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Roles of VAMP3 in melanoma cell migration and integrin trafficking.

Yaman Babi 1981-
University of Louisville

ROLES OF VAMP3 IN MELANOMA CELL MIGRATION AND INTEGRIN TRAFFICKING

By

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BSc. University of Sharjah, 2004

A Thesis Submitted to the Faculty of the School of Medicine at University of Louisville in partial Fulfillment of the Requirements for the degree of

Master of Science

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A thesis approved on

December 21, 2010

By the following thesis committee

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Thesis Director (Chuan Hu)

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Barbara J. Clark

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Thomas Geoghegan

……

David Powell

DEDICATION

This thesis is dedicated to:

My dearest parents, brothers and sisters,

My husband,

And my friends

For their patient support and continuous encouragement.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my thesis advisor, Dr. Chuan Hu. His encouragement and assistance over the past two years is greatly appreciated. I would like also to thank my committee members Dr. Barbara Clark, Dr. Thomas Geoghegan and Dr. David Powell for their support and advice. I also would like to thank the member's of Dr. Hu lab, Dr. Nizarul Hasan , Dr. Krista Riggs and Mrs. Deborah Corbin for their guidance, assistance and friendship. Moreover, I would like to thank my family for their support and encouragement as this educational opportunity became part of my life for two and a half years.

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ABSTRACT

ROLES OF VAMP3 IN MELANOMA CELL MIGRATION AND INTEGRIN TRAFFICKING

Yaman Babi

Dec 21, 2010

Integrins are major receptors for cell adhesion to the extracellular matrix, and play key roles in various cellular processes including adhesion, migration, proliferation and survival. Apart from developmental and physiological events, integrins are involved in many pathological conditions including cancer metastasis. During cell migration, the exocytosis of integrins at the cell front contributes to the formation and stabilization of protrusions. Previous studies showed that VAMP3, a SNARE protein that mediates exocytosis, is important in integrin trafficking and cell migration. However, the mechanism by which VAMP3 participates in integrin trafficking is not clear. Since VAMP3 is over expressed in melanoma, the current study determines the roles of VAMP3 in melanoma cell migration and integrin trafficking. shRNA-induced silencing of VAMP3 inhibited the migration of B16F10 melanoma cells by more than 60% without affecting cell proliferation. VAMP3 knockdown diminished cell adhesion to Matrigel and fibronectin. Furthermore, VAMP3 silencing resulted in the accumulation of β1, α3 and α5 integrins in lysosomes, indicating that VAMP3 mediates vesicle trafficking of α3β1

and α5β1 integrins. For the first time, this study examined the effect of VAMP3 knockdown on metastasis of melanoma *in vivo* using an experimental metastasis assay in nude mice. It seemed that melanoma cells can metastasize to the lung even when VAMP3 is depleted and we suggest more quantitative experiments to determine the effect of VAMP3 knockdown in melanoma pulmonary metastasis *in vivo*.

TABLE OF CONTENT

LIST OF FIGURES

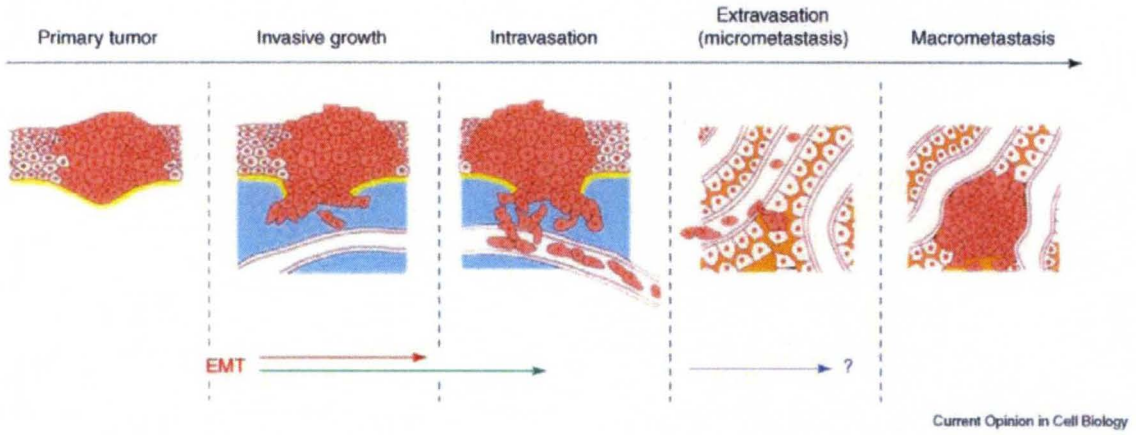
CHAPTER I: INTRODUCTION

Metastasis and cell migration

Metastasis (Greek: *displacement, μετά=next + στάσις=placement*, plural: *metastases*) is the process of spreading a disease from one organ to another non-adjacent organ. Metastases are responsible for about 90% of the deaths in cancer patients. Cancer metastasis by definition consists of a series of sequential steps in which some cancer cells escape from the original tumor site and migrate to other parts of the body through the bloodstream, or the lymphatic system. Metastasis start when some malignant cells break away from the primary tumor and degrade the proteins that make up the surrounding basement membrane which separates the tumor from adjoining tissues. By degrading these proteins, cancer cells are able to breach the ECM and invade (1) (Fig 1). Cell migration is a fundamental step in cancer metastasis.

Cell migration in specific direction and to a specific site in the body is required in wound healing, immune responses and embryonic tissue formation and development. Errors during cell migration have serious consequences, including mental retardation, vascular diseases and cancer metastasis. An understanding of the mechanism by which cells migrate might lead to the discovery of novel therapeutic strategies, that can help in controlling invasive tumour cells.

Figure 1. Cancer metastasis cascade. Cancer metastasis consists of a series of sequential steps, including local tumor growth, invasion by transmigration through basement membranes and surrounding tissues, intravasation into blood vessels, dissemination and survival in the bloodstream, extravasation and re-establishment of tumor colonies at distant sites.



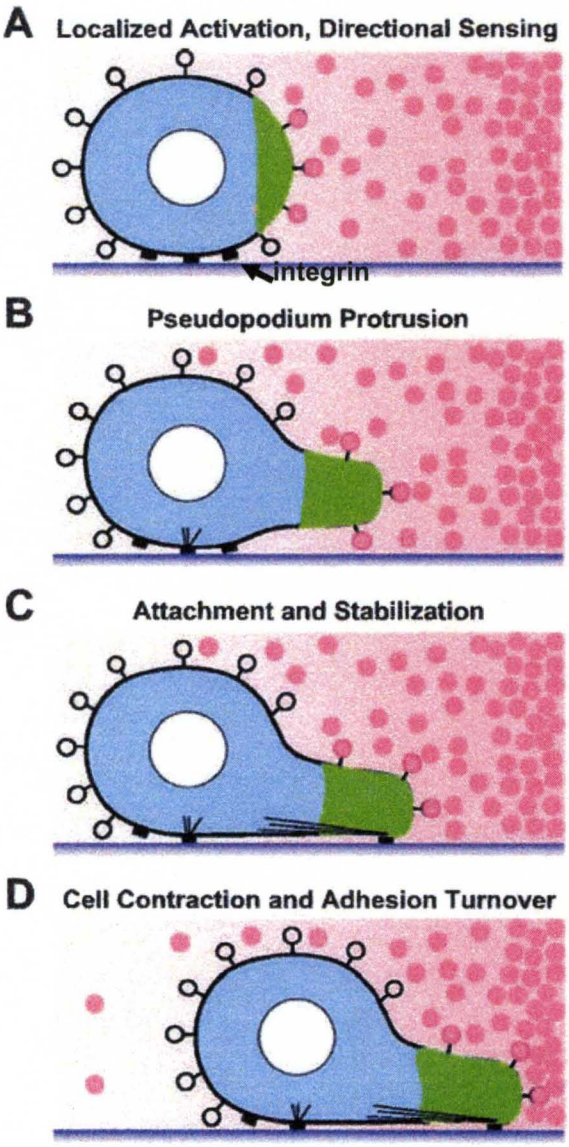
Huber, M.A., 2005. *Curr Opin Cell Biol* 17:548-558.

Chemotaxis is the process by which cells migrate in animal tissue toward a specific external signal (2) (Figure 2). The migration of single mammalian cell can be viewed under the microscope as the cells move randomly on a glass slide. By observing that, changes in the moving cell shape is noticed and its leading front has a characteristic behavior. This part of the cell is highly active, sometimes spreading forwards quickly, sometimes retracting, othertimes ruffling, or bubbling. It is widely considered that the leading front is the main engine which moves the cell forward (3).

There is still great uncertainty on how cell migration really works. However, because the locomotion of all mammalian cells has several common features, the underlying cell migration processes are believed to be similar. The two main constant features are: 1) the behavior of the leading part of the cell; 2) the dorsal surface of the cell moves backwards and towards its trailing part. The latter feature is most easily observed when aggregates of a surface molecule are cross-linked with a fluorescent antibody or when small beads become artificially bound to the front of the cell (2).

Studies have demonestrated that cell front is the site where membrane is recycled to the cell surface from internal membrane pools at the end of the endocytic cycle. This has led to the understanding that the extension of the leading edge occurs when new membrane is added at the front of the cell. If so, the actin filaments which form at the front might stabilize the added membrane so that a structured extension, or lamella, is formed rather than the cell

Figure 2. Chemotactic cell migration. (A) The cell is attached to the extracellular matrix through integrin receptors on the cell surface. Upon exposure to a chemoattractant, directionality is established by localized receptor binding that leads to activation and accumulation of signaling events on the side of the cell facing the highest chemottractant concentration. (B) The activated signaling events facilitate localized F-actin polymerization leading to membrane protrusion that is independent of integrins and the ECM. (C) The protruding psedopodium is stabilized by integrin-mediated cell adhesion. (D) A forward translocation of the cell is achieved as the cell body contracts and releases attachment sites at the rear of the cell.



Chodniewicz, D.,2004. Exp Cell Res 301:31-37.

blowing bubbles at its front. Integrins, mediate cell adhesion to the ECM at the leading edge to stabilize the lamella and serve as traction points. It is likely that these integrins "feet" are brought to the front by added membrane at this site. The rest of the cell is simply dragged forward. Although not clearly established, there are suggestions that the nucleus and perhaps other large structures inside the cell may also be pulled forward by actin filaments. In addition, it may be that the rear of the cell actively contracts (2).

Integrins

Integrins mediate cell adhesion to extracellular matrix (ECM) proteins such as fibronectin, laminin, collagen and vitronectin (4). Integrins define cellular shape, mobility, and regulate the cell cycle. Some integrins play a role in the attachment of cells to other cells. Besides the attachment role, integrin also plays a role in signal transduction, a process by which a cell transforms one kind of signal or stimulus into another.

Integrins are heterodimers containing two chains: the α (alpha) and β (beta) subunits. In mammals, eighteen α and eight β subunits have been characterized and twenty-four α/β combinations have been described (4). The α and β subunits contain two separate tails, both of which span the plasma membrane and possess small cytoplasmic domains. Integrin subunits penetrate the plasma membrane and have very short cytoplasmic domains of about 40–70 amino acids. The exception is the beta-4 subunit, which has a cytoplasmic domain of 1088 amino acids, one of the largest known cytoplasmic domains of any membrane protein. Outside the cell plasma membrane, the α

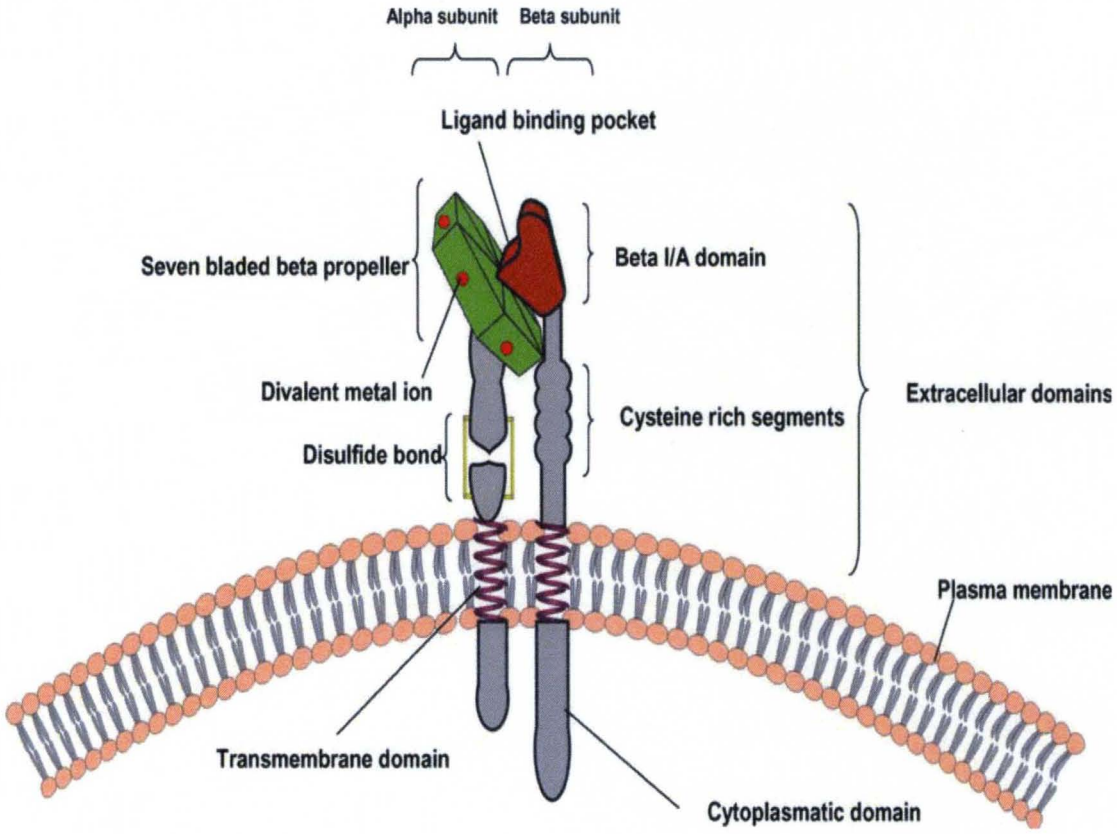
and β chains lie close together along a length of about 23 nm; the final 5 nm N-termini of each chain forms a ligand-binding region for the extracellular matrix (Figure 3).

Integrin expression pattern on the cell surface makes the cell fit into its surrounding environment. During embryonic development, integrins are expressed very neatly in time and space to guide the cells and make them detect where exactly they are and where to attach. However, integrins that show modulated interactions with their environment can transfer dramatic and far-reaching consequences upon cells. This can give the cells the tendency to loose their original ties, recognize a different ECM substrate and reconfigure them with features that give them the capacity to metastasize (5). Indeed, many studies have shown that the upregulation of expression of specific integrins is associated with the acquisition of a more metastatic phenotype (6).

Vesicle trafficking of integrins in cell migration

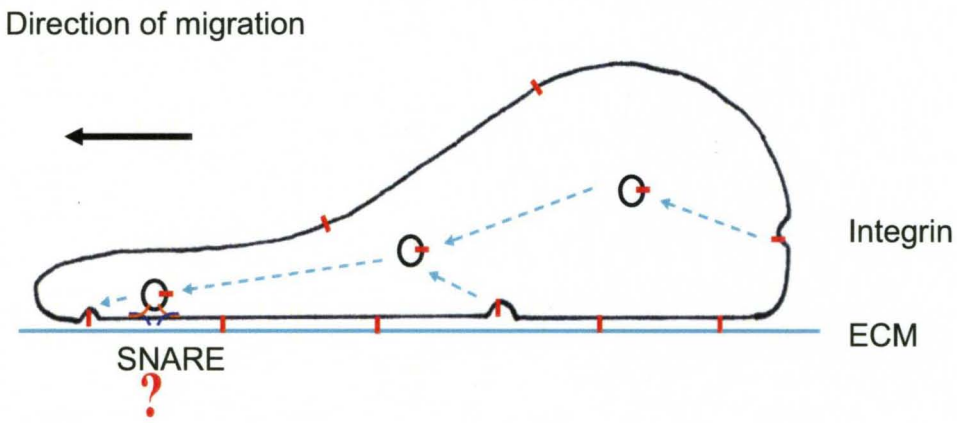
Emerging evidence indicates that intracellular vesicle trafficking exerts temporal control over integrin functions (3). As transmembrane proteins, the α and β subunits of integrins are synthesized and paired in the endoplasmic reticulum (7, 8), transported in vesicles and delivered to the plasma membrane or the cell surface by exocytosis. In migrating cells, the cell forward movement would lead to an accumulation of integrins towards the cell rear (Fig 4). To enhance adhesion at the cell front or the leading edge, integrins at the plasma membrane are endocytosed, transported forward by vesicles and exocytosed at the cell front (9-11). Protein kinase Cα(PKCα) and PKCε regulate the exocytosis of integrins (12, 13).

Figure 3. Structure of integrin receptors. Integrin are heterodimers of α and β subunits. Both the α and β subunits, except the α4 subunit, have large ligand-binding extracellular domains and short cytoplasmic domains. Integrins link ECM proteins on the extracellular face of the plasma membrane to the cytoskeletal filaments on the cytoplasmic face. Modified from (14).



Kuphal, S., Bauer, 2005. Cancer Metastasis Rev 24:195-222.

Figure 4. SNARE-mediated polarized exocytosis in cell migration. Integrins and other plasma membrane proteins are endocytosed and transported by vesicles to the cell front. SNARE proteins such as VAMP3 mediate the fusion of transport vesicles with the plasma membrane. Accumulation of integrins at the cell front promotes migration.

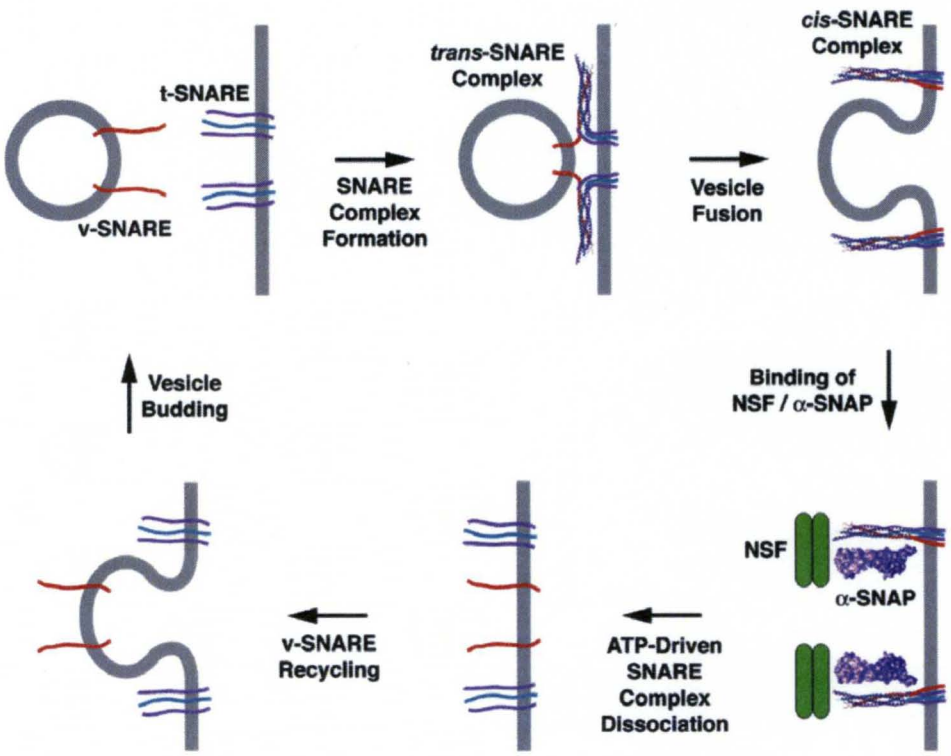

Direction of migration
Integrin
ECM
SNARE
?

Accordingly, Rab GTPases which control the targeting and tethering of transport vesicles have been shown to modulate integrin trafficking and cell mobility (11, 15). However, not much is known about the molecular mechanism of the integrin exocytosis, *i.e* fusion of integrin-containing vesicles with the plasma membrane.

SNARE proteins

SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) are a large protein family consisting of more than 35 members in humans. SNAREs can be divided into two main categories: *vesicle* or *v-SNAREs*, which are incorporated into the membranes of transport vesicles during budding, and *target* or *t-SNAREs*, which are found in the membranes of target compartments. Recent classification divides SNARE proteins according to their structural features into R-SNAREs and Q-SNAREs (16). Research in the vesicle trafficking field has demonstrated that the interactions between v-SNAREs and t-SNAREs drive intracellular vesicle fusion (17-19). Most SNAREs are C-terminally anchored transmembrane proteins, with their N-terminal functional domains facing the cytosol. These cytoplasmic domains of v- and t-SNAREs form an extremely stable four-helix bundle (20). Energy made available from the assembly of the SNARE complex is used to bring the vesicular and target membranes into close proximity and drive membrane fusion (21-23) (Figure 5). What exactly not clear is, which SNARE proteins mediate the trafficking of integrins to the plasma membrane.

Figure 5. SNARE proteins mediate intracellular vesicle fusion. A trans-SNARE complex assembles when t-SNARE in the target membrane bind to the v-SNARE in the vesicle by forming a stable 4-helix bundle which drive membrane fusion. This results in cis–SNARE complex in the fused membrane. The adaptor protein α-SNAP binds to the cis-SNARE complex and recruits N-ethylmaleimide sensitive fusion (NSF), which hydrolysis ATP to dissociate the SNARE complex. The v-SNARE is then recycled for the next round of vesicle fusion.



Juan S. Bonifacino., Cell, Vol. 116, 153–166, January 23, 2004.

VAMP3

Vesicle associated membrane proteins (VAMP) are v-SNAREs found in various post-Golgi vesicular compartments. VAMP3 or cellubrevien is a member of this family. It is expressed in tissues and is localized to recycling endosomes and endosome-derived vesicles (24, 25). VAMP3 has been involved in the exocytosis of α-granules in platelets (26) as well as the recycling of endocytosed transferrin receptors (27) to the cell surface. Recent studies suggest that VAMP3 is implicated in integrin trafficking and cell migration (16, 28, 29). Studies have shown that tetanus neurotoxin-mediated cleavage of VAMP3 disrupts epithelial cell migration and integrin-dependent cell adhesion (29). A recent study from our lab showed that VAMP3-dependent integrin trafficking is crucial in cell migration and cell adhesion to laminin (30). The goal of my thesis is to further analyze the role of VAMP3 in integrin trafficking and cell migration, using melanoma cells as a model.

Melanoma

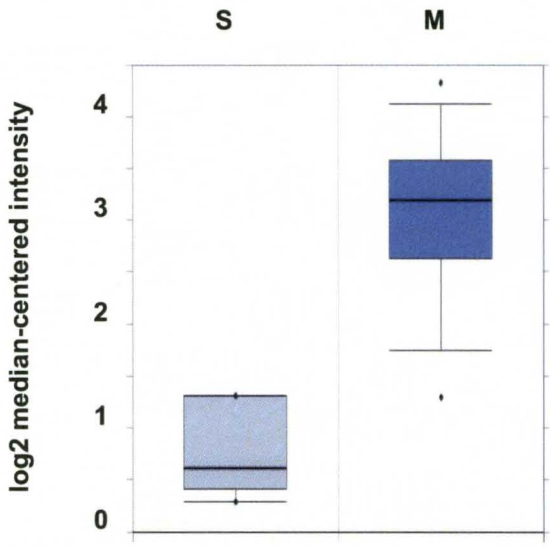
About 75% of death in skin cancer patients is referred to melanoma. According to a prediction by the American Cancer Society, in 2009 about 68,720 people in the United States will be newly diagnosed with melanoma and about 8,650 will die of the disease. In the United States, the percentage of people who have developed melanoma has more than doubled in the past 30 years. The early stage melanoma is cured by surgery in most cases, however, once melanoma cells metastasize, it is almost always fatal. Currently there is no

effective therapy for metastatic melanoma. The median survival time of melanoma patients with distant metastases has been reported to be 7.5 months. Therefore, new therapeutic strategies that inhibit metastasis are urgently needed to overcome melanoma.

Melanoma is an attractive model for studying integrin roles in cancer progression because it generally follows a sequential series of definable stages (31). During the melanoma progression, modulations in integrin expression and signaling change the ability of the cancer cells to interact with the environment and convert melanoma cells from a stationary to a migratory and invasive phenotype. The expression levels of $\alpha v\beta 3$, $\alpha 3\beta 1$, $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins are increased in melanoma (14). The $\alpha v\beta 3$ integrin plays an important role during the transition of melonoma cells from the radial growth stage to the vertical growth stage. Moreover, further progression leading to metastases may require changes in the cell's integrins that would enhance their ability to escape from the primary tumor, and help in their ability to invade and ultimately form metastases. It is also conceivable that the $\alpha v\beta 3$ integrin is reexpressed during various stages of metastatic dissemination and in particular during tumor reestablishment (31). However, it has to be pointed out that there are some differences in integrin expression patterns depending on *in vivo* (tissue) or *in vitro* (cell culture) analysis (14).

As shown in the microarray dataset developed by Talantov *et al.* (32) (Fig. 6), VAMP3 is highly over expressed in melanoma. It is ranked in the top 1% in the Over

Figure 6. Over expression of VAMP3 in melanoma. VAMP3 gene expression in 45 melanoma specimens (M) was analyzed using the Affymetrix human genome U133A array, and compared to the expression in 7 normal skin (S). The original microarray dataset was reported by Talantov *et al.* Box plot was generated at www.oncomine.org.



www.oncomine.org

expression Gene Rank (P = 2.86E-11). In this thesis, I hypothesize that VAMP3 mediates integrin trafficking.

CHAPTER II: RESULTS AND DISCUSSION

The exocytosis of integrins at the cell front contributes to the formation and stabilization of protrusions in cell migration. Previous studies have shown that VAMP3 is involved in vesicle trafficking of integrins and cell migration (30). However, the mechanism by which VAMP3 participates in integrin trafficking is not well elucidated. Interestingly, VAMP3 is over expressed in melanoma (Fig. 6). The specific aims of my dissertation are to: 1) determine the role of VAMP3 in melanoma cell migration and integrin trafficking; 2) examine the role of VAMP3 in melanoma metastasis *in vivo*. Using the widely used B16F10 melanoma cells as a model, I developed stable cell lines in which VAMP3 is silenced. Using transwell migration assay and integrin immunostaining, I analyzed the function of VAMP3 in cell migration and integrin trafficking. Using a B16F10 experimental metastasis assay in nude mice, I examined if VAMP3 knockdown inhibits the metastasis of melanoma *in vivo*.

i. Determine the role of VAMP3 in migration and integrin trafficking of melanoma cells

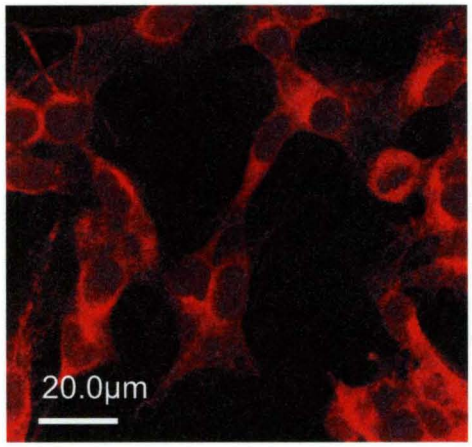
Silencing of VAMP3 inhibits cell migration

Immunostaining revealed that VAMP3 is present in intracellular vesicles in the B16F10 mouse melanoma cells (Fig 7A). To examine the role of VAMP3 in integrin

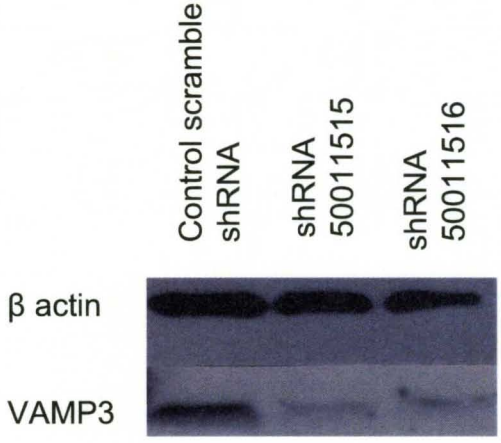
trafficking and cell migration, I depleted VAMP3 expression by shRNA gene silencing. As shown by immunoblotting analysis (Fig 7 B), within 72 h after transfection of shRNAs

Figure 7. Silencing of VAMP3 in B16F10 cells. (A) Subcellular distribution of VAMP3. B16F10 cells were fixed, permeabilized and stained with a rabbit polyclonal antibody to VAMP3. Representative Confocal images are shown. Arrow indicates VAMP3 concentrated in intracellular vesicles close to the nucleus. Bar represents 20 μm. **(B)** B16F10 cells were transfected with two shRNAs to VAMP3: 50011515 and 50011516. 72 h later, whole cell lysates of the transfected cells were analyzed by immunoblotting with the antibody to VAMP3. The same membrane was probed with an antibody to anti-β-actin as loading control. Shown is a representative western blot of two independent experiments.

A


20.0µm

B


Control scramble shRNA
shRNA 50011515
shRNA 50011516
β actin
VAMP3

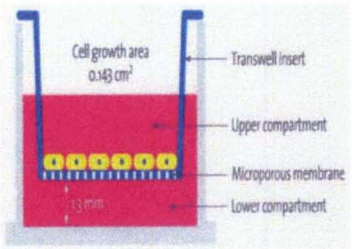
against VAMP3, the expression of VAMP3 protein in B16F10 cells was depleted by more than 90%. The large reduction of VAMP3 protein indicated high efficiency of shRNA transfection and interference. Transfection of shRNA did not modulate the expression of β-actin (Fig. 7B).

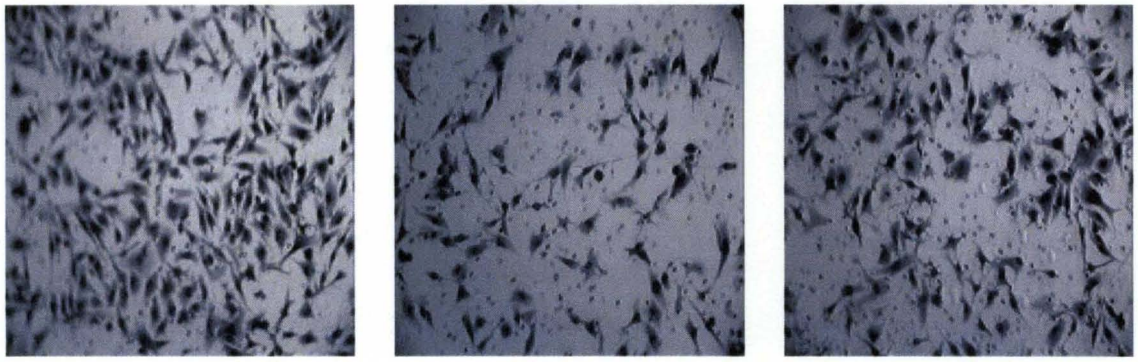
The effect of VAMP3 knockdown on cell mobility was analyzed using transwell migration assay (33). Matrigel, which consists mainly of laminin and collagen, was used as the chemoattractant. When loaded to the upper chambers of the transwells, B16F10 cells migrated efficiently across the membranes in a relatively homogeneous fashion (Fig 8 A). Interestingly, cells transfected with VAMP3 shRNAs had much reduced mobility. Compared with the cells transfected with non-targeting scramble shRNA control, the number of migrated cells transfected with the VAMP3 shRNA 50011515 decreased by 50.5% and the number of migrated cells transfected with the VAMP3 shRNA 50011516 decreased by 26% (Fig 8 B), indicating that VAMP3 diminished the chemotactic migration of melanoma cells.

Because the shRNA 50011515 silenced VAMP3 (Fig. 7B) and inhibited cell migration (Fig. 8), we developed B16F10 cell lines which stably express 50011515 to further analyze the role of VAMP3 in integrin trafficking and migration of melanoma cells. Since the shRNA is encoded in a vector that contains the puromycin resistant gene, B16F10 stable cells were selected in cell culture medium with puromycin (Fig 9 A). We expanded 24 B16F10 clones that are puromycin resistant and used immunoblotting to determine VAMP3 expression in the 24 clones (part of the immunoblotting is shown in (Fig 9 B)). VAMP3 protein was depleted

Figure 8. VAMP3 knockdown inhibits cell migration. (A) 72 h after transfection with the VAMP3 shRNAs 50011515 and 50011516, or a scrambled control shRNA, 5 x 10^5 of B16F10 cells were harvested and loaded to the top chambers of transwells. Matrigel (20 μg/ml) was included in the lower chambers as a chemoattractant. After 24 h, unmigrated cells were removed and migrated cells were Giemsa stained. Representative images of migrated cells are shown. **(B)** Random images were taken for each transwell. The number of migrated cells in each image was qualified using the ImageJ software then averaged for each experimental group. The number of migrated cells transfected with the VAMP3 shRNAs was normalized to the number of migrated cells transfected with the scramble shRNA. Error bar represent standard deviation of 3 independent experiments in which two transwells were used for each transfection. ***$P < 0.001$ vs. control shRNA, **$P < 0.01$ vs. control shRNA.

A





Scramble shRNA | VAMP3 shRNA 50011515 | VAMP3 shRNA 50011516

B

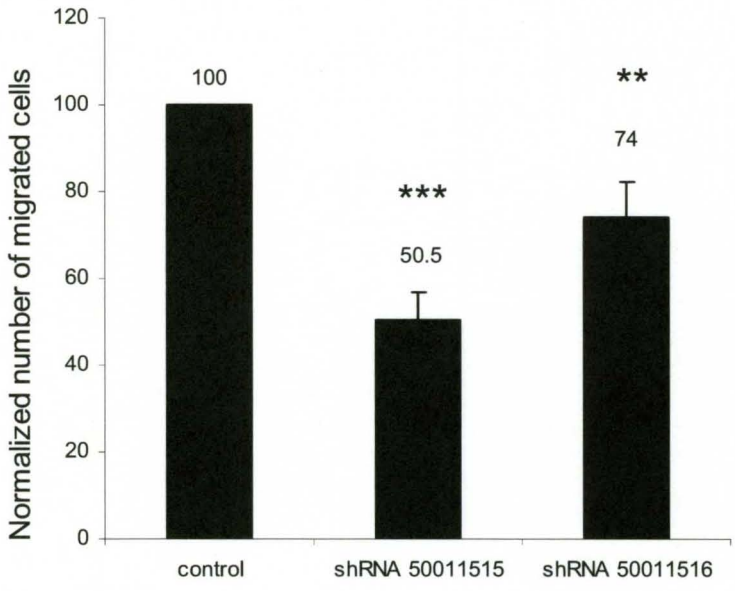
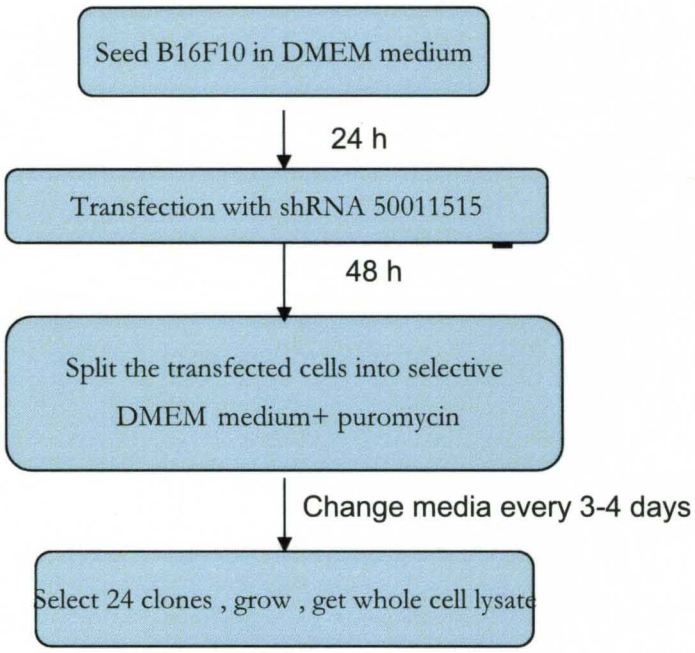
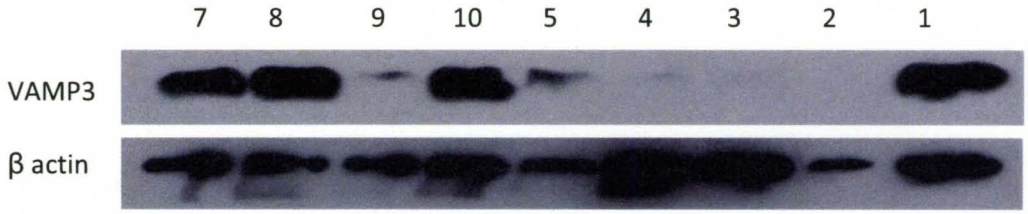


Figure 9. Development of stable cell lines that express a VAMP3 shRNA. (A) B16F10 cells were transfected with the shRNA 50011515. The transfected cells were split into selective medium containing puromycin (1 μg/ml). After 2 weeks, 24 stable clones were picked, expanded and screened for silencing of VAMP3 by immunoblotting. **(B)** Whole cell lysates of 9 of the clones were immunoblotted with a VAMP3 antibody. The same membrane was probed with an antibody to anti-β-actin as loading control.

A


Seed B16F10 in DMEM medium
24 h
Transfection with shRNA 50011515
48 h
Split the transfected cells into selective DMEM medium+ puromycin
Change media every 3-4 days
Select 24 clones , grow , get whole cell lysate

B


7
8
9
10
5
4
3
2
1
VAMP3
β actin

almost completely in Clones 3 and 4. These two cell lines were expanded and further immunoblotting analysis confirmed the depletion of VAMP3 in Clone 3 and Clone 4 to undetectable level compared with wild type B16F10 cells (Fig. 10). These data indicate VAMP3 in B16F10 melanoma cells is silenced efficiently using shRNA.

Then we assessed the effect of VAMP3 knockdown on the proliferation of B16F10 cells. Clearly, the stable cell line Clone 3 proliferated at the same rate as the wild type cells (Fig 11 B). This was an indication that VAMP3 was not required for cell survival or proliferation, and ruled out the possibility that the observed reduction in the number of migrated cells (Fig 8 A & B) was a result of decreased cell viability. Microscopic images of the wild type, Clone 3 and Clone 4 cells showed no obvious morphological differences (Fig 11 A). However, the pellets of Clone 3 and Clone 4 cells look darker in color compared to the wild type cells (data not shown), suggesting that secretion of the melanin pigment in the VAMP3 knockdown melanoma cells was disrupted.

The transwell migration assay performed on the wild type and Clone 3 and Clone 4 cells showed 54% reduction of migration of Clone 3 cells and 39% reduction of migration of Clone 4 cells compared with the wild type cells (Fig 12 B). When Clone 3 cells were transfected with a plasmid that encodes human VAMP3, cell motility was rescued back to the wild type level (Fig 12 C), further indicating that migration of Clone 3 cells was inhibited due to the knockdown of VAMP3. Due to the fact that Clone 3 cells have more reduced cell migration than Clone 4 cells, Clone 3 cells were used in later studies to determine the effects of VAMP3 knockdown on melanoma metastasis *in vivo*.

Figure 10. VAMP3 expression in stable cell lines that express VAMP3 shRNA. Whole cell lysate of wild-type B16F10, Clone 3 and Clone 4 cells were immunoblotted with an antibody to VAMP3. The same membrane was blotted with an antibody to β-actin as a loading control.

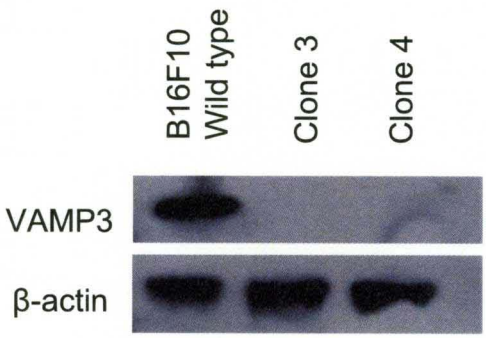
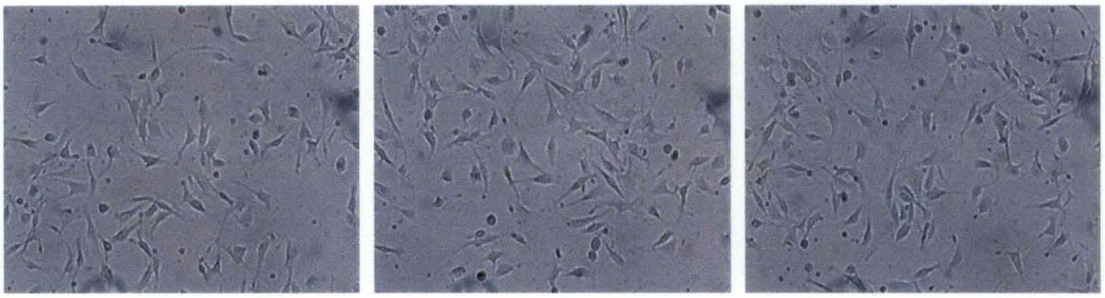

B16F10
Wild type
Clone 3
Clone 4
VAMP3
β-actin

Figure 11. VAMP3 silencing has no effect on cell morphology and proliferation. **(A)** Representative light microscopy images for B16F10 wild type cells and clones 3 and 4. **(B)** VAMP3 silencing has no effect on cell proliferation. Same amount of wild-type and Clone 3 cells were seeded in 24 well plate. At 0, 24, 48, or 72 h after seeding, the number of living B16F10 wild type and clone3 were measured using the CellTiter 96 A Queous One Solution Cell Proliferation Assay by absorbance at 490nm. The error bars represent standard deviation of three independent experiments.

A



Wild type Clone 3 Clone 4

B

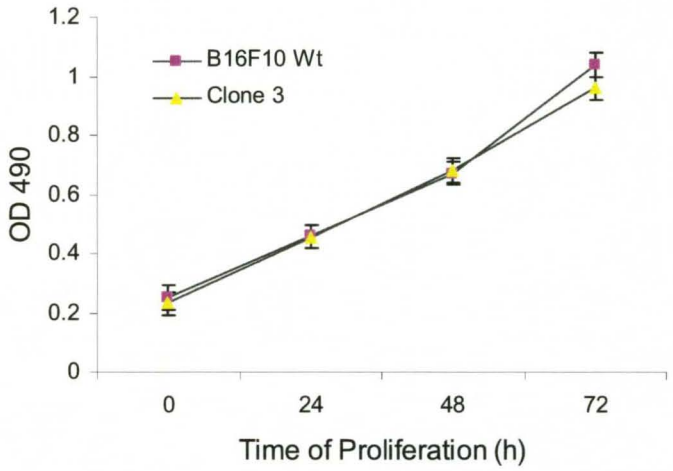
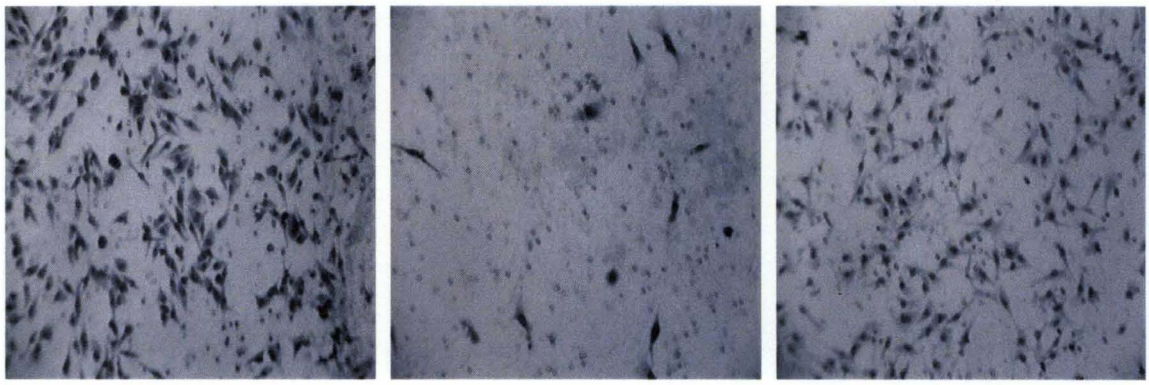
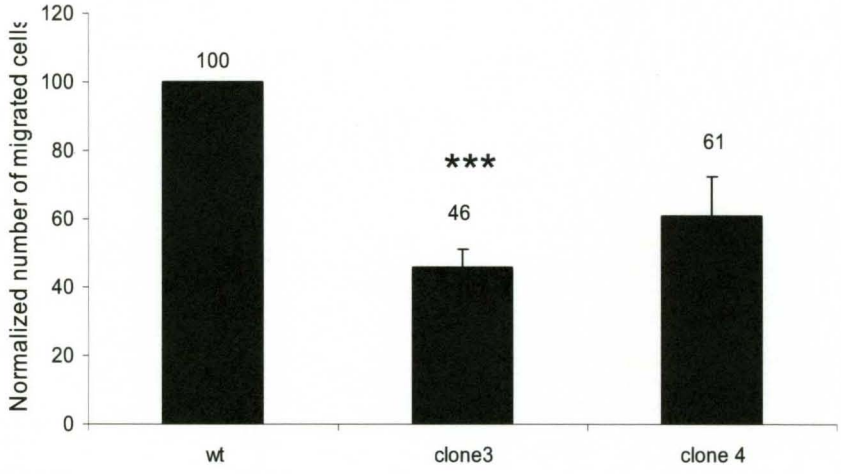


Figure 12. . Migration is inhibited in Clone 3 and Clone 4. (A) B16F10 while type cells and Clone 3 and 4 were harvested and loaded to the top chambers of transwells. Matrigel (20 μg/ml) was included in the bottom chamber as chemoattractant. After 24h at 37C unmigrated cells were removed and migrated cells were Giemsa stained. Representative images of migrated cells are shown. **(B)** Random images were taken for each transwell and the number of migrated cells in each image was quantified using the ImageJ software then averaged for each experimental group. The number of migrated cells in Clone 3 and Clone 4 were normalized to the number of migrated cells of B16F10 wild type. Error bar represents standard deviation of 4 independent experiments. **(C)** Expression of human VAMP3 rescues the inhibition of cell migration by VAMP3 shRNA. The motility of wild-type, Clone 3 and Clone 3 cells transfected with a plasmid that encodes human VAMP3 was compared using the transwell migration assay. Error bars represent standard deviation of 4 independent experiments. *** $P < 0.001$ vs. wild type B16F10 cells.

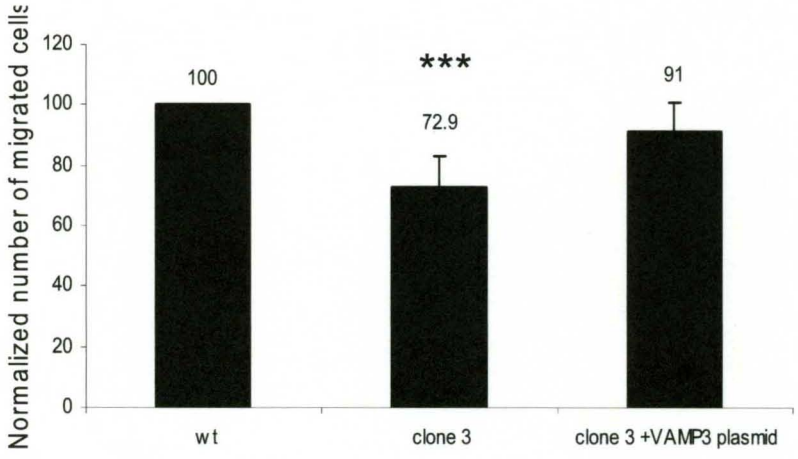
A


Wild type
Clone 3
Clone 4

B


Normalized number of migrated cells
120
100
80
60
40
20
0
100
***
46
61
wt
clone3
clone 4

C


Normalized number of migrated cells
120
100
80
60
40
20
0
100
***
72.9
91
wt
clone 3
clone 3 +VAMP3 plasmid

Different from the transwell migration assay, which examines chemotactic cell migration, wound healing assay (34) analyzes 2-dimensional random migration. The wound healing assay was used to analyze if VAMP3 silencing affects 2-dimensional migration (Fig 13). Monolayers of B16F10 wild type and Clone 3 cells were wounded, and migration of the cells into the wounded areas was photographed until the wounds were closed. Both the wild type and Clone 3 cells entered the wounded space within 24 h, but Clone 3 cells showed ~ 10% narrower wound. 48 h after wounding, both the wild type cells and Clone 3 cells have closed ~90% of the wounded areas. The slight difference in migration through the wound between the wild type and Clone 3 cells at 24 h, and the similar migration of both cells at 48 h, conclude no obvious difference of 2-dimensional migration between the two cell lines in the absence of chemoattractant.

Silencing of VAMP3 inhibits cell adhesion to fibronectin and Matrigel

Having shown that VAMP3 is required in chemotactic migration of B16F10 cells, I sought to measure the effect of VAMP3 knockdown on cell adhesion to the ECM proteins Matrigel and fibronectin. Compared with wild type cells, at 10 min, 30 min and 60 min, adhesion of Clone 3 cells to Matrigel was attenuated by 13%, 20% and 40%, respectively (Fig 14 A). At 60 min and 90 min, adhesion of Clone 3 cells to fibronectin was decreased by 35% and 32%, respectively (Fig 14 B). These data indicate that silencing of VAMP3 suppressed adhesion of melanoma cells to Matrigel and fibronectin.

Figure 13. Wound healing assay. B16F10 cells wild type and Clone 3 was seeded at 5 x 10^4 cells/well in a 6-well plate. At 90% confluency, a wound was made through the cell monolayers using a pipette tip. After 0, 6, 24 and 48 h, images were taken on a light microscope. Representative images of two experiments are shown.

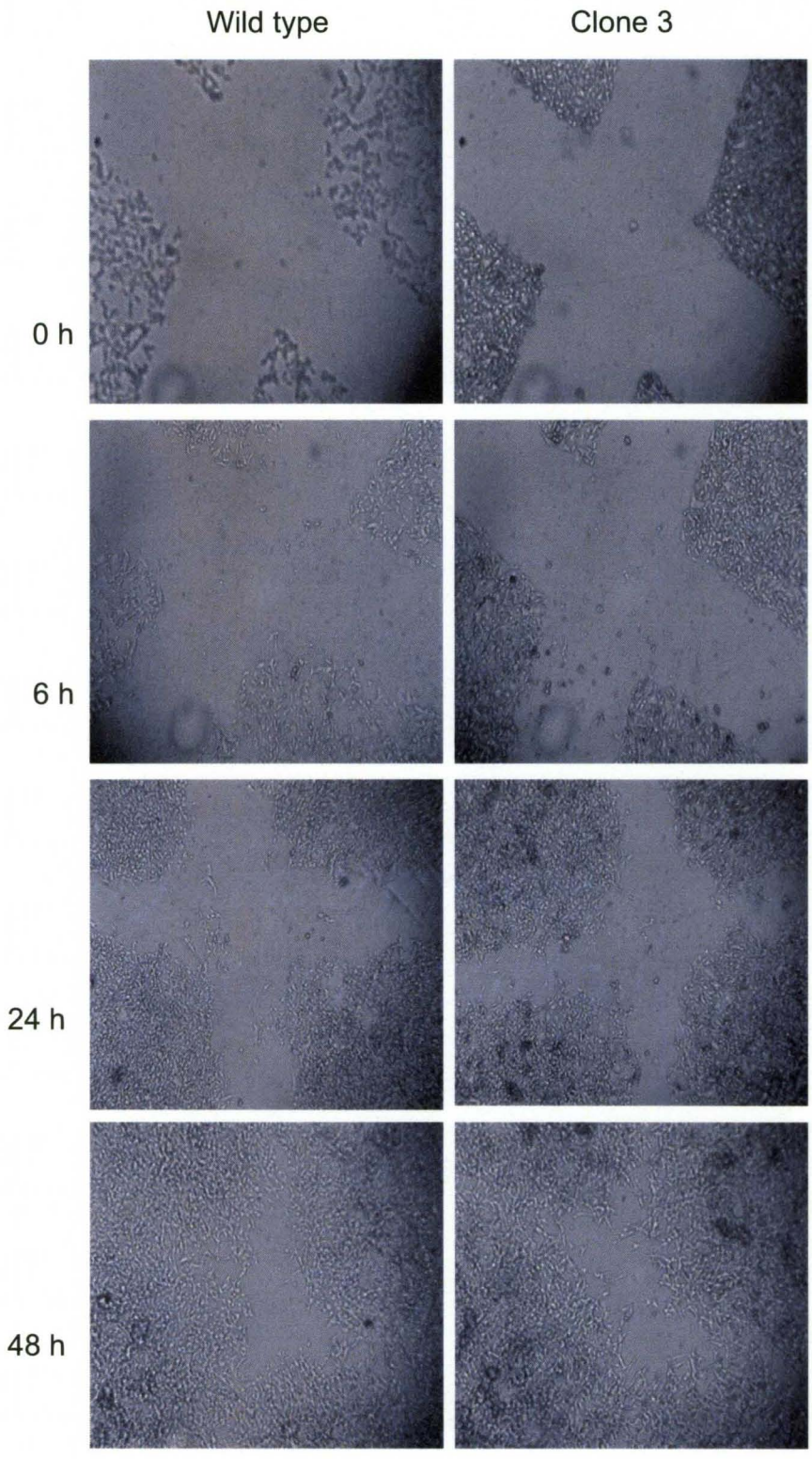
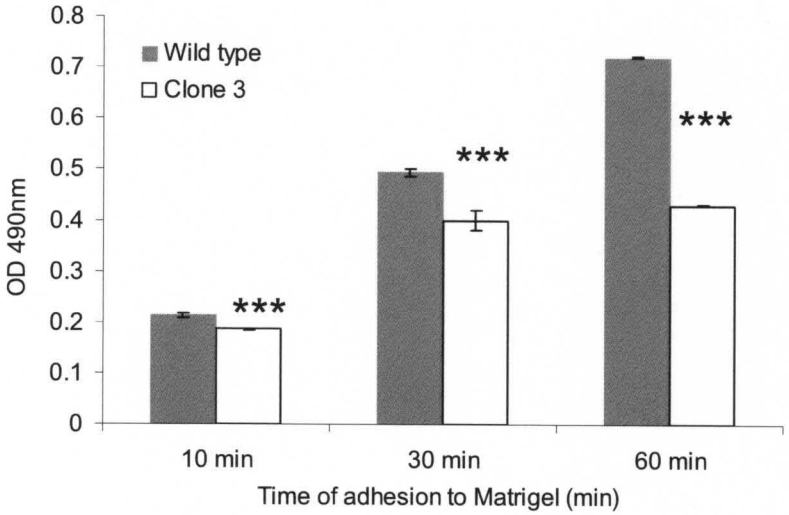
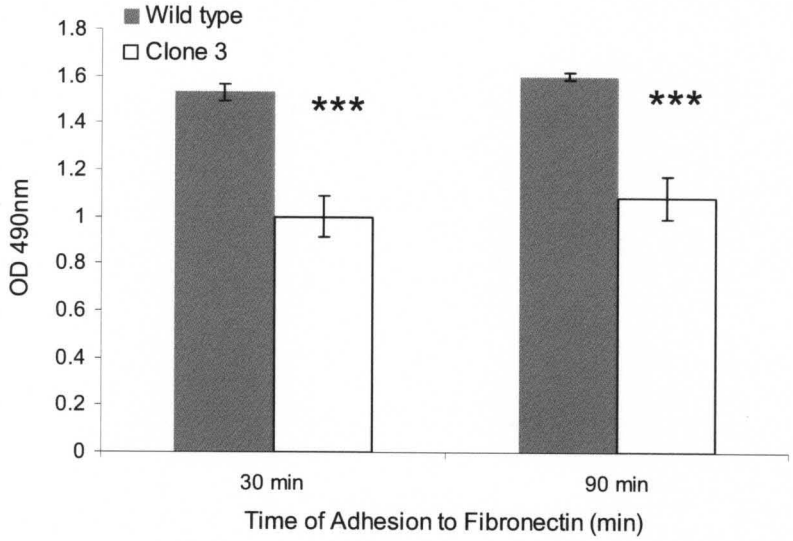

Wild type
Clone 3
0 h
6 h
24 h
48 h

Figure 14. Silencing of VAMP3 decreases cell adhesion to Matrigel and fibronectin. B16F10 wild type cells and clone3 were added to **(A)** Matrigel-coated plates **(B)** or fibronectin-coated plate. At different time points, unattached cells were gently washed away, and the number of adherent cells was measured using a colorimetric assay by absorbance at 490nm. Error bars represent standard deviation of two independent experiments. ***$P < 0.001$ vs. wild type B16F10 cells.

A


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 490nm
Wild type
Clone 3
***
***
***
10 min
30 min
60 min
Time of adhesion to Matrigel (min)

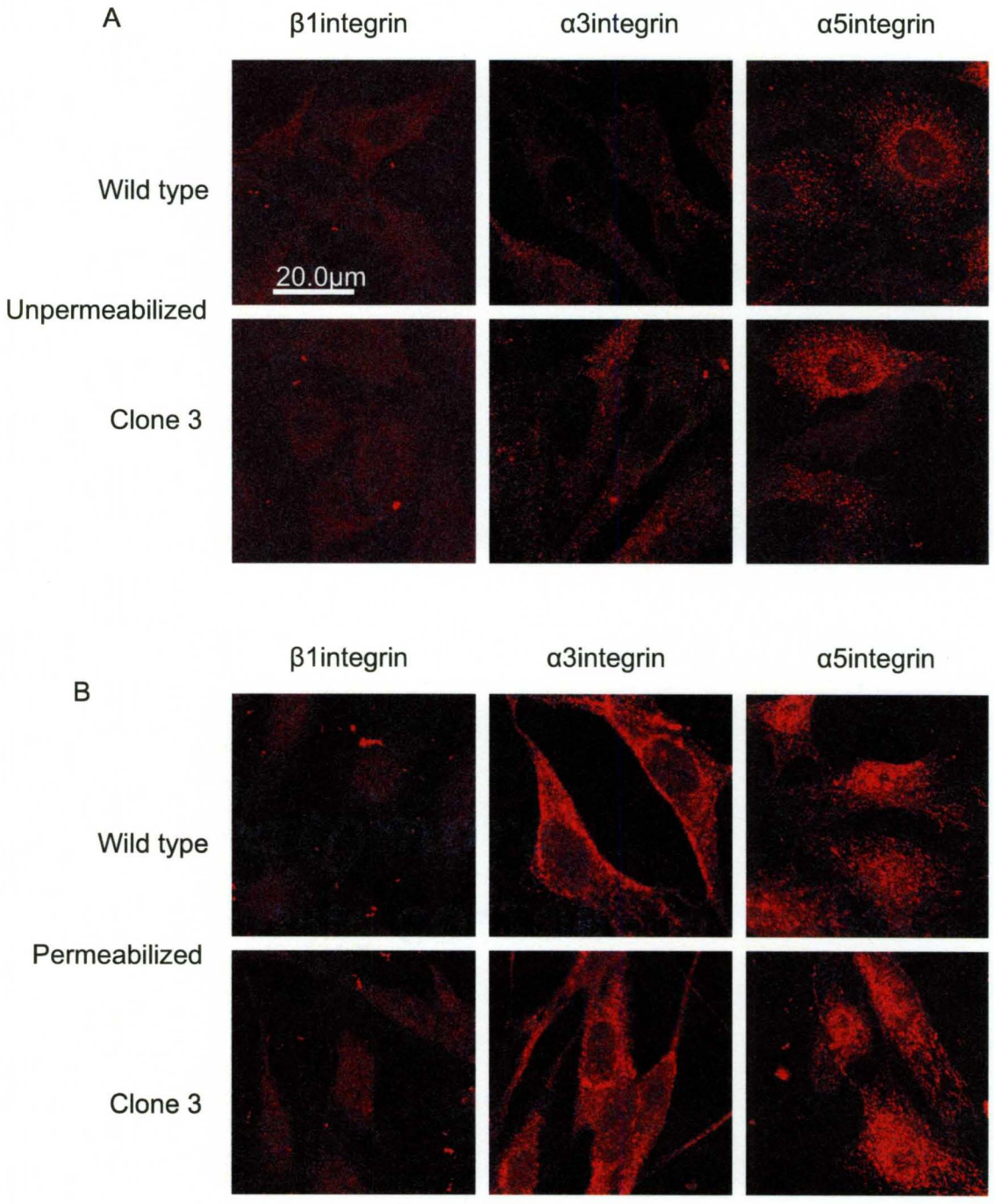
B


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 490nm
Wild type
Clone 3
***
***
30 min
90 min
Time of Adhesion to Fibronectin (min)

Effects of VAMP3 silencing on integrin trafficking in B16F10 cells

Cell adhesion to fibronectin is mediated by integrins. The chief components of Matrigel are ECM proteins such as laminin and collagen. In particular, the β 1 integrin pairs with α5, αV or α8 integrin subunits to form heterodimeric fibronectin receptors, with α3, α6 or α7 to form laminin receptors, or with α1, α2, α10 or α11 to form collagen receptors (4). The inhibitory effects of VAMP3 knockdown on adhesion to fibronectin and Matrigel suggest that VAMP3 knockdown disrupted the trafficking of integrins to the cell surface. We postulated that if VAMP3 mediates integrin trafficking, silencing of VAMP3 would reduce cell surface integrin expression and result in accumulation of integrins in intracellular vesicles. However, when B16F10 wild type and Clone 3 cells were stained with polyclonal antibodies against β1, α3 and α5 integrins (Fig 15 A and B), we were unable to detect obvious effects of VAMP3 depletion on subcellular distribution of those integrins. The best integrin antibodies that are used in our lab to stain α3, α5 and β1 (30, 35) are mouse monoclonal antibodies. Because B16F10 cells are mouse melanoma cells, those monoclonal antibodies were not suitable for the detection of integrins in B16F10 cells. The specificity of the rabbit and goat polyclonal antibodies used in (Fig. 15) needs further validation. To circumvent this problem to determine which integrin receptors are delivered by VAMP3, we decided to use the human cancer cell lines HeLa (cervical cancer) and H1299 (lung cancer).

Figure15. VAMP3 depletion has no obvious effect on integrins distribution. (A) Unpermeabilized B16F10 wild type and Clone 3 cells were stained with polyclonal antibodies to β1, α3 or α5 integrins. **(B)** Permeabilized B16F10 wild type and clone 3 cells were stained with polyclonal antibodies to β1, α3 or α5 integrins. Bar represents 20.0 μm. Representative confocal images of five experiments are shown.


A
β1integrin
α3integrin
α5integrin
Wild type
20.0μm
Unpermeabilized
Clone 3
B
β1integrin
α3integrin
α5integrin
Wild type
Permeabilized
Clone 3

ii. Determine the role of VAMP3 in integrin trafficking in HeLa and H1299 cells

Silencing of VAMP3 inhibits cell migration

siRNAs against VAMP3 were used to silence VAMP3 in HeLa cells. Immunoblotting analysis showed that within 72 h after siRNA transfection, the expression of VAMP3 protein in HeLa cells was depleted by more than 95% (Fig 16 A). Transfection of VAMP3 siRNA did not modulate the expression of β-actin.

The effect of VAMP3 knockdown on HeLa cell mobility was analyzed using the transwell migration assay and matrigel as the chemoattractant. When loaded to the upper chambers, HeLa cells migrated efficiently across the membranes (Fig 16 B). HeLa cells transfected with VAMP3 siRNA had much reduced mobility. Compared with the cells transfected with a non-targeting control siRNA, the number of migrated VAMP3 knockdown cells decreased by 65% (Fig 16 C), indicating that VAMP3 was required in the migration of HeLa cells.

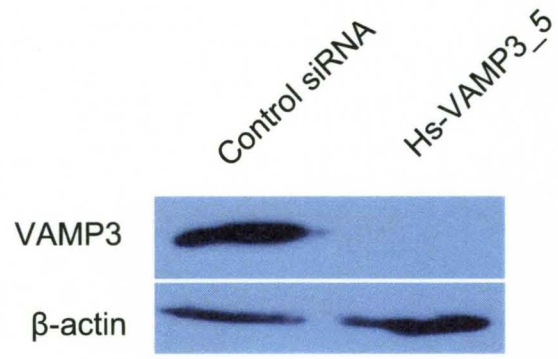
Silencing of VAMP3 inhibits cell adhesion to fibronectin and laminin

The next step was to determine the effects of VAMP3 silencing on HeLa cell adhesion to ECM proteins. At 10 min, 20 min and 30 min, VAMP3 silencing inhibited cell adhesion to fibronectin by 10%, 20% and 35% respectively (Fig 17 A). In addition, VAMP3 silencing inhibited adhesion to laminin by 20-25 % (Fig 17 B). These data suggested that VAMP3 participated in the trafficking of the integrin receptors for fibronectin and laminin.

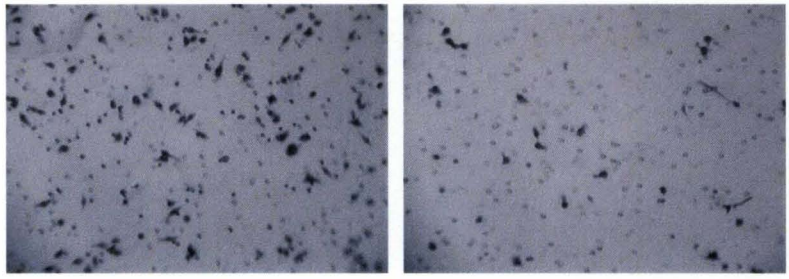
Figure 16. VAMP3 knockdown inhibits migration of HeLa cells. (A) HeLa cells were transfected with negative siRNA control or a siRNA against VAMP3 (Hs-VAMP3_5).

72h after transfection whole cell lysates were immunoblotted with antibodies to VAMP3. The same membrane was blotted with antibodies to β-actin as a loading control. **(B)** 72h after transfection with negative siRNA control or VAMP3 siRNA, HeLa cells were harvested and loaded in the top chamber of transwells. Matrigel (20μg/ml) was included in the lower chamber as chemoattractant. After 24h at 37◦C unmigrated cells were removed while the migrated cells were Giemsa stained. Representative images of the migrated cells are shown. **(C)** Random images were taken for each transwell, and the number of migrated cells in each image was quantified using the ImageJ software then averaged for each experimental group. The number of migrated cells transfected with VAMP3 siRNA was normalized to the number of migrated cells transfected with negative siRNA control. Error bar represents standard deviation of two independent experiments in which two transwells were used for each transfection.

A


Control siRNA
Hs-VAMP3_5
VAMP3
β-actin

B


Control siRNA
Hs-VAMP3_5

C

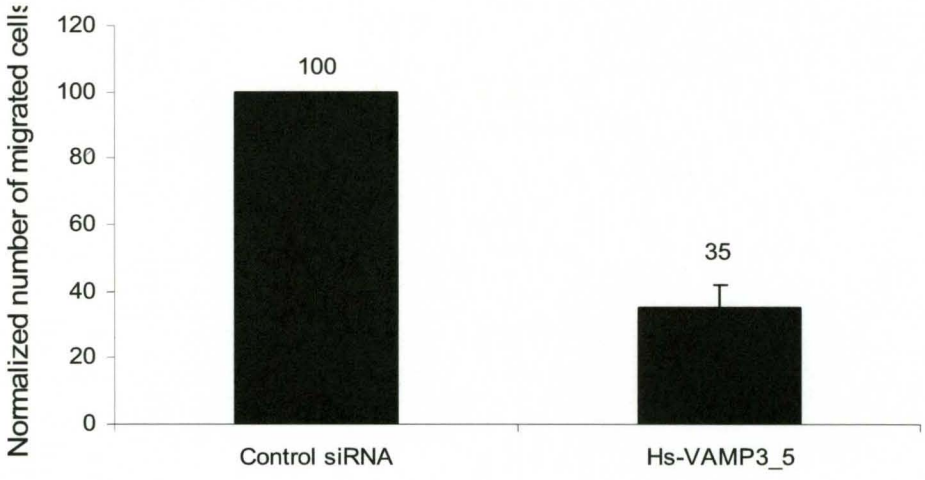
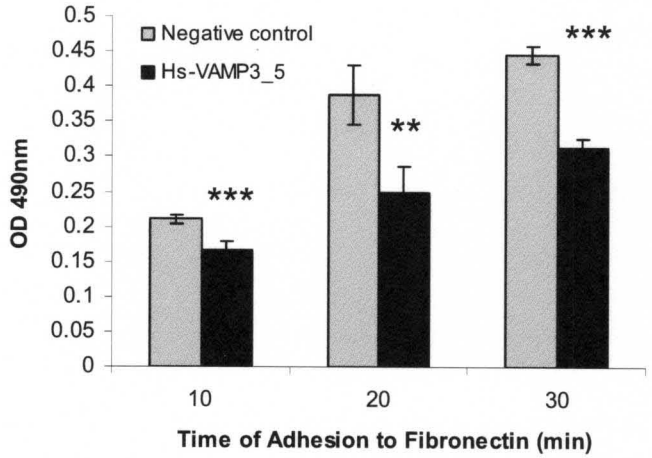
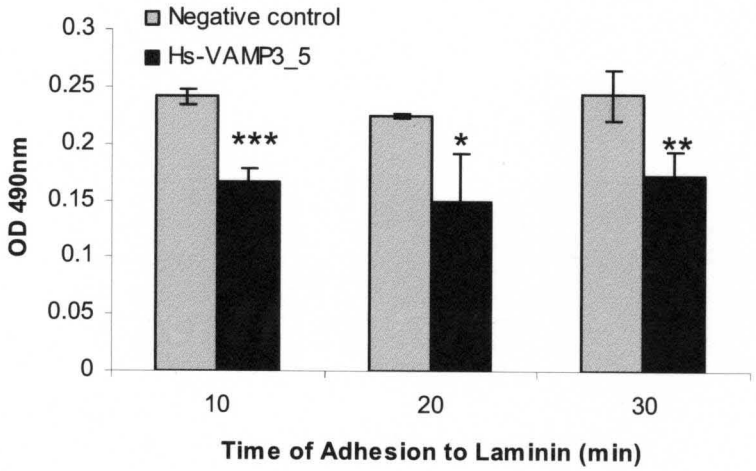

Normalized number of migrated cells
120
100
80
60
40
20
0
100
35
Control siRNA
Hs-VAMP3_5

Figure 17. Effects of VAMP3 knockdown on the adhesion of HeLa cells to fibronectin and laminin. 72h after transfection with negative control siRNA (gray columns) or VAMP3 siRNA (black columns), HeLa cells were added to fibronectin-coated plates **(A)** or laminin-coated plates **(B).** At different time points, unattached cells were washed away, and the number of adherent cells was measured using a colorimetric assay by absorbance at 490nm. Error bars represent standard deviation of three independent experiments. * $P < 0.05$ vs. cells transfected with negative control siRNA, ** $P < 0.01$ vs. cells transfected with negative control siRNA, *** $P < 0.001$ vs. cells transfected with negative control siRNA.

A


Negative control
Hs-VAMP3_5
OD 490nm
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
***
**
***
10
20
30
Time of Adhesion to Fibronectin (min)

B


Negative control
Hs-VAMP3_5
OD 490nm
0.3
0.25
0.2
0.15
0.1
0.05
0
***
*
**
10
20
30
Time of Adhesion to Laminin (min)

VAMP3 is required for trafficking of β1, α3 and α5 integrins to the cell surface

To analyze the role of VAMP3 in vesicular trafficking of integrins, we determined the effects of VANP3 silencing on subcellular distribution of α3β1 and α5β1 integrins by immunostainig and confocal microscopy. Silencing of VAMP3 in HeLa cells resulted in the accumulation of β1, α3 and α5 integrins in intracellular vesicles as shown by staining of permeabilized cells (Fig 18 A). Surprisingly, VAMP3 knockdown had no obvious effects on cell surface expression of β1, α3 and α5 integrins (Fig 18 B). The accumulation of β1, α3 and α5 integrins inside the cells indicated that VAMP3 knockdown disrupted the trafficking of β1, α3 and α5 integrins to the plasma membrane, suggesting that VAMP3 mediates the delivery of α3β1 and α5β1 inetgrins to the cell surface. Disrupted integrin trafficking is consistent with reduced chemotactic migration and adhesion to fibronectin (a α5β1 ligand) and laminin (a α3β1 ligand) of VAMP3 knockdown in HeLa cells (Figs 16 C and 17) in the VAMP3 knockdown cells.

Further investigation was performed to track integrins inside the VAMP3 knockdown cells. Permeabilized HeLa cells transfected with the control siRNA or VAMP3 siRNA were immunostained for β1, α3 and α5 integrins after incubation with Lyso Tracker Red DND-99 which tags the lysosomes. As shown by confocal microscopy (Fig 19), in VAMP3 knockdown cells a large fraction 40%, 60% and 20% of α3, α5 and β1 integrins (respectively) were localized in lysosomes. In the control cells (Fig. 19), few lysosomes were detected, whereas in the VAMP3 knockdown cells, the number of lysosomes was increased at least

Figure 18. VAMP3 is required for the trafficking of α3, α5 and β1 integrins to the cell surface. (A) 72h after transfection with negative siRNA control or VAMP3 siRNA, permeabilized HeLa cells were stained with monoclonal antibodies to α3, α5 and β1 integrins. Representative Confocal images of two experiments are shown. **(B)** Unpermeabilized HeLa cells were stained with monoclonal antibodies to α3, α5 and β1 integrins. Representative confocal images of two experiments are shown. Arrows indicate intracellular vesicles.

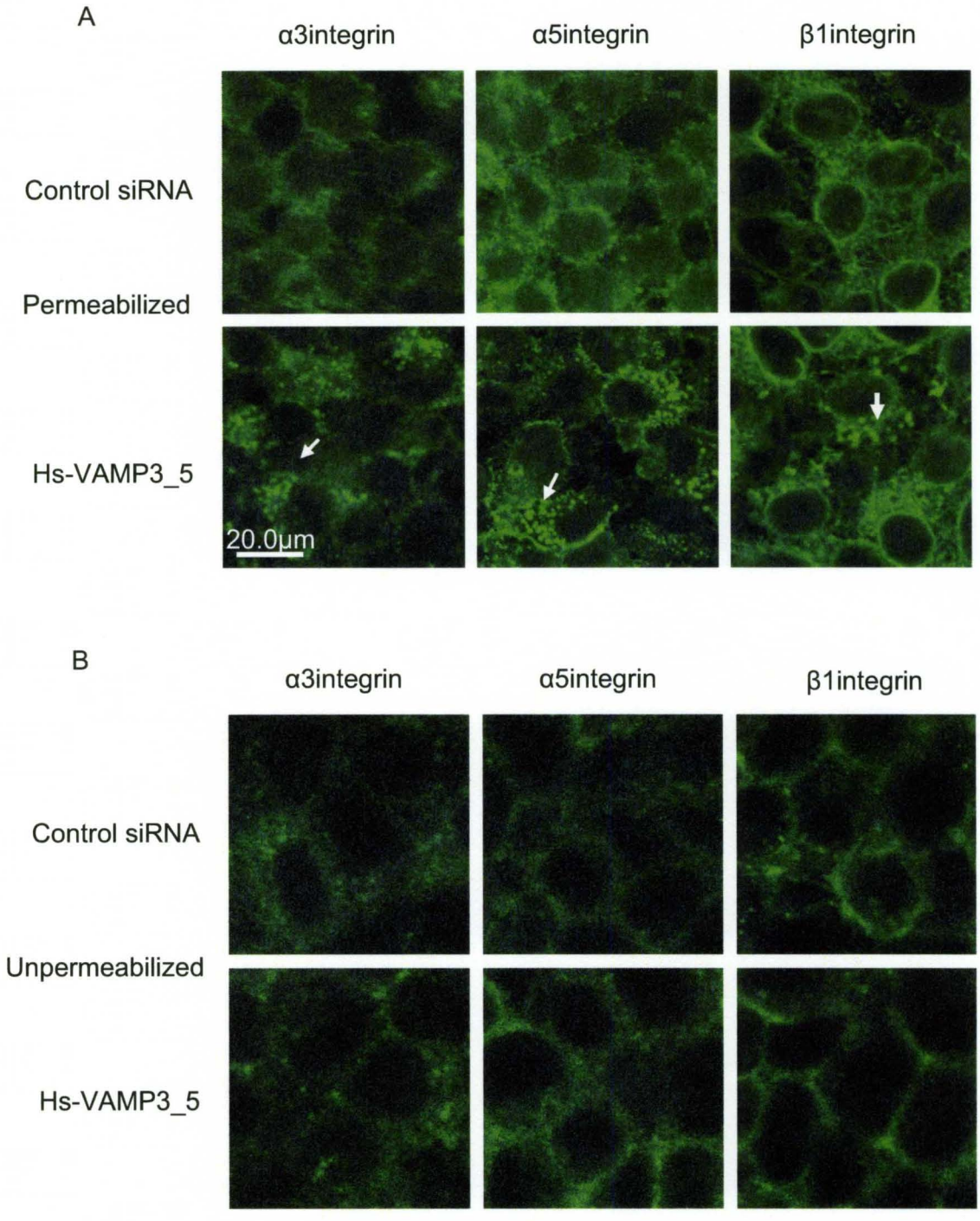
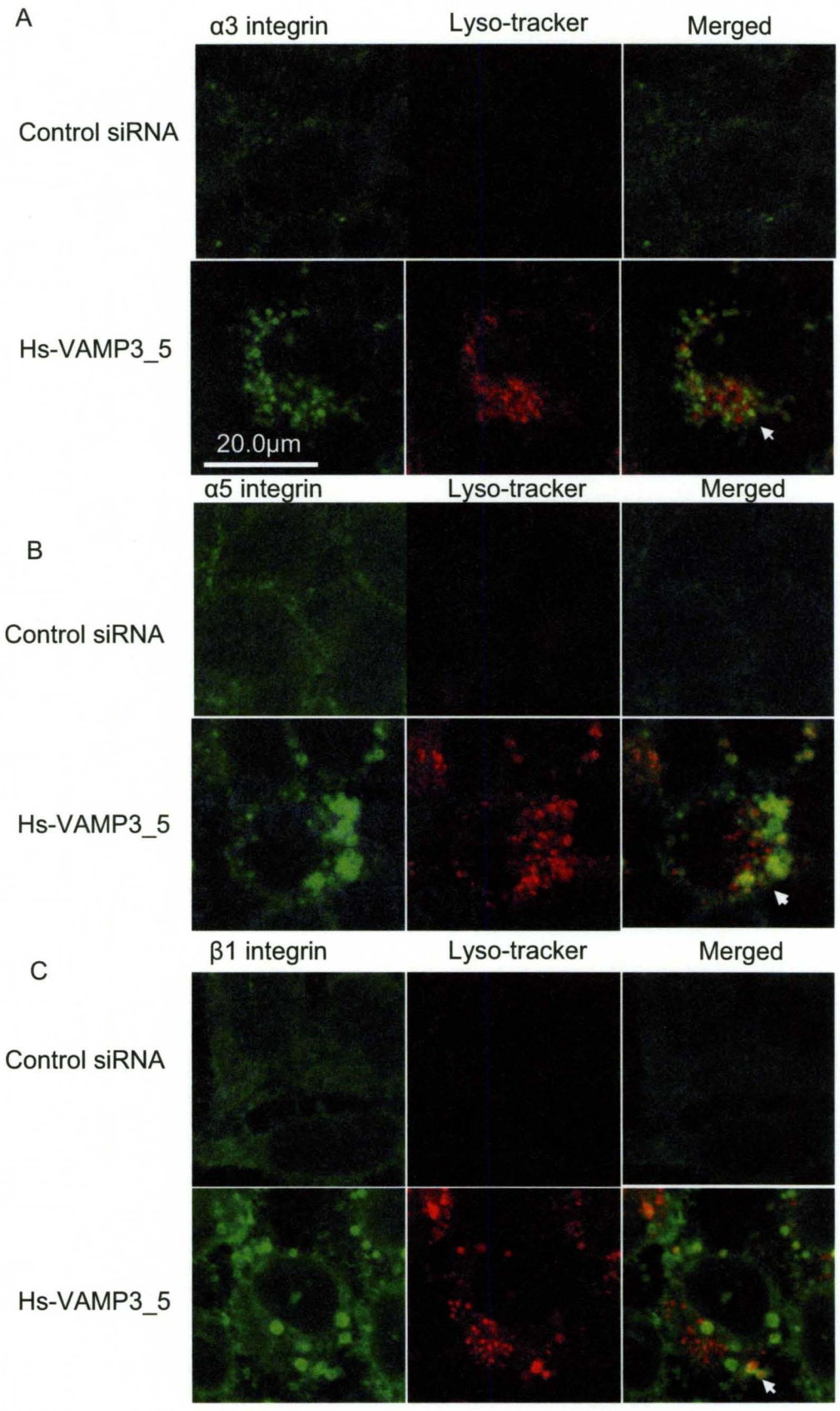

A
α3integrin
α5integrin
β1integrin
Control siRNA
Permeabilized
Hs-VAMP3_5
20.0μm
B
α3integrin
α5integrin
β1integrin
Control siRNA
Unpermeabilized
Hs-VAMP3_5

Figure 19. In VAMP3 knockdown cells integrins are accumulated in lysosomes. 72h after transfection with negative control or VAMP3 siRNAs, HeLa cells was incubated with Lyso Tracker Red DND-99, fixed, permeabilized and stained with antibodies (green) to α3 integrin **(A)**, α5 integrin **(B)** or β1 integrins **(C).** Representative confocal images of two experiments are shown. Arrows indicate integrins localization to lysosomes.


A
α3 integrin
Lyso-tracker
Merged
Control siRNA
Hs-VAMP3_5
20.0μm
α5 integrin
Lyso-tracker
Merged
B
Control siRNA
Hs-VAMP3_5
β1 integrin
Lyso-tracker
Merged
C
Control siRNA
Hs-VAMP3_5

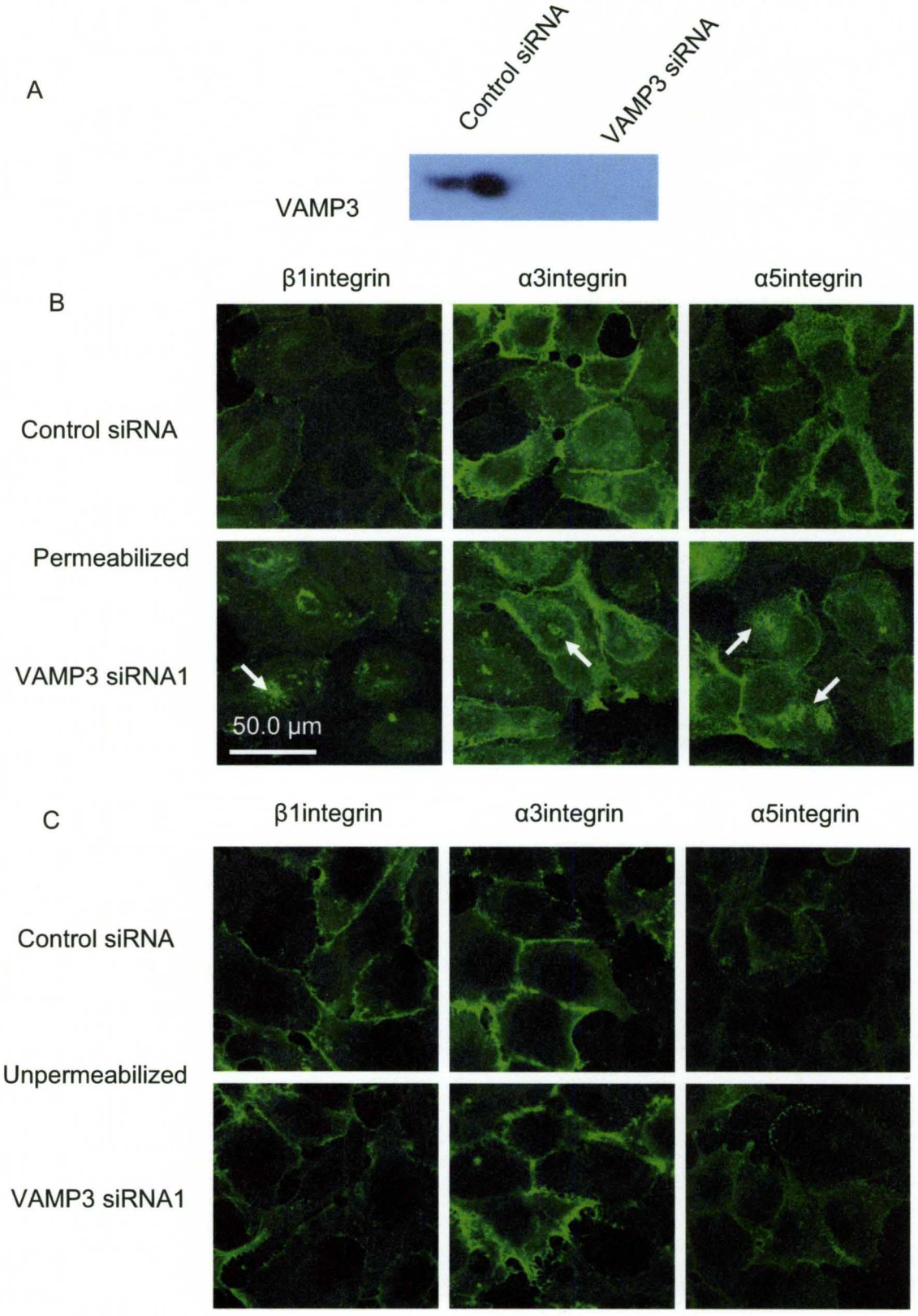
several folds. Together, these data suggested that when trafficking of β1, α3 and α5 integrins to the plasma membrane is inhibited, the integrins are targeted to lysosomes for degradation.

To determine if VAMP3 mediates the trafficking of α3β1 and α5β1 integrins in other cells, the H1299 lung cancer cell line was used. Immunoblotting analysis showed that within 72 h after transfection of siRNA against VAMP3, the expression of VAMP3 protein in H1299 cells was depleted by more than 95% (Fig 20 A). Like HeLa cells, silencing of VAMP3 in H1299 cells led to the accumulation of β1, α3 and α5 integrins in intracellular vesicles (arrows in Fig 20 B). In contrast, VAMP3 knockdown had no effect on cell surface expression of β1, α3 and α5 integrins (Fig 20 B & C). The accumulation of β1, α3 and α5 integrins inside the VAMP3 knockdown cells indicates that VAMP3 mediates the trafficking of β1, α3 and α5 integrins in H1299 cells.

iii. Examine the role of VAMP3 in melanoma metastasis *in vivo*

VAMP3 is over expressed in melanoma (Fig. 7). Data presented in this thesis suggest that silencing of VAMP3 inhibited chemotactic migration of melanoma cells in vitro (Fig. 8 and 12). These findings prompted us to examine whether metastasis of melanoma in vivo can be inhibited by silencing VAMP3. B16F10 melanoma cells are widely used as a syngeneic model to study melanoma metastasis. Preliminary studies in our lab have showed that B16F10 cells injected in the tail vein of nude mice metastasize to the lung and form tumor lesions. In the present study we used nude mice to study the effects of VAMP3 silencing on the growth and metastasis of B16F10 tumor cells to the lung.

Figure 20: VAMP3 is involved in the trafficking of β1, α3 and α5 integrins in H1299 cells. **(A)** 72h after transfection with negative control siRNA or VAMP3 siRNA 1, permeabilized H1299 cells were stained with antibodies to β1, α3 or α5 integrins. Representative Confocal images of two experiments are shown. **(B)** Staining of unpermeabilized H1299 cells show no effect of VAMP3 knockdown on surface β1, α3 or α5 integrins. Representative Confocal images of two experiments are shown. Arrows indicates intracellular vesicles.


A
Control siRNA
VAMP3 siRNA
VAMP3
B
β1integrin
α3integrin
α5integrin
Control siRNA
Permeabilized
VAMP3 siRNA1
50.0 μm
C
β1integrin
α3integrin
α5integrin
Control siRNA
Unpermeabilized
VAMP3 siRNA1

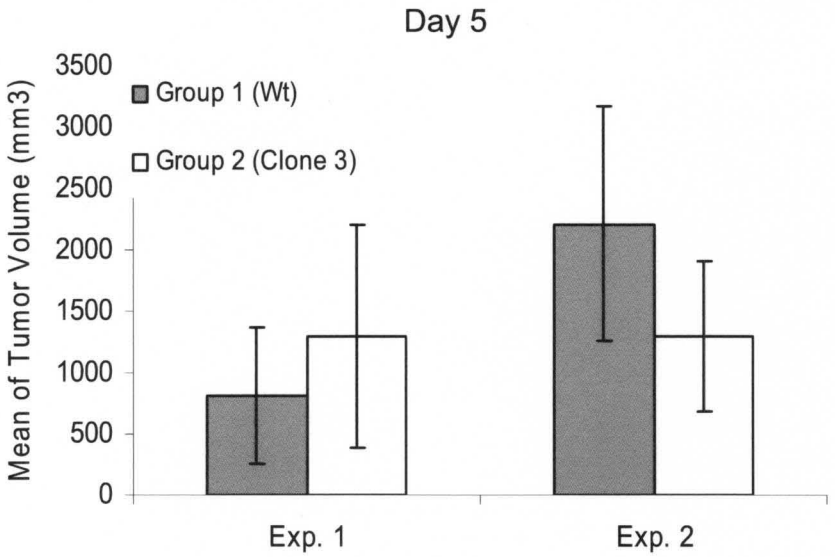
VAMP3 knockdown had no effect on the growth of melanoma

To investigate if tumor growth *in vivo* is affected by VAMP3 knockdown, parental B16F10 and the Clone 3 cells that express a VAMP3 shRNA were injected subcutaneously into the anterior flank of nude mice. B16F10 cells form tumor rapidly. Within 5 days after injection, measurable tumors were detected (Fig. 21). 12 days after injection most tumors have reached 1.5 cm in diameter, and the experiment had to be terminated. In two independent experiments, no statistically significant differences were observed between the Clone 3 tumors and the wild-type B16F10 tumors (Fig 21), suggesting that silencing of VAMP3 has no effect on melanoma tumor growth. These results are consistent with the observation that VAMP3 knockdown has no effect on B16F10 proliferation in vitro (Fig. 11).

Effects of VAMP3 silencing on melanoma metastasis

The metastatic B16F10 melanoma cells injected through the tail vein of nude mice results in pulmonary metastases (36). In preliminary experiments, we found that two weeks after injection of wild type B16F10 cells, lung metastases were developed in nude mice (data not shown). To test the possible inhibitory effect of VAMP3 knockdown on metastasis, the same number of B16F10 and Clone 3 cells were injected into nude mice. Two weeks later, the mice were sacrificed and the lung tissues were collected and examined. In the first experiment, (Fig 22 A) all three mice injected with B16F10 cells developed pulmonary metastases, whereas only two out of four mice received the Clone 3 cells developed metastases,

Figure 21: Subcutaneous tumor growth assay. Nude mice model was used to investigate whether tumor growth in vivo can be inhibited by VAMP3 knockdown. B16F10 cells and Clone 3 expressing VAMP3 knockdown were implanted subcutaneously into the anterior flank skin of nude mice, and tumor growth was compared in the two groups of animals (each group include 5 mice). In two independent experiments, measurable tumors were detected within 5 days after injection, (Fig. 21). 12 days after injection most tumors have reached the size of 1.5 cm in diameter, and the experiment had to be terminated. . Tumors were measured by calipers and the tumor's volume was calculated using the following formula: Tumor volume (mm^3) = ½ (Length x width^2). No significant effect of VAMP3 knockdown on tumor growth was observed. ***P <0.001 vs. the mice injected with wild type B16F10 cells.


Day 5
Mean of Tumor Volume (mm3)
3500
3000
2500
2000
1500
1000
500
0
Group 1 (Wt)
Group 2 (Clone 3)
Exp. 1
Exp. 2

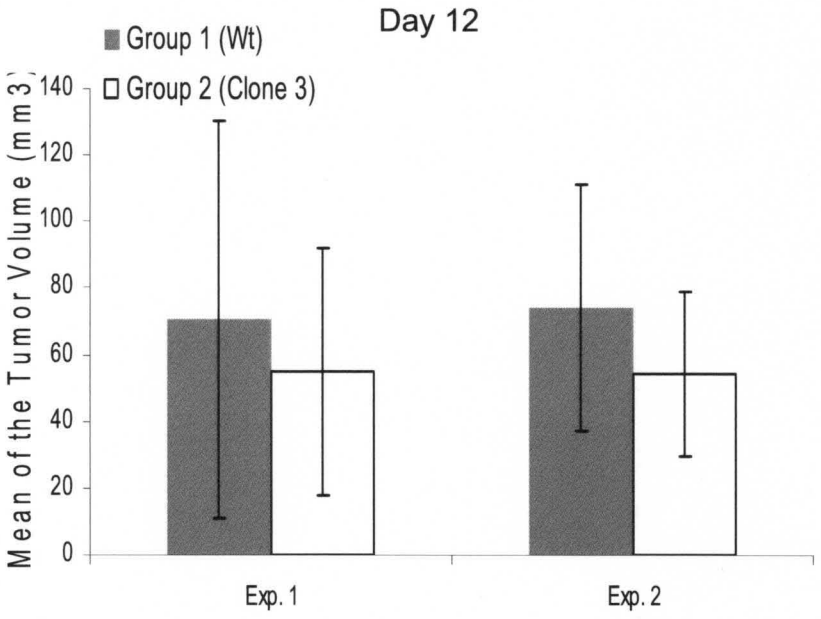
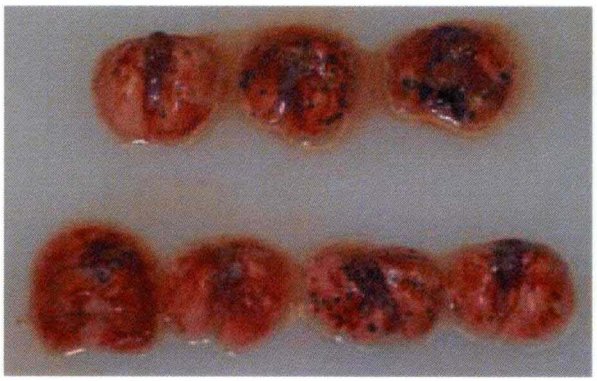

Day 12
Group 1 (Wt)
Group 2 (Clone 3)
Mean of the Tumor Volume (mm3)
140
120
100
80
60
40
20
0
Exp. 1
Exp. 2

Figure 22. Experimental lung metastasis assay. B16F10 wild type cells and Clone 3 were injected into the tail veins of a group of nude mice. Each cell line was injected in 4 mice in two independent experiments. Two weeks after tumor cell injection, mice were sacrificed to examine the inhibitory effect on lung metastasis of VAMP3 knockdown. Representative lungs are shown in (Fig 22 A and B). Extensive tumor metastasis was found in the wild type and Clone 3 groups and the lungs from mice injected with both cell lines showed cancer lesions indicated by arrows.

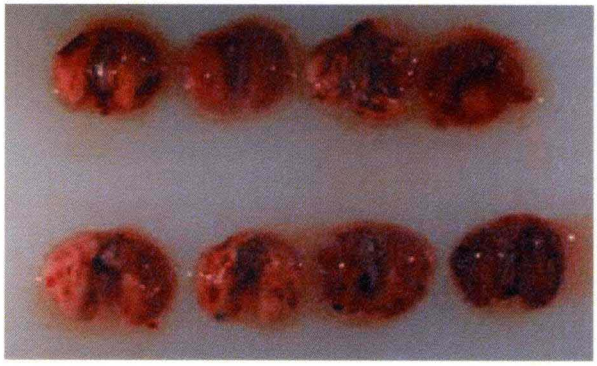
A



Wild type (3/3)

Clone 3 (2/4)

B



Wild type (4/4)

Clone 3 (4/4)

suggesting that in this experiment silencing of VAMP3 may have an inhibitory effect on metastasis of melanoma to the lung. However, in another experiment (Fig. 22 B), Clone 3 cells metastasized to the lung comparable to wild type B16F10 cells. From these experiments we conclude that melanoma cells can still metastasize even when VAMP3 is depleted. Further experiments are needed to determine quantitatively if VAMP3 knockdown has inhibitory effects on metastasis. In addition, we may need to sacrifice the mice at a shorter time point to detect any differences between the two cell lines before they reach the same degree of metastasis.

Discussion

In migrating cells, integrins are transported in vesicles and exocytosed actively at the cell front (37). Although it is well established that SNAREs mediate intracellular vesicle fusion (18, 19), it is not clear which SNARE proteins mediate the trafficking of integrins to the plasma membrane. Using RNA interference, we show here that silencing of the v-SNARE VAMP3 effectively inhibit both melanoma cell migration, as well as, melanoma cell adhesion to Matrigel and fibronectin without affecting cell proliferation. VAMP3 silencing led to the accumulation of β1, α3 and α5 integrins in vesicles in HeLa and H1299 cells, indicating that VAMP3 is required for trafficking of these integrins to the plasma membrane. Together, these data suggest that VAMP3 plays an important role in migration and adhesion of melanoma cells and that VAMP3 mediates the trafficking of α3β1 and α5β1 integrins.

By expression of catalytic light chain of tetanus toxin (TeTx-LC), a protease that specifically cleaves and inactivates VAMP1, VAMP2 and VAMP3, several studies show

that inhibition of VAMP3 function interferes with cell motility and integrin functions. In CHO cells, expression of TeTx-LC impairs cell migration, reduces cell surface α5β1 integrin, and inhibits cell spreading but not adhering to fibronectin (16, 28). In MDCK cells, TeTx-LC expression reduces cell motility and disrupts the recycling of β1 integrin (29). Since among the three VAMP proteins, VAMP3 is the only one expressed in CHO cells and MDCK cells (16, 29), these results suggest that VAMP3 participates in integrin trafficking and cell migration. In the present study, we used shRNA and siRNA to silence VAMP3 and found that VAMP3 silencing inhibited the migration of B16F10 and HeLa cells by more than 60% and effectively disrupted integrin trafficking. This work provides further evidence that VAMP3-dependent integrin trafficking plays an important role in cell migration. Although trafficking of α3β1 and α5β1 integrin is disrupted, cell surface expression of the integrins in the VAMP3 knockdown cells is not altered. This observation is not surprising, since our lab has shown that other v-SNAREs such as VAMP2 also mediates vesicle trafficking of integrins to the cell surface.

Like other receptors, integrins at the cell surface are continually endocytosed from the plasma membrane, transported into endosomal compartments and then recycled back to the cell surface (38). Most endocytosed integrin molecules are recycled, rather than being targeted to lysosomes for degradation (3, 12). The endocytosis and recycling of integrins is particularly important during cell migration. In migrating cells, integrins act as the "feet" to support cell adhesion to the ECM. The net forward movement of the cell leads to an accumulation of integrins towards the cell rear. To provide a fresh source of integrins at the cell front, integrins are endocytosed and recycled to the leading edge (9, 10). This thesis indicates that although cell surface expression of integrins in VAMP3

knockdown cells is not altered, cell migration is inhibited dramatically. These data are in accordance with recent findings from other groups showing that vesicle trafficking of integrins but not the levels of integrins at the cell surface plays a major role in cell migration (4). These data emphasize the importance of VAMP3-mediated integrin trafficking in cell migration.

VAMP3 is over expressed in melanoma. To our knowledge, this is the first systematic study on the role of VAMP3 in migration of melanoma cells. Our data indicate that VAMP3 is required for chemotactic migration of melanoma cells *in vitro*. However, *in vivo* analysis using the experimental lung metastasis assay has yielded variable results. One experiment showed that VAMP3 knockdown inhibited melanoma metastasis to the lung, whereas another experiment showed no such effect. To metastasize to the lung, the B16F10 cells need to survive in the blood stream, migrate out of the blood vessels and form colonies in the lung. The fact that we detect pulmonary metastases of melanoma cells depleted of VAMP3 indicates that VAMP3 is not required for the aforementioned processes. More experiments are needed to determine if there is a quantitative difference of metastasis between wild type and VAMP3-depleted melanoma cells.

CHAPTER III: METHODS

Cell culture

B16-F10, H1299 and HeLa cells were obtained from the American Type Culture Collection (ATCC). B16-F10 cells were cultured in Dulbecco's Modified Eagle medium (DMEM) with 4.5 g/l glucose and 10% fetal bovine serum (FBS). H1299 cells were cultured in RPMI medium 1640 with 2.5 g/L glucose, 1% sodium pyruvate, 1 mM HEPES and 10% FBS. HeLa cells were cultured in Minimum Essential Medium α (MEMα) media with 10% FBS. The cells were maintained at 37°C with 5% CO_2. All cell culture media were manyfactured by Invitrogen.

To split cells, the culture medium was aspirated, and the cell culture dishes were washed once with PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) containing 0.5 mM EDTA (PBS-EDTA). 2 ml of trypsin (0.05% in PBS-EDTA) was added to each dish. After incubation at 37°C for 3-5 min, 10 ml of complete medium was added to quench the trypsin reaction and the cells were dispersed by repeated pipetting. The cells were transferred to 15 ml conical tubes, and centrifuged at 200 g for 10 min. The medium was aspirated from the conical tubes and the cells were re-suspended gently in culture medium and plated.

To store cells, cells were trypsinized and centrifuged as above, re-suspended at a density of 1×10^6-1×10^7 cells/ml in FBS supplemented with 5% DMSO (Sigma), and transferred to cryovials. The vials were placed in a Nalgene Cryo freezing container,

frozen at -80°C for 24 h, and then transferred to a liquid nitrogen container. To recover, cells were thawed by warming the vials at 37°C until the frozen pellet had nearly melted. The cells were slowly added to a 15 ml conical tube containing 10 ml of culture medium. Cells were then centrifuged and plated as above.

Transfection of plasmids and siRNAs

The scramble shRNA, and the shRNAs 50011515 and 50011516 which target mouse VAMP3 were obtained from Sigma. A 21 nucleotide siRNA oligo that targets human VAMP3 sequence TCAAGCTTACCTACTGTTA was synthesized by Dharmacon Thermo Scientific. The AllStars Negative control siRNA was obtained from QIAGEN. The day before transfection, B16-F10, HeLa or H1299 cells were seeded into 6-well plates (5×10^5 cells per well) or 24-well plates (5×10^5 cells per well). 1 μg of shRNAs were transfected into the cells grown in each well of 6-well plates using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). The control and VAMP3 siRNAs were transfected at 10 nM using Lipofectamine RNAiMAX.

Immunoblotting

Whole cell lysates were obtained by lysing cells in 2× SDS-PAGE sample buffer. 45 μl of whole cell lysates were loaded in each well of 15% SDS-PAGE gels. After electrophoresis, proteins were transferred to Hybond-LFP (PVDF) membranes (GEHealthcare) using 20V overnight at 4°C or to Trans-Blot Nitrocellulose Membranes (BIO-RAD) at 0.25A at room temperature for 2 h. The membranes were preincubated with 5% Milk in TBS (20 mM Tris-HCl pH7.6, 140 mM NaCl) with agitation overnight

4°C or 1 h at room temperature. The membranes were then incubated with rabbit polyclonal antibodies to VAMP3 (Synaptic Systems, 1:1,000 dilution) for 2 h at room temperature. The membranes were washed three times with TBS and once with TBS-T (0.05% Tween-20 in TBS), and then incubated with HRP-conjugated goat anti rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, 1:10,000 dilution) for 1 h. After the antibody solution was removed, the membranes were washed as described above. Bound antibodies were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce). The membranes were exposed to Kodak BioMax autoradiography films, and films were developed using HOPE micro-max developer located in the Department of Biochemistry and Molecular Biology at the University of Louisville. The same membranes were labeled with a mouse monoclonal antibody to β-actin as loading control.

Development of stable cell lines that express VAMP3 shRNAs

B16-F10 cells were transfected with the shRNA 50011515 using Lipofectamin 2000. 72 h after transfection, the cells were splitted into selective DMEM medium containing 1 ug/ml puromycin. After two weeks, 24 clones were picked, transferred to 24-well plates and grown in puromycin-containing medium. Whole cell lysates of the clones were analyzed by immunoblotting for VAMP3 expression. Clones that had reduced VAMP3 expression were expanded. Clones 3 and 4 were chosen for further studies because they showed knockdown of VAMP3, but not β-actin.

Immunocytochemistry

The day before transfection, cells were seeded on sterile 12-mm glass cover slips contained in 24-well plates. 72 h after transfection, the cells were fixed with 4% paraformaldehyde in PBS++ (PBS supplemented with 0.1 g/l CaCl2 and 0.1 g/ l MgCl2) for 10 min. Cells in some wells were permeabilized with 0.2% Triton X-100 in PBS++ for 5 min. After washing, the cells were blocked in 10% FBS in PBS++ for 30 min. Primary antibodies were incubated with the cells at the following dilutions: anti-β1 integrin mouse monoclonal antibody P5D2, neat conditioned culture medium of the hybridoma cells; anti-α3 integrin mouse monoclonal antibody (Chemicon International), 1:50; anti-α5 integrin mouse monoclonal antibody (Chemicon International), 1:50; anti-α3 integrin goat polyclonal antibody (Santa Cruz Biotechnology), 1:50; anti-α5 integrin rabbit polyclonal antibody (Santa Cruz Biotechnology), 1:50. FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at a dilution of 1:500. For double staining, cells were incubated first with Lyso Tracker© Red DND-99 (Invitrogen) (50 nM or 100 nM) for 45 minutes before fixation. Confocal images were collected at 60× on an Olympus laser scanning confocal microscope located in the Department of Biochemistry and Molecular Biology. Images were processed with Adobe Photoshop software.

Cell adhesion assay

Cell adhesion assay was performed as described (35). Each well of 24-well plates was coated with 20 μg of fibronectin, Matrigel or laminin for 1 h at room temperature. The plates were washed once with PBS and blocked with 2% heat

inactivated BSA in PBS. 72 h after transfection, cells were harvested with trypsin/EDTA, counted with a hemacytometer and re-suspended in serum free medium supplemented with 0.5% BSA (SFM-BSA). 2×10^5 cells were added to each well and allowed to adhere to the coated plates for various time at 37 °C. After incubation, non-attached cells were removed by 3 gentle washes with SFM-BSA medium. Phenol red free medium and CellTiter 96 Aqueous One Solution Reagent (Promega) were then added to measure the number of living cells. After incubation at 37 °C for 90 min, absorbance of the medium at 490 nm was measured in a 96-well ELISA plate reader. Absorbance from wells containing only the medium but no cells was taken as blank reading.

Transwell migration assay

Transwell migration assay was performed as described (35).Serum-free medium (SFM) containing 20 μg/ml of growth factor-reduced Matrigel was added to the lower chambers of the 12-well format transwells (8 μm-pore, BD Biosciences) as chemoattractant. 72 h after transfection, cells were harvested with trypsin/EDTA, re-suspended in SFM, and added to the upper chambers at 5×10^4 cells per transwell. After 24 h at 37 °C, the transwells were fixed in methanol, and stained with Giemsa Stain solution (Sigma). The unmigrated cells were removed from the top of the membranes using cotton swabs. The membranes were detached from the transwells then affixed to glass slides using Permount mounting medium (Fisher Scientific). To quantify the number of migrated cells, five to ten random images were taken at 10× on a light microscope for each transwell. The number of migrated cells per image was counted using the Particle analysis function in ImageJ software.

Cell proliferation assay

B16F10 cells or Clone 3 cells were seeded in 24-well plates at 5×10^4 cells per well. 0, 24, 48, and 72 h later, cell culture medium was replaced with phenol red free medium, and the CellTiter 96 Aqueous One Solution Reagent was added to measure the number of living cells. After incubation at 37°C for 90 min, absorbance of the medium at 490 nm was measured in a 96-well ELISA plate reader. Absorbance from wells containing only the medium but no cells was taken as blank reading.

Wound healing assay

B16F10 cells or Clone 3 cells were seeded in 12-well plate at $5x10^4$ cells per well. After 48 h, a cross wound was made in the cell monolayers using a 200 μl pipette tip. The cells were washed once with cell culture medium to remove cellular debris and returned to 37°C culture. After 0, 6, 24 or 48 h, images of the wounded areas were taken using a light microscope.

Experimental pulmonary metastasis assay

The experimental pulmonary metastasis assay was performed as described (36) with modifications. 6 weeks old male nude mice were obtained from the National Cancer Institute and kept in barrier facility in the Delia Baxter Building. Wild-type B16-F10 and Clone 3 cells were detached from cell culture plates by trypsinization and suspended in PBS at 5×10^6 cells/ml. The cell suspension was injected into the tail's vein of nude mice using 27 gauge butterfly needles. Each mouse received approximately 0.1 ml of cell

suspension. The mice were checked every other day. After two weeks, the mice were euthanized and the lungs were harvested, examined and pictured for the presence of tumor metastases. Each experimental group consisted of 4-5 mice.

Subcutaneous tumor growth assay

Wild-type B16F10 and Clone 3 cells were injected subcutaneously into the anterior flank of nude mice at 1×10^6 cells/mice. The tumor diameters were measured once weekly with a calipers and the tumor volume was calculated using the formula: Tumor volume (mm^3) = 1/2(length × $width^2$) (39). Each experimental group consisted of 5 mice.

REFERENCES

1. Huber, M.A., Kraut, N., and Beug, H. 2005. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 17:548-558.
2. Chodniewicz, D., and Klemke, R.L. 2004. Guiding cell migration through directed extension and stabilization of pseudopodia. *Exp Cell Res* 301:31-37.
3. Bretscher, M.S. 1996. Getting membrane flow and the cytoskeleton to cooperate in moving cells. *Cell* 87:601-606.
4. Hynes, R.O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell* 110:673-687.
5. Caswell, P.T., Vadrevu, S., and Norman, J.C. 2009. Integrins: masters and slaves of endocytic transport. *Nat Rev Mol Cell Biol* 10:843-853.
6. Guo, W., and Giancotti, F.G. 2004. Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol* 5:816-826.
7. Lenter, M., and Vestweber, D. 1994. The integrin chains beta 1 and alpha 6 associate with the chaperone calnexin prior to integrin assembly. *J Biol Chem* 269:12263-12268.
8. Rigot, V., Andre, F., Lehmann, M., Lissitzky, J.C., Marvaldi, J., and Luis, J. 1999. Biogenesis of alpha6beta4 integrin in a human colonic adenocarcinoma cell line involvement of calnexin. *Eur J Biochem* 261:659-666.
9. Lawson, M.A., and Maxfield, F.R. 1995. Ca(2+)- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature* 377:75-79.
10. Laukaitis, C.M., Webb, D.J., Donais, K., and Horwitz, A.F. 2001. Differential dynamics of alpha 5 integrin, paxillin, and alpha-actinin during formation and disassembly of adhesions in migrating cells. *J Cell Biol* 153:1427-1440.
11. Woods, A.J., White, D.P., Caswell, P.T., and Norman, J.C. 2004. PKD1/PKCmu promotes alphavbeta3 integrin recycling and delivery to nascent focal adhesions. *EMBO J* 23:2531-2543.
12. Ng, T., Shima, D., Squire, A., Bastiaens, P.I., Gschmeissner, S., Humphries, M.J., and Parker, P.J. 1999. PKCalpha regulates beta1 integrin-dependent cell motility through association and control of integrin traffic. *EMBO J* 18:3909-3923.
13. Ivaska, J., Whelan, R.D., Watson, R., and Parker, P.J. 2002. PKC epsilon controls the traffic of beta1 integrins in motile cells. *EMBO J* 21:3608-3619.
14. Kuphal, S., Bauer, R., and Bosserhoff, A.K. 2005. Integrin signaling in malignant melanoma. *Cancer Metastasis Rev* 24:195-222.
15. Caswell, P.T., Spence, H.J., Parsons, M., White, D.P., Clark, K., Cheng, K.W., Mills, G.B., Humphries, M.J., Messent, A.J., Anderson, K.I., et al. 2007. Rab25 associates with alpha5beta1 integrin to promote invasive migration in 3D microenvironments. *Dev Cell* 13:496-510.
16. Tayeb, M.A., Skalski, M., Cha, M.C., Kean, M.J., Scaife, M., and Coppolino, M.G. 2005. Inhibition of SNARE-mediated membrane traffic impairs cell migration. *Exp Cell Res* 305:63-73.

17. Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362:318-324.
18. Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature* 372:55-63.
19. Jahn, R., and Scheller, R.H. 2006. SNAREs--engines for membrane fusion. *Nat Rev Mol Cell Biol* 7:631-643.
20. Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature* 395:347-353.
21. Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H., and Rothman, J.E. 1998. SNAREpins: minimal machinery for membrane fusion. *Cell* 92:759-772.
22. McNew, J.A., Weber, T., Parlati, F., Johnston, R.J., Melia, T.J., Sollner, T.H., and Rothman, J.E. 2000. Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors. *J Cell Biol* 150:105-117.
23. Hu, C., Ahmed, M., Melia, T.J., Sollner, T.H., Mayer, T., and Rothman, J.E. 2003. Fusion of cells by flipped SNAREs. *Science* 300:1745-1749.
24. Steegmaier, M., Klumperman, J., Foletti, D.L., Yoo, J.S., and Scheller, R.H. 1999. Vesicle-associated membrane protein 4 is implicated in trans-Golgi network vesicle trafficking. *Mol Biol Cell* 10:1957-1972.
25. McMahon, H.T., Ushkaryov, Y.A., Edelmann, L., Link, E., Binz, T., Niemann, H., Jahn, R., and Sudhof, T.C. 1993. Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature* 364:346-349.
26. Polgar, J., Chung, S.H., and Reed, G.L. 2002. Vesicle-associated membrane protein 3 (VAMP-3) and VAMP-8 are present in human platelets and are required for granule secretion. *Blood* 100:1081-1083.
27. Galli, T., Chilcote, T., Mundigl, O., Binz, T., Niemann, H., and De Camilli, P. 1994. Tetanus toxin-mediated cleavage of cellubrevin impairs exocytosis of transferrin receptor-containing vesicles in CHO cells. *J Cell Biol* 125:1015-1024.
28. Skalski, M., and Coppolino, M.G. 2005. SNARE-mediated trafficking of alpha5beta1 integrin is required for spreading in CHO cells. *Biochem Biophys Res Commun* 335:1199-1210.
29. Proux-Gillardeaux, V., Gavard, J., Irinopoulou, T., Mege, R.M., and Galli, T. 2005. Tetanus neurotoxin-mediated cleavage of cellubrevin impairs epithelial cell migration and integrin-dependent cell adhesion. *Proc Natl Acad Sci U S A* 102:6362-6367.
30. Luftman, K., Hasan, N., Day, P., Hardee, D., and Hu, C. 2009. Silencing of VAMP3 inhibits cell migration and integrin-mediated adhesion. *Biochem Biophys Res Commun* 380:65-70.
31. Seftor, R.E., Seftor, E.A., and Hendrix, M.J. 1999. Molecular role(s) for integrins in human melanoma invasion. *Cancer Metastasis Rev* 18:359-375.

32. Talantov, D., Mazumder, A., Yu, J.X., Briggs, T., Jiang, Y., Backus, J., Atkins, D., and Wang, Y. 2005. Novel genes associated with malignant melanoma but not benign melanocytic lesions. *Clin Cancer Res* 11:7234-7242.
33. Powelka, A.M., Sun, J., Li, J., Gao, M., Shaw, L.M., Sonnenberg, A., and Hsu, V.W. 2004. Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. *Traffic* 5:20-36.
34. Rodriguez, L.G., Wu, X., and Guan, J.L. 2005. Wound-healing assay. *Methods Mol Biol* 294:23-29.
35. Hasan, N., and Hu, C. Vesicle-associated membrane protein 2 mediates trafficking of alpha5beta1 integrin to the plasma membrane. *Exp Cell Res* 316:12-23.
36. Brooks, S.A. 2001. Metastasis Research Protocols. 205-213.
37. Caswell, P.T., and Norman, J.C. 2006. Integrin trafficking and the control of cell migration. *Traffic* 7:14-21.
38. Bretscher, M.S. 1983. Distribution of receptors for transferrin and low density lipoprotein on the surface of giant HeLa cells. *Proc Natl Acad Sci U S A* 80:454-458.
39. Jensen, M.M., Jorgensen, J.T., Binderup, T., and Kjaer, A. 2008. Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. *BMC Med Imaging* 8:16.

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Hasan, N., Babi, Y., and Hu, C. 2008. Depletion of VAMP2 diminishes the exocytosis of $\alpha 5\beta 1$ integrin. James Graham Brown Cancer Center Annual Retreat.

Hasan, N., Babi, Y., and Hu, C. 2009. Depletion of VAMP2 diminishes the exocytosis of $\alpha5\beta1$ Integrin. IMD^3 Annual Symposium.