THE ROLE OF VAPB IN BREAST CANCER

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To my loving parents

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LIST OF ABBREVIATIONS

AKAP	A-kinase anchor proteins
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
ARF	ADP ribosylation factor
BFA	brefeldin A
CC	coiled-coil
CERT	ceramide transfer protein
COP	cotamer protein
DCIS	ductal carcinoma *in situ*
DMEM	Dulbecco's modified eagle's medium
EGF	epidermal growth factor
ECM	extracellular matrix
EMT	epithelial to mesenchymal transition
EPH	Erythopoietin Producing Hepatoma
ER	endoplasmic reticulum
ERBB2	erythroblastic Leukemia Viral Oncogene Homolog 2
ERGIC	ER-Golgi-intermediate complex
ERK	extracellular signal-regulated kinases
FBS	fetal bovine serum
GLUT4	glucose transporter 4
HCV	hepatitis C virus
HER2	Human Epidermal Growth Factor Receptor 2
IDC	invasive ductal carcinoma
IHC	immunohistochemistry
LPT	lipid transfer protein
MMP	matrix metalloproteinase
MMTV	mouse mammary tumor virus
MSP	major sperm protein
NE	nuclear envelope
OSBP	oxysterol binding protein
PCNA	proliferating cell nuclear antigen
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-trisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKA	protein kinase A
RTK	receptor tyrosine kinase
SCS2	suppressor of choline sensitivity 2
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
siRNA	silencing RNA

SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TGFα	Transforming growth factor-alpha
TGN	trans golgi network
TM	transmembrane
VAMP	vesicle associated membrane protein
VAPB	VAMP-associated protein B
VSVG	Vesicular stomatitis viral protein G

CHAPTER I

INTRODUCTION

Overview

Vesicle associated membrane protein (VAMP), associated protein B (VAPB) is highly conserved type II, integral membrane protein that belongs to the VAP protein family. VAPs function in diverse cellular processes such as lipid transport and synthesis, vesicle trafficking, the unfolded protein response and neurotransmitter release. Genome-wide microarray analysis revealed that VAPB is overexpressed in human breast cancer cells lines and correlates with poor clinical outcome. Despite aberrant expression in human cancer, the function of VAPB in tumor cells is poorly understood. Our results presented herein investigate how VAPB regulates breast tumor cell proliferation, invasion and *in vivo* tumor growth. Specifically, we demonstrate that VAPB promotes proliferation, at least, in part through the AKT pathway. In addition to enhanced cell growth, VAPB elevates the invasive potential in breast tumor cells. We also identified novel VAPB interacting proteins that potentially contribute to tumor growth and invasion. Moreover, our *in vivo* studies show that VAPB deficiency reduced tumor burden in the MMTV-Neu mouse model for spontaneous breast cancer. Collectively, these genetic, functional and mechanistic studies demonstrate that VAPB as a tumor-promoting factor in breast cancers.

VAPs: Highly conserved from yeast to mammals

The first VAP protein (VAP-33) was identified in *Aplysia californica* as a VAMP/synaptobrevin-associated protein using a yeast two-hybrid screen (Skehel et al, 1995). In this study, VAP-33 was proposed to function in neurotransmitter release. Since the identification of VAP-33, homologs in yeast and mammals were discovered. The *S. cerevisiae* homolog of VAP-33, *SCS2* was identified as a suppressor of inositol auxotrophy and functions in regulation of inositol metabolism (Nikawa et al, 1995). Two mammalian homologues (VAPA and VAPB) have been cloned in rat, mouse and human and share high sequence homology (Nishimura et al, 1999; Skehel et al, 2000; Soussan et al, 1999; Weir et al, 1998). VAPA and VAPB of most species share a primary organization consisting of three domains: a highly conserved N-terminal major sperm protein (MSP) domain, a coiled-coil (CC) domain, and a transmembrane (TM) domain (Figure 1.1B). VAPC is a splice variant of VAPB, lacking both the CC and TM domains. Limited data exists regarding VAPC expression and function. A VAP consensus sequence within the N- terminal MSP domain is conserved across species and isoforms as highlighted in Figure 1.1A (Kagiwada et al, 1998; Nikawa et al, 1995).

VAP Protein Domains

Major Sperm Protein (MSP) Domain

The MSP domain of VAPB is in the amino-terminus, which faces into the cytosol as determined by protease protection assays (Soussan et al, 1999). A schematic representation of VAP is shown in Figure 1.1B. Of the three structural components of VAP proteins (MSP, CC and TM), the MSP domain is the most conserved among species (Figure 1.1A), with 22% sequence homology with nematode MSP, a protein mediating ameboid-like motility in sperm cells (Roberts & Stewart, 2000) and also functions in fertility (Miller et al, 2001). The MSP domain of rat VAPA has a protein crystal structure similar to that of nematode MSP (Kaiser et al, 2005) further demonstrating protein conservation.

The MSP domain of VAPs is an important interface for cytosolic protein interaction as well as targeting FFAT-motif proteins to the ER. Within the highly conserved VAP consensus sequence of the MSP domain is the binding site for FFAT-containing proteins. FFAT stands for diphenylalanine [FF] in an acidic tract, consisting of the amino acid consensus sequence EEFFDAxE (Loewen et al, 2003). This motif is a targeting signal responsible for localizing a number of proteins to the cytosolic surface of the endoplasmic reticulum (ER) and to the nuclear membrane (Kaiser et al, 2005).

The majority of FFAT-containing proteins are involved in lipid-sensing and lipid transport (Lev et al, 2008). More recently, one study defined FFAT-like

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motifs more widely than before and identified novel proteins whose functions have not been previously been directly linked to the endoplasmic reticulum (ER), such as PKA anchoring proteins (AKAPS). (Mikitova & Levine, 2012). However the functional significance of these new candidate FFAT-motif proteins is not well understood.

Coiled-coil (CC) Domain

The 50 amino acid stretch between the MSP and TM domains is predicted to form a coiled-coil like structure (Nishimura et al, 1999). In general, the α-helical coiled-coil is one of the principal dimerization motifs in proteins (Kapinos et al, 2011). Therefore, the requirement of VAPB CC domain to facilitate dimerization was tested using VAPB-ΔCC mutants. While the mutant retained proper ER localization, it failed to interact with WT VAPB, demonstrating that the CC domain was necessary for VAP dimers (Kim et al, 2010). The VAP CC domain also resembles that of many SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins and therefore provides a potential interface for VAMP proteins.

Transmembrane (TM) Domain

The TM domain is the C-terminal portion of VAPs. This domain is critical for localizing and docking VAPs to ER membranes. A TM domain-truncated mutant of VAP localized to the cytosol rather than the ER membranes. Although

Figure 1.1. VAP protein homology and primary structure (A) Multiple sequence alignment of VAP proteins from yeast to humans was generated by CLUSTALW. Residues of high conservation are shaded in grey. The major sperm protein (MSP) domain, coiled-coil (CC) domain and transmembrane (TM) domain are outlined in red, blue and green, respectively [adapted from (Lev et al, 2008)]. (B) A schematic representation of VAP dimers [image taken from (Tsuda et al, 2008)].

the G*XXX*G dimerization motif within the TM domain is sufficient for driving

dimerization of TM domains alone, it is not sufficient for dimerization of full length

VAPs (Kim et al, 2010). However, the CC domain was found to be more

important for VAP dimerization. Thus the domains have the following order of

importance for driving dimerization: CCD > TM > MSP (Kim et al, 2010).

Taken together, the VAP domains play important roles in both structure

and function by targeting cytosolic proteins to the ER (MSP), hetero- and homo-

dimerization (CC) and VAP cellular localization (TM).

Cellular Functions of VAP proteins

VAPs participate in a diverse array of cellular processes such as vesicle

trafficking, lipid metabolism, lipid transfer, ER stress response, viral replication,

and signal transduction [Reviewed in (Lev et al, 2008)] as suggested by the

identification, characterization and functional analysis of VAP-interacting proteins

in various cell types and organisms. A summary of VAP interacting proteins is

provided in Table 1. We explore the reported VAP cellular functions below.

Vesicle Trafficking

The first VAP identified, VAP-33, provides evidence that VAPs function in

vesicle trafficking. VAP-33 localized to plasma membrane regions of neurons and

was proposed to function in late secretory trafficking events (Skehel et al, 1995).

Injection of antibodies against VAP-33 inhibited synaptic transmission,

Table 1. Summary of VAP functions based on protein interactions.

	VAP Interacting Proteins	Reference	Function
Vesicle Trafficking	VAMP/ synaptobrevin VAMP1, VAMP2 αSNAP, NSF	(Skehel et al, 1995) (Weir et al, 2001) (Weir et al, 1998, 2001)	SNARE- mediated vesicle fusion
	Naked2-vesicles	(Cao et al, 2008)	Naked2 coats exocytic vesicles that contain TGFα
	COPI	(Soussan et al, 1999)	Coatamer protein that initiates vesicle budding on cis-golgi
Lipid Metabolism	Opi1p	(Loewen et al, 2003)	Transcriptional repressor of INOS regulated genes involved in phospholipid synthesis *S. cerevisiae*.
Non- vesicular Lipid Transport	Osh OSBP/ Related ORPs	(Loewen et al, 2003; Stefan et al, 2011) (Jansen et al, 2011; Peretti et al, 2008; Perry & Ridgway, 2006; Wyles et al, 2002)	Highly conserved family of sterol binding/ transfer proteins that contain the FFAT-motif
	Nir2	(Amarilio et al, 2005)	FFAT-motif containing PI/ PC transfer protein
	CERT	(Peretti et al, 2008; Perry & Ridgway, 2006)	FFAT-motif containing ceramide transfer protein
	GLTP	(Tuuf et al, 2009)	Glycolipid transfer protein
UPR	ATF6 IRE1	(Gkogkas et al, 2008) (Chen et al, 2010; Kanekura et al, 2006)	Receptors are activated in reponse to misfolded proteins in the ER
Viral Replication	NS5A, NS5B	(Gao et al, 2004; Tu et al, 1999)	Key factors in HCV replication
Signaling	Eph RTK	(Miller et al, 2003; Tsuda et al, 2008)	Secreted MSP domain of VAPB activates Eph receptor signaling

suggesting that VAP-33 functions in neurotransmitter release. Protein interactions with VAMP/synaptobrevin (Skehel et al, 1995) and several v-SNARES and t-SNARES further implicate VAPs in vesicle docking at the plasma membrane (Weir et al, 2001). Along these lines, the VAP-33/SNARE interaction is important for insulin-dependent GLUT4 traffic (Foster et al, 2000). Other studies show the presence of human VAPA on TGFα-containing exocytic vesicles suggesting that VAPs may play a role in vesicle targeting to the plasma membrane (Cao et al, 2008). Furthermore siRNA-mediated depletion of both VAPA and VAPB inhibited trans-golgi network (TGN)-to plasma membrane transport (Peretti et al, 2008).

While these results propose VAPs function in late secretory events from the TGN to plasma membrane, other studies show VAPs also accumulate at the ER-Golgi-intermediate complex (ERGIC), suggesting a role in early secretory vesicle trafficking events (Moumen et al, 2011; Skehel et al, 2000). The ER is the primary site for maturation, modification and packaging of proteins destined for secretion or membrane insertion (Schmidt & Stephens, 2010). Vesicle transport in the protein secretory pathway depends on early steps, such as the formation of coated vesicular intermediates, known as COPI-and COPII vesicles (Nickel & Rabouille, 2009). These coated vesicles are critical for the budding of vesicles within the ER-Golgi network and vesicle transport, both in retrograde movement of Golgi components back to the ER (COPI) and anterograde movement of cargo outward from the ER to Golgi and plasma membrane (COPII) (Spang, 2002).

Although Prosser et al. provide evidence that overexpression of VAPA blocks ER-Golgi transport by inhibiting cargo loading into COPII vesicle (Prosser et al, 2008), the rat homolog of VAPB, EGR-30, was shown to promote secretory protein transport mediated by COPI vesicles. Antibody inhibition of VAPB, prohibited transport between Golgi cisternae and the ER as measured by the accumulation of COPI coated vesicles (Soussan et al, 1999). Collectively, these results suggest that VAPs are involved at early and late stages of the secretory pathway, affecting vesicle trafficking events occurring at the ER/ Golgi network and the plasma membrane.

Lipid Metabolism

The yeast homolog of VAP proteins, *SCS2* was originally identified as a suppressor of inositol auxotrophy in both *cse1* and *ire15* mutant strains of *S. cerevisiae* (Nikawa et al, 1995). Both mutants cannot activate the expression of *INO1*, which encodes inosotiol-1 phosphate synthase, an essential enzyme for myo-inositol biosynthesis is yeast cells and is also the structural basis for important secondary messengers such as phosphatidylinositol and its various phosphates (Dean & Beaven, 1989). Analysis of scs2Δ mutants, demonstrate its function in phospholipid biosynthesis such that scs2Δ mutants have reduced levels of phosphatidylinositol (Kagiwada & Zen, 2003), but other phospholipids, such as phosphatidylcholine, were not affected (Kagiwada & Zen, 2003).

Mechanistic studies revealed that SCS2 binds and sequesters a transcriptional repressor of *INO1,* known as Opi1p. Therefore, SCS2 prevents Opi1p's nuclear translocation allowing for *INO1* transcription (Kagiwada et al, 1998; Nikawa et al, 1995). The dynamics of this repression are modulated by phosphatidic acid. Opi1p's transcriptional repressor activity is inhibited by binding phosphatidic acid on the ER, facilitated by interactions with SCS2's MSP domain. Since phosphatidic acid is shunted toward the production of inositol products, Opi1p can rapidly translocate into the nucleus in response to consumption of phosphatidic acid induced by inositol (Loewen et al, 2004; Loewen et al, 2003). Thus, the Opi1p/ SCS2 interaction is an integral part of a negative feedback mechanism to shut off *de novo* inositol lipid synthesis in the presence of exogenous inositol.

Non-vesicular Lipid Transfer

The ER-localization of VAPs and its interaction with lipid transfer proteins (LTPs) suggest that VAPs facilitate the transfer of lipids across membranes. LTPs function to move hydrophobic lipids across the aqueous environment of the cytoplasm, using non-vesicular mediated trafficking (Wirtz, 1997). LTPs primarily localize membrane contact sites (MCSs), which are specialized gaps (10-20nm) between the ER membranes and other membrane bound organelles such as the mitochondria, the Golgi apparatus, endosomes, lipid droplets as well as the

plasma membrane (Holthuis & Levine, 2005). Thus, LTPs are hypothesized to modulate the lipid composition of various intracellular membranes.

The most well-characterized VAP-LTP interactions include oxysterol binding protein (OSBP), Nir2 (membrane-associated phosphatidylinositol transfer protein 1), and ceramide transfer protein (CERT) (Amarilio et al, 2005; Peretti et al, 2008). These proteins interact with VAPs through their FFAT-motif. Nir2 is a phosphoinositol/ phosphotidylcholine transfer protein that affects DAG levels at the Golgi membranes (Litvak et al, 2005). Another VAP-interacting LTP is CERT, which transfers newly synthesized ceramide from the ER to the Golgi for subsequent synthesis into sphingomyelin by SM synthase (Kumagai et al, 2005). OSBP, which localizes to the ER and Golgi, functions in sterol-transfer between the ER and Golgi apparatus and is coupled to activation of CERT (Perry & Ridgway, 2006).

There is much evidence to suggest that VAPs are critical to the lipid transfer function of Nir2, CERT and OSBP. Using cell-free biochemical assays, it was shown that mutations in the FFAT motif of CERT impair its ceramide transfer activities, underscoring the importance of the VAP-CERT interaction in the ER and Golgi. Knockdown of mammalian VAPA and VAPB reduced Golgi targeting of Nir2, CERT, and OSBP (Litvak et al, 2005; Peretti et al, 2008). Given the lipid transfer functions of these proteins, the mis-targeting of these proteins affected the lipid composition at different MCSs such as the Golgi, as shown by reduced levels of PIP4, DAG and SM. Taken together, the coordinated function of LTPs

such as Nir2, OSBP, and CERT and VAP proteins at the ER–Golgi MCSs are important maintain lipid composition and thus the structural and functional integrity of the ER–Golgi complex (Peretti et al, 2008).

The Unfolded Protein Response (UPR)

The UPR is an evolutionary conserved ER-stress response pathway activated by accumulation of misfolded proteins in the ER lumen (Walter & Ron, 2011). In mammalian cells, there are three major UPR sensory receptors IRE1, ATF6, and PERK. In yeast, only *IRE1* is present (Cox et al, 1993). IRE1 is an ER receptor with dual kinase and endonuclease activities such that upon activation, it initiates unconventional mRNA splicing of the transcriptional activator, Hac1p (Cox & Walter, 1996). In mammals Hac1p is known as XBP1 (Yoshida et al, 2001). The yeast homolog of VAP, *SCS2* was identified as a suppressor of inositol auxotrophy in *ire15/ hac1p* mutants (Nikawa et al, 1995). Moreover, scs2Δ mutants have increased tunicamycin-induced cell death, and reduced activation of IRE1, suggesting the requirement of *SCS2* for UPR activation (Kagiwada et al, 1998).

In mammalian cells, current data suggests that UPR receptors are differentially activated by VAPs. For instance, VAPB overexpression attenuates the ATF6-regulated transcription (Gkogkas et al, 2008). Another study demonstrates that VAPB expression enhances IRE1 endonuclease activity. Conversely, VAPB depletion attenuates this response (Kanekura et al, 2006).

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Whether VAPB is a positive or negative regulator of the UPR remains to be determined.

HCV Replication

There is also evidence to suggest that VAPs function in viral replication. VAPA and VAPB have been shown to interact with non-structural phosphoproteins NS5A and NS5B of the Hepatitis C virus (HCV), which is a single-stranded RNA virus primarily affecting the liver (Gao et al, 2004; Tu et al, 1999). The HCV non-structural proteins are important for viral replication and production (Cao et al, 2009). Studies show that VAPB neutralizing antibodies suppressed HCV RNA replication in a cell-free assay. Overexpression of full length VAPB, but not a mutant VAPB lacking the TM domain, enhanced NS5A and NS5B expression and HCV RNA replication in hepatocyte cell lines (Hamamoto et al, 2005). Knockdown of VAPB by small interfering RNA decreased expression of NS5B, but not of NS5A (Hamamoto et al, 2005). These results demonstrate the potential importance of VAPs in viral replication.

Eph Receptor Signaling

The MSP protein in *C. elegans* shares high sequence homology to the MSP domain of VAPs. In addition to nematode MSP's intracellular function in cytoskeletal networks (Roberts & Stewart, 2000), the MSP protein also has extracellular function during fertilization, inducing oocyte maturation (Miller et al,

2001). Additional studies demonstrate that MSP binds to Eph-related receptor protein tyrosine kinase in *C. elegans* (VAB-1) to activate ERK/ MAPK pathways (Kosinski et al, 2005; Miller et al, 2003). Furthermore, Tsuda *et al.* has shown that *Drosophila* VAPB is cleaved, secreted and acts as a ligand for Eph receptor tyrosine kinases (RTKs) (Tsuda et al, 2008). Eph receptors are a highly conserved class of receptor tyrosine kinases that bind to ephrin ligands. Eph receptors have important roles in development, signal transduction and pathological conditions such as cancer (Pasquale, 2008; Vaught et al, 2008). Secreted MSP directly binds to Eph receptors and compete ephrins for binding (Tsuda et al, 2008). Both secreted MSP from *Drosophila* and human VAPB are able to activate Eph signaling suggesting that VAP's extracellular signaling activities are evolutionarily conserved (Tsuda et al, 2008).

VAPB in Human Disease

As opposed to the restricted neuronal expression of VAP-33 in *Aplysia californica*, mammalian VAPs are ubiquitously expressed in multiple tissue types (Nishimura et al, 1999; Skehel et al, 2000; Soussan et al, 1999; Weir et al, 1998) suggesting that VAP function is not limited to neurotransmitter release, but many other cellular processes as described above. Moreover, there is emerging evidence suggesting an important role for VAPB in human disease pathologies, specifically neurodegenerative disorders and cancer.

VAPB in Amyotrophic Lateral Sclerosis (ALS)

A single missense mutation within the human VAPB gene is associated with a familial form of atypical amyotrophic lateral sclerosis (ALS). ALS is a progressive neurodegenerative disorder causing death in nerve cells and loss of motor control in patients. The identification of the P56S mutation in VAPB's MSP domain and subsequent mechanistic studies has given insight into VAPB's cellular function in pathological conditions (Nishimura et al, 2004).

Several studies have shown that expression of VAPB P56S in cell lines, causes insoluble protein aggregates VAPB and cytosolic mis-localization rather than the ER (Nishimura et al, 2004; Prosser et al, 2008). The proline residue resides in a highly conserved 16 amino acid stretch near the amino-terminus (Figure 1.1A). A proline to serine substitution causes conformational changes exposing VAPB's hydrophobic patch to the cytosol, increasing the propensity for aggregation (Kim et al, 2010). Furthermore, VAPB P56S acts in a dominant negative fashion by sequestering WT VAPB (Suzuki et al, 2009).

VAPB P56S fails to interact with FFAT-motif containing proteins, which mainly function in non-vesicular lipid transfer across membranes. The P56S VAPB mutation also disrupts calcium homeostasis affecting mitochondrial movement (De Vos et al, 2012), nuclear envelope (NE) architecture and localization of NE proteins (Tran et al, 2012). It should be noted that an additional mutation (T46I) in the MSP domain was identified in another patient group with

familial ALS, which also causes protein aggregates when expressed in cells (Chen et al, 2010).

Protein inclusion bodies such as those induced by mutant VAPB are characteristic features of neurodegenerative disorders such as Huntington's, Parkinson's and Alzheimer's diseases. Whether ALS disease pathogenesis is caused by the direct loss of VAPB function due to the dominant-negative action of VAPB P56S, or the mere presence of mutant protein aggregates remains to be determined.

VAPB in Human Breast Cancer

Genomic abnormalities are known to function in cancer progression. In breast cancer alone, there are thousands of genetic aberrations (Korkola & Gray, 2010). Interestingly, there are several reports that indicate aberrant gene expression of VAPB human breast cancer cells. Using genome copy number and gene expression analysis of both clinical breast tumor samples and breast tumor cell lines, it was shown that VAPB is one of 66 genes overexpressed by genomic copy number abnormalities (Chin et al, 2006; Neve et al, 2006). VAPB was amplified or overexpressed in eight out of fifty breast cancer cell lines. Notably, a VAPB-IKZF3 fusion protein was identified in BT474 and shown to enhance cell proliferation (Edgren et al, 2011). While the VAPB- expressing breast cancer cell lines vary in intrinsic molecular subtype, five of the eight cell lines were HER2

Table 2. VAPB-expressing human breast cancer cell lines. Cell lines are classified as luminal or basal genetic subtype, estrogen (ER), progesterone (PR) or HER2 positive. Results were gathered from microarray analysis from (Neve et al, 2006) or western analysis of protein lysates (Figure 1.2 D)

	Gene Cluster	ER	PR	HER2
MCF7	Luminal	+	+	-
T47D	Luminal	+	+	-
HCC1428	Luminal	+	+	-
BT474	Luminal	+	+	+
SUM52PE	Luminal	+	-	+
UACC812	Luminal	+	-	+
SKBR3	Luminal	-	-	+
HCC1954	Basal A	-	-	+
MDA MB 157	Basal B	-	-	-

gene amplified (Table 2). Interestingly, a study found recurrent amplification of *VAPB* in *HER2-* amplified invasive breast cancers (Arriola et al, 2008).

VAPB was also identified as a gene that was upregulated upon transition from a benign to malignant phenotype (Rizki et al, 2008). The acquisition of invasiveness is critical to the metastatic process. Breast cancer progression is a multi-step process beginning with localized, neoplastic growth, loss of polarity, increased motility, and ability to invade the surrounding extra-cellular matrix (Eckhardt et al, 2012). Microarray analysis on isogenic "pre-invasive" and "invasive" cell lines showed the upregulation of genes belonging to several functional groups including protein kinases, transcription factors, and intracellular vesicle transport, including elevated VAPB expression.

Taken together these observations suggest that VAPB has an important role in breast cancer. To complement the published data and demonstrate VAPB's clinical significance, we investigated VAPB expression in a published human breast cancer microarray dataset from a panel of 295 patient samples (van de Vijver et al, 2002). In collaboration with the Vanderbilt-Ingram Cancer Center Biostatistics Shared Resource, we categorized each patient's gene expression values as "high" (top 25%) or "low" (bottom 25%) and found that high levels of VAPB significantly associated with worse recurrence-free survival than in patients with low VAPB expression, based on Kaplan-Meier survival analysis (Figure 1.2A). Similar results were also achieved using a second, independent dataset (Figure 1.2B). Complementary to this approach, we assessed VAPB

Figure 1.2. Analysis of VAPB expression in human breast cancer VAPB expression levels were analyzed in two published breast cancer microarray datasets. Correlation of VAPB expression with clinical outcome was analyzed by Kaplan-Meier survival curve using the (A) van de Vijver dataset (van de Vijver et al, 2002) and the (B) Wang dataset (Wang et al, 2005). (C) VAPB protein expression was analyzed in human invasive ductal carcinoma samples on commercial tissue microarrays by immunohistochemistry using a previously validated anti-VAPB antibody, as described in Materials and Methods (Chapter II). (D) Western analysis of human and mouse tumor cell lysates.

A

Recurrence Free Survival
(van di Vijver, et al, 2002)

B

Recurrence Free Survival
(Wang, et al, 2005)

C

VAPB IHC

Normal

Primary

Metastatic

D

Human

Mouse
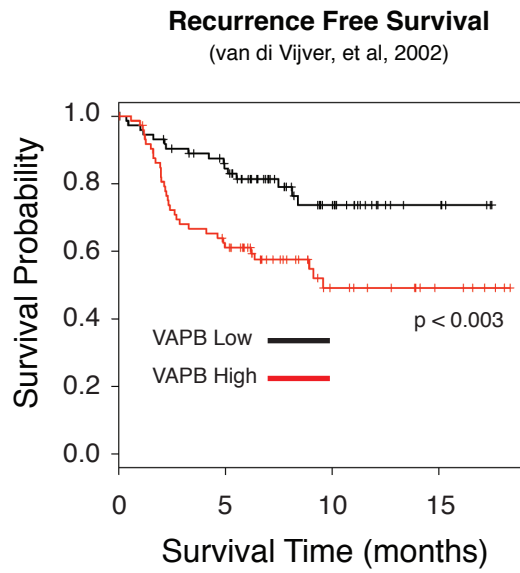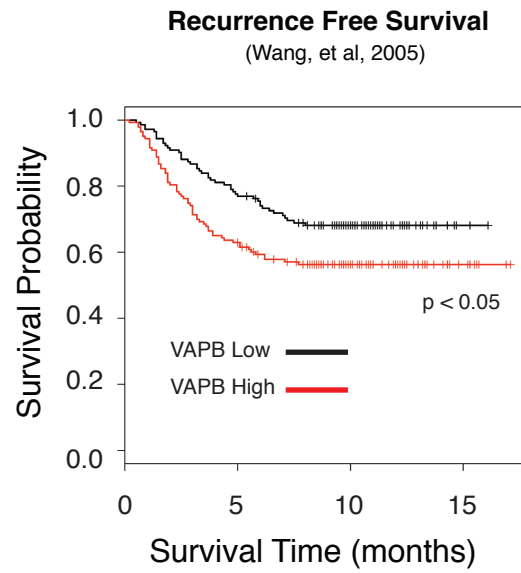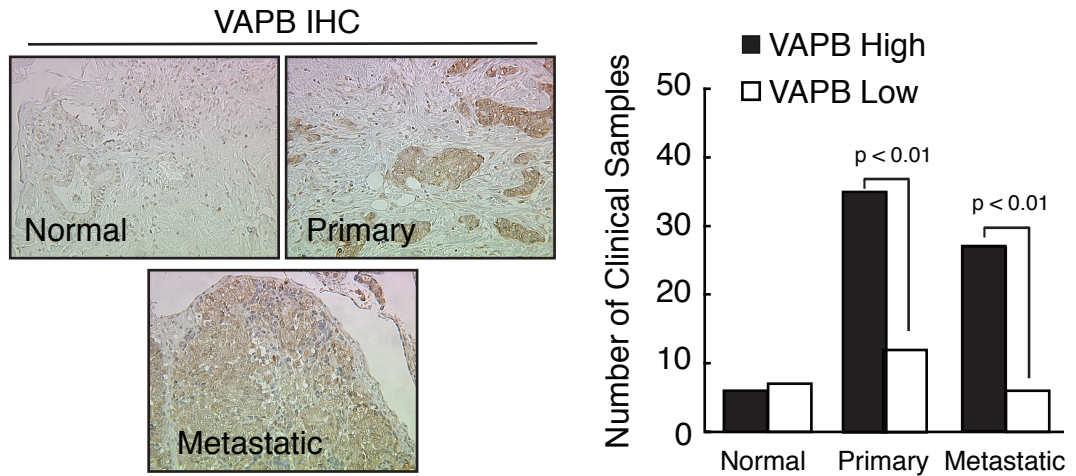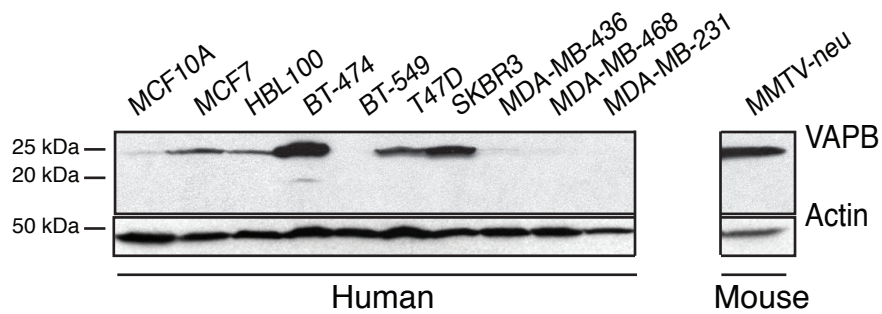
protein expression in human breast cancer tissue microarrays (TMA). We observed a significant increase in the number of VAPB-positive invasive ductal carcinoma (IDC) and metastasis samples compared to normal tissue (Figure 1.2C). Taken together, these data highlight the clinical relevance of VAPB in breast cancer and solicit more functional studies to understand VAPB's role in breast tumor cell growth and invasion.

Summary

VAPB was originally defined as VAMP binding protein, also known as VAPs. Numerous studies implicate VAPs in a wide range of cellular processes, demonstrated in different species and cell types. VAPs function in, but are not limited to, neurotransmitter release, vesicle trafficking, lipid metabolism and transfer, the unfolded protein response (UPR), viral replication, and Eph receptor tyrosine kinase (RTK) signaling.

VAPB is one of three mammalian homologues of VAPs. The other members include VAPA and VAPC (a VAPB splice variant) (Nishimura et al, 1999). VAPA and VAPB share high sequence homology and primary organization, consisting of three conserved domains: an N-terminal major sperm domain (MSP), a coiled-coil (CC) domain and a C-terminal transmembrane domain (TM), with each domain mediating cytosolic protein interactions, dimerization, and ER localization, respectively. Within the MSP domain, a missense mutation (P56S) in human VAPB is associated with atypical

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amyotrophic lateral sclerosis (ALS). This mutation disrupts the normal localization of VAPB, forming inclusion bodies with functional consequences on calcium homeostasis and NE and ER architecture.

Not only are VAPB mutations detrimental to VAPB function, abnormal expression levels of VAPB are also critical to other human pathologies such as cancer. Result from other laboratories as well as our own, show VAPB as aberrantly expressed in human breast cancer. Combined analysis of genome copy number and gene expression analysis of 145 clinical specimens and 50 human breast cancer cell lines revealed that *VAPB*, in addition to *HER2*, is one of 66 genes overexpressed by genomic copy number abnormalities. Another study revealed recurrent amplification of VAPB in HER2-amplifed invasive breast cancer patient samples. Moreover, VAPB mRNA expression is also upregulated in a breast tumor cell model of pre-invasive to invasive transition, suggesting that VAPB enhances tumor cell invasion. Our own data corroborates with these studies. We show that high VAPB expression significantly correlates with poor clinical outcome and increased VAPB protein expression in human breast tumor samples. Despite VAPB's clinical relevance and its role in diverse cellular processes, the precise function of VAPB in breast cancer is poorly understood.

Thesis Project

In our studies proposed herein, we demonstrate the causal role between VAPB and breast cancer, specifically in three areas (1) cell growth (2) invasion

and (3) tumor growth *in vivo*. Our results show that VAPB is important for breast tumor cell growth, affecting proliferation both *in vitro* and in mammary gland transplantation experiments. We also provide evidence that VAPB elevates the invasive potential of mammary epithelial cells. These phenotypes are, at least, partly mediated through enhanced AKT activation, a critical mitogenic signaling pathway in breast cancer. We have also identified novel proteins found in-complex with VAPB. The functional groups associated with VAPB-interacting proteins suggest that multiple pathways converge through VAPB to promote tumor proliferation and invasion. Additionally, we generated VAPB-deficient mice that overexpress the rat homologue of HER2 specifically in the mammary gland and scored spontaneously arising tumors for size and proliferation. Collectively, the genetic, functional and mechanistic analyses suggest an important function of VAPB in human breast cancer.

CHAPTER II

VAMP-ASSOCIATED PROTEIN B (VAPB)

PROMOTES BREAST TUMOR GROWTH BY

MODULATION OF AKT ACTIVITY

Introduction

Vesicle associated membrane protein associated protein B (VAPB) is a highly conserved type II integral membrane protein that belongs to the VAP protein family (Nishimura et al, 1999; Weir et al, 1998) and primarily localizes to the endoplasmic reticulum (ER) and cis-Golgi (Moumen et al, 2011; Skehel et al, 2000). Studies of VAP-interacting proteins in yeast and in higher organisms implicate VAP proteins in a diverse array of cellular processes. VAP proteins function in the regulation of neurotransmitter release, vesicle trafficking, lipid binding and transfer proteins, maintainance of ER/ golgi architecture and the unfolded protein response (UPR) (Gkogkas et al, 2008; Loewen et al, 2003; Skehel et al, 1995; Soussan et al, 1999). Recent studies in *C. elegans* and *Drosophila* discovered that the Major Sperm Protein (MSP) domain of VAPB can be cleaved, secreted, and act as a ligand for Eph receptor tyrosine kinases (Tsuda et al, 2008). A single missense mutation within the human *VAPB* gene is associated in a familial form of atypical amyotrophic lateral sclerosis (ALS) (Kanekura et al, 2006; Nishimura et al, 2004; Suzuki et al, 2009), triggering a

renewed interest in the VAPB protein and its cellular function in human pathologies.

In addition to familial ALS, VAPB expression has been observed in breast cancer. A genome-wide microarray analysis of 50 human breast cancer cell lines and 145 clinical specimens revealed that VAPB is overexpressed in breast cancer. Similarly, identification of a VAPB-IKZF3 fusion protein and high *VAPB* genomic amplification in breast cancer cells (Kao et al, 2009) suggest that VAPB plays in important role in cancer cell growth. Furthermore, as VAPB also functions in protein secretion and vesicle trafficking, tumor cells may rely on this pathway for receptor localization and growth factor secretion in order to sustain growth (Hanahan & Weinberg, 2011). Despite strong indications of the consequences of VAPB expression in cancer, a direct role of VAPB in tumor growth has not been investigated.

In this report, we studied the role of VAPB in breast cancer. We analyzed the expression of VAPB in breast cancer tissue microarray and publically available large breast cancer RNAmicroarray datasets, correlating VAPB expression with clinical outcomes. To determine the causal role of VAPB in cancer, we overexpressed VAPB in mammary epithelial cells or stably knocked down VAPB in tumor cells. Cell proliferation, apoptosis, spherical growth in 3D culture, and tumor growth *in vivo* were analyzed. Finally, we identified molecular mechanisms by which VAPB regulates tumor cell proliferation.

Materials and Methods

Antibodies and Reagents

Antibodies against the following proteins were used: anti-VAPB (K-16), anti-actin (I-19), anti-β-tubulin (Santa Cruz Biotechnologies), and anti-PCNA (NeoMarkers), anti-Myc, anti-pThr308AKT, anti-pSer473AKT, anti-pERK, anti-AKT, anti-ERK (Cell Signaling Technologies). Lentiviral control and *VAPB* shRNA plasmids KD# 1 (5′-GCACACACAAATATAGCATAA-3′) and KD# 2 (5′-CGGAAGACCTTATGGATTCAA-3′) and *VAPB* cDNA were obtained from OpenBiosystems. Growth factor-reduced Matrigel and TO-PRO3 was purchased from BD Biosciences and Invitrogen, respectively. The AKT 1/2 inhibitor 5J8/0360263-1 was produced by the Vanderbilt University Department of Chemistry as described previously (Lindsley et al, 2005). Erlotinib was a generous gift from the laboratory of Dr. William Pao (Vanderbilt University).

DNA constructs

VAPB mutants PCR products were sub-cloned into the pCLXSN retroviral expression vector. VAPB-ΔMSP was constructed using the following forward and reverse primers: 5′ - CGGAATTCATGGAGAATGATAAACCACATGAT -3′, 5′-CGGGATCCTCAATGGTGATGGTGATGATGACC-3′. The ΔCC mutant was constructed as previously described (Kim et al, 2010). The ΔCC (aa Δ159–196) mutant was constructed using three PCR steps. The first two PCR products

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correspond to the 5′ and 3′ DNA sequences upstream and downstream of the CCD. Each of these PCR products consists of complementary sequences flanking the boundaries of the CCD. Annealing of these two PCR products and further amplification with primers corresponding to the 5′ and 3′ sequence of the full-length VAP-B results in the production of the ΔCC mutant. The following set of sense and antisense primers have been used: for 5′ amplification: 5′-CCGGGATCCGCGAAGGTGGAGCA-3′; 5′-CTGTCTTCCTCATCCGACTCAGAGACTTAGACAC-3′; for 3′ amplification: 5′-GTGTCTAAGTCTCTGAGTCGGATGAGGAAGACAG-3′ and 5′-CGGGATATCCTACAAGGCAATCTTCCCAAT-3′. For full-length amplification: 5′-CCGGGATCCGCGAAGGTGGAGCA-3′ and 5′-CGGGATATCCTACAAGGCAATCTTCCCAAT-3′. VAPB sMSP was constructed by flanking the amino acids (1-132) with EcoRI and BamHI. The resultant PCR product was sub-cloned into pCLXSN.

Human mRNA expression profiling and tissue microarray protein analyses

Analysis of *VAPB* mRNA expression in human breast cancer datasets (van de Vijver et al, 2002; Wang et al, 2005) was performed in collaboration with the Vanderbilt-Ingram Cancer Center's Biostatistics Core Resource. *VAPB* "high" or "low" expression was defined as top or bottom quartiles of tumors expressing *VAPB*. Expression levels were analyzed in relation to overall and/or recurrence-free survival using Log Rank and Cox analyses. Statistical analyses were

performed using Software: R2.12.1. The survival curves from Kaplan-Meier was created and plotted by the function "survfit" under R package "survival." P values shown on KM plots were calculated based on log rank test between two survival curves of high or low expression groups.

Breast tumor spectrum Tissue Microarrays (TMAs; BR480) were purchased from US Biomax, Inc. (Rockville, MD). Immunohistochemical staining for VAPB was performed as described previously (Brantley et al, 2002; Brantley-Sieders et al, 2008) using a previously validated rabbit anti-VAPB antibody (Amarilio et al, 2005). Briefly, tumor sections were re-hydrated and subjected to thermal antigen retrieval in citrate buffer (2 mM citric acid, 10 mM sodium citrate, pH 6.0) using a PickCell Laboratories 2100 Retriever as per manufacturer's instructions. Endogenous peroxidases were quenched by incubation in 3% H_2O_2 solution for 30 minutes. After blocking, sections were incubated with anti-VAPB (1:25, produced in the Lev lab) overnight at 4°C, followed by a biotinylated goat anti-rabbit secondary antibody (1:200, BD Pharmingen, San Diego, CA), an avidin-peroxidase reagent (Life Technologies/Molecular Probes, Carlsbad, CA), and stained with liquid 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Life Technologies/Zymed Laboratories). Relative expression in TMAs was scored using a continuous scale (Amarilio et al, 2005) as follows: 0 = 0-10% positive tumor epithelium, 1 = 10-25% positive tumor epithelium, 2 = 25-50% positive tumor epithelium, and 3 = >50% positive tumor epithelium/core. Differential expression between tissue samples was quantified and analyzed

statistically (Chi square analysis).

Cell culture and generation of stable VAPB knockdown or overexpression cell populations

MCF10A, MCF7, HBL100, BT-474, BT-549, T47D, SKBR3, MDA-MB-436, MDA-MB-468, MDA-MB-231 were obtained from American Type Culture Collection (ATCC) and grown in culture per ATCC protocol. MMTV-Neu tumor cells were isolated as previously described (Muraoka et al, 2003) and grown in DMEM/F-12 (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone), 5ng/mL estrogen (Sigma-Aldrich), 5ng/mL progesterone (Sigma-Aldrich), 5ng/mL EGF (PreproTech), 5μg/ mL insulin (Sigma-Aldrich) with penicillin/streptomycin. For generation of stable VAPB knockdown cells, Vector-only (pLKO.1) or *VAPB* shRNA lentivirus was produced by co-transfecting HEK-293T cells with pLKO.1 or pLKO.1-shRNAs, along with packaging plasmids psPAX2 and pMD.2G, using Lipofectamine. Viral supernatants were collected 48 and 72 hours post transfection and used to infect MMTV-Neu tumor cells. MMTV-Neu cells were selected for plasmid inclusion by culturing cells in growth media containing 5μg/mL of puromycin (Sigma-Aldrich). For re-expression of VAPB, *VAPB* cDNA (open reading frame) was subcloned into pCLSXN retroviral vector that contains a Neomycin resistance gene, allowing for G418 (300μg/mL for 6 days) selection in puromycin-resistant populations. Viral supernatant was collected as described above from HEK 293T cells that were co-transfected with

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pCLSXN-Vector (10μg) only or pCLSXN-VAPB (10μg) and pCLAmpho plasmids (10μg) using Lipofectamine 2000.

MCF10A cells were obtained from American Type Culture Collection (ATCC) and cultured as previously described (Debnath et al, 2003). MCF10A-HER2 cells were generated via retroviral transduction of the HER2 proto-oncogene (Ueda et al, 2004). Overexpression of VAPB in MCF10A-HER2 cells was achieved by infecting cells with pCLXSN-Vector or -VAPB retrovirus and selected in media containing 300μg/mL G418 for 6 days.

Analysis of 3-dimensional spheroid culture

MMTV-Neu tumor cells or MCF10A-HER2 cells were plated on a solidified layer of Matrigel (2 mm thick) using an 8-well, glass chamber slide (Thermo Scientific) as previously described (Brantley-Sieders et al, 2008; Debnath et al, 2003). Fresh MMTV-Neu growth media or MCF10A-HER2 Assay Media (Debnath et al, 2003) was replaced every 48 hours. Cultures were maintained for 9 days prior to photo-documentation. Digital images were taken in 4 random fields/ well (3-4 spheroids/ field). Total spheroid area was calculated using NIH Image J software. For spheroid proliferation analysis, cultures were fixed at day 5 in a 1:1 methanol: acetone solution for 10 minutes at -20°C and co-stained with anti-PCNA (1:1000) and nuclear stain TO-PRO3 (1:2000) as previously described (Debnath et al, 2003). Confocal images of 15 spheroids per experiment were taken at random and percentage of PCNA-positive nuclei was

quantified. For AKT inhibitor-treated spheroid cultures, cells were allowed to grow for 48 hours, followed by AKT-inhibitor or vehicle control treatment starting on day 3. Fresh media containing inhibitor or control was added every 48 hours. For each analysis described above, at least two to three independent experiments were performed.

In vivo tumor studies

All animals were housed under pathogen-free conditions, and experiments were performed in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. FVB female recipient animals (3-4 weeks of age) were cleared of endogenous epithelium as described previously (Brantley et al, 2001) and one million MMTV-Neu tumor cells were injected orthotopically. A total of 8-10 animals per group were analyzed in 2 independent experiments. Resulting tumors were harvested 4-5 weeks after injection for analysis of tumor volume (volume = length x width^2 x 0.52). Harvested tumors were further processed for H&E staining and immunohistochemistry analysis of proliferation (Ki67) or apoptosis (cleaved caspase-3). Proliferating tumor cells were quantified by enumerating Ki67 positive nuclei in 4 random fields per tumor and presented as a percentage of Ki67+nuclei/total nuclei. A total of 8-10 tumors were quantified in two independent experiments. Statistical significance was assessed by single factor ANOVA.

Immunoblotting

For immunoblot analysis of VAPB knockdown or overexpression, cells were washed with PBS and lysed in Radioimmunoprecipitation Assay (RIPA) buffer (50mM Tris pH 7.4, 150mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitor tablets (Roche). Approximately 30-50ug of total cell lysate was separated by SDS-PAGE and probed with anti-VAPB (1:1000) overnight in 5% nonfat dry milk/ TBS-Tween (0.05%) and detected with anti-goat HRP (1:1000). For analysis of pAKT levels in VAPB expressing cells, cells were serum starved overnight and stimulated with DMEM/F-12 with 20ng/mL of EGF for the indicated time points or in the presence of erlotinib (1μM). For some experiments, an AKT inhibitor (0.1μM or 1μM) was added under normal growth conditions for 3 hours prior to cell lysis. Data are a representation of three independent experiments.

Statistical analysis

Results are presented as mean values±standard error. *P*-values are given in the figure legends, and values of $P < 0.05$ were considered to be statistically significant. Statistical analyses were performed by single factor ANOVA and unpaired, two-tailed Student *t*-test using PRISM software (GraphPad Sofware, La Jolla, CA, USA). For TMAs, statistical significance of VAPB expression between patient samples was determined by Chi-Square analysis.

VAPB is overexpressed in breast cancer and negatively correlates with patient survival

Several studies reported an increase in *VAPB* gene copy number or VAPB expression in breast cancer (Arriola et al, 2008; Chin et al, 2006; Edgren et al, 2011; Kao et al, 2009; Neve et al, 2006; Rizki et al, 2008). To determine the impact of VAPB expression on clinical outcome in human breast cancer, we analyzed *VAPB* mRNA expression in a published human breast cancer microarray dataset from a panel of 295 patient samples (van de Vijver et al, 2002). Kaplan-Meier analysis revealed that high levels of *VAPB* mRNA were significantly associated with poorer recurrence free survival (Figure 1.2A) than in patients with low *VAPB* mRNA expression. Similar results were obtained from a second, independent patient data set (n = 286) (Figure 1.2B) (Wang et al, 2005). To assess VAPB protein expression in human breast cancer, we performed immunohistochemistry in breast cancer tissue microarrays (TMA, n=84) with a previously validated anti-VAPB antibody (Amarilio et al, 2005). As shown in Figure 1.2C, we observed a significant increase of VAPB expression in invasive ductal carcinoma and lymph node metastasis samples, compared to normal tissue (p < 0.01, Chi-square test). We also observed differential VAPB protein expression in breast tumor cell lines, with higher VAPB expression in HER2-

positive breast cancer cell lines (Figure 1.2D). Taken together these data highlight the clinical relevance of high VAPB expression in human breast cancer.

VAPB promotes tumor spheroid growth by enhancing tumor cell proliferation

Because the VAPB is often overexpressed in human breast cancer (Arriola et al, 2008; Chin et al, 2006; Neve et al, 2006), we sought to determine if elevated VAPB expression enhances tumor cell growth. MCF10A-HER2 cells, an immortalized mammary epithelial cell line with low level of endogenous VAPB, were transduced to express human VAPB protein (Figure 2.1A). Tumor spheroid growth was quantified in a three-dimensional culture. As previously reported, control MCF10A-HER2 cells formed large acinar-like structures with a filled lumen (Debnath et al, 2003). Expression of VAPB in MCF10A-HER2 cells led to larger, irregular structures with invasive protrusions and a significant increase of spheroid size relative to parental and vector controls (Figure 2.1B).

To investigate which VAPB domains are critical for enhanced spheroid growth, we constructed several VAPB truncation mutants as illustrated in Figure 2.9A. Upon stable expression of VAPB mutants in MCF10A-HER2 cells (Figure 2.9B), we measured spheroid growth in 3-dimensional culture. Interestingly, all three mutants impaired spheroid growth as compared with WT VAPB (Figure 2.10). Given that the MSP, CC and TM domains facilitate cytosolic protein

Figure 2.1. VAPB expression enhances spheroid size and proliferation in mammary epithelial cells. (A) VAPB was stably expressed in MCF10A-HER2 cells via retroviral transduction, as judged by western blot analysis. (B) MCF10A-HER2-VAPB spheroids were cultured in three-dimensional Matrigel. Spheroid size was quantified and presented as average pixel area per spheroid ($p < 0.01$, ANOVA). (C) Cell proliferation in spheroid culture was determined by immunofluorescence using an anti-PCNA antibody (red). Cell nuclei were stained blue by TO-PRO-3 nuclear stain. PCNA-positive nuclei were quantified from an average of 15 random spheroids for each of two experiments ($p<0.05$, t test). Arrows: PCNA positive cells.

A

Parental Vector VAPB

25 kDa —   VAPB

50 kDa —   Actin

200 kDa —
150 kDa —   HER2

MCF10A-HER2

B

p < 0. 01

Average Spheroid Size
(Pixel Area x 100)/ 10x Field

140
120
100
80
60
40
20
0

Parental   Vector   VAPB

Parental

Vector

VAPB

C

p < 0.05

% PCNA+ Nuclei/ Total Nuclei

40

30

20

10

0

Vector   VAPB

Vector
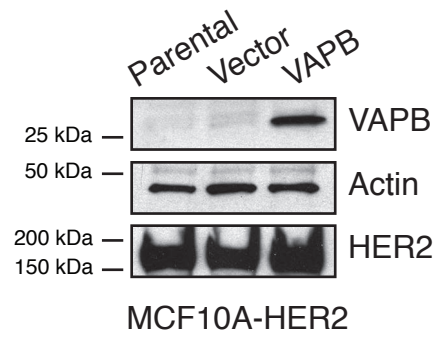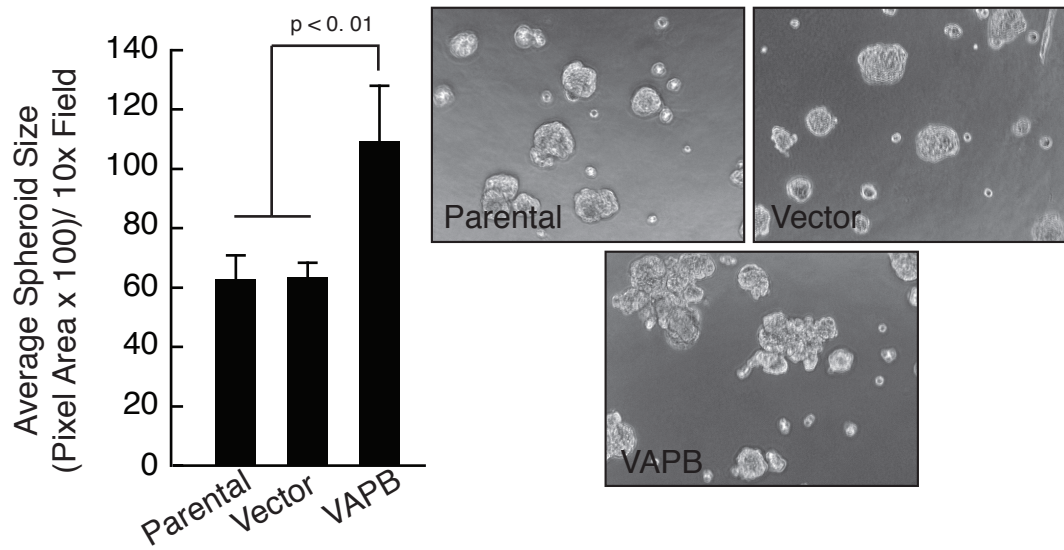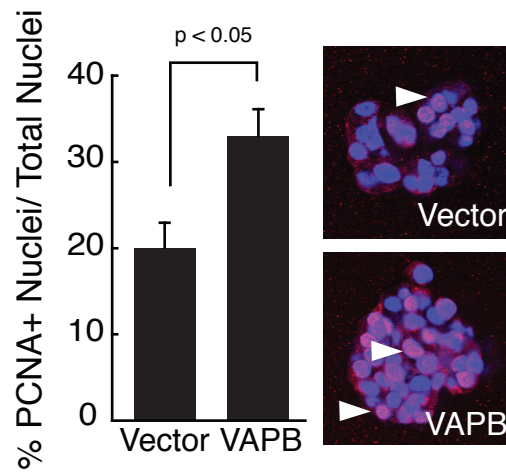
VAPB

interactions, VAPB self-dimerization, and VAPB localization to the ER respectively, this suggests that these functions are important for VAPB-mediated spheroid growth.

To determine whether increased tumor spheroid size is due to increased cell proliferation, the tumor spheroids were stained for proliferating cell nuclear antigen (PCNA), a cell proliferation marker. As shown in Figure 2.1C, VAPB expression elevated the number of PCNA-positive nuclei per spheroid, suggesting that VAPB regulates cell proliferation. Apoptosis, as measured by cleaved caspase 3 staining of 3D spheroid cultures, was not significantly changed (Figure 2.5A).

As an independent approach to determine whether VAPB is necessary for tumor cell growth, we knocked down VAPB in MMTV-Neu cells. These cells are derived from spontaneously arising mammary tumors from the *MMTV-Neu* mouse model (Muraoka et al, 2003). Stable expression of two independent shRNAs significantly reduced VAPB protein levels (Fig. 2.2A) but not the related protein VAPA (Figure 2.2C), suggesting that shRNAs specifically downregulated VAPB expression. In contrast to the morphology of vector control spheroids, which displayed disorganized and irregular structures with protrusions, knockdown of VAPB resulted in much smaller and more compact tumor spheroids (Figure 2.3A). Since one of the VAPB shRNA sequences (KD #1)

A — MMTV-Neu Tumor Cells

Lanes: Parental, Vector, KD #1, KD #2

37 kDa —, 25 kDa — : VAPB

50 kDa — : Actin

B — MMTV-Neu VAPB KD

Lanes: Vector, KD #1, KD #1_Vector, KD #1_VAPB

37 kDa —, 25 kDa — : VAPB

50 kDa — : Actin

C

Lanes: Parental, Vector, KD #1, KD #2

37 kDa —, 25 kDa — : VAPA

50 kDa — : Actin
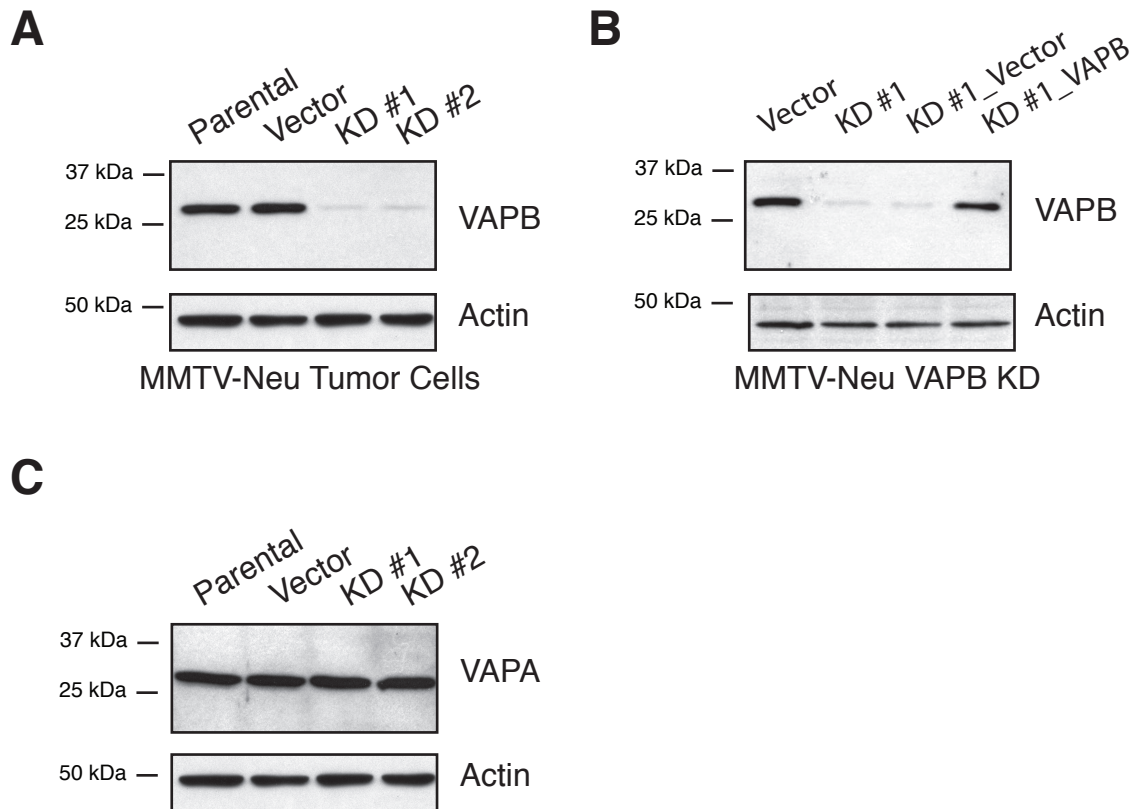
Figure 2.2. Specificity of VAPB shRNA knockdown. (A) VAPB expression was silenced in MMTV-Neu tumor cells by two independent lentiviral-mediated shRNAs, as judged by western blot analysis. (B) VAPB knockdown (KD #1) cells were rescued by re-expressing a full-length human VAPB cDNA via retroviral transduction. (C) VAPA protein expression was not affected by VAPB shRNA, as judged by western blot analysis.
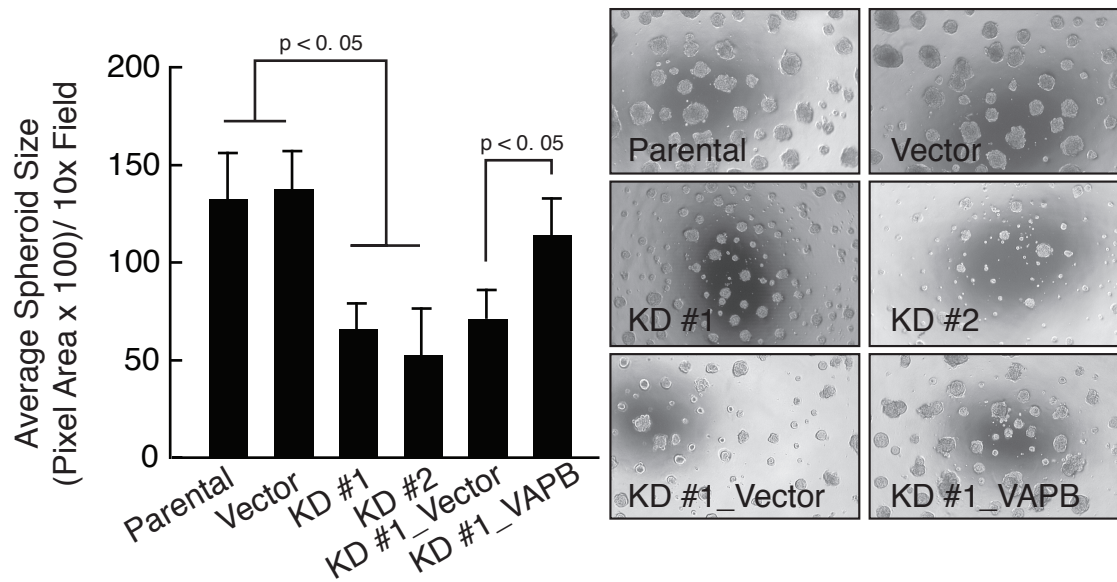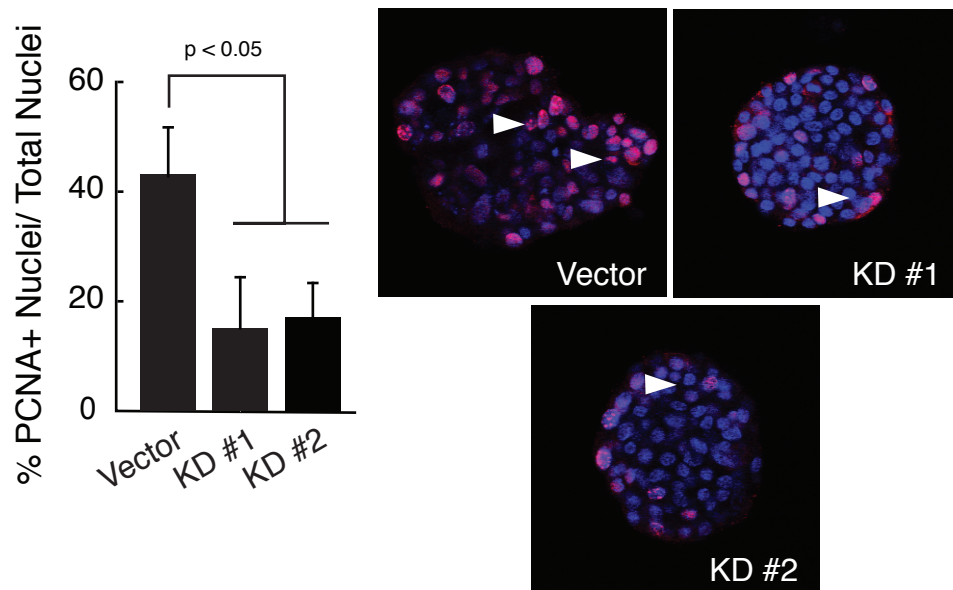
Figure 2.3. VAPB knockdown impairs mammary tumor spheroid growth and cell proliferation. (A) MMTV-Neu spheroids were cultured in three-dimensional Matrigel. Spheroid size was quantified and presented as average pixel area per spheroid (p < 0.01, ANOVA). (B) MMTV-Neu cell proliferation in spheroid culture was determined by immunofluorescence using an anti-PCNA antibody (red). PCNA-positive nuclei were quantified from an average of 15 random spheroids for each of two experiments (p<0.05, t test). Arrows: PCNA positive cells.

targeted the 3′ UTR, we were able to re-express full-length human VAPB protein (Figure 2.2B). Re-expression of VAPB restored tumor spheroid size and irregular morphology, demonstrating that phenotypes induced by shRNAs are VAPB specific and not due to off-target effects (Figure 2.3A). Knockdown of VAPB significantly reduced proliferation in spheroid cultures as quantified by PCNA staining (Figure 2.3B), without affecting apoptosis as measured by cleaved caspase 3 staining of 3D spheroid cultures (Figure 2.5B). Together, these data indicate that VAPB regulates tumor spheroid size by increased tumor cell proliferation.

VAPB promotes tumor growth in an orthotopic mammary tumor model

Next, we investigated the role of VAPB in tumor growth *in vivo* in a mammary tumor orthotopic transplantation model (Brantley-Sieders et al, 2008). One million *MMTV-Neu* control or VAPB knockdown cells were injected into cleared mammary gland fat pads of FVB recipient female mice. VAPB knockdown tumor cells failed to form tumors or formed very small, non-palpable tumors at five weeks post-transplantation, compared to parental or vector controls (Figure 2.4A). While parental or vector control tumors display densely packed tumor cells, VAPB knockdown tumors exhibit a reduced mammary tumor cell content (Figure 2.4B). To examine changes within the tumor epithelium, we assessed tumor proliferation and apoptosis in tissue sections by staining for Ki67 and cleaved caspase-3, respectively. We observed a significant decrease in
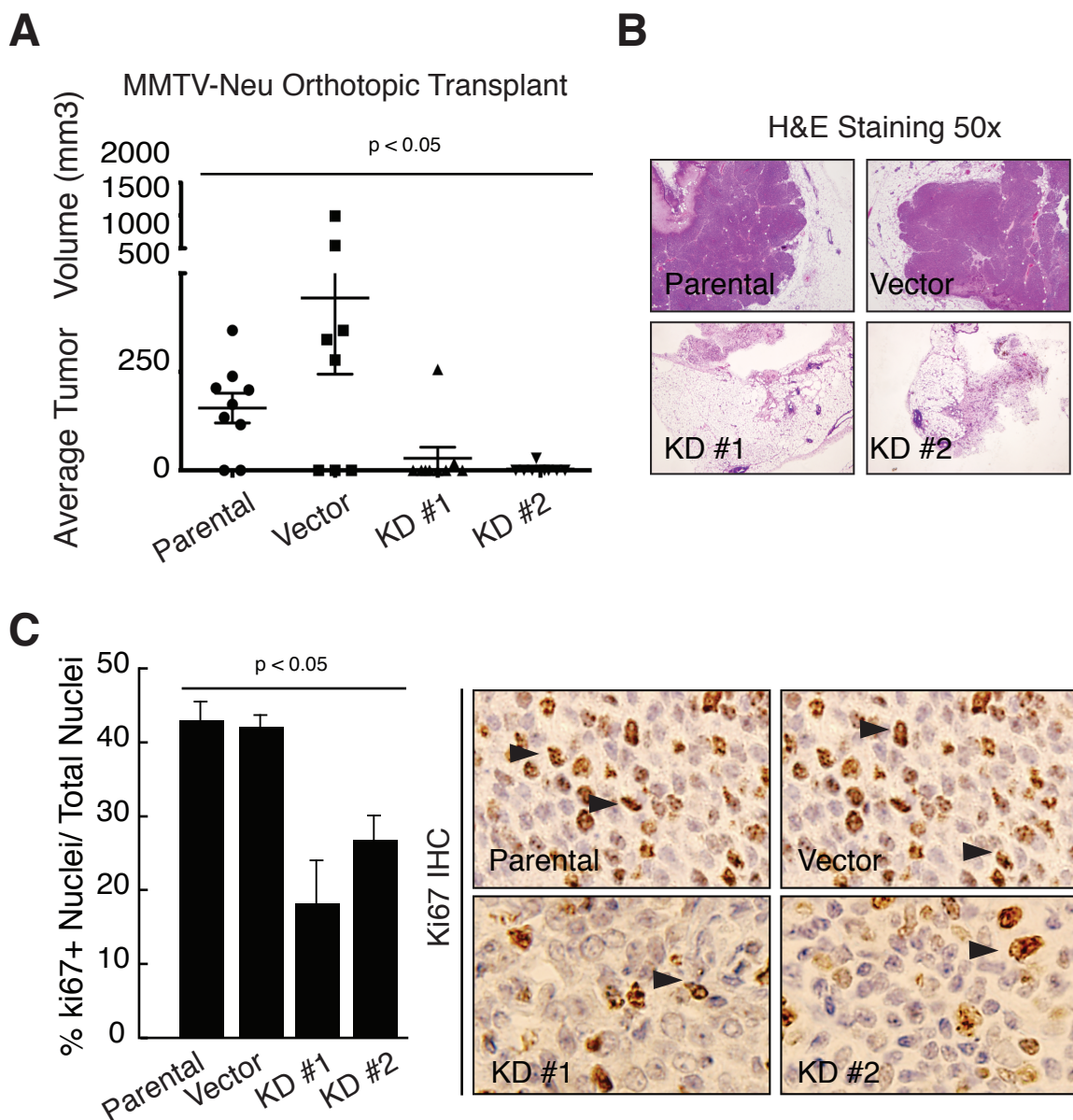
Figure 2.4. VAPB is required for tumor growth in vivo. (A) One million of MMTV-Neu control or VAPB knockdown cells were injected into cleared mammary gland fat pads of 3-week old FVB recipient female mice (n=8-10/experimental condition). Tumors were harvested 5 weeks after transplantation. Tumor size was measured by a caliper and tumor volumes were calculated. ($p < 0.05$, ANOVA). (B) Tumors were processed, sectioned and stained with H&E (B) or Ki67, a proliferation marker (C). Proliferating tumor cells were quantified by enumeration of Ki67+ nuclei and presented as a percentage of Ki67+ nuclei/total nuclei. VAPB knockdown cells showed a significant decrease in proliferation ($p < 0.05$, ANOVA). Arrows: Ki67 positive cells.
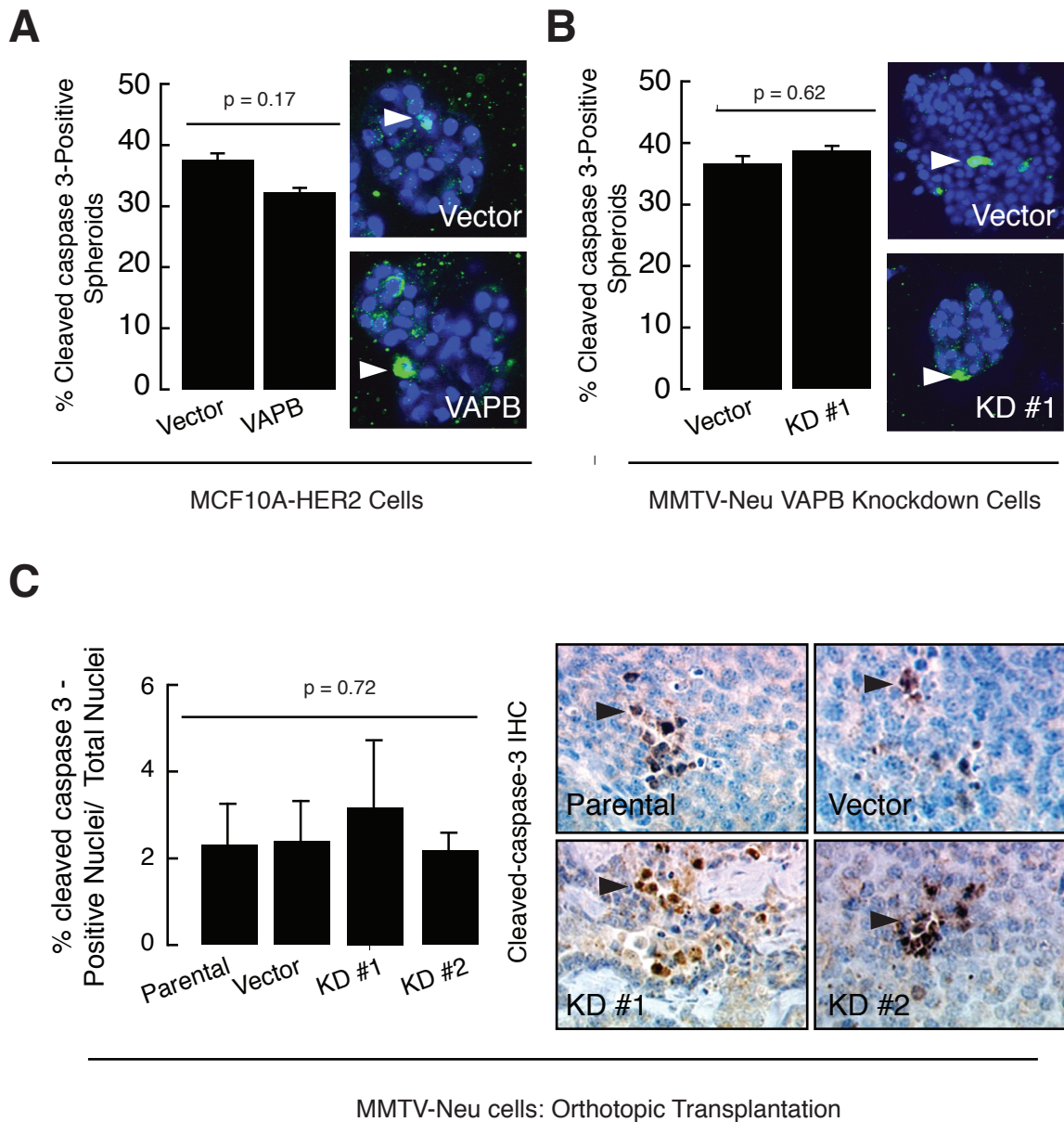
Figure 2.5. VAPB expression does not affect apoptosis. (A) MCF10A-HER2 or (B) MMTV-Neu knockdown cells were stained for cleaved caspase-3 at day 8 in 3-dimensional culture. Cleaved caspase-3 positive spheroids (green) were quantified by confocal microscopy analysis. No significant changes were observed in both cell lines. (C) *in vivo* analysis of apoptosis was measured by cleaved caspase-3 staining of tumor sections. Cleaved caspase -3 positive nuclei were quantified. VAPB deficiency does not significantly affect apoptosis in vivo. Arrows: cleaved caspase-3 positive cells.

tumor cells with Ki67 nuclear staining (Figure 2.4 C). Whereas cleaved caspase-3 levels were unaffected (Figure 2.5C). These data suggest that loss of VAPB inhibits HER2-initiated mammary tumor proliferation *in vivo*.

VAPB-induced cell proliferation is mediated by elevated AKT activity

To understand the mechanisms through which VAPB enhances proliferation, we investigated potential links between VAPB and signaling molecules relevant to tumor growth. Because ERK and AKT signaling pathways are two major players in regulating cell proliferation in mammary tumor cells, we assessed their activities by western blot analysis. MCF10A-HER2 or VAPB knockdown MMTV-Neu cells carrying either wild-type VAPB expression construct or the control vector were serum-starved overnight and stimulated with EGF at the indicated time points. In response to EGF stimulation, there is a rapid increase in phosphorylation of AKT at both Thr308 and Ser473 residues, indicating activation of its kinase activity. The phospho-AKT levels were significantly increased in MCF10A-HER2 cells overexpressing VAPB than those carrying the control vector (Figure 2.6A). Re-expression of VAPB in knockdown cells restored AKT phosphorylation to control levels (Figure 2.6B). In contrast, ERK phosphorylation levels were not changed between two cell populations (Figure 2.6). These results indicate that AKT may play a key role in regulating cell growth in these cells.

Figure 2.6. Analysis of AKT and ERK activities in VAPB expressing cells.
(A) MCF10A cells expressing VAPB or carrying control vector were serum starved and stimulated with 20ng/mL EGF at the indicated time points. As a negative control for EGF stimulation, cells were treated for 60 minutes with EGFR inhibitor, erlotinib (1μM). Phospho-AKT and phospho-ERK levels in MCF10A-HER2 cells were measured by western blot analysis and quantified. Representative blots from 3 independent experiments are shown. (B) VAPB was knocked down in MMTV-Neu cells and re-expressed via retroviral transduction. Cells were stimulated as in (A) and phospho-AKT and phospho-ERK levels were measured by western blot analysis and quantified. Representative blots from 3 independent experiments are shown.

A MCF10A-HER2 Cells

Vector   VAPB

Minutes (EGF)   0 5 20 60 *Erlotinib*   0 5 20 60 *Erlotinib*

p-Thr308 Akt — 50 kDa
p-Ser473 Akt — 50 kDa
Total Akt — 50 kDa
p-Erk — 37 kDa
Total Erk — 37 kDa

p < 0.05 *

Percent pTh308 AKT of Total AKT (0–120)
EGF (Min): 0 5 20 60

Percent pERK of Total ERK (0–300)
EGF (Min): 0 5 20 60
□ Vector ■ VAPB

B MMTV-Neu VAPB Knockdown Cells

Vector   VAPB

Minutes (EGF)   0 5 20 60 *Erlotinib*   0 5 20 60 *Erlotinib*

p-Thr308 Akt — 50 kDa
p-Ser473 Akt — 50 kDa
Total Akt — 50 kDa
p-Erk — 37 kDa
Total Erk — 37 kDa

* p < 0.05

Percent pTh308 AKT of Total AKT (0–250)
EGF (Min): 0 5 20 60

Percent pERK of Total ERK (0–180)
EGF (Min): 0 5 20 60
□ Vector ■ VAPB

45

Figure 2.7. VAPB-dependent cell growth is mediated through AKT activity. Pharmacologic AKT inhibition significantly impaired VAPB-mediated spheroid growth in (A) MCF10A-HER2 cells and (C) MMTV-Neu VAPB knockdown cells rescued with VAPB re-expression ($p < 0.05$). Inhibition of AKT activity was confirmed by western blot analysis for phospho-AKT in (B) MCF10A-HER2 cells and (D) MMTV-Neu knockdown cells.
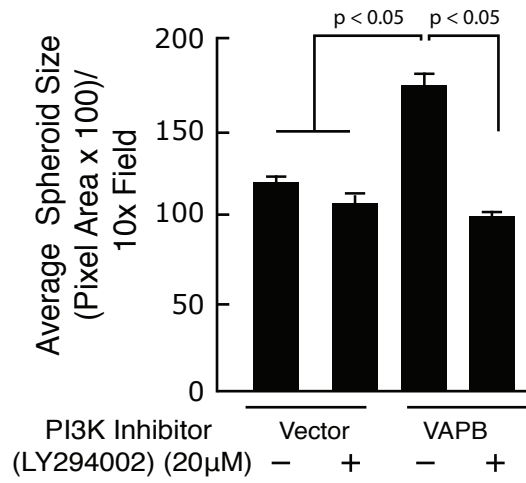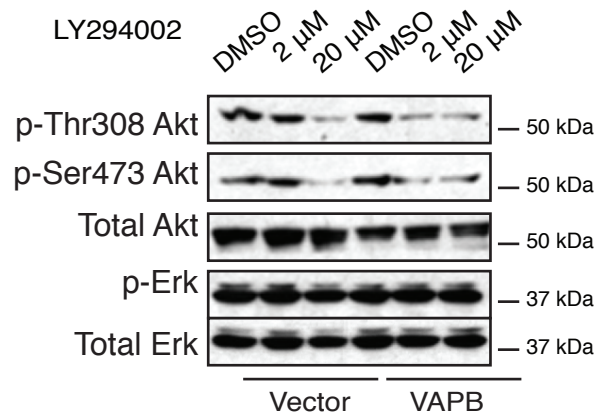
Figure 2.8. PI3K inhibition attenuates VAPB dependent spheroid growth.
(A) Cells were cultured in 3D Matrigel for 2 days and then treated with
LY294002 (20µM) or vehicle control every 2 days. Tumor cell spheroids were
quantified at day 8. (B) Inhibition of AKT activity was confirmed by western blot
analysis for phospho-AKT levels. Shown were representative blots from two
independent experiments.

A

B
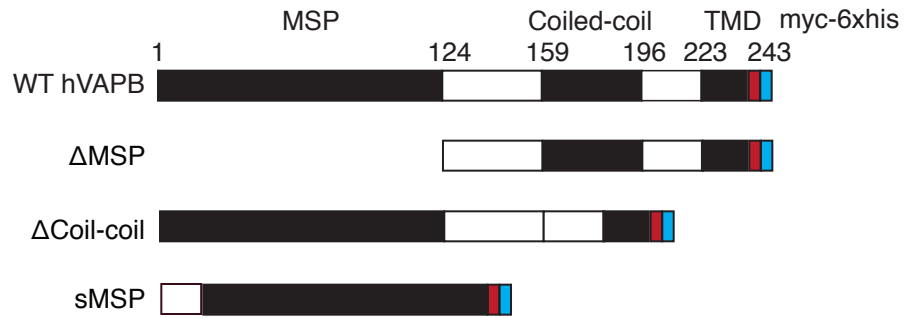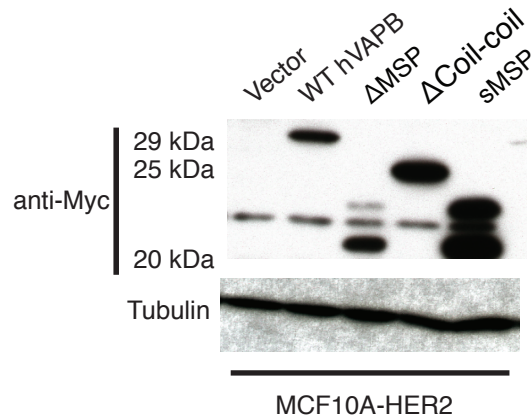
MCF10A-HER2

Figure 2.9. VAPB mutant panel. (A) Schematic of human VAPB wildtype or mutant tagged with Myc/ 6x His. Sequences were inserted into pcLSXN_Neo retroviral vector. (B) MMTV-Neu VAPB knockdown tumor cells or MCF10A-HER2 cells were infected and cell lysates were subjected to western analysis to confirm protein expression.
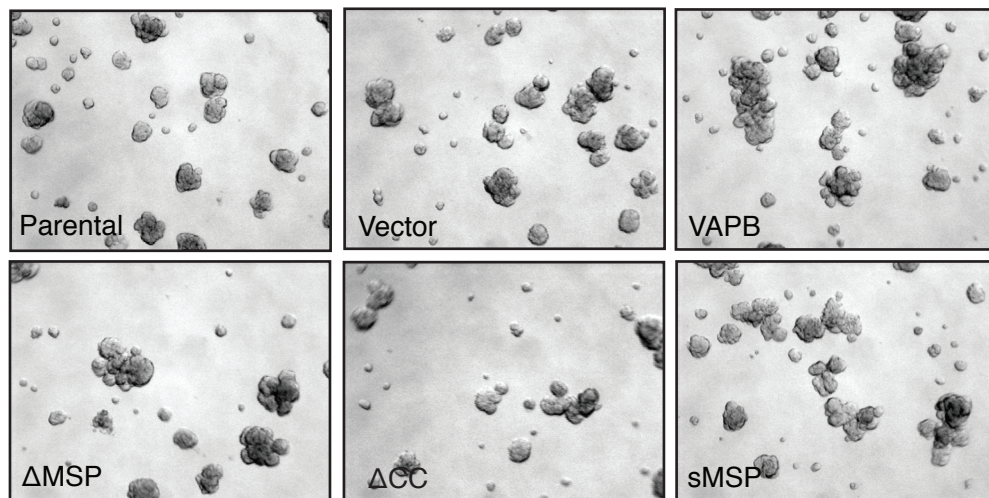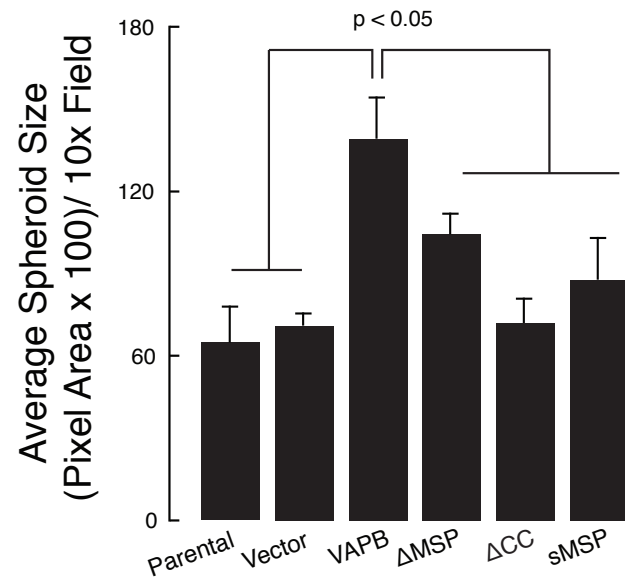
Figure 2.10. Mutant VAPB expression impairs spheroid growth. MCF10A-HER2 cells expressing WT or mutant VAPB were grown in 3D culture. The size was quantified at day nine. Expression of mutant VAPB significantly impaired spheroid size when compared with WT VAPB ($p < 0.05$ Single Factor ANOVA).

To test if VAPB-mediated spheroid growth is dependent on AKT phosphorylation, cells were treated with an allosteric AKT inhibitor (Lindsley et al, 2005). AKT inhibition significantly reduced spheroid growth of VAPB expressing cells (Figure 2.7A and 2.7C). As expected the AKT inhibitor reduced phosphorylated AKT, while phospho-ERK was unaffected (Figure 2.7 B and 2.7 D). Similar results were obtained using the LY294002, a PI3K inhibitor (Figure 2.8) in MCF10A-HER2 cells. Although these results do not rule out the contribution of other signaling pathways, our findings suggest that VAPB-induced proliferation is mediated, at least in part, through activation of the AKT pathway.

Discussion

VAPB was originally identified as one of the vesicle-associated membrane protein (VAMP) associated proteins. Although VAPB was implicated in a wide range of cellular processes, its function in cancer has not been characterized. In this report, we provide evidence that VAPB regulates mammary tumor growth and proliferation via activation of AKT activity. VAPB protein expression is elevated in primary and metastatic tumor specimens, and *VAPB* mRNA expression levels correlated negatively with patient survival in two large breast tumor datasets. Overexpression of VAPB increased spheroid size and proliferation in MCF10A-HER2 cells. Conversely, knockdown of VAPB in MMTV-Neu mammary tumor cells inhibited tumor cell proliferation in 3-D culture *in vitro* and suppressed tumor growth in orthotopic mammary tumor allografts. The

growth regulation of mammary tumor cells controlled by VAPB appears to be mediated, at least in part, by modulation of AKT activities. Collectively, the genetic, functional and mechanistic analyses suggest a role of VAPB in tumor promotion in human breast cancer.

Approximately 30% of all breast tumors have mutations in one or more components of the PI3K/AKT pathway (Stemke-Hale et al, 2008). Two lines of evidence suggest that VAPB expression modulates AKT activation. First, overexpression of VAPB in MCF10A-HER2 cells enhanced phosphorylation of AKT, whereas knockdown of VAPB in MMTV-Neu tumor cells inhibited pAKT levels. Furthermore, the addition of an allosteric AKT inhibitor significantly reduced 3D spheroid growth induced by VAPB. It is currently unclear how VAPB regulates PI3K/AKT activity. Since VAPB does not contain known enzymatic activities, it is likely that its action is mediated by its interaction with other proteins.

Major subcellular compartments where VAPB is localized are the ER and Golgi, where secretory and membrane proteins are synthesized and transported to the cell surface (Moumen et al, 2011; Skehel et al, 2000). Several studies show that VAPB is required for neurotransmitter release and functions in early secretory transport events (Skehel et al, 1995; Soussan et al, 1999). Therefore it is possible that VAPB may regulate the protein secretion. Indeed, when VAPB is overexpressed in MCF10A-HER2 cells, transport of VSV-G-GFP ts045, a protein used to monitor vesicle traffic from ER/Golgi to the plasma membrane, is

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markedly enhanced (Figure 4.3), consistent with a previous report that show VAPB deficiency inhibited VSV-G-GFP ts045 to the plasma membrane in HeLa cells (Peretti et al, 2008). Accordingly, one mechanism by which VAPB promotes tumor cell proliferation could be through secretion of growth factors, cytokines, matrix metalloproteinases, as well as delivery of receptors to the cell surface (Hanahan & Weinberg, 2011). This possibility is further strengthened by the fact that VAPB physically interacts with Arf1 (Figure 4.4A) and Rab1 (Figure 4.6), two small GTPases that are known to play critical roles in regulation of vesicle trafficking in the secretary pathway (D'Souza-Schorey & Chavrier, 2006; Gillingham & Munro, 2007; Plutner et al, 1991; Spang, 2002; Stenmark, 2009). Interestingly, Arf1 has also been implicated in recruitment of p85 subunits of PI3K to EGFR in breast cancer cells (Boulay et al, 2008), providing an additional possible mechanism by which VAPB could regulate AKT activities.

Aside from interacting with Arf1/Rab1, we and others also found VAPB in complex with other proteins including lipid transfer binding proteins such as Nir2 (Table 3) (Amarilio et al, 2005; Peretti et al, 2008), a phosphoinositol/ phosphotidyalcholine transfer protein. Deletion of *scs2*, the yeast homolog of VAPs, was reported to reduce phosphoinosotide levels (Kagiwada & Zen, 2003). Since AKT phosphorylation is dependent on recruitment to the plasma membrane through interaction between the PH domain and PIP3, such alterations in phosphoinositols at the plasma membrane could affect ultimately the activation of AKT. Given the diverse function of VAPB-interacting proteins

(Table 3), it is likely that multiple pathways converge through VAPB to enhance the AKT pathway and affect tumor cell proliferation.

Our results suggest that VAPB enhances breast tumor cell proliferation is mediated through the AKT pathway. However we cannot rule out other functions of VAPB that also may contribute to the phenotypes observed in this study, such as lipid sensing and transport (Jansen et al, 2011; Kawano et al, 2006; Loewen & Levine, 2005) ER and Golgi architecture (Amarilio et al, 2005; Peretti et al, 2008), and the unfolded protein response (Gkogkas et al, 2008; Kanekura et al, 2006). Therefore further studies are needed to address whether perturbation in these mechanisms alters the phenotypes of tumor cells in relation to VAPB expression. Furthermore, although full-length VAPB localizes to the ER, Golgi, and membrane-bound vesicles (Lapierre et al, 1999; Moumen et al, 2011), a number of studies showed that the MSP domain of VAPB can be secreted and act as an antagonist for Eph receptor tyrosine kinases in *C. elegans* and *Drosophila* (Miller et al, 2003; Tsuda et al, 2008). Secreted MSP has also been detected in human serum (Tsuda et al, 2008). Because ligand-dependent EphA2 receptor signaling has been associated with tumor suppression whereas EphA2 ligand-independent signaling promotes tumor initiation and malignancy in breast cancer (Chen et al, 2008; Vaught et al, 2008), it is tempting to speculate that the secreted MSP domain may compete with ligand for binding to EphA2 receptor, thereby blocking EphA2-tumor suppressive function as hypothesized in Figure 6.1B. However, although tumor cells do secrete MSP (Figure 6.1A), thus far we are unable to

detect any biological activities of soluble MSP, using either conditioned media or purified recombinant soluble MSP proteins. Further investigations are required to test this hypothesis.

In summary, we identified a functional role of VAPB in promoting tumor cell proliferation in breast cancer. While the expression of VAPB in other cancers has not been systematically investigated, this discovery opens up a number of exciting avenues for future studies of both full length VAPB and the secreted MSP domain. As VAPB overexpression is associated with poor patient survival, targeting VAPB-associated protein secretory pathway may provide novel targets for future pharmacological strategies in breast cancer therapy.

CHAPTER III

VAMP-ASSCOCIATED PROTEIN B (VAPB)

ELEVATES THE INVASIVE POTENTIAL OF

MCF10A-HER2 CELLS

Introduction

Metastasis is the major cause of death in breast cancer patients (Amercian-Cancer-Society, 2012). The metastatic cascade begins with tumor cells becoming increasingly mobile and breaking through the basement membrane to invade surrounding tissues. Eventually the disseminated tumor cells enter the blood stream (or lymphatics) and migrate to distant organs, leading to advanced stage breast cancer. Therefore, it is critical to understand the molecular alterations that contribute to tumor cell invasiveness, a critical step in the metastatic process (Chambers et al, 2002).

Recently, Rizki et al., found elevated VAPB expression from microarray analysis on "pre-invasive" versus "invasive" cell lines derived from sub-culturing basal-like HMT-3522 cells in a 3-dimensional environment that mimics the extracellular matrix (ECM) (Rizki et al, 2008). Additional evidence to implicate VAPB in tumor cell invasion includes recurrent genomic amplification of VAPB in *HER2* amplified invasive breast cancers (Arriola et al, 2008). Furthermore, our own data shows that VAPB protein is elevated in metastatic breast tumor

samples over control tissue (Figure 1.2C). These observations suggest that VAPB may promote invasiveness in breast cancer cells, thus contributing to increased malignancy and poor outcome associated with aberrant expression (Figure 1.2).

Normal mammary glands contain highly polarized luminal epithelial cells, surrounded by a layer of basal epithelial cells in addition to various stromal cell types (Watson & Khaled, 2008). MCF10A cells are a valuable *in vitro* tool for modeling breast cancer genes and pathways in the context of invasion (Debnath et al, 2003; Lee et al, 2007). In three-dimensional cell culture, MCF10A cells mimic the highly organized acini-like structures found in mammary glands *in vivo.* Moreover, MCF10A cells secrete laminin V protein, a major component of the basement membrane (Debnath et al, 2003). The basement membrane encapsulates the assembled mammary epithelial structure, and plays an important role in maintaining cell polarity and serves as a barrier, preventing tumor cells from invading the surrounding tissue (Slade et al, 1999).

Here we demonstrate that VAPB expression enhances the invasive behavior of MCF10A cells that express HER2. We found that VAPB expression led to abnormal acini morphology and laminin V localization in three-dimensional culture, suggesting a loss of basement membrane integrity. Furthermore, our data also show that VAPB increases growth factor-induced invasion, but not migration suggesting that VAPB promotes the early dissemination of cells.

Materials and Methods

<u>Antibodies and Reagents</u>

Antibodies against the following proteins were used: anti-VAPB (K-16), anti-E-cadherin (BD Biosciences), and anti-laminin5γ2 (Santa Cruz Biotechnologies). Growth factor-reduced Matrigel and TO-PRO3 was purchased from BD Biosciences and Invitrogen, respectively.

<u>Transwell assays</u>

Transwell filters (8 µm pore) (Corning Incorporated) were blocked with 1% BSA (Sigma) for 30 min at 37°C. Blocking solution was removed and filters were coated with a 1:20 dilution of Matrigel (BD Biosciences) in DMEM/F-12 plus Penicillin/ streptomycin (Invitrogen) for 2 hours at 37°C. 100,000 MCF10A-HER2 control or VAPB expressing cells were seeded (200µL volume) on the top of the coated (invasion assay) or uncoated (migration assay) transwell filter in serum-free and EGF-free DMEM/F-12. Immediately, the transwells were placed in 24-well plates containing DMEM/F-12 media with or without EGF (20ng/mL) and were incubated at 37°C for 24 hours. For inhibitor studies, AKT 1/2 inhibitor 5J8/0360263-1 (Vanderbilt University Department of Chemistry) was added to the bottom chamber at the indicated concentrations. Filters were fixed in 10% NBF and stained with crystal violet. Cells on the lower surface were counted in three random fields from each well, with duplicate samples per condition.

Cell culture and analysis of VAPB acini morphology

MCF10A-HER2 cells were plated on a solidified layer of Matrigel (2 mm thick) using an 8-well, glass chamber slide (Thermo Scientific) as previously described (Brantley-Sieders et al, 2008; Debnath et al, 2003). Fresh MCF10A-HER2 Assay Media (Debnath et al, 2003) was replaced every 48 hours. Cultures were maintained for nine days prior to photo-documentation. Quantification of invasive protrusions was performed as previously described (Chatterjee et al, 2012) such that acini with one or more invasive protrusion was counted as "positive". For spheroid morphology analysis, cultures were fixed at day nine in a 1:1 methanol: acetone solution for 10 minutes at -20°C and co-stained with anti-E-cadherin (1:1000), anti-laminin5γ2 (1:500) and TO-PRO3 nuclear stain (1:2000) as previously described (Debnath et al, 2003). For laminin V quantification, MCF10A-HER2 cells were stained with anti-laminin5γ2 at day five and nine. Confocal images of 30 randomly selected spheroids per experiment were taken and percentage of laminin staining surrounding each spheroid was quantified by NIH Image J software.

VAPB immunofluorescence analysis

MMTV-Neu or MCF10A-HER2 cells were plated on glass slides and incubated in growth media in a 6-well dish overnight. At 40 – 50 % confluence, the cells were fixed in 10% NBF for 15 minutes at room temperature. Slides were rinsed three times in PBS for 5 minutes each, then blocked in PBS/5% goat

rinsed three times in PBS for 5 minutes each, then blocked in PBS/5% goat serum for 1 hour at room temperature. Anti-VAPB was diluted 1:1000 in PBS containing 1% BSA/ 0.3% Triton-X (Sigma) overnight at 4°C. After washing, AlexaFluor (Invitrogen) secondary antibody (1:2000) was incubated for 4 hours at room temperature followed with TO-PRO-3 nuclear stain. Slides were mounted and analyzed by confocal microscopy. For "scratch" assays, MMTV-Neu tumor cells were cultured to confluence. After overnight serum starvation, cells were scratched with 200μL micro-pipet tip and normal growth media was replaced. Following a 24-hour incubation, cells were stained as described above.

Results

VAPB alters the morphology of MCF10A-HER2 acini.

While we demonstrated that ectopic expression of VAPB in MCF10A-HER2 cells increased acini growth in 3-dimensional culture (Figure 2.1B), we also observed gross morphological changes in VAPB-expressing acini. Consistent with previous reports, parental and vector control MCF10A-HER2 cells formed non-invasive acini structures (Figure 3.1A) and intact basement membrane structures as seen with laminin V immunofluorescence staining (Figure 3.1B) (Lu et al, 2009; Muthuswamy et al, 2001). However with the expression of VAPB, we observed an increase in invasive growth into the surrounding matrix as quantified in Figure 3.1A. Further analysis of the VAPB-

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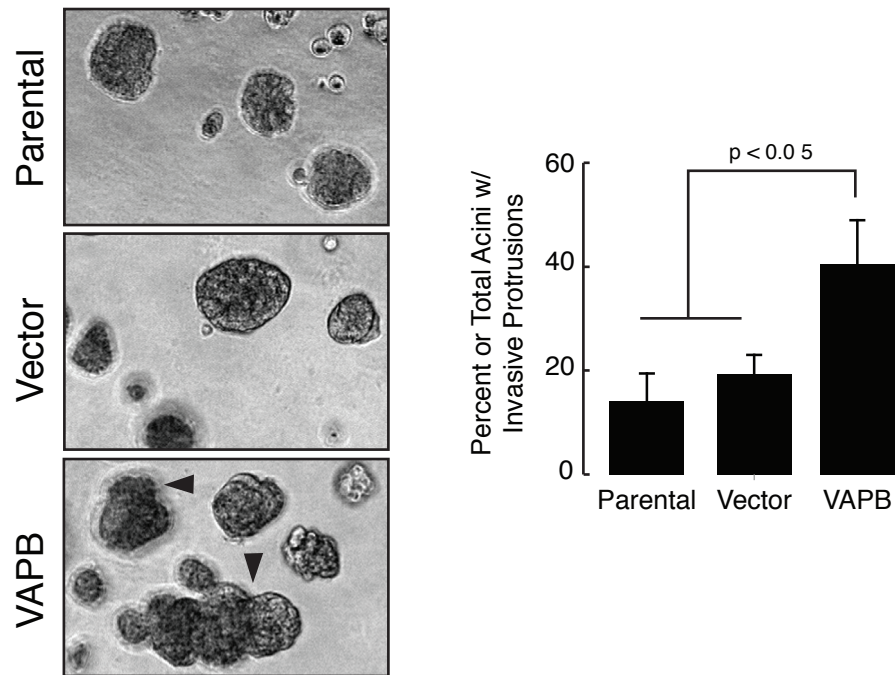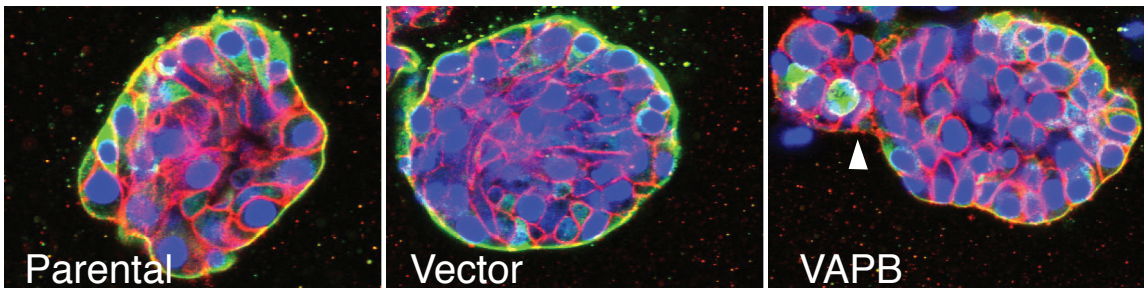
Figure 3.1. VAPB induces invasive-like growth into the extracellular matrix. (A) MCF10A-HER2-VAPB cells were cultured in Matrigel. At day 9, cultures were photodocumented and scored for invasive growth (black arrows) as previously described (Chatterjee et al, 2012). Percentage of acini displaying invasive protrusions were quantified from three independent experiments ($p < 0.05$, t-test) (B) Acini were stained at day 9 for TO-PRO-3 nuclear stain (blue), E-cadherin (red) and laminin Vγ2 (green) and imaged by confocal microscopy. Confocal analysis revealed MCF10A-HER2 VAPB acini displayed normal E-cadherin localization at the cell junction, but diminished laminin V localization at areas of invasive growth (white arrow).

mediated invasive acini growth with confocal microscopy revealed a strong presence of E-cadherin at cell junctions but weak laminin V staining at the area of invasive growth into the matrix (white arrow) (Figure 3.1B).

To address the observations seen in Figure 3.1B, we examined the effects of VAPB expression on basement membrane integrity by monitoring laminin V deposition. MCF10A cells normally deposit laminin V at the basal surface of acini by day five (Debnath et al, 2003), creating a continuous ring that surrounds each structure. Upon ectopic expression of VAPB in MCF10A-HER2 cells we observed a significant decrease in laminin V circumscribing each acini as analyzed by confocal microscopy at day nine. At day 5, both vector and VAPB-expressing cells have a continuous laminin V layer surrounding the acini, suggesting that laminin V is sufficiently produced in early stages of acinar growth. The results at day five and nine are quantified in the lower panel. These data support the idea that VAPB is an important factor that promotes the irregularity and invasive behavior of HER2-driven acini growth.

VAPB expression elevates the invasive potential of MCF10A-HER2 cells

A critical step in the metastatic cascade is the acquisition of invasive characteristics such as increased tumor cell motility and the ability to break down ECM components (Hanahan & Weinberg, 2011). Therefore, we tested whether VAPB allowed for these characteristics in MCF10A-HER2 cells, using Transwell (Boyden chamber) assays. As seen in Figure 3.3A, VAPB expression
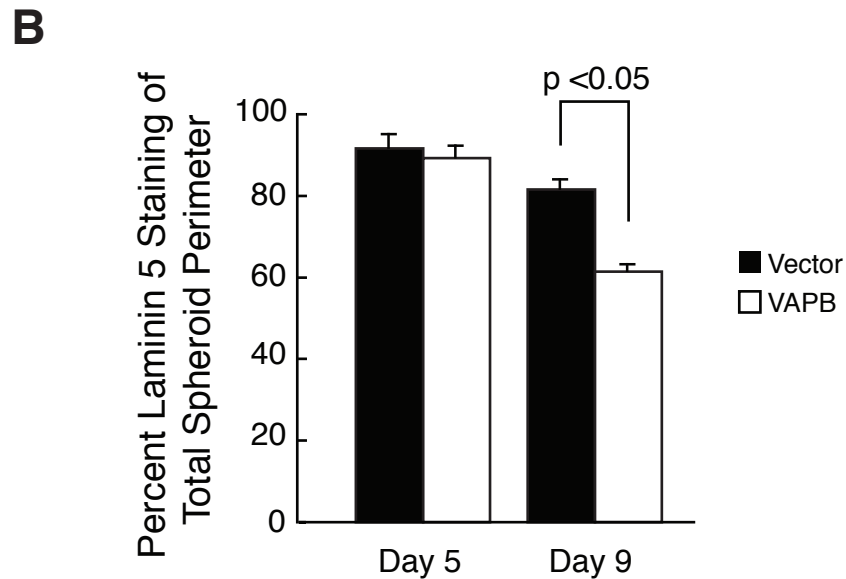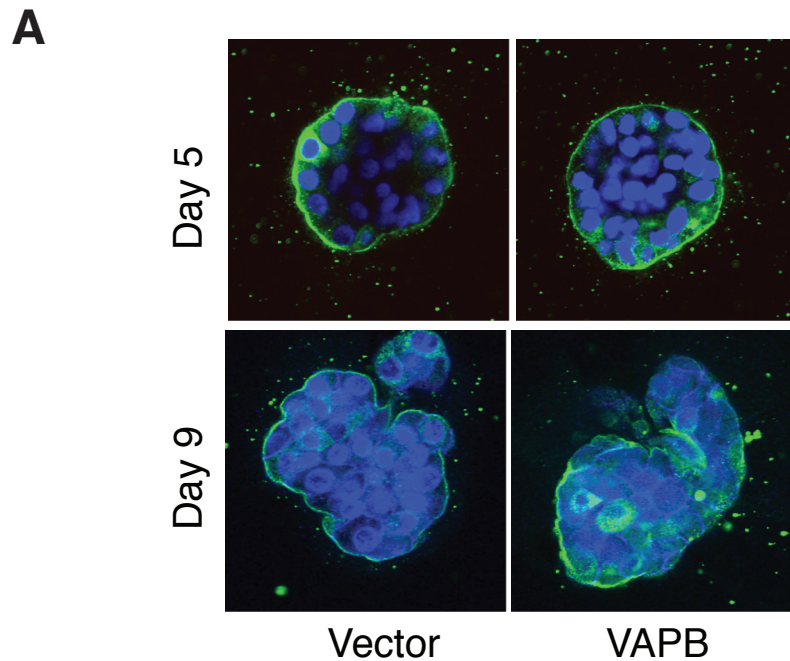
Figure 3.2. VAPB expression disrupts basement membrane integrity. (A) MCF10A-HER2 cells were cultured in Matrigel and stained at day five and day nine for lamininVγ2 (green) and TO-PRO-3 nuclear stain (blue) and analyzed by confocal microscopy. At day five both vector and VAPB expressing cells displayed continuous laminin V staining around each spheroid which was significantly reduced by day nine in VAPB-expressing cells only. (B) Quantification of 30 randomly selected spheroids from two independent experiments is shown in the lower panel ($p < 0.05$ ANOVA).

Figure 3.3. VAPB elevates the invasive potential of MCF10A-HER2 cells. (A) VAPB significantly increased EGF-induced (20ng/mL) invasion through Matrigel-coated, Boyden chambers (Transwells). (B) EGF- induced migration was assayed using uncoated Transwell filters. No significant difference was observed between vector and VAPB-expressing cells. (C) Pharmacologic inhibition of AKT significantly impaired EGF-induced invasion in VAPB expressing cells. Inhibition of AKT activity was confirmed by western blot analysis as shown in Figure 2.7 B. All results were quantified from three independent experiments ($p < 0.05$ ANOVA).
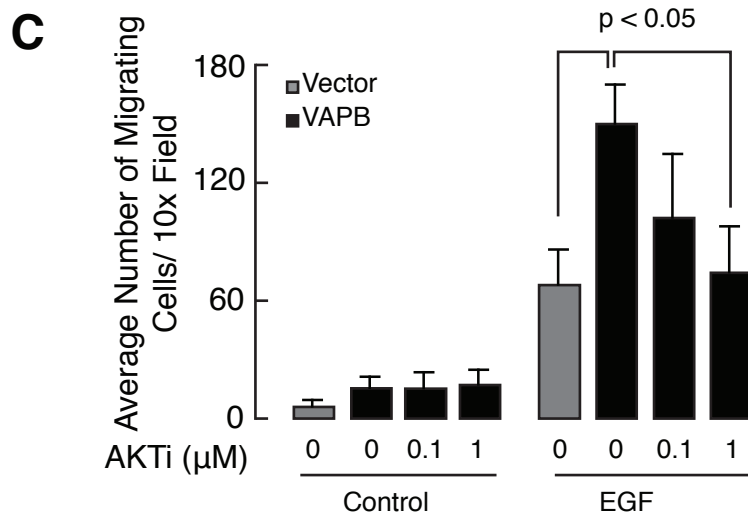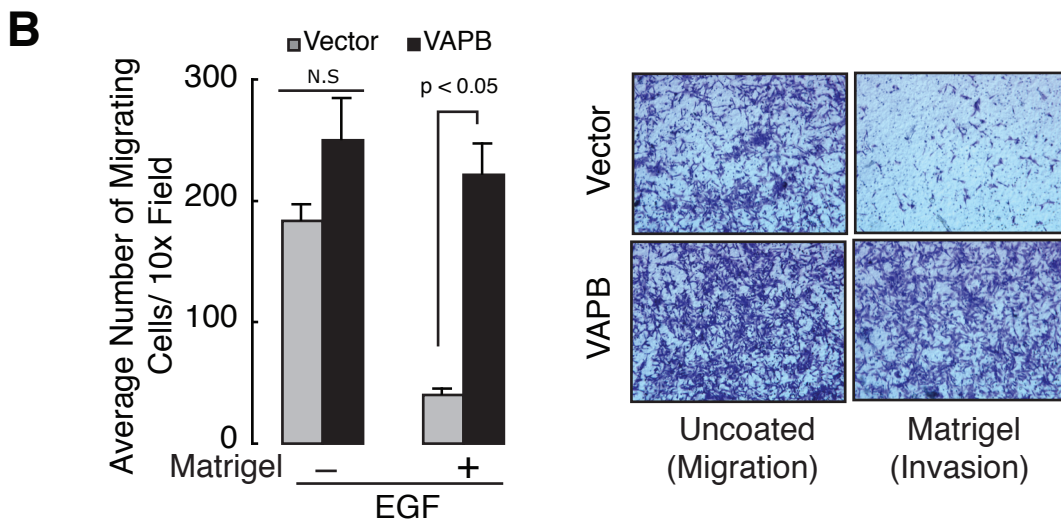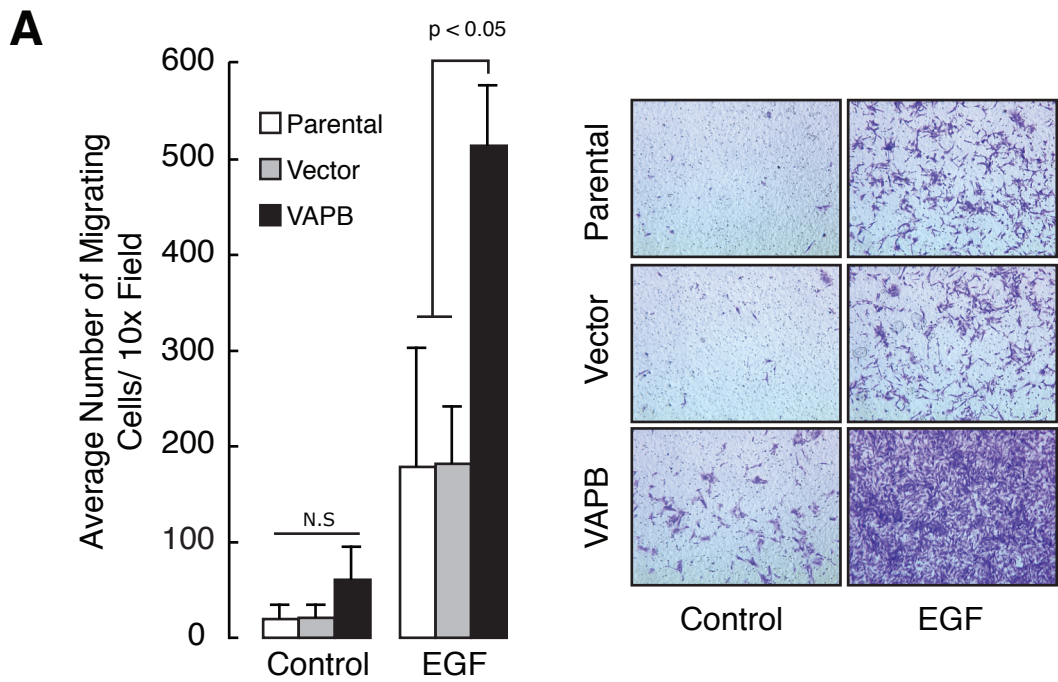
significantly increased MCF10A-HER2 invasion through the Matrigel-coated Transwell filters in response to EGF (Figure 3.3A), demonstrating that VAPB imparts to MCF10A-HER2 cells the ability to breakdown the Matrigel layer, prior to migration through the membrane pores. However the results presented in Figure 3.3B suggest that VAPB does not affect cell motility because the differences seen between vector and VAPB-expressing are diminished when using uncoated Transwell filters, which tests for directional cell migration. Thus, it is likely that VAPB enhances the invasive capabilities of MCF10A-HER2 cells at the level of ECM breakdown, rather than increasing cell motility.

Based on our earlier studies, we found that VAPB expression increases tumor cell proliferation through enhanced AKT activation (Chapter II). In addition to proliferation, invasion is also a process modulated by the AKT pathway (Chin & Toker, 2009). Therefore, we used the same allosteric AKT inhibitor as in Figure 2.7, to test whether VAPB-induced invasion is dependent on AKT. Indeed, we find that pharmacologic inhibition of AKT significantly reduced invasion in VAPB-expressing cells (Figure 3.4C).

VAPB localization at the leading edge of cells

Although VAPB primarily localizes to the ER, there is data to support that VAPs also localize to other intracellular membranes such as recycling endosomes, tight junctions and plasma membrane [Reviewed in (Lev et al, 2008)]. Along these lines of data we also observed VAPB localization at the

leading edge of cells (Figure 3.5). Our data shows a distinct pattern of VAPB in both randomly migrating cells (Figure 3.5A and B) and in directional migration in MMTV-Neu tumor cells (Figure 3.5C) by immunofluorescence analysis. This VAPB staining pattern suggests the possibility of distinct interplay between VAPB and proteins that mediate cell motility or invasion at the plasma membrane.

Discussion

The acquisition of invasiveness is a critical step during breast cancer metastasis. Our data demonstrate that VAPB expression elevates the invasive potential of HER2-expressing tumor cells. First, we found that VAPB increases the formation invasive protrusions in MCF10A cells engineered to express HER2. Upon closer examination of invasive growth by confocal microscopy, we observed normal E-cadherin localization at cell junctions, however, the laminin V staining was markedly diminished. Next, we found that VAPB-expressing cells had significantly reduced laminin accumulation around each acinus, signaling a loss of basement membrane integrity. These observations are in agreement with the data from our Transwell assays, showing that VAPB significantly increases invasiveness in MCF10A-HER2 cells and is, at least in-part, mediated through AKT signaling.

Interestingly we observe no significant difference in EGF-induced cell migration between vector and VAPB-expressing cells, suggesting that VAPB has little effect on cell motility. While it is tempting to speculate that VAPB increases

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A

Vector   VAPB

MCF10A-Her2 Cells

B   **C**

MMTV-Neu Tumor Cells
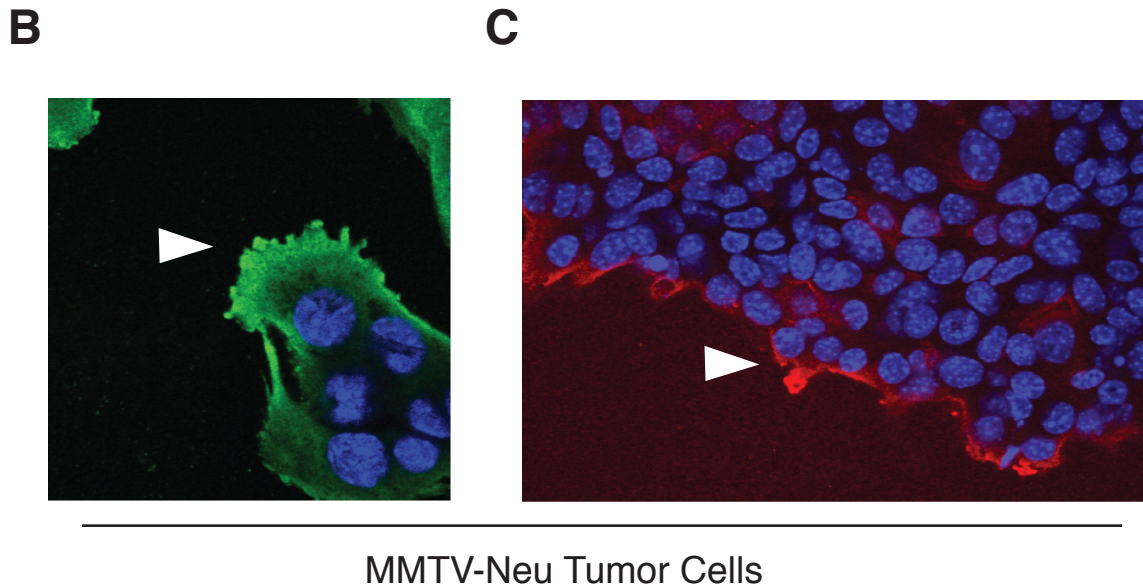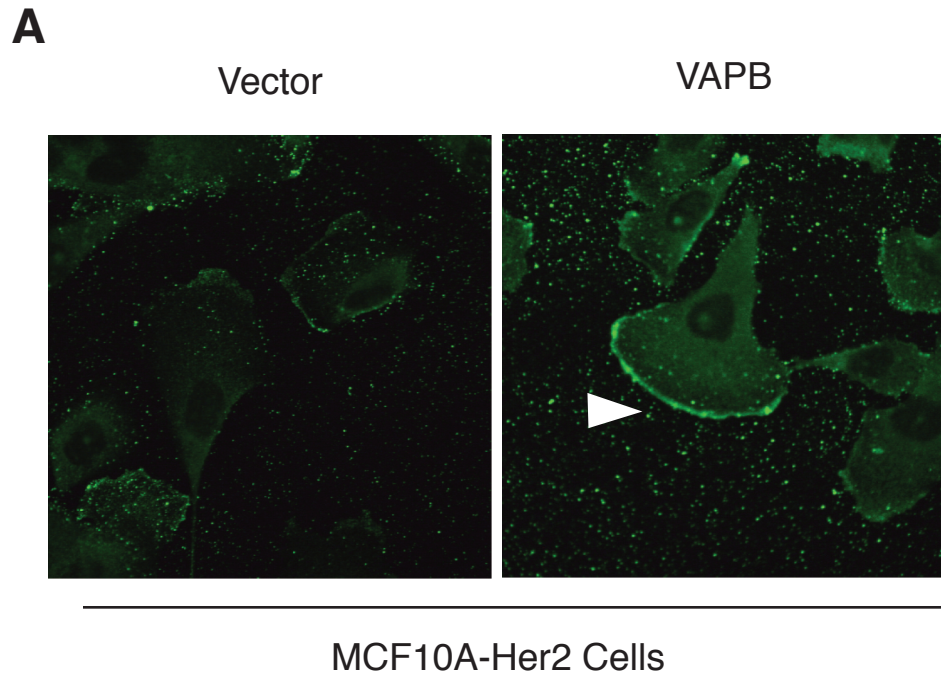
Figure 3.4. VAPB localizes to the leading edge of cells. Immunofluorescence analysis of VAPB protein revealed a distinct staining pattern at the leading edge of randomly migrating (A) MCF10A-HER2 VAPB cells and (B) MMTV-Neu tumor cells (white arrows). (C) MMTV-Neu tumor cells were subjected to a "scratch" assay to ensure directional migration of cells. A similar VAPB staining pattern was observed (white arrows).

invasive potential by inducing epithelial-to-mesenchymal transition (EMT), a process by which polarized cells gain motility, characterized by loss of E-cadherin expression (Thiery, 2002), our data show clear junction staining of E-cadherin with or without VAPB expression. This suggests that downregulation of E-cadherin may not be the primary mechanism promoting invasion in the context of VAPB. However, a more thorough analysis of EMT markers such as Vimentin, Snail and Twist is needed (Thiery, 2002).

Rather than enhanced cell motility as a primary factor in VAPB-induced invasion, another potential mechanism that warrants further investigation is the role of matrix metalloproteinases (MMPs). MMPs are a family of proteins that remodel the ECM through proteolytic degradation its components, including collagens and laminin. MCF10A-HER2 cells secrete specific MMPs such as MMP-2 and MMP-9 that break down ECM components and facilitate invasion (McCawley & Matrisian, 2000). Interestingly it has been shown that AKT activation can increase the expression and activity of both MMP-2 and MMP-9, leading to enhanced invasive capabilities (Jin et al, 2007; Kim et al, 2009; Kwiatkowska et al, 2011). Therefore, future studies are needed to explore the possibility that the invasive phenotypes observed with VAPB expression are dependent on MMPs.

While the potential involvement of MMPs suggests that VAPB promotes the breakdown the basement membrane, it is equally likely that laminin V is being mis-localized. This alternative hypothesis is supported by the fact that there are

centrally-located cells with cytoplasmic localization of laminin V in VAPB-expressing structures (Figure 3.1B and 3.2A). This is also consistent with the fact that VAPB has reported functions in vesicle trafficking [Reviewed in (Lev et al, 2008)].

Many reports suggest that other players work in conjunction with HER2 to promote invasion like TGFβ (Chow et al, 2011; Seton-Rogers & Brugge, 2004), 14-3-3ζ (Lu et al, 2009), EphA2 (Brantley-Sieders et al, 2008) and Integrin β4 (Guo et al, 2006). While the status of these proteins is unknown in VAPB-expressing cells, our observations that VAPB localizes at the leading edge suggests a sub-cellular localization where VAPB can foster interactions with the above-mentioned proteins. These data sparks interesting questions for future studies: (1) Which proteins does VAPB interact with at the leading edge of cells? (2) How do these interactions relate to the invasive potential of tumor cells?

The data presented here also highlight several areas of interest in clinical breast cancer. An important feature of non-invasive DCIS is the structural integrity of the basement membrane, where tumor cells are encapsulated and restricted to the mammary gland. The transition into IDC is defined by basement membrane breakdown and tumor cells invasion into neighboring tissues. The fact that VAPB evokes similar behaviors in breast tumor cells (i.e. loss of basement membrane integrity and increased cell invasion) warrants further investigation of VAPB function during metastatic progression *in vivo*.

CHAPTER IV

VAMP-ASSOCIATED PROTEIN B (VAPB) PROMOTES

BREAST TUMOR GROWTH BY MODULATING THE

PROTEIN SECRETORY PATHWAY

Introduction

Aberrant vesicle trafficking affects pathogenesis of many diseases, including cancer (Aridor & Hannan, 2000). Dysregulated endocytosis of growth factor receptors and cadherin based adhesion complexes often promotes tumor growth (Mosesson et al, 2008). Conversely, the protein secretory pathway is increasingly recognized for its importance in tumor initiation and progression (Chan & Weber, 2002; Palmer et al, 2002; Vadlamudi et al, 2000). Secretion of growth factors, cytokines, matrix metalloproteinases, and delivery of receptors to the cell surface, facilitate tumor growth and invasion (Hanahan & Weinberg, 2011), and all rely on protein trafficking. The dependence of tumor cells on protein and vesicle trafficking provides unique opportunities for cancer therapeutics aimed at mediators of trafficking (Wright, 2008).

Most eukaryotic proteins that are destined for release into the extracellular space or for insertion into the plasma membrane are transported via an ER/Golgi-dependent vesicle secretory pathway. Small GTPases such as Rabs

and Arfs have emerged as key regulators in vesicle trafficking, involved in the multi-step process of vesicle formation, scission, motility, tethering, and fusion as diagrammed in Figure 4.1 (Segev, 2011). Select members of the Arf family function to recruit effector proteins, such as coat proteins, to newly budding vesicles in the ER and Golgi (Gillingham & Munro, 2007), whereas Rab proteins target cargo-loaded vesicles to the correct membrane destination (Stenmark, 2009). Although Arf/Rab proteins can function in both endocytosis and exocytosis, the primary function of Arf1 and Rab1 appears to regulate vesicle-mediated protein secretion (D'Souza-Schorey & Chavrier, 2006; Stenmark, 2009).

Vesicle associated membrane protein associated protein B (VAPB) is a highly conserved type II integral membrane protein that belongs to the VAP protein family (Nishimura et al, 1999; Weir et al, 1998) and primarily localizes to the ER and cis-Golgi (Moumen et al, 2011; Skehel et al, 2000). Although the yeast homolog of VAPB was shown to play critical roles in lipid homeostasis (Kagiwada et al, 1998), multiple studies in higher eukaryotic species demonstrate a function in protein transport along the secretory pathway. The first VAP protein (VAP-33) was proposed to function in neurotransmitter exocytosis (Skehel et al, 1995) and further studies demonstrated the presence of VAP-33 on TGFα containing exocyst vesicles (Cao et al, 2008). The rat homolog of VAP-33, EGR-30, was shown to function in early secretory protein transport mediated by COPI vesicles (Soussan et al, 1999). Despite the importance of vesicle-mediated

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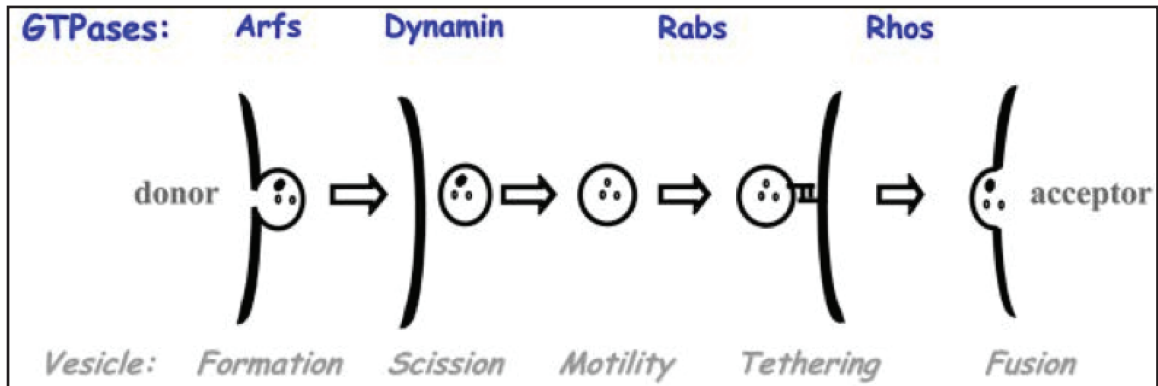
Figure 4.1. A diagram of GTPase families that regulate vesicle trafficking sub-steps. Vesicle formation is regulated by Arfs; vesicle scission by dynamin; vesicle motility by Rabs; vesicle tethering by Rabs and Rhos, and vesicle fusion by Rhos. [Image reproduced from (Segev, 2011)].

protein secretion in tumor growth, the role of VAPB in cancer has not been investigated thus far. However, a genome-wide microarray analysis of 50 human breast cancer cell lines and 145 clinical specimens revealed that VAPB is often amplified or overexpressed in breast cancer (Chin et al, 2006; Neve et al, 2006), suggesting the potential importance of VAPB function in human cancer.

In this report, we tested the potential contribution of protein secretion in VAPB's growth-enhancing effects in breast tumor cells. We found that VAPB regulates breast tumor cell proliferation by modulating vesicle-mediated transport from the ER/Golgi network to the plasma membrane, partly through an Arf1 small GTPase-dependent mechanism. This data highlights the potential for targeting vesicle trafficking as a novel therapeutic approach in cancer.

Materials and Methods

Antibodies and Reagents

Antibodies against the following proteins were used: anti-Arf1 (Arfs1A9/5), and agarose-congugated anti-HA for immunoprecipitation studies (Santa Cruz Biotechnologies), anti-HA (Sigma), anti-MYC, anti-cleaved caspase-3 (Cell Signaling Technologies) and anti-VSVG (8G5F11) (KeraFast). C-terminus tagged VAPB-Myc or Arf1-HA and FLAG-Rab1b was subcloned into pCLSXN_Neo (Addgene) or pCDNA3. 1 (Invitrogen) using cDNAs obtained from the Vanderbilt Microarray Core. Growth factor-reduced Matrigel were purchased from BD

Biosciences. Cell permeable crosslinkers (DSP and DTME) were purchased from Thermo Scientific.

Cell culture

Stable knockdown of VAPB in MMTV-Neu tumor cells was previously described (Chapter II). For re-expression of mutant VAPB, mutant constructs were subcloned into pCLSXN retroviral vector that contains a Neomycin resistance gene, allowing for G418 (300μg/mL for 6 days) selection in puromycin-resistant populations. Viral supernatant was collected as described above from HEK 293T cells that were co-transfected with pCLSXN-Vector (10μg) only or pCLSXN-VAPB (10μg) and pCLAmpho plasmids (10μg) using Lipofectamine 2000.

MCF10A cells were obtained from American Type Culture Collection (ATCC) and cultured as previously described (Debnath et al, 2003). MCF10A-HER2 cells were generated via retroviral transduction of the HER2 proto-oncogene (Ueda et al, 2004). Overexpression of WT or mutant VAPB in MCF10A-HER2 cells was achieved by infecting cells with pLXSN-Vector or -VAPB retrovirus and selected in media containing 300μg/mL G418 for 6 days.

Identification of VAPB-interacting proteins

Proteins in complex with VAPB were identified using ReCLIP (Reversible Cross-Link Immuno-Precipitaiton) technology as previously described (Smith et

al, 2011). Briefly, to preserve physiological complexes, MMTV-Neu Vector or VAPB knockdown cells (KD #1) were treated with membrane permeable cross-linkers (DSP and DTME) prior to lysis. DSP and DTME were first dissolved in DMSO (20nM) and then diluted in PBS (2mM). DSP and DTME were mixed in a 1:1 ratio, with a final concentration of 0.4mM in PBS. Cells were washed twice with PBS and incubated with the cross-linker mixture for 30 minutes at room temperature. The reaction was quenched for 10 minutes and cells were subsequently lysed and subjected to co-immunoprecipitation with anti-VAPB conjugated Protein G Dynabeads (Invitrogen). VAPB and its binding partners were eluted by incubating the beads at 37°C for 30 minutes in the presence of 100mM DTT. For mass spectrometry analysis, the eluates were boiled for 5 minutes in freshly prepared Laemmli sample buffer. Eluted and boiled proteins from MMTV-Neu vector and VAPB knockdown samples were trichloroacetic acid -precipitated and processed for shotgun analysis using Multidimensional Protein Identification Technology (MudPIT). Mass spectra data was analyzed using IDPICKER [2] software, an open-source protein assembly tool that derives a minimum protein list from peptide identifications filtered to a specified False Discovery Rate of less that 5%. This service was provided by the Mass Spectrometry Research Center at Vanderbilt University.

VSVG-GFP ts045 trafficking assays

MCF10A-HER2 cells were transfected with VSV-G-GFP ts045 (generous gift from Dr. Jennifer Lippencott-Swartz) using Lipofectamine 2000. After 3 hours, transfection media was replaced with growth media and immediately incubated at 40°C for an additional 16 hours. Cells were then treated with 100ug/mL cyclohexamide (Sigma-Aldrich) for 30 minutes prior to shift to 32°C at the indicated time points. For immunofluorescence analysis of VSVG-GFP, cells were plated on glass cover slips were rinsed in PBS, then fixed without permeabilization in 2% PFA for 15 minutes at room temperature and probed with ectodomain- specific anti-VSVG (1:1000).

For biochemical analysis of surface VSVG-GFP, cells were labeled with Sulfo- NHS-LC-Biotin (Thermo Scientific) and processed as previously described [3]. Surface labeled proteins were pulled down using Streptavidin-agarose resin (Thermo Scientific) and subjected to western blot analysis. Cell surface and total VSVG protein was probed with monoclonal anti-VSVG (1:1000) and detected with anti-mouse HRP. Densitometry analysis for band intensity was performed using NIH Image J software. Cell surface VSVG was calculated as a percent of total VSVG. Data are derived from two independent experiments.

Immunoblot, co-immunoprecipitation, and Arf1 pull-down assays

For immunoblot analysis of VAPB knockdown or overexpression, cells were washed with PBS and lysed in Radioimmunoprecipitation Assay (RIPA)

buffer (50mM Tris pH 7.4, 150mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitor tablets (Roche). Approximately 30-50ug of total cell lysate was separated by SDS-PAGE and probed with anti-VAPB (1:1000) overnight in 5% nonfat dry milk/ TBS-Tween (0.05%) and detected with anti-goat HRP (1:1000). VAPB and Arf1 co-immunoprecipitation in HEK 293T cells was performed with the addition of 2mM DSP (dithiobis (succinimidyl propionate), a reversible chemical cross linker and processed as previously described (Amarilio et al, 2005).

For Arf1 activity assay, 2×10^6 MCF10A-HER2 vector or VAPB-expressing cells were plated into 10 cm dishes and serum-starved for 6 hours. Cells were briefly rinsed once with PBS and then lysed in ice-cold lysis buffer. Samples were briefly vortexed, incubated for 15 minutes on ice, then centrifuged at 16,000 x g for 15 min at 4°C. GST-GGA3-PBD coupled to glutathione-Sepharose 4B was added to each tube, and the samples were rotated at 4 °C overnight. Proteins were eluted in 50 µl of SDS-sample buffer and boiled for 5 min. Proteins were separated on a 12% SDS-PAGE gel. Detection of Arf1-GTP was performed with immunoblot analysis using anti-Arf1. Densitometry analysis of percent Arf1-GTP was calculated using NIH Image J software.

Results

Functional diversity of candidate VAPB-interacting proteins

Many of VAPB's proposed cellular functions are in relation to VAPB-interacting proteins as summarized in Table 1. To gain molecular insights on how VAPB functions in breast tumor cells, we utilized the ReCLIP-coupled mass spectrometry-based proteomics approach (Multidimensional Protein Identification Technology) to identify novel VAPB interacting proteins. This approach not only preserves labile protein complexes but also greatly reduces background prior to proteomics analysis (Smith et al, 2011). Figure 4.2A is the western analysis of VAPB immunoprecipitation that was used for mass spectrometry.

Using PANTHER (Mi & Thomas, 2009) we discovered functional diversity among the 170 candidate VAPB-interacting proteins (Figure 4.2B) with 11 distinct biological processes including protein modification, carbohydrate metabolism, and lipid transport. These proteins were selected based on a greater than four-fold enrichment in the vector control cells relative to VAPB knockdown cells. These proteins include previously reported VAPB-interacting proteins, such as membrane associated phosphatidylinositol transfer protein 1 (Nir2) (Amarilio et al., 2005) and Oxysterol binding protein 1 (OSBP) (Wyles et al., 2002) (Table 3), thus validating the approach. We identified several biological processes including vesicle trafficking, protein modification, and cell-cell/matrix adhesion. Additionally,

A

B

Biological Process

- Protein metabolism and modification
- Intracellular protein transport
- Carbohydrate metabolic process
- Lipid metabolism
- Signal Transduction
- Lipid transport
- Cell Motion
- Apoptosis
- Cell cyle
- Nucleoside, nucleotide, and nucleic acid metabolism
- Other
- Unclassified
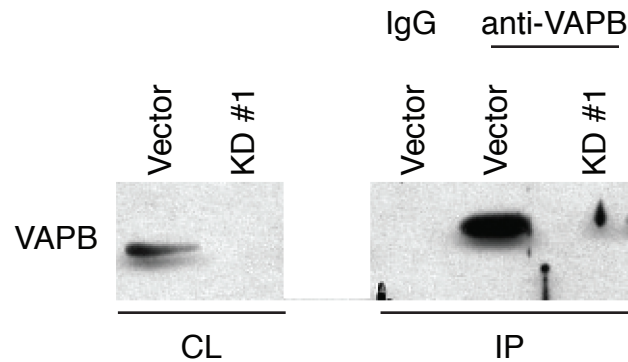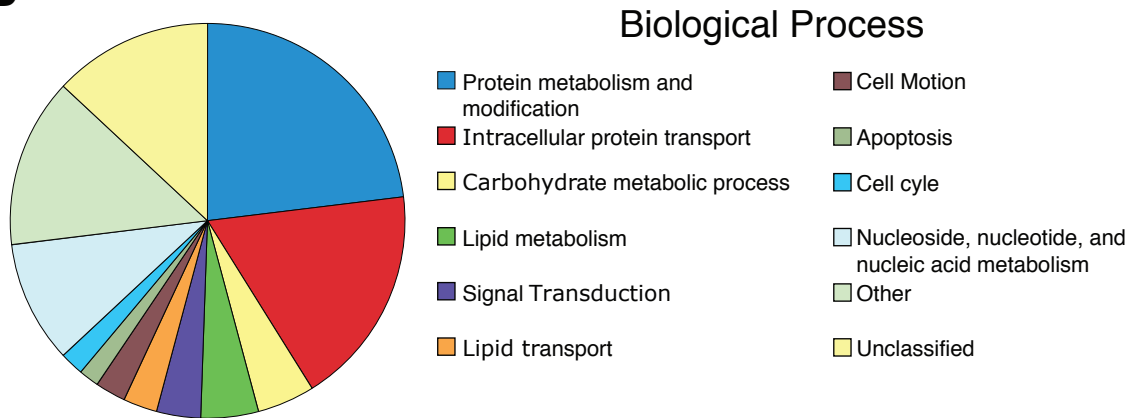
Figure 4.2. Functional diversity of candidate VAPB-binding proteins.
MMTV-Neu control or VAPB knockdown cells were treated with chemical cross-linkers prior to lysis. (A) MMTV-Neu tumor cell lysates were immunoprecipitated with anti-VAPB conjugated beads and the resulting protein complexes were subjected to mass spectrometry analysis. The following criteria were used for selection of candidate proteins: (1) spectral counts ≥ 5 and (2) the ratio of vector/ knockdown ≥ 4. (B) Candidate proteins were classified based on biological processes as annotated in PANTHER (Mi & Thomas, 2009). Selected functional groups and proteins are listed in Table 3.

Table 3. Selected functional groups of candidate VAPB-interacting proteins

Ras Family of Small GTPases	Vector	KD #1
ADP-ribosylation factor 1;Arf1	22	5
ADP-ribosylation factor 3;Arf3	22	5
Ras-related protein Rab-7a;Rab7a	21	2
ADP-ribosylation factor 5;Arf5	19	2
Ras-related protein Rab-18;Rab18	18	1
Ras-related protein Rab-1A;Rab1A	12	3
Ras-related protein Rab-5C;Rab5c	11	2
Ras-related protein Rab-1B;Rab1b	10	2
Ras-related protein Rab-5A;Rab5a	9	2
Ras-related protein Rab-5B;Rab5b	8	1
Ras-related protein Rab-8A;Rab8a	7	1
Ras-related protein Rap-1b;Rap1b	6	1
Ras-related protein Rap-1A;Rap1a	6	1

Vesicle Trafficking Related Proteins	Vector	KD #1
VAMP-associated protein B;Vapb	127	31
VAMP-associated protein A;Vapa	39	1
Vesicle-trafficking protein SEC22b;Sec22b	12	1
Rab GDP dissociation inhibitor beta;Gdi2	10	0
ADP-ribosylation factor-like protein 1;Arl1	6	0
Sec1 family domain-containing protein 1;Scfd1	5	0

Lipid Transport	Vector	KD #1
Oxysterol-binding protein 1;Osbp	19	3
1-acylglycerophosphocholine O-acyltransferase 1;Lpcat1	13	3
Membrane-associated phosphatidylinositol transfer protein 1;Pitpnm1 (Nir2)	9	0
Phosphatidylinositol transfer protein beta isoform;Pitpnb	8	1
Long-chain fatty acid transport protein 4;Slc27a4	6	0

Protein Modification	Vector	KD #1
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2;Rpn2	26	17
Protein disulfide-isomerase A6;Pdia6	24	6
E3 ubiquitin-protein ligase NEDD4;Nedd4	18	2
UDP-glucose:glycoprotein glucosyltransferase 1;Ugcgl1	18	2
Phosphatidylinositide phosphatase SAC1;Sacm1l	14	2
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit;Ddost	10	2
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A;Stt3a	9	8
Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform;Ppp3ca	5	0
Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform;Ppp2cb	5	0
Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform;Ppp2ca	5	0
Serine/threonine-protein kinase 3;Stk3	5	1
Glycylpeptide N-tetradecanoyltransferase 1;Nmt1	5	0

Cell-cell or Cell-Matrix Adhesion and Motility	Vector	KD #1
Profilin-1;Pfn1	24	4
Integrin beta-4;Itgb4	12	0
Ezrin;Ezr	9	1
Inverted formin-2;Inf2	8	2
Integrin beta-1;Itgb1	6	0

analysis revealed that VAPB interacts with a group of small GTPases, including Arf1, Arf3, Arf5, Rab1, and Rab8 proteins (Table 3).

VAPB facilitates the transport of proteins to cell surface

Because the primary functions of Arf1/3 (Spang, 2002; Stearns et al, 1990) and Rab1/8 (Huber et al, 1993; Plutner et al, 1991) small GTPases are to regulate vesicle trafficking in the secretory pathway, we assessed whether VAPB regulates vesicle-mediated protein transport from the ER/Golgi to the plasma membrane. Control or VAPB expressing MCF10A-HER2 cells were transfected with a temperature sensitive mutant of vesicular stomatitis viral protein G (ts045-VSVG)-GFP (Presley et al, 1997), commonly used to visualize secretory protein transport (Griffiths et al, 1985). At higher temperatures (40°C), ts045-VSVG-GFP mis-folds and is retained in the ER. Upon temperature reduction (32°C), VSVG-GFP folds rapidly and is transported from the ER/Golgi network to plasma membrane. As shown in Figure 4.2, MCF10A-HER2 cells that overexpress VAPB displayed enhanced VSVG-GFP trafficking to the plasma membrane. By 30 minutes MCF10A-HER2-VAPB cells display VSVG protein at the cell surface (red) as indicated using an antibody that specifically recognizes extracellular VSVG (Figure 4.3A). Surface biotinylation assays revealed a significant increase in amount of VSVG protein at the cell surface in VAPB overexpressing cells compared to vector control cells (Figure 4.3B). Taken together these data

Figure 4.3. VAPB facilitates the transport of secretory proteins to cell surface. (A) MCF10A-HER2-VAPB expressing cells were transfected with the ts045 temperature sensitive vesicular stomatitis viral glycoprotein (VSVG) GFP and incubated at 40°C for 16 hours to accumulate misfolded VSVG protein in the ER. Following a 30-minute incubation with cyclohexamide, the cells were shifted to a permissive temperature (32°C) to allow transport along the secretory pathway. Total VSVG was visualized by GFP fluorescence (green) and cell surface VSVG was detected using an antibody against VSVG ectodomain (red) under non-permeable condition. (B) The kinetics of appearance of VSVG-GFP at the cell surface was measured by cell-surface biotinylation and subsequent quantification of immnoblots with anti-VSVG in two independent experiments ($p < 0.05$, unpaired t test).

A

Vector — GFP, anti-VSVG (Ectodomain), Merge

VAPB — GFP, anti-VSVG (Ectodomain), Merge

0 min, 15 min, 30 min

B

Surface VSVG (Percent of Total) vs Minutes

Vector, VAPB

$p < 0.05$
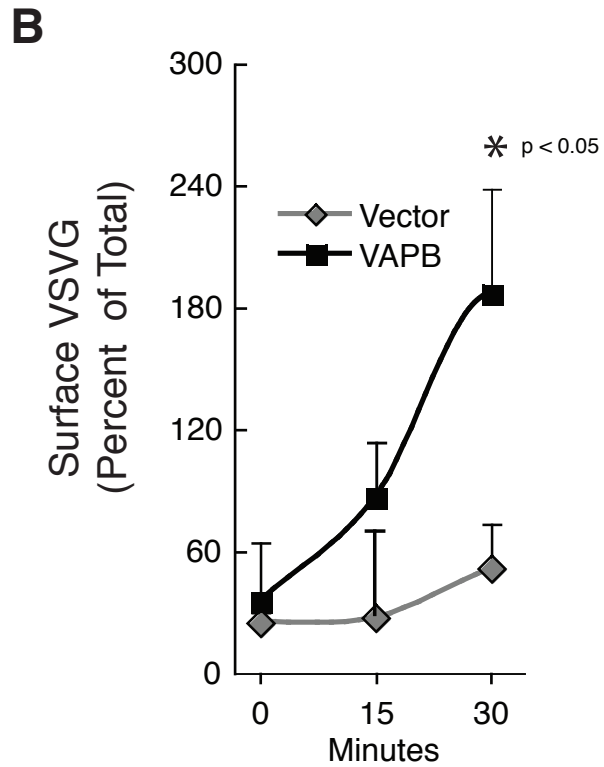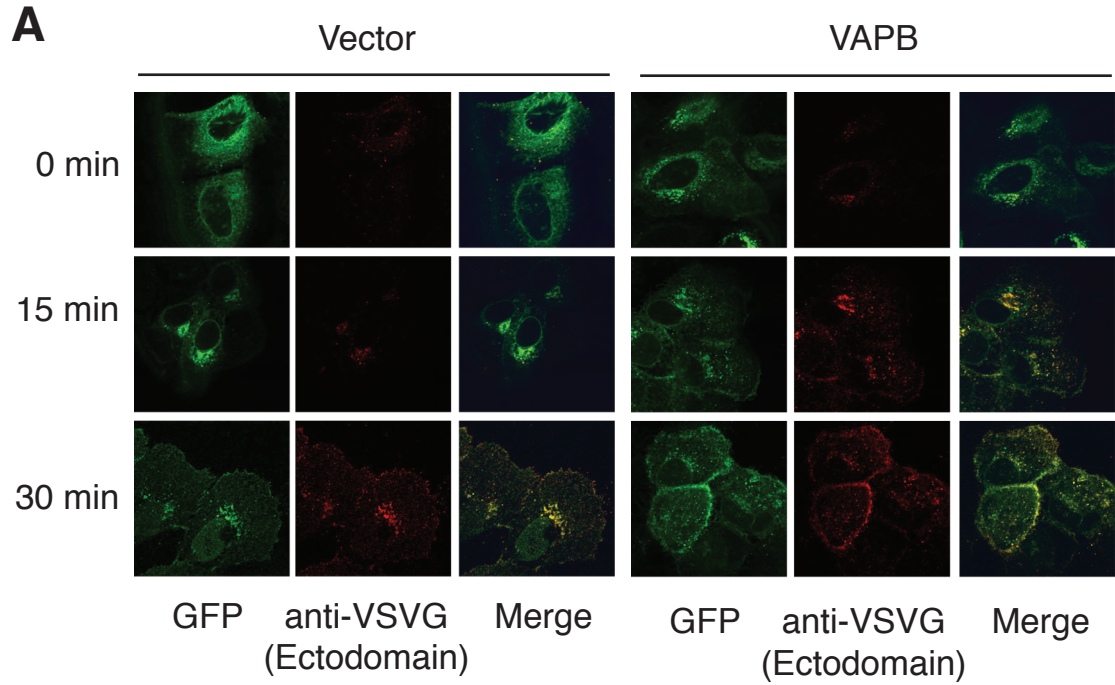
indicate that VAPB expression mediates protein transport to the plasma membrane.

VAPB-dependent tumor growth is partly mediated by Arf1 small GTPase.

To dissect molecular mechanisms by which VAPB regulates vesicle-mediated protein secretion in tumor cells, we focused on small GTPase Arf1 because of Arf1's well-recognized function in secretory transport (Spang, 2002). To test physical interaction between VAPB and Arf1, we transfected 293T cells with Myc-tagged VAPB and HA-tagged Arf1 and immunoprecipitated cell lysates with anti-Myc-conjugated beads. As shown in Figure 4.4A, the Arf1 protein was readily detected in anti-Myc immunoprecipitates. The co-immunoprecipitation of Arf1 with VAPB was dependent on the expression of both VAPB and Arf1, was reduced markedly in cells transfected with Arf1 only, and was undetectable in control cells (left panel). Conversely, VAPB was also readily detected in anti-HA immunoprecipitates in cells transfected with both VAPB and Arf1, but not in those transfected with VAPB alone or control cells (right panel).

Next, we determined whether Arf1 activity is affected by VAPB expression. Similar to most small GTPases, Arf1 cycles between a GTP-bound "active" state and GDP-bound "inactive state. Active Arf1 has several downstream effectors, including coatamer proteins that facilitate budding of transport vesicles. Using a GST fusion protein of the GGA3, a known Arf-GTP binding protein, we were able to "pull-down" activated Arf1-GTP in MCF10-HER2 cells. Overexpression of
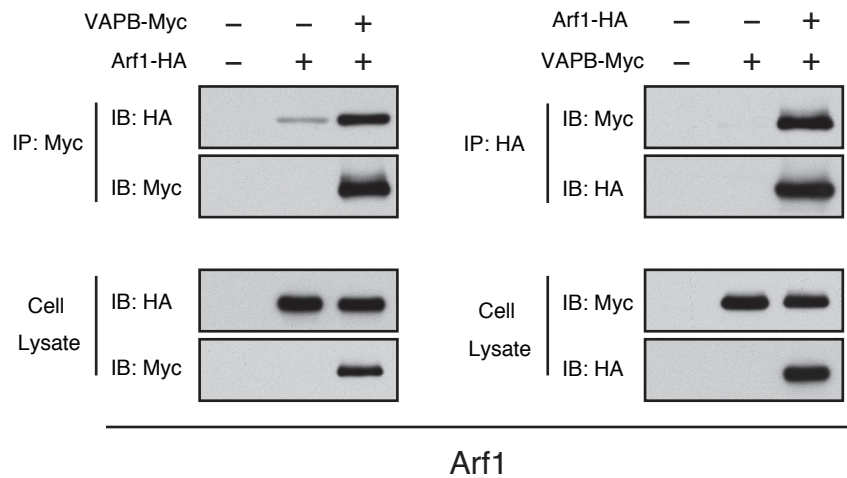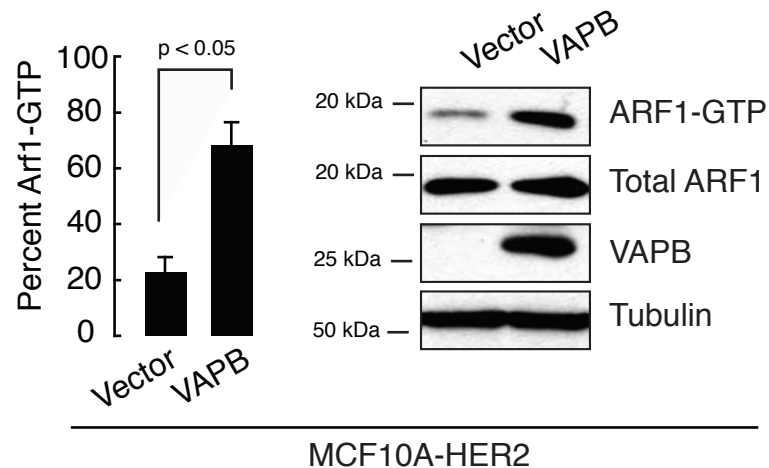
Figure 4.4. VAPB physically interacts with Arf1 GTPase and increases Arf1 activity. (A) HEK 293T cells were transfected with VAPB-Myc and/ or Arf1-HA expression constructs. VAPB or Arf1 was immunoprecipitated from cell lysates and proteins were analyzed for Arf1 or VAPB, respectively. (B) Arf1-GTP was measured by an effector pull-down assay using GST-GGA3-PBD glutathione sepharose beads. Elevated Arf1-GTP level was observed in MCF10A-HER2 cells overexpressing VAPB relative to vector control cells. Two independent experiments were performed ($p < 0.05$, t test).

VAPB was sufficient to increase Arf1-GTP levels (Figure 4.4B), suggesting that VAPB regulates Arf1 activity.

To investigate whether Arf1 is involved in VAPB-mediated protein secretion and tumor cell growth, we used an Arf inhibitor, Brefeldin A (BFA). BFA is a fungal metabolite that inhibits Arf guanine nucleotide exchange factors and prevents Arf-GTP formation (Donaldson et al, 1992). It is also well documented that BFA-treated cells reversibly inhibits secretory protein transport (Klausner et al, 1992). Accordingly, we investigated the effects of BFA treatment on spheroid growth in VAPB expressing cells. Two days after plating cells in 3D Matrigel culture, MMTV-Neu tumor cells or MCF10A-HER2 Vector or VAPB expressing cells were treated with vehicle or BFA. As expected, short-term treatment of cells with BFA significantly inhibited Arf1-GTP levels in MMTV-Neu cells (Figure 4.5A) and MCF10A-HER2 cells (Figure 4.5D). BFA treatment also significantly decreased spheroid size in both MMTV-Neu (Figure 4.5B) and MCF10A-HER2 VAPB (Figure 4.5E) expressing cells and cell proliferation as quantified by Ki67 staining at day 5 (Figure 4.5C and 4.5F). These results suggest that VAPB-dependent tumor growth is mediated partly by Arf1 small GTPase.

Discussion

VAPB was originally identified as one of the vesicle-associated membrane protein (VAMP) associated proteins, which functions in a wide range of cellular processes. In mammalian cells, VAPB appears to be involved in lipid transfer and

Figure 4.5. Arf inhibitor Brefeldin A reduces spheroid size in VAPB expressing tumor cells. MMTV-Neu and MCF10A-HER2 cells grown in 3D Matrigel were treated with BFA for 9 days at the concentration of 500nM and 125nM, respectively. Arf1-GTP levels were measured by GST-GGA3-PBD pull-down assay (A&D). Tumor cell spheroid size was quantified using Image J. ($p < 0.05$; single factor ANOVA) (B&E). Tumor cell proliferation was measured by immunofluorescence using an antibody against PCNA, a cell proliferation marker (C&F). Percentage of PCNA positive nuclei (red) was quantified at day 5 in MMTV-Neu cells (C) and MCF10A-Her2 cells (F).

A

BFA

— +

ARF1-GTP

Total ARF1

B

Average Spheroid (Pixel Area x 100)/ 20x Field

p < 0.05

BFA (125nM)   —   +

C

% PCNA Positive Nuclei/ spheroid

p < 0.05

BFA (125nM)   —   +

Control

BFA 125

MMTV-Neu Tumor Cells

D

BFA

— +

ARF1-GTP

Total ARF1

E

Average Spheroid (Pixel Area x 100)/ 20x Field

p < 0.05
*

BFA (25nM)   Vector —   VAPB —   VAPB +

F

% PCNA Positive Nuclei/ spheroid

p < 0.05
*

BFA (25nM)   Vector —   VAPB —   VAPB +

Vector Control   VAPB Control   VAPB BFA 25nM

MCF10A-HER2 Cells

88
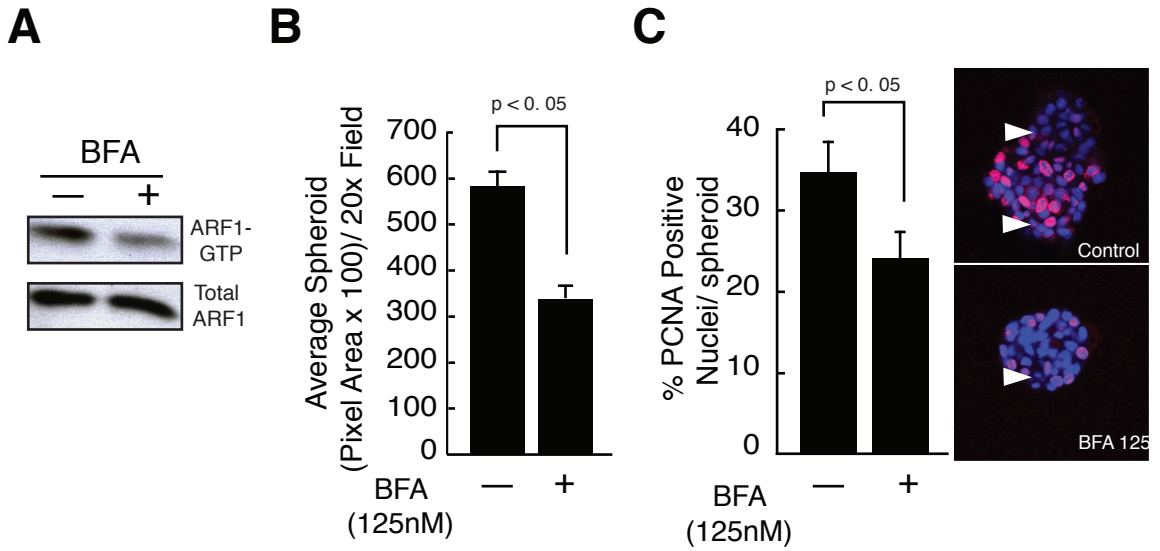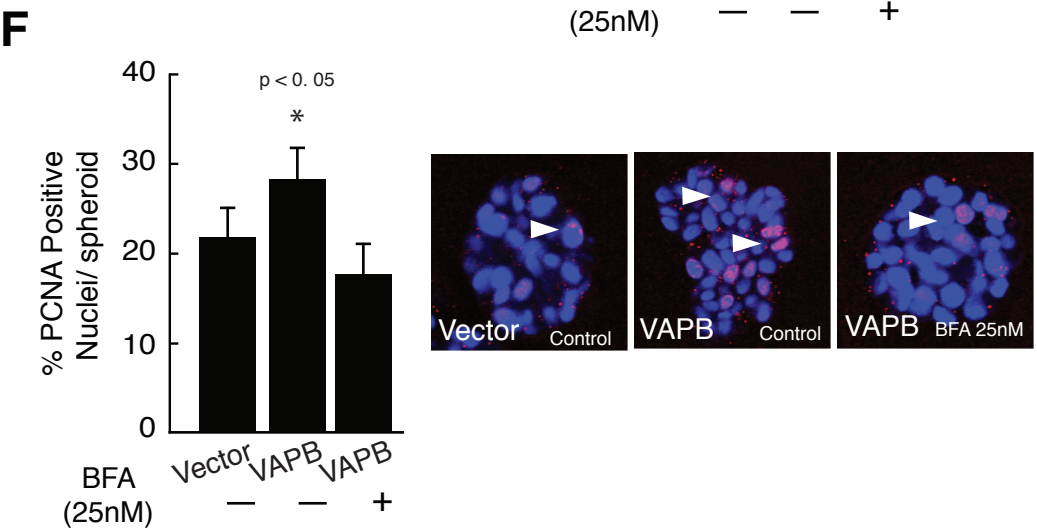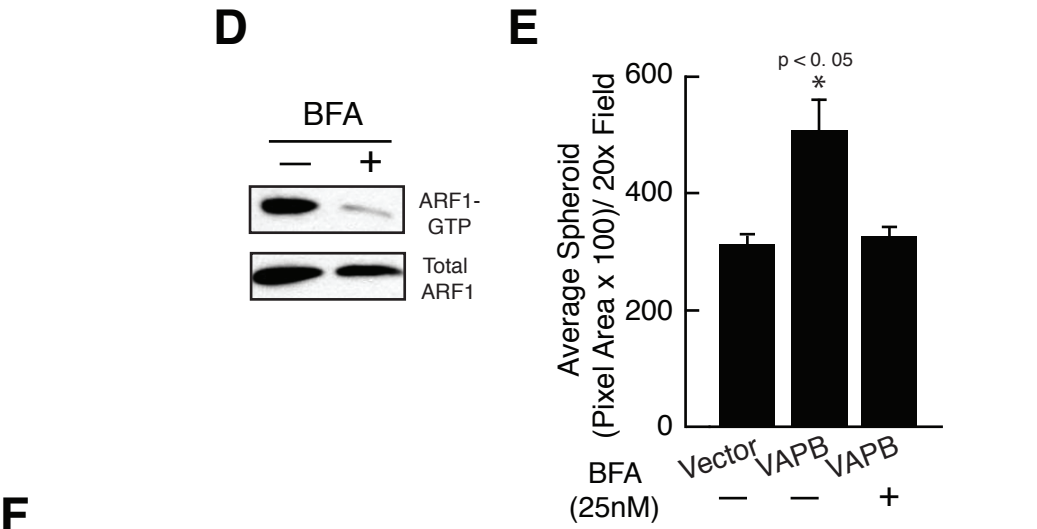
vesicle trafficking (Amarilio et al, 2005; Lev et al, 2008; Peretti et al, 2008). Increased copy numbers and expression levels of the VAPB gene were reported in human breast cancer cells and clinical tumor specimens (Arriola et al, 2008; Chin et al, 2006; Neve et al, 2006). However, VAPB function in tumor cells is poorly understood. In this study, we showed that VAPB regulates tumor cell growth in both cell culture and animal models (Chapter II). Moreover, our data indicate that one mechanism leading to the phenotype is modulation of Arf1 small GTPase-dependent vesicle-mediated protein secretion by VAPB. Collectively, the analyses provide genetic, functional and mechanistic evidence that VAPB promotes tumor cell proliferation by regulating vesicle-mediated protein traffic from ER-Golgi to plasma membrane.

Studies in *S. cerevisiae* have established the importance of Arf1 in secretory membrane transport (Stearns et al, 1990). Subsequent studies showed that Arf1 GTPase regulates coat assembly during vesicle formation, both from the Golgi to the ER and between Golgi cisternae (D'Souza-Schorey & Chavrier, 2006; Gillingham & Munro, 2007; Spang, 2002). Several lines of evidence suggest that one mechanism by which VAPB regulates vesicle-dependent protein secretion is through modulating Arf1 activity. First, Arf1 can physically interact with VAPB and regulate the levels of Arf1-GTP, which serves to recruit effectors, such as coatamer protein I, AP1 and GGAs to the Golgi and mediate vesicle formation (Spang, 2002). In addition, Brefeldin A, which inhibits the formation of Arf-GTP and secretory protein transport, inhibited tumor cell

proliferation in 3-dimentional cell culture. It is also possible that VAPB regulates more than one of these small GTPases. We found that VAPB was also able to interact with Rab1b in HEK 293T cells, another mediator of secretory vesicle formation (Dugan et al, 1995) (Figure 4.6). Aside from Arf1 and Rab1b, Mass Spectrometry analysis also identified Arf3, Arf5, and Rab8 small GTPases as potential VAPB-interact partners, all of which have proposed functions secretory vesicle traffic. (Bonifacino & Glick, 2004; D'Souza-Schorey & Chavrier, 2006; Huber et al, 1993; Plutner et al, 1991). Given that tumor cells heavily rely on growth factor secretion and receptor localization to the plasma membrane the functional significance of these interactions needs further investigation.

While, our results suggest that VAPB enhances breast tumor cell proliferation through enhanced secretory vesicle trafficking, we cannot rule out the possibility that aberrant VAPB expression dysregulates other vesicle trafficking events such as endocytosis. From our screen, we also identified Rab5 (Table 3), a small GTPase enriched on early endosomes (EE) which are focal points of the endocytic pathway [Reviewed in (Jovic et al, 2010)]. Rab5 was also found to regulate endocytosis and recycling of β-integrins (Pellinen et al, 2006). Interestingly, we identified Integrinβ1 and Integrinβ4 as a candidate VAPB interacting proteins (Table 3). The fact that VAPB also localizes plasma membrane (Figure 3.5), where endocytosis is often initiated, only strengthens the rationale for investigating the functional significance VAPB-Rab5 or VAPB-β-Integrin interactions in breast tumor proliferation and invasion.
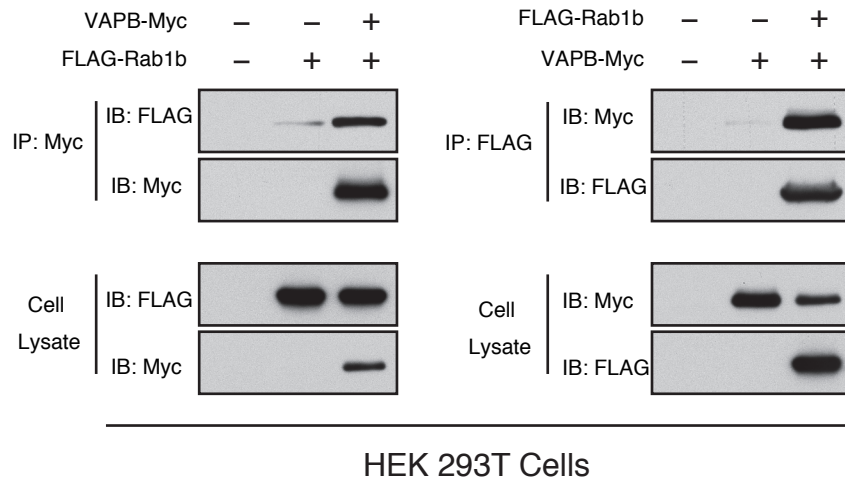
Figure 4.6. VAPB interacts with Rab1 in HEK 293 cells. HEK 293T cells were transfected with VAPB-Myc and/ or FLAG-Rab1 expression constructs. VAPB or Rab1 was immunoprecipitated from cell lysates and the resulting protein complexes were analyzed by western blot for Rab1 or VAPB, respectively. VAPB physically associated with Rab1 when both VAPB and Rab1 were expressed in 293 cells.

Our previous studies demonstrated a functional role of VAPB in promoting tumor cell proliferation in breast cancer (Chapter II). Moreover we have identified various VAPB interacting proteins of diverse function. It is tempting to speculate that VAPB may represent a common mechanism by which many pathways including secretory protein transport converge to promote tumor growth. This discovery opens up a number of exciting avenues for future studies of VAPB in cancer. Additionally targeting the VAPB-associated protein secretory pathway may provide novel targets for future pharmacological strategies in cancer therapy.

CHAPTER V

THE ROLE OF

VAMP-ASSOCIATED PROTEIN B (VAPB)

IN TUMOR GROWTH, *IN VIVO*

Introduction

Analysis of human breast cancer dataset and tissue microarray indicates that VAPB is often overexpressed in tumors and that high VAPB-expression is associated with poor patient survival (Figure 1.2). Consistent with our data, several other laboratories also demonstrated VAPB gene amplification in patient breast cancer samples (Chin et al, 2006; Neve et al, 2006). VAPB expression is also elevated in a breast cancer cell culture model of pre-invasive to invasive transition (Rizki et al, 2008). This evidence highlights the clinical relevance of VAPB in human breast cancer.

Although our previous study shows that VAPB enhances tumor growth *in vitro* (Chapter II)*,* functional validation of VAPB in a mouse model of breast cancer is needed. At present VAPB animal models are limited to the understanding of neurodegenerative disorders. Tudor et al., generated two transgenic mouse lines that express WT-VAPB or P56S-VAPB specifically in motor neurons. Analyses of both sets of mice revealed no overt motor or behavioral dysfunction (Tudor et al, 2010). However the mutant mice developed early signs of neuronal abnormalities associated with ALS, marked by P56S-

VAPB protein aggregates similar to results found in cell culture (Tudor et al, 2010).

To investigate the consequences of VAPB deficiency in transgenic models of breast cancer, we crossed VAPB knockout/LacZ knockin (VAPB KO) mice with the MMTV-NeuT mouse-model of spontaneous mammary tumor formation. In this model, the MMTV-LTR drives the expression of activated *Neu*, the rat homolog of ErbB2 with a single amino acid substitution, specifically in the mammary gland epithelium (Muller et al, 1988). This model recapitulates multi-step tumor progression similar to that observed in human breast cancer. Given that VAPB deficiency reduces growth in MMTV-Neu tumor cells, we anticipate that VAPB deficiency will impair tumor growth in MMTV-NeuT/VAPB KO mice. Indeed our supporting data will show decreased tumor number and tumor burden in VAPB KO animals, highlighting the importance of VAPB in breast cancer.

Materials and Methods

Mice and *in vivo* tumor studies

All animals were housed under pathogen-free conditions, and experiments were performed in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval. VAPB knockout/ LacZ knock-in mice were generated by ingenious Targeting Laboratories (Ronkonkoma, NY). The VAPB allele was disrupted with the insertion of a lacZ reporter gene in. VAPB KO/ LacZ knockin clones (129/ SvEv

ES cells) were selected and injected into C57BL/6 mouse blastocysts. 129/ Bl6-hybrind, VAPB KO mice were backcrossed with FVB animals for three generations prior to crossing with MMTV-NeuT mice (generous gift from Dr. Guido Fiorni), inbred in an FVB background. VAPB WT or KO mice were identified by PCR analysis of genomic DNA isolated from ear digests using the following primers: WT (forward) - TCG AGC CAC AAC ACG AGC TCA - 3′; KO (forward) - GCA TAA GCT TGG ATC CGT TCT TCG GAC - 3′; WT or KO (reverse) 5′ - AGT GTG CAC GGG CTT CGT GG - 3′. The *NeuT* transgene was detected by PCR using primers (forward 5′ - CCC CGG GAG TAT GTG AGT GA - 3′; reverse 5′ - TGA GCT GTT TTG AGG CTG ACA - 3′) under conditions recommended by Jackson Laboratories (Bar Harbor, ME). Littermate NeuT-positive VAPB WT and KO mice were sacrificed at 5 months for tumor analysis. Tumors were collected, enumerated and dimensions were measured by caliper. Tumor volume was calculated as previously described (Brantley-Sieders et al, 2008).

Histological analysis

Mammary glands were harvested and fixed in 10% NBF. Whole-mount H&E analysis of mammary glands was performed as previously described (Brantley-Sieders et al, 2008). Whole-mount hematoxylin staining of mammary glands was performed by fixing the number 4 inguinal mammary glands in 10% buffered formalin (Fisher, SF93-4) overnight at 4°C. The glands were incuvated in acetone in order to dissolve the fat, equilibrated into 100% ethanol, and stained in Mayer's hematoxylin solution (VWR Scientific, West Chester, PA) for

one hour at room temperature, light protected. Glands were de-stained in tap water, followed by 50% ethanol acidified with hydrochloric acid (0.05M). The glands were then dehydrated in a graded ethanol series followed by xylenes, and mounted on slides for photodocumentation. Branch points were quantified as previously described (Vaught et al, 2009)

<u>Western analysis of mammary glands and tumors</u>

Harvested mammary glands or tumors were mechanically homogenized in Radioimmunoprecipitation Assay (RIPA) buffer (50mM Tris pH 7.4, 150mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitor tablets (Roche). Protein were resolved on SDS page gel and analyzed by western blot for VAPB, actin, or ErbB2 (Neu).

Results

VAPB deficient mice exhibit normal mammary gland architecture.

Our own studies revealed that VAPB promotes tumor cell proliferation in cell culture (Chapter II). However whether these phenotypes are reproducible in an animal model of breast cancer remain unknown. Therefore we generated (inGenious Targeting Laboratory) VAPB KO mice in order to test the function of VAPB in tumor cell growth *in vivo.* The targeting strategy and confirmation of genotype by southern blot are shown in Figure 5.1A and B. After backcrossing mice into the FVB background, we confirmed VAPB deficiency by western
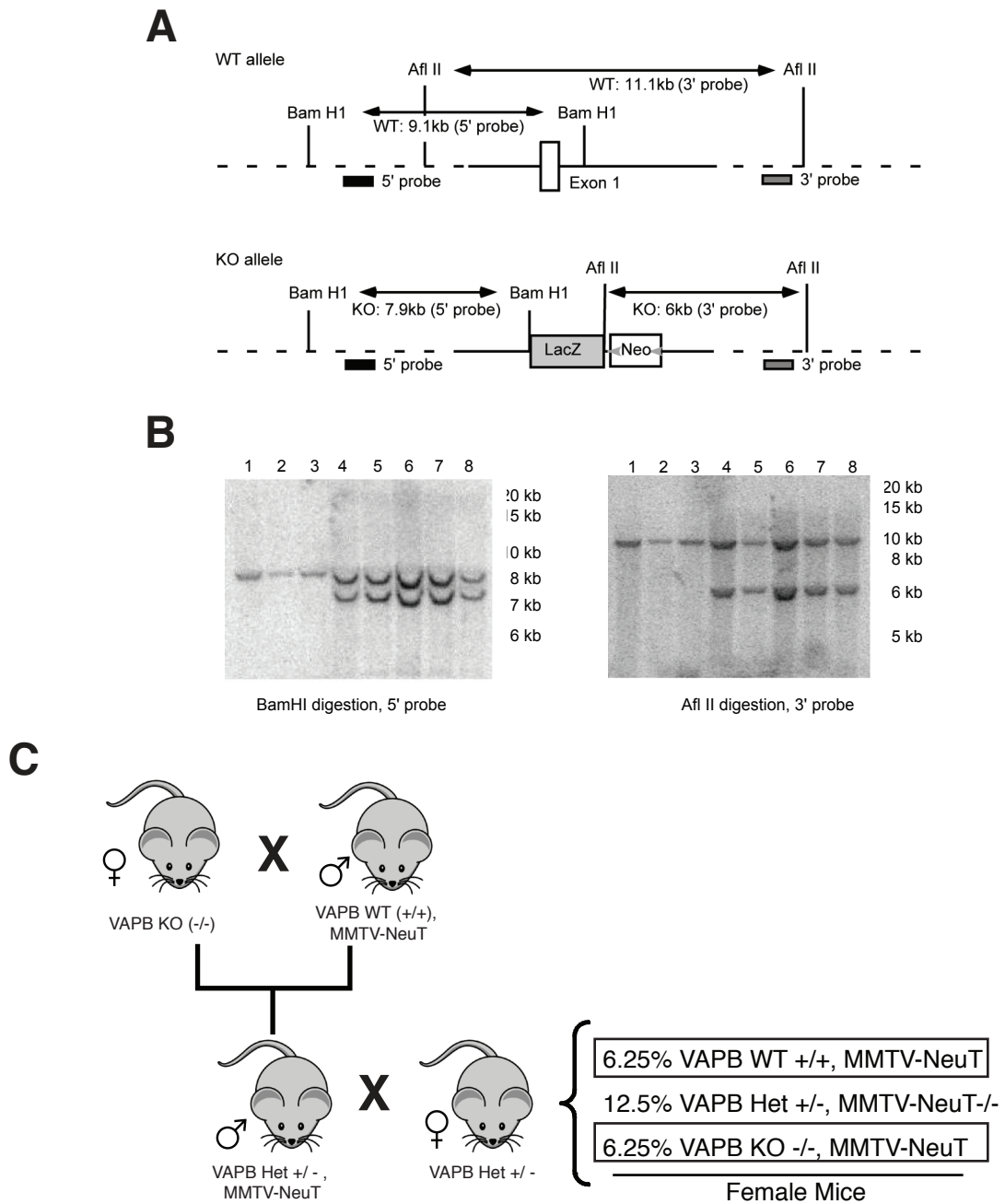
Figure 5.1. Generation of VAPB knockout/ LacZ knockin mice. (A) Targeting strategy. (B) Southern blotting analysis to reconfirm positive clones first identified by PCR in ES cells. Lanes 1-3 is DNA from C57Bl(6), 129/ SzEv, and B6 X 129 hybrid, respectively. (C) Breeding strategy to generate VAPB WT or VAPB KO mice that are positive for the NeuT transgene.

analysis. As expected VAPB protein was undetectable from whole mammary gland lysates from VAPB KO mice when compared with littermate WT controls (Figure 5.2 A).

To determine whether VAPB deletion affects mammary gland architecture, we performed whole mount analysis of WT and VAPB KO mammary glands at 8 weeks of age. We observed no gross morphological changes in the outgrowth of epithelial cells into the fat pad (Figure 5.2B) and in number of epithelial branch points as quantified in Figure 5.2 C. These results suggest that VAPB deficiency is not critical to mammary gland development under these conditions.

Reduced tumor number in MMTV/NeuT VAPB KO mice

To investigate the influence of VAPB expression on mammary tumor formation, we crossed VAPB knockout mice (FVB background) with MMTV-NeuT transgenic mice as diagrammed in Figure 5.1C. In this model, MMTV-LTR drives the expression *Neu,* the rat homolog of ErbB2 (HER2) along containing an activating mutation, specifically in the mammary gland. In this model, virgin, female mice develop tumors between 4-5 months of age (Muller et al, 1988). At five months, tumors that were harvested from MMTV-NeuT-positive, VAPB WT or KO mice were enumerated and scored for size. Not only do we see a significant decrease in the number of tumors formed per mouse (Figure 5.3A), we also observed a reduction in overall tumor burden in VAPB KO mice relative to WT controls (Figure 5.3 B). Western analysis of tumor lysates show that VAPB depletion in KO tumors, however protein expression levels of ErbB2 were not
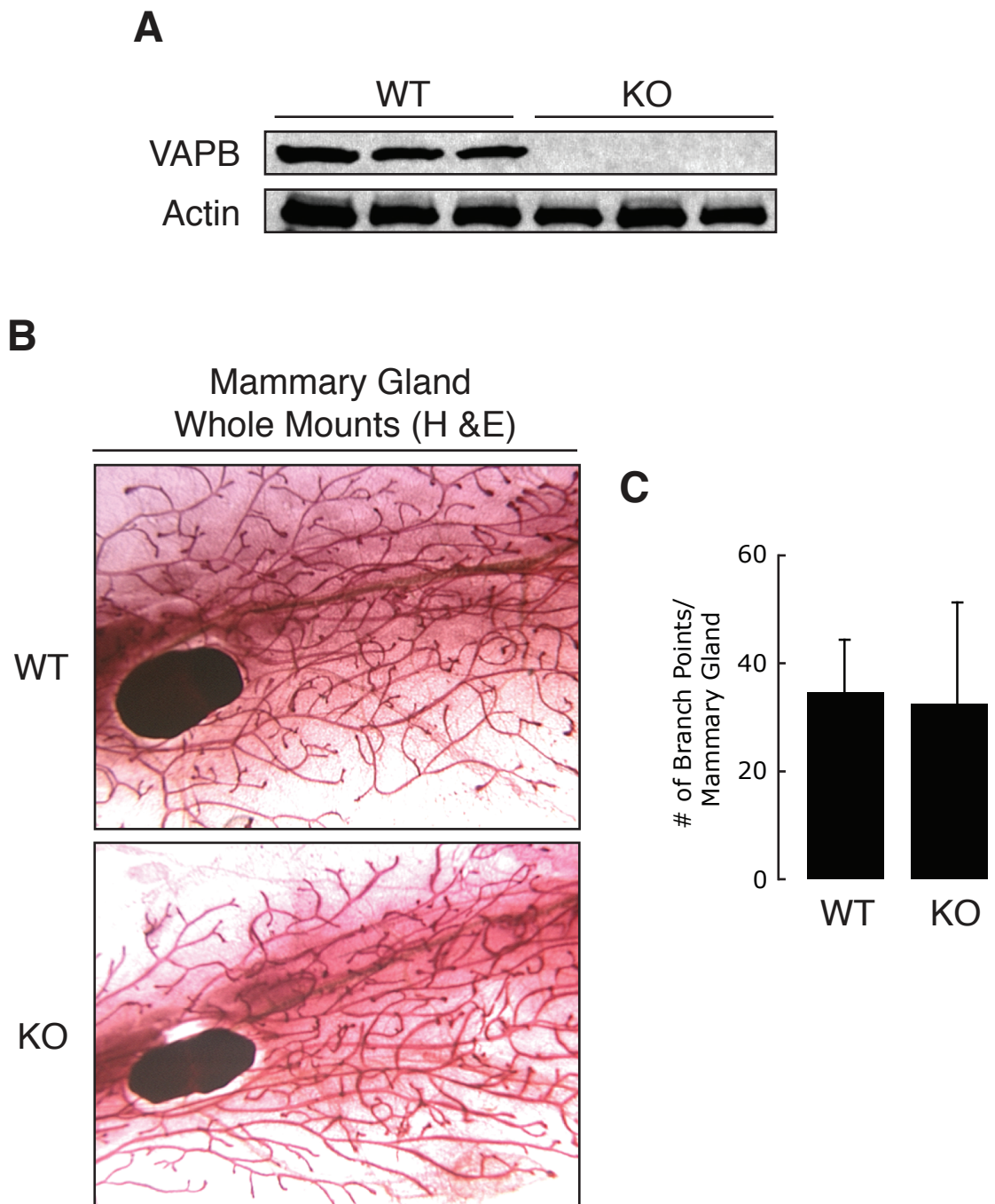
Figure 5.2. Normal mammary gland morphology in VAPB deficient mice.
(A) VAPB protein expression from mammary gland lysates was undetectable in KO mice when compared to WT controls. (B) Whole-mount hematoxylin staining of number 4 inguinal mammary glands collected from VAPB WT and KO VAPB female animals at 8 weeks after birth and was quantified for number of branch points (C).

A

B

C

WT   KO   WT   KO

VAPB

Actin

Neu (ErbB2)

Tumor Lysates
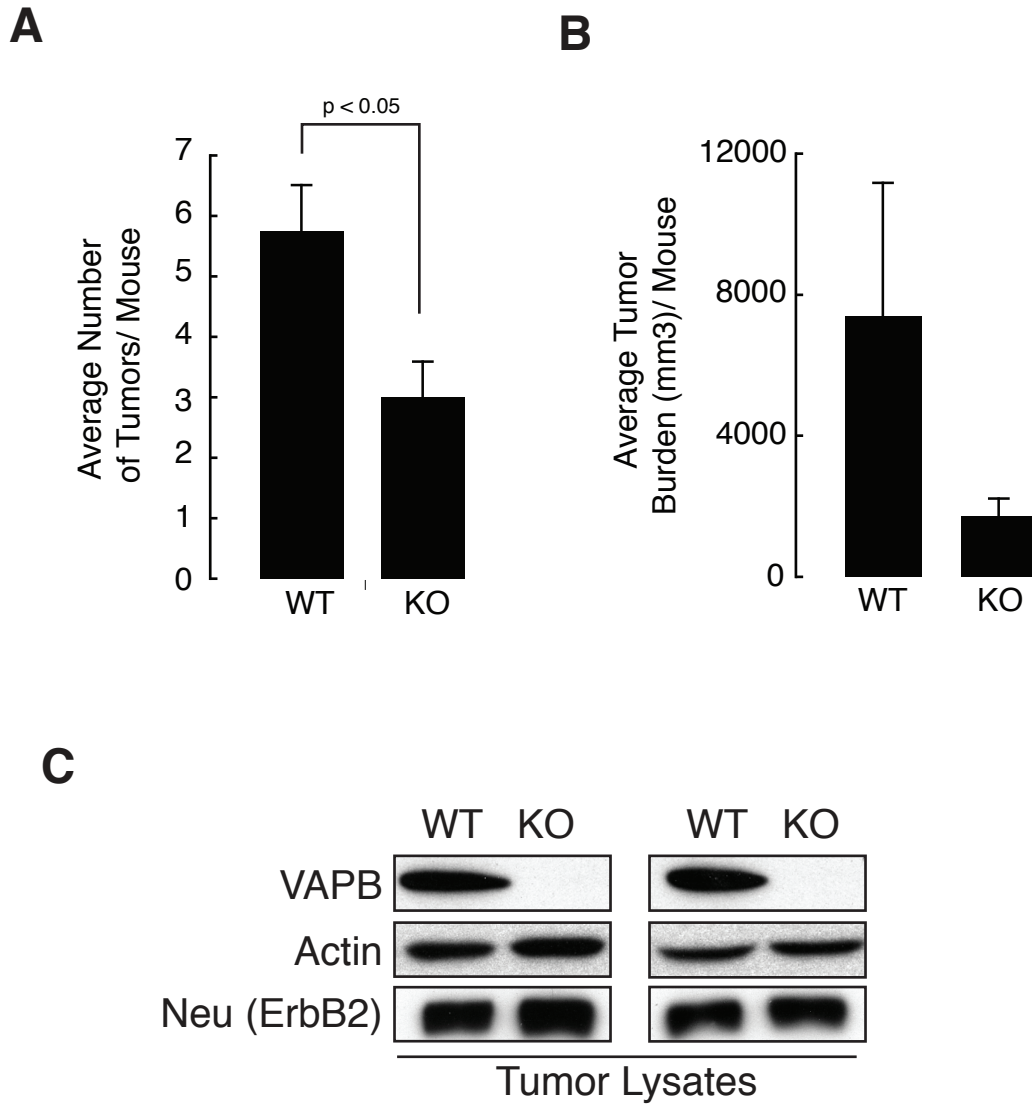
Figure 5.3. VAPB deficiency results in reduced tumor number and burden. (A) MMTV-NeuT/ VAPB WT or KO mice were sacrificed at 5 months of age. Tumors were harvested, enumerated and scored for size (n=4). (A) Average number of tumors per mouse was significantly reduced with VAPB deficiency ($p < 0.05$ unpaired t-test). (B) Reduced tumor burden per mouse was observed in MMTV-NeuT/ VAPB KO mice. (C) Confirmation of VAPB protein in tumor lysates.

affected by VAPB deficiency (Figure 5.3 B). Our *in vivo* results are consistent with our observations that VAPB deficiency also reduces spheroid growth (Figure 2.3 A) in cell culture.

Discussion

Data from our cell culture models demonstrate that VAPB functions to promote tumor cell proliferation (Chapter II). Additionally, high VAPB expression correlates with poor prognosis in human breast cancers (Figure 1.2), demonstrating VAPB's clinical significance. As an extension of these results and observations, we investigated the function of VAPB in a mouse model of spontaneous breast cancer, using the MMTV-NeuT model to maintain consistency with our cell culture models. Importantly, these mice form tumors in a manner similar to that seen in HER2-amplified human breast cancers (Muller et al, 1988). MMTV-NeuT/VAPB KO, female mice exhibited a reduction in tumor number and burden, consistent with reduced rate of tumor cell proliferation upon knockdown of VAPB in cell culture (Figure 2.3A).

While these data are consistent with the hypothesis that VAPB promotes tumor growth, further studies are required to understand the role of VAPB in early and late stages of breast tumor progression. To address the function of VAPB in tumor initiation, it would be of interest to examine pre-malignant changes in the mammary gland of MMTV-NeuT VAPB KO mice, including epithelial hyperplasia, proliferation and apoptosis prior to tumor formation. Along these lines tumor latency can be addressed by monitoring tumor formation in MMTV-NeuT, VAPB

KO mice by palpation. Since the VAPB knockout allele contains a LacZ knockin, one can determine the temporal and cell type expression of VAPB during tumor progression by staining tumor sections with X-gal from MMTV-NeuT mice.

Additionally, we also have data to suggest that VAPB promotes invasive behavior of breast tumor cells (Chapter III). At later stages of tumor progression, MMTV-NeuT mammary tumors progress from hyperplasia to metastatic carcinoma as tumor cells become increasingly motile and enter the blood stream or lymphatics, developing secondary metastatic tumors in the lung (Guy et al, 1992). While our data presented here only implicates VAPB is important for tumor growth *in vivo,* further investigation is required to see VAPB expression is required for metastatic tumor growth.

Because the MMTV-NeuT VAPB-KO mice generated are whole-body knockouts, we cannot fully dissect the contribution of the tumor microenvironment to the reduced burden observed with VAPB deficiency. Therefore additional experiments are needed to address the function of VAPB in cell types that make up the tumor microenvironment. However, our *in vivo* results thus far provide strong rationale to pursue the above-mentioned experiments.

CHAPTER VI

DISCUSSION AND

FUTURE DIRECTIONS

Discussion

We recently observed that high expression VAPB in clinical breast cancer samples significantly correlated with reduced overall and relapse-free survival. Other laboratories have also reported similar results. A genome–wide microarray analysis of 50 human breast cancer cell lines and 145 clinical specimens revealed that VAPB is often amplified or overexpressed in breast cancer, and functions in metastasis initiation. VAPB is a member of the VAP family proteins that interact with a large number of intracellular proteins and known VAP functions include vesicle trafficking, lipid metabolism and transfer, and the unfolded protein response (UPR). In addition to VAPB's intracellular function, it is cleaved and secreted, acting as a ligand for Eph receptor tyrosine kinases. Despite VAPB's clinical relevance and its role in diverse cellular processes, the precise function of VAPB in breast cancer was poorly understood. Our work presented herein has advanced our understanding of VAPB in breast tumor cell proliferation and invasion. We identified two potential mechanisms by which VAPB promotes tumor growth and invasion (1) increased AKT activity and (2) enhanced vesicle secretion.

Many breast cancers depend on the hyperactivation of AKT-PI3K for growth, evasion of apoptosis, and invasion (Miller et al, 2011; Vivanco & Sawyers, 2002). We provide evidence that VAPB regulates breast tumor cell proliferation and invasion through AKT activation. Overexpression of VAPB in MCF10A-HER2 cells enhances phosphorylation of AKT. In contrast, knockdown of VAPB in MMTV-Neu tumor cells diminishes pAKT levels. Moreover, pharmacological inhibition of AKT significantly reduced three-dimensional spheroid growth induced by VAPB and invasion through Transwell filters. Although these results do not rule out the contribution of other signaling pathways that modulate cell proliferation, our evidence suggests that VAPB induces growth and invasion through AKT activation.

We also report that VAPB modulates vesicle-mediated protein secretion. In doing so, it is possible that VAPB directly enhances tumor growth. Tumor cells rely on vesicle-mediated transport for receptor localization and growth factor secretion. Immunoprecipitation-coupled mass spectrometry analysis identified candidate VAPB-interacting proteins in cancer cells, including Arf1, a small GTPase involved in secretory protein transport. VAPB physically interacts with Arf1 and VAPB overexpression enhances trafficking of vesicular stomatitis virus glycoprotein (VSVG) to the cell surface. Inhibiting the secretory pathway with an Arf inhibitor, Brefeldin A, reduced tumor spheroid size and proliferation. While these data suggest that Arf1 is at least one major component of VAPB-mediated

vesicle trafficking in breast cancer, they do not rule out the possibility of other vesicle trafficking pathways such as endocytosis.

Dysregulated endocytosis of growth factor receptors and cadherin-based adhesion complexes often promotes tumor growth (Mosesson et al, 2008). Further investigation is needed to determine whether VAPB expression can prevent receptor downregulation, potentially sustaining mitogenic signaling pathways. It would also be of great interest to determine whether increased secretory transport observed with VAPB expression also contributes to AKT activation, potentially through autocrine or paracrine mechanisms.

Future Directions

While our work implicates two mechanisms by which VAPB promotes breast tumor growth or invasion, further investigation is required to reveal VAPB's full potential in cancer. We are confident that continued work in this field of study will provide novel insights into breast cancer progression. We attempt to speculate on a few possibilities below.

Does secreted VAPB (MSP) contribute to breast tumor growth?

MSP was discovered in *C. elegans*, where it was associated with both cell motility and fertility (Miller et al, 2001). In humans, the Major Sperm Protein (MSP) domain of VAPB was shown to be cleaved, secreted and able to bind to Eph receptors (Tsuda et al, 2008). We were able to detect soluble MSP in the

conditioned media in several breast cancer cell lines by western analysis (Figure 6.1A). Furthermore, MSP is detectable in human serum (Tsuda et al, 2008).

The Eph family of RTKs has recently been shown to play an important role in breast cancer (Duxbury et al, 2004; Zelinski et al, 2001). Eph receptors are divided into Class A and Class B receptors, based on their binding affinity to two distinct types of membrane bound ligands known as ephrins. Specifically, the EphA2 receptor is aberrantly over-expressed in 60-80% of mammary carcinomas ~~verses~~ relative to normal breast tissue, and EphA2 overexpression is associated with poor patient survival (Fournier et al, 2006; Martin et al, 2008). One model suggests that in normal cells, intact cell-cell contact allows EphA2 to bind with its ligand, ephrinA1. This interaction promotes phosphorylation and activates EphA2, reducing ERK phosphorylation. ERK signaling is a major pathway regulating cell proliferation. In breast cancer, EphA2 overexpression and lack of interaction with ephrinA1 can promote tumor growth by activating MAPK. EphA2's oncogenic effects appear to be ligand independent. However, the recent discovery of active soluble ephrin-A1 ligand in tumor cells poses a challenge to this model (Wykosky et al, 2008), as soluble ligand should be able to activate receptor in the absence of cell-cell contact. Accordingly, it is possible that tumor cells may secrete an antagonist to compete with ephrin-A1 ligand for binding to the EphA2 receptor as modeled in Figure 6.1B. Therefore further studies are needed to dissect the effects of MSP versus full-length VAPB on breast tumor cells.
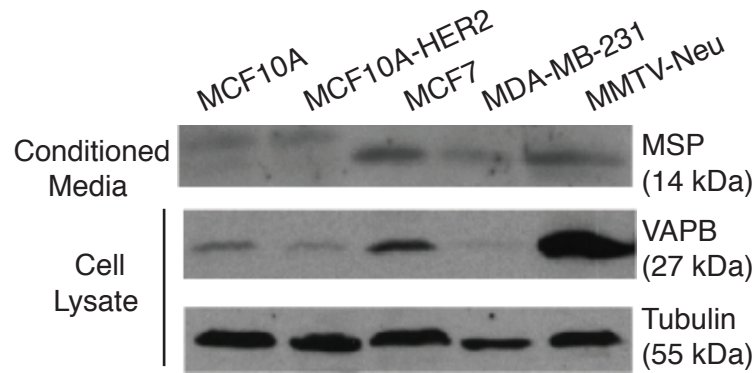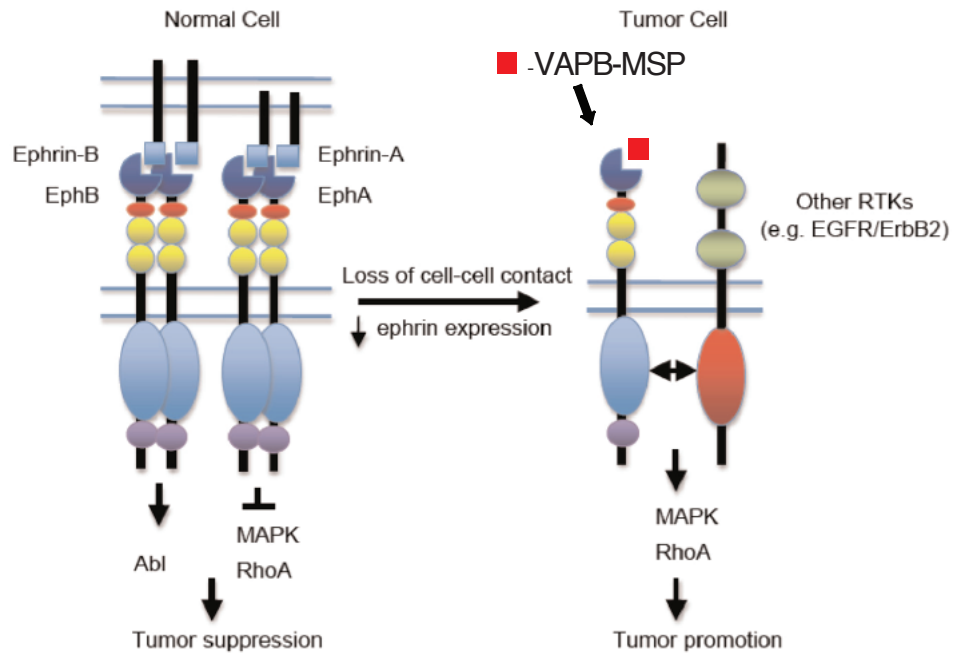
Figure 6.1. Potential role of secreted VAPB (MSP) in cancer. (A) VAPB-MSP is detectable in tumor cell conditioned media, using the same VAPB antibody used to detect full-length VAPB protein. (B) Working model for VAPB-MSP in Eph receptor function of tumor promotion versus tumor supression [Adapted from (Vaught et al., 2008)].

How does VAPB modulate AKT activity?

Although we demonstrate that VAPB enhances breast tumor cell proliferation and invasion, at least in-part, through the AKT pathway, the exact mechanism by which this happens remains unknown. There are several steps leading to AKT phosphorylation, starting with phosphoinositide 3-kinase (PI3K) activation upon recruitment to autophosphorylated, ligand-activated receptor tyrosine kinases (RTKs) (Vivanco & Sawyers, 2002). PI3K is a lipid kinase that catalyzes the synthesis of membrane phosphatidylinositol-3,4,5-P_3 (PIP3) from phosphatidylinositol-4,5-P_2 (PIP2). The generation of PIP3 on the inner leaflet of the plasma membrane recruits AKT by direct interaction with its PH domain (Vivanco & Sawyers, 2002). At the membrane, another PH-domain-containing serine/threonine kinase named PDK1 phosphorylates AKT on Thr308 (Stokoe et al, 1997) and while mTORC2 mediates phosphorylation of Ser473 (Sarbassov et al, 2005).

Given the effects of VAPB on breast tumor cell proliferation and invasion is dependent on AKT activation, it would be interesting to examine how VAPB modulates upstream activators of AKT, such as PI3K, both at the level of recruitment to RTKs and PI3K activity. In this study it is also noteworthy that VAPB interacts with Arf1, a small, Ras-family GTP-ase. Interestingly, Arf1 has been implicated in the recruitment of the p85 subunit of PI3K to EGFR in breast cancer cells (Boulay et al, 2008). This raises the possibility that the VAPB/ Arf1 interaction enhances PI3K activity by promoting recruitment of its p85 subunit.

On the other hand, if VAPB does not affect AKT activation at the level of PI3K, it is possible that VAPB alters the availability of PI3K's intracellular substrate PIP2 at the plasma membrane. We can speculate on this idea because we, and others, have found that VAPB interacts with Nir2 (PITPB2), a phosphoinositol transfer protein [Table 3; (Litvak et al, 2005; Peretti et al, 2008)]. These proteins facilitate the transfer of phosphoinositols between various cell membrane interfaces. Nir2 has been proposed to target PIs from the ER to *trans*-Golgi PI4-kinases (Schaaf et al, 2008) ultimately generating PtdIns(4)*P*s at the Golgi. However, recent studies indicate that the majority of PtdIns(4)*P* resides in the plasma membrane (Hammond et al, 2009), which is then converted to PIP2 by phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks) (Fruman et al, 1998). It is tempting to speculate that VAPB promotes AKT by elevating levels of PIP2 substrate for PI3K, perhaps through localized targeting of phosphoinositide (PIs) intermediates by Nir2.

Does VAPB relieve ER stress during tumor proliferation?

Proliferative tumor cells often hijack various cellular processes in order to support growth, exerting stress on organelles such as the ER which is the site of protein folding and secretion, lipid biosynthesis and calcium homeostasis (Tsai & Weissman, 2010). Tumor cells face extracellular stresses such as hypoxia, nutrient deprivation or growth constraints by the extracellular matrix (ECM) as well as intracellular stresses such as high protein demand for growth factors and

receptors (Tsai & Weissman, 2010). Such harsh conditions activate the UPR through one or all three receptors (IRE1, ATF6 and PERK).

Evidence has emerged that VAPB activates the IRE1-XBP1 arm of the UPR (Kanekura et al, 2006). Furthermore sustained IRE1-XBP1 activation has been shown to help cells enhance proliferation and evade cell death (Lin et al, 2007; Lin et al, 2009). Interestingly the IRE1-XBP1 pathway has been implicated in breast cancer such that XBP1 expression is upregulated in certain breast cancers and correlates with poor prognosis in patients (Davies et al, 2008; Fujimoto et al, 2003). Additionally, *in vitro* studies have demonstrated that XBP1 confers resistance to apoptosis in breast cancer cell lines (Gomez et al, 2007). A role for the IRE1-XBP1 pathway in breast cancer is further supported by *in vivo* bioluminescence imaging of breast tumor cells expressing a XBP1-luciferase reporter in which luciferase is expressed only when XBP1 is spliced by activated IRE1 (Spiotto et al, 2010; Tsai & Weissman, 2010), which also provides a useful mouse model to address the role of IRE1-XBP1 in VAPB-mediated breast tumor proliferation and invasion.

VAPB's therapeutic potential in breast cancer?

Our data presented thus far demonstrate that VAPB functions to increase breast tumor cell and invasion through enhanced AKT activity. This pathway is commonly dysregulated in breast cancer and often promotes resistance to current therapies (Miller et al, 2011), such as those that target HER2. Treatment

of VAPB-expressing cells with two different PI3K-AKT inhibitors reduced spheroid size to a greater extent than non-VAPB-expressing cells. This suggests that VAPB is more dependent on AKT signaling and may increase sensitivity to AKT inhibitor-based therapies. Another step to determine the therapeutic potential of targeting AKT in VAPB-expressing cancers is to correlate high VAPB expression with elevated AKT activity in human breast tumor samples. The results from such studies would help stratify patient populations into those that would benefit most from AKT-targeted therapies.

Moreover, our results also demonstrate that VAPB enhances secretory vesicle transport. Secretion of growth factors, cytokines, matrix metalloproteinases, as well as delivery of receptors to the cell surface, facilitate tumor growth and invasion (Hanahan & Weinberg, 2011). The high demand for such proteins to maintain tumor growth provides unique opportunities for targeting vesicle trafficking in cancer therapeutics (Wright, 2008).

Furthermore, our observations that high VAPB expression correlates with poor prognosis and the ability to detect its secreted form (MSP) in human serum (Tsuda et al, 2008) suggests that VAPB can be used as a potential biomarker in breast cancer diagnostics, both in tumor tissue and in serum. Despite numerous advances in mechanism-based therapies, breast cancer morbidity rates remain high. Therefore, it is imperative that alternate cellular processes, such as those modulated by VAPB, be further investigated as potential targets for breast cancer chemotherapy.

Final Remarks

Since the cloning of VAPB 15 years ago, significant progress has been made to understand the importance of VAPB in multiple cellular processes. The data presented in my dissertation demonstrate the impact of VAPB expression in human breast cancer through genetic, biochemical analyses using 3-dimensional cell culture and animal models. We suggest two mechanisms by which VAPB affects breast tumor cells: (1) increased AKT activation and (2) enhanced secretory vesicle transport. It is likely that both pathways converge to support VAPB function in tumor cells. The diversity of VAPB functions and their impact on cellular processes such as secretory protein transport, lipid metabolism and lipid transfer represent several pathways that could be potentially exploited by breast tumor cells to promote growth and invasion. We anticipate that new insights regarding how VAPB promotes breast cancer will emerge in the coming years as well as clarify the role of the extracellular, secreted form of VAPB. More importantly, this work will trigger renewed interest regarding VAPB in other cancer types in the hope that future mechanistic studies will help understand VAPB's role in various human pathologies.

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