr Opin Cell Biol*, **17**, 610-6.
127. Cerretti, D.P., Kozlosky, C.J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T.A., March, C.J., Kronheim, S.R., Druck, T., Cannizzaro, L.A. and et al. (1992) Molecular cloning of the interleukin-1 beta converting enzyme. *Science*, **256**, 97-100.
128. Li, J., Brieher, W.M., Scimone, M.L., Kang, S.J., Zhu, H., Yin, H., von Andrian, U.H., Mitchison, T. and Yuan, J. (2007) Caspase-11 regulates cell migration by promoting Aip1-Cofilin-mediated actin depolymerization. *Nat Cell Biol*, **9**, 276-86.
129. Barbero, S., Mielgo, A., Torres, V., Teitz, T., Shields, D.J., Mikolon, D., Bogyo, M., Barila, D., Lahti, J.M., Schlaepfer, D. and Stupack, D.G. (2009) Caspase-8 association with the focal adhesion complex promotes tumor cell migration and metastasis. *Cancer Res*, **69**, 3755-63.
130. Frisch, S.M. (2008) Caspase-8: fly or die. *Cancer Res*, **68**, 4491-3.

131. Zhang, C., Li, H., Zhou, G., Zhang, Q., Zhang, T., Li, J., Zhang, J., Hou, J., Liew, C. and Yin, D. (2007) Transcriptional silencing of the TMS1/ASC tumour suppressor gene by an epigenetic mechanism in hepatocellular carcinoma cells. *J Pathol*, **212**, 134-42.
132. Bryan, N.B., Dorfleutner, A., Rojanasakul, Y. and Stehlik, C. (2009) Activation of inflammasomes requires intracellular redistribution of the apoptotic speck-like protein containing a caspase recruitment domain. *J Immunol*, **182**, 3173-82.
133. Fernandes-Alnemri, T., Wu, J., Yu, J.W., Datta, P., Miller, B., Jankowski, W., Rosenberg, S., Zhang, J. and Alnemri, E.S. (2007) The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell Death Differ*, **14**, 1590-604.
134. Sieg, D.J., Hauck, C.R. and Schlaepfer, D.D. (1999) Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J Cell Sci*, **112 (Pt 16)**, 2677-91.
135. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*, **377**, 539-44.
136. Totsukawa, G., Wu, Y., Sasaki, Y., Hartshorne, D.J., Yamakita, Y., Yamashiro, S. and Matsumura, F. (2004) Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. *J Cell Biol*, **164**, 427-39.
137. Glading, A., Bodnar, R.J., Reynolds, I.J., Shiraha, H., Satish, L., Potter, D.A., Blair, H.C. and Wells, A. (2004) Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. *Mol Cell Biol*, **24**, 2499-512.
138. Seeley, E.S. and Nachury, M.V. (2009) Constructing and deconstructing roles for the primary cilium in tissue architecture and cancer. *Methods Cell Biol*, **94**, 299-313.
139. Christensen, S.T., Pedersen, L.B., Schneider, L. and Satir, P. (2007) Sensory cilia and integration of signal transduction in human health and disease. *Traffic*, **8**, 97-109.
140. Schneider, L., Stock, C.M., Dieterich, P., Jensen, B.H., Pedersen, L.B., Satir, P., Schwab, A., Christensen, S.T. and Pedersen, S.F. (2009) The Na+/H+ exchanger NHE1 is required for directional migration stimulated via PDGFR-alpha in the primary cilium. *J Cell Biol*, **185**, 163-76.
141. Ma, R., Li, W.P., Rundle, D., Kong, J., Akbarali, H.I. and Tsiokas, L. (2005) PKD2 functions as an epidermal growth factor-activated plasma membrane channel. *Mol Cell Biol*, **25**, 8285-98.
142. Satir, P. and Christensen, S.T. (2007) Overview of structure and function of mammalian cilia. *Annu Rev Physiol*, **69**, 377-400.
143. Nigg, E.A. and Raff, J.W. (2009) Centrioles, centrosomes, and cilia in health and disease. *Cell*, **139**, 663-78.
144. Azimzadeh, J. and Bornens, M. (2007) Structure and duplication of the centrosome. *J Cell Sci*, **120**, 2139-42.

145. Xie, Z., Sanada, K., Samuels, B.A., Shih, H. and Tsai, L.H. (2003) Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. *Cell*, **114**, 469-82.
146. Endoh-Yamagami, S., Karkar, K.M., May, S.R., Cobos, I., Thwin, M.T., Long, J.E., Ashique, A.M., Zarbalis, K., Rubenstein, J.L. and Peterson, A.S. A mutation in the pericentrin gene causes abnormal interneuron migration to the olfactory bulb in mice. *Dev Biol*, **340**, 41-53.
147. Torres, V.A., Mielgo, A., Barbero, S., Hsiao, R., Wilkins, J.A. and Stupack, D.G. Rab5 mediates caspase-8-promoted cell motility and metastasis. *Mol Biol Cell*, **21**, 369-76.
148. Simpson, K.J., Selfors, L.M., Bui, J., Reynolds, A., Leake, D., Khvorova, A. and Brugge, J.S. (2008) Identification of genes that regulate epithelial cell migration using an siRNA screening approach. *Nat Cell Biol*.
149. Yamaguchi, H., Wyckoff, J. and Condeelis, J. (2005) Cell migration in tumors. *Curr Opin Cell Biol*, **17**, 559-64.
150. Richards, N., Schaner, P., Diaz, A., Stuckey, J., Shelden, E., Wadhwa, A. and Gumucio, D.L. (2001) Interaction between pyrin and the apoptotic speck protein (ASC) modulates ASC-induced apoptosis. *J Biol Chem*, **276**, 39320-9.
151. Nikolic, M. (2004) The molecular mystery of neuronal migration: FAK and Cdk5. *Trends Cell Biol*, **14**, 1-5.
152. Waite, A.L., Schaner, P., Hu, C., Richards, N., Balci-Peynircioglu, B., Hong, A., Fox, M. and Gumucio, D.L. (2009) Pyrin and ASC co-localize to cellular sites that are rich in polymerizing actin. *Exp Biol Med (Maywood)*, **234**, 40-52.
153. Ley, R., Ewings, K.E., Hadfield, K. and Cook, S.J. (2005) Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. *Cell Death Differ*, **12**, 1008-14.
154. Zhao, Y., Sui, X. and Ren, H. From procaspase-8 to caspase-8: revisiting structural functions of caspase-8. *J Cell Physiol*, **225**, 316-20.
155. Agostini, L., Martinon, F., Burns, K., McDermott, M.F., Hawkins, P.N. and Tschopp, J. (2004) NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity*, **20**, 319-25.
156. Masumoto, J., Taniguchi, S., Nakayama, K., Ayukawa, K. and Sagara, J. (2001) Murine ortholog of ASC, a CARD-containing protein, self-associates and exhibits restricted distribution in developing mouse embryos. *Exp Cell Res*, **262**, 128-33.
157. Masumoto, J., Taniguchi, S. and Sagara, J. (2001) Pyrin N-terminal homology domain- and caspase recruitment domain-dependent oligomerization of ASC. *Biochem Biophys Res Commun*, **280**, 652-5.
158. Liepinsh, E., Barbals, R., Dahl, E., Sharipo, A., Staub, E. and Otting, G. (2003) The death-domain fold of the ASC PYRIN domain, presenting a basis for PYRIN/PYRIN recognition. *J Mol Biol*, **332**, 1155-63.
159. Moriya, M., Taniguchi, S., Wu, P., Liepinsh, E., Otting, G. and Sagara, J. (2005) Role of charged and hydrophobic residues in the oligomerization of the PYRIN domain of ASC. *Biochemistry*, **44**, 575-83.

160. Cheng, J., Waite, A.L., Tkaczyk, E.R., Ke, K., Richards, N., Hunt, A.J. and Gumucio, D.L. Kinetic properties of ASC protein aggregation in epithelial cells. *J Cell Physiol*, **222**, 738-47.
161. Palazzo, A.F., Eng, C.H., Schlaepfer, D.D., Marcantonio, E.E. and Gundersen, G.G. (2004) Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. *Science*, **303**, 836-9.
162. LaFlamme, S.E., Nieves, B., Colello, D. and Reverte, C.G. (2008) Integrins as regulators of the mitotic machinery. *Curr Opin Cell Biol*, **20**, 576-82.
163. Reverte, C.G., Benware, A., Jones, C.W. and LaFlamme, S.E. (2006) Perturbing integrin function inhibits microtubule growth from centrosomes, spindle assembly, and cytokinesis. *J Cell Biol*, **174**, 491-7.
164. Goetz, S.C. and Anderson, K.V. The primary cilium: a signalling centre during vertebrate development. *Nat Rev Genet*, **11**, 331-44.
165. Praetorius, H.A. and Spring, K.R. (2001) Bending the MDCK cell primary cilium increases intracellular calcium. *J Membr Biol*, **184**, 71-9.
166. Balci-Peynircioglu, B., Waite, A.L., Schaner, P., Taskiran, Z.E., Richards, N., Orhan, D., Gucer, S., Ozen, S., Gumucio, D. and Yilmaz, E. (2008) Expression of ASC in renal tissues of familial mediterranean fever patients with amyloidosis: postulating a role for ASC in AA type amyloid deposition. *Exp Biol Med (Maywood)*, **233**, 1324-33.
167. Kaelin, W.G., Jr. (2008) The von Hippel-Lindau tumour suppressor protein: O2 sensing and cancer. *Nat Rev Cancer*, **8**, 865-73.
168. Schermer, B., Ghenoiu, C., Bartram, M., Muller, R.U., Kotsis, F., Hohne, M., Kuhn, W., Rapka, M., Nitschke, R., Zentgraf, H., Fliegauf, M., Omran, H., Walz, G. and Benzing, T. (2006) The von Hippel-Lindau tumor suppressor protein controls ciliogenesis by orienting microtubule growth. *J Cell Biol*, **175**, 547-54.
169. Lutz, M.S. and Burk, R.D. (2006) Primary cilium formation requires von hippel-lindau gene function in renal-derived cells. *Cancer Res*, **66**, 6903-7.
170. Hergovich, A., Lisztwan, J., Barry, R., Ballschmieter, P. and Krek, W. (2003) Regulation of microtubule stability by the von Hippel-Lindau tumour suppressor protein pVHL. *Nat Cell Biol*, **5**, 64-70.
171. Vojtek, A.B. and Cooper, J.A. (1995) Rho family members: activators of MAP kinase cascades. *Cell*, **82**, 527-9.
172. Toure, A., Dorseuil, O., Morin, L., Timmons, P., Jegou, B., Reibel, L. and Gacon, G. (1998) MgcRacGAP, a new human GTPase-activating protein for Rac and Cdc42 similar to Drosophila rotundRacGAP gene product, is expressed in male germ cells. *J Biol Chem*, **273**, 6019-23.
173. Raymond, K., Bergeret, E., Avet-Rochex, A., Griffin-Shea, R. and Fauvarque, M.O. (2004) A screen for modifiers of RacGAP(84C) gain-of-function in the Drosophila eye revealed the LIM kinase Cdi/TESK1 as a downstream effector of Rac1 during spermatogenesis. *J Cell Sci*, **117**, 2777-89.
174. Giot, L., Bader, J.S., Brouwer, C., Chaudhuri, A., Kuang, B., Li, Y., Hao, Y.L., Ooi, C.E., Godwin, B., Vitols, E., Vijayadamodar, G., Pochart, P., Machineni, H., Welsh, M., Kong, Y., Zerhusen, B., Malcolm, R., Varrone, Z., Collis, A., Minto, M., Burgess, S., McDaniel, L., Stimpson, E., Spriggs, F., Williams, J., Neurath, K., Ioime, N., Agee, M., Voss, E., Furtak, K., Renzulli, R., Aanensen, N.,

Carrolla, S., Bickelhaupt, E., Lazovatsky, Y., DaSilva, A., Zhong, J., Stanyon, C.A., Finley, R.L., Jr., White, K.P., Braverman, M., Jarvie, T., Gold, S., Leach, M., Knight, J., Shimkets, R.A., McKenna, M.P., Chant, J. and Rothberg, J.M. (2003) A protein interaction map of Drosophila melanogaster. *Science*, **302**, 1727-36.

175. Casci, T., Vinos, J. and Freeman, M. (1999) Sprouty, an intracellular inhibitor of Ras signaling. *Cell*, **96**, 655-65.
176. Guy, G.R., Jackson, R.A., Yusoff, P. and Chow, S.Y. (2009) Sprouty proteins: modified modulators, matchmakers or missing links? *J Endocrinol*, **203**, 191-202.
177. Torii, S., Nakayama, K., Yamamoto, T. and Nishida, E. (2004) Regulatory mechanisms and function of ERK MAP kinases. *J Biochem*, **136**, 557-61.
178. Giannoni, E., Fiaschi, T., Ramponi, G. and Chiarugi, P. (2009) Redox regulation of anoikis resistance of metastatic prostate cancer cells: key role for Src and EGFR-mediated pro-survival signals. *Oncogene*, **28**, 2074-86.
179. Mirza, S., Sharma, G., Prasad, C.P., Parshad, R., Srivastava, A., Gupta, S.D. and Ralhan, R. (2007) Promoter hypermethylation of TMS1, BRCA1, ERalpha and PRB in serum and tumor DNA of invasive ductal breast carcinoma patients. *Life Sci*, **81**, 280-7.
180. Hoque, M.O., Prencipe, M., Poeta, M.L., Barbano, R., Valori, V.M., Copetti, M., Gallo, A.P., Brait, M., Maiello, E., Apicella, A., Rossiello, R., Zito, F., Stefania, T., Paradiso, A., Carella, M., Dallapiccola, B., Murgo, R., Carosi, I., Bisceglia, M., Fazio, V.M., Sidransky, D. and Parrella, P. (2009) Changes in CpG islands promoter methylation patterns during ductal breast carcinoma progression. *Cancer Epidemiol Biomarkers Prev*, **18**, 2694-700.
181. Kerlikowske, K., Molinaro, A.M., Gauthier, M.L., Berman, H.K., Waldman, F., Bennington, J., Sanchez, H., Jimenez, C., Stewart, K., Chew, K., Ljung, B.M. and Tlsty, T.D. Biomarker expression and risk of subsequent tumors after initial ductal carcinoma in situ diagnosis. *J Natl Cancer Inst*, **102**, 627-37.
182. Kapoor-Vazirani, P., Kagey, J.D., Powell, D.R. and Vertino, P.M. (2008) Role of hMOF-dependent histone H4 lysine 16 acetylation in the maintenance of TMS1/ASC gene activity. *Cancer Res*, **68**, 6810-21.
183. Reagan-Shaw, S. and Ahmad, N. (2007) The role of Forkhead-box Class O (FoxO) transcription factors in cancer: a target for the management of cancer. *Toxicol Appl Pharmacol*, **224**, 360-8.
184. Sabbatini, P. and McCormick, F. (1999) Phosphoinositide 3-OH kinase (PI3K) and PKB/Akt delay the onset of p53-mediated, transcriptionally dependent apoptosis. *J Biol Chem*, **274**, 24263-9.
185. Fu, Z. and Tindall, D.J. (2008) FOXOs, cancer and regulation of apoptosis. *Oncogene*, **27**, 2312-9.
186. Sunters, A., Fernandez de Mattos, S., Stahl, M., Brosens, J.J., Zoumpoulidou, G., Saunders, C.A., Coffer, P.J., Medema, R.H., Coombes, R.C. and Lam, E.W. (2003) FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem*, **278**, 49795-805.
187. Nadal, A., Marrero, P.F. and Haro, D. (2002) Downregulation of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by insulin: the role of the forkhead transcription factor FKHRL1. *Biochem J*, **366**, 289-97.

188. Allen, I.C., TeKippe, E.M., Woodford, R.M., Uronis, J.M., Holl, E.K., Rogers, A.B., Herfarth, H.H., Jobin, C. and Ting, J.P. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med*, **207**, 1045-56.
189. Kim, I.S. and Baek, S.H. Mouse models for breast cancer metastasis. *Biochem Biophys Res Commun*, **394**, 443-7.
190. Bissell, M.J. and Radisky, D. (2001) Putting tumours in context. *Nat Rev Cancer*, **1**, 46-54.
191. Lee, G.Y., Kenny, P.A., Lee, E.H. and Bissell, M.J. (2007) Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods*, **4**, 359-65.
192. Debnath, J., Muthuswamy, S.K. and Brugge, J.S. (2003) Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods*, **30**, 256-68.
193. Whelan, K.A., Caldwell, S.A., Shahriari, K.S., Jackson, S.R., Franchetti, L.D., Johannes, G.J. and Reginato, M.J. Hypoxia Suppression of Bim and Bmf Blocks Anoikis and Luminal Clearing During Mammary Morphogenesis. *Mol Biol Cell*.
194. Debnath, J. and Brugge, J.S. (2005) Modelling glandular epithelial cancers in three-dimensional cultures. *Nat Rev Cancer*, **5**, 675-88.
195. Cherish, D. (2007) *Methods in Enzymology*. Elsevier, Pasadena.
196. Sung, Y.M., Xu, X., Sun, J., Mueller, D., Sentissi, K., Johnson, P., Urbach, E., Seillier-Moiseiwitsch, F., Johnson, M.D. and Mueller, S.C. (2009) Tumor suppressor function of Syk in human MCF10A in vitro and normal mouse mammary epithelium in vivo. *PLoS One*, **4**, e7445.
197. Swamydas, M., Eddy, J.M., Burg, K.J. and Dreau, D. Matrix compositions and the development of breast acini and ducts in 3D cultures. *In Vitro Cell Dev Biol Anim*, **46**, 673-84.
198. Chen, J., Miller, E.M. and Gallo, K.A. MLK3 is critical for breast cancer cell migration and promotes a malignant phenotype in mammary epithelial cells. *Oncogene*, **29**, 4399-411.