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**AN INTEGRAL ROLE OF ARRDC3 IN STEM
CELL MIGRATION AND BREAST CANCER
PROGRESSION**

A Dissertation Presented

By

Kyle Margaret Draheim

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

(March, 2nd 2010)

Cancer Biology

AN INTEGRAL ROLE OF ARRDC3 IN STEM CELL MIGRATION AND BREAST
CANCER PROGRESSION

A Dissertation Presented by

Kyle Margaret Draheim

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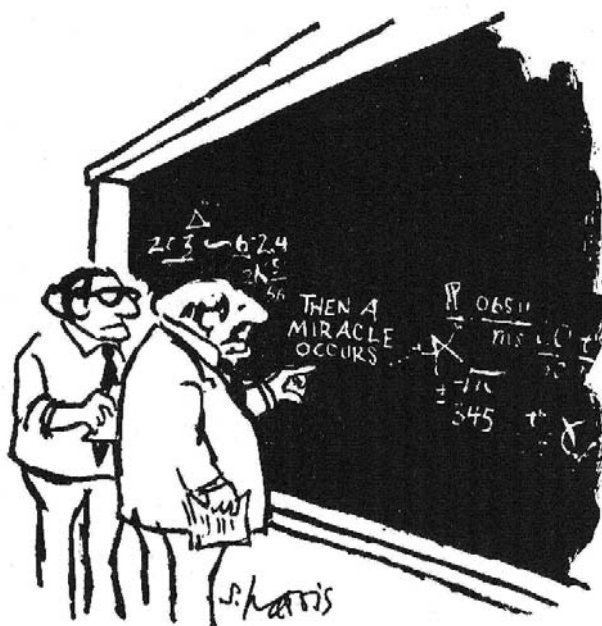
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Cancer Biology Program
March, 2nd 2010

DEDICATION



I would like to dedicate my thesis work in memory of my father, James E. Draheim, Ph.D., who has been a major source of both personal and professional inspiration in my life. I will forever be trying to figure out step two.



"I think you should be more explicit here in step two."

Acknowledgements

First and foremost, I would like to thank my parents, James & Barbara Draheim for their unwavering belief in me throughout my entire life. For as long as I can remember, they taught me to ask questions, critically evaluate everything and most of all, not to be afraid to challenge myself. Without their inspiration and support I would not have accomplished all that I have in my life. I will always take the comment “You are just like your mom/dad” as the highest of compliments.

I would next like to thank my brother and sister, Thaddeus and Meghan, for always knowing when to get excited and when to groan sympathetically even when they had no idea what I was talking about.

I would also like to thank the members of the Lyle laboratory, Dr. Zhiru Guo, Amy Chen, Nathan Moore, Michael Roche and Liz Hamir, and the Cancer Department as a whole for their support and guidance. No experiment is too overwhelming when there is always someone around to help if you need it. I would like to extend special appreciation towards Nathan Moore. It is rare to have someone you trust enough to help with critical assays and I am very fortunate to have Nate to count on.

I must also thank the members of my committee, Drs. JeanMarie Houghton, Arthur Mercurio, Alonzo Ross and Leslie Shaw. Their knowledge and suggestions were invaluable in guiding and helped push my project further than I realized possible.

I would like to thank my advisor, Dr. Stephen Lyle for being willing to take a chance on me. I would not come this far without his instruction and guidance. As a scientist, he had knowledge and ability to guide this project wherever it took us, even

when that wasn't so obvious. As my PI, he allowed me the freedom to explore different aspects of my project, but was always there to provide encouragement and suggestions when things seemed like they would never work. And as a mentor, he showed me how to think, read and write as a scientist, to keep evaluating the data, and to keep re-writing/re-submitting the papers; no matter how hard it might be.

Lastly, I thank my husband, Jason O'Brien for his support throughout my whole graduate school experience. For understanding that a "15 minute stop" in lab on a Sunday night really meant closer to an hour and having dinner ready for me even when I came home crazy late. I will *always* be grateful for your support. I love you, A&F.

Statement of Contribution

The subtraction hybridization screen identifying ARRDC3 was performed by Xiaowei Xu in the department of pathology at the University of Pennsylvania in Philadelphia. The data presented in figures 2-1A, 2-3A, 2-8A and 3-5A was performed by Qingfeng Tao, a former research assistant in our lab. HongBing (Amy) Chen, a research associate in our lab, is responsible for the work presented in figures 2-9A&B, 2-10, 2-11A&B, 2-12, 3-6A&B, 3-7, 3-8A&B, and 3-9. Dr. Keith Merdek, a former postdoctoral associate in Dr. Arthur Mercurio's lab, assisted in the optimization of the ARRDC3/ITG β 4 immuno co-precipitation. Graduate student in our lab Nathan Moore helped with analysis any time a "blind" analysis was needed. He also assisted in the xenograph studies. Tissue bank research technicians Elizabeth Hamir and Michael Roche obtained all the human tissue used in this thesis and cut all slides used in the primary tissue immuno-fluorescence, immuno-histochemistry, and H&E analyses. I have acknowledged all the individuals who have shared their reagents in the Materials and Methods section.

Abstract

Despite the importance of integrins in epithelial cell biology surprisingly little is known about their regulation. It is known that they form hemidesmosomes (HDs), are actively involved in cell contacts during cell migration/invasion, and are key signaling molecules for survival and growth. However, there has been a distinct lack of understanding about what controls the dynamic integrin localization during cell activation and movement. Growth factors, such as EGF, are elevated during wound healing and carcinoma invasion leading to phosphorylation of ITG β 4 and the disassembly of the HD and mobilization of ITG β 4 to actin-rich protrusions. More recently the phosphorylation of a novel site on ITG β 4 (S1424) was found to be distinctly enriched on the trailing edge of migrating cells, suggesting a possible mechanism for the dissociation of ITG β 4 from HDs.

Arrestin family member proteins are involved in the regulation of cell surface proteins and vesicular trafficking. In this study, we find that over-expression of arrestin family member ARRDC3 causes internalization and proteasome-dependent degradation of ITG β 4, while decreased levels of ARRDC3 stabilizes ITG β 4 levels. These results lead us to a new mechanism of ITG β 4 internalization, trafficking and degradation. During migration, ARRDC3 co-localizes with ITG β 4 on the lagging edge of cells but has a distinct distribution on the leading edge of cells. Additional immuno co-precipitation experiments demonstrate that ARRDC3 preferentially binds to ITG β 4 when phosphorylated on S1424. Using confocal microscopy, we show that the expression pattern of ARRDC3 on the lagging edge of a migrating cell is identical to the expression

pattern of ITG β 4-pS1424. We demonstrate that ARRDC3 expression represses cell proliferation, migration, invasion, growth in soft agar and tumorigenicity.

Collectively, our data reveals that ARRDC3 is a negative regulator of β 4 integrin and demonstrates how this new pathway impacts biologic processes in stem cell and cancer biology. Additionally, as ARRDC3 is highly expressed in several tissues and conserved across species, our results are likely to be translated to other models.

Table of Contents

Dedication.....	iii
Acknowledgements.....	iv
Statement of Contribution.....	vi
Abstract.....	vii
Table of Contents.....	ix
List of Tables.....	xii
List of Figures.....	xiii
List of Abbreviations.....	xvi
Chapter 1: Introduction.....	1
Section I: Adult Stem Cells.....	2
<i>Functions of Adult Stem Cells</i>	2
<i>Stem Cell Niche</i>	3
<i>The Epidermis & Skin Stem Cells</i>	4
The Hair Follicle Bulge.....	5
Signaling Pathways.....	9
Adhesion & Motility.....	10
Section II: Stem Cells & Cancer Cells.....	12
<i>Self-Renewal</i>	12
<i>Importance of Environment</i>	13
Section III: Integrins.....	14
<i>Adhesion/Cell Migration</i>	16
<i>Survival</i>	17
<i>Proliferation</i>	18
<i>Activation</i>	19
<i>Trafficking</i>	19
<i>Integrin $\beta 4$</i>	21
Role in Hemidesmosomes.....	21
Signaling/Role in Cancer.....	22

In Breast Cancer.....	25
In the Epidermis.....	26
Section IV: Arrestins.....	27
<i>Family Members</i>	27
<i>Role in GPCR signaling</i>	28
<i>Beyond GPCR regulation</i>	30
<i>Role in non-GPCR signaling</i>	32
<i>ARRDC3</i>	33
Chapter 2: The Arrestin Protein Family Member ARRDC3 Controls Stem Cell	
Motility through $\beta 4$ Integrin Regulation.....	36
Introduction.....	37
Results.....	40
<i>ARRDC3 is expressed in skin stem cells</i>	40
<i>ARRDC3 sequence structure and analysis</i>	42
<i>Expression pattern of ARRDC3 in human tissues</i>	42
<i>ARRDC3 co-localizes with endosomal markers</i>	43
<i>ARRDC3 interacts with ITG$\beta 4$</i>	44
<i>ARRDC3 regulates ITG$\beta 4$ protein levels</i>	45
<i>ITG$\beta 4$ regulation by ARRDC3 is proteasome dependent</i>	46
<i>ARRDC3 controls stem cell motility</i>	47
<i>ARRDC3 interacts with phosphorylated ITG$\beta 4$</i>	48
<i>ARRDC3 does not affect multi-potency</i>	49
<i>ARRDC3 expression preserves an undifferentiated state in skin stem</i> <i>cells</i>	49
<i>Loss of ARRDC3 causes abnormal whisker follicles and loss of fur in</i> <i>Mice</i>	50
Discussion.....	52
Materials & Methods.....	56
Chapter 3: ARRDC3 is a Novel Regulator of Breast Cancer Progression.....	86

Introduction.....	87
Results.....	90
<i>ARRDC3 expression is down-regulated during tumor progression.....</i>	<i>90</i>
<i>ARRDC3 affects in-vitro human cancer cell tumorigenicity.....</i>	<i>91</i>
<i>ARRDC3 suppresses in-vivo tumorigenicity.....</i>	<i>92</i>
<i>ARRDC3 negatively regulates ITGβ4.....</i>	<i>94</i>
<i>ARRDC3 regulates ITGβ4 in a proteasome dependent manner.....</i>	<i>94</i>
<i>ARRDC3 directly interacts with activated ITGβ4.....</i>	<i>95</i>
<i>ARRDC3 specifically targets ITGβ4.....</i>	<i>97</i>
<i>Effects of ARRDC3 on in-vitro tumorigenicity is dependent on ITGβ4.....</i>	<i>97</i>
<i>ARRDC3 down-regulation and coordinate ITGβ4 up-regulation in human breast tumors.....</i>	<i>98</i>
Discussion.....	100
Materials & Methods.....	105
Chapter 4: Final thoughts and Future directions.....	137
REFERENCES.....	159
Appendix I: A DNA binding mutant of TAL1 cooperates with LMO2 to cause T cell leukemia in mice.....	179
Appendix II: Targeting the Notch1 pathway in a Mouse T-ALL Model.....	196

List of Tables

Table 3-1: Expression of ARRDC3 in human breast tumors.....135

Table 4-1: Loss of ARRDC3 in mice appears to have an aging phenotype.....157

List of Figures:

Figure 1-1: Diagram of the hair follicle stem cell niche.....	8
Figure 1-2: Diversity of integrins.....	15
Figure 1-3: Schematic of Arrestin mediated GPCR regulation and GPCR independent signaling.....	29
Figure 2-1: ARRDC3 is preferentially expressed in skin stem cells.....	63
Figure 2-2: ARRDC3 is not a stem cell marker.....	65
Figure 2-3: Structure analysis of ARRDC3.....	66
Figure 2-4: ClustalW alignment on the amino acid sequence of ARRDC3.....	67
Figure 2-5: Putative 3D structure of ARRDC3.....	69
Figure 2-6: Expression patterns of ARRDC3.....	70
Figure 2-7: ARRDC3 localizes in the vesicular trafficking pathway.....	71
Figure 2-8: ARRDC3 directly interacts with ITG β 4.....	72
Figure 2-9: ARRDC3 negatively regulates ITG β 4.....	74
Figure 2-10: The regulation of ITG β 4 by ARRDC3 is post-transcriptional.....	75
Figure 2-11: The regulation of ITG β 4 by ARRDC3 is dependent on the proteasome.....	76
Figure 2-12: Decrease of ITG β 4 protein levels by ARRDC3 over-expression is not dependent on the lysosomes.....	77
Figure 2-13: ARRDC3 expression regulates cell motility.....	78
Figure 2-14: ARRDC3 co-localizes with ITG β 4 on the lagging edge of cells.....	81
Figure 2-15: ARRDC3 expression does not affect the multi-potency of skin stem cells..	82
Figure 2-16: ARRDC3 levels changes upon differentiation.....	83

Figure 2-17: Gross examination of ARRDC3 null mice.....	84
Figure 2-18: ARRDC3 null mice have morphologically abnormal whisker follicles and fewer hair follicles.....	85
Figure 3-1: Down-regulation of ARRDC3 is an early event in carcinogenesis.....	110
Figure 3-2: Expression of ARRDC3 effects in-vitro tumorigenicity.....	111
Figure 3-3: ARRDC3 negatively regulates in-vivo tumorigenicity.....	113
Figure 3-4: Xenograph tumors display an undifferentiated morphology.....	115
Figure 3-5: ARRDC3 repression increases cell survival after stress.....	116
Figure 3-6: ARRDC3 negatively regulates ITG β 4.....	118
Figure 3-7: The regulation of ITG β 4 by ARRDC3 is post translational.....	119
Figure 3-8: ARRDC3 negatively regulates ITG β 4 protein levels in a mechanism dependent on the proteasome.....	120
Figure 3-9: The regulation of ITG β 4 by ARRDC3 is not dependent on the lysosomes..	121
Figure 3-10: Arrdc3 appears to preferentially interact with ITG β 4 when phosphorylated on serine-1494 and/or when ubiquitinated.....	122
Figure 3-11: The regulation of ITG β 4 by ARRDC3 is specific and not a product of increased random endocytosis.....	124
Figure 3-12: The creation of stable cell lines with varied expression of ARRDC3.....	125
Figure 3-13: Effects of ARRDC3 on in-vitro tumorigenicity is dependent on ITG β 4....	126
Figure 3-14: Expression of ARRDC3 in human breast tumors.....	128
Figure 3-15: Expression levels of ARRDC3 in normal human breast tissue.....	129
Figure 3-16: Expression levels of ARRDC3 and ITG β 4 in Grade 1 Tumors.....	130

Figure 3-17: Expression levels of ARRDC3 and ITGβ4 in Grade 2 Tumors.....	131
Figure 3-18: Expression levels of ARRDC3 and ITGβ4 in Grade 3 Tumors.....	132
Figure 3-19: Expression levels of ARRDC3 and ITGβ4 in breast cancer metastatic lesions.....	133
Figure 3-20: Expression levels of ARRDC3 and ITGβ4 in basal-like breast cancers.....	134
Figure 4-1: Model.....	152
Figure 4-2: Histological analysis of the cancer found in ARRDC3 heterozygous mice.	153
Figure 4-3: ClustalW alignment of the interacting region on ITGβ4.....	154
Figure 4-4: Changes in <i>ARRDC3</i> mRNA expression in Hypoxia.....	155
Figure 4-5: Down-regulation of ARRDC3 expression in basal like vs. non-basal like tumors.....	156

List of Abbreviation

7TMR- 7 transmembrane receptor
AIG- anchorage independent growth
AIP4- atrophin interacting protein 4
ARRDC3- arrestin domain containing protein 3
ASK1- apoptosis signal-regulating kinase 1
BMP- bone morphogenic factor
Cdc42- cell division control protein 42 homolog
CHIP- c-terminus of Hsc70-interacting protein
CHX- cyclohexamide
CREB- cAMP response element binding
DAPI- 4',6-diamidino-2-phenylindole
DP- Dermal Pappilae cells
Dsh- Dishevelled
ECM- extracellular matrix
EGF- epidermal growth factor
ER- estrogen receptor
ERK- extracellular signal regulated kinase
FAK- focal adhesion kinase
FITC- fluorescein isothiocyanate
Fzd- Frizzled
GPCRs- G-protein coupled receptors
GRKs- G-protein coupled receptor kinases
GSK3- Glycogen synthase kinase-3
H&E- hematoxylin & eosin
HD- Hemidesmosome
HER2- human epidermal growth factor receptor 2
Hh- Hedgehog
HIF1- hypoxia inducible factor 1

IF- immuno-fluorescence
IFE- inter-follicular epidermis
IHH- indian hedgehog
IP- immuno-precipitation
I κ B- inhibitor of κ B
ITG β 4- integrin β 4
JEB- junctional epidermolysis bullosa
JNK- c-Jun N-terminal kinase
KGF- keratinocytes growth factor
LEF1- lymphoid enhancer binding factor 1
MAPK- Mitogen Activated Protein Kinase
MEK- MAP/ERK kinase
MKK- map kinase kinase
MTT- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEDD- neuronal precursor cell-expressed developmentally down-regulated
NF- κ B- nuclear factor kappaB
NFAT- nuclear factor of activated t-cells
OCT- optimal cutting temperature compound
ORS- outer root sheath
PE- phycoerythrin
PI3K- phosphoinositide 3-kinase
PKA- protein kinase A
PKC- protein kinase C
PLA- proximity ligation assay
PMA- phorbol 12-myristate 13-acetate
PP2A- protein phosphatase 2A
PR- progesterone receptor
Ptc- Patched receptor
RTKs- receptor tyrosine kinases

SHH- sonic hedgehog

Smo- Smoothened

TGF β - Transforming growth factor β

TRAF- tumor necrosis factor receptor-associated factor

CHAPTER I:
Introduction

Adult Stem Cells

An adult stem cell, also termed somatic stem cell or tissue specific stem cell, is defined as an undifferentiated cell, found among differentiated cells in a tissue or organ, that can self-renew and differentiate to yield some or all of the major specialized cell types of the tissue or organ (Evans and Potten, 1991; Moore and Lemischka, 2006; Morasso and Tomic-Canic, 2005). The primary role of adult stem cells in a living organism is to maintain and repair the tissue in which they are found. Unlike embryonic stem cells, which are defined by their origin (the inner cell mass of the blastocyst), the origin of adult stem cells in most mature tissues is still under investigation. Adult stem cells have two general properties: they are capable of long-term self-renewal and they are undifferentiated (Morrison and Spradling, 2008; Potten and Morris, 1988). However, whereas embryonic stem cells are considered to be pluripotent and therefore capable of differentiation into almost all cells from the three germ layers; adult stem cells are multipotent and only capable of producing a limited range of differentiated cells, typically confined to those of a single tissue (Morrison and Spradling, 2008; Potten and Morris, 1988).

Functions of Adult Stem Cells

Adult stem cells are identified as the cells responsible for the regeneration of the tissues in which they reside. They are generally considered to be capable of differentiating into the multiple cell types within the tissue in which they reside (Spradling et al., 2001). This is vital in the overall homeostasis of the tissue as well as injury repair. In order for stem cells to fulfill these critical functions, they have to

maintain several properties. Upon division, stem cells need to maintain the stem cell pool (self-renewal) as well as derive more differentiated cells. This is generally believed to be accomplished through asymmetrical cell division, in which one daughter cell retains the stem cell characteristics while the other daughter cell continues down a path of hierarchical differentiation. However, current data does not conclusively exclude other mechanisms, such as interspersed divisions of stem cells resulting in either self-renewal or differentiation. Stem cells are generally slow-cycling *in-vivo* but give rise to highly proliferative transit-amplifying cells, the cells which ultimately differentiate to replenish lost cells (Potten, 1974; Potten et al., 1974). This system is believed to minimize the number of times the stem cell needs to undergo DNA synthesis, thus limiting the introduction of mutations.

Stem Cell Niche

Due to the essential role of adult stem cells in tissue regeneration and wound repair, the proper upkeep of these cells is crucial. Maintenance is thought to be achieved through the interactions between the stem cells and their specialized microenvironment known as the niche, first postulated by Schofield in 1978 (Schofield, 1978). Stem cell functions (i.e. the balance between self-renewal and differentiation) are primarily controlled by extracellular cues from the niche, although the natures of many of these signals are still unclear. Comparison of the most understood stem cell niches (mammalian bone marrow, skin, intestine and testis) reveals several common properties (reviewed in (Li and Xie, 2005; Moore and Lemischka, 2006)). The niche is typically an asymmetric structure; cells supporting the undifferentiated state are located on one end with cells

promoting differentiation on the other. This asymmetry likely helps to ensure the asymmetric division of the stem cells. Also, stem cells will tightly adhere themselves within the niche by increasing expression of adhesion molecules such as cadherins and integrins. Strong adherence makes it difficult for physical damage to dislodge the adult stem cells from their protective environment. Additionally, all known niches contain a variety of support cells that are essential in producing extrinsic factors, which both positively and negatively regulate the growth-restrictive and un-differentiated state. The loss of the niche has severe consequences. Excessive self renewal at the expense of differentiation leads to aberrant expansion and even tumorigenesis as well as degeneration of the tissue. Conversely, excessive differentiation at the expense of self-renewal will deplete the stem cell pool leading to a reduction in the regenerative capacity of tissues, affecting both normal homeostasis as well as wound healing.

The Epidermis & Skin Stem Cells

The mammalian skin represents a physical barrier between the body and its external environment, receiving most of the damage brought on by physical trauma and mutagenic UV radiation. The skin is a multi-layered epithelium that is comprised of sebaceous glands, hair follicles and the interfollicular epidermis. In order to protect against the accumulation of mutations, the epidermis has a rapid cellular turnover, renewing itself in humans every 2-3 weeks (Potten, 1975a; Potten, 1975b). This regeneration is sustained by different populations of epidermal stem cells which additionally participate in the repair of the skin after injuries. These cells are quiescent but upon injury can be mobilized into an extensive and sustained self-renewal capacity.

There are two main methods used to identify epidermal stem cells. The first makes use of the slow-cycling nature that defines all stem cells in a pulse-chase assay. Actively proliferating cells are pulse-labeled with the introduction of a DNA precursor like $^3\text{H-TdR}$ or BrdU. For *in-vivo* studies, mice are injected at post-natal day 3 or shortly after the engraftment of human skin onto an adult nude mouse. The DNA precursor can be added into the media for *in-vitro* studies. The labeling period is then followed by an extended chase period (4-10 weeks); proliferating cells will dilute the label by half with every cell division. Over time, the rapidly proliferating cells, such as the transit amplifying cells, will no longer contain detectable levels of label but the stem cells will (Bickenbach and Mackenzie, 1984; Braun et al., 2003; Cotsarelis et al., 1990). Inducible GFP and β -galactosidase have also been used as pulse-labeling reagents in adapted methods (Tumbar et al., 2004). The second method used for identification of epidermal stem cells involves the use of stem cell markers. Current markers include ITG α 6, ITG β 1, CD71^{lo}, CD34, CD200, KRT15 and KRT19 (Cotsarelis, 2006; Jih et al., 1999; Lyle et al., 1998; Lyle et al., 1999; Trempus et al., 2003; Van Waes et al., 1991). Unfortunately, most of the markers identified lack specificity to epidermal stem cells alone. However, these markers in combination with knowledge of the approximate physical location relative to histological markers will allow for accurate isolation through micro-dissection and laser capture. A combination of cell labeling and immuno-marking techniques identified epidermal stem cells located in the hair follicle bulge as well as within the interfollicular region of the skin.

The Hair Follicle Bulge

The bulge region of the hair follicle represents the best characterized epidermal stem cell population described to date, but there is evidence of other stem populations in the interfollicular epidermis and the sebaceous glands (Bieniek et al., 2007; Kaur, 2006; Tiede et al., 2007). In human fetal and murine follicles, the bulge is a morphologically prominent outgrowth of epithelial cells below the opening of the sebaceous gland, marking the lower end of the permanent portion of the follicle (Akiyama et al., 1995). In contrast, this morphological structure is not typically seen in adult human follicles (only at the onset of anagen) and therefore the location of the bulge is estimated to be roughly located at the insertion of the arrector pili muscle (Figure 1-1). These bulge cells have been demonstrated to possess stem cell properties *in-vivo* and *in-vitro* (Cotsarelis et al., 1990; Kobayashi et al., 1993). In addition to the *in-vitro* studies, the bulge cells possess the *in-vivo* proliferative behavior expected of stem cells. While the stem cells are generally slowly cycling, as determined in label-retaining studies, cell proliferation analysis shows that the stem cells of the mouse bulge are activated to transiently proliferate at the onset of anagen (Wilson et al., 1994) starting the hair cycle as characterized in the “bulge activation hypothesis” (Cotsarelis et al., 1990).

Similar to other epithelial tissues (Cotsarelis et al., 1989; Lavker and Sun, 1982) the bulge has all the features expected of a stem cell niche and the bulge cells display the characteristics of stem cells. The bulge represents the permanent portion of the hair follicle; while the lower follicle undergoes apoptosis and degenerates during catagen. Bulge cells are tightly adherent to the basement membrane and are protected from accidental loss to plucking (Cotsarelis et al., 1990). Plucking of human follicles, which

can remove a majority of the hair follicle epithelium below the level of the bulge, will still result in hair regeneration (Moll, 1995). The basement membrane zone of the bulge also appears specialized for its role in protecting the bulge cells. K-laminin and type VII collagen are much more highly expressed in the basement membrane zone of the bulge than in the lower portion of the outer root sheath (ORS), allowing for increased adhesion to the niche (Akiyama et al., 1995). Additionally, the vasculature of the bulge provides nourishment for this important area and the bulge-containing isthmus region is the most richly innervated skin area (Schneider et al., 2009). The bulge area also contains a concentrated number of Langerhans cells, a cell type thought to help maintain the epidermal stem cell niche (Allen and Potten, 1974; Potten and Allen, 1976).

There are three major epithelial cell types in which bulge cells can contribute: sebaceous glands, epidermis and the whole hair follicle. Bulge cells are primarily responsible for the regeneration of a new lower follicle and the start a new hair cycle (Liu et al., 2003; Lyle et al., 1998; Oshima et al., 2001). In addition, these cells can contribute to the sebaceous gland and epidermis as well (Ito et al., 2005; Morris et al., 2004; Oshima et al., 2001). The main role for inter-follicular (IF) stem cells seems to be replenishing the inter-follicular epidermis (IFE); however these cells are capable of giving rise to cells of multiple lineages including the hair (Ferraris et al., 1997; Reynolds and Jahoda, 1992; Silva-Vargas et al., 2005). It is likely that during normal tissue homeostasis, both inter-follicular and bulge stem cells respond to niche derived signals and give rise to restricted subsets of progeny (Ferraris et al., 1997; Niemann et al., 2002). However, after an event

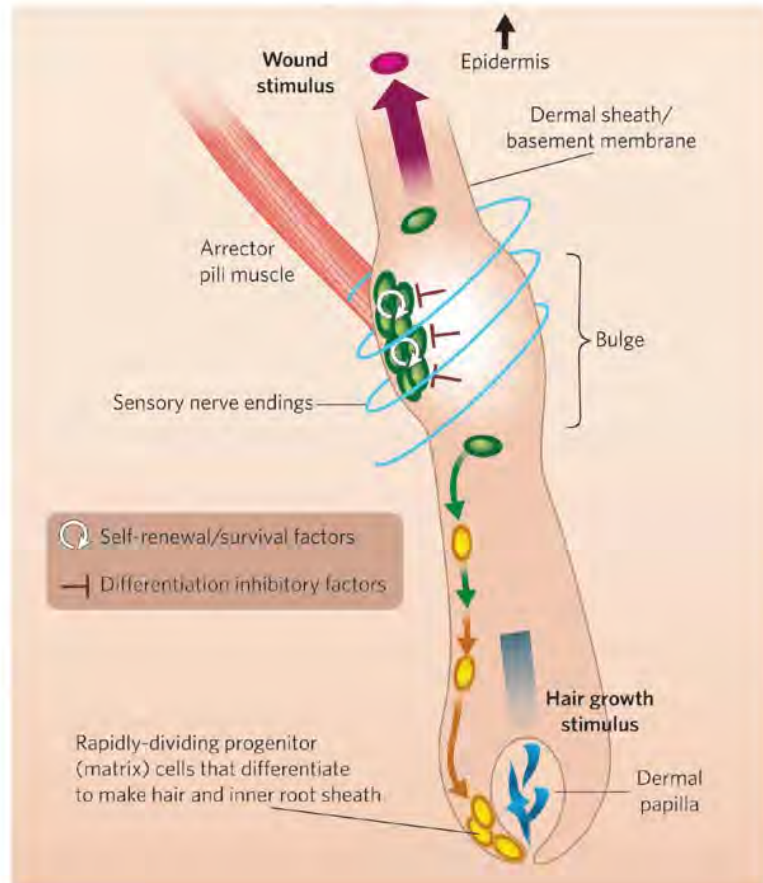


Figure 1-1: Diagram of the hair follicle stem cell niche. The bulge contains infrequently cycling, label-retaining cells, which include multipotent stem cells (green) that can generate the new hair follicle during cycling and repair the epidermis on injury. The bulge is in a specialized niche, surrounded by other cell types, which together provide cues that maintain these cells in an undifferentiated and quiescent state. For stem cells to be activated, the niche environment must change.

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Scratching the surface of skin development. Elaine Fuchs

that disrupts normal tissue homeostasis (such as wounding or engraftment), all epidermal stem cells may be stimulated to produce daughter cells of all the epidermal lineages (Blanpain et al., 2004; Ito et al., 2005; Morris et al., 2004; Tumber et al., 2004).

Skin Stem Cell Signaling Pathways

There are several signaling pathways critical in the regulation of bulge stem cells, but the Wnt/ β -catenin pathway is perhaps the best characterized. Repressing this pathway with dominant negative forms of either β -catenin or the downstream transcription factor Lef1 results in the loss of hair follicles and formation of cysts of IFE with associated sebocytes (Huelsenken et al., 2001; Zhu and Watt, 1999). Additionally, mice expressing either dominant negative β -catenin or dominant negative Lef1 can form sebaceous and hair tumors (trichofolliculomas and pilomatricomas) (Huelsenken et al., 2001; Takeda et al., 2006). Conversely, constitutively active β -catenin or Lef1 results in de novo hair morphogenesis at the expense of sebaceous differentiation (Gat et al., 1998; Huelsenken et al., 2001; Zhu and Watt, 1999). Activation of β -catenin can additionally induce bulge stem cells to re-enter the cell cycle in a Hedgehog (Hh) dependent manner (Lowry et al., 2005; Niemann, 2006).

Hh signaling is also important for lineage determination of bulge stem cells. Where Sonic Hh (SHH) has been shown to be important for hair follicle development and basal cell carcinogenesis, Indian Hh (IHH) is involved in the growth and differentiation of sebocytes in normal skin and in the formation of sebaceous tumors of human and mice (Adolphe et al., 2004; Niemann et al., 2003).

The appropriate timing of c-Myc expression is also important for the self-renewal/differentiation balance. When c-Myc is mis-expressed in bulge stem cells, they will aberrantly differentiate into inter-follicular epidermis and sebocytes (Arnold and Watt, 2001; Waikel et al., 2001). However, it is believed that c-Myc actually regulates the departure of the bulge stem cells from their niche, and it is the absence of the restrictive environment that subsequently causes proliferation and differentiation (Frye et al., 2007; Watt et al., 2008).

Finally, bone morphogenic protein (BMP) signaling is critical in maintaining the quiescence of the bulge stem cells (Andl et al., 2004; Kobiela et al., 2003; Kobiela et al., 2007). Bulge stem cell activation responds to cyclic BMP2 and BMP4 expression within the dermis (Plikus et al., 2008). Dermal BMP signals progressively diminish during the resting period of the hair follicle cycle promoting the switch from quiescent to activated bulge stem cells (Plikus et al., 2008).

Adhesion & Migration of Skin Stem Cells

The adhesion molecules of skin stem cells are very important in their regulation. Interactions with support cells and the extra-cellular matrix (ECM) via adhesion molecules establish and maintain niche architecture, generate and transmit regulatory signals and control the frequency and nature of stem cell division. There are several ECM proteins and adhesion molecules up-regulated in skin stem cells (when compared to differentiated keratinocytes) including: TNC, COL6A1, COL18A1, FBLN1, SPARC, ITG β 1, ITG β 4, ITG β 6 and P-cadherin (Fuchs, 2008; Jones et al., 2007; Morris et al., 2004; Rhee et al., 2006; Tumber et al., 2004). The importance of these adhesion

molecules is highlighted by a study which deleted ITG β 1 and α -catenin from basal keratinocytes and resulted in random orientation of cell division and alterations in epidermal homeostasis (Lechler and Fuchs, 2005). These results support the hypothesis that signals generated from the proper interaction between adhesion molecules and the environment should either promote directional migration (out of niche for regeneration) or inhibit motility (keeping the stem cells within the niche).

Under normal circumstance, bulge stem cells have very limited motility as it is crucial for the cells to remain in the niche (Roh et al., 2005). During normal tissue regeneration transit-amplifying (TA) cells migrate out of the niche so it is not surprising that these daughters of bulge stem cells exhibit a high motility (Roh et al., 2005). However, bulge stem cells can be induced to migrate downward to the hair follicle as well as outward to resurface the epidermis in response to wounding (Taylor et al., 2000). After injury or during normal turnover, keratinocytes become activated; simultaneously secreting and responding to growth factors and cytokines (Rizvi and Wong, 2005; Tomic-Canic et al., 1998; Watt et al., 2006). After mechanical injury, bulge stem cells will start migrating and proliferating, generating TA cells (Blanpain and Fuchs, 2009; Owens and Watt, 2003). The cells undergo significant changes to their junction and adhesion molecules, a critical part of this process. Importantly, hemidesmosomes (HDs) are dissolved to allow for migration. These migrating keratinocytes produce a different set of membrane molecules, such as vitronectin and fibronectin receptors (importantly integrin α 5 β 1), which replace the collagen receptor (integrin α 2 β 1) and further permit migration (Cavani et al., 1993; Haapasalmi et al., 1996; Singer and Clark, 1999). The growth

factors active during wound healing such as epidermal growth factor (EGF), keratinocyte growth factor (KGF) and TGF β are all potential regulators of these processes (Nanney et al., 1996; Werner et al., 1994; Zambruno et al., 1995).

Stem Cells and Cancer Cells

Similarities between stem cells and cancer cells have long been recognized as both have the capacity for self renewal and unlimited replication. The identification of key pathways for stem cell regulation and cancer progression (such as Notch, Hedgehog and Wnt) reveals further parallels. Stem cells are increasingly becoming recognized as a model system for properties exhibited by cancer cells as well as potential targets of carcinogenic pathways.

Self-renewal

Self-renewal is the process in which a stem cell divides asymmetrically or symmetrically to generate one or two daughter stem cells that have a developmental potential similar to the mother cell. As stem cells and cancer cells share the ability to self-renew, it is reasonable to propose that newly arising cancer cells appropriate (or “hijack”) the self-renewing cell division machinery normally expressed in stem cells. There is evidence showing that many pathways classically associated with cancer also regulate normal stem cell development. For example, the prevention of apoptosis by forced expression of the oncogene *bcl-2* results in increased numbers of hematopoietic stem cells (HSCs) *in-vivo*, suggesting that cell death has a role in regulating the homeostasis of HSCs (Domen et al., 1998; Domen and Weissman, 2000). Additionally, the potent oncogene c-Myc controls the balance between stem cell self-renewal and

differentiation, presumably by regulating the interaction between hematopoietic stem cells and their niche (Sato et al., 2004; Wilson et al., 2004). Other signaling pathways associated with oncogenesis have been shown to play a role in stem cell self renewal; such as the Notch (Dontu et al., 2004; Hitoshi et al., 2002; Shen et al., 2004; Vercauteren and Sutherland, 2004), SHH (Clement et al., 2007; Liu et al., 2006), and Wnt signaling pathways (Dravid et al., 2005; Reya et al., 2003).

Importance of Environment

As previously discussed, the primary function of the niche is to maintain undifferentiated stem cells, although the precise mechanisms are still unclear. Stephen Paget's "Seed and Soil" Hypothesis, introduced over a hundred years ago, postulates that the location of secondary tumors reflect the "fertile soil" that site/organ provides for the "seeds" of cancer cells (Paget, 1889). However, the role of stroma in the tumor microenvironment has only recently become recognized as a major influencing factor of cancer progression. Normal fibroblastic stroma preserves epithelial cell quiescence and effectively inhibits epithelial cell transformation (and consequent tumorigenesis) demonstrating a stromal dominance over neoplastic cells (Kenny and Bissell, 2003; Kuperwasser et al., 2004; Maffini et al., 2004). Correspondingly, activated stroma (typically damaged by age, genetic mutation, injury/inflammation, or cancer cell influence) can provide a more supportive microenvironment for expanded tumor growth (Barcellos-Hoff and Ravani, 2000; Kurose et al., 2002; Kurose et al., 2001; Li et al., 2007; Olumi et al., 1999).

Normal epithelium interacts with the underlying stromal compartment through a basement membrane composed mainly of collagen IV and laminin. Upon carcinoma progression, the previously uncompromised basement membrane is degraded and malignant epithelial cells invade into the underlying reactive stroma (Mueller and Fusenig, 2004). Invasive carcinoma cells then directly interact with activated fibroblasts (referred to as tumor-associated fibroblasts (TAFs), carcinoma-associated fibroblasts (CAFs) or reactive stroma) which remain in a chronic state of activation and ultimately support tumor progression (Kunz-Schughart and Knuechel, 2002a; Kunz-Schughart and Knuechel, 2002b; Orimo et al., 2005). Stromal cells influence epithelial cell behavior by secreting various extra-cellular matrix proteins, chemokines, cytokines, growth factors, proteases, and protease inhibitors. Altogether the tumor micro-environment, similar to the stem cell niche, creates a specialized environment which is permissive for cancer progression.

Integrins

Integrins make up a large family of heterodimeric transmembrane cell-matrix receptors composed of an α and a β subunit. 18 α and 8 β subunits have been described and they can assemble to form 24 different heterodimers, each with a different specificity to extra-cellular matrix proteins such as fibronectin, laminin, vitronectin, collagen and thrombospondin (Figure 1-2). Each of the 24 integrins appears to have unique functions made evident by their ligand specificities and varying phenotypes of the knockout mice.

Integrins form a functional link between the extra-cellular matrix and the intracellular signaling pathways which influence cell morphology, survival, motility,

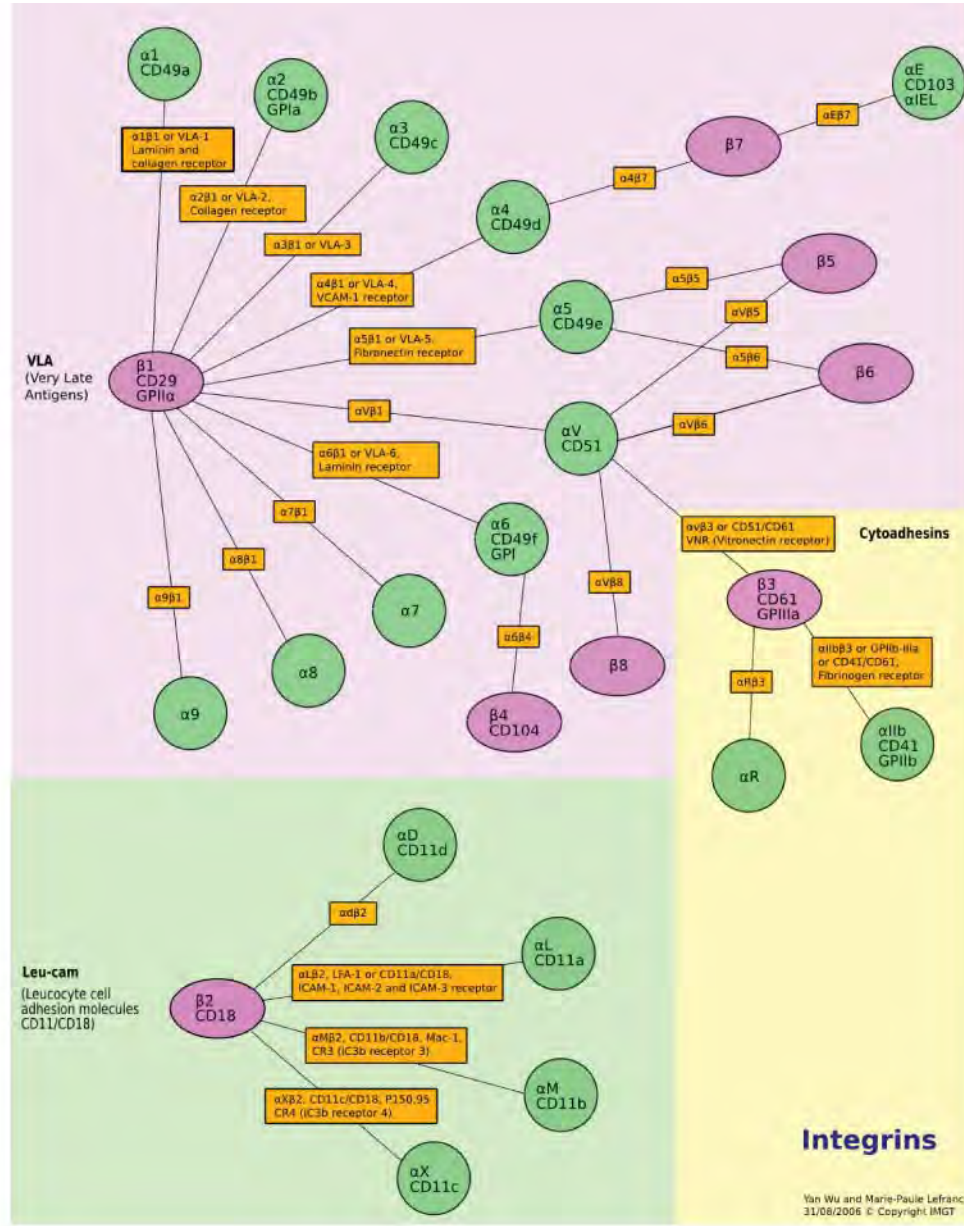


Figure 1-2: Diversity of integrins. 18 α and 8 β subunits have been described and they can assemble to form 24 different heterodimers, each with a different specificity to extra-cellular matrix proteins.

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Yan Wu and Marie-Paule Lefranc

proliferation, migration and invasion (Aplin et al., 1999). Binding of integrins to the extra-cellular matrix causes a conformation change allowing signals to be transmitted into the cell (in what is known as “outside-in signaling”) activating several downstream signaling events (Juliano et al., 2004). Since integrins do not have an intrinsic catalytic activity, any downstream signaling must be modulated by integrin-associated proteins. Some of the more important integrin-mediated signals rely on the recruitment of the Src and FAK families of protein tyrosine kinases (Arias-Salgado et al., 2003; Arias-Salgado et al., 2005). There are three main signaling pathways activated by integrins that are critical for both cancer cells and stem cells: cytoskeletal organization (adhesion/cell migration), cell proliferation and cell survival pathways.

Adhesion/Cell Migration

In cells, adhesion and actin polymerization are dependent on one another. Adhesions provide nucleation points which support actin polymerization, but actin polymerization determines rates of adhesion and possibly nucleates adhesions containing activated integrins (Butler et al., 2006). Following the binding of ECM proteins, α -actinin and talin are recruited to the β -integrin cytoplasmic tail rapidly followed by the actin-binding protein vinculin, altogether forming a stable focal adhesion (Martin et al., 2002). Removal of any of the proteins comprising this linkage leads to the disruption of the entire adhesion structure (Vicente-Manzanares et al., 2009). These integrin-mediated adhesions serve as traction points for contractile or tensional forces through their interaction with the actin cytoskeleton.

Integrin clustering by ligand binding also results in the oligomerization of focal adhesion kinase (FAK). Both FAK and vinculin can interact with the Arp2/3 complex, which controls the assembly of a branched actin filament network in the lamellipodium (Butler et al., 2006). Additionally, autophosphorylation of FAK results in the recruitment of Src through the SH2 domain and ultimate activation of GTPases RhoA, Rac1 and Cdc42. These GTPases, by way of a variety of effectors, lead to actin nucleation, cytoskeletal contractility, branched F-actin growth and thus membrane protrusion and migration (Huveneers and Danen, 2009; Kiyokawa et al., 1998; ten Klooster et al., 2006).

Cell Survival

Most adherent cell types depend on integrin mediated adhesion for survival (Giancotti and Ruoslahti, 1999). Loss of adhesion causes cells to undergo a form of programmed cell death referred to as anoikis (Frisch and Screaton, 2001). Anoikis likely prevents cells from growing in inappropriate sites after losing adhesion from their original surroundings, thus maintaining the integrity of tissues. Integrin-mediated cell adhesion in 2-dimensional culture systems stimulates Bcl-2 expression and PKB/AKT activity, which in turn mediates survival signals (Giancotti and Ruoslahti, 1999). In the absence of serum factors, integrin-mediated adhesion to fibronectin enhances survival by activating JNK in a FAK dependent manner (Almeida et al., 2000). Alternatively, ligand-free integrins can trigger apoptosis of fully adherent cells via the recruitment and activation of caspase-8. This suggests that a given integrin expression profile renders a cell dependent on a specific ECM environment for its survival (Stupack et al., 2001; Varner et al., 1995).

Proliferation

Through the activation of Rac and ERK, integrin-mediated cell adhesion in normal untransformed cells also regulates the G1 phase of the cell cycle (Assoian and Schwartz, 2001; Schwartz and Assoian, 2001). Activated Rac and ERK contribute to the induction of cyclin D1 in mid-G1 phase. Ultimately, these events lead to the phosphorylation of the retinoblastoma protein (pRb) and entry into S phase. RTK stimulation and integrin-mediated adhesion can each independently activate ERK explaining the transcriptional regulation of cyclin D1. However, it is the combination of RTK and integrin signaling (i.e. when adherent cells are stimulated) which creates strong and sustained ERK activity necessary for cell cycle progression (Chen et al., 1996; Renshaw et al., 1997).

Integrin-mediated adhesion, through ERK signaling, also leads to the induction of p21^{cip1} in early G1 phase and suppression or re-localization of the cyclin dependent kinase inhibitors p21^{cip1} and p27^{kip1} in mid-late G1 phase, coincident with activation of cyclin-E-Cdk2. Additionally, increased expression of c-Myc through activation of c-Src in response to integrin-mediated adhesion drives entry into S-phase (Benaud and Dickson, 2001). The organization of the actin cytoskeleton by integrins is also essential for adhesion-regulated proliferation. Integrin mediated control of Rho GTPases activity (enzymes critically involved in actin cytoskeletal organization) is an important aspect of adhesion mediated regulation of cyclin D1 and cdk-inhibitors.

Activation

Current information regarding the regulation of integrin signaling focuses on conformational changes, which affect the affinity of the integrin to its ligand. Many integrins are expressed and remain in a low-affinity binding state until cellular stimulation transforms them into a high-affinity form, an event known as integrin activation, which can modify cell adhesion (Qin et al., 2004). Because information travels from the inside to the outside of the cell, this is often referred to as ‘inside-out’ signaling (Hynes, 2002). Although the details are still unclear, inside-out signaling typically leads to the binding of talin to the β -integrin cytoplasmic tail, followed by a conformational change of the integrin and finally an increased affinity of the integrin to the ligand (Anthis et al., 2009; Ling et al., 2003; Ye et al.). Other signaling events modulating integrin signaling (either activating or deactivating) mainly either manipulate talin-binding or cause valency changes of the integrin (which affects ligand affinity) (Carman and Springer, 2003; Hynes, 2002). This regulation of integrins is essential for their biological functions made most obvious in platelet aggregation. If the affinity of the major platelet integrin (α IIb β 3) is not tightly controlled, the effects on the clotting process would be disastrous, leading to either thrombosis or bleeding disorders (Banno and Ginsberg, 2008; Kim et al., 2009).

Trafficking

It has been known for almost 20 years that integrins are continuously internalized and recycled back to the cell surface (Bretscher, 1989; Bretscher, 1992). The trafficking of integrins by the endosomal pathway can influence their functions, polarize

distributions and affect the signaling of other associated growth factor receptors. Integrin endocytosis and recycling has been described as a process which moves the adhesion receptors from the back to the front of a migrating cell (Bretscher, 1996); however, there is still no evidence for such long-ranging machinery.

Depending on the heterodimer, integrin internalization occurs through both clathrin-dependent and -independent mechanisms. However, not all integrins have been characterized leaving much still unclear (known data summarized in (Caswell et al., 2009)). It has been speculated that the same integrin can be internalized by different mechanisms in response to various environmental cues (Ramsay et al., 2007). It has also been suggested that the mechanism of internalization will affect the trafficking route and subsequently the state of the cell (Caswell and Norman, 2006).

Following endocytosis, the fate of the internalized integrin is determined in the early endosome: degradation, short-loop recycling or long-loop recycling. Most of the evidence accumulated thus far links integrins to the long-loop recycling; passing internalized integrins (such as $\alpha 5\beta 1$, $\alpha 2\beta 1$, $\alpha 6\beta 1$, $\alpha L\beta 2$, $\alpha V\beta 3$) from the early endosome to the perinuclear recycling compartment before returning to the plasma membrane via a Rab11-dependent mechanism (Caswell and Norman, 2006). This recycling path is dependent on other signaling molecules such as PKB/Akt, GSK3 β and PKC ϵ (Roberts et al., 2004). So far only $\alpha V\beta 3$ has been shown to be recycled in the short-loop pathway via Rab4, a pathway dependent on the recruitment of PKD1 (Roberts et al., 2001; Woods et al., 2004).

Integrin β 4

The β 4 integrin subunit (ITG β 4) was initially identified as a tumor-associated antigen (TS180) associated with metastasis (Falcioni et al., 1989; Falcioni et al., 1988). ITG β 4 was soon identified as a partner for the α 6 integrin subunit, to which it exclusively binds (Hemler et al., 1989; Kennel et al., 1989). A majority of integrins link the ECM to the actin cytoskeleton (Geiger et al., 2009), however α 6 β 4 integrin is a laminin receptor which mediates the hemidesmosome (HD) formation linking the ECM with the intermediate filament cytoskeleton (Borradori and Sonnenberg, 1996; Green and Jones, 1996; Nievers et al., 1999). The α 6 β 4 integrin is unique in other ways; most obvious is that while all other integrins consist of a very short cytoplasmic domain of ~50 amino acids, the cytoplasmic tail of α 6 β 4 (specifically the tail of ITG β 4) is 20 times as long (Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990). The extended tail is characterized by two pairs of fibronectin type III repeats separated by a connecting sequence. This cytoplasmic domain is crucial for the formation of HDs as well as any downstream signaling of α 6 β 4 integrin stimulation (Merdek et al., 2007; Murgia et al., 1998; Nikolopoulos et al., 2004; Nikolopoulos et al., 2005).

Role in hemidesmosomes

The importance of ITG β 4 is highlighted in the knockout mice which suffer from junctional epidermolysis bullosa (blistering of the skin) in response to mechanical stress and die shortly after birth (Dowling et al., 1996; Fuchs et al., 1997; Georges-Labouesse et al., 1996). The blistering is due to the loss of functional hemidesmosomes (HDs) leading to the detachment of the skin from the basement membrane. There are currently two

different types of HDs described in the literature; classic or type I HD which contain $\alpha 6\beta 4$ integrin, BPAG 180 and 230, HD1/plectin and CD151 (Litjens et al., 2006; Nievers et al., 1999) and type II HDs which lack the BPAG proteins (Uematsu et al., 1994). The linkage of integrin $\alpha 6\beta 4$ to the intermediate filament through HD1/plectin is critical for both types of HD formation (Niessen et al., 1997; Rezniczek et al., 1998; Sanchez-Aparicio et al., 1997).

Although it is important for HDs to provide strength and stability to the cell, they must also be dynamic and disassemble for cell migration during wound closure or carcinoma invasion (Daisuke Tsuruta, 2003; Geuijen and Sonnenberg, 2002; Ito et al., 2005; Levy et al., 2007). Although the process is still unclear, studies investigating the mechanism of HD disassembly have found that growth factor induced phosphorylation of several sites within the ITG $\beta 4$ cytoplasmic tail plays a crucial role in this process. EGF signaling triggers PKC dependent phosphorylation on S1356, S1360, S1364 and S1424, all of which can individually lead to at least partial HD disassembly (Germain et al., 2009; Rabinovitz et al., 2004; Santoro et al., 2003; Wilhelmsen et al., 2007).

Signaling/Role in Cancer

Although integrin $\alpha 6\beta 4$ is polarized to the basal region of normal epithelia, an early observation described its localization in several types of carcinoma cells as diffuse (Tennenbaum et al., 1993). This phenomenon supports the hypothesis that $\alpha 6\beta 4$ switches from a mechanical adhesive device into a signaling competent receptor during the progression from normal epithelium to invasive carcinoma (Lipscomb and Mercurio, 2005; Santoro et al., 2003). Integrin $\alpha 6\beta 4$ plays a role in a wide variety of intracellular

signaling, but it contains no inherent kinase activity and the precise mechanisms are still unclear. There are currently two different but not mutually exclusive hypotheses: the $\beta 4$ intracellular domain functions as a signaling adaptor and/or the localization of $\alpha 6\beta 4$ with tetraspanin-enriched membrane micro-domains enhances signaling abilities (Trusolino et al., 2001; Yang et al., 2004). The mechanisms of $\alpha 6\beta 4$ signaling in cancer are made even more complex considering there is both ligand-dependent and -independent activation (O'Connor et al., 1998).

Integrin $\alpha 6\beta 4$ signaling initiates a number of signaling cascades such as MAPK, PI3K/Akt and Shc/Src. These signals modulate many cellular behaviors like adhesion, proliferation, survival, motility and gene expression. Following stimulation, the cytoplasmic tail of ITG $\beta 4$ can be phosphorylated on several different tyrosine and serine residues. Y1494 is located in the consensus SH2 binding motif which binds phosphatase SHP2 (and possible SHP1) and activates Src (Bertotti et al., 2006; Merdek et al., 2007; Shaw, 2001; Unkeless and Jin, 1997). Studies found that Y1494 phosphorylation is necessary for the integrin $\alpha 6\beta 4$ activation of PI3K (Dutta and Shaw, 2008; Shaw, 2001). PI3K activation can then lead to Akt/PKB and Rac stimulation (Shaw et al., 1997). Y1526 is another crucial site for integrin $\alpha 6\beta 4$ signaling. When phosphorylated, Y1526 can recruit Shc, Grb2 and Sos thus activating Ras and stimulating the MAPK, Jnk and Erk signaling cascades (Mainiero et al., 1997). Studies have also shown that integrin $\alpha 6\beta 4$ regulated Rac activity in mammary epithelial cells leads to NF κ B activation (Weaver et al., 2002; Zahir et al., 2003).

Integrin $\alpha 6\beta 4$ can also indirectly control the expression of various genes critical for cancer progression. Activation of the PI3K/Akt pathway leads to the phosphorylation of 4E-BP1 by mTor. 4E-BP1 phosphorylation releases the repression on translation factor eIF-4E leading to the up-regulation of VEGF as well as other target genes (Chung et al., 2002). Additionally, integrin $\alpha 6\beta 4$ can activate the transcription factors c-Jun and STAT3 through ErbB2 (Guo et al., 2006), NF κ B through EGFR (Weaver et al., 2002; Zahir et al., 2003) and NFAT through PI3K (Jauliac et al., 2002). The introduction of ITG $\beta 4$ into MDA-MB-435 cells leads to a drastic change in the genetic profile including the up-regulation of S100A4/Metastasin, HDAC4 and SFRP1 and the down-regulation of FOS, EGR1 and GADD45A (Chen et al., 2009).

The culmination of these signaling events leads to the promotion of characteristics essential for cancer, such as survival, proliferation, motility, invasion and anchorage independent growth. PI3K promotes survival as well as motility and invasion in carcinoma cells and angiogenesis within a tumor (Baril et al., 2007; Lee et al., 2008; Nikolopoulos et al., 2004; O'Connor et al., 1998; Rabinovitz and Mercurio, 1996; Rossen et al., 1994; Shaw et al., 1997; Wei et al., 1998). An increase in NF κ B signaling leads to survival in addition to anchorage independent growth (Weaver et al., 2002; Zahir et al., 2003). The previously mentioned Shp2/Src signaling pathway aids in anchorage-independent growth when activated through integrin $\alpha 6\beta 4$ (Bertotti et al., 2006). Altogether, integrin $\alpha 6\beta 4$ signaling facilitates the survival and growth of carcinoma cells, especially when in a stressful environment like hypoxia (Baril et al., 2007; Chen et al., 2009; Lipscomb and Mercurio, 2005; Lipscomb et al., 2005). Studies found that cell

surface expression of integrin $\alpha 6\beta 4$ is up-regulated in hypoxic environments, indicating how crucial this signaling is for cancer cell survival (Yoon et al., 2005).

In addition to the signaling capabilities, integrin $\alpha 6\beta 4$ can also support cell migration and invasion through mechanical means. Antibodies specific for integrin $\alpha 6\beta 4$ inhibit carcinoma cell migration on laminin-1 as well as the formation of filopodia and lamellipodia (Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999). Integrin $\alpha 6\beta 4$ interacts with the F-actin in filopodia, lamellipodia and the retraction fibers of invasive cells (O'Connor et al., 1998; Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999). Further studies demonstrated that traction forces on the base of the lamellae are exerted directly through integrin $\alpha 6\beta 4$ in cells plated on either laminin or anti- $\alpha 6\beta 4$ antibodies (Rabinovitz et al., 2001).

In Breast Cancer

Although ITG $\beta 4$ expression is also linked to poor patient prognosis in a variety of cancers (Raymond et al., 2007), its role in breast cancer has been of particular interest. Similar to other integrins, integrin $\alpha 6\beta 4$ associates and cooperates with a variety of growth factor receptors, such as EGFR (Mariotti et al., 2001), ErbB2 (Gambaletta et al., 2000), and Met (Chung et al., 2004; Trusolino et al., 2001). Although all of these receptors have known roles in cancer, ErbB2 is a dominant oncogene in breast cancer and is amplified in 25-30% in human breast tumors (Muthuswamy, 2006). Further studies of human samples found that ITG $\beta 4$ expression is correlated to breast cancer size and nuclear grade (Diaz et al., 2005), and significantly correlates with basal-like breast cancer (Lu et al., 2008). More recently, EGFR was also found to be over-expressed in the basal-

like subgroup of breast-cancer and $\alpha 6\beta 4$ was identified as important for its distribution and function (Gilcrease et al., 2009).

Overall, these data demonstrate that integrin $\alpha 6\beta 4$ plays a critical role in cancer and any further insight into the regulation of this molecule may prove invaluable towards the development of a targeted therapy.

In the Epidermis

The role of ITG $\beta 4$ in skin is moderately well defined; however there are several seemingly conflicting reports. As previously mentioned, the skin of mice nullizygous for ITG $\beta 4$ fails to remain attached to the basement membrane causing the mice to die shortly after birth. In humans, this disease is called junctional epidermolysis bullosa (JEB) or epidermolysis bullosa lethalis because of the high mortality rate associated with the disorder. A majority of JEB cases involve either homozygous or compound heterozygous mutations in the laminin 5 chain (the ligand for $\alpha 6\beta 4$), but some compound heterozygous mutations have also been detected in the *ITG $\beta 4$* gene (Niessen et al., 1996; Vidal et al., 1995). When samples from skin lacking ITG $\beta 4$ were examined histologically, integrin $\alpha 6$ was barely detectable, suggesting that it is unstable in the absence of ITG $\beta 4$ (Dowling et al., 1996; van der Neut et al., 1996). Some studies find mice lacking ITG $\beta 4$ maintain normal skin morphogenesis and show no defects in keratinocyte proliferation, survival or differentiation (DiPersio et al., 2000; Raymond et al., 2005). However, others report degeneration of basal keratinocytes and defective epidermal growth and migration in cells expressing a signaling defective ITG $\beta 4$ (Dowling et al., 1996; Nikolopoulos et al., 2005). Supporting the latter finding, the cytoplasmic domain of ITG $\beta 4$ can bind to the

adaptor protein Shc and thus activate Ras/MAPK contributing to keratinocytes proliferation (Mainiero et al., 1997). Follow-up studies found that ITG β 4 needs to physically interact with an EGF-R family member to amplify pro-proliferative signals (Guo et al., 2006). These seemingly conflicting findings regarding the role of ITG β 4 in proliferation may be the product of the method used to nullify the signal. Signaling defective ITG β 4 may compete for binding sites on proliferation-promoting proteins; whereas this would not be a concern in ITG β 4 null cells. This would explain why the signaling defective ITG β 4 cells show a decrease in proliferation (Dowling et al., 1996; Nikolopoulos et al., 2005), whereas the ITG β 4 null cells do not (DiPersio et al., 2000; Raymond et al., 2005). It is possible that integrin β 1, the other binding partner of integrin α 6, provides a functionally redundant signal in regards to proliferation.

Arrestins

Family Members

There are four known and well studied genes encoding arrestins. The expression patterns of arrestin 1 (SAG/visual arrestin) and arrestin 4 (ARR3/cone arrestin) are restricted, primarily localized to the visual sensory tissue where they regulate rhodopsin photoreceptor signaling. However, arrestin 2 (ARRB1/ β -arrestin-1) and arrestin 3 (ARRB2/ β -arrestin-2) are ubiquitously expressed and interact with the vast majority of other GPCRs. However, recent phylogenetic analysis has identified another 6 members of the arrestin family in humans: ARRDC1, ARRDC2, ARRDC3, ARRDC4, ARRDC5 and TXNIP (Alvarez, 2008). This same study also identified VPS26 as a close relative to the family.

Role in GPCR regulation

Arrestins were first discovered to have a role in a conserved two-step mechanism for regulating G protein-coupled receptors (GPCRs) (Gurevich and Gurevich, 2006b). In response to a stimulus, GPCRs activate heterotrimeric G proteins, but in order to turn off this response, the activated receptors need to be silenced. Receptor silencing is initiated by phosphorylation by a class of serine/threonine kinases called G protein coupled receptor kinases (GRKs). GRK phosphorylation creates a high-affinity binding site for arrestins on the GPCR; for example, the affinity of the β 2 receptor-arrestin 2 interaction increases 10-30 fold after phosphorylation by GRK2 (Lohse et al., 1992). GPCR phosphorylation recruits arrestins from the cytoplasm to the plasma membrane, a binding which blocks further G protein-mediated signaling and redirects signaling to alternative G protein-independent pathways (Figure 1-3).

Arrestins block GPCR coupling to G proteins via two mechanisms: desensitization and sequestration. The arrestin binds to the cytoplasmic tip of the receptor and masks the binding site for the heterotrimeric G-protein (preventing activation); this is called desensitization. However, β -arrestins can further suppress signaling by linking the receptor to elements of the internalization machinery (clathrin and clathrin adaptor AP2). This promotes receptor internalization via clathrin coated pits and subsequent transport to an internal compartment, known as the endosome. Subsequently, the receptor could be either targeted for degradation (typically via the lysosome) or recycled back to the plasma

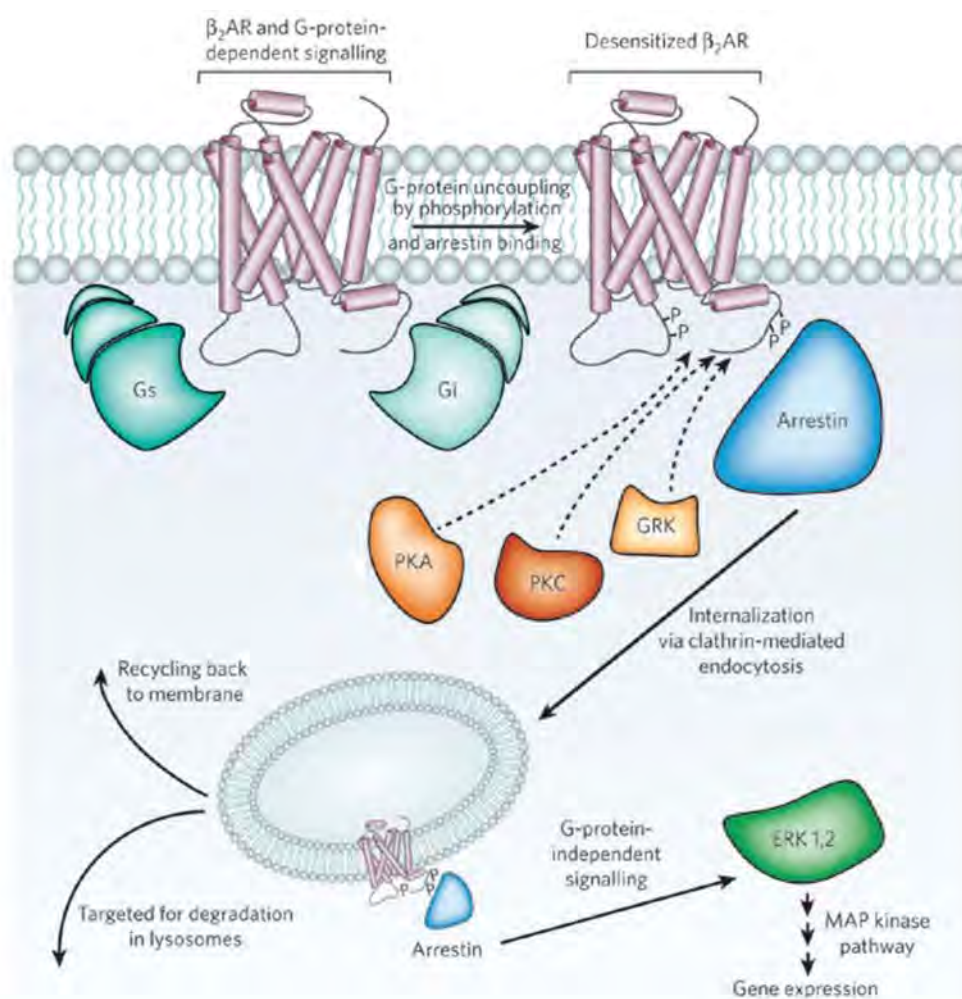


Figure 1-3: Schematic of Arrestin mediated GPCR regulation and GPCR-independent signaling. Stimulation of the receptor leads to phosphorylation by a G-protein-coupled receptor kinase (GRK) and subsequent coupling to arrestin preventing further signaling through the receptor. Arrestins can also promote the internalization of the receptor through clathrin coated pits. Arrestins also act as scaffolding proteins and promote the activation of ERK (as well as other signaling pathways).

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 The structure and function of G-protein-coupled receptors
 Daniel M. Rosenbaum, Søren G. F. Rasmussen & Brian K. Kobilka

membrane where it can signal again (re-sensitization). The strength of arrestin-receptor interaction seems to play a role in this choice: tighter complexes tend to increase the probability of receptor degradation, whereas more transient complexes typically favor recycling (Gurevich and Gurevich, 2006b). Also important in the post-endocytotic sorting of the internalized proteins is the ubiquitination of the receptor and/or arrestin (Bhandari et al., 2007; Shenoy and Lefkowitz, 2003; Shenoy et al., 2001; Shenoy et al., 2008). Receptor and self ubiquitination can also be controlled by the arrestin and will be further discussed shortly.

Beyond GPCR regulation

In addition to GPCRs, arrestins can bind to several other signaling proteins and thus affect signaling (Gurevich and Gurevich, 2004). Arrestins can act as scaffolding or bridging proteins which brings together signaling partners and increases efficiency. There is a dramatic conformational change between free and receptor-bound arrestin and this can influence the affinity of these other signaling molecules. Receptor-bound arrestins serve as scaffolds for MAP kinase cascades, bringing together apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinase 3 (JNK3), as well as the kinase c-Raf-1 and extracellular signal-regulated kinase 2 (ERK2), thereby facilitating signaling in the ASK1-Map kinase kinase 4 (MKK4)-JNK3 and c-Raf-1-MAP/ERK kinase 1 (MEK1)-ERK2 pathways (Luttrell et al., 2001; McDonald et al., 2000). Additionally, arrestin3 binds both PKB/Akt and its negative regulator protein phosphatase 2A (PP2A), permitting the deactivation of Akt (Beaulieu et al., 2005). Arrestin3 also facilitates the deactivation of JNK3 by recruiting the dual-specificity phosphatase MKP7 (Willoughby

and Collins, 2005). When ERK2 and JNK3 are activated by the arrestin-receptor complex they stay bound to the endosomes and thus cannot translocate to the nucleus, affecting transcription (Luttrell et al., 2001; McDonald et al., 2000).

Arrestins can influence gene transcription in other ways. Both arrestin2 and arrestin3 interact directly with I κ B α , an inhibitor of NF- κ B, following stimulation of the β 2-adrenergic receptors. This interaction prevents its phosphorylation and degradation and thereby repressing the activity of NF- κ B (Gao et al., 2004; Witherow et al., 2004). These arrestins regulate NF- κ B signaling in another way; by interacting with the tumor necrosis factor receptor-associated factor 6 (TRAF6), preventing its auto-ubiquitination and the subsequent activation of NF- κ B (Wang et al., 2006). There is further evidence that arrestins can effect gene transcription more directly by entering the nucleus.

Arrestins 2 and 3 can be present in both the cytoplasm and nucleus, depending on various post-translational modifications (Wang et al., 2003). After translocation into the nucleus, arrestins can associate with transcription factors such as p300 and CREB and modulate transcription (Kang et al., 2005; Ma and Pei, 2007).

In addition to signaling molecules, arrestins also recruit ubiquitin ligases to the receptors: the E3 ubiquitin ligase Mdm2 is mobilized by mammalian β -arrestins and ubiquitinates GPCRs (Shenoy et al., 2001), and the E3 ligase Deltex mobilized by Kurtz (an arrestin specific to *Drosophila*) ubiquitinates the Notch receptor (Mukherjee et al., 2005). Through proteins known as ARTs (*arrestin related trafficking adaptors*), arrestins can recruit other E3 ligases including CHIP, NEDD4 and ITCH/AIP4 (Bhandari et al.,

2007; Shenoy et al., 2008; Zhang et al., 2009). This function allows the arrestin to have even more control over the fate of its target.

Role in non-GPCR signaling

Arrestins can bind membrane proteins not part of the GPCR super-family, some of which are important for development and cancer. These include both receptor and non-receptor tyrosine kinases, non-classical 7TMRs (like Smoothed and Frizzled), ion channel receptors, and cytokine receptors. Similar to their interactions with GPCRs, arrestins can act as a scaffolding protein (creating new signaling complexes), control internalization/cellular localization or promote degradation with the non-GPCR targets.

In response to IGF stimulation, β -arrestin acts as an E3 ligase adaptor. After IGF binds to the tetrameric IGF1R, β -arrestin recruits Mdm2 to the receptor. Mdm2 ubiquitinates IGF1R, thus leading to its internalization. Once internalized, β -arrestin is part of a new “signalsome” and mediates the activation of ERK, which then translocates to the nucleus and activates transcription. IGF1R is ultimately degraded by the proteasome (Lin et al., 1998).

β -arrestin 2 binds to the type III TGF β receptor, a co-receptor that contributes to TGF β signaling through currently unknown mechanisms (Chen et al., 2003).

Downstream of TGF β -binding, β -arrestin is essential for the activation of Cdc42, which is then responsible for actin reorganization leading to chemotaxis and filipodial extension (Mythreya and Blobel, 2009). β -arrestin can also internalize the bound TGF- β receptor, attenuating TGF β -mediated SMAD signaling (Chen et al., 2003).

In the Hedgehog (Hh) pathway, arrestins play a role in receptor signaling as well as sub-cellular localization. Upon Hh binding, Patched (Ptc) repression is relieved and Smoothed (Smo) is phosphorylated by GRK-2. This initiates the formation of a complex with β -arrestins and the molecular motor Kif3A. The Smo- β -arrestin-Kif3A complex translocates Smo to the primary cilium where Smo cleaves Gli into its active form. Active Gli then translocates down the primary cilium and into the nucleus where it activates transcription of downstream targets (Kovacs et al., 2008; Molla-Herman et al., 2008). Smo activation and phosphorylation by GRK2 can also recruit β -arrestin 2 to the cell membrane which promotes clathrin mediated endocytosis (Chen et al., 2004).

Relevant to epidermal stem cell regulation, β -arrestins have been implicated as important mediators of both canonical and non-canonical Wnt signaling, but in different capacities. β -arrestin 1 interacts with phosphorylated Dishevelled 1/2 (Dsh1 & Dsh2) (Chen et al., 2001), and was shown to synergistically enhance LEF-mediated transcription when co-expressed with either Dsh proteins. In canonical Wnt signaling, β -arrestins also bind to Frizzled (Fz, receptor for Wnts) through Dsh and sequester the Axin/GSK3 destruction complex away from β -catenin, thus promoting its stabilization (Bryja et al., 2007). During non-canonical Wnt signaling, β -arrestins will complex with Dsh and AP-2 subsequently activating RhoA and Rac1 (Barnes et al., 2005).

ARRDC3

Arrestin domain containing protein 3 (ARRDC3, KIAA1376, TLIMP, ADC3) contains structural homology to β -arrestins which play an essential role in G protein-coupled receptors (GPCRs) signaling and internalization (Alvarez, 2008; Seachrist and

Ferguson, 2003). Although ARRDC3 is classified as an α -arrestin, there is a cluster of acidic and hydrophobic residues in the C-terminus that may bind to clathrin, suggesting a possible role in membrane protein internalization (Alvarez, 2008).

Prior to the work discussed in this thesis, there had been only one paper which looked at the function of ARRDC3 directly (Oka et al., 2006). The work of Oka et al. found that ARRDC3 is localized to the cell membrane, endosomes and lysosomes. This study also found that over-expression of ARRDC3 decreased cell proliferation and inhibited anchorage independent growth. Due to homology with thioredoxin binding protein 2 (TBP2 or vitamin D3 up-regulated protein-1), they investigated whether vitamin D3 affects ARRDC3 levels. Oka et al found the expression of ARRDC3 is induced in cells treated with vitamin D3 and, to a lesser extent, PMA. Interestingly, ARRDC3 expression was up-regulated when cells were treated with PPAR γ agonists troglitazone and pioglitazone, but not PPAR α agonist clofibrate. However, increases in ARRDC3 expression lead to decreases in PPAR γ signaling. These data suggest that ARRDC3 is part of a negative-feedback regulatory pathway of PPAR γ .

Although only directly investigated in the work previously mentioned, there have been a few passing references to ARRDC3, mostly in genetic screens. ARRDC3 was recently found within a cluster on chromosome 5 deleted in 17% of basal-like breast cancers (compared to 0% in luminal breast-cancers) suggesting a role as a tumor suppressor (Adelaide et al., 2007). When comparing pre-eclamptic placentas to normal, ARRDC3 expression was significantly higher in the pre-eclamptic samples. ARRDC3 expression was also high in the STOX1 (Storkhead Box 1, a transcription factor in the

Forkhead-family) over-expression model of preeclampsia and 4 Forkhead binding sites were identified and verified (Rigourd et al., 2008). This data suggests that ARRDC3 may be regulated in response to stress by the members of the forkhead family of transcription factors, proteins with know roles in development as well as cancer.

Although limited, the current data suggests that ARRDC3, similar to other arrestin family members, regulates signaling pathways and subsequently effects cellular behaviors such as proliferation, anchorage independent growth and survival.

CHAPTER II:

The Arrestin Protein Family Member ARRDC3 Controls Stem Cell Motility through β 4 Integrin Regulation

Parts of this chapter represent work submitted as:

The Arrestin Protein Family Member ARRDC3 Controls Stem Cell Motility through β 4
Integrin Regulation

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Abstract:

Control of stem cell migration is essential for organogenesis, tissue homeostasis and response to injury, but the molecular mechanisms governing this process within the stem cell niche are still unclear. In this study, we show that *ARRDC3* (*KIAA1376*, *TLIMP*) is more highly expressed within human adult skin stem cells when compared to daughter transit-amplifying cells. We demonstrate that *ARRDC3* is a novel post-translational regulator of ITG β 4 (ITG β 4). During dynamic regulation of ITG β 4 in migrating cells, *ARRDC3* interacts with the cytoplasmic domain of serine phosphorylated ITG β 4 on the lagging edge and targets it for internalization and proteasome dependent degradation. Over-expression of *ARRDC3* inhibits stem cell motility while down-regulation induces active migration, but does not diminish multipotency. Our results identify a novel mechanism of integrin regulation that controls the transition from anchored stem cells to migrating daughter cells.

Introduction:

Adult epithelial stem cells are generally found within distinct locations tightly adherent to the basement membrane (Raymond et al., 2009). Within skin, multipotent stem cells are located in the hair follicle bulge region, while daughter transit-amplifying (TA) cells are present in the hair bulb (Cotsarelis et al., 1990; Lyle et al., 1998; Morris et al., 2004). Several studies have identified genes over-expressed in the stem cell compartment of skin that help define stem cells and their niche (Morris et al., 2004; Ohyama et al., 2006; Tumber et al., 2004). The challenge has now become trying to understand the functions of differentially expressed genes in maintaining stem cell

properties such as cell migration. During normal tissue homeostasis and tissue regeneration of wound healing, stem cells remain as permanent residents of the stem cell niche, while their progeny TA cells migrate away from the niche to repopulate the tissue (Adams and Watt, 1991; Ito et al., 2005; Roh et al., 2005). While a great deal is known about the molecular mechanisms of cell migration, these pathways have not been fully analyzed in the context of epithelial stem cells and it is also clear that additional key regulatory molecules have yet to be characterized.

Members of the arrestin family of proteins represent components of the transmembrane receptor-binding complex that have been shown to regulate a number of cellular processes including cell migration (reviewed in (Kendall and Luttrell, 2009)). Recently, new members of the arrestin family have been identified, called “Arrestin Domain Containing Proteins” (ARRDC1-5, also ADC1-5) that have unidentified targets and functions (Aubry et al., 2009). ARRDC3 contains structural homology to the well-characterized β -arrestins that play an essential role in G protein-coupled receptor (GPCR) signaling and internalization (Alvarez, 2008; Seachrist and Ferguson, 2003). Other arrestin family members form complexes that co-localize with key vesicular trafficking proteins as well as endosomes (McDonald et al., 1999). Although ARRDC3 is classified as an α -arrestin, there is a cluster of acidic and hydrophobic residues in the C-terminus that may bind to clathrin, suggesting a role in membrane protein internalization (Alvarez, 2008). Although prior work has suggested a role for ARRDC3 in PPAR γ signaling and endosomal functions (Oka et al., 2006), no link to surface molecules has been demonstrated.

Integrins are a class of cell surface receptors that are known to play an important role in epithelial cell adhesion and migration (Rabinovitz and Mercurio, 1997; Streuli, 2009; Vicente-Manzanares et al., 2009), but there is still much to learn about the regulatory networks that control integrin function. Several integrins are expressed in skin stem cells, including the $\alpha 6\beta 4$ heterodimer (here out referred as ITG $\beta 4$) (Watt, 2002). ITG $\beta 4$ is the core component of hemidesmosomes that anchor cytoplasmic keratin filaments to the extracellular basement membrane (Rezniczek et al., 1998; Schaapveld et al., 1998). Humans with ITG $\beta 4$ mutations suffer from a disease known as junctional epidermolysis bullosa (blistering of the skin) which demonstrates its significant role in tissue homeostasis (Gil et al., 1994; Niessen et al., 1996; Vidal et al., 1995). ITG $\beta 4$ null mice also demonstrate junctional epidermolysis bullosa in response to mechanical stress and die shortly after birth (Dowling et al., 1996; Fuchs et al., 1997; Georges-Labouesse et al., 1996). However, the role of ITG $\beta 4$ in the skin stem cells beyond adhesion to the basement membrane is currently unclear.

In addition to adhesion, ITG $\beta 4$ confers the ability of cells to migrate and resist apoptotic stimuli, (Lipscomb and Mercurio, 2005; Lipscomb et al., 2005; Rabinovitz and Mercurio, 1996; Wilhelmsen et al., 2006). Migration of stem cell progeny is critical for normal tissue homeostasis and wound healing as demonstrated by the re-epithelialization process after the wounding of skin (Ito and Cotsarelis, 2008; Ito et al., 2005; Levy et al., 2007). During keratinocyte migration, hemidesmosomes, containing ITG $\beta 4$, are disassembled by a mechanism that has not been well-characterized (Borradori and Sonnenberg, 1999; Litjens et al., 2006; Margadant et al., 2008). During wound healing, as

well as in carcinoma invasion, growth factors such as EGF are elevated and lead to hemidesmosome disassembly (Mainiero et al., 1996; Rabinovitz et al., 1999). EGF stimulation induces phosphorylation of ITG β 4 on a variety of serine and tyrosine residues (Germain et al., 2009; Mainiero et al., 1996; Rabinovitz et al., 2004) resulting in hemidesmosome disassembly and mobilization of ITG β 4 to actin-rich protrusions (Germain et al., 2009; Rabinovitz et al., 1999; Rabinovitz et al., 2004; Wilhelmsen et al., 2007). More recently, phosphorylation of ITG β 4-S1424 was found enriched on the trailing edge of migrating cells and implicated in the dissociation of ITG β 4 from hemidesmosomes (Germain et al., 2009). This dynamic regulation of ITG β 4 is such a process that could potentially mediate the transition from anchored stem cells to actively migrating TA cells in human skin.

In this study we demonstrate that a new arrestin family member, ARRDC3, is up-regulated in skin stem cells. Using in vitro assays of stem cell behavior, we show that ARRDC3 has significant effects on cell migration. Our molecular studies establish that ARRDC3 affects stem cell function through a novel regulatory pathway involving the internalization and degradation of ITG β 4.

Results:

ARRDC3 is expressed in skin stem cells.

We previously demonstrated key functional differences (such as cell migration and self-renewal) between keratinocyte stem cells and TA cells of the human hair follicle (Roh et al., 2005). We next wished to determine differences in the genetic expression profiles of these two cell types in an effort to elucidate the mechanism for the functional

changes. An enriched stem cell population was obtained by micro-dissection of telogen hair follicle bulges, while TA cell enriched population was dissected from anagen hair follicle bulbs (Figure 2-1A). RNA isolated from these cell populations was then used for subtraction hybridization. Stem cell-enriched cDNAs were then cloned and sequenced, identifying ARRDC3 as a differentially expressed gene.

Real-time PCR analysis shows that ARRDC3 expression is 3.41 fold higher in telogen bulge cells when compared to anagen bulb cells (Figure 2-1B). Interestingly, the anagen bulge has a 2.03 fold higher level of ARRDC3 expression when compared to anagen bulb (Figure 2-1B); suggesting high ARRDC3 levels are maintained throughout the hair cycle and are increased during stem cell activation of telogen follicles.

Immunofluorescence and immunohistochemistry of adult human skin sections and hair follicles confirmed that ARRDC3 protein is increased in keratinocyte stem cells of the bulge when compared to cells in the lower follicle (Figures 2-1C&D). As shown in tissue sections, cells in the basal layer of the bulge region demonstrate the most intense staining with some apparent expression in suprabasal cells as well. Altogether these data demonstrate that ARRDC3 is highly expressed in epithelial stem cells and down-regulated in daughter/TA cells.

Interestingly, when co-stained for the skin stem cell marker keratin 15 (K15) (Lyle et al., 1998), only a subset of ARRDC3 positive cells are positive. This data, along with the staining from the skin sections, suggests that although ARRDC3 is more highly expressed in the stem cell region of the skin, it is not a stem cell marker. In addition, only a subset of keratin 15 positive cells also expressed ARRDC3 (Figure 2-2).

ARRDC3 sequence and structure analysis

ARRDC3 has a high degree of sequence homology throughout several species, suggesting a similarly important role. ClustalW alignment on the amino acid sequence of ARRDC3 from *G. gallus*, *E. caballus*, *R. norvegicus*, *M. musculus*, *P. troglodytes*, *P. abelii*, *H. sapiens*, and *B. taurus* show at least a 98.3% similarity to the human sequence (Figures 2-3A & 2-4). Primary sequence analysis of ARRDC3 using secondary structure prediction algorithm PSPIRED found secondary structure elements consistent with other arrestin family members (Figure 2-3B). 3D-Jigsaw (Bates et al., 2001) generated a putative tertiary structure of ARRDC3 (Figure 2-5). Using GenThreader, we found that ARRDC3 is predicted to be a close structural homologue to VPS26 (vacuolar protein sorting) with an identical probability value of $5e-11$ (Jones, 1999; McGuffin and Jones, 2003; Shi et al., 2006). VPS26 is a key part of the retromer, a complex of proteins involved in endocytotic trafficking, endosomal sorting and the transcytosis of polarized cells (Alvarez, 2008; Bonifacino and Hurley, 2008; Bonifacino and Rojas, 2006; Haft et al., 2000; Seaman, 2005). Comparing the known structure of VPS26 and the projected structure of ARRDC3 reveals marked similarities (Figure 2-5). Mutations made in the domains of VPS26 homologous to ARRDC3 cause an abrogation of its functions (Reddy and Seaman, 2001), suggesting ARRDC3 also plays a role in endocytosis or vesicular trafficking.

Expression Pattern of ARRDC3 in Human Tissues

ARRDC3 mRNA levels were analyzed in a variety of normal human tissues using northern blot analysis (Figure 2-6A). We found that ARRDC3 expression levels vary in

different tissue types. ARRDC3 expression is exceptionally high in the placenta, but is also high in skeletal muscle, kidney, liver and lung. ARRDC3 is weakly expressed in brain, skeletal muscle, colon, thymus, and small intestine. Moderate expression is seen in the heart and peripheral blood leukocytes (Figure 2-6A). Although widely expressed in human tissues, the expression patterns within specific cell types of a tissue are yet to be determined. Specifically, it is unclear whether ARRDC3 expression is restricted to cells within the adult stem cell niche.

We next analyzed the cellular localization of ARRDC3 using a monoclonal antibody generated in our lab for immunofluorescence. Confocal microscopy images of hair follicle stem cells show endogenous ARRDC3 is weakly distributed throughout the cytoplasm, but is concentrated at certain sections of the cell membrane. The presence of ARRDC3 foci near the cell membrane suggests an association with intracellular vesicles (Figure 2-6B). This observation further validates the notion that ARRDC3 regulates a cell-surface protein.

ARRDC3 co-localizes with endosomal markers

Confocal microscopy images of hair follicle stem cells show that endogenous ARRDC3 concentrates at certain sections of the cell membrane. The presence of ARRDC3 foci near the cell membrane suggests an association with intracellular vesicles (Figures 2-6B & 2-7A). To confirm this, we examined the co-localization of ARRDC3 with endosomal markers using a proximity ligation assay (PLA). ARRDC3 strongly co-localizes with EEA1 and Clathrin, while only minimal signal is seen with CAV1 (Figure 2-7B&C). To further visualize positioning within the cell, images obtained from confocal

microscopy were used to generate a 3D reconstruction of the cells. This reconstruction demonstrated that ARRDC3 co-localizes with endosomal proteins evenly throughout the cell. Overall, these data indicate ARRDC3 primarily associates with endosomes which are internalized in a clathrin dependent manner.

ARRDC3 interacts with ITG β 4

Due to the homology with other arrestin proteins (Figure 2-4B) (Luttrell and Lefkowitz, 2002), we hypothesized that ARRDC3 has similar biological functions. To determine potential molecular targets, a yeast-two hybrid library screen using full length ARRDC3 as bait was performed. This screen identified a fragment of the cytoplasmic tail of ITG β 4 (Figure 2-8A). This interaction can be seen endogenously in telogen cells by immuno co-precipitating ITG β 4 with an anti-ARRDC3 antibody (Figure 2-8B). Phosphatase inhibitors are necessary to see this interaction, suggesting that ITG β 4 requires phosphorylation for binding to occur. Since ITG β 4 has several critical serine and tyrosine phosphorylation sites within the cytoplasmic tail, we tested for the enrichment of phospho-ITG β 4 after using an anti-ARRDC3 antibody for immuno co-precipitation (Figure 2-8B&C). Quantification using densitometry revealed that anti-ARRDC3 antibody precipitated approximately 53% of the phospho-ITG β 4 compared to the amount precipitated with ITG β 4.

Immunofluorescence of skin stem cells demonstrated co-localization of ARRDC3 and ITG β 4 within the cell (Figure 2-8C). Interestingly, there were areas of the cell that appeared to show a deliberate segregation of the proteins, specifically near cell-cell boundaries. These data suggest that the interaction between ARRDC3 and ITG β 4 is

dynamic and dependent on the relative position within the cell and/or phosphorylation status. To further investigate the intercellular positioning of the ARRDC3/ITG β 4 complex, we generated a 3D construction of a PLA from confocal images. Cultured stem cells showed a polarized localization of ARRDC3/ITG β 4 on the basal layer of the cell (Figure 2-8D), suggesting that ARRDC3 interacts with ITG β 4 while in HDs. Giving further support to this theory, PLA analysis of ARRDC3 with HD marker PLEC1 found the same pattern of co-localization in the basal layer of the cell (Figure 2-8E).

ARRDC3 regulates ITG β 4 protein levels

Since β -arrestins target GPCRs for internalization/degradation (Luttrell and Lefkowitz, 2002), we hypothesized that ARRDC3 has a similar effect on ITG β 4. Using two different siRNAs to down-regulate ARRDC3 in cultured skin stem cells, we found an inverse relationship between ARRDC3 expression and ITG β 4 protein levels (Figure 2-9A). To further verify the effects of altered ARRDC3 on ITG β 4, we infected skin stem cells with an adenovirus co-expressing ARRDC3 and GFP and then looked at ITG β 4 levels using immunofluorescence. ITG β 4 was lost from ARRDC3-GFP over-expressing cells while there was no change in either neighboring uninfected cells or control-GFP infected cells (Figure 2-9B). Using flow cytometry on non-permeabilized cells, we found cell surface levels of ITG β 4 were completely lost in cells over-expressing ARRDC3, although a residual amount appeared to be retained within the cytoplasm when examined by immunofluorescence (Figure 2-9C).

ITGβ4 regulation by ARRDC3 is proteasome dependent

As ITGβ4 affects numerous signaling pathways, it was unclear whether the decreased protein level was an immediate response to the interaction with ARRDC3 or caused by a feedback pathway leading to a decrease in mRNA and subsequently protein level. To test this, we transfected the skin stem cells with either a control siRNA or an ARRDC3 targeting siRNA. RT-PCR analysis showed no changes in *ITGβ4* mRNA levels, demonstrating the regulation of ITGβ4 by ARRDC3 is post-transcriptional (Figure 2-10A). To exclude the possibility that protein synthesis was necessary for the increase in ITGβ4 after ARRDC3 repression, we treated cells with cyclohexamide and transfected with either control or *ARRDC3* targeting siRNAs. Cells analyzed using immunofluorescence and western blot analysis revealed the treatment of cyclohexamide did not prevent an increase in ITGβ4 (Figures 2-10B). This further supports the notion that ARRDC3 regulates ITGβ4 protein directly and not through its synthesis.

We next wanted to determine the mechanisms of ITGβ4 protein degradation and found that ARRDC3-mediated ITGβ4 protein degradation is inhibited in the presence of proteasome inhibitor lactacystin. This is demonstrated by both immunofluorescence and western blot analysis (Figure 2-11A&B). ITGβ4 expression was not affected by the GFP control virus, either with or without proteasome inhibitor. When cells are examined using flow cytometry, the addition of the proteasome inhibitor does not prevent ITGβ4 removal from the cell surface (Figure 2-11C), supporting the notion that interaction with ARRDC3 causes the initial internalization of ITGβ4, a process that would be unaffected by downstream proteasome inhibition. The addition of lysosomal inhibitor chloroquin did

not affect the ARRDC3-induced decrease in ITG β 4 levels (Figure 2-12), suggesting that the degradation is solely dependent on the proteasome.

ARRDC3 controls stem cell motility

Stem cells are anchored within the niche whereas their progeny must be able to leave the niche and replenish tissues. Knowing ITG β 4 is involved in cell motility, we suspected that ARRDC3 affects the motility of skin stem cells. Stem cells from human skin (Roh et al., 2008) were infected with either a control siRNA, an siRNA targeting ARRDC3, or an adenovirus expressing ARRDC3 and then used to conduct a scratch assays. The wound widths were measured in Adobe Photoshop (arbitrary units) from images taken using a 4X objective. After 48 hours, cells with decreased ARRDC3 had almost a complete wound closure (0.91 to 0.24) whereas the control cells did not (0.95-0.66). Additionally, the wound width for skin stem cells over-expressing ARRDC3 was only minimally decreased (0.95-0.82) (Figure 2-13A). Motility in transit-amplifying cells isolated from the hair bulb was assayed in the same manner (Figure 2-13B). TA cells with low ARRDC3 demonstrated a faster wound closure rate (0.99-0.05) compared to control TA cells (0.97-0.17). TA cells engineered to over-express ARRDC3 that showed a significantly slower rate of migration (0.94-0.50) ($p < 0.0006$).

Our data so far suggests that ARRDC3 plays a role in controlling skin stem cell migration by regulating ITG β 4. To understand how ARRDC3 behaves in migrating stem cells, *in-vitro* scratch assays were used to identify actively migrating cells and the endogenous expression pattern of ITG β 4 and ARRDC3 was examined. Interestingly, ARRDC3 and ITG β 4 maintained co-localization only on the lagging edge of the

migrating cells (Figure 2-14A). Furthermore, when the leading edge of the cell was examined, the basal plane of the cell attached to the substratum showed high levels of ITG β 4 while ARRDC3 was present in the apical (top) plane. This suggests ARRDC3 interacts with ITG β 4 in HDs about to be dissolved, but not in newly formed ITG β 4 focal adhesions.

ARRDC3 interacts with ITG β 4 when phosphorylated at serine-1424

A novel phosphorylation site on ITG β 4 was recently identified as important in HD disassembly and shown to be enriched on the trailing edge of a migrating cell (Germain et al., 2009). As this phenotype mirrored our observations of ARRDC3, this residue was a good candidate for the phosphorylation site which facilitates interaction between ITG β 4 and ARRDC3. The relative distributions of endogenous ARRDC3, ITG β 4 and ITG β 4-pS1424 in migrating skin stem cells were examined using confocal microscopy. Supporting our hypothesis, we found almost a complete overlap of ARRDC3 and ITG β 4-pS1424 localization in migrating cells (Figure 2-14B).

We then determined that ARRDC3 physically interacts with ITG β 4-pS1424 by endogenous immuno co-precipitation of skin stem cell lysates (Figure 2-8B).

Densitometric analysis demonstrates that ~68% of ITG β 4-pS1424 is immuno co-precipitated with an ARRDC3 antibody when compared to an ITG β 4 antibody. The densitometric ratio of precipitated pS1424/ITG β 4 is 6.6 fold higher in the ARRDC3 immuno co-precipitation when compared to the ITG β 4 immuno co-precipitation. Collectively, these data suggest that following phosphorylation of ITG β 4-S1424, ARRDC3 binds to the cytoplasmic tail and promotes internalization of ITG β 4. This

occurs during HD disassembly either while the cell is migrating (a process localized on the lagging edge of cells) or during normal HD turnover (a perpetual event in resting cells) (Daisuke Tsuruta, 2003; Geuijen and Sonnenberg, 2002).

ARRDC3 does not affect multi-potency

To test whether ARRDC3 affected the multi-potency of stem cells, we generated stable human skin stem cell lines with altered ARRDC3 expression. The lines generated were analyzed for ARRDC3 and ITG β 4 levels using western blot analysis (Figure 2-15A). We then induced the altered skin stem cells lines down epidermal, hair or sebaceous lineages. After induction, cells were examined for morphology and expression of differentiation markers.

After sebocyte differentiation was induced, each cell line showed a marked increase in oil red O staining (not shown) and an increase in KRT7 expression when compared to the un-induced control (Figure 2-15B), indicating that ARRDC3 expression levels do not affect the ability of stem cells to undergo sebocyte differentiation.

Epidermal markers are increased in all stem cell lines after epidermal differentiation indicating that ARRDC3 expression does not affect epidermal differentiation (Figure 2-15C). Each skin stem cell line showed an up-regulation of K6HF after hair induction compared to the control, confirming that ARRDC3 expression does not affect the ability of stem cells to undergo hair differentiation (Figure 2-15D).

ARRDC3 expression preserves an undifferentiated state in skin stem cells

It was interesting to note that expression of ARRDC3 appeared to shift undifferentiated cells towards the sebaceous lineage. A low level of spontaneous

differentiation occurs in the cultured Telogen E6/E7 cells seen by the faint presence of keratin markers when the cells are undifferentiated. However, expression of keratin 1, involucrin and keratin k6hf are decreased in the skin stem cells over-expressing ARRDC3 and expression of keratin 7 is slightly increased. This suggests that ARRDC3 may influence the spontaneous differentiation of telogen cells down the sebaceous lineage (Figure 2-15).

To test how differentiation affected ARRDC3 protein levels, we analyzed lysates from the parental skin stem cell line after differentiation along with the respective controls. Western blot analysis shows ARRDC3 levels decrease with sebocyte and hair differentiation but increase with skin differentiation. These changes did not correlate to the changes in ITG β 4 expression suggesting that it is unlikely that ARRDC3 is the only factor regulating ITG β 4 during skin stem cell differentiation (Figure 2-16).

Loss of ARRDC3 causes abnormal whisker follicles and loss of fur in mice

In an effort to determine the role of ARRDC3 *in vivo*, we generated a knock-out mouse model using mouse embryonic stem cells, on a 129 background, generated by Sanger Institute (cell line CG0361, created using gene-trap technology). C57Bl6/129 chimeras were created with these cells and mated to C57Bl6 mice to test for germline transmission, with the presence of the gene-trap verified by PCR amplification of the NEO-cassette.

Unfortunately, all of our efforts to create a genotyping protocol which distinguishes between the homozygous and heterozygous null mice have been unsuccessful. SIGTR performed 5' RACE on ES cell RNA and determined the gene-trap

is located between exons 1 and 2 (in intron 2) of the ARRDC3 locus. We initially tried to use forward and reverse PCR primers (positioned every ~200 bp in the 3.5kb intron) in the intron paired with a reverse primer on the 5' end of the genetrap or a forward primer on the 3' end of the genetrap (respectively) to amplify the insertion site. Unfortunately, there was no amplification. We next tried to generate a Southern genotyping protocol. However, all attempts to PCR amplify this region of the wild-type strand (in order to create a probe) have failed. Consultations with the trouble-shooting departments of Genewiz and Genetyper indicate that this locus is “mostly inaccessible” likely due to supercoiling and/or CpG methylation. Both companies indicated that these difficulties could eventually be overcome with the right PCR conditions. For the mice discussed in this thesis, heterozygotes and homozygous null mice were distinguished by phenotype.

ARRDC3 heterozygous mice are viable, develop normally and are fertile.

However, as the mice age, they seem to progressively lose their fur (Figure 2-17A). Additionally, ARRDC3 null mice are born without whiskers and lose their fur much faster when compared to the heterozygotes (Figure 2-17B). It was interesting to note that although all mice start to lose their fur, the rate and pattern of the loss appears to be different for each mouse. Some had large patchy losses whereas some presented with an overall thinning of the fur. However, as mice were not housed separately it is possible this phenotype is actually due to mice barbering one another. Histological examination of the whisker pads from ARRDC3 null mice show morphological abnormalities in the whisker follicles (Figure 2-18A). Additionally, the dermis is much thinner in the null mice and there are fewer hair follicles present compared to wild-type littermates (Figure

2-18B). It is possible the loss of ARRDC3 led to increased migration of hair follicle stem cells out of the niche. This would lead to stem cell pool exhaustion and the loss of hair follicle homeostasis (i.e. decreased hair follicles). As hair follicles provide structure to the skin, which helps to maintain the thickness of the dermis, their loss is likely the cause of the thin epidermal layer. However, all of these observations are preliminary as the mice are still on a mixed background and only a few mice have been examined.

Discussion:

In the present study, we demonstrate that ARRDC3 is highly expressed in the stem cell compartment of human skin. As it does not affect differentiation, the main function of ARRDC3 appears to be suppressing motility. This is especially critical at the time of stem cell activation when the stem cells divide asymmetrically to self-renew and produce a committed daughter TA cell. Since the stem cell must remain behind while the TA cells migrate away, high levels of ARRDC3 are likely needed within the stem cells to prevent migration. Indeed, we saw the highest levels of ARRDC3 in the hair follicle stem cell compartment in the telogen phase when stem cells are in the early stages of being activated. We saw elevated, but comparatively lower levels of ARRDC3 in stem cells of anagen stage follicles when the stem cells are quiescent and stable.

Although the role of ITG β 4 in cell migration has been studied extensively, there is yet to be a clear understanding of how HD regulation is involved in this process. In cultured keratinocytes, there is a constant turnover of HDs (Daisuke Tsuruta, 2003; Geuijen and Sonnenberg, 2002). It is thought that this dynamic state helps to maintain cells competent for migration, when HDs need to be rapidly disassembled to allow for

detachment from the basement membrane. This hypothesis explains why skin stem cells maintain high expression of both ARRDC3 and ITG β 4.

Our data introduces a new hypothesis for HD disassembly during cell migration. In this mechanism, ARRDC3 interacts with ITG β 4 phosphorylated on S1424 by PKC α after the cell has received migration stimuli (Germain et al., 2009). Upon this interaction, ITG β 4 located on the lagging edge of the cell becomes endocytosed, dissolving the HD, and sent to the proteasome for degradation. This disruption of adhesion molecules at the lagging edge allows the cell to migrate. Although our data clearly demonstrates that ARRDC3 promotes the internalization of ITG β 4, it is unclear whether it is directly involved in the trafficking to the proteasome. Although over-expression of ARRDC3 leads to degradation of ITG β 4, it is possible that there is a point after internalization where other signals from the cell could contribute to its fate; either degradation or trafficking. It is unclear whether ARRDC3 also plays a role in re-localizing ITG β 4 to the leading edge of the cell where it helps form and stabilize motility structures (filopodia and lamellae) (Lipscomb and Mercurio, 2005; Mercurio et al., 2001b; Rabinovitz et al., 2004; Santoro et al., 2003). When treated with a proteasome inhibitor, cells over-expressing ARRDC3 have slightly higher ITG β 4 surface protein levels compared to untreated cells; suggesting at least a small fraction of internalized protein is returned to the cell surface when it cannot be degraded. Additionally, the basal ITG β 4 and apical ARRDC3 staining patterns seen on the leading edge of the cells suggests a role beyond degradation. It would be interesting to test whether an ITG β 4-binding deficient mutant of ARRDC3 still affects ITG β 4 protein levels and cell motility.

While the ARRDC3-induced changes in ITG β 4 protein levels are modest, the affects on cell motility are pronounced suggesting the balance between stationary and motile states in skin stem cells is delicate. Motility has significant implications for stem cell biology because stem cells need to stay within the growth and differentiation restricted environment of the niche. We did not see an effect of ARRDC3 expression on *in-vitro* differentiation, but it is possible that aberrant differentiation or tissue homeostasis would be seen *in-vivo* due to mis-localization of the cells. This study also does not imply that ITG β 4 signaling or ARRDC3 expression does not influence the differentiation of adult keratinocyte stem cells, only that altered ARRDC3 levels do not prevent induced differentiation *in-vitro*. Indeed, skin stem cells expressing ARRDC3 tended to have a lower level of spontaneous differentiation as measured by marker expression in the un-induced cells (Figure 2-15B-D). As VPS26 is involved in receptor transcytosis in polarized cells, it is also possible that ARRDC3 plays a role in polarizing the skin stem cells during asymmetric cell division (Eaton, 2008).

Our findings may also have a significant implication in wound healing. Upon injury, keratinocytes secrete a number of cytokines and growth factors (Jones et al., 1998; Litjens et al., 2006; Margadant et al., 2008; Wilhelmsen et al., 2007). Depending on the type of injury keratinocytes will differentiate, proliferate or migrate. During tissue repair, HDs must be deconstructed to permit migration and thus regulation of ARRDC3 would likely be required for the ability to heal wounds. These data are also relevant to cancer biology as many aspects of wound healing (cell migration, loss of cell polarity, de-differentiation, and proliferation) are also characteristics of aggressive cancer cells.

Additionally, ITG β 4 increases the migration and invasion properties in cancer cells, thus any mechanism involved in its regulation could be used and/or altered in cancer cells.

The necessity of phosphatase inhibitors to observe the interaction between ARRDC3 and ITG β 4 suggests that phosphorylation of ITG β 4 is either necessary for or stabilizes the interaction. The enrichment of phosphorylated forms of ITG β 4 in co-immunoprecipitation experiments further supports this. The identical cellular distribution of ARRDC3 and ITG β 4-pS1424 in both migrating cells and resting cells (Figure 2-14B and data not shown) identifies ITG β 4-pS1424 as a candidate phosphorylation site required for ARRDC3 interaction. This phosphorylation enhances the interaction between ARRDC3 and ITG β 4 although it is still unclear whether this occurs before or after the disassembly of the HD. ITG β 4-S1424 is important to HD dynamics (Germain et al., 2009) and it is possible that this phosphorylation allows ARRDC3 to interact with and then causes the internalization of ITG β 4. As S1424 is not within the interacting fragment of ITG β 4 identified in the yeast-two hybrid screen, the phosphorylation needs to either cause a conformation change or recruit other proteins thus allowing the interaction to occur. Our data does not exclude other possible important phosphorylation events during ITG β 4 internalization; S1356, S1360 and S1364 are also significant during HD disassembly (Germain et al., 2009; Rabinovitz et al., 2004; Wilhelmssen et al., 2007). It is also possible that ARRDC3 only interacts with ITG β 4 after HD disruption and the increased ARRDC3/ITG β 4-pS1424 is a spurious correlation, not a causative one. Indeed data discussed in Chapter 3 showing the ARRDC3/ITG β 4 interaction in cells lacking HDs supports this.

Altogether, our data suggests that ARRDC3 plays a role in ITG β 4 trafficking, a process critical in the regulation of skin stem cell migration. Although not required for development, the importance of ARRDC3 is highlighted by the loss of fur and whiskers in knock-out mice. Based on our data, it is likely that the skin stem cells in ARRDC3-null mice exhibit an increase in motility, causing them to migrate out of the niche escaping its growth and differentiation inhibitive environment. This would ultimately lead to exhaustion of the stem cell pool and defective homeostasis, explaining why the phenotype becomes more pronounced as the mouse ages.

An interesting question raised by our data is whether ARRDC3-mediated internalization is the result of a specific chemotactic stimulation or if it is an inherent feature of the skin stem cell which is up-regulated during migration. The latter is supported by the co-localization seen in resting cells (Figure 2-8C) and the fact that keratinocytes in culture maintain a constant HD turn-over even in the absence of migration.

The discovery of a new protein that plays a role in migration and potentially trafficking generates many questions. Little is understood about integrin internalization or trafficking and less is known about the events at the lagging edge of migrating cells. Our findings open doors to further both lines of research.

Methods:

Dissection of Anagen Bulb and Telogen Bulge: Keratinocytes were isolated and cultured as described previously (Roh et al., 2004). Briefly, fresh adult human scalp skin from plastic surgical procedures was obtained from the Cooperative Human Tissue Network

(funded by National Cancer Institute) with Institutional Review Board approval, according to the Declaration of Helsinki Principles. One portion of the skin (1 × 2 cm) was treated with 4 mg per mL Dispase (Sigma-Aldrich, St Louis, Missouri) in Dulbecco's modified Eagle's medium (Invitrogen–Gibco, Carlsbad, California) overnight at 4°C. Using forceps, hair follicles were plucked from the Dispase-treated skin, and segregated into telogen club hairs based on their morphology under a dissecting microscope. Plucked anagen follicles were dissected to remove the upper outer root sheath corresponding to the bulge region. A second portion of the skin was dissected without Dispase treatment to obtain the matrix area at the bottom of the hair bulb. The telogen and anagen bulb hair fragments were either used as explant cultures directly or were digested with 0.05% trypsin–ethylene diamine tetra-acetic acid (EDTA) (Gibco) for 10 min, and then Versene (Gibco) was added (1.33:1) for an additional 10 min. Cells were centrifuged and plated onto tissue culture plastic dishes (Falcon, St Louis, Missouri). The isolated cells and tissue fragments were then cultured in keratinocyte medium (KCM) (Roh et al., 2004) on a feeder layer of J2-3T3 fibroblasts that had been pre-treated with mitomycin C (15 µg per mL) for 2 h. Media were changed every other day. For subsequent experiments, the trypsinization method was used to culture keratinocytes from the hair follicles. For gene-expression analysis, the anagen bulb and telogen bulge were isolated using a dissecting microscope. The tissue was rapidly frozen on dry ice and stored at -80 C. Total RNA from telogen bulge and anagen bulb was extracted using the guanidine isothiocyanate method.

Subtraction Hybridization: The Clontech PCR-Select Differential Screening Kit was purchased from Clontech (cat# 637403). Manufacturer's recommended protocol was followed.

Co-immunoprecipitation: Cells were solubilized at 4 °C for 10 min in NP-40 lysis buffer (Boston Bioproducts) containing 1 mM sodium orthovanadate, 1 mM NaF, and protease inhibitors (Complete mini tab; Roche Applied Science). Aliquots of cell extracts containing equivalent amounts of protein were incubated overnight at 4°C with antibodies and protein-A/G-Sepharose (Santa Cruz) with constant agitation. Immune complexes were resolved by SDS-PAGE and transferred to nitro-cellulose.

Northern Blotting Analysis: Pre-quantified RNA from BD Biosciences were subjected to agarose gel electrophoresis and transferred on to a nylon membrane. A fragment of DIG-labeled ARRDC3 RNA was used for hybridization. Membrane was washed and DIG was detected immunologically and exposed on film.

siRNA Sequence and Transfection: Three different siRNA oligos were designed targeting the following sequences in ARRDC3: 1- AAACACGGCCTTCTATGCCA, 2- AAAGGCGGAACAATCTTGAC, and 3- AATCTTGACACCAGTGAGTGCT. siRNA duplexes against these target sequences were ordered from Qiagen along with three control target sequences. siRNA duplexes were transfected into cells using TransIT-TKO transfection reagent (Mirus) following the manufacturer's recommended protocol. For experiments shown in this paper, duplexes 1 and 3 were pooled for maximum knockdown of ARRDC3.

Cloning and Generation of Stable Lines: pSuper vectors (Oligoengine) containing control shRNA (shCtl target sequence: TTCTCCGAACGTGTCACGT) and shRNAs targeting ARRDC3 (shARRDC3 target sequence A:GGCCTTGGCTACTACCAGT; shARRDC3 target sequence C: GCGTGGAATATTCATAAT) were generated in our lab per manufacturers recommended protocol. Full length human ARRDC3 cDNA and Flag-LacZ fusion cDNA were cloned into the pBABE-puro expression vector (Addgene). Cells were transfected with TransIT Keratinocyte Reagent (Mirus) following manufacturers recommended protocol. Stable transformants were selected using puromycin and pooled.

Adenoviral Preparation: Full length human ARRDC3 cDNA was initially cloned into pLEGFP (Clontech). The fragment containing ARRDC3+GFP (or GFP alone) was then cloned into Adeno-X LP CMV (Clontech) using the Adeno-X Expression System 2 kit (Clontech). Adeno-X maxi purification kit (Clontech) was used to isolate/purify the adenovirus. For all steps, the manufacturers recommended protocol was followed. The amount of virus needed was determined empirically.

Proximity Ligation Assay: The PLA system from Olink was purchased (Mouse MINUS, Rat MINUS and Rabbit PLUS probe sets as well as the 613 detection kit). Telogen cells were cultured on poly-D lysine coated coverslips and fixed with acetone. PLA was performed following the manufacture's recommended protocol.

Yeast-2 Hybrid: The BD Matchmaker Yeast-2-Hybrid System (BD Biosciences) was used as per manufacturers recommended protocol, using full length ARRDC3 as bait and cultured telogen stem cell RNA.

Adenovirus Production: A GFP-tagged ARRDC3 adenoviral construct was generated using the AdenoX expression system from Clontech. Adenovirus was prepared using the AdenoX Maxi purification kit as per manufacturers recommended protocol.

Western Blotting Analysis: Cell extracts were prepared from cultured cells lysed in NP-40 lysis buffer (Boston Bioproducts) for 15 minutes and then clarified by centrifugation. Extracts containing equivalent amounts of total protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for an hour using a 50 mM Tris buffer (pH 7.5) containing 0.15 mM NaCl and 0.05% Tween 20 and 5% (w/v) Blocker (BioRad). Membranes were incubated overnight at 4°C in the same buffer containing primary antibodies (rabbit polyclonal targeting ARRDC3 (Abcam ab64817) or rabbit polyclonal targeting ITGβ4 (505, a generous gift from Arthur Mercurio, UMMS Worcester, MA or phospho-specific antibody pS1424 a generous gift from Isaac Rabinovitz, BIDMC & HMS Boston, MA). Proteins were detected by enhanced chemiluminescence (Pierce). For phospho-immunoblots, the blocking buffer for the primary antibodies contained 5% (w/v) BSA.

Immunofluorescence Staining: Cells were cultured on coverslips and fixed with acetone. Cells were permeabilized by incubating with PBS+Triton and blocked with 5% goat serum. Coverslips were incubated with primary antibody (mouse monoclonal ARRDC3 generated by our lab, rat monoclonal ITGβ4 439-9b (BD-Pharmingen cat# 555719) and rabbit polyclonal ITGβ4-pS1424 (generous gift from Isaac Rabinovitz, BIDMC & HMS Boston, MA) for 2 hr at room temperature or overnight at 4°C. After being washed, cells were then stained with secondary antibodies conjugated with either FITC or Texas-Red

(Vector) for 2 hours at room temperature. After washing, cover-slips were mounted on slides using vectashield (Vector) and slides were analyzed using a fluorescence microscope.

Wound Assay: 8×10^5 Telogen or TA cells were plated in a 6-well plate in Keratinocytes SFM (Gibco). 24 hours after plating, cells were either transfected with siRNAs using TransIT-TKO (Mirus) or infected with adenovirus. 48 hours post transfection/infection; the cells were treated with $15 \mu\text{g/ml}$ mitomycin C for two hours at 37°C . The monolayer was scratched with a P200 pipette tip and washed 3 times to remove floating cells. The wound closure was then monitored by digital photography.

Cell Culture and In-vitro differentiation: **Hair Differentiation-** Cells were plated at a density of $5,000 \text{ cells/cm}^2$. Cells were allowed to attach overnight in KCM without EGF and then changed to fresh EGF-containing KCM along with 3T3-J2 inserts. After overnight incubation, half of the 3T3-J2 inserts were replaced with DP inserts and all cells were fed with fresh media. Transwell inserts (Corning) for 12-well plates were prepared with 20,000 mitomycin C-treated 3T3-J2 cells per insert in KCM or 20,000 DP cells per insert in Chang medium C (Irvine scientific) supplemented with 10% FBS and P/S. **Epidermal Differentiation-** Cells are plated at a density of $20\text{--}25,000 \text{ cells/cm}^2$ and allowed to attach overnight, and then changed to low-calcium medium, KGM (keratinocyte growth medium, Cellntec, Bern, Switzerland). After overnight incubation, keratinocytes were fed with fresh KGM supplemented with additional 1.5 mM CaCl_2 and incubated for the indicated times (1-4 days). **Sebaceous Differentiation-** Cells were plated at a density of $20\text{--}25,000 \text{ cells/cm}^2$ and allowed to attach overnight in sebocyte

media (DMEM and Ham F-12 (1:1, Gibco), 6% FBS (Gibco), 2% human serum (sigma), P/S, and EGF (10 nM, Sigma)] as describe previously (Akimoto et al., 2005). Cells were induced for sebocyte differentiation with sebocyte differentiation media containing 10 μ M Arachidonic acid (Sigma) in sebocyte media for 2-4 days. Cultured sebocytes were washed with propylene glycol twice for 5 minutes each, stained with 0.7% (w/v) Oil Red O (Sigma) in propylene glycol for 7 minutes with agitation, washed once with 85% propylene glycol in distilled water, and then rinsed in distilled water. Oil Red O staining was viewed with a light microscope.

Generation of ARRDC3 -/- mice: ES cells on a 129 background with a gene trap inserted between exons 1 and 2 of ARRDC3 were purchased from SIGTR (cell line CG0361). Cells heterozygous for the targeted mutation were microinjected into C57BL/6 blastocysts to produce chimeric offspring. Chimeras were mated to wild-type C57BL/6 and tested for germline transmission using PCR amplification of the NEO cassette found in the genetrapp. Heterozygotes were interbred to obtain homozygous mice. Homozygous mice were identified phenotypically.

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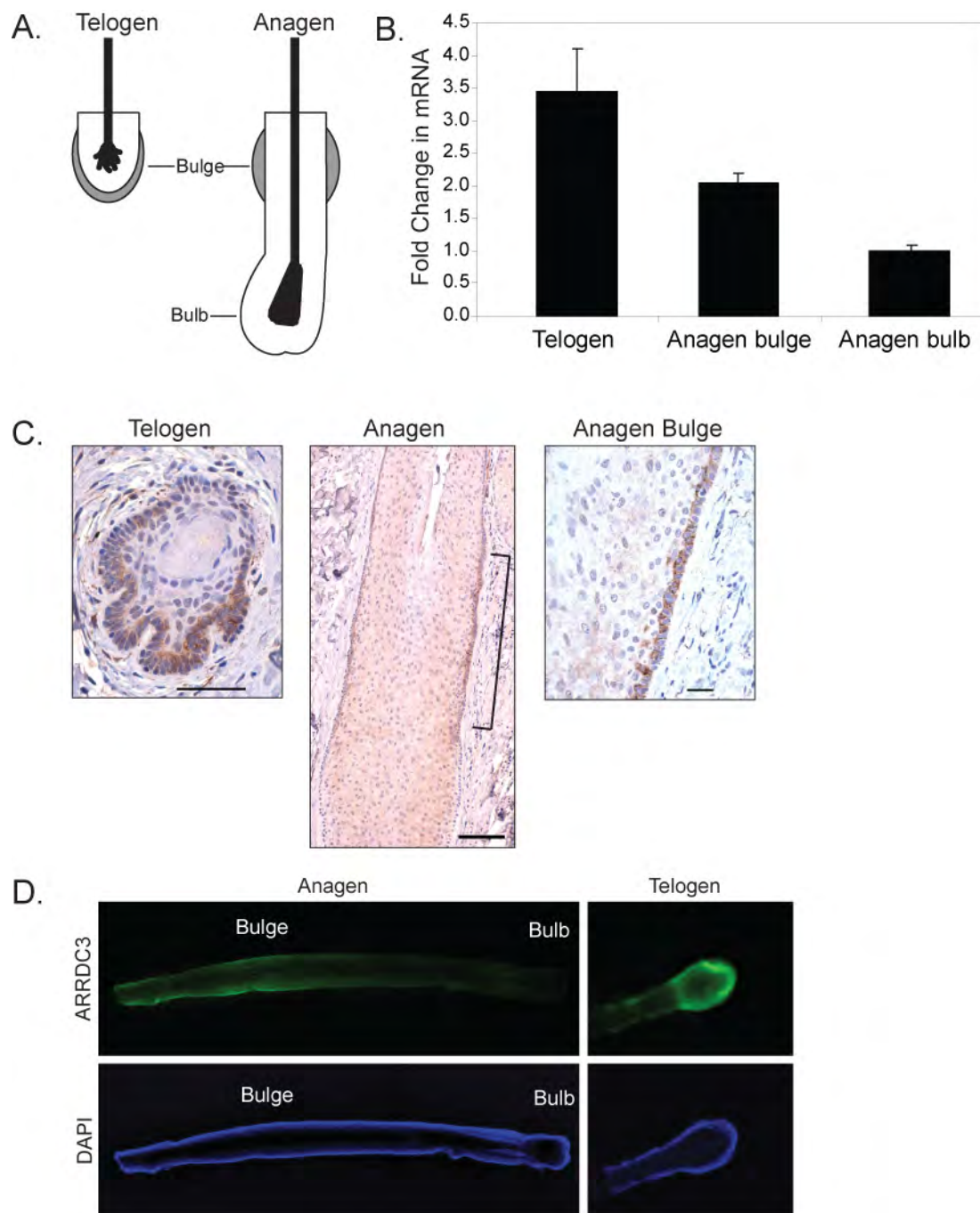


Figure 2-1: ARRDC3 is preferentially expressed in skin stem cells. (A) Schematic representation of a telogen and anagen hair follicles. Quiescent epidermal stem cells are localized in the bulge regions, although they proliferate at the end of telogen. Conversely, the cells within the anagen bulb are the highly proliferative transit-amplifying (TA) cells. (B) Real-time PCR analysis was performed on 3 separate RNA-isolations. ARRDC3 values were normalized to GAPDH to get the relative expression. Relative expression values were then standardized to anagen bulb to obtain fold change. Data are represented as mean +/- SEM). Fold change is statistically significantly as determined using the student's t-test, $p=0.0017$ and $p=0.0066$ for Telogen and Anagen bulge respectively. (C) Immunohistochemistry for ARRDC3 in adult human skin. Positive cells (brown stain) are seen in the basal layer of telogen hairs (left). Anagen follicles (center) show positive cells in the basal layer of the outer root sheath of the bulge region (brackets), seen in higher power in the right panel. (D) Whole mount immuno-fluorescence of anagen and telogen hair follicles. ARRDC3 is more highly expressed in the anagen bulge when compared to the anagen bulb. The telogen follicle bulge also highly expresses ARRDC3 (each channel had equivalent exposure times throughout the different images).

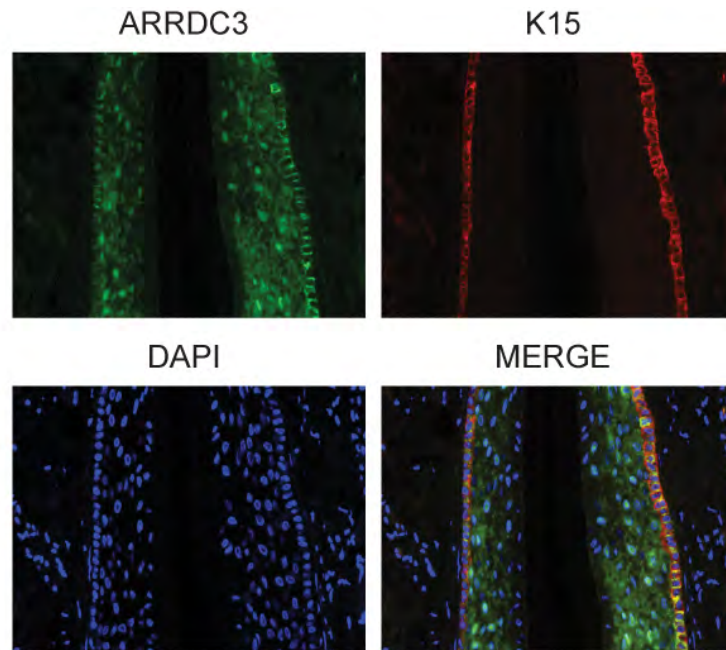


Figure 2-2: ARRDC3 is not a stem cell marker. Comparative expression of ARRDC3 and skin stem cell marker KRT15. ARRDC3 is expressed in KRT15 positive epidermal stem cells in addition to other bulge cells.

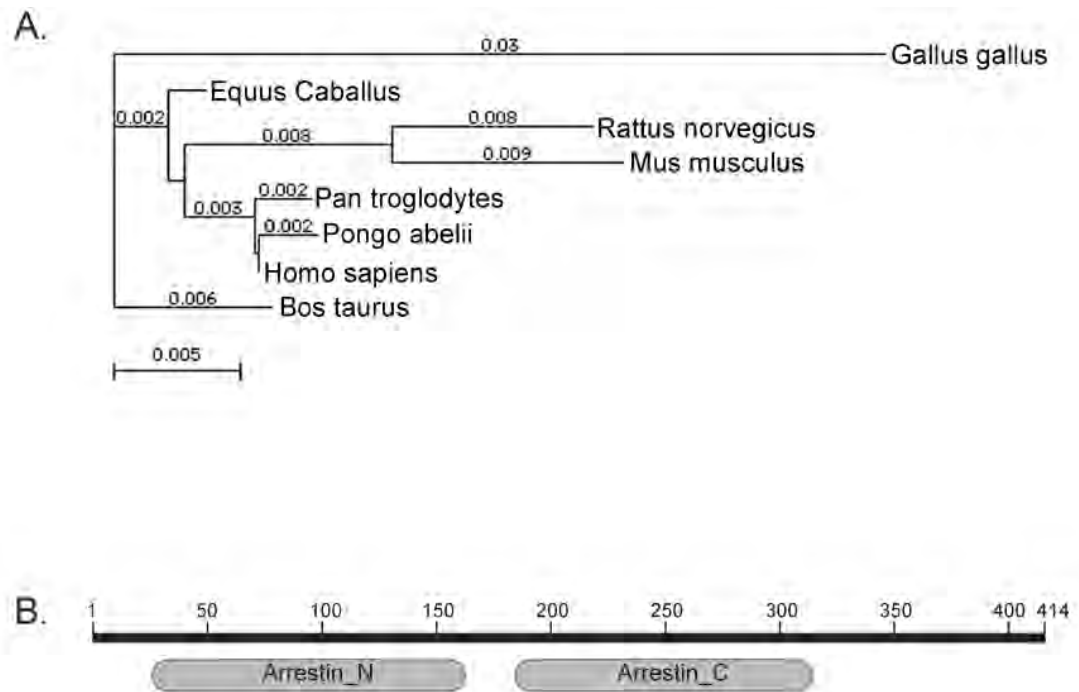


Figure 2-3: Structure analysis of ARRDC3. (A) Phylogenetic tree of ARRDC3 amino acid sequence demonstrating a high conservation between species. ARRDC3 maintains at least a 98.3% similarity compared to the human amino-acid sequence. Tree was generated in MacVector using the Neighbor-Joining algorithm and proportional gap distribution. (B) The ARRDC3 protein sequence consists of 414 amino acid and contains two Arrestin domains. The Arrestin N and Arrestin C domains form beta-sandwiches separated by a hinge region. The C-terminus is projected to have very little structure.

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Gallus gallus      1 MVLGKVKSLTISFDCLNDSNVPVYSSGDTVSGRVNLEVTGEIRVKS LKIH 50
Pongo abelii      1 MVLGKVKSLTISFDCLNDSNVPVYSSGDTVSGRVNLEVTGEIRVKS LKIH 50
Rattus norvegicus 1 MVLGKVKSLTISFDCLNDSNVPVYSSGDTVSGRVNLEVTGEIRVKS LKIH 50
Pan troglodytes   1 MVLGKVKSLTISFDCLNDSNVPVYSSGDTVSGRVNLEVTGEIRVKS LKIH 50
Homo sapiens      1 MVLGKVKSLTISFDCLNDSNVPVYSSGDTVSGRVNLEVTGEIRVKS LKIH 50
Mus musculus      1 MVLGKVKSLTISFDCLNDSNVPVYSSGDTVSGRVNLEVTGEIRVKS LKIH 50
Bos taurus        1 MVLGKVKSLTISFDCLNDSNVPVYSSGDTVSGRVNLEVTGEIRVKS LKIH 50
Equus caballus    1 MVLGKVKSLTISFDCLNDSNVPVYSSGDTVSGRVNLEVTGEIRVKS LKIH 50
*****

Gallus gallus      51 ARGHAKVRWTESRNAGSNTAYTQNYTEEVEYFNHKDVLIGHERDDD NSEE 100
Pongo abelii      51 ARGHAKVRWTESRNAGSNTAYTQNYTEEVEYFNHKDILIGHERDDD NSEE 100
Rattus norvegicus 51 ARGHAKVRWTESRNAGSNTAYTQNYTEEVEYFNHKDILIGHERDDD NCEE 100
Pan troglodytes   51 ARGHAKVRWTESRNAGSNTAYTQNYTEEVEYFNHKDILIGHERDDD NSEE 100
Homo sapiens      51 ARGHAKVRWTESRNAGSNTAYTQNYTEEVEYFNHKDILIGHERDDD NSEE 100
Mus musculus      51 ARGHAKVRWTESRNAGSNTAYTQNYTEEVEYFNHKDILIGHERDDD NSEE 100
Bos taurus        51 ARGHAKVRWTESRNAGSNTAYTQNYTEEVEYFNHKDILIGHERDDD NSEE 100
Equus caballus    51 ARGHAKVRWTESRNAGSNTAYTQNYTEEVEYFNHKDILIGHERDDD NSEE 100
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Gallus gallus      101 GLHTIPSGRHEYAFS FELPQTPLATSFEGRHGSVRYWVKAE LHRPWLLPV 150
Pongo abelii      101 GFHTIHSGRHEYAFS FELPQTPLATSFEGRHGSVRYWVKAE LHRPWLLPV 150
Rattus norvegicus 101 GFSTIHSGRHEYAFS FELPQTPLATSFEGRHGSVRYWVKAE LHRPWLLPV 150
Pan troglodytes   101 GFHTIHSGRHEYAFS FELPQTPLATSFEGRHGSVRYWVKAE LHRPWLLPV 150
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Bos taurus        151 KLKKEFTVFEHIDINTPSLLSPQAGTKEKTLCCWFCTSGPISLSAKIERK 200
Equus caballus    151 KLKKEFTVFEHIDINTPSLLSPQAGTKEKTLCCWFCTSGPISLSAKIERK 200
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Bos taurus        201 GYTPGESIQIFAEIENCSSRMVVPKAAIYQTQAFYAKGKMKEVKQLVANL 250
Equus caballus    201 GYTPGESIQIFAEIENCSSRMVVPKAAIYQTQAFYAKGKMKEVKQLVANL 250
*****

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Rattus norvegicus 251 RGESLSSGKTETWDGKLLKIPVSPSILDCSII RVEYSLMVYVDIPGAMD 300
Pan troglodytes   251 RGESLSSGKTETWNGKLLKIPVSPSILDCSII RVEYSLMVYVDIPGAMD 300
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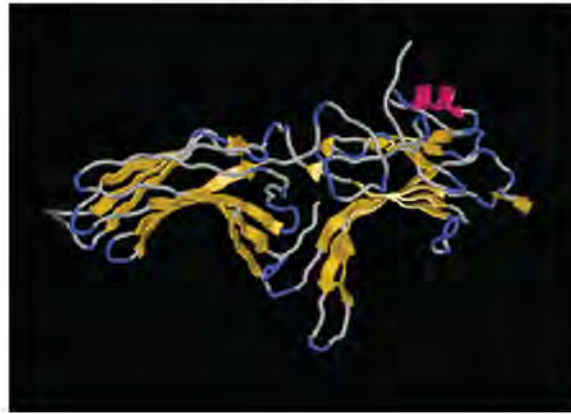
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Equus caballus    351 EVVTEEQRRNNLAPVSACDDFERALQGGLFAYIQEFRFLPPPLYSEIDPN 400
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Rattus norvegicus 401 PDQSSEDRPSPSPSR 414
Pan troglodytes   401 PDQSADDRPSPSPSR 414
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Mus musculus      401 PDQSSEDRPSPSPSR 414
Bos taurus        401 PDQPADDRPSPSPSR 414
Equus caballus    401 PDQSADDRPSPSPSR 414
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Figure 2-4: ARRDC3 ClustalW alignment. Alignment of the amino acid sequence of ARRDC3 from several different species shows significant homology.

ARRDC3



VPS26a



Figure 2-5: Putative 3D structure of ARRDC3. Putative protein structure of ARRDC3 as determined by comparative modeling (top) and the crystal structure of VPS26a (bottom) (Shi et. al., 2006). Each protein has two arrestin domains (seen here as the yellow beta-sandwiches). Both proteins have several beta-turns and a small alpha-helix above one of the arrestin domains. The structural homology indicates ARRDC3 has similar functional roles.

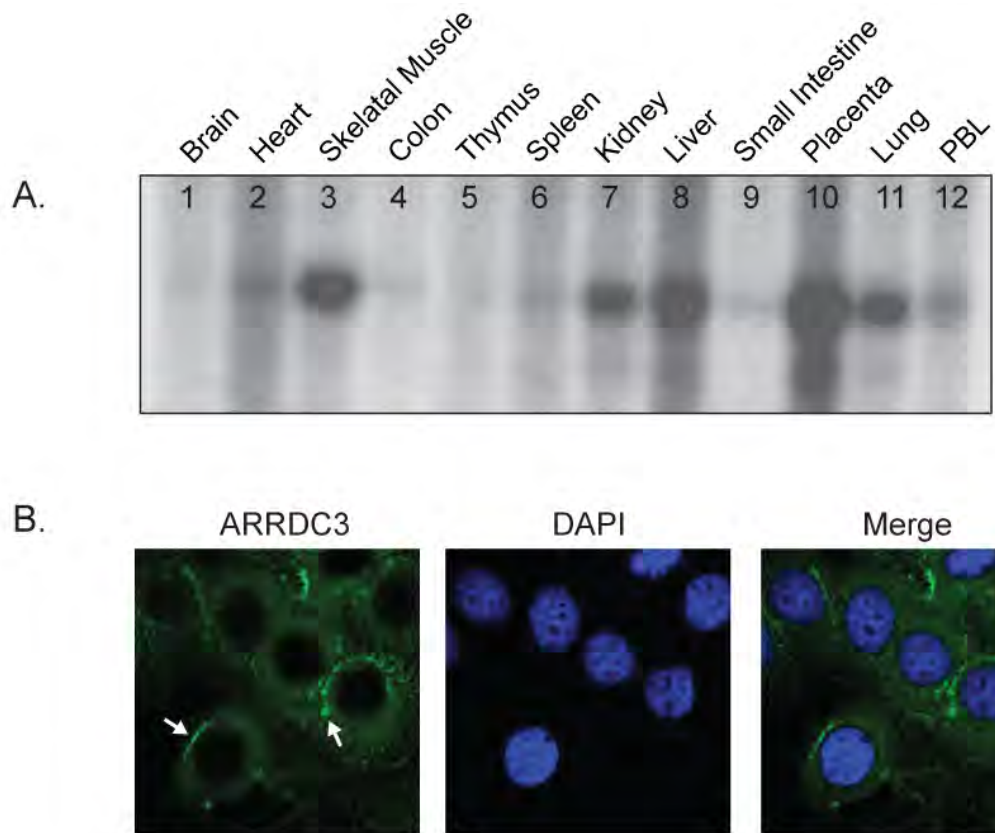


Figure 2-6: Expression patterns of ARRDC3. (A) Northern blot of probed with radio-labeled ARRDC3 cDNA demonstrates expression of ARRDC3 in a variety of human tissues: 1-brain, 2-heart, 3-skelatal muscle, 4-colon, 5-thymus, 6-spleen, 7-kidney, 8-liver, 9-small intestine, 10-placenta, 11-lung, 12-peripheral blood leukocyte. (B) Confocal images of resting telogen cells reveal ARRDC3 localizes to the cell membrane (left arrow) and in punctate likely representing intra-cellular vesicles (up arrow).

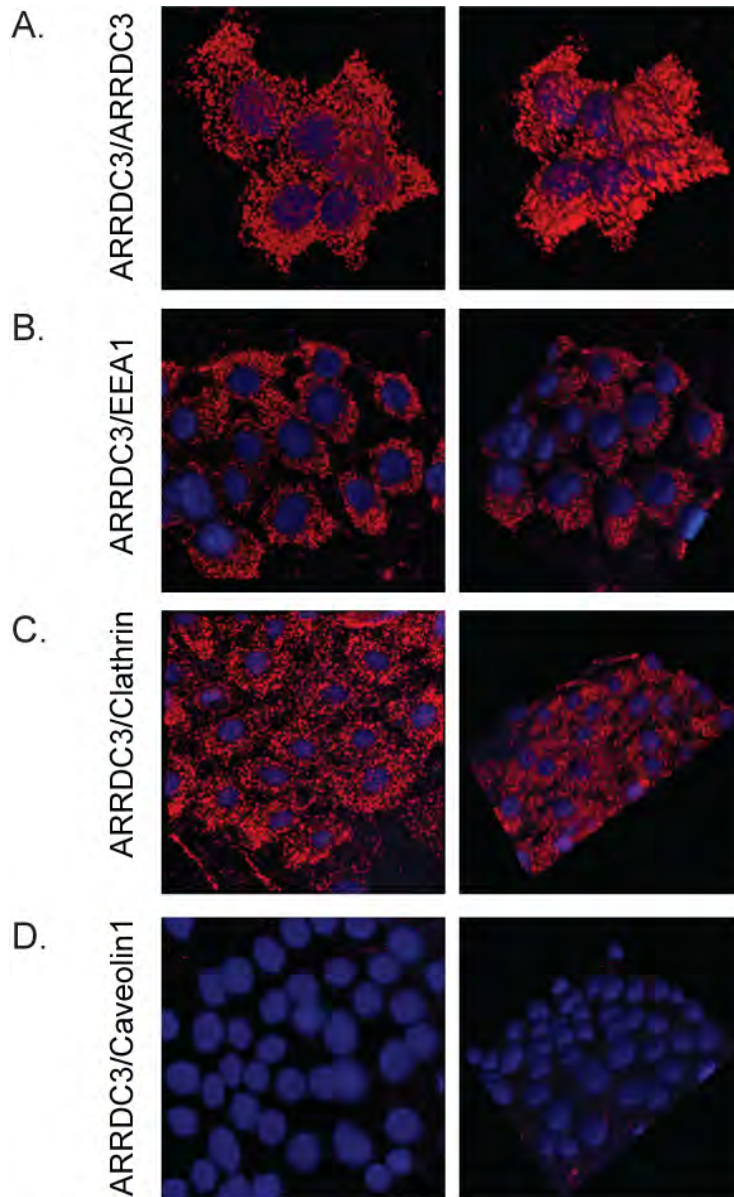


Figure 2-7: ARRDC3 localizes within the vesicular trafficking pathway of telogen cells 3D reconstruction from confocal imaging of PLA using (A) two different antibodies recognizing ARRDC3. ARRDC3 is evenly distributed throughout the cell near the cell membrane. (B) ARRDC3 and EEA1. ARRDC3/EEA1 co-localization is more highly prevalent at the basal pole of the cell. (C) ARRDC3 and Clathrin. ARRDC3/Clathrin co-localization is evenly distributed throughout the cell. (D) ARRDC3 and Caveolin1. ARRDC3/Caveolin is mostly absent. Left panel is the top view of the 3D reconstruction. Right panel is the same image rotated to get a side view.

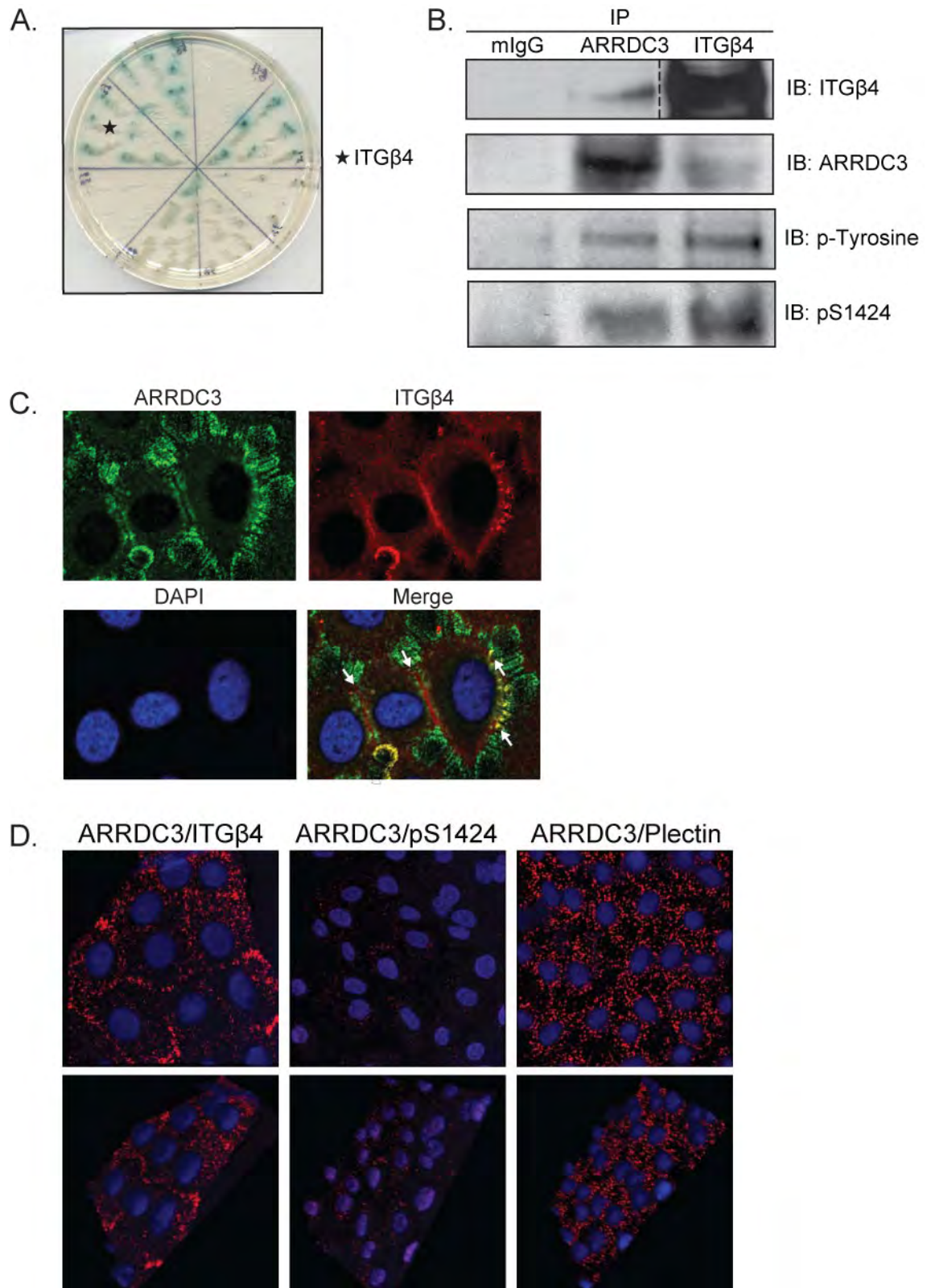


Figure 2-8: ARRDC3 directly interacts with ITG β 4. (A) Yeast-2 hybrid analysis identifies ITG β 4 as a binding partner of ARRDC3. Star represents yeast co-expressing ARRDC3 and ITG β 4 fragment; isolated from -His plate and re-streaked on plates containing X-gal. (B) Endogenous coIP using antibodies for ARRDC3 and ITG β 4. Dashed line represents a band from a non-adjacent lane on the same film. Bands seen in the phospho-tyrosine and phospho-S1424 immunoblots were identical in size to ITG β 4 bands. (C) Confocal imaging revealed regions of co-localization and segregation of ARRDC3 and ITG β 4 within the cell. Some cell boundaries exhibited a stripe of ITG β 4 bordered by stripes of ARRDC3 (down arrows). Others showed a co-localization of ITG β 4 and ARRDC3 (up arrows). (D) 3D reconstruction from confocal imaging of PLA images. The ARRDC3/ITG β 4 co-localization occurs non-uniformly throughout the basal layer of the cell. The ARRDC3/ ITG β 4-pS1424 co-localization is rarer, but also present non-uniformly throughout the basal layer of the cell. Additionally, ARRDC3 localizes with hemidesmosome protein plectin on the basal layer of the cell. Top panel is the top view of the 3D reconstruction. Bottom panel is the same image rotated to get side view.

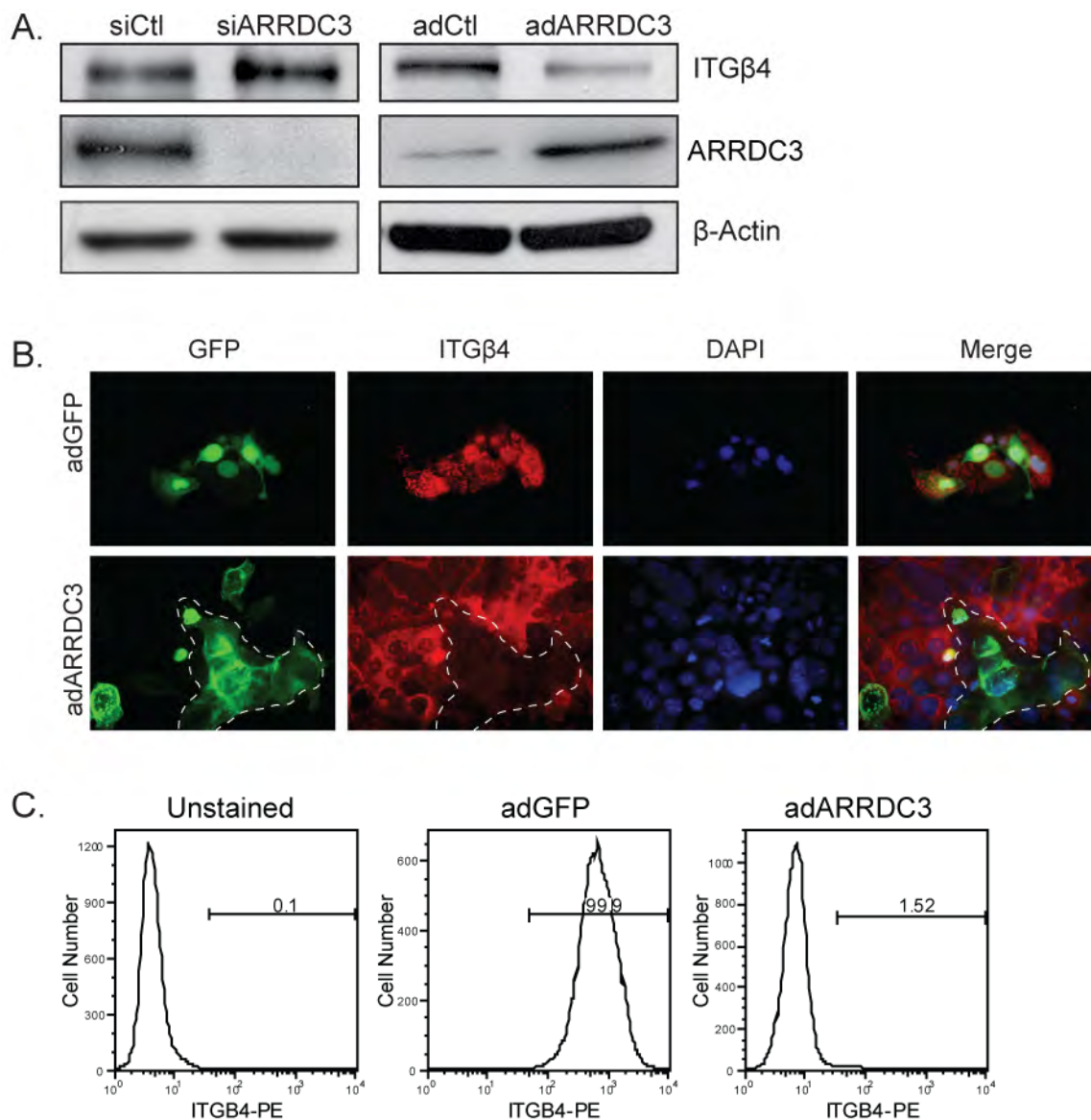


Figure 2-9: ARRDC3 negatively regulates ITGβ4. (A) ITGβ4 expression is inversely correlated to ARRDC3 expression. Repression of ARRDC3 in skin stem cells (siARRDC) caused an increase in ITGβ4 protein when compared to control cells (siCtl). Conversely, when skin stem cells over-express ARRDC3 (adARRDC3), protein levels of ITGβ4 are decreased when compared to control cells (adCtl). (B) Immuno-fluorescence of adult skin stem cells after infection with either a control GFP or ARRDC3-GFP expressing adenovirus (C) ARRDC3 causes a near complete removal of ITGβ4 from the cell surface. The cell surface of live cells were stained with an ITGβ4 antibody and analyzed by flow cytometry. Infected cells are outlined with a dashed line.

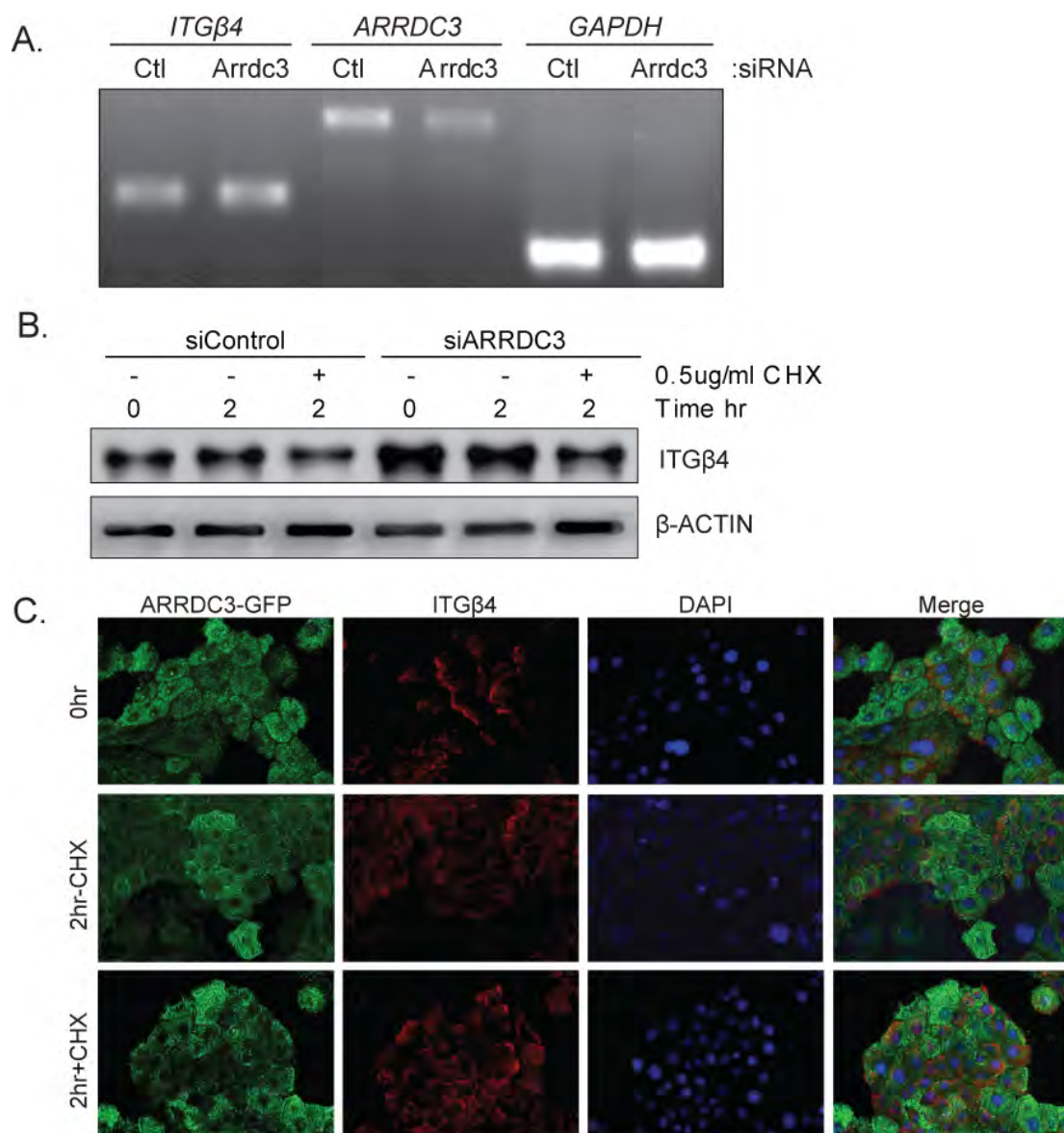


Figure 2-10: The regulation of ITGβ4 by ARRDC3 is post-translational. (A) Repression of ARRDC3 expression by RNAi does not affect ITGβ4 mRNA levels. RNA was isolated from cells transfected with either a scrambled siRNA (Ctl) or an siRNA targeting ARRDC3, and analyzed by RT-PCR using gene specific primers: ITGβ4 (lanes 1-2), ARRDC3 (lanes 3-4) and GAPDH (lanes 5-6). (B-C) Western blot and immuno-fluorescence of adult skin stem cells after transfected with either a control siRNA or siRNA targeting ARRDC3 with and without treatment of 0.5μg/mL of cyclohexamide (CHX). Inhibition of translation does not prevent the up-regulation of ITGβ4 induced by ARRDC3 knock-down.

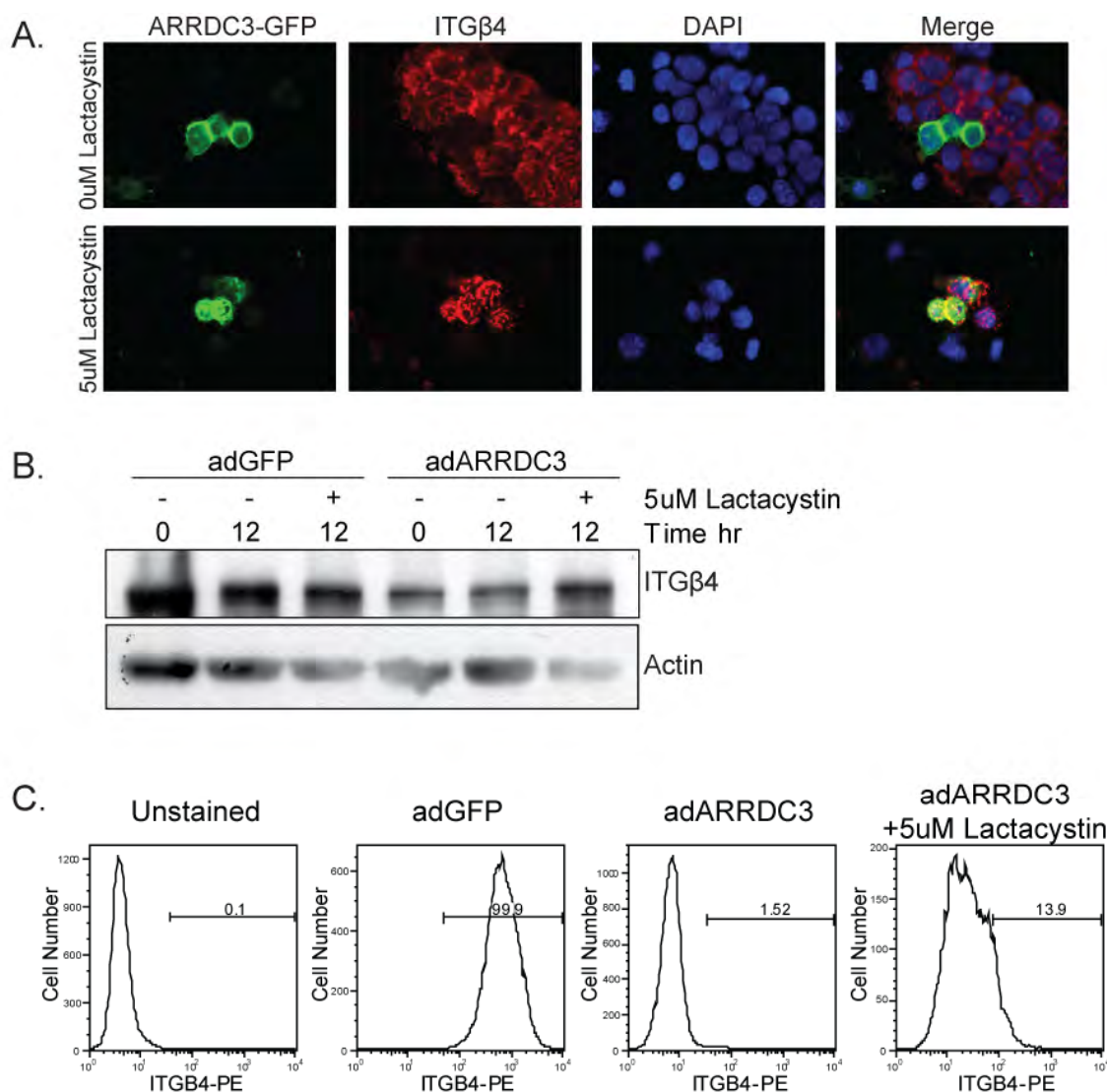


Figure 2-11: The regulation of ITG β 4 protein levels by ARRDC3 is dependent on the proteasome. (A-B) Decrease of ITG β 4 by ARRDC3 is dependent on the proteasome. Epidermal stem cells infected with either a control GFP-expressing adenovirus (adGFP) or a GFP-expressing adenovirus over-expressing ARRDC3 (adARRDC3). Cells can maintain high expression of ITG β 4 after ARRDC3 over-expression if treated with proteasome inhibitor Lactacystin. This is visualized both with immuno-fluorescence (A) and western blotting (B). (C) The removal of cell surface ITG β 4 after ARRDC3 over-expression is marginally rescued with proteasome inhibition. The cell surface of live cells were stained with an ITG β 4 antibody and analyzed by flow cytometry. GFP negative cells and dead cells were gated out before ITG β 4 levels were examined.

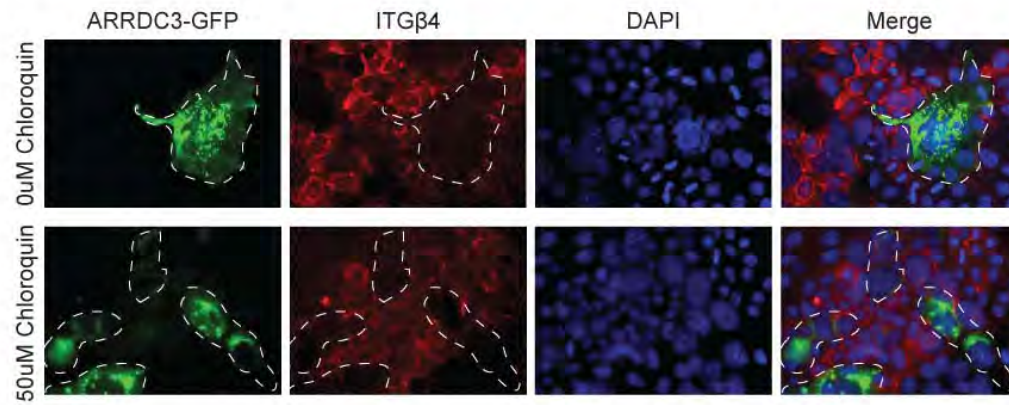
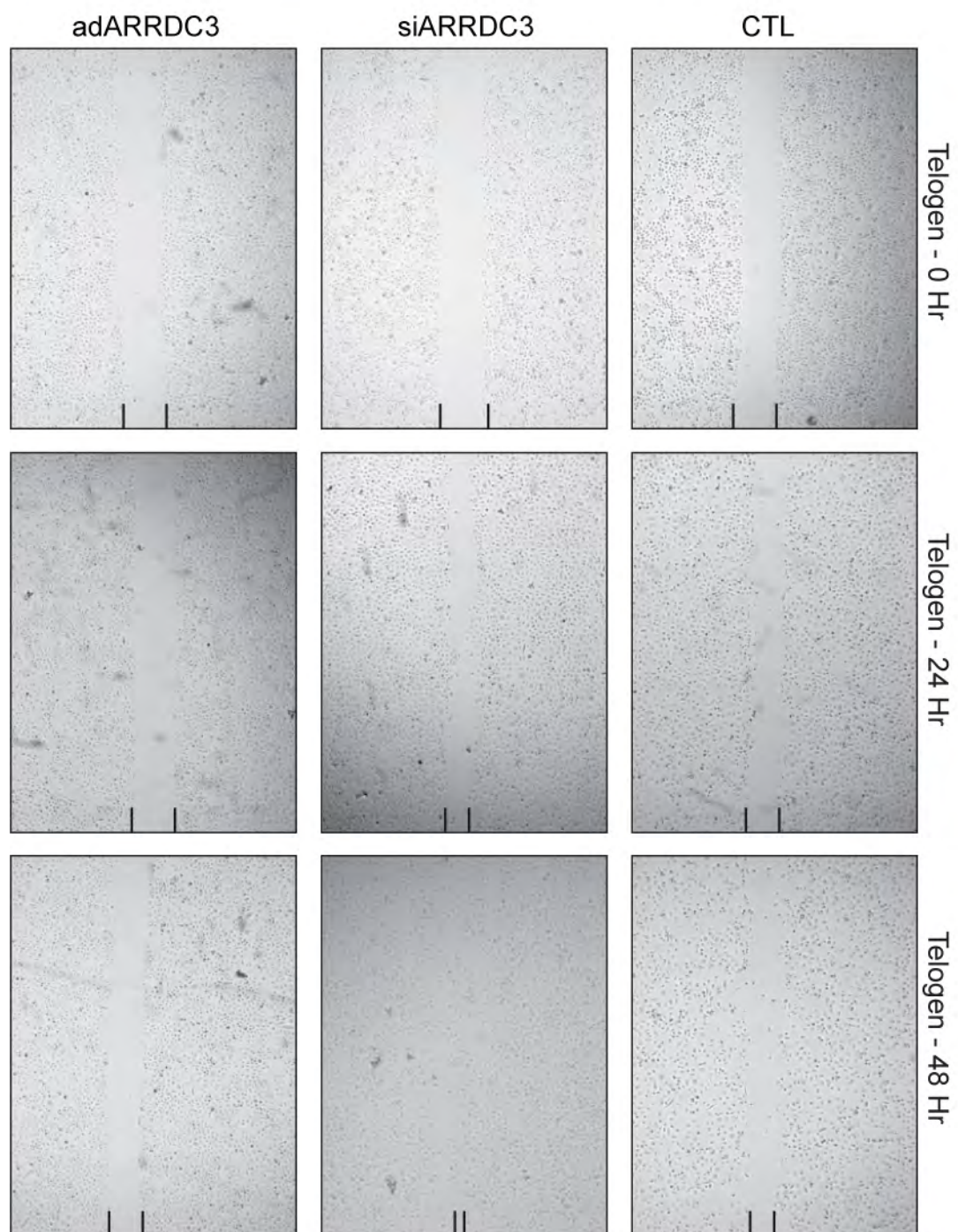
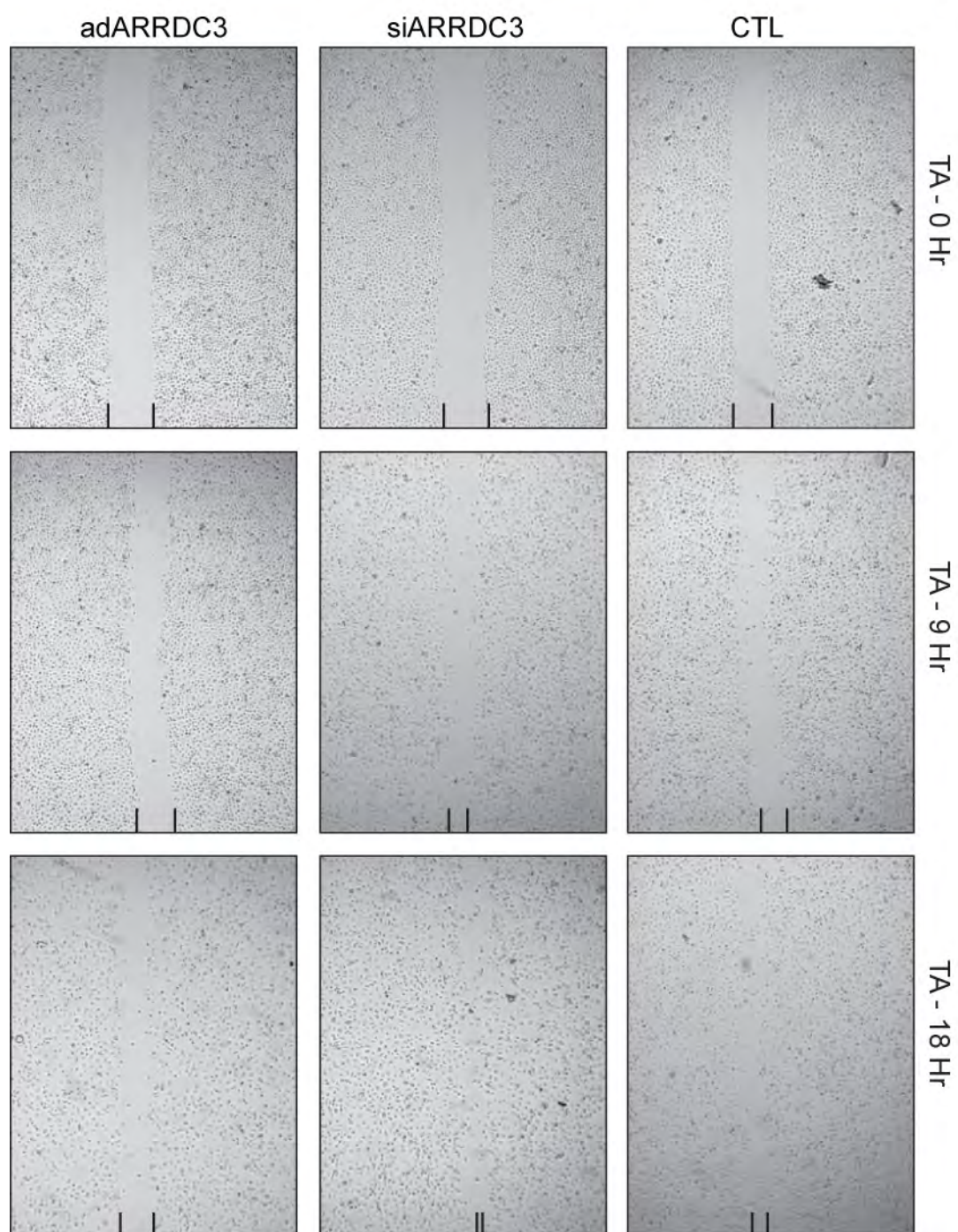


Figure 2-12: Decrease of ITG β 4 protein levels by ARRDC3 over-expression is not dependent on the lysosome. Immuno-fluorescence of adult skin stem cells infected with an adARRDC3-GFP with and without treatment of lysosomal inhibitor chloroquin. Chloroquin treatment does not prevent ARRDC3-mediated decrease in ITG β 4 levels. Infected cells are outlined with a dashed line.

A.



B.



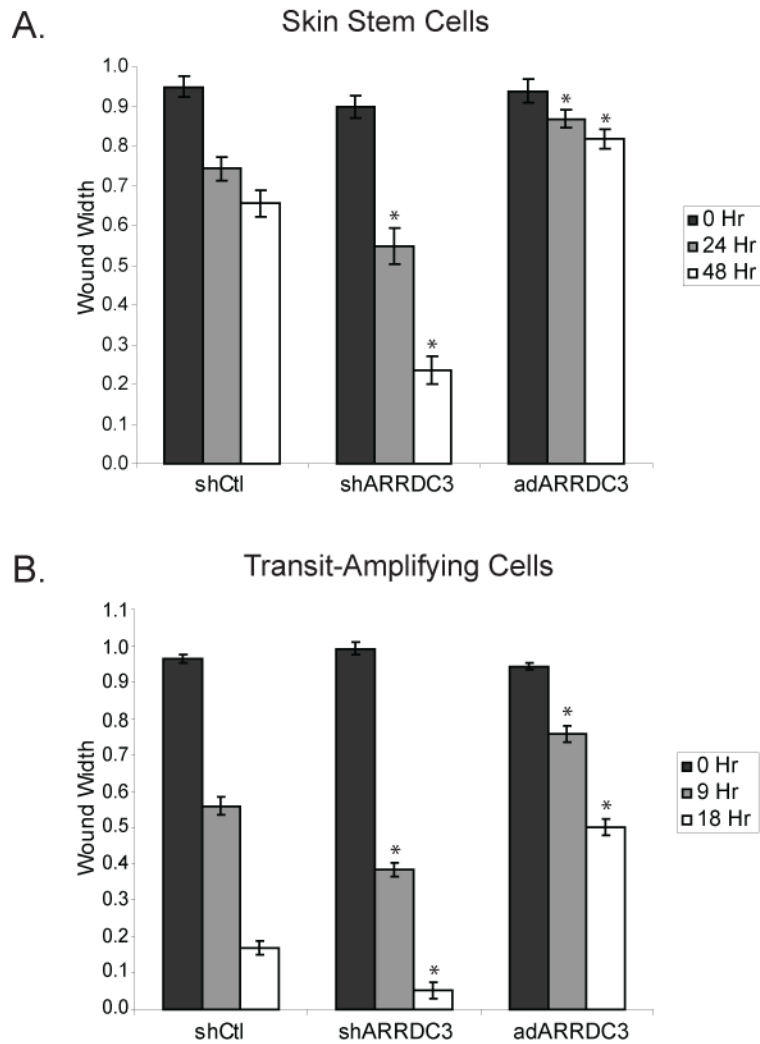


Figure 2-13: ARRDC3 expression regulates cell motility. (A) Wound assay of epidermal stem cells (Telogen) and (B) transit-amplifying cells (TA). ARRDC3 over-expression (adARRDC3) decreases cell motility whereas knockdown of ARRDC3 (siARRDC3) increases cell motility. Average position of each side of the wound is designated with a hatch mark. Results are statistically significant when wound widths are quantified. Asterisks indicate $p < 0.0025$. Shown is a representation of 2 separate shRNAs targeting ARRDC3 that were tested.

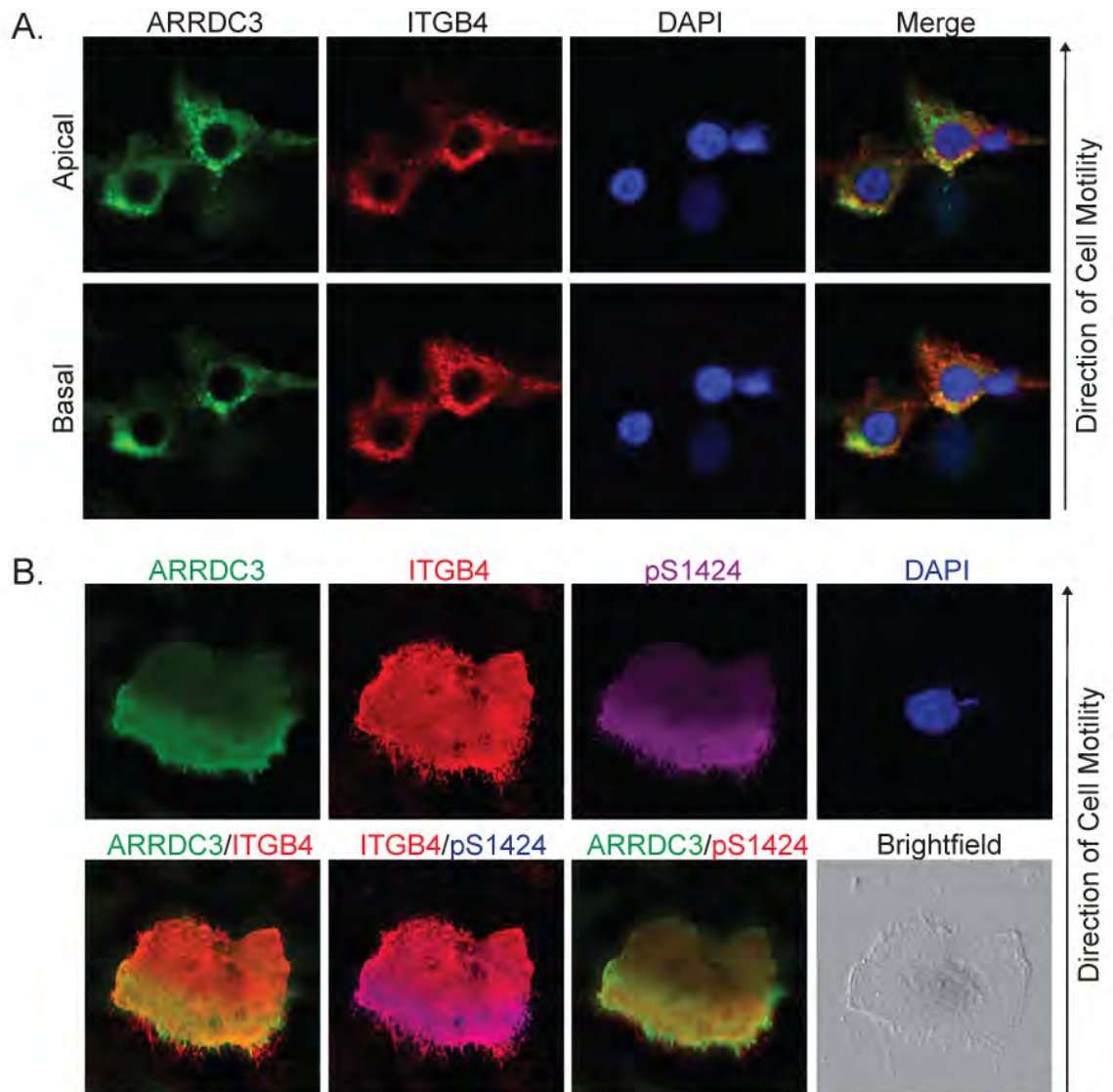


Figure 2-14: ARRDC3 co-localizes with ITG β 4 on the lagging edge of cells. (A) Confocal images of migrating skin stem cells. ARRDC3 co-localizes with ITG β 4 in the basal pole of the lagging edge and segregates at the leading edge where ARRDC3 is more highly present in the apical pole of the cells. Optical image through the apical pole is 0.9 μ m above the basal pole of the cells. (B) Confocal images of migrating epidermal stem cells showing co-localization of ARRDC3 (green) and ITG β 4-pS1424 (purple) at the lagging edge of the cell. Total ITG β 4 is shown in red. ARRDC3, ITG β 4 and ITG β 4-pS1424 images are from the basal plane of cells. DAPI image was taken from apical plane 2.0 μ m above the basal pole of the cells.

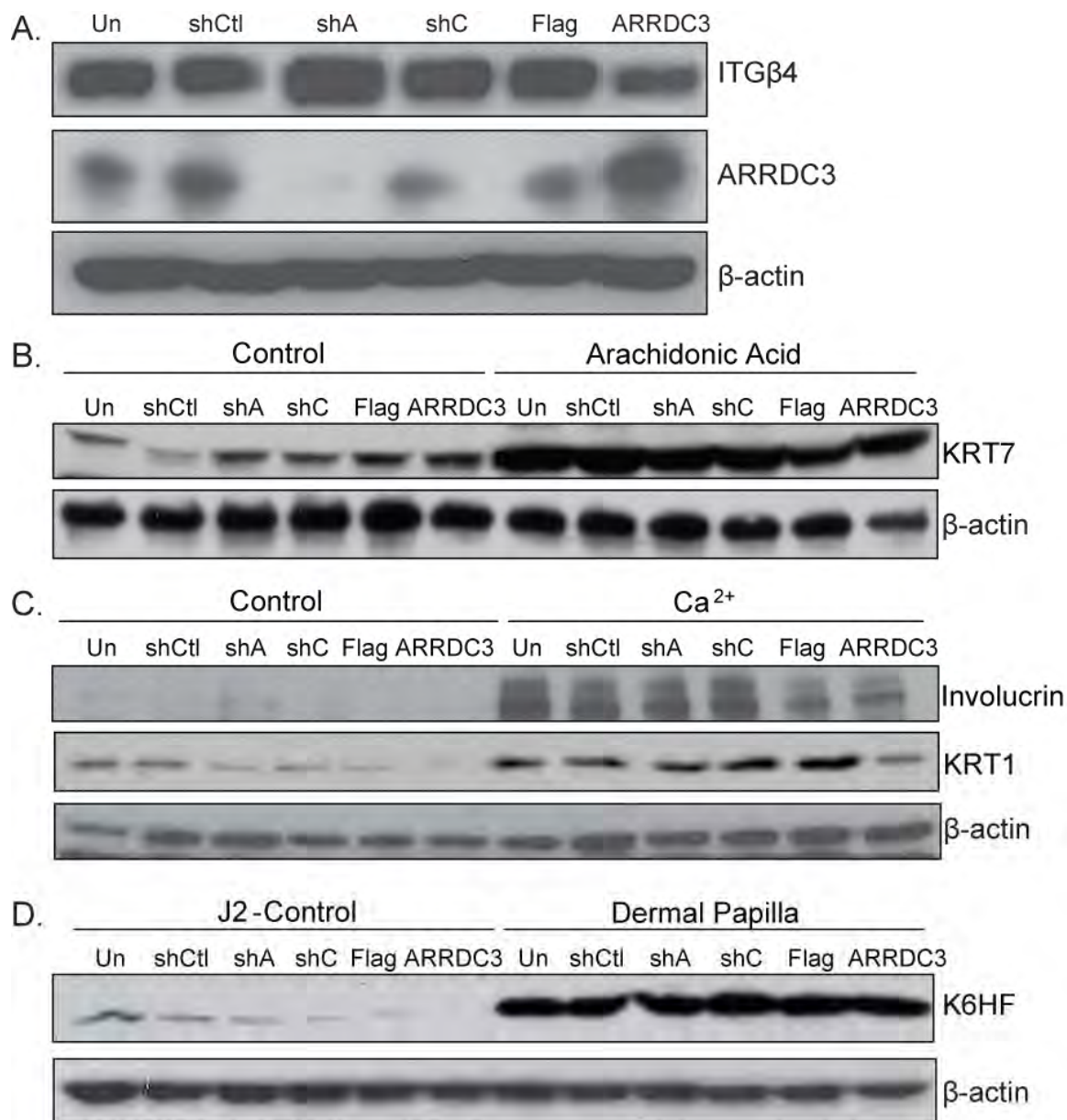


Figure 2-15: ARRDC3 expression does not affect the multi-potency of skin stem cells. (A) Lysates from epidermal stem cell lines show ARRDC3 can be stably modulated and the inverse correlation on ITGβ4 is maintained. (B-D) Lysates from control-treated and differentiated epidermal stem cells were analyzed for the up-regulation of differentiation markers. (B) Arachidonic acid induces sebaceous differentiation and KRT7 expression (C) calcium induces epithelial differentiation and Involucrin and KRT1 expression (D) and co-culture with dermal papilla cells induces hair differentiation and K6HF expression. Alterations in ARRDC3 did not affect the ability of cells to undergo in-vitro differentiation.

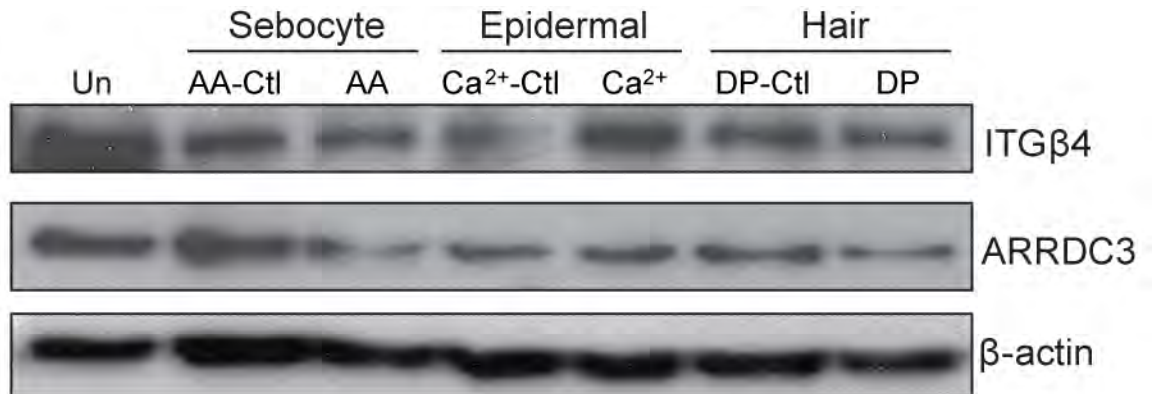


Figure 2-16: ARRDC3 levels changes upon differentiation. Skin stem cells were differentiated in vivo and whole cell lysates were prepared. Western blot analysis was used to determine relative expression levels of ARRDC3 after differentiation. ARRDC3 levels decrease upon sebocyte and hair differentiation and increase with skin differentiation

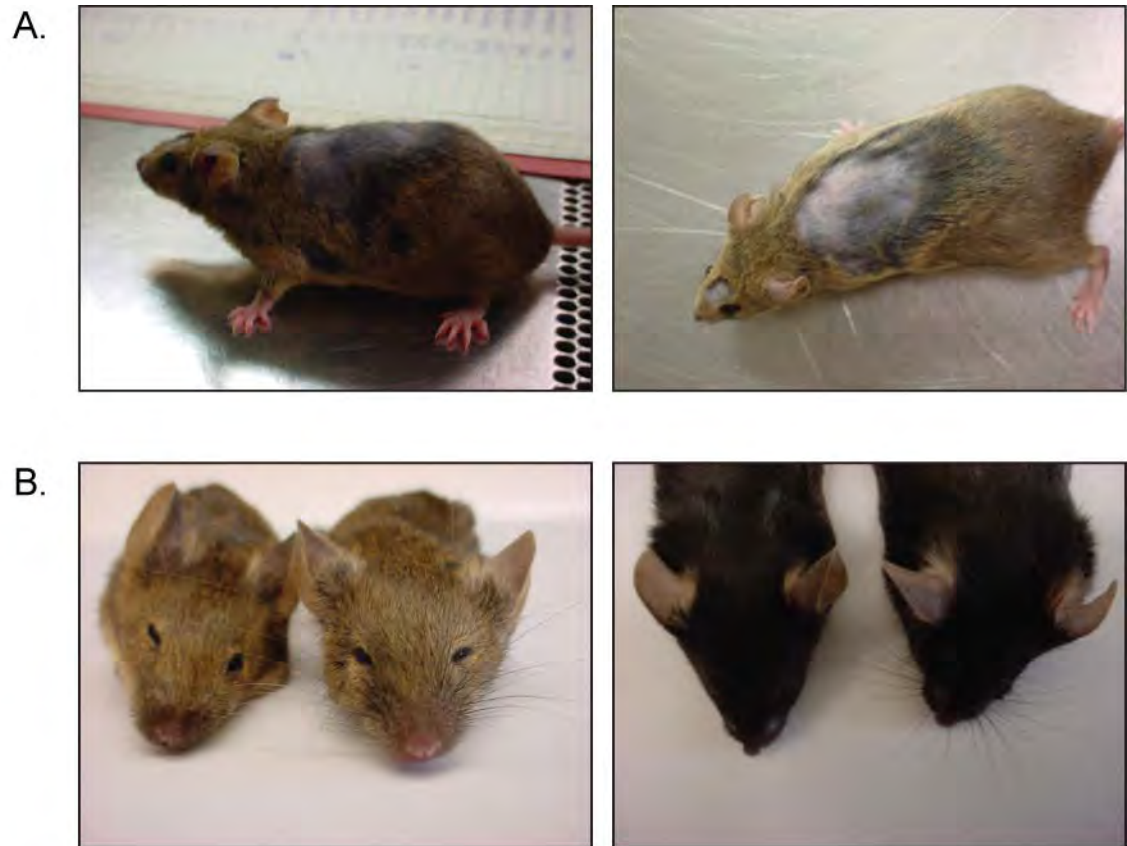


Figure 2-17: Gross examination of ARRDC3 null mice. (A) 4-month old ARRDC3 null mouse displays a patchy loss of fur. (B) 4 week old ARRDC3 null (left) and wild-type littermates (right). Mice null for ARRDC3 lack whiskers.

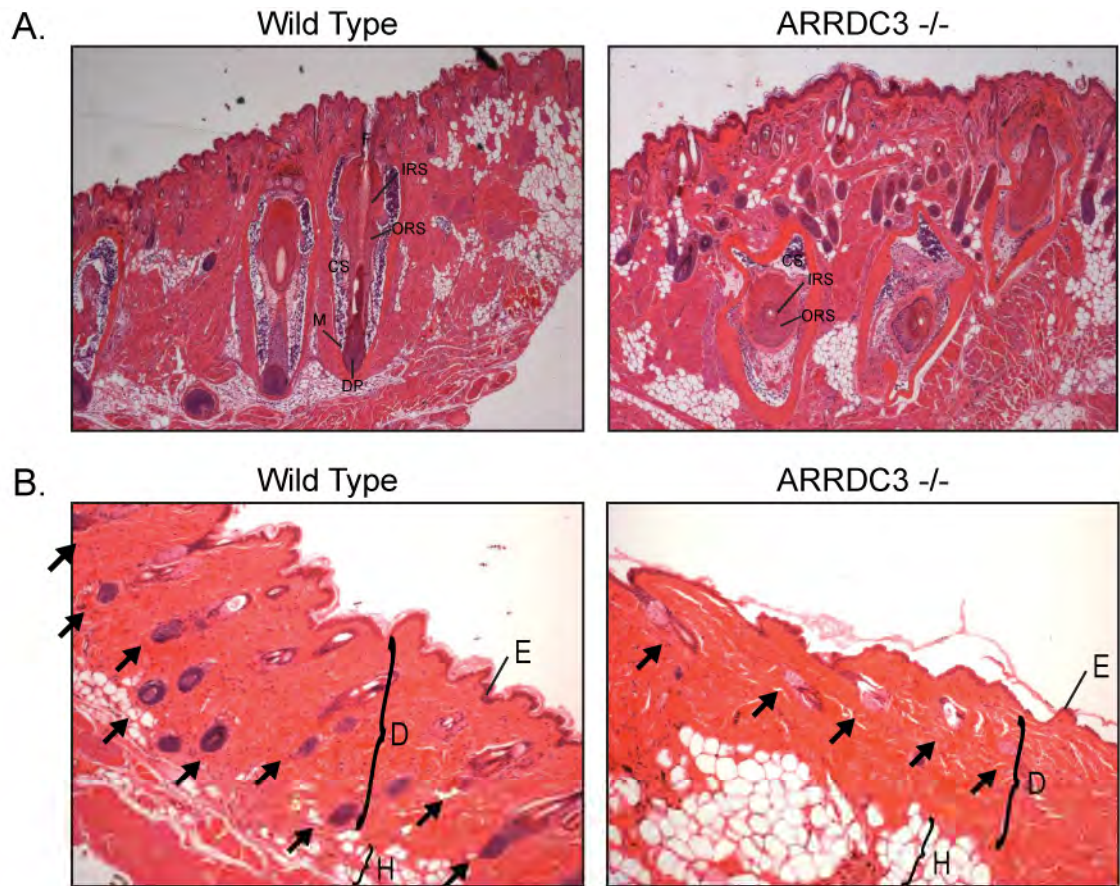


Figure 2-18: ARRDC3 null mice have morphologically abnormal whisker follicles and fewer hair follicles. (A) Whisker pads from 4-week old ARRDC3 null and wild-type littermates. ARRDC3 null mice have abnormally shaped whisker follicles when compared to wild-type littermate. Wild type whisker follicles are shaped similarly to hair follicle (see Figure 1-1) with a bulb (consisting of matrix and dermal papillae cells) deep into the dermis, blood vessels and nerves (CS) surrounding the follicle (F) which is surrounded by the inner and outer root sheaths. Mutant whisker pads lack most of these structures. F-whisker follicle, IRS- inner-root sheath, ORS- outer root sheath, CS- cavernous sinus, M-matrix, DP- dermal papillae. (B) Skin from 4 month old ARRDC3 null and wild type littermates. The images were taken under the same magnification. The dermis from the ARRDC3 null mice is substantially thinner than that of the wild type. Additionally, there are much fewer hair follicles (arrows) in the ARRDC3 null mice when compared to wild type control. E-epidermis (stratified epithelium), D-dermis (area with collagen fibers) and H-hypodermis (identified by the presence of adipose tissue).

CHAPTER III:

ARRDC3 is a Novel Regulator of Breast Cancer Progression

Parts of this chapter represent work submitted as:

ARRDC3 Suppresses Breast Cancer Growth by Negatively Regulating Integrin β 4

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ABSTRACT

Large-scale genetic analyses of human tumor samples have been used to identify novel oncogenes, tumor suppressors and prognostic factors, but the functions and molecular interactions of many individual genes have not been determined. In this study we examine the cellular effects and molecular mechanism of the arrestin family member, ARRDC3, a gene preferentially lost in a subset of breast cancers. Oncomine data reveals that expression of ARRDC3 decreases with tumor grade, metastases and recurrences. ARRDC3 over-expression represses cancer cell proliferation, migration, invasion, growth in soft agar and *in vivo* tumorigenicity while down-regulation of ARRDC3 has the opposite effects. Mechanistic studies demonstrate that ARRDC3 acts in a novel regulatory pathway that controls the cell surface adhesion molecule, beta-4 integrin (ITG β 4), a protein associated with aggressive tumor behavior. Our data indicates ARRDC3 directly binds to a phosphorylated form of ITG β 4 leading to its internalization, ubiquitination and ultimate degradation. The results identify the ARRDC3-ITG β 4 pathway as a new therapeutic target in breast cancer and demonstrate the importance of connecting genetic arrays with mechanistic studies in the search for new treatments.

Key Words: ARRDC3/Breast Cancer/Integrin β 4/TLIMP

INTRODUCTION

The basal-like subset of breast cancer was first identified as tumors lacking estrogen and progesterone receptors and HER2 amplification (“triple negative”) with a gene expression profile similar to basal/myoepithelial cells of the breast (Perou et al., 2000). Basal-like breast cancers account for 8-37% of all breast cancers (depending on

the definition criteria) and are associated with a poor prognosis; increased development of distant metastasis, decreased survival rate and increased mortality (reviewed in (Rakha and Ellis, 2009; Rakha et al., 2008; Voduc and Nielsen, 2008)). Since these tumors lack expression of estrogen and progesterone receptors as well as HER2, there are limited treatment options and these are largely ineffective in treating patients suffering from basal-like breast cancer.

Large-scale genetic analyses of human tumor samples have generated a wealth of molecular information and have identified potential tumor suppressors, oncogenes and prognostic factors (Perou et al., 2000; Sørlie et al., 2001; Sotiriou and Pusztai, 2009). The challenge now is to study the function of these new genes and understand their mechanisms of action in order to validate their clinical utility and confirm their potential use as targets for intervention. The arrestin family member ARRDC3 is one such gene that was recently found within a cluster on chromosome 5 deleted in 17% of basal-like breast cancers (compared to 0% in luminal breast-cancers) suggesting a role as a tumor suppressor (Adelaide et al., 2007). ARRDC3 contains structural homology to the arrestin family of proteins, which play an important role in the internalization and subsequent regulation of G protein-coupled receptors (GPCRs). Although ARRDC3 has been classified as an α -arrestin, there is a cluster of acidic and hydrophobic residues in the C-terminus that may bind to clathrin, suggesting a role in membrane protein internalization (Alvarez, 2008). A role for ARRDC3 as a negative regulator of PPAR γ signaling and endosomal functions has been suggested (Oka et al., 2006).

Integrins are cell surface adhesion molecules that mediate cell-extracellular matrix and cell-cell interactions. Binding of integrins to their ligands initiates a number of signaling events that modulate cellular behaviors such as adhesion, proliferation, survival, motility, gene expression and differentiation (Arnaout et al., 2005; Dowling et al., 1996; Fuchs et al., 1997; Germain et al., 2009; Hynes, 2002; Lipscomb and Mercurio, 2005; Lipscomb et al., 2005; Mercurio et al., 2001a; Vicente-Manzanares et al., 2009; Watt, 2002; Wilhelmsen et al., 2006). The ITG β 4 subunit was initially identified in cancer as a tumor-related antigen associated with metastasis (Falcioni et al., 1989; Falcioni et al., 1988) and was later found to promote motility and invasion in carcinoma cells (O'Connor et al., 1998; Rabinovitz and Mercurio, 1996; Rossen et al., 1994; Shaw et al., 1997; Wei et al., 1998). ITG β 4 signaling increases invasive potential and sustains the survival of carcinoma cells in stressful environments (Baril et al., 2007; Chen et al., 2009; Lipscomb and Mercurio, 2005; Yoon et al., 2005). Recent studies in human samples found ITG β 4 expression is correlated to breast cancer size and nuclear grade (Diaz et al., 2005), and significantly associates with basal-like breast cancer (Lu et al., 2008). ITG β 4 expression is also linked to poor patient prognosis in a variety of other cancers (Raymond et al., 2007). Despite its significance in tumor progression, surprisingly little is known about the regulation of ITG β 4 at the protein level. It is phosphorylated during signal transduction and is expected to be internalized, trafficked throughout the cell, and either recycled or degraded (Caswell and Norman, 2008; Caswell and Norman, 2006; Dutta and Shaw, 2008; Germain et al., 2009; Hemler, 2001; Rabinovitz et al., 2004; Wilhelmsen et al., 2007; Yoon et al., 2005). It has been hypothesized that after hemidesmosome

disruption, the newly liberated ITG β 4 is rendered capable for signaling. Activation results in the release of ITG β 4 from interactions with the keratin cytoskeleton and allows for de novo interaction with the actin cytoskeleton and signaling molecules (Lipscomb and Mercurio, 2005; Mitra and Schlaepfer, 2006; Rabinovitz and Mercurio, 1996; Wilhelmsen et al., 2007; Yoon et al., 2005). However, little is known about the mechanism by which this occurs.

In order to understand the function of ARRDC3, we used over-expression and shRNA-mediated down-regulation studies in human breast cancer cells. We demonstrate significant effects on breast cancer cell migration and growth that coincided with dramatic effects on the cell surface protein ITG β 4. We also show that ARRDC3 expression is inversely correlated to breast tumor grade. In this study we demonstrate that ARRDC3 acts as a novel regulator of tumor progression in breast cancer due to its effects on ITG β 4 internalization and degradation.

RESULTS

ARRDC3 expression is down-regulated during tumor progression

To investigate how ARRDC3 expression is altered during carcinogenesis, we used Oncomine to analyze published microarray data. Levels of *ARRDC3* mRNA are lower in breast cancer tissues compared to normal mammary gland tissue (Figure 3-1A). As normal mammary tissue is mostly fat whereas tumors contain varying levels of stroma, this alone is not conclusive. However, levels of *ARRDC3* mRNA decrease upon the transformation of purified and cultured human mammary epithelial cells (Figure 3-1B). Oncomine data also reveal that there is a decrease in *ARRDC3* mRNA in metastatic

tumors when compared to the primary tumor (Figure 3-1C). Additionally, *ARRDC3* expression is lower in breast cancer patients that relapsed within 5 years of initial diagnosis compared to tumors from patients that remained disease free for >5 years (Figure 3-1D). Combined, these data indicate that *ARRDC3* repression occurs early in oncogenesis, decreases throughout tumor progression and indicates a poor prognosis.

ARRDC3 affects in-vitro human cancer cell tumorigenicity

To evaluate the potential role of *ARRDC3* as a suppressor of tumor growth, we generated stable cancer cell lines either over-expressing or repressing *ARRDC3* in the basal-like breast cancer cell line MDA-MB-231. We first noticed a change in the comparative growth rates. When quantitated, *ARRDC3* over-expression causes a decrease in cell growth rates whereas the repression of *ARRDC3* increases cell growth (Figure 3-2A).

To investigate the affect of *ARRDC3* on invasiveness, the sub-lines were used in a Matrigel chemoinvasion assay. Over-expression of *ARRDC3* in MDA-MB-231 cells caused a 50% reduction in the number of invasive cells where the repression of *ARRDC3* caused a two fold increase (Figure 3-2B).

The sub-lines were then used in a scratch assay to test whether *ARRDC3* affects cancer cell migration. We found a dramatic decrease in cell migration in the *ARRDC3* over-expressing sub-lines (Figure 3-2C). The sub-lines with repressed *ARRDC3* exhibited a significant increase in migration rate.

We next evaluated the role of *ARRDC3* in anchorage independent growth using the sub-lines. After 4-weeks growth in soft agar, colony number and size were calculated

using ImageJ. There was a significant decrease in colony number in the ARRDC3 over-expressing sub-lines. The sub-lines with repressed ARRDC3 demonstrated a significant increase in colony number (Figure 3-2D). ARRDC3 expression also affected the size of the colonies; sub-lines with repressed ARRDC3 had a higher percentage of colonies >200 μ m whereas lines over-expressing ARRDC3 had a lower percentage of colonies >200 μ m (Figure 3-2E).

To evaluate the effect of ARRDC3 on viability, MTT assays were used to analyze MDA-MB-231 cells with either repressed ARRDC3 or over-expression of ARRDC3. Cells with altered ARRDC3 expression demonstrated no changes in cell viability when MTT signal was normalized to the total number of cells. However, when cells were assayed day 4 after mitomycin C treatment, cells with repressed ARRDC3 had increased viability while cells over-expressing ARRDC3 demonstrated decreased viability when compared to day 0 untreated cells (Figure 3-2F).

ARRDC3 suppresses in-vivo tumorigenicity

To assess the role of ARRDC3 in tumor development *in-vivo*, 1×10^6 cells of the stable sub-clones of MDA-MB-231 cells were injected into the mammary fat pad of nude mice. All sub-clones formed tumors efficiently by 7-weeks in 85-100% of the mice suggesting that ARRDC3 does not affect *in-vivo* tumor incidence. However, the sub-lines with repressed ARRDC3 formed measurable tumors more quickly (2 weeks) when compared to the control line (3 weeks). In contrast, the formation of tumors from the ARRDC3 over-expressing line was delayed (4 weeks) when compared to the control line (3 weeks). Seven weeks post injection, all mice were euthanized and the tumors were

dissected and measured. ARRDC3 significantly suppressed tumor growth *in-vivo* as determined by the weekly and final tumor volume measurements (Figure 3-3A-C).

To determine whether there was a difference in the proliferation of the tumor cells, sections from the xenograft tumors (n=5 tumors for each cell line) were stained for Ki67. As expected from the *in-vitro* studies, repressing ARRDC3 increases the percent of Ki67 positive cells by almost 2-fold, while over-expressing ARRDC3 decreases the number of Ki67 positive cells by approximately 3-fold (Figure 3-3D). When quantitated, these differences were statistically significant.

When examined microscopically, all tumors displayed an undifferentiated solid tumor morphology (Figure 3-4). To assess the contribution of ARRDC3 to *in-vivo* tumor cell survival, H&E stained sections of the xenograft tumors were quantitatively evaluated for central necrosis using ImageJ software. Although larger tumors are generally more necrotic, tumors derived from the shARRDC3 lines contained significantly less necrosis when compared to control tumors (Figure 3-5B). In contrast, tumors from the ARRDC3 over-expressing cell line, although smaller, were highly necrotic (Figure 3-5B). When quantitated, these differences were statistically significant. When TUNEL staining was performed on sections from the xenograft tumors, the expression of ARRDC3 appeared to have no effect on the number of apoptotic cells (Figure 3-5C), suggesting that ARRDC3 acts independently from the apoptotic pathway. Altogether, these data demonstrate that ARRDC3 suppresses *in-vivo* tumor cell growth and possibly affects the viability of tumor cells.

ARRDC3 negatively regulates ITGβ4

Our early adeno-viral mediated over-expression experiments caused detachment of MDA-MB-231 cells from the substratum after one week in culture suggesting a defect in cell adhesion. The MDA-MB-231 human cancer cell depends on the cell surface adhesion molecule ITGβ4 for cell adhesion to laminin, survival, migration and invasion (Lee et al., 2008; Mercurio and Rabinovitz, 2001; Shaw et al., 1997). To determine whether ARRDC3 expression directly influences ITGβ4 levels in breast cancer cells, transient over-expression and RNAi analyses were performed using the MDA-MB-231 cell line. Over-expression of ARRDC3 causes a significant decrease in ITGβ4 protein levels by Western blot. Conversely, cells transfected with a siRNA targeting ARRDC3 demonstrate a marked increase in ITGβ4 (Figure 3-6A). To verify that ITGβ4 was only affected in cells with altered ARRDC3, MDA-MB-231 cells were infected with an ARRDC3 and GFP co-expressing adenovirus and evaluated for ITGβ4 levels using immunofluorescence. Only GFP⁺ cells demonstrated a decrease in ITGβ4 surface expression while there was no change in ITGβ4 protein levels in control GFP-only infected cells (Figure 3-6B). Therefore, it is unlikely that the decrease in ITGβ4 levels is in response to a paracrine signaling pathway as ARRDC3 over-expression does not affect neighboring cells. Flow cytometric analysis showed that over-expression of ARRDC3 induces a complete lack of ITGβ4 protein at the cell surface (Figure 3-6C).

ARRDC3 regulates ITGβ4 protein levels in a proteasome-dependent manner

To help determine whether ARRDC3 regulates ITGβ4 at the level of mRNA expression or post-translationally, MDA-MB-231 cells were treated with cyclohexamide

after infection with either an adenoviral ARRDC3 & GFP virus (adARRDC3) or a control GFP-only virus (adGFP). Down-regulation of ITG β 4 in adARRDC3 infected cells was detected by western blot analysis and immunofluorescence despite treatment with cyclohexamide (Figure 3-7). These data demonstrate that ARRDC3 regulation of ITG β 4 protein levels is a post-translational process leading to increased degradation that does not require transcription of new genes.

We next wanted to determine the mechanism of ITG β 4 degradation in breast cancer cells. MDA-MB-231 cells were treated with the proteasome inhibitor lactacystin prior to infection with adARRDC3. The addition of lactacystin prevented the ARRDC3 mediated decrease in ITG β 4 protein levels as determined by immunofluorescence and Western blots (Figure 3-8A&B). Since proteasome inhibition should not affect internalization, lactacystin treatment does not prevent the significant decrease in surface ITG β 4 as seen by flow cytometry (Figure 3-8C). To exclude the possibility of lysosomal contribution to the protein degradation, the experiment was repeated using chloroquin instead of lactacystin prior to infection. Chloroquin treatment did not prevent ITG β 4 reduction after infection with adARRDC3 (Figure 3-9A&B).

ARRDC3 directly interacts with activated ITG β 4

A novel phosphorylation site on ITG β 4, serine-1424, was recently identified as important in hemidesmosome disassembly and shown to be enriched on the trailing edge of migrating cells (Germain et al., 2009). This phosphorylation, along with the phosphorylation of other serines, results in the disassembly of the hemidesmosome and mobilization of ITG β 4 to actin-rich protrusions (Germain et al., 2009; Rabinovitz et al.,

1999; Rabinovitz et al., 2004; Wilhelmsen et al., 2007). To determine if this site is important in ARRDC3 mediated ITG β 4 internalization during breast cell migration, endogenous expression patterns of ITG β 4, ITG β 4-pS1424 and ARRDC3 were examined using confocal microscopy on migrating cancer cells. ARRDC3 co-localizes with ITG β 4 only on the lagging edge of the cell, where ITG β 4-pS1424 is enriched (Figure 3-10A). We then determined that ARRDC3 physically interacts with ITG β 4-pS1424 by endogenous immuno co-precipitation on lysates prepared from MDA-MB-231 cells pre-treated with phosphatase inhibitors (Figure 3-10B). Densitometric analysis demonstrates ~70% of ITG β 4-pS1424 is immuno co-precipitated by an ARRDC3 antibody when compared to an ITG β 4 antibody. The densitometric ratio of precipitated pS1424/ITG β 4 is 3.5 fold higher for the ARRDC3 immuno co-precipitation when compared to the ITG β 4 immuno co-precipitation suggesting, ARRDC3 preferentially interacts with this “activated” (i.e. engaged in a signaling cascade) form of ITG β 4.

As the regulation of ITG β 4 by ARRDC3 is dependent on the proteasome, we next wondered whether ARRDC3 interacts with ITG β 4 after it is targeted for degradation. In untreated MDA-MB-231 cells, there is an undetectable amount of ubiquitinated ITG β 4, even after ITG β 4 immuno co-precipitation. However, when cells were pre-treated with proteasome inhibitor lactacystin, higher molecular weight bands of ITG β 4 were detected after immuno co-precipitation with both ARRDC3 and ITG β 4 antibodies (Figure 3-10C). Immuno-blotting the same membranes for Ubiquitin revealed almost equal amounts of ubiquitinated- ITG β 4 is immuno co-precipitated with the ARRDC3 antibody when

compared to the ITG β 4 immuno co-precipitation (Figure 3-10C). This suggests that ARRDC3 maintains in complex with ITG β 4 after it is targeted for degradation.

ARRDC3 specifically targets ITG β 4

Cancer cells frequently have defects within vesicular trafficking and/or endocytotic pathways (reviewed in (Mosesson et al., 2008)). To demonstrate that ARRDC3 is specifically targeting ITG β 4, rather than stimulating generalized endocytosis, we examined a variety of cell surface proteins after cells (MDA-MB-231) were infected with either adGFP or adARRDC3. Analysis of non-permeabilized cells by flow cytometry shows that ARRDC3 over-expression did not affect surface levels of ITG β 1, CD44 and EpCam (Figure 3-11). These data indicate that ITG β 4 is specifically targeted by ARRDC3.

Effects of ARRDC3 on in-vitro tumorigenicity is dependent on ITG β 4

To determine whether ITG β 4 was required to mediate the effects of ARRDC3 on cancer cell behavior, we used MDA-MB-435 (a cancer line which does not express ITG β 4) and MDA-MB-435+ β 4 cells (a daughter cell line engineered to over-express ITG β 4), to create lines with altered ARRDC3 levels and assayed for tumorigenicity. When analyzed by western blot, the inverse relationship between ARRDC3 and ITG β 4 protein levels were maintained (Figure 3-12).

Similar to MDA-MB-231 cells, the MDA-MB-435+ β 4 cells showed marked changes in proliferation, migration, invasion and anchorage independent growth. Over-expression of ARRDC3 in MDA-MB-435+ β 4 cells caused decreased proliferation and migration, while repression of ARRDC3 lead to increased proliferation and migration

(Figure 3-13A&B). Soft agar assays again showed that over-expression of ARRDC3 inhibits anchorage independent growth, while repressed ARRDC3 promotes tumorigenicity in the MDA-MB-435+ β 4 cells (Figure 3-13C&D). Cell invasion assays demonstrated a 50% decrease in the number of invasive cells in ARRDC3 over-expressing cells whereas repression of ARRDC3 caused a two-fold increase (Figure 3-13E). In contrast to the ITG β 4-positive cell lines, ARRDC3 had marginal effects on the parental, ITG β 4-negative MDA-MB-435 cell line (Figure 3-13A-E). Interestingly, ARRDC3 over-expression reduced the tumorigenic properties of the MDA-MB-435+ β 4 cells to that of the parental line. Altogether, these data show that ARRDC3 affects *in-vitro* tumorigenicity, principally in an ITG β 4-dependent fashion. However, the data also shows that ARRDC3 also has an ITG β 4-independent effect on *in-vitro* tumorigenicity, but the physiological relevance of this pathway is unclear.

ARRDC3 down-regulation and coordinate ITG β 4 up-regulation in human breast tumors

Since there is an extensive connection between ITG β 4 and breast carcinogenesis, we examined protein levels of ARRDC3 and ITG β 4 in normal breast tissue and primary human breast cancers (invasive ductal carcinomas) obtained from *UMass Cancer Center Tissue and Tumor Bank*. As expected, ARRDC3 expression appeared to be inversely correlated to ITG β 4. It is interesting to note that moderate expression of ARRDC3 is not sufficient to completely abrogate ITG β 4 (Figure 3-14).

We next examined expression of ARRDC3 and ITG β 4 in normal breast tissue sections using immuno-fluorescence. As expected, ITG β 4 positive cells were located in the basal layer of normal ducts. Conversely, ARRDC3 was more highly expressed in

luminal cells and breast stroma (where ITG β 4 expression is negligible) but weakly expressed in basal cells (where ITG β 4 expression is highest) (Figure 3-15).

The combination of our data and the current knowledge of ITG β 4 strongly suggested that repression of ARRDC3 would lead to a more aggressive/metastatic phenotype. To analyze the expression pattern of ARRDC3 in tumors, 52 human breast tumors of varied grade and ER/PR/HER2 receptor status (obtained from *UMass Cancer Center Tissue and Tumor Bank*) were used for ITG β 4 & ARRDC3 co-immunofluorescence. Expression of ARRDC3 was inversely correlated to tumor Grade (Table 3-1 & Figures 3-16 through 3-18). Within grade 1 tumors, 5/6 demonstrated high ARRDC3 staining while the outlier exhibited strong ITG β 4 staining (Figure 3-16). Interestingly, although expression of ARRDC3 varied greatly in grade 2 tumors, levels were inversely correlative to ITG β 4 expression (Figure 3-17). Grade 3 tumors generally expressed low or undetectable levels of ARRDC3. Only 2/22 grade 3 tumors displayed high expression of ARRDC3 (Figure 3-18). Although 11/52 tumors expressed no/low levels of ARRDC3 and ITG β 4, only 1/52 tumors had intense staining for both ARRDC3 and ITG β 4. Matched primary/metastatic tumor samples showed a further repression of ARRDC3 level in the metastatic lesion (Figure 3-19).

The combination of our data and the current knowledge of ITG β 4 strongly suggested that repression of ARRDC3 would lead to a more aggressive/metastatic phenotype. Overall, the data support the hypothesis that ARRDC3, probably through its effects on ITG β 4, acts as a regulator of breast cancer.

DISCUSSION

In the present study, we describe a novel regulatory pathway involving the internalization and degradation of the cell surface protein ITG β 4, which has significant effects on breast cancer cell growth and tumorigenicity. We demonstrate that ARRDC3, previously identified by genetic screening as a potential tumor suppressor (Adelaide et al., 2007), is a new regulator of breast cancer progression. We show that ARRDC3 directly binds to ITG β 4, specifically when phosphorylated at S1424, and ultimately leads to proteasome dependent degradation. The subsequent change in ITG β 4 protein levels significantly affects *in-vitro* tumorigenic properties such as proliferation, migration, invasion and growth in soft agar. *In-vivo* analyses demonstrate that ARRDC3 expression inversely correlates with tumor growth in nude mice and viability of tumor cells under stressed environments. Additionally, data from human breast cancer samples show that ARRDC3 expression is, in general, inversely correlated to ITG β 4 protein levels.

ITG β 4 is part of a genetic signature correlated to basal-type breast cancer (Lu et al., 2008). It was therefore very interesting to note that ARRDC3 is part of a cluster on chromosome 5 deleted in 17% of the same basal-type breast cancer subset (compared to 0% in luminal breast-cancers) (Adelaide et al., 2007). We found that ARRDC3 protein was low or absent in 5 of 11 human infiltrating ductal carcinomas of the breast, and these tumors had high levels of ITG β 4. Although we do not know the mechanism by which ARRDC3 is down-regulated within these tumors, we suspect that chromosome deletion is not the only cause of ARRDC3 deficits. Further genetic, epigenetic and mutational analyses of the ARRDC3 locus are needed to more fully investigate the transcriptional

regulation of ARRDC3 within tumors. All basal-like breast cancer samples we analyzed had little to no expression of ARRDC3 (Figure 3-20), suggesting that ARRDC3 may be inactivated by additional mechanisms other than chromosomal deletion. These data also suggest the loss of ARRDC3 is more critical in basal-type breast cancers, which typically express high levels of ITG β 4. Understanding the mechanism of ITG β 4 regulation by ARRDC3 may lead to improved therapies for this aggressive subset of breast cancer that currently has a very poor prognosis. However, it should be noted that neither ARRDC3 repression nor ITG β 4 expression was exclusive to basal-like breast tumors meaning that potential therapies designed around this process would be beneficial to other cancer types as well.

Interestingly, some tumors we evaluated maintained expression of both ARRDC3 and ITG β 4. It is possible that the effects of ARRDC3 on ITG β 4 are simply dose dependent and moderate expression of ARRDC3 maintains intermediate levels of ITG β 4. Low or moderate levels of ARRDC3 may also allow for the recycling of ITG β 4 similar to the effects of β -arrestin on GPCRs (Luttrell and Lefkowitz, 2002). It is worth speculating how one tumor we evaluated maintained high expression of both ARRDC3 and ITG β 4. If the pathway in which ARRDC3 targets ITG β 4 for degradation is somehow altered, it is possible that ARRDC3 could allow for the mobilization and recycling of ITG β 4 from hemidesmosomes at an increased rate. The presence of increased surface ITG β 4 we observed after treatment with a proteasome inhibitor may be evidence of such a mechanism (Figure 3-8C). This would facilitate the speed in which filamentous actin protrusions form, thus promoting tumor progression. However, further trafficking studies

are necessary to determine whether ITG β 4 is actively being recycled to the cell surface in the absence of degradation.

ITG β 4 is the core component of hemidesmosomes which anchor keratin filaments within the cell to the basement membrane (Borradori and Sonnenberg, 1999; Jones et al., 1998; Wilhelmsen et al., 2006). Hemidesmosomes mediate stable adhesion but are highly dynamic structures that can quickly disassemble under conditions in which detachment from the sub-strata is required, such as during cell migration or carcinoma invasion (Geuijen and Sonnenberg, 2002; Tsuruta et al., 2003). Previous investigations of the mechanism of hemidesmosome disassembly revealed the importance of several phosphorylation sites on ITG β 4 (Germain et al., 2009; Mainiero et al., 1996; Rabinovitz et al., 1999; Rabinovitz et al., 2004; Wilhelmsen et al., 2007). Our data reveal a novel intermediate in hemidesmosome disassembly during cell migration. After the cell receives migration stimuli, ARRDC3 directly interacts with ITG β 4 phosphorylated on S1424 by PKC α (Germain et al., 2009). As this phosphorylation event only occurs on the lagging edge of the cell, only ITG β 4 located there becomes endocytosed and sent to the proteasome, ultimately dissolving the hemidesmosome. This disruption of adhesion molecules at the lagging edge allows the cell to migrate. Although we demonstrated that ARRDC3 preferentially binds to the pS1424 form of ITG β 4, it is possible that other phosphorylation sites are mediators of ARRDC3 binding.

Until recently, there have been relatively few investigations of the factors involved in integrin endocytosis. Internalization of integrins α 5 β 1, α v β 6, α v β 3, α 6 β 1 have been associated with clathrin-mediated endocytosis (Caswell and Norman, 2008;

Ezratty et al., 2009), whereas integrins $\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha 2\beta 1$ (Caswell and Norman, 2008; Shi and Sottile, 2008) have been connected to caveolae dependent endocytosis. Integrin $\alpha 6\beta 4$ has been shown to associate with lipid rafts (Gagnoux-Palacios et al., 2003) suggesting that caveolae may play a role in internalization. However, not all integrin $\alpha 6\beta 4$ was found associated with lipid rafts. Our data provides additional insight to the area of integrin endocytosis. Since ARRDC3 contains clathrin binding motifs it is possible that it functions by a clathrin-dependent mechanism although more investigation of the protein complexes within this pathway is needed. Lastly, ARRDC3 does not appear to interact with $\beta 1$ integrins (Fig. 3-11) and because of the unique long cytoplasmic tail of ITG $\beta 4$, ARRDC3 is likely specific for this integrin.

It has been hypothesized that during the progression from normal epithelium to invasive carcinoma, the function of ITG $\beta 4$ switches from a mechanical adhesive device into a signaling-competent receptor. In this case, ITG $\beta 4$ needs to be liberated from hemidesmosome where it can then be trafficked to actin-rich motility structures (filopodia and lamellae) (Lipscomb and Mercurio, 2005; Mercurio et al., 2001b; Santoro et al., 2003). Our findings highlight the importance of the ITG $\beta 4$ -pS1424 site in this process as endogenous ARRDC3 co-localizes with this phosphorylated form of ITG $\beta 4$, likely causing internalization. ITG $\beta 4$ -pS1424 has been identified as a critical residue in HD disassembly; however, MDA-MB-231 cells do not form HD. It is possible that the site is still phosphorylated prior to internalization of ITG $\beta 4$ regardless whether HDs need to be dissolved. It is also possible that the real phosphorylation event increasing the ARRDC3/ITG $\beta 4$ interaction is caused by the same signals leading to ITG $\beta 4$ -S1424

phosphorylation. It would be interesting to test whether S→D (which mimics phosphorylation) and S→A mutants (which cannot be phosphorylated) of residue S1424 affect the ARRDC3/ITGβ4 interaction.

It is clear from our data that ARRDC3 plays a role in the regulation of ITGβ4 protein levels and likely contributes to the control of ITGβ4 function during breast cancer progression. The interaction between ARRDC3 and ITGβ4 may represent a new therapeutic target for basal-like breast cancers. Disruption of this interaction, by peptides or small molecules, may stabilize ITGβ4 in hemidesmosomes and block its growth and pro-survival effects. These data also highlight the importance of ITGβ4-blocking antibodies as a potential therapy.

Our data does not exclude the possibility that ARRDC3 has an ITGβ4 independent mechanism of action in cancer cells or in normal cells. The effects of ARRDC3 on cancer cells lacking ITGβ4 had marginal statistical significance and the overall effects were small (Figure 3-13). It is therefore unclear whether these effects are biologically significant. Considering its similarity to β-arrestins, ARRDC3 may also regulate G-protein coupled receptors (GPCRs). Possible targets could include Smoothed and Frizzled receptors (Class-6 GPCRs) or chemokine receptors (Class-1 GPCRs); which all have extensive implications in cancer in addition to potential therapeutic targets.

Although our data demonstrate tumor growth suppressor functions of ARRDC3 in breast cancer, it should not be taken for granted that this will readily translate to other tissues. ITGβ4 can play a dichotomous role in certain tissue types, either positively or

negatively regulating tumor progression (Giancotti, 2007; Rabinovitz and Mercurio, 1996; Raymond et al., 2007). The overall role of ARRDC3 in a specific cancer would depend on the role of the target proteins (like ITG β 4) in the cancer cells.

In summary, our data identifies ARRDC3 as a novel regulator of breast cancer progression that targets ITG β 4 for internalization and proteasome dependent degradation. We reveal several oncogenic properties affected by changes in ARRDC3 expression and identify a correlation between ARRDC3 expression and human breast tumor grade/aggressiveness. Our research also raises additional questions. What other proteins work with ARRDC3 in ITG β 4 internalization? Is ARRDC3 involved in other steps of integrin trafficking? How is ARRDC3 regulated? What is the biological significance of possible ITG β 4-independent pathways? What other cancers show deregulated ARRDC3 expression? Our data open several avenues of future research in cancer biology, which may ultimately lead to new treatment strategies.

METHODS

Cells and Reagents. MDA-MB-435 human breast carcinoma cells expressing wild-type ITG β 4 were generated previously (Shaw et al., 1997). MDA-MB-231 human breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository (Georgetown University).

siRNA Sequence and Transfection. Three different siRNA oligos were designed targeting the following sequences in ARRDC3: 1- AAACACGGCCTTCTATGCCA, 2- AAAGGCGGAACAATCTTGAC, and 3- AATCTTGCACCAGTGAGTGCT. siRNA duplexes against these target sequences were ordered from Qiagen along with three

control target sequences. siRNA duplexes were transfected into cells using HiPerFect transfection reagent (Qiagen) following the manufacturer's recommended protocol. For experiments shown in this paper, duplexes 1 and 3 were pooled for maximum knockdown of ARRDC3.

Cloning and Generation of Stable Lines. pSuper vectors (Oligoengine) containing control shRNA (shCtl target sequence: TTCTCCGAACGTGTCACGT) and shRNAs targeting ARRDC3 (shARRDC3 target sequence A:GGCCTTGGCTACTACCAGT; shARRDC3 target sequence C: GCGTGGAATATTCACTAAT) were generated in our lab per manufacturers recommended protocol. Full length human ARRDC3 cDNA and Flag-LacZ fusion cDNA were cloned into the pBABE-puro expression vector (Addgene). Cells were transfected with Fugene HD (Roche) following manufacturers recommended protocol. Stable transformants were selected using puromycin and pooled.

Adenoviral Preparation. Full length human ARRDC3 cDNA was initially cloned into pLEGFP (Clontech). The fragment containing ARRDC3+GFP (or GFP alone) was then cloned into Adeno-X LP CMV (Clontech) using the Adeno-X Expression System 2 kit (Clontech). Adeno-X maxi purification kit (Clontech) was used to isolate/purify the adenovirus. For all steps, the manufacturers recommended protocol was followed. The amount of virus needed for each cell type was determined empirically.

Immunofluorescence. Cells were cultured on coverslips and fixed with acetone. Cells were permeabilized by incubating with PBS+Triton and blocked with 5% goat serum (Gibco). Coverslips were incubated with primary antibody (rabbit polyclonal ARRDC3 (Abcam) and/or ITG β 4 439-9b (BD-Pharmingen)) for 2 hr at room temperature or

overnight at 4°C. After being washed, cells were then stained with secondary antibodies conjugated with either FITC or Texas-Red (Vector Laboratories) for 2 hours at room temperature. After washing, cover-slips were mounted on slides using Vectashield with DAPI (Vector Laboratories) and slides were analyzed using a fluorescence microscope.

Wound Assay. 5×10^5 cells were evenly plated in a 6-well plate. 24 hours after plating, the 100% confluent cells were treated with 15 µg/mL mitomycin C (Roche) for 90 minutes at 37°C. The monolayer was scratched with a P200 pipette tip and washed 3 times to remove floating cells. The wound closure was then monitored by digital photography.

Growth in Soft Agar. 1.0×10^3 cells were suspended in 2 mL of serum-containing medium containing 0.3% agar and overlaid on a 1 mL base layer of 0.75% agar in six-well plates. The soft agar was overlaid with complete medium (0.5 mL/well), which was changed every 2 days. After 3-4 weeks of incubation, viable colonies were stained by adding MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) into the covering media. Images were captured using bright-field microscopy and the number and size of the colonies were analyzed using ImageJ software. Only colonies with a diameter of $>50 \mu\text{m}$ were counted.

Invasion. Matrigel invasion assays were performed as described previously using 6.5-mm Transwell chambers (8-µm pore size, CoStar) (Shaw et al., 1997). After 4 hours, the cells that had invaded to the lower surface of the filters were fixed in methanol for 10 minutes. The fixed membranes were mounted on glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories). Invasion was quantified by counting the

number of stained nuclei in five independent fields in each transwell using ImageJ software.

Xenograft Mouse Studies. Animals' care was in accordance with guidelines approved by IACUC. Cells were trypsinized, washed five times with sterile PBS, and re-suspended in 35 μ L phenol red-free Matrigel immediately before injection. Female immunocompromised mice (*nu/nu*; National Cancer Institute) at 9 wk of age were anesthetized briefly with isoflurane and cells were injected into the #3 and #8 mammary fat pad (1×10^6 cells per injection in 50 μ L of Matrigel, two injection sites per mouse). Estimated tumor volume was determined using the following formula: $(4/3) \pi (1/2 \times \text{smaller diameter})^2 (1/2 \times \text{larger diameter})$. At the final time point, mice were euthanized using isoflurane and cervical dislocation. Final tumor volume (after dissection) was determined using the following formula: $(4/3) \pi (1/2 \times \text{length}) (1/2 \times \text{width}) (1/2 \times \text{height})$.

Tumor Analysis. All tumors were cut in half lengthwise. One piece was then imbedded in paraffin after formalin fixation, where the remaining piece was cut in two for fixation in O.C.T. and protein extraction. Sections of paraffin-embedded tumor were used for H&E staining, Ki67-IHC and TUNEL staining. All of the staining was performed by the DERC-Histology facility at UMass Medical School.

Western Blot Analysis. Cell extracts containing equivalent amounts of total protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for an hour using a 50 mM Tris buffer (pH 7.5) containing 0.15 mM NaCl and 0.05% Tween 20 and 5% (w/v) Blocker (BioRad) in PBS+ Tween 20. Membranes were

incubated overnight at 4°C in the same buffer containing primary antibodies (rabbit pAB targeting ARRDC3 (Abcam ab64817) or rabbit pAB targeting ITGβ4 (505, a generous gift from Arthur Mercurio, UMMS Worcester, MA). Proteins were detected by enhanced chemiluminescence (Pierce). For phospho-immunoblots, the blocking buffer for the primary antibodies contained 5% (w/v) BSA.

STATISTICAL ANALYSIS: All values in the present study were expressed as mean ± SEM unless otherwise noted. The significant differences between the groups were analyzed by a Student's *t* test and a *P* value of <0.05 was considered significant.

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(<http://www.umassmed.edu/cancercenter/tissuebank/index.aspx>) has provided with obtaining primary human breast tumor samples.

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AUTHOR CONTRIBUTIONS: The experiments were designed and performed by K.M.D., H.C., Q.T., N.F.M, M.R. and S.R.L. The manuscript was written by K.M.D. and S.R.L. The principle investigator is S.R.L.

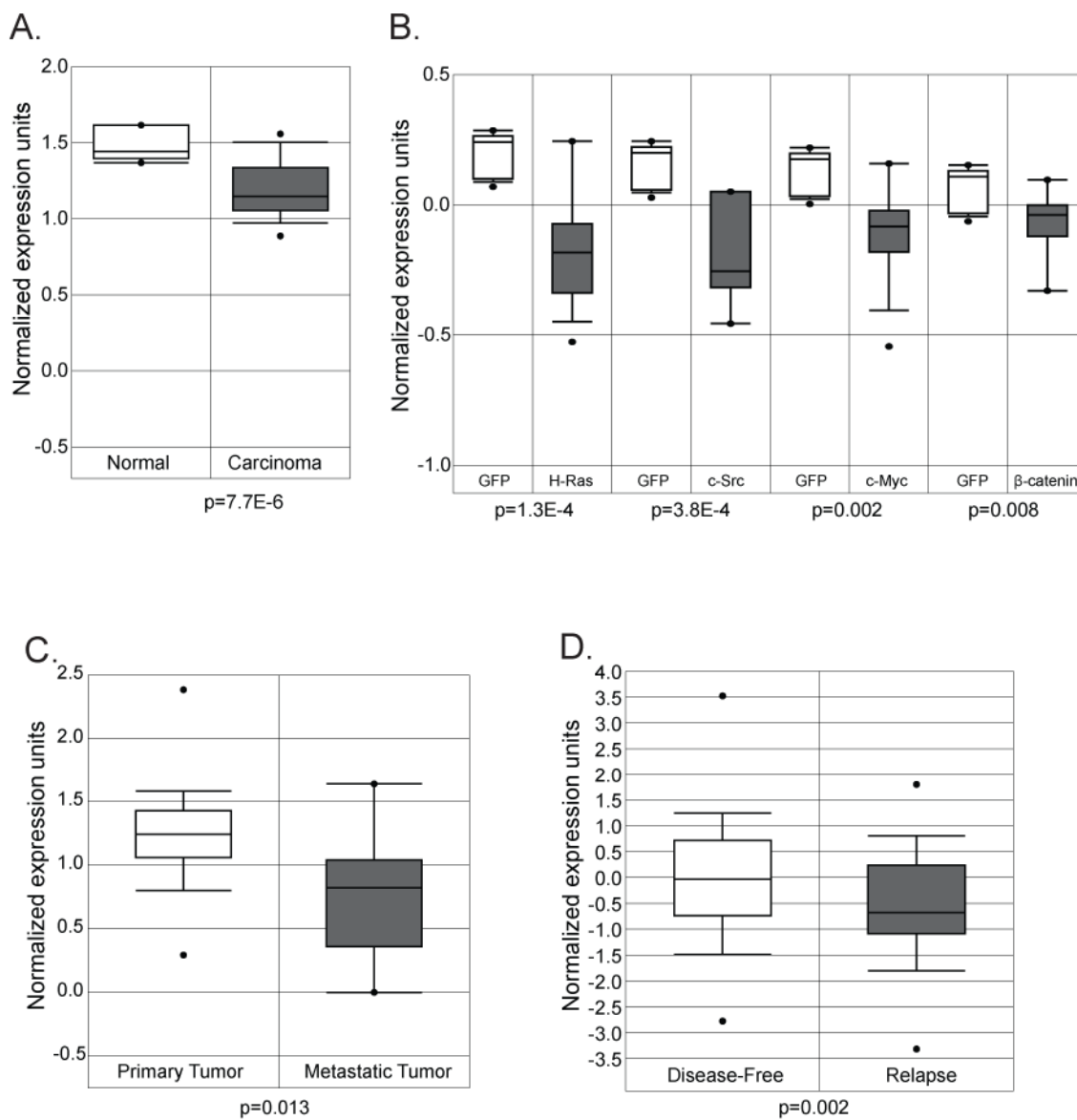


Figure 3-1: Down-regulation of ARRDC3 is an early event in carcinogenesis. (A) Levels of ARRDC3 mRNA are decreased in human breast cancers when compared to normal breast tissue. (B) ARRDC3 mRNA levels decrease in human mammary epithelial cells after transformation with various oncogenes. (C) Levels of ARRDC3 mRNA is decreased in metastatic lesions when compared to the primary tumor in the same patient. (D) Expression of ARRDC3 mRNA is decreased in the initial ER+ tumors from patients that have relapsed when compared to tumors 5-year disease-free patients. All data, including p -values, were calculated from Oncomine.

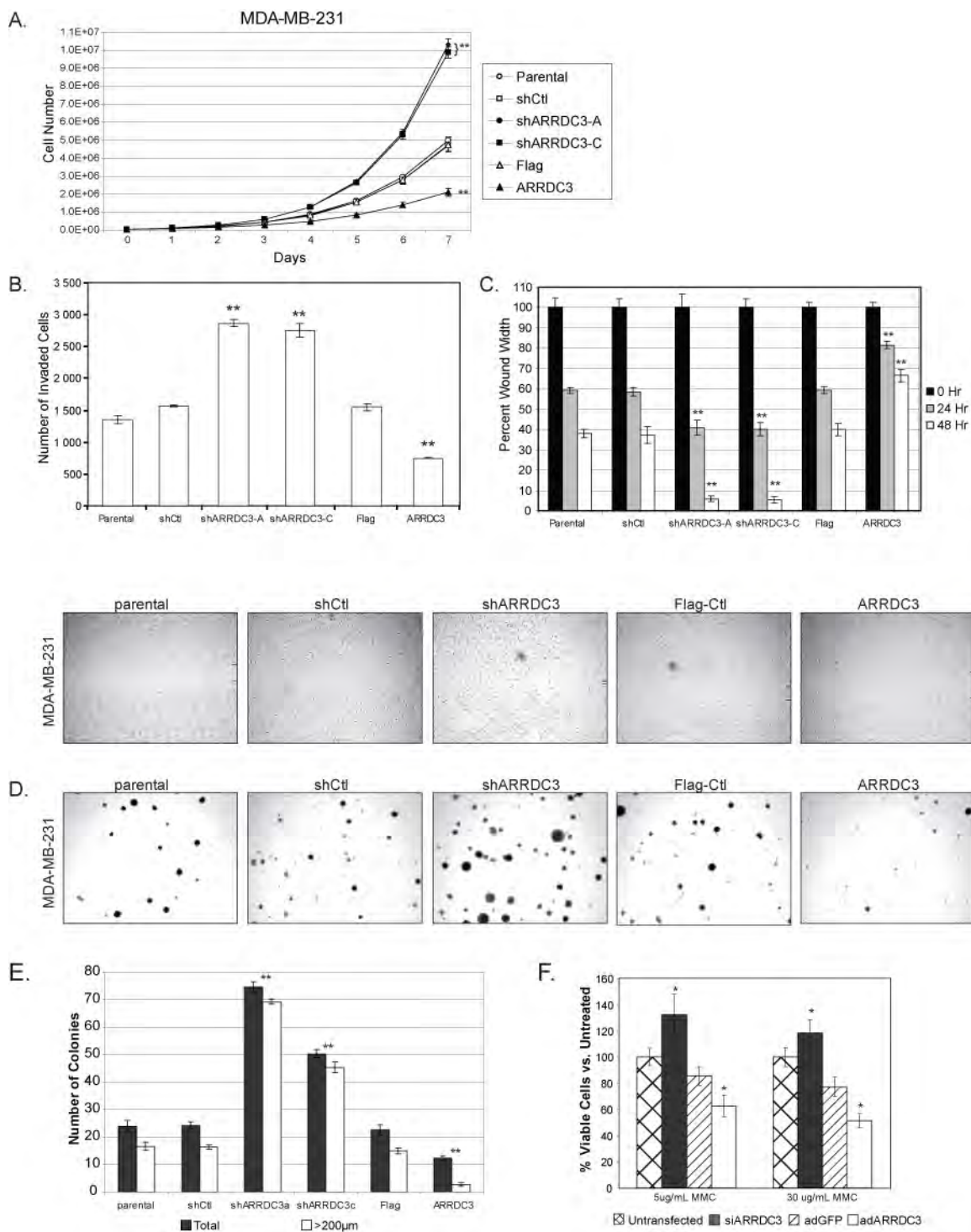


Figure 3-2: Expression of ARRDC3 effects in-vitro tumorigenicity. Each line was tested at least in triplicate and data bars represent mean +/- SEM. Single asterisk represents $p < 0.05$ whereas a double asterisk represents $p < 0.001$ as determined by student's t-test. (A) Growth curves from the stable lines demonstrate that expression of ARRDC3 is inversely correlated to cellular proliferation. (B) Matrigel chemo-invasion assay demonstrate that expression of ARRDC3 is inversely correlated to invasiveness. (C) Wound assay of mitomycin-C treated cells shows that over-expression of ARRDC3 leads to a decrease in cell migration whereas RNAi-mediated repression of ARRDC3 increases cell migration. (D) Expression of ARRDC3 is inversely correlated to anchorage independent growth both in colony number and colony size (E) Dark gray bars represent total colonies (at least $50\mu\text{m}$) whereas light gray bars represent colonies larger than $200\mu\text{m}$. (F) MDA-MB-231 cells were transfected with either siRNA targeting ARRDC3 or a vector over-expressing ARRDC3. Cells were then treated with mitomycin C for 2.5hrs. Viability was determined by MTT absorbance/cell number after 4 days and normalized to untransfected Day 0 viability. Repression of ARRDC3 promotes viability of cells after stress. Hatched, dark grey, lined and white boxes represents untransfected, control, siARRDC3, and ARRDC3 transfected cells respectively.

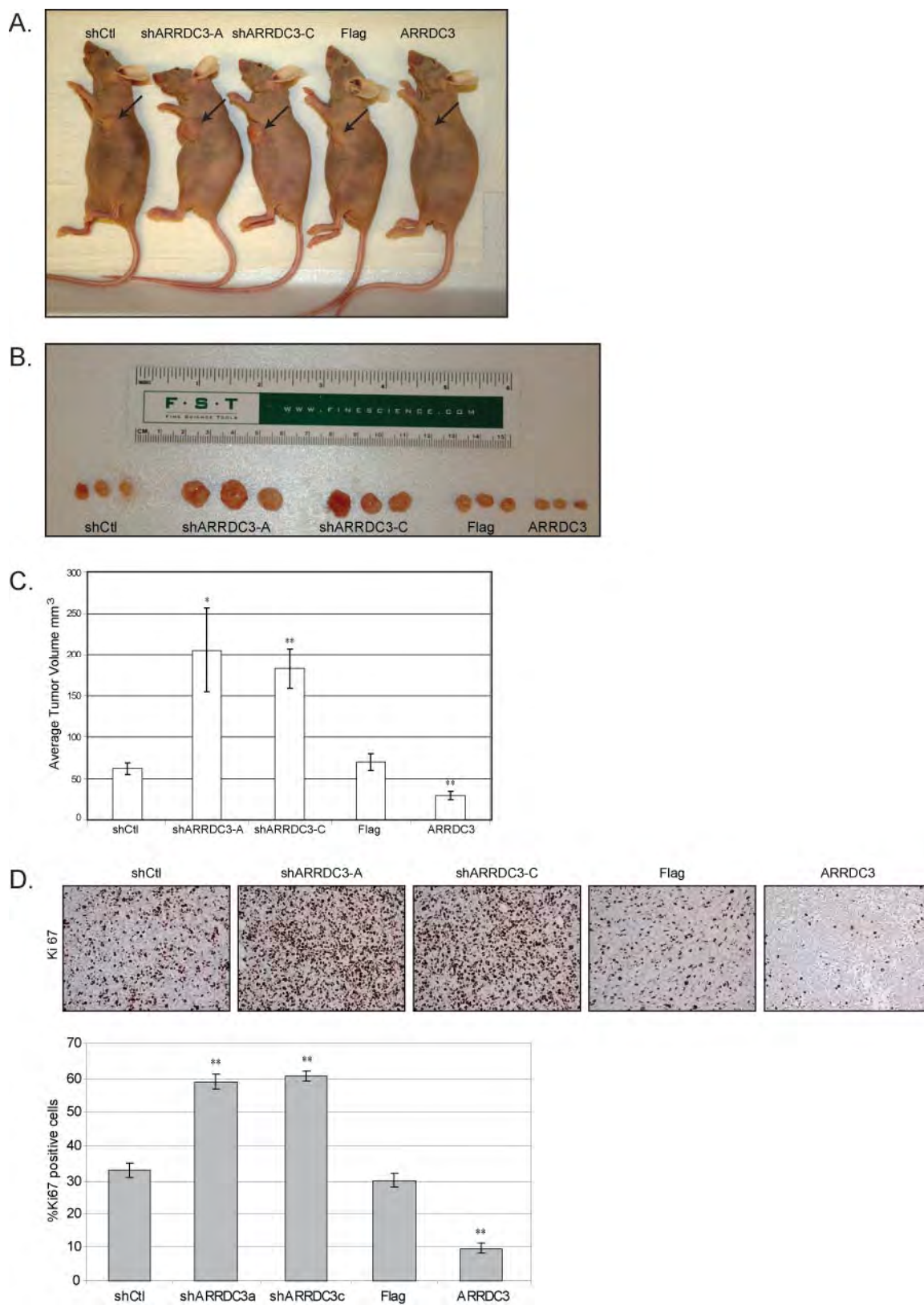


Figure 3-3: ARRDC3 negatively regulates in-vivo tumorigenicity. (A-B) Over-expression of ARRDC3 in MDA-MB-231 cells led to a decrease in in-vivo tumor size whereas repression of ARRDC3 in MDA-MB-231 cells led to an increase in in-vivo tumor size 7-weeks after xenograft. (C) When quantitated, the differences in final tumor volume were statistically significant compared to scrambled shRNA and empty vector controls. (D) ARRDC3 affects the in-vivo proliferation of tumor cells as determined by percentage of Ki67 positive cells. Xenograft tumor sections were analyzed for Ki67 using IHC. Over 1500 cells per tumor were counted and scored either Ki67+ or Ki67-. The percentage of Ki67+ cells was then calculated. 4-5 tumors per group was analyzed and data bars represent mean +/- SEM. Single asterisk represents $p < 0.05$ whereas a double asterisk represents $p < 0.001$ as determined by student's t-test.

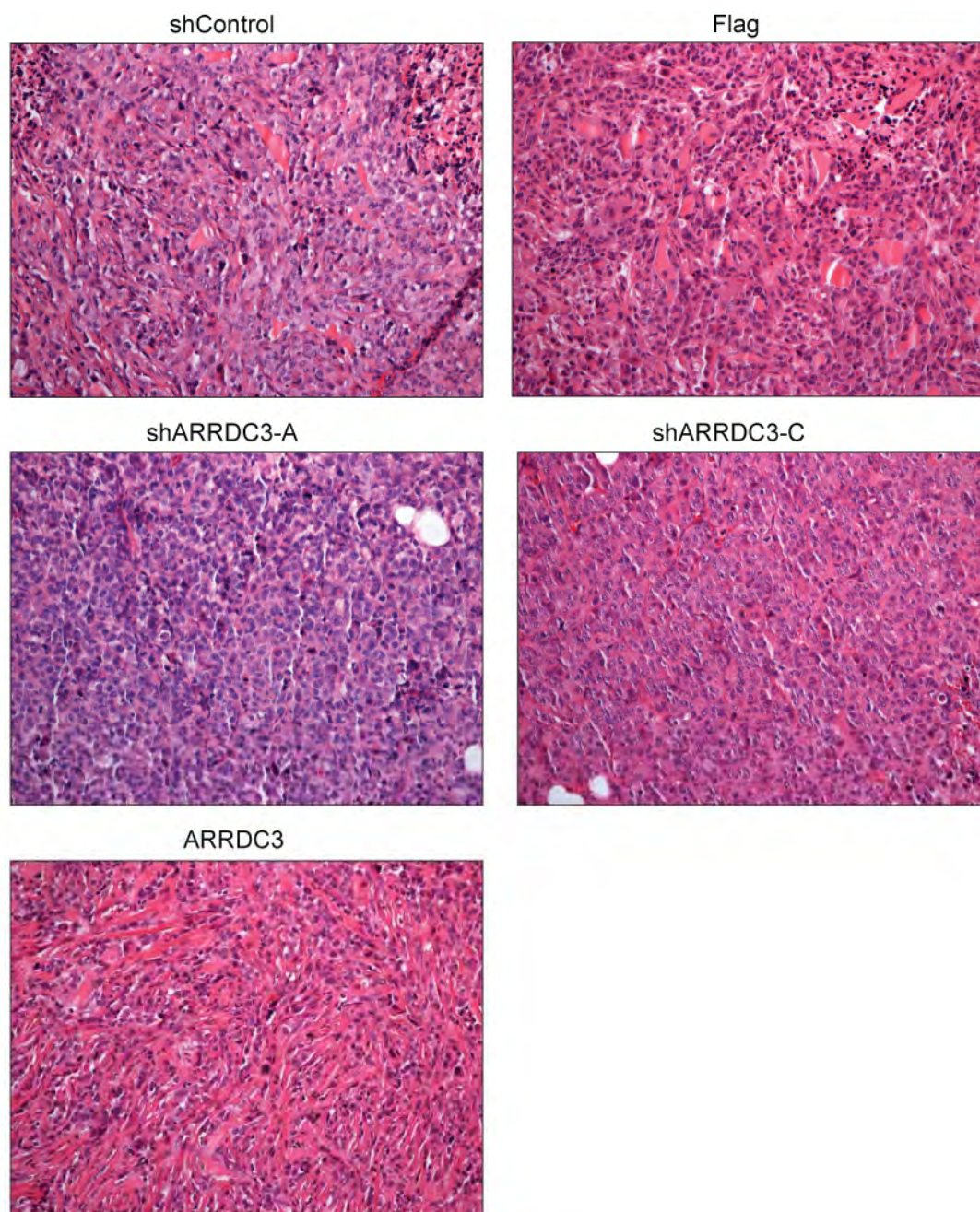


Figure 3-4: Xenograph tumors display an undifferentiated morphology. H&E stained sections from xenograph tumors resembled poorly differentiated human breast carcinomas and displayed a close relationship between polygonal carcinoma cells and spindly fibroblasts. Morphology between the tumors generated from different cell lines was not appreciably different.

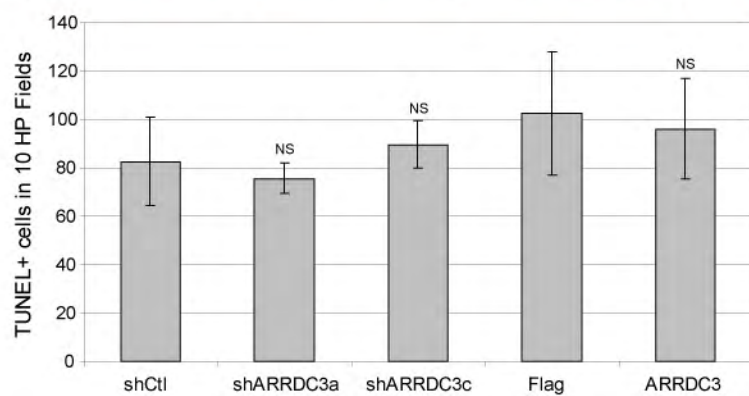
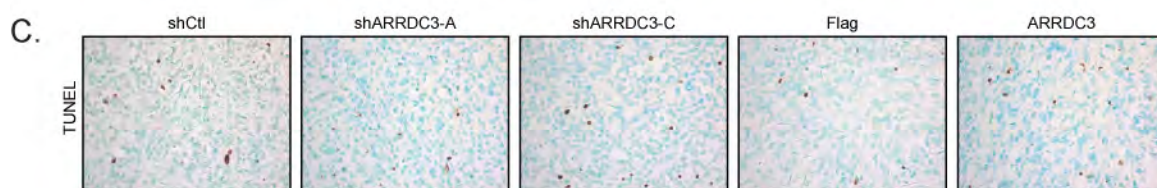
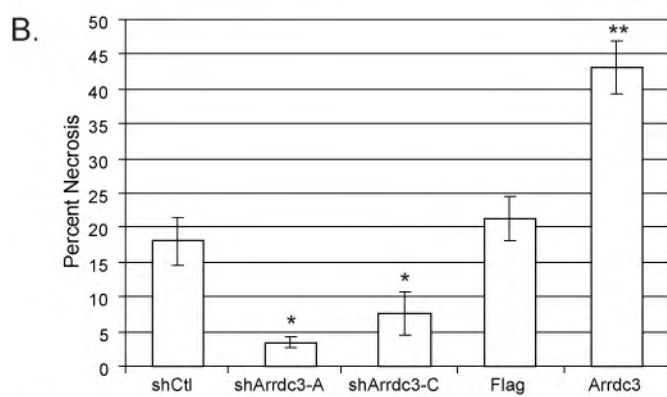
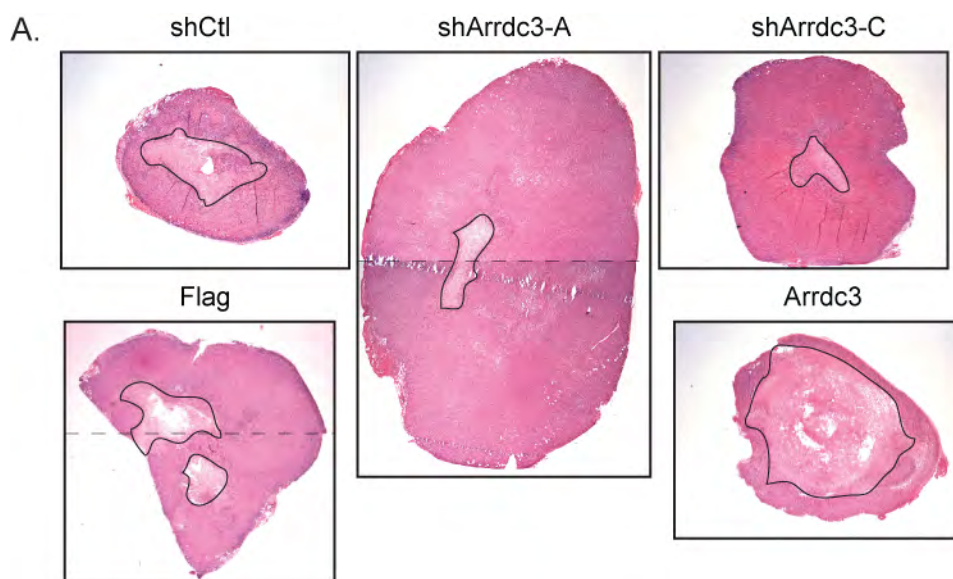


Figure 3-5: ARRDC3 repression promotes in vivo cell survival. (A-B) Repression of ARRDC3 leads to a decrease in xenograft tumor necrosis and over-expression of ARRDC3 leads to an increase in xenograft tumor necrosis (outlined in black).

Dashed line represents the point where two images were merged to visualize the entire tumor section. Tumors were cut in half along the longest axis therefore the center-most section of the tumor was used to make slides. Changes in necrosis are more evident when tumors of comparable size are analyzed. At least 5 tumors from each line were analyzed and the area of necrosis quantitated; results were statistically significant. (C) ARRDC3 does not affect the number of apoptotic cells in xenograft tumors. TUNEL analysis was performed on tumor sections and the number of positive cells in 10 high-powered fields (40X) was counted. 5 tumors per group were analyzed and data bars represent mean \pm SEM. Data bars represent mean \pm SEM. Single asterisk represents $p < 0.05$ whereas a double asterisk represents $p < 0.001$ as determined by student's t-test. NS signifies not significant.

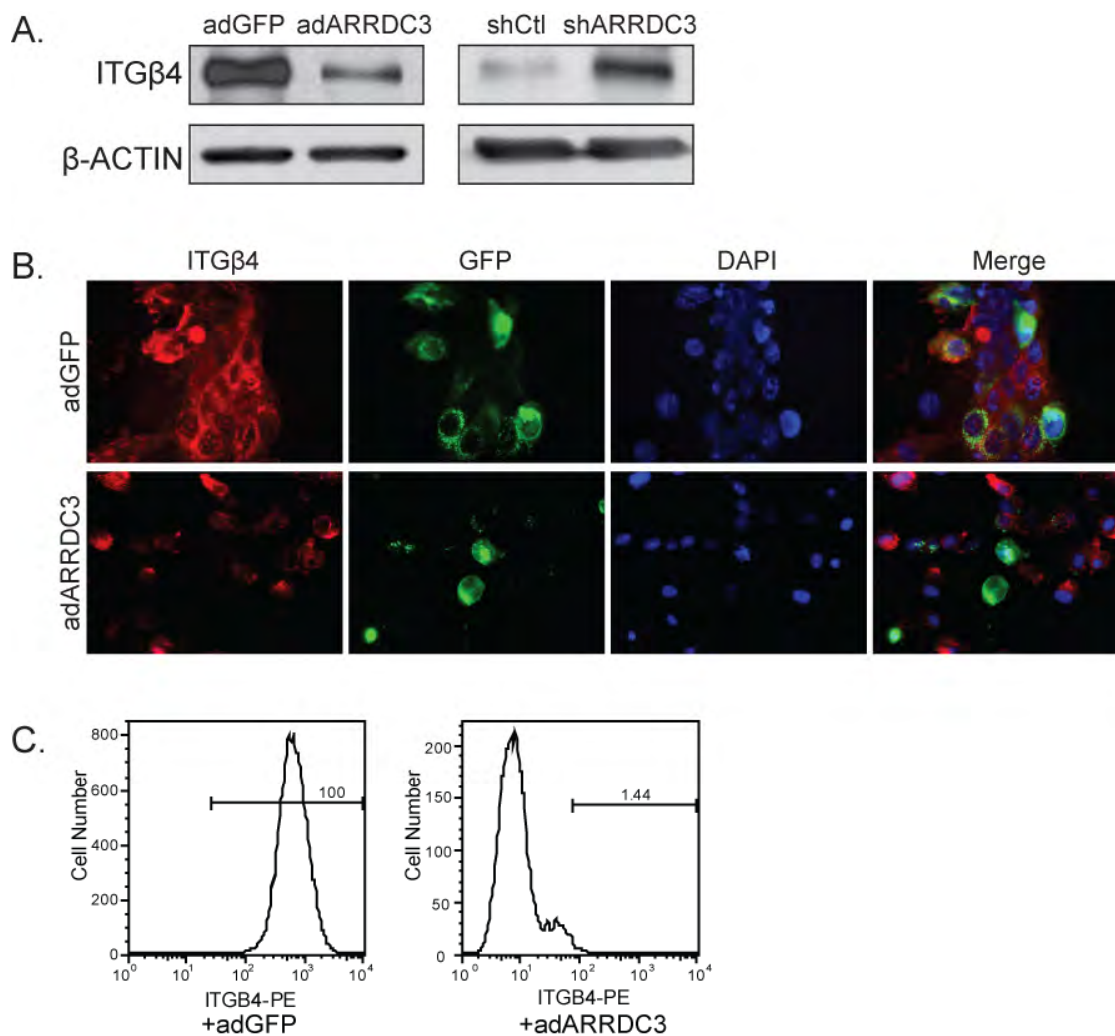


Figure 3-6: ARRDC3 negatively regulates ITGβ4 in MDA-MB-231 cells. (A) Western blot analysis shows ITGβ4 expression is inversely correlated to ARRDC3 expression. ARRDC3 repression using RNAi (shARRDC3) caused an increase in ITGβ4 protein when compared to control cells (shCtl). Conversely, when cells were made to over-express ARRDC3 (adARRDC3), ITGβ4 levels decreased when compared to control cells (adGFP). (B) Immuno-fluorescence demonstrate that when MDA-MB-231 cells are made to over-express ARRDC3 using a GFP-expressing adenovirus (adARRDC3), ITGβ4 levels decrease when compared to uninfected cells (GFP-) and cells infected with a control GFP-expressing adenovirus (adCtl). (C) ARRDC3 causes a complete removal of ITGβ4 from the cell surface. The cell surface of live cells were then stained with an ITGβ4 antibody and analyzed by flow cytometry.

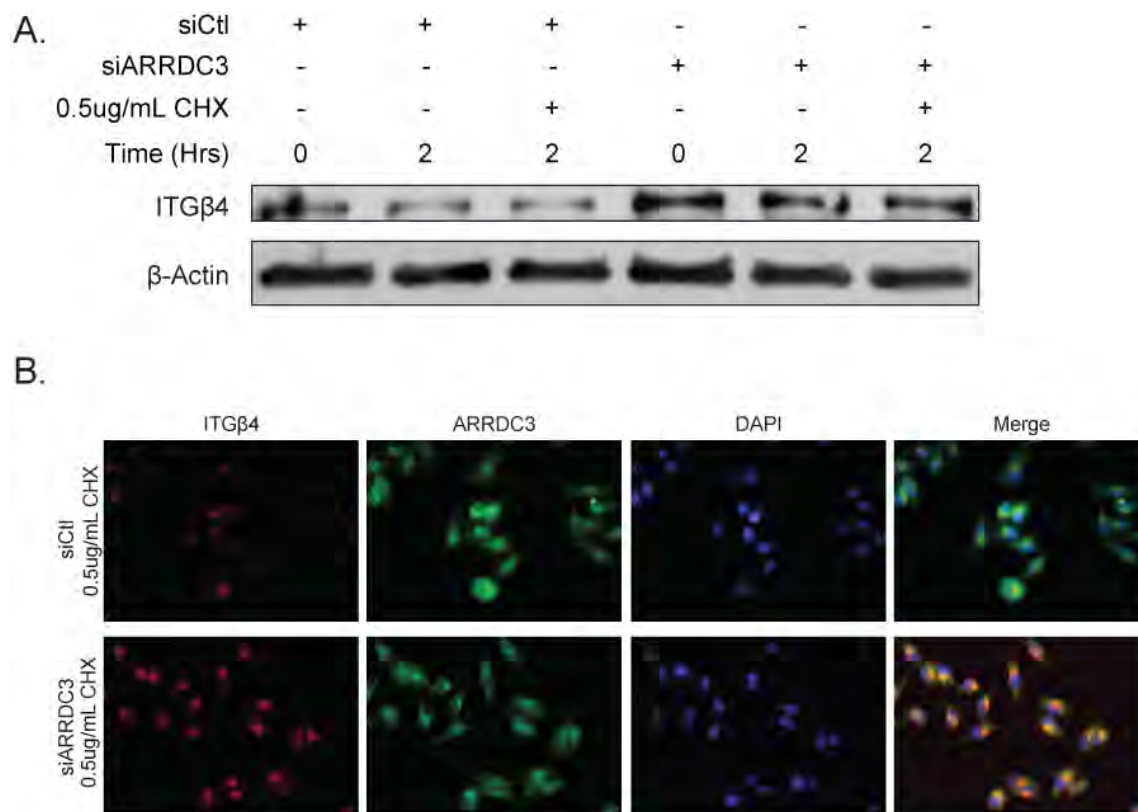


Figure 3-7: The regulation of ITGβ4 by ARRDC3 is post translational. (A) Western blot of MDA-MB-231 cell lysates after transfection with either a control siRNA or siRNA targeting ARRDC3 with and without treatment of 0.5μg/mL of cyclohexamide (CHX). Inhibition of translation does not prevent the up-regulation of ITGβ4 induced by ARRDC3 knock-down. (B) Immuno-fluorescence of MDA-MB-231 cells transfected with either control siRNA or siRNA targeting ARRDC3 and treated 0.5μg/mL of cyclohexamide (CHX). The increase ITGβ4 mediated by the repression of ARRDC3 is unaffected by the addition of the protein synthesis inhibitor.

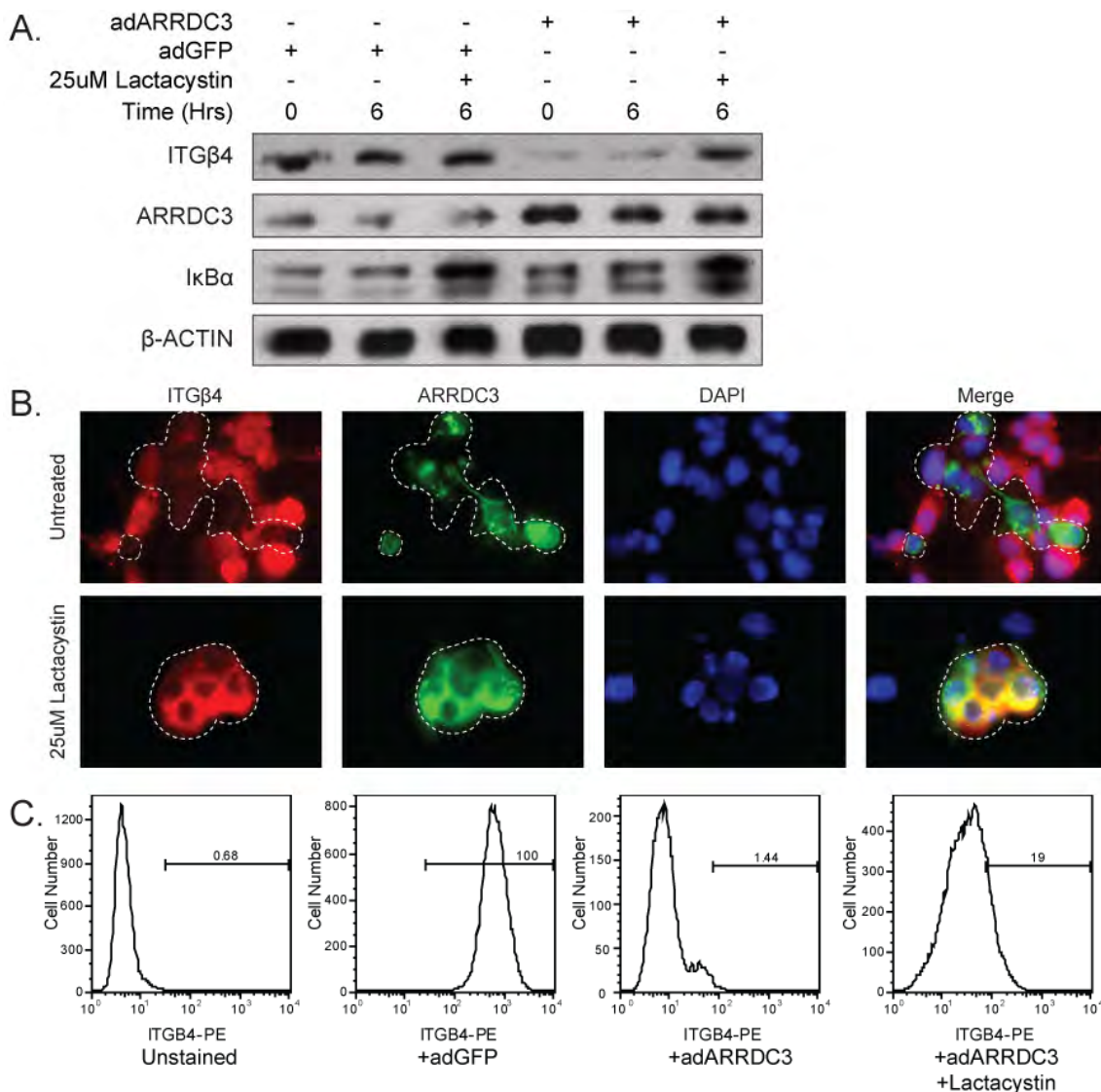


Figure 3-8: ARRDC3 negatively regulates ITGβ4 protein levels in a mechanism dependent on the proteasome. (A-B) Cells can maintain high levels of ITGβ4 after ARRDC3 over-expression if treated with proteasome inhibitor Lactacystin for 6 hours. (A) Western blot analysis shows ITGβ4 expression after ARRDC3 over-expression is restored when the proteasome is inhibited. Inhibition of the proteasome is demonstrated by the accumulation of IκBα. (B) Immunofluorescence demonstrates that ITGβ4 levels are retained after ARRDC3 over-expression if cells are treated with proteasome inhibitor. Cells positive for adARRDC3 (GFP+) are outlined with a dashed line. (C) ARRDC3 causes a complete removal of ITGβ4 from the cell surface which is partially rescued with proteasome inhibition. The cell surface of live cells were then stained with an ITGβ4 antibody and analyzed by flow cytometry. Uninfected GFP negative cells were gated out before ITGβ4 levels were examined.

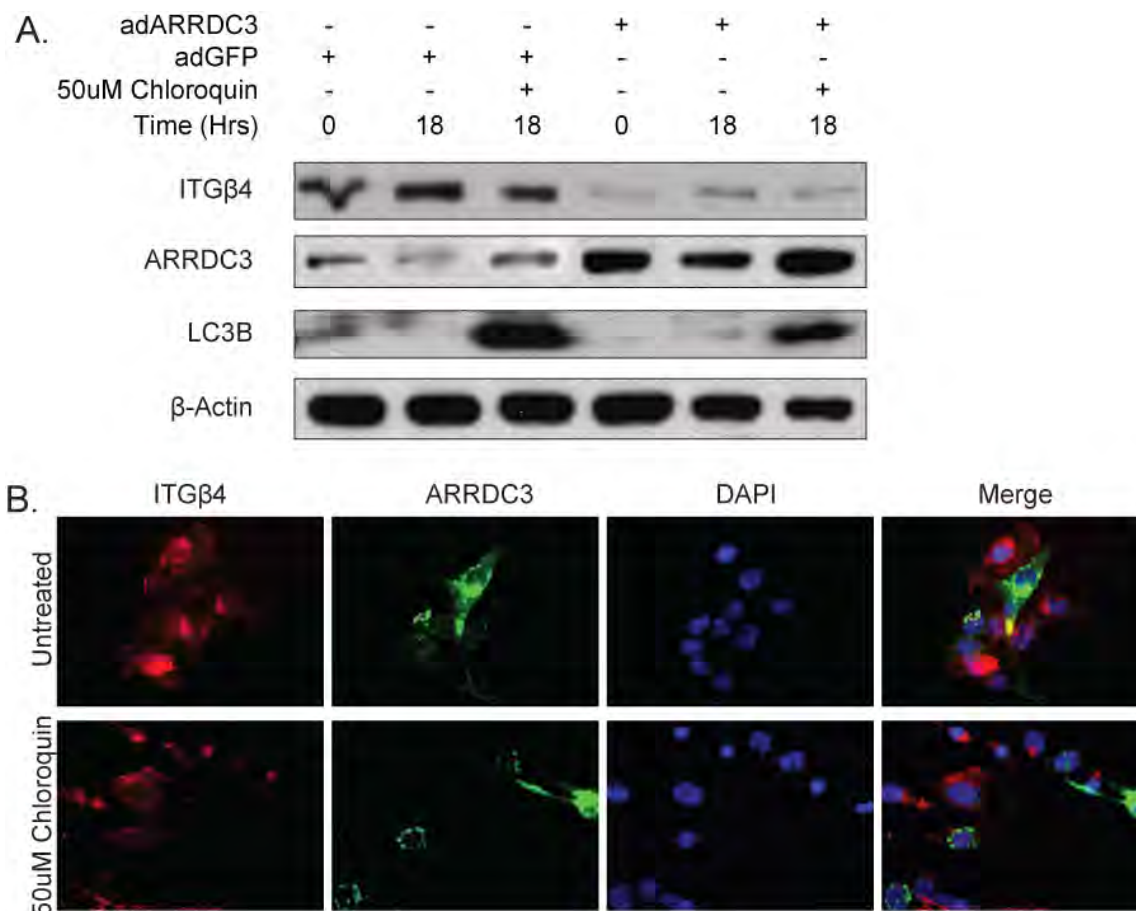


Figure 3-9: The regulation of ITGβ4 by ARRDC3 is not dependent on the lysosome. (A) Western blot comparing MDA-MB-231 infected with either adGFP or adARRDC3-GFP and treated with lysosome inhibitor chloroquin or left untreated. Inhibition of the lysosome is demonstrated by the accumulation of LC3B. The ARRDC3-mediated decrease of ITGβ4 is not affected by the inhibition of the lysosomes. (B) Immuno-fluorescence of MDA-MB-231 cells infected with an adenovirus expressing ARRDC3 with and without treatment of 50μM chloroquin demonstrate that lysosomes inhibition does not affect the loss of ITGβ4 in ARRDC3 overexpressing cells.

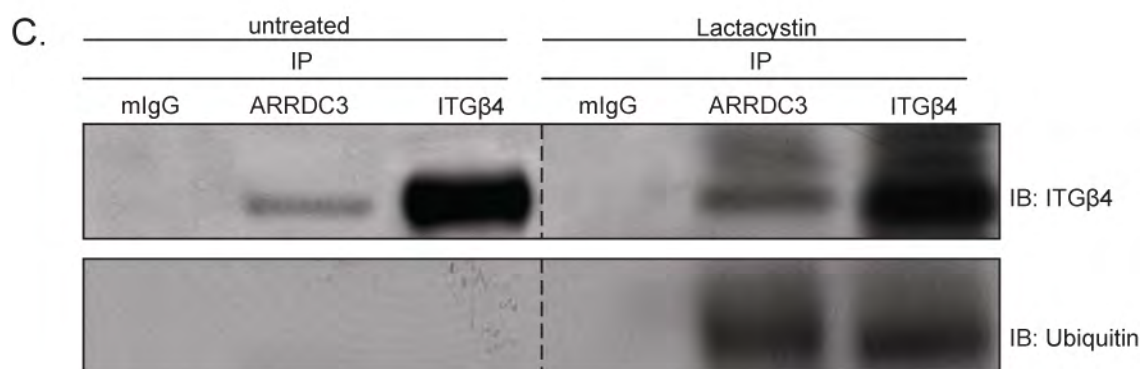
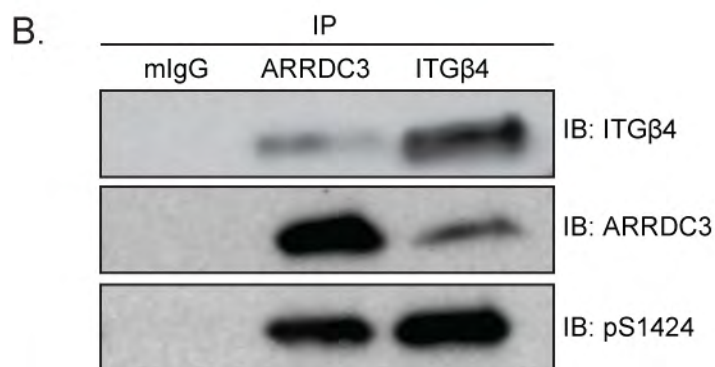
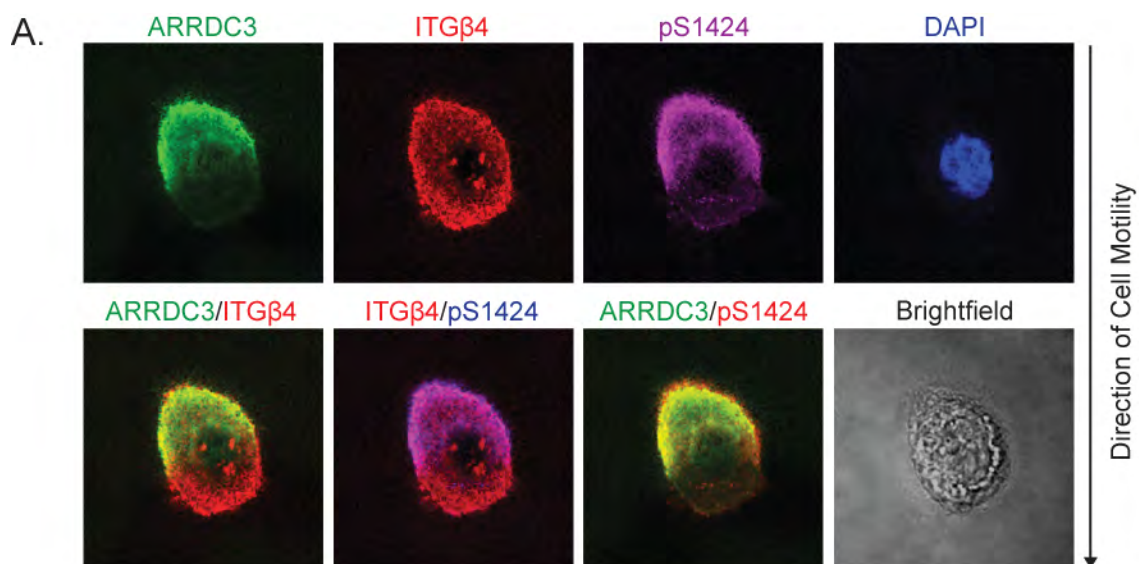


Figure 3-10: Arrdc3 appears to preferentially interact with ITG β 4 when phosphorylated on serine-1494 and/or when ubiquitinated (A) Confocal images of a migrating MDA-MB-231 cell after wounding. Endogenous expression of ARRDC3, ITG β 4 and ITG β 4-pS1424 was detected. ARRDC3 co-localizes with ITG β 4 on the lagging edge of the cell, where ITG β 4-pS1424 is located. (B) MDA-MB-231 cells were treated with phosphatase inhibitors 1 μ M Sodium Vanadate and 1 μ M Sodium Fluoride for 3 hours. Lysates were then prepared from the cells and used in endogenous immuno co-precipitation with antibodies targeting ARRDC3 and ITG β 4. ARRDC3 enriches ITG β 4-pS1424 when compared to total ITG β 4. Bands seen in the phospho-S1424 immuno-blots were identical in size to ITG β 4 bands. (C) Pre-treatment of MDA-MB-231 cells with proteasome inhibitor lactacystin prior to endogenous immuno co-precipitation with antibodies targeting ARRDC3 and ITG β 4 enriches ubiquitinated forms of ITG β 4. Bands seen in the ubiquitin immuno-blots were identical in size to ITG β 4 bands.

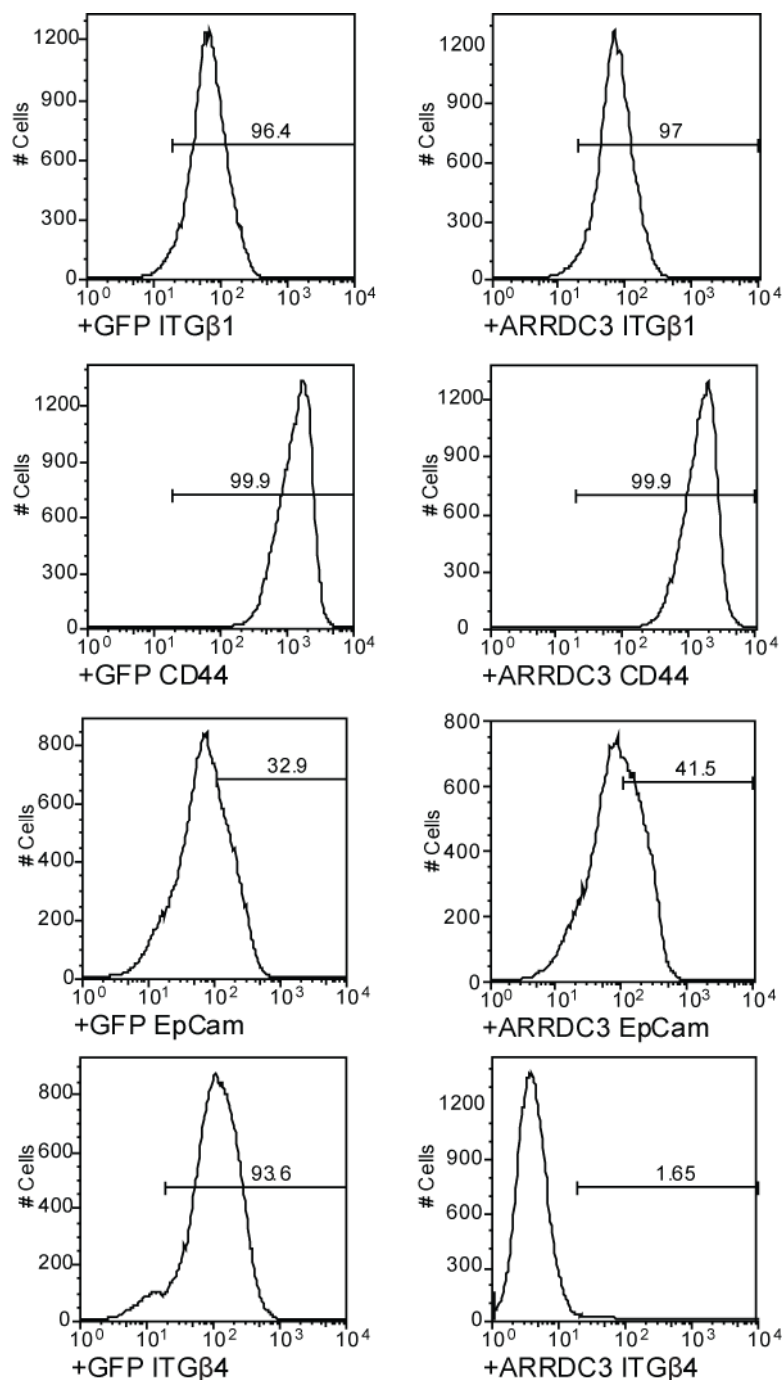


Figure 3-11: The regulation of ITGβ4 by ARRDC3 is specific and not a product of increased random endocytosis. MDA-MB-231 cells were infected with either a GFP-expressing adenovirus or an ARRDC3-expressing adenovirus. Non-permeabilized cells were then analyzed by flow cytometry for various cell surface proteins while cell lysates were used in western blot analysis. ARRDC3 over-expression affected neither surface nor total levels of ITGβ1, CD44 or EpCam.

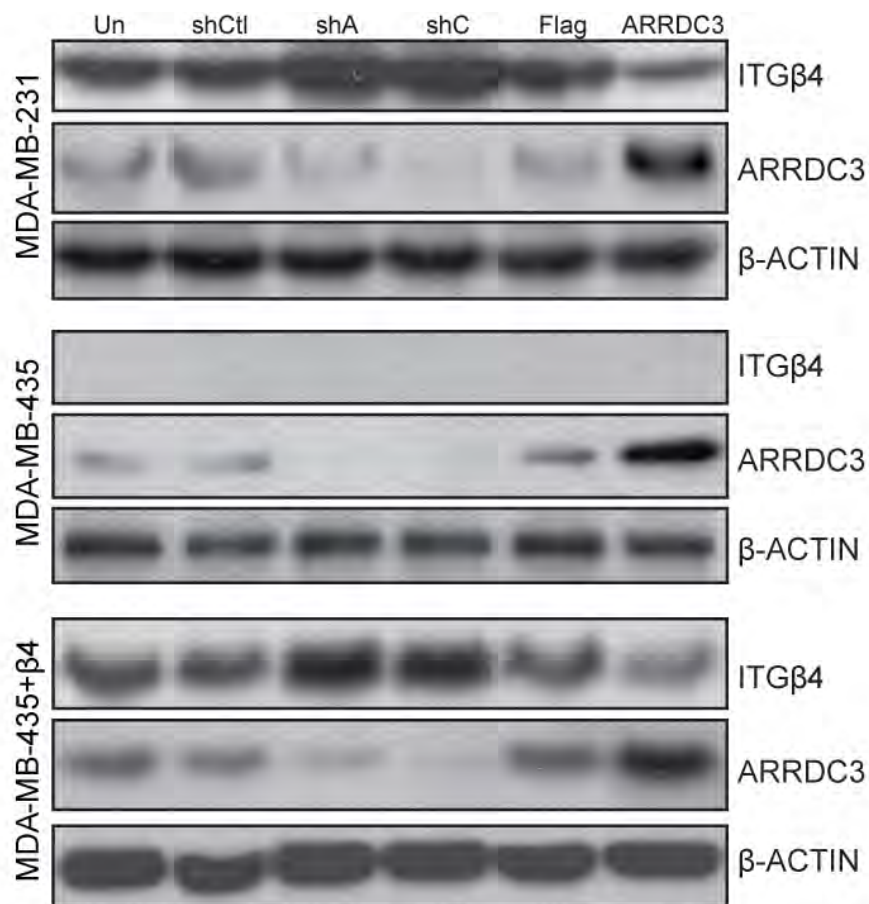


Figure 3-12: The creation of stable cell lines with varied expression of ARRDC3. Western blot demonstrating stable alterations of ARRDC3 expression in MDA-MB-231, MDA-MB-435 and MDA-MB-435+β4 cell lines. Two different shRNA sequences targeting ARRDC3 were used (shA and shC) and compared to a scrambled sequence (shCtl). The ARRDC3 overexpression cells were compared to empty vector control cells (Flag). All cell lines were compared to the uninfected parental cells (Un).

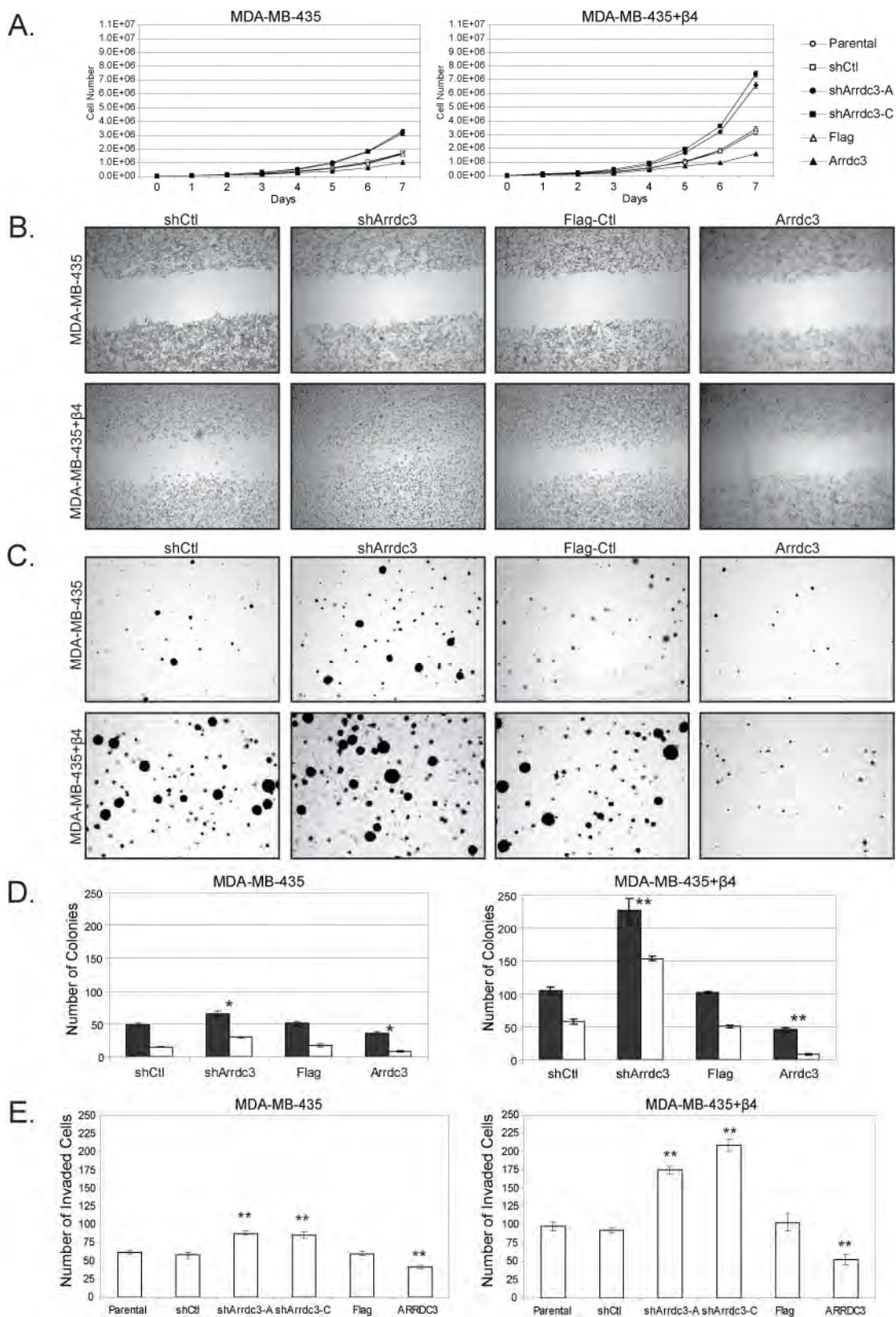


Figure 3-13: Effects of ARRDC3 on in-vitro tumorigenicity is dependent on ITG β 4. Each line was tested at least in triplicate and data bars represent mean \pm SEM. Single asterisk represents $p < 0.05$ whereas a double asterisk represents $p < 0.001$ as determined by student's t-test. (A) Growth curves from the stable lines demonstrate that expression of ARRDC3 is inversely correlated to cellular proliferation. (B) Matrigel chemo-invasion assay demonstrate that expression of ARRDC3 is inversely correlated to invasivity. (C) Wound assay of mitomycin-C treated cells shows that over-expression of ARRDC3 leads to a decrease in cell migration whereas RNAi-mediated repression of ARRDC3 increases cell migration. (D) Expression of ARRDC3 is inversely correlated to anchorage independent growth both in colony number and colony size (E) Dark gray bars represent total colonies (at least 50 μ m) whereas light gray bars represent colonies larger than 200 μ m.

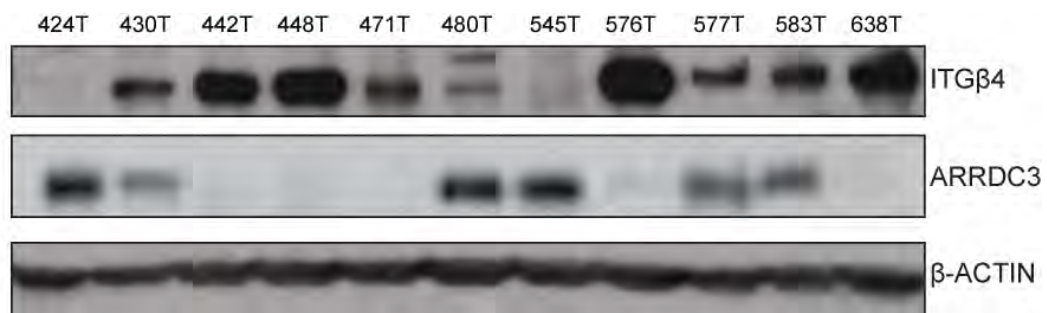


Figure 3-14: Expression of ARRDC3 in human breast tumors Western blot analysis of protein extracts from snap-frozen pieces of primary human breast tumors (invasive ductal carcinomas). ARRDC3 and ITGβ4 expression are inversely correlative.

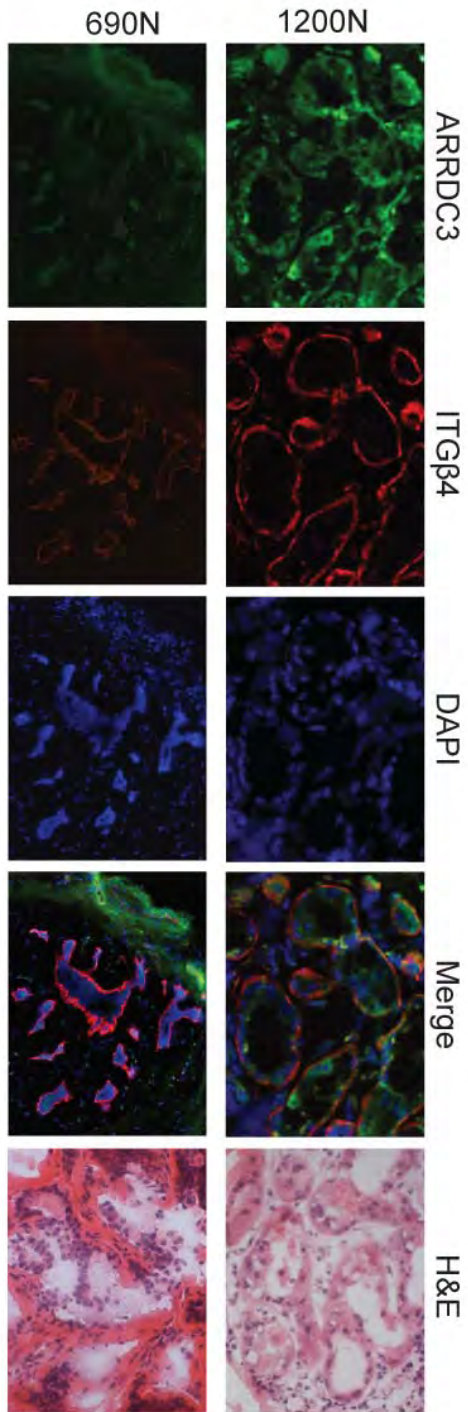


Figure 3-15: Expression levels of ARRDC3 in normal human breast tissue. ARRDC3 is primarily expressed in the luminal cells in normal breast tissue where ITGβ4 is expressed in the basal (myo-epithelial) cells.

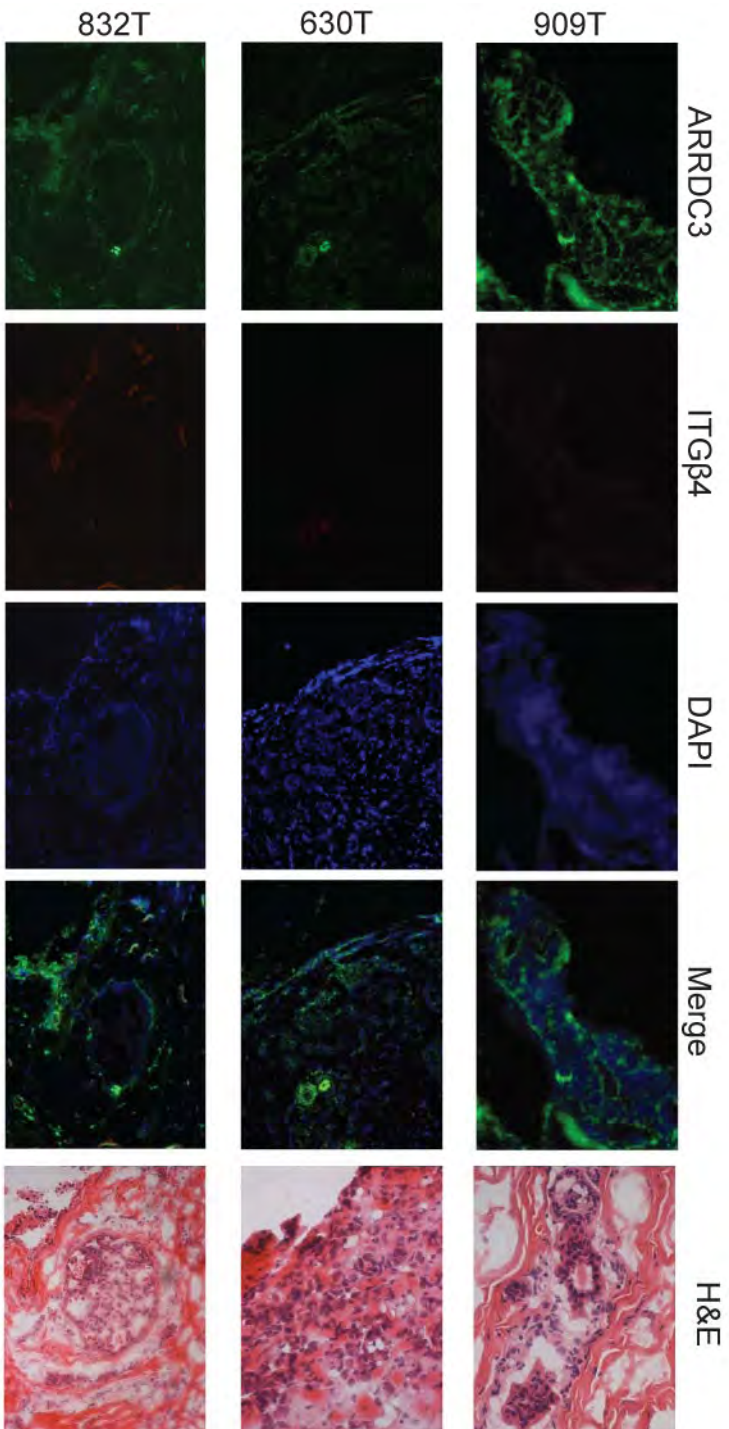


Figure 3-16: Expression levels of ARRD3 and ITGB4 in grade 1 tumors. Grade 1 breast tumors (as determined by a pathologist) tend to express high levels of ARRD3 and low levels of ITGB4. Residual breast architecture is an indication of a Grade 1 tumor.

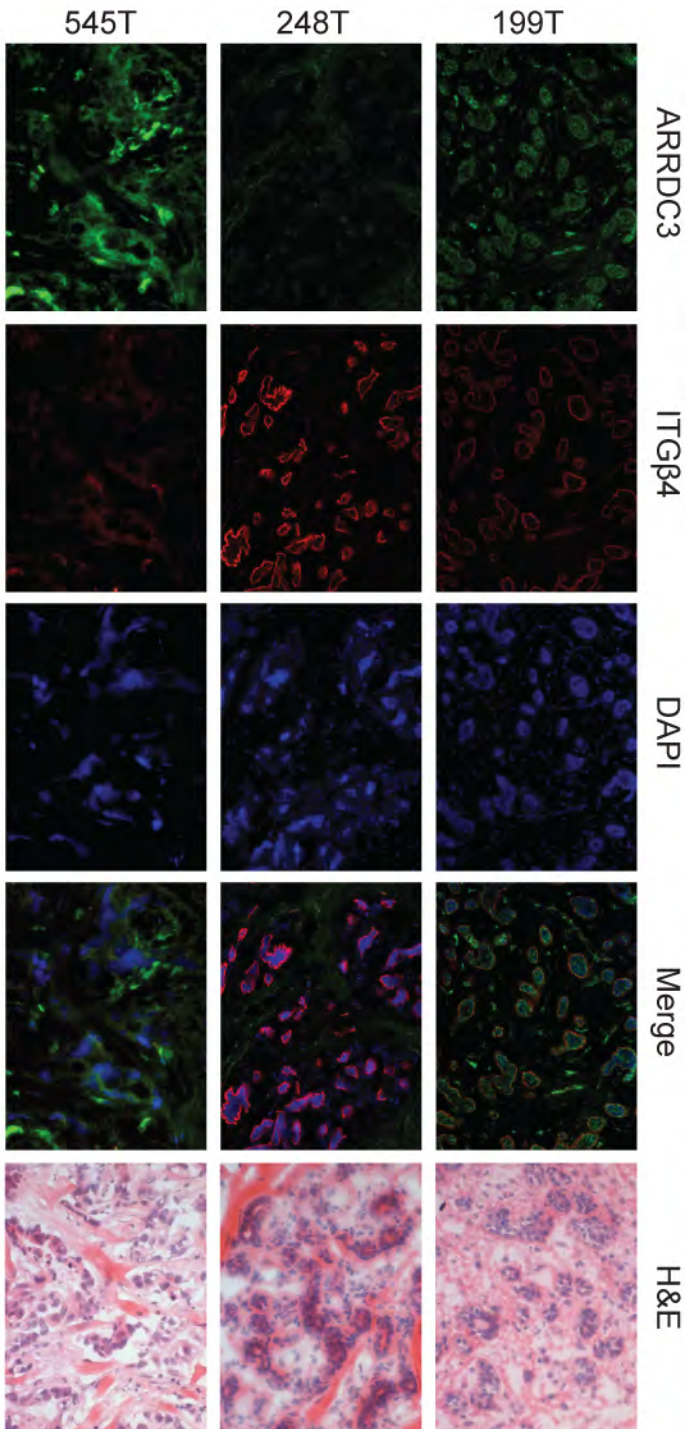


Figure 3-17: Expression levels of ARRD3 and ITGB4 in grade 2 tumors. Grade 2 human breast tumors (as determined by a pathologist) display varied expression levels of ARRD3. Expression is always the inverse of ITGB4 expression.

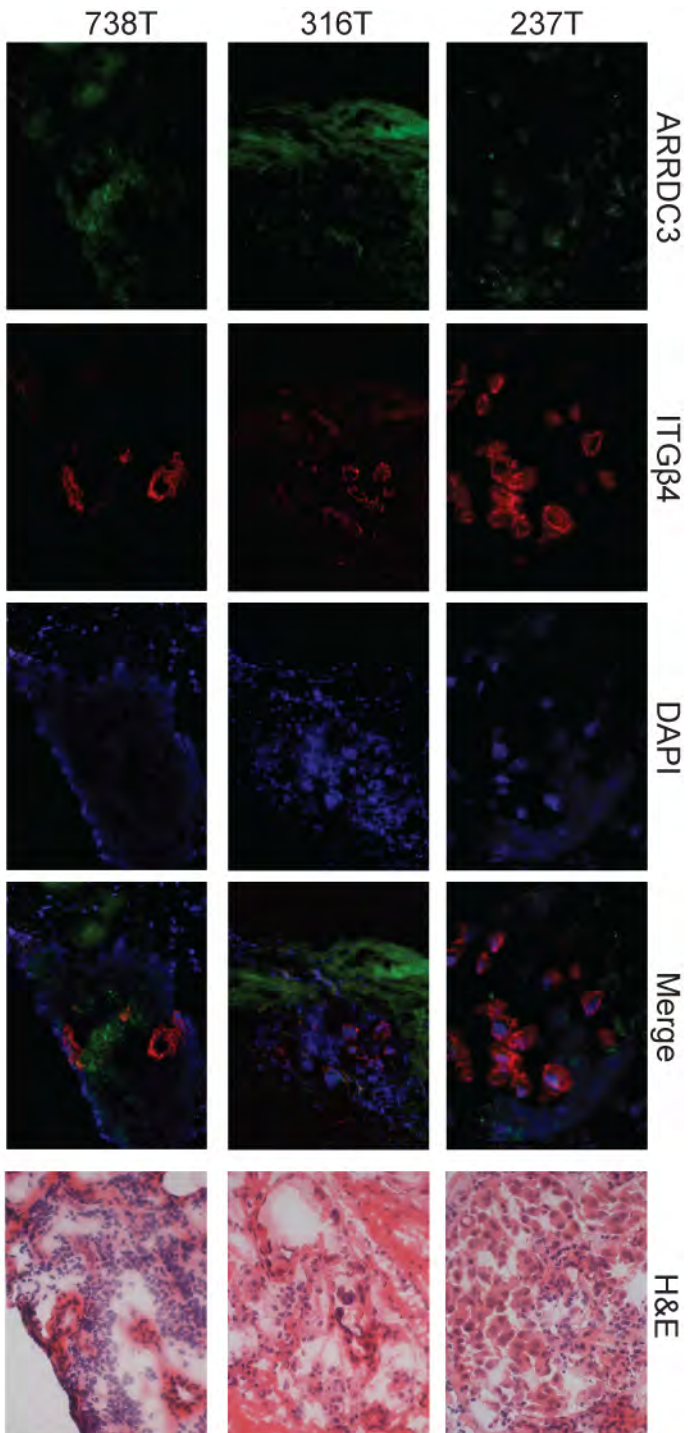


Figure 3-18: Expression levels of ARRDC3 and ITGB4 in grade 3 tumors. Grade 3 breast tumors (as determined by a pathologist) tend to express low or undetectable levels of ARRDC3 and high levels of ITGB4. Loss of any normal breast architecture in addition to the presence of infiltrated cancer cells in the connective tissue are indicators of Grade 3 carcinoma.

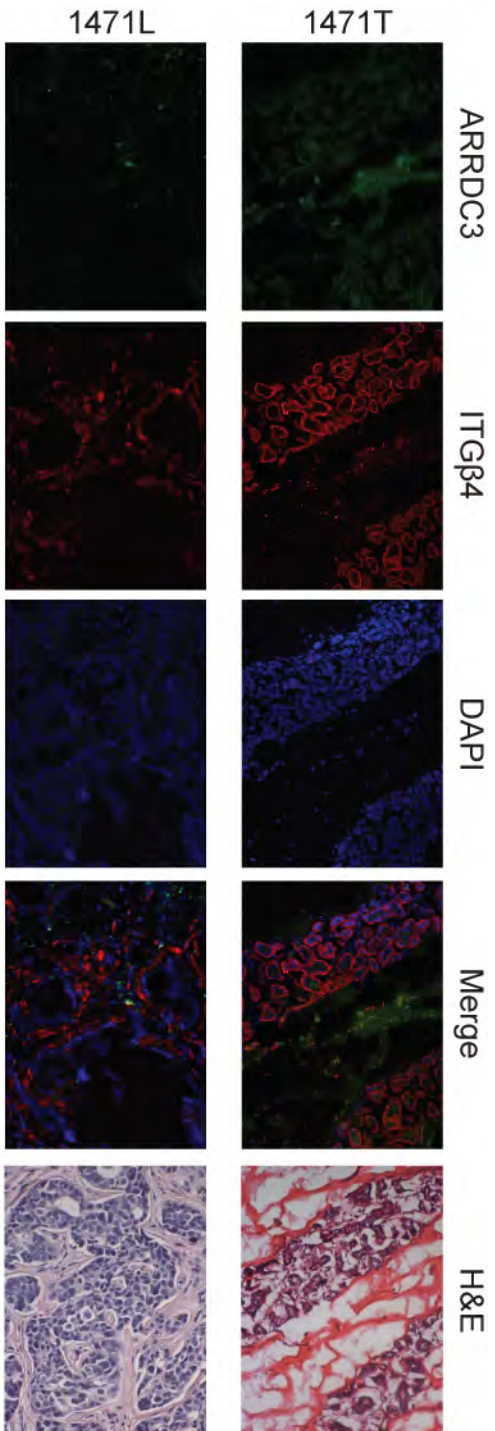


Figure 3-19: Expression levels of ARRDC3 and ITGB4 in breast cancer metastatic lesions. A metastatic lesion demonstrates decreased ARRDC3 expression when compared to the primary tumor (1471T). Both samples were from biopsies from the same patient, 1471T is from the primary tumor and 1471L is from an affected lymph node.

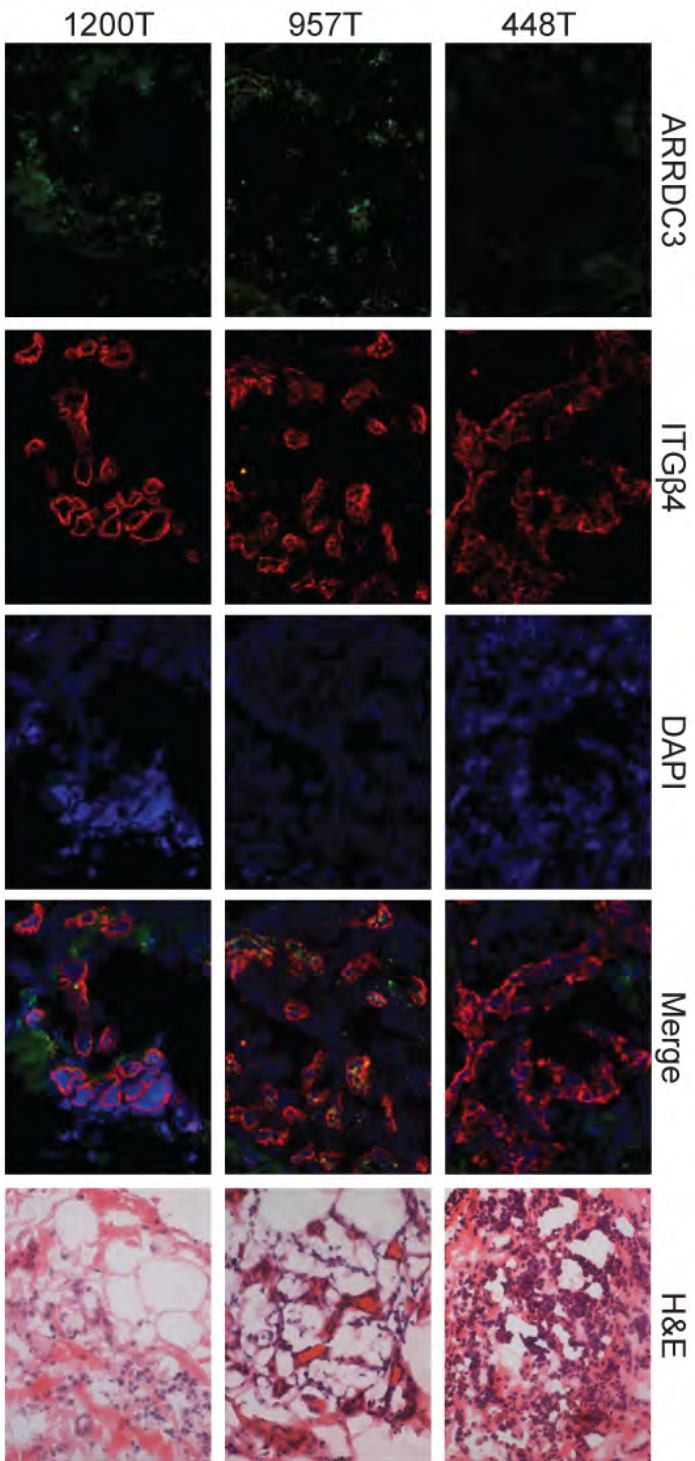


Figure 3-20: Expression levels of ARRDC3 and ITG β 4 in basal-like breast cancers. Basal breast cancers with high ITG β 4 expression express very low/no levels of ARRDC3. These tumors are samples previously identified as basal-like breast tumors (Lu et. al, 2008).

Table 3-1: Expression of ARRDC3 in Human Breast Tumors

ID	HER2	ER	PR	Cancer Type	Grade	ITGβ4	ARRDC3
0630T	-	+	weak	Lobular Carcinoma	1	+	+++
0832T	-	+	-	Ductal Carcinoma	1	+	+++
0885T	-	+	+	Ductal Carcinoma	1	+	++
0909T	-	+	weak	Ductal Carcinoma	1	-	+++
1334T	-	+	+	Lobular Carcinoma	1	+++	+
1640T	-	+	-	Ductal Carcinoma	1	++	++
0199T	+	+	+	Ductal Carcinoma	2	++	++
0248T	-	+	+	Ductal Carcinoma	2	+++	+
0327T	+	+	+	Ductal Carcinoma	2	++	++
0424T	-	+	+	Papillary Carcinoma	2	-	+
0448T	-	-	-	Ductal Carcinoma	2	+++	+
0462T	-	+	-	Ductal Carcinoma	2	++	++
0480T	-	+	+	Ductal Carcinoma	2	+	+
0545T	+	+	-	Ductal Carcinoma	2	++	++
0576T	-	+	-	Ductal Carcinoma	2	+++	+
0638T	-	+	-	Ductal Carcinoma	2	+	++
0837T	weak	-	-	Ductal Carcinoma	2	++	+
0884T	-	+	-	Lobular Carcinoma	2	++	+
0934T	+	-	-	Ductal Carcinoma IS	2	+++	+
0960T	+	+	+	Ductal Carcinoma	2	+	+
1003T	-	+	+	Ductal Carcinoma	2	-	++
1099T	-	+	-	Ductal Carcinoma	2	+	+
1281T	-	+	+	Lobular Carcinoma	2	++	-
1376T	-	+	+	Lobular Carcinoma	2	+++	+
1403T	+	+	+	Ductal Carcinoma	2	++	++
1404T	-	+	+	Lobular Carcinoma	2	+++	+
1471L	-	+	+	Carcinoma	2	+++	+
1471T	-	+	+	Ductal Carcinoma	2	+++	+
1502T	-	+	+	Ductal Carcinoma	2	++	++
1569T	-	+	+	Ductal Carcinoma	2	+	+++
0237T	+	-	-	Ductal Carcinoma	3	+++	-
0316T	+	-	+	Ductal Carcinoma	3	+++	+
0442T	+	-	-	Ductal Carcinoma	3	+++	+
0454T	-	+	+	Lobular Carcinoma	3	+++	++
0471T	+	+	-	Lobular Carcinoma	3	-	+
0489T	-	+	+	Lobular Carcinoma	3	+	-
0577T	-	+	-	Lobular Carcinoma	3	+++	+
0690T	-	-	-	Ductal Carcinoma	3	-	+
0738T	-	-	+	Ductal Carcinoma	3	+++	+
0839T	-	-	-	Ductal Carcinoma	3	-	-
0902T	-	-	-	Ductal Carcinoma	3	-	++
0957T	-	-	-	Ductal Adenocarcinoma	3	+++	+
0959M	-	+	-	Carcinoma	3	-	+

0959T	-	+	-	Ductal Carcinoma	3	+	+
1060T	-	+	-	Ductal Carcinoma	3	+	+
1097T	weak	+	weak	Ductal Carcinoma	3	++	+
1200T	-	-	-	Ductal Carcinoma	3	+++	-
1342M	-	-	-	Ductal Carcinoma	3	+++	+
1550T	-	+	+	Lobular Carcinoma	3	+++	+
1623T	-	+	+	Ductal Carcinoma	3	+++	+
1645T	NT	+	+	Lobular Carcinoma	3	++	-
1659T	-	+	-	Ductal Carcinoma	3	+++	+

Table 3-1: Expression of ARRDC3 in human breast tumors. 52 human breast tumors were analyzed for ARRDC3 and ITG β 4 expression using immuno-fluorescence. ID refers to the UMass Tissue Bank tumor number. HER2, ER and PR statuses, cancer type and grade are those determined at the time of diagnosis. A – indicates no detectable expression, + is low expression, ++ is moderate expression and +++ is high expression. All samples were processed at the same time using the same master-mix of primary and secondary antibodies. Images from each sample were taken all on the same day using the same exposure times for each channel. Expression levels were determined by a person blinded to the sample information.

CHAPTER IV:

Final Thoughts and Future Directions

The arrestin family of gene products was originally discovered as molecules that desensitize, or turn off, classical G protein-coupled receptor (GPCR) signaling (Hausdorff et al., 1990). Upon ligand binding, GPCRs are phosphorylated on many sites within an intracellular loop by GPCR kinases (GRKs). This modification leads to enhanced β -arrestin binding, physically blocking further signaling to G proteins and thus leading to receptor desensitization. Arrestins can also mediate internalization of receptors, leading to numerous physiological outcomes including receptor degradation, receptor recycling, and the generation of “signalosomes” where arrestins scaffold various proteins to potentiate distinct downstream signaling events (Figure 1-2). In recent years, these multifunctional adaptor proteins have also come to be appreciated as important mediators of core signaling pathways used in growth, differentiation, homeostasis and cancer. This includes the Hedgehog, Wnt, Notch, and TGF β signaling pathways. Arrestin proteins are also key regulators of endocytosis: a complex cellular program, through which cells can regulate signaling, modulate adhesion and become polarized.

The culmination of the data presented in my thesis underlines a mechanism by which ITG β 4 is regulated by ARRDC3. Through this mechanism, ARRDC3 can directly bind to ITG β 4, the phosphorylation of which seems to increase the affinity of the interaction. Preliminary data indicates that ARRDC3 preferentially binds to ITG β 4-pS1424, although it is still unclear whether S1424 is the key site mediating the interaction. Upon this interaction, ITG β 4 becomes internalized and sent to the proteasome for degradation (Figure 4-1A). In a migrating cell these events appear to be restricted to the lagging edge of the cell on the basal layer, where the adhesion structures

are likely about to become disrupted (Figure 4-1B). This specific localization is likely critical in cell motility as cells cannot migrate without the disruption of these adhesion molecules. This regulation plays a critical role in all cellular functions affected by ITG β 4 signaling; such as adhesion, migration, survival, invasion and proliferation.

ARRDC3 deficient mice

The importance of this regulation is best highlighted in our preliminary observations of ARRDC3 null mice. Although these mice develop normally, they display what we consider to be an “aging phenotype” compared to their wild-type littermates. This includes a loss of fur, decreased wound healing and a higher disposition towards lymphoma (Table 4-1). Mice heterozygous for ARRDC3 display a phenotype that is intermediate between the null and wild-type mice.

Null mice exhibited patchy fur loss by 4 months of age and progressed as the mice aged (Figure 2-17). Additionally, these mice appeared more prone towards dermatitis and wounding (as reported by the animal facility veterinarians) than their wild-type or even heterozygous littermates. Although fighting wounds are not uncommon in mice, a disproportionate number of ARRDC3 null and heterozygous mice needed to be euthanized due to their injuries compared to wild-type littermates (Table 4-1). In addition, in each of four separate mating cages (all containing one ARRDC3 null female, one ARRDC3 heterozygous female and one wild-type male) only the ARRDC3 null females presented with injuries on their hindquarter regions. This suggests that the loss of ARRDC3 renders the mouse either more likely to become wounded and/or decreases the rate at which the wounds are repaired. As the dermis is much thinner in the null mice

compared to wild-type littermates (Figure 2-18B), it is probable that ARRDC3 null mice wound more easily. However, this does not exclude the possibility that there is an impairment of the wound healing process. A decrease in the number of stem cells would affect homeostasis as well as prolong the wound healing process. Altogether, our observations support the notion that although ARRDC3 does not affect differentiation, its loss does allow for an increase in the migration of skin stem cells outside of the niche likely leading to an exhaustion of the stem cell pool and defective skin homeostasis. As these thoughts are generated from general observations outside of a well controlled experiment, definitive conclusions cannot be made. A formal study where a full-thickness skin biopsy is performed on each mouse and the wound-healing rates and histology are monitored would help to answer this question.

Additionally, ARRDC3 null and heterozygous mice seemed to have an increased disposition towards developing cancer. All ARRDC3 null mice (not euthanized for lethal wounds) died before 11 months of age. Post-mortem necropsy of all mice revealed both splenomegaly as well as large masses within (but not part of) the intestines. Histological analyses of the spleen and the masses found an abundance of plasma cells indicating these mice suffered from a lympho-proliferative disease, most likely multiple myeloma based on the presence of “clock-face” cells (Figure 4-2A&B). This predisposition towards lymphoma was also evident in the heterozygous mice, although survival for this genotype exceeded that of the null mice. However, it is impossible to distinguish the affects of ARRDC3 from the normal pathology of 129; B6 mice. Studies indicate that only 44.5% of 129;B6 mice are alive at 2 years and 54% of these mice develop

lymphoma (Haines et al., 2001). However, the 129; B6 mice had an average disease latency of 15 months whereas the ARRDC3 null mice all developed lymphoma before 1 year. This decreased latency suggests a tumor suppressive role for ARRDC3, but as wild-type littermates were not monitored for lymphoma, we cannot be sure. Interestingly, a single 19-month old ARRDC3 heterozygote generated a mass in the mammary tissue. Histological analysis revealed that this mass was a well-differentiated mammary adenocarcinoma (Figure 4-2C). However, as mammary carcinomas can spontaneously form in <1% of wild-type mice (albeit at a latency of 24+ months), it is possible that the formation of this tumor is unrelated to ARRDC3 deficiency. The analysis of a larger cohort of ARRDC3 $-/-$ and ARRDC3 $+/-$ on a pure background is needed to determine whether these mice are more cancer-prone.

Impact in hemidesmosome regulation & cell migration

While the ARRDC3 induced changes in ITG β 4 protein levels are modest, the affects on cell motility are pronounced (Figures 2-9A & 2-13). This suggests the balance between stationary and motile states of both skin stem cells and cancer cells is delicate. Motility has significant implications for both cancer and stem cells. Stem cells need to stay within the growth and differentiation restricted environment of the niche whereas increased migration of cancer cells is a hallmark characteristic of metastasis. Hemidesmosomes (HDs) are dynamic structures which are constantly turning over, even in resting cells (Daisuke Tsuruta, 2003; Geuijen and Sonnenberg, 2002). This phenomenon is thought to keep the cell in a “primed” state allowing it to respond more quickly to migratory stimuli. It is likely that the internalization of ITG β 4 by ARRDC3 is

part of the normal machinery involved in HD turnover. Our data showing the co-localization of ARRDC3 with ITG β 4 and plectin on the basal layer of resting cells supports this hypothesis (Figure 2-8C&D). It would be interesting to investigate whether the ARRDC3 and plectin co-localization also becomes polarized within a migrating cell.

It is important to remember that our studies examining the localization of ITG β 4 and ARRDC3 (for both resting and migrating cells) were performed using cells cultured in their cell-line specific media. Although this media contains EGF, the concentration (10ng/mL) is 10-fold lower than that used when stimulating cells to undergo HD disassembly (Germain et al., 2009; Rabinovitz et al., 2004; Wilhelmson et al., 2007). To study the role of ARRDC3 in hemidesmosome disassembly further, the cells would need to be stimulated by concentrations of EGF closer to 100ng/mL and re-analyzed using either proximity ligation analysis (PLA) or immuno-fluorescence (IF).

It would be interesting to evaluate the importance of the S1424 site in ITG β 4 regulation by ARRDC3. Our data suggest that phosphorylation on this site enhances the interaction between ARRDC3 and ITG β 4 (Figures 2-8B, 2-14B, and 3-10A&B). However, the fragment of ITG β 4 used in the original yeast-2 hybrid screen only included amino acids 987-1120 (Figure 4-3A). It is possible that S1424 provides a binding site for another protein that either hinders ARRDC3 interaction when un-phosphorylated or stabilizes the interaction when phosphorylated. It would also be interesting to test whether the phosphorylation status of S1356, S1360, and S1364 impact ARRDC3 mediated internalization of ITG β 4. These sites are phosphorylated in response to EGF (a key growth factor in HD disassembly as well as cell migration) and are crucial in HD

disassembly (Rabinovitz et al., 2004; Wilhelmsen et al., 2007). If ARRDC3 plays a role in normal HD disassembly, it would be logical that the phosphorylation of these sites also affect the affinity between ARRDC3 and ITG β 4. However, it is possible that ARRDC3 can only interact with ITG β 4 after it is liberated from the HD therefore the increased ARRDC3/ITG β 4-pS1424 interaction is only correlative.

It is also feasible that these phosphorylation events are effects of ARRDC3 binding rather than vice versa. For other family members, the phosphorylation of the receptor allows for the binding of the arrestin at the site of phosphorylation (Gurevich and Gurevich, 2006b; Luttrell and Lefkowitz, 2002). Arrestins can then act as scaffolding proteins or bridging factors which recruit other signaling molecules including kinases (Buchanan and DuBois, 2006; Gurevich and Gurevich, 2006a). ARRDC3 may be the molecule responsible for recruiting PKC and PKA (the kinases responsible for phosphorylating S1356, S1360, S1364 and S1424) to the cytoplasmic tail of ITG β 4 (Germain et al., 2009; Rabinovitz et al., 1999; Rabinovitz et al., 2004; Wilhelmsen et al., 2007). It is possible that the ARRDC3-interaction region on ITG β 4 is upstream of all of these key phosphorylation sites to allow access for any kinases it recruits (Figure 4-3B). We know that phosphatase inhibition allowed us to see the interaction between ARRDC3 and ITG β 4, but more specific mutational analysis would need to be performed to know which sites are critical for binding.

Potential Regulation of ARRDC3

Further insight into the transcriptional regulation of ARRDC3 is another area of interest. One study suggests that ARRDC3 is under the control of the Forkhead

transcription factor family member STOX1 (Rigourd et al., 2008). It is therefore possible that other Forkhead family members can also regulate *ARRDC3*. Some of these have also been found to repress cancer progression (Ahmad et al., 2009; Rayoo et al., 2009; Zhang and Sun, 2009), whereas others seem to promote cancer progression (Han et al., 2008; Storz et al., 2009).

As the surface expression of ITG β 4 increases in a hypoxic environment (Yoon et al., 2005), we sought out to investigate whether *ARRDC3* was repressed in hypoxia versus normoxia. Contrary to our expectations, *ARRDC3* mRNA actually increased in cells cultured in a hypoxic environment (Figure 4-4). This data corresponded to information found on Oncomine (Figure 4-4). When we examined the *ARRDC3* promoter, we found 2 conserved HRE (hypoxia response elements). However, chromatin immuno-precipitation would need to be performed to determine whether *ARRDC3* is actually a direct target of HIF1. Regardless, it is difficult to reconcile how increased ITG β 4 surface expression occurs after an increase in *ARRDC3* mRNA. This suggests that *ARRDC3* has a role in ITG β 4 regulation beyond degradation.

Integrin Recycling

We show that *ARRDC3* promotes the internalization of ITG β 4 and subsequent degradation via the proteasome (Figures 2-11C & 3-8C). It is worth speculating how some of the tumors analyzed maintained expression of both *ARRDC3* and ITG β 4 (Figure 3-14). It is possible that the effects of *ARRDC3* on ITG β 4 are simply dose dependent and moderate expression of *ARRDC3* maintains intermediate levels of ITG β 4. Low or moderate levels of *ARRDC3* may also allow for the recycling of ITG β 4 similar to the

effects of β -arrestin on GPCRs (Luttrell and Lefkowitz, 2002). It is conceivable that lower levels of ITG β 4 would indicate less ligand-independent signaling therefore less stimulated ITG β 4 needed to endocytose. However, this would not explain the one tumor we evaluated which maintained high expression of both ARRDC3 and ITG β 4 (Table 3-1). If the pathway in which ARRDC3 targets ITG β 4 for degradation is somehow altered, ARRDC3 expression may allow for an increase in the mobilization rate (and hence recycling rate) of ITG β 4. This would facilitate the speed in which filamentous actin protrusions form, thus promoting tumor progression. However, further trafficking studies are necessary to determine whether ITG β 4 is actively being recycled to the cell surface in the absence of degradation.

Integrin Trafficking

Although our data clearly demonstrates that ARRDC3 actively promotes the internalization of ITG β 4, it is unclear whether it is directly involved in the trafficking to the proteasome. Although over-expression of ARRDC3 leads to degradation of ITG β 4, it is possible that there is a point after internalization where other signals from the cell could contribute to its fate; either degradation or recycling. The ubiquitination state of ITG β 4 likely plays a role in this fate decision. We show that ITG β 4 in complex with ARRDC3 is ubiquitinated (Figure 3-10C). As arrestins can behave as scaffolding molecules, ARRDC3 may potentially play a role in the recruitment of the ubiquitination machinery necessary for modifying ITG β 4. The C-terminus of ARRDC3 contains a PPXY motif that can interact with HECT ubiquitin ligases such as Smurf1, WWP1/2, Itch and the NEDD family proteins (Ingham et al., 2004; Martin-Serrano et al., 2005;

Sangadala et al., 2007). As with the integrin recycling, more definitive trafficking studies would need to be performed to determine whether ARRDC3 plays a role beyond the internalization of ITG β 4.

Although our data demonstrates that ARRDC3 over-expression leads to the degradation of ITG β 4, this is not necessarily its role in the cell when expressed at endogenous levels. It is possible that ARRDC3 also plays a role in re-localizing ITG β 4 to either the cell surface or the leading edge of the cell where it helps form and stabilize motility structures (filopodia and lamellae) (Lipscomb and Mercurio, 2005; Mercurio et al., 2001b; Rabinovitz and Mercurio, 1997; Santoro et al., 2003). When treated with a proteasome inhibitor, cells over-expressing ARRDC3 have slightly higher ITG β 4 surface protein levels compared to untreated cells; suggesting at least a small fraction of internalized protein is returned to the cell surface when it cannot be degraded (Figure 2-11C & 3-8C). Additionally, the basal ITG β 4 and apical ARRDC3 staining patterns seen on the leading edge of the cells suggests a role beyond degradation (Figure 2-14A).

Although we show that ARRDC3 and ITG β 4 directly interact, it is important to realize that we do not show whether any of the ARRDC3-induced affects on ITG β 4 are a result of this interaction. Although it is probable that ARRDC3 and ITG β 4 need to physically interact to cause internalization and degradation, we would need to generate an ITG β 4-binding deficient form of ARRDC3 and test whether it affects ITG β 4 protein levels and cellular process like migration. This mutant could also be used to determine the relevance of other ARRDC3 targets in cell migration, invasion, etc.

It is also interesting to speculate what proteins (besides ARRDC3) make up the machinery involved in ITG β 4 regulation. Until recently, there have been relatively few investigations of the proteins involved in integrin endocytosis. Internalization of integrins α 5 β 1, α v β 6, α v β 3, α 6 β 1 have been associated with clathrin-mediated endocytosis (Caswell and Norman, 2008; Ezratty et al., 2009), whereas integrins α 5 β 1, α v β 3, α 2 β 1 (Caswell and Norman, 2008; Shi and Sottile, 2008) have been connected to caveolae dependent endocytosis. ITG β 4 has been shown to compartmentalize in lipid rafts to permit association with a palmitoylated Src family kinase (allowing for mitogenic signaling) (Gagnoux-Palacios et al., 2003) suggesting that caveolae may play a role in internalization. However, our data suggested the endocytosis of ITG β 4 is through a clathrin-dependent pathway (Figure 2-7C). Further evidence supporting our data is the clathrin binding motifs within ARRDC3. However, this does not exclude the possibility that ARRDC3 can promote non-clathrin mediated endocytosis. Also, as not all integrin α 6 β 4 is found associated with lipid rafts, it is possible that another protein may be responsible for either internalizing ITG β 4 while in lipid rafts or removing ITG β 4 from the lipid raft. Since the mechanism of internalization is thought to contribute to the fate of the endosome, ARRDC3 might differentially co-localize with clathrin or caveolin in response to different stimuli. As our data only looked at normal resting cells, it is possible that we would see a different set of machinery in migrating or cancer cells. Further experiments would need to be done to investigate this possibility.

Implications in Cancer

It has been hypothesized that the function of ITG β 4 switches from a mechanical adhesive device into a signaling-competent receptor during the progression from normal epithelium to invasive carcinoma (Lipscomb and Mercurio, 2005; Mercurio et al., 2001b; Santoro et al., 2003). It would be interesting to see if expression of ARRDC3 inhibits or promotes this process. Relatively high expression of ARRDC3 in grade one tumors suggests that its loss is not necessary for tumorigenesis, although it seems to turn off as the tumor progresses. It is clear from our data that ARRDC3 plays a role in the regulation of ITG β 4 protein levels and likely contributes to the control of ITG β 4 function during breast cancer progression. However, as we are unclear whether ARRDC3 plays a role in the trafficking and/or recycling of ITG β 4, it is hard to determine whether its expression in the early stages of cancer would be beneficial or detrimental. This could be determined by crossing our ARRDC3 null and heterozygous mice to a mammary tumor model (such as *Brcal^{fl/fl}; MMTV-Cre; Trp53^{+/-}*) and determine if there is any affect on either the penetrance or the latency of disease. Approximately 73% of these mice form mammary carcinomas by 6-8 months of age allowing room to show both acceleration and inhibition of carcinogenesis (Xu et al., 1999).

Another question raised by our experiments concerns the effect of ARRDC3 expression on metastasis. Although we examined the lungs of mice from the xenograph study and only found micro-metastases in the shARRDC3 tumors, we cannot draw any conclusions as the tumors from each sub-line were of different sizes. It is possible that if permitted to grow to as large a volume, the tumors with over-expressed ARRDC3 would metastasize to the same extent. Additionally, the *MMTV-PyMT* transgenic mice form

highly aggressive tumors which metastasize to the lungs (Guy et al., 1992); crossing them with the ARRDC3 *-/-* null mice would help to answer whether ARRDC3 expression affects metastasis. However, the combined knowledge that over-expression of ARRDC3 decreased *in-vitro* invasion, repressed ARRDC3 promoted *in-vitro* invasion and ITG β 4 is known to promote metastasis; it is probable that ARRDC3 is a negative regulator of metastasis as well.

The interaction between ARRDC3 and ITG β 4 may represent a new therapeutic target for basal-like breast cancers that can be manipulated by peptides or small molecules. These data also highlight the importance of ITG β 4-blocking antibodies as a potential therapy in breast cancer. However, it should not be taken for granted that this will readily translate to other tissues. ITG β 4 can play a dichotomous role in various tissue types, either positively or negatively regulating tumor progression (Giancotti, 2007; Rabinovitz and Mercurio, 1996; Raymond et al., 2007). The overall role of ARRDC3 in a specific cancer would depend on the role of its target protein(s) in that particular cancer type.

Another question arising from our human breast cancer analysis concerns the significance of genes differentially expressed in cancer stroma versus the malignant cells. When assigning scores for ARRDC3 and ITG β 4 expression, only the malignant glands were evaluated. A serial section for each sample was used for H&E analysis and this was referenced throughout the scoring to ensure only tumor tissue was being examined. Interestingly, the tumor stroma, especially in grade 3 tumors, tended to express higher levels of ARRDC3, even when the breast cancer cells did not (an example can be seen in

Figure 3-18). Although it is possible the staining is non-specific, I feel that is unlikely as not all tumor stroma stained positive for ARRDC3. Without further knowledge of the roles for ARRDC3 beyond that of ITG β 4 regulation, it is impossible to hypothesize the significance of its expression in stroma. It would be interesting to test whether ARRDC3 null mice show changes in *in vivo* tumor formation in a breast cancer xenograph study when compared to wild type mice.

Interestingly, although ARRDC3 is preferentially lost in basal like breast cancers (Adelaide et al., 2007), data obtained from Oncomine revealed that basal like breast cancers demonstrate no change in *ARRDC3* mRNA levels when compared to non-basal like cancers (Figure 4-5). This could indicate that the loss of ARRDC3 does not influence the tumor classification. However, it is also possible the samples used in the microarray analysis were not purely cancer cells, but had contaminating stroma. In order to definitively answer this question, tumors with known classification would need to be analyzed for ARRDC3 expression using either IHC/IF on tumor sections or real-time PCR from laser-captured tumor cell RNA.

Other potential targets of ARRDC3

Our data does not exclude the possibility that ARRDC3 has targets other than ITG β 4 in either cancer cells or in normal cells. ARRDC3 could be similar to β -arrestins (which have a vast array of targets) or visual arrestins (which are only responsible for regulating rhodopsin in photoreceptors of the eye). As the expression of ARRDC3 is not nearly as restricted as the expressions of visual arrestins (Figure 2-3A); I believe it is probable that targets other than ITG β 4 exist. Supporting this notion, we see statistically

significant effects on tumorigenicity when modulating ARRDC3 in cancer cells lacking ITG β 4 (Figure 3-13). Unfortunately, as overall effects were small, it is unclear whether these targets are biologically significant in cancer. Considering its similarity to β -arrestins, ARRDC3 may also regulate G-protein coupled receptors (GPCRs). Possible targets could include Smoothed and Frizzled receptors (Class-6 GPCRs) or RhoA/C (Class-1 GPCRs); which all have extensive implications in cancer and skin stem cells. Although our data does not exclude the possibility that other integrins are regulated by ARRDC3, this is unlikely. Our data shows that ARRDC3 expression does not appear to affect β 1 integrin (Fig. 3-11). Additionally, the region in which ARRDC3 interacts with ITG β 4 is within the unique long cytoplasmic tail which does not share homology with other integrins.

Final Thoughts

The research presented in this thesis provides insight into the regulation of ITG β 4 and how it affects systems within stem cell and cancer biology. This research lays the groundwork for a lot of future research as well. ARRDC3 may be critical for maintaining adult stem cells (other than skin) within their niche. Also, any protein with a potential role in aging is of profound interest because it may provide information into the ultimate deterioration of organ systems. Additionally, ARRDC3 may be the key in therapeutic targeting of ITG β 4 in cancer; however a deeper understanding of the mechanism in which it regulates ITG β 4 is needed before this potential can be realized.

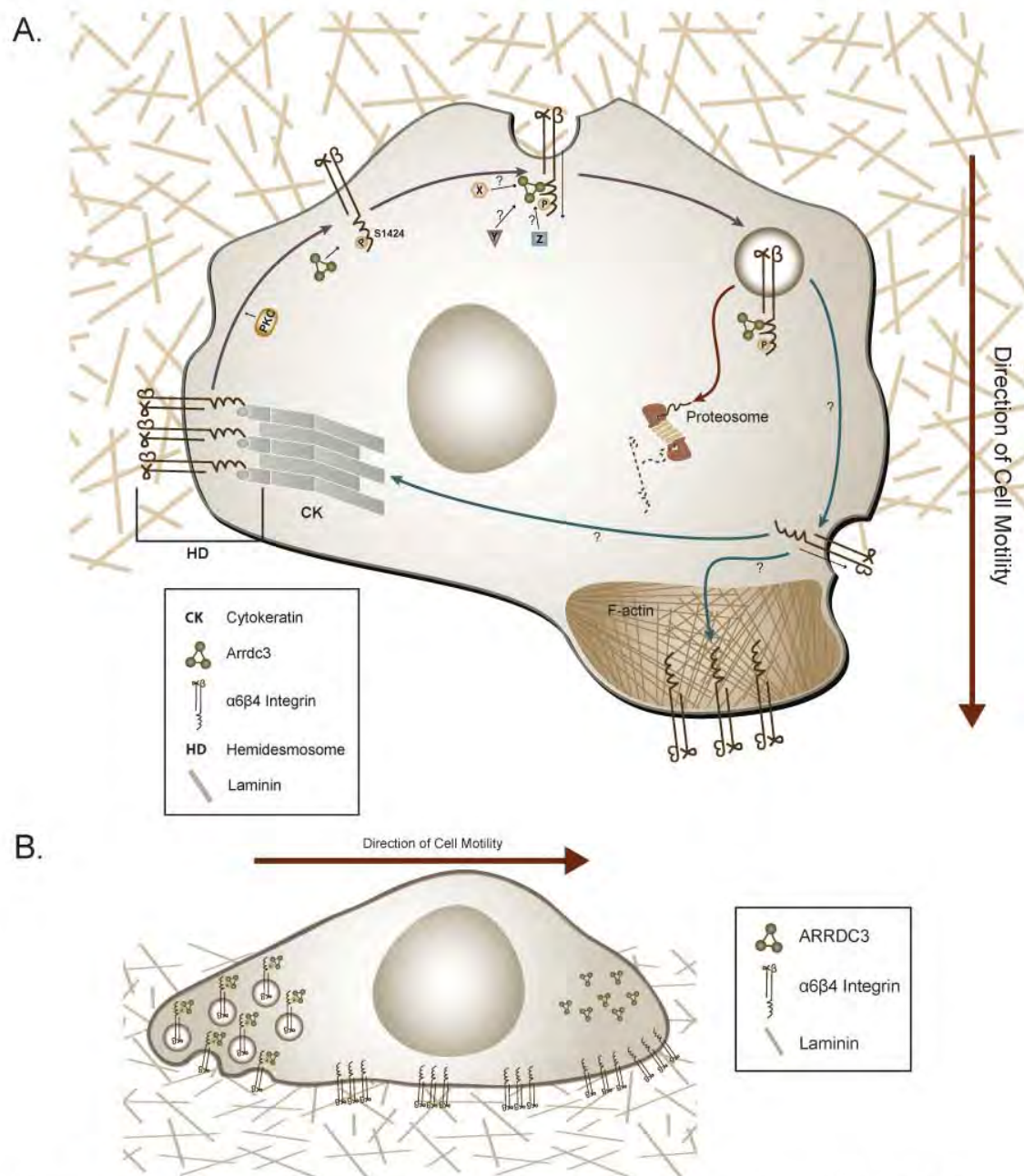


Figure 4-1: Schematic representation of how ARRDC3 regulates ITG β 4 in a migrating cell. (A) Following stimulation, ITG β 4 is phosphorylated on S1424 by PKC allowing for an interaction with ARRDC3. The interaction between ITG β 4 and ARRDC3 causes the internalization of ITG β 4, likely with the assistance of other proteins. Once internalized, ITG β 4 is degraded by the proteasome. However, it is possible that ITG β 4 may be recycled back to the cell membrane if degradation does not occur. ITG β 4 could then be either returned to hemidesmosomes or sent to motility structures at the leading edge. (B) Side view.

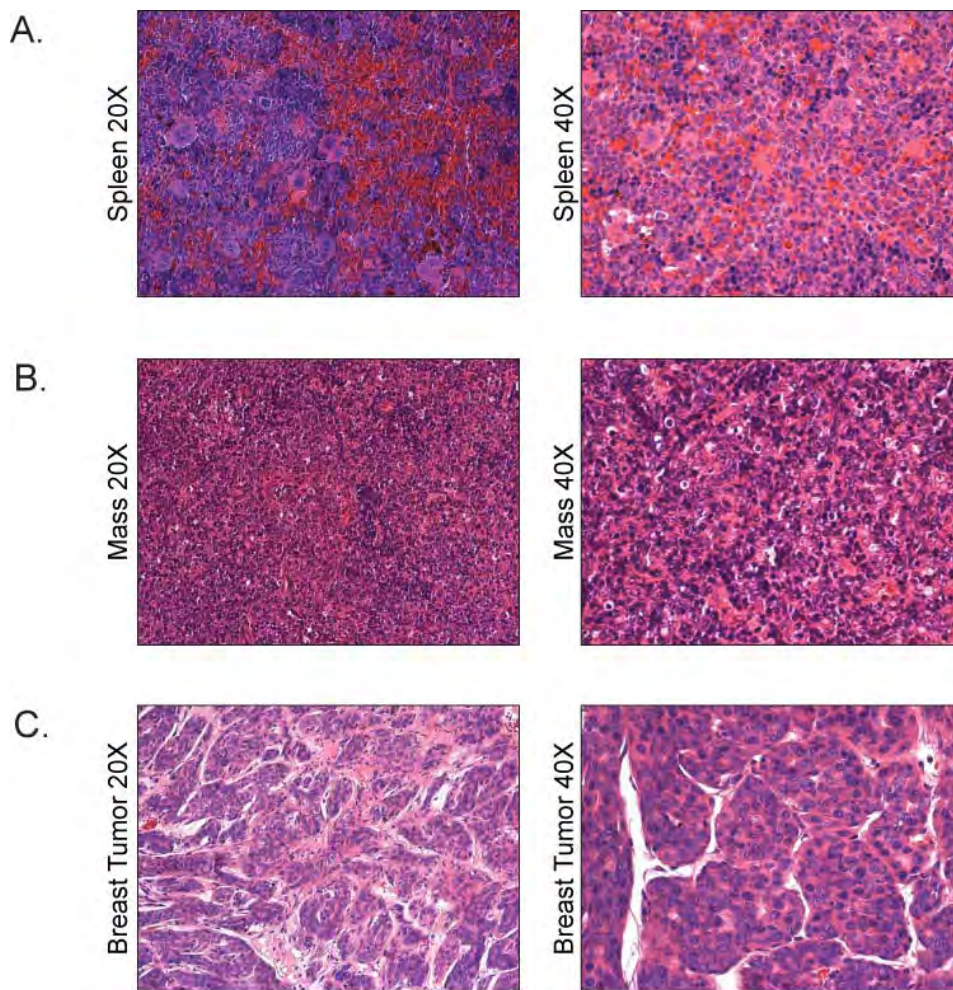


Figure 4-2: Histological analysis of the cancer found in ARRDC3 heterozygous mice. (A) Spleen from an 18-month ARRDC3 heterozygous mouse. Present is an increase in fully differentiated B-cells (plasma cells) as well as evidence of extra-medullary hematopoiesis. (B) Mass found within (but not attached to) the intestines of the same mouse. Presence of all the lymphoid cells suggest the mass was originally a gut-associated lymph node. Also contains a large number of plasma cells. Overall, splenomegaly and increased plasma cells suggest multiple myeloma. Similar observations were found in ARRDC3 null and other ARRDC3 heterozygous mice. (C) Histology of the breast tumor found in the ARRDC3 heterozygous mouse. Tumor is a grade 2 adenocarcinoma.

A.

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Homo sapiens      1 ARDVVSFEQPEFSVSRGDQVARIPVIRRVLDGGKSQVSYRTQDGTAGQNR  50
Equus caballus   1 ASGIVSFEQPEYLVSGGEQVARI PVVRRILDNGKSQVSYRTQDNTAQGNR  50
Mus musculus     1 ASGVVSFEQPEYSVSRGDQVARIPVIRHILDNGKSQVSYSTQDNTAHGHR  50
Rattus norvegicus 1 ASGIVSFEQPEYSVSRGDQVARIPVIRHILDNGKSQVSYSTQDNTAHGHR  50
Pan troglodytes  1 ARDVVSFEQPEFSVSRGDQVARIPVIRRVLDGGKSQVSYRTQDGTAGQNR  50
Bos taurus       1 GRGIVSFEQPEYLVSGGEHVARI PVVRRILDGSGKSQVSYRTQDNTAKANR  50
subseq          1 ARDVVSFEQPEFSVSRGDQVARIPVIRRVLDGGKSQVSYRTQDGTAGQNR  50
                  .*****. ** *..*****. . . * ***** ***. ** . . *

Homo sapiens      51 DYIPVEGELLFQPGEAWKELQVKLLELQEVDSLRLRGRQVRRFHVQLSNPK 100
Equus caballus   51 DYIPVEGELLFQPGEMWKELQVKLLELQEMDSLRLRGRQTRHFHIQLSNPK 100
Mus musculus     51 DYVPVEGELLFHPGETWKELQVKLLELQEVDSLRLRGRQVRRFQVQLSNPK 100
Rattus norvegicus 51 DYVPVEGELLFYPGETWKELQVKLLELQEVDSLRLRGRQVRRFQVQLSNPK 100
Pan troglodytes  51 DYIPVEGELLFQPGEAWKELQVKLLELQEVDSLRLRGRQVRRFHVQLSNPK 100
Bos taurus       51 DYIPMEGELLFQPGETWKELQVKLLELQEMDSLRLRGPQTRRFYIQLSNPK 100
subseq          51 DYIPVEGELLFQPGEAWKELQVKLLELQEVDSLRLRGRQVRRFHVQLSNPK 100
                  **.*.***** ** * **********.***** * *.*.*****

Homo sapiens      101 FGAHLGQPHSTTIIIRDPELDRSFTSQMLSSQ 133
Equus caballus   101 FGARLGQPHSATVIIGDPDELDRNLISQTVSSP 133
Mus musculus     101 FGARLGQPSTTTVIL---DETDRSLINQTLSSP 133
Rattus norvegicus 101 FGARLGQPNTATVIIGEQDETDRSLINEISASP 133
Pan troglodytes  101 FGAHLGQPHSTTIIIRDPELDRSFTSQMLSSQ 133
Bos taurus       101 FGARLGQPQCATVITGDRDELDRNVMNQTVSSP 133
subseq          101 FGAHLGQPHSTTIIIRDPELDRSFTSQMLSSQ 133
                  ***.***** ..*.* ** ** . . *

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B.

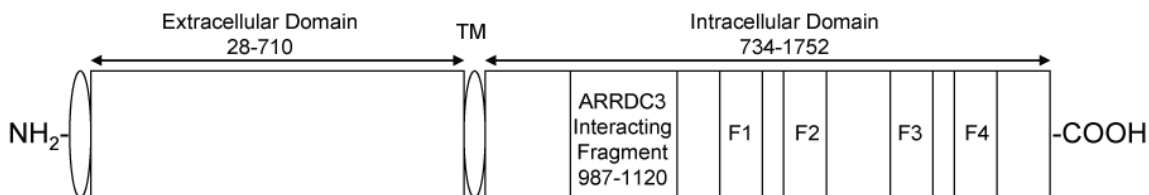


Figure 4-3: Analysis of the region on integrin $\beta 4$ in which ARRDC3 interacts. (A) ClustalW alignment of the sequence corresponding to the interacting amino acid fragment on the integrin $\beta 4$ cytoplasmic tail. The region is relatively well conserved throughout mammals and includes serines and tyrosine residues which are possible phosphorylation sites. (B) A map of integrin $\beta 4$ illustrating the known domains and the region where ARRDC3 binds. It is part of the cytoplasmic domain but upstream of the two pairs of fibronectin type III repeats.

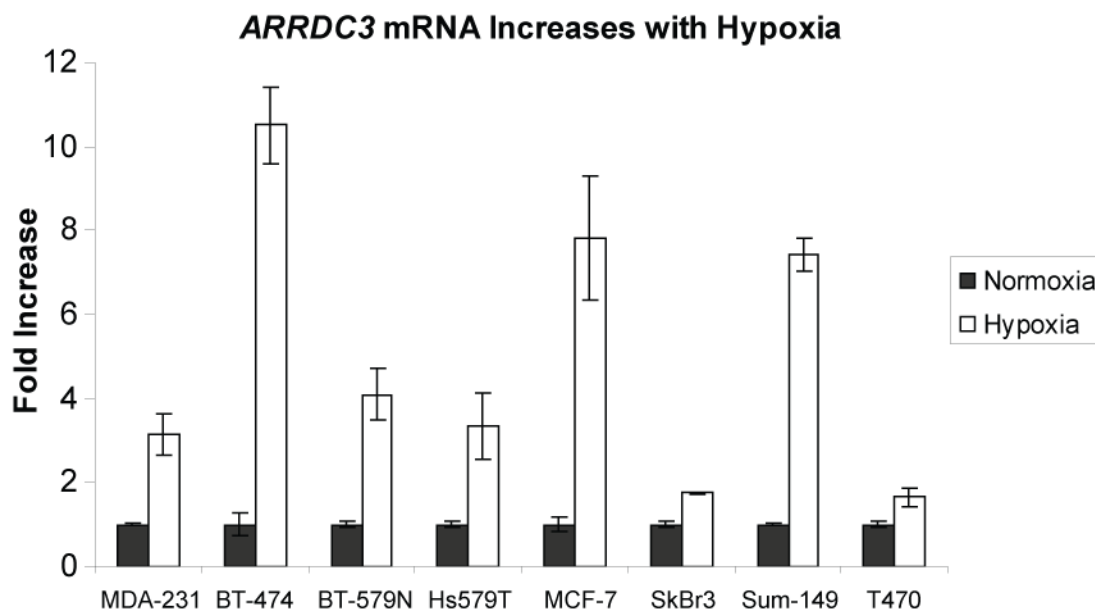


Figure 4-4: Changes in ARRDC3 mRNA expression in Hypoxia. Multiple breast cancer cell lines were cultured in hypoxic conditions (0.5% oxygen) for 24 hours before RNA was isolated. Real-Time PCR analysis determined relative *ARRDC3* mRNA levels. All samples were normalized to actin. RNA from cells cultured in normal oxygen conditions (normoxia) was used for the calibrator of each cell line.

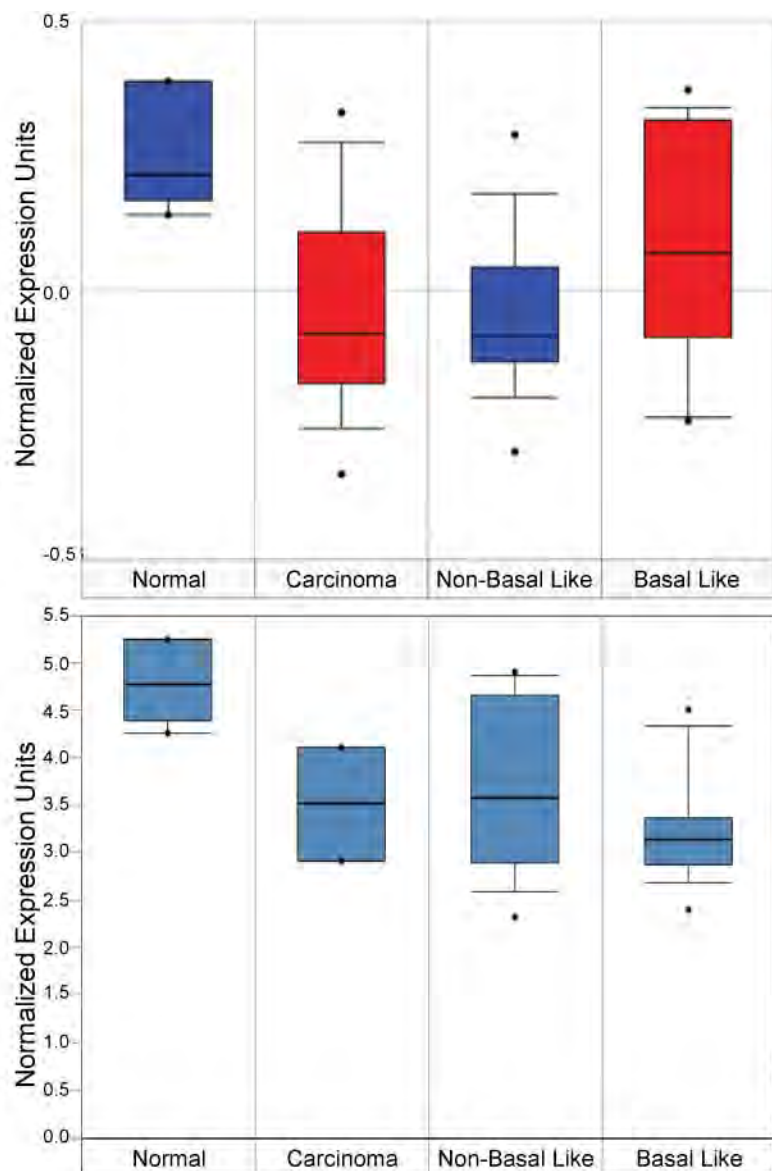


Figure 4-5: Downregulation of ARRDC3 expression in basal like vs. non-basal like tumors. Two different datasets on Oncomine were analyzed for relative ARRDC3 in basal-like and non-basal like tumors. Although ARRDC3 levels were always lower in tumor vs. normal, expression differences between basal like and non-basal like were not consistent. This data indicates that ARRDC3 is not further repressed in basal like breast cancers.

Number	DOB	Genotype	DOD	Age (Days)	Comments
71	22-Jan-08	+/+	4-Jan-10	713	
72	22-Jan-08	+/+	4-Jan-10	713	
73	22-Jan-08	+/+	4-Jan-10	713	
74	22-Jan-08	+/+	4-Jan-10	713	
75	22-Jan-08	+/+	4-Jan-10	713	
76	22-Jan-08	+/+	4-Jan-10	713	
77	22-Jan-08	+/+	4-Jan-10	713	
78	22-Jan-08	+/+	4-Jan-10	713	
79	22-Jan-08	+/+	4-Jan-10	713	
80	22-Jan-08	+/-	16-Sep-09	603	dermatitis
81	22-Jan-08	+/-	15-Feb-09	390	dead from fighting wounds
82	22-Jan-08	+/-	26-Nov-08	309	dead from fighting wounds
83	22-Jan-08	+/-	13-Mar-09	416	found dead; cancer
84	22-Jan-08	+/-	23-Nov-09	671	found dead; cancer
85	22-Jan-08	+/-	6-Jun-09	501	dead from fighting wounds
86	3-Mar-08	+/-	2-Dec-09	639	breast cancer
87	3-Mar-08	+/-	30-Aug-09	545	found dead
88	3-Mar-08	+/+	N/A	N/A	
89	3-Mar-08	-/-	7-Jul-08	126	dead from fighting wounds
90	3-Mar-08	-/-	8-Jul-08	127	dead from fighting wounds
91	3-Mar-08	+/+	11-Jan-10	679	
92	3-Mar-08	+/-	6-Apr-09	399	found dead
93	3-Mar-08	+/+	11-Jan-10	679	
94	3-Mar-08	-/-	12-Dec-08	284	found dead; cancer
95	3-Mar-08	-/-	29-Sep-08	210	found dead; cancer
96	3-Mar-08	+/-	N/A	N/A	dermatitis & fur loss
97	3-Mar-08	+/-	N/A	N/A	dermatitis & fur loss
98	3-Mar-08	+/+	N/A	N/A	
99	3-Mar-08	+/+	N/A	N/A	
100	3-Mar-08	+/+	1-Dec-08	273	dead from fighting wounds
101	3-Mar-08	+/+	N/A	N/A	
102	3-Mar-08	+/-	N/A	N/A	dermatitis
103	3-Mar-08	+/-	N/A	N/A	dermatitis
104	3-Mar-08	-/-	22-Oct-08	233	found dead; cancer
105	3-Mar-08	+/-	N/A	N/A	dermatitis
106	3-Mar-08	+/+	N/A	N/A	
107	3-Mar-08	+/-	N/A	N/A	fur loss
108	3-Mar-08	+/-	N/A	N/A	fur loss
109	3-Mar-08	+/+	N/A	N/A	
110	8-Mar-08	+/-	N/A	N/A	fur loss
111	8-Mar-08	+/-	1-Dec-08	268	dead from fighting wounds
112	8-Mar-08	+/-	1-Dec-08	268	dead from fighting wounds
113	8-Mar-08	+/-	N/A	N/A	fur loss
114	8-Mar-08	+/+	N/A	N/A	
115	8-Mar-08	+/+	N/A	N/A	
116	8-Mar-08	+/+	N/A	N/A	

Table 4-1: Loss of ARRDC3 in mice appears to have an aging phenotype. Four different litters of ARRDC3 wild type, heterozygous and null mice were observed over a phenotype. All ARRDC3 null mice lacked whiskers from birth, yet ARRDC3 heterozygous mice did not appear to have this defect. Mice lacking ARRDC3 seemed more prone to develop cancer (specifically lymphoma) or obtain lethal wounds from fighting. All mice are on a 129; B6 background.

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**Appendix 1: A DNA binding mutant of TAL1 cooperates with LMO2 to
cause T cell leukemia in mice**

This chapter represents work submitted as:

A DNA binding mutant of TAL1 cooperates with LMO2 to cause T cell leukemia in mice.

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Abstract:

The most common translocation in childhood T cell acute lymphoblastic leukemia (T-ALL) involves the LMO2 locus, resulting in ectopic expression of the LMO2 gene in human thymocytes. The LMO2 gene was also activated in 3/20 patients with X-linked Severe Combined Immune Deficiency (X-SCID) treated with gene therapy due to retroviral insertion in the LMO2 locus. The LMO2 insertions predisposed these children to T-ALL, yet how LMO2 contributes to T cell transformation remains unclear. The LIM domain containing LMO2 protein regulates erythropoiesis as part of a large transcriptional complex consisting of LMO2, TAL1, E47, GATA1 and LDB1 that recognizes bipartite E box-GATA1 sites on target genes. Similarly, a TAL1/E47/LMO2/LDB1 complex is observed in human T-ALL and Tal1 and Lmo2 expression in mice results in disease acceleration. To address the mechanism(s) of Tal1/Lmo2 synergy in leukemia, we generated Lmo2 transgenic mice and mated them with mice that express wild type Tal1 or a DNA binding mutant of TAL1. Tal1/Lmo2 and MutTAL1/Lmo2 bitransgenic mice exhibit perturbations in thymocyte development due to reduced E47/HEB transcriptional activity and develop leukemia with identical kinetics. These data demonstrate that the DNA binding activity of Tal1 is not required to cooperate with Lmo2 to cause leukemia in mice and suggest that Lmo2 may cooperate with Tal1 to interfere with E47/HEB function(s).

Introduction:

LMO2 was first implicated in leukemogenesis in 1992 when found associated with a t(11;14)(p13;q11) and t(7;11)(q34;p13) chromosomal translocations (Boehm et al., 1990; Garcia et al., 1991). The LMO1 and LMO2 proteins are found expressed in human T-ALL and LMO proteins are co-expressed in approximately 80% of TAL1+ human T-ALL patients (Ferrando et al., 2002; Rabbitts, 1994; Wadman et al., 1994). Interest in LMO2-mediated leukemogenesis was reignited in 2002, when 4/5 children in 2 X-SCID gene therapy trials developed T-ALL due to retroviral insertion in the LMO2 locus (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). Two leukemic patients exhibited evidence of TAL1 activation and 3 patients developed NOTCH1

mutations (Hacein-Bey-Abina et al., 2008; Howe et al., 2008). Studies in mouse T-ALL models revealed that Tal1 and Lmo proteins cooperate to cause leukemia in mice (Larson et al., 1996; Wadman et al., 1994), but precisely how LMO proteins contribute to leukemogenesis remains unclear.

LMO2 is part of the “LIM only” family of proteins that are thought to serve as bridging factors in transcriptional complexes (Wadman et al., 1997). LIM-domains are cysteine-rich zinc-binding domains that are structurally similar to DNA-binding GATA finger domains; however, currently there is no evidence to suggest that LMO proteins bind DNA. LMOs are thought to mediate protein:protein interactions via their LIM domains and evidence suggests that LMOs act as transcriptional co-regulators. There are 4 human LMO proteins (LMO1-4) and all four LMO proteins have been associated with oncogenesis (Aoyama et al., 2005; Fisch et al., 1992; Visvader et al., 2001).

LMO2 is a nuclear protein that in erythroid cells forms a multi-protein complex which includes TAL1, E47, GATA-1, and LDB1 (Valge-Archer et al., 1994). These proteins form a functional transcriptional complex and recognize a bipartite DNA sequence consisting of an Ebox and a GATA site separated by one helix turn (Wadman et al., 1997). The Tal1/E47/Lmo2/Ldb1/GATA-1 complex regulates the expression of genes important in erythroid or megakaryocytic differentiation, including p4.2, glycophorin A, c-kit, p21CIP and the transcription factors Gfi-1b and ek1f (Goardon et al., 2006; Kros1 et al., 1998; Lahlil et al., 2004; Lecuyer et al., 2002; Xu et al., 2006). Lmo2 is thought to bridge the TAL1/E47 heterodimer to GATA-1, and by mechanisms not fully understood, enhance transcription (Xu et al., 2003). Based on their roles in regulating gene expression during hematopoiesis, Tal1 and Lmo2 have been thought to contribute to leukemia by transactivating the expression of genes such as retinaldehyde dehydrogenase, the transmembrane protein TALLA1, the receptor tyrosine phosphatase IA2, cyclin D2, heat shock cognate 73 and a bub-like gene (Davenport et al., 2000; Ono et al., 1997).

An alternative model posits that in leukemic cells the Tal1/Lmo2 complex interferes with the transcriptional activity of E47/HEB, by sequestering E47 and HEB proteins and/or by recruiting co-repressors to E-box regulated loci (Herblot et al., 2000;

O'Neil et al., 2004). The E47/HEB complex directs the expression of several genes essential for proper lymphoid development including the preT α chain of the pre-TCR, the Rag1 and Rag2 recombinases, CD3, CD4, CD5 and the T cell receptor α/β genes (Greenbaum and Zhuang, 2002a; Greenbaum and Zhuang, 2002b). A HEB or E47 deficiency disrupts thymocyte development and E47-deficient mice are predisposed to the development of T cell leukemia (Bain et al., 1997). Our published work provides genetic evidence to support the inhibition model of TAL1-mediated leukemogenesis. We demonstrate that a DNA binding mutant form of TAL1 (R188G;R189G) (referred to here as mutTAL1) is able to induce T-ALL-like disease in mice and T cell leukemogenesis is accelerated when Tal1 is expressed on an E2A or HEB heterozygous background (O'Neil et al., 2001; O'Neil et al., 2004). Moreover, the functions of Tal1 in early hematopoietic development also do not appear to require the DNA binding properties of Tal1 (Schlaeger et al., 2004). More recently, the ETO-2 repressor has been identified as a novel component of the TAL1/LMO2 complex in erythroid cells (Goardon et al., 2006). Thus, Tal1/E47 or HEB/Lmo2 complexes may also actively repress the expression of E-box containing genes during normal erythroid differentiation and in leukemia.

To determine the molecular basis of the observed Tal1/Lmo2 synergy and to distinguish between the transcriptional activation and inhibition models, we asked whether the DNA binding properties of Tal1 were required to collaborate with Lmo2 to cause T cell leukemia in mice. To test this model, we generated bitransgenic mice co-expressing LMO2 with wild type Tal1 or with a DNA binding TAL1 mutant. We observed disease acceleration in both the Tal1/Lmo2 and mutTAL1/Lmo2 transgenic mouse lines, indicating that the DNA binding properties of TAL1 are not required to cooperate with Lmo2 to cause disease in mice. Moreover, thymocyte development was inhibited in pre-leukemic Tal1/Lmo2 and mutTAL1/Lmo2 mice due to reduced expression of E47/HEB-regulated genes important for thymocyte differentiation. These data indicate that Lmo2 may accelerate Tal1-mediated leukemogenesis by enhancing the sequestration and/or inhibition of the E47/HEB heterodimer.

Results and Discussion:***Generation of transgenic mice expressing a DNA binding mutant of TAL1 and Lmo2***

Approximately 80% of TAL1 expressing T-ALL patients also express LMO proteins (Ferrando et al., 2002). Tal1 and Lmo expression in the mouse results in dramatic acceleration of disease (Aplan et al., 1997; Larson et al., 1996). To determine how Lmo2 contributes to TAL1-mediated leukemogenesis and to understand the basis for the TAL1 and LMO oncogenes cooperativity, we asked whether the DNA binding properties of TAL1 were required to cooperate with Lmo2 to cause disease in mice. To express both oncogenes in developing mouse thymocytes, we generated transgenic mouse lines where the mouse Lmo2 cDNA was expressed under the control of the proximal lck promoter (Figure 1A). The 3' untranslated regions of this construct contain introns, exons and the poly A addition site of the human growth hormone gene (Abraham et al., 1991). The prox lck Lmo2 construct was microinjected into the pronuclei of fertilized FVB/N oocytes. Sixteen founder mice were identified initially by Southern blot analysis and two founder lines (Fo5 and 19) with variable expression levels were expanded for further study (Figure 1B).

The Lmo2 transgenic mouse lines were mated with transgenic mice expressing wild type Tal1 or a DNA binding mutant of TAL1. The DNA binding mutant mice express a form of TAL1 where the contact residues of the basic DNA binding domain, arginine 188 and 189 have been mutated to glycines (designated R188G;R189G) (Hsu et al., 1994). This mutant form of TAL1 heterodimerizes with E47 or HEB proteins but fails to stably bind E box binding sequences in-vitro and in vivo (Hsu et al., 1994; O'Neil et al., 2001). Like Tal1 transgenic mice, expression of a TAL1 DNA binding mutant perturbs thymocyte development and approximately half of the mice develop a T-ALL-like disease (O'Neil et al., 2001).

To determine whether the DNA binding activities of TAL1 were required to cooperate with Lmo2 to cause disease in mice, we generated a cohort of 15 Tal1/Lmo2 and 11 mutTAL1/Lmo2 bitransgenic mice and monitored the animals daily for development of disease. Both Lmo2 transgenic lines were included in the study and both

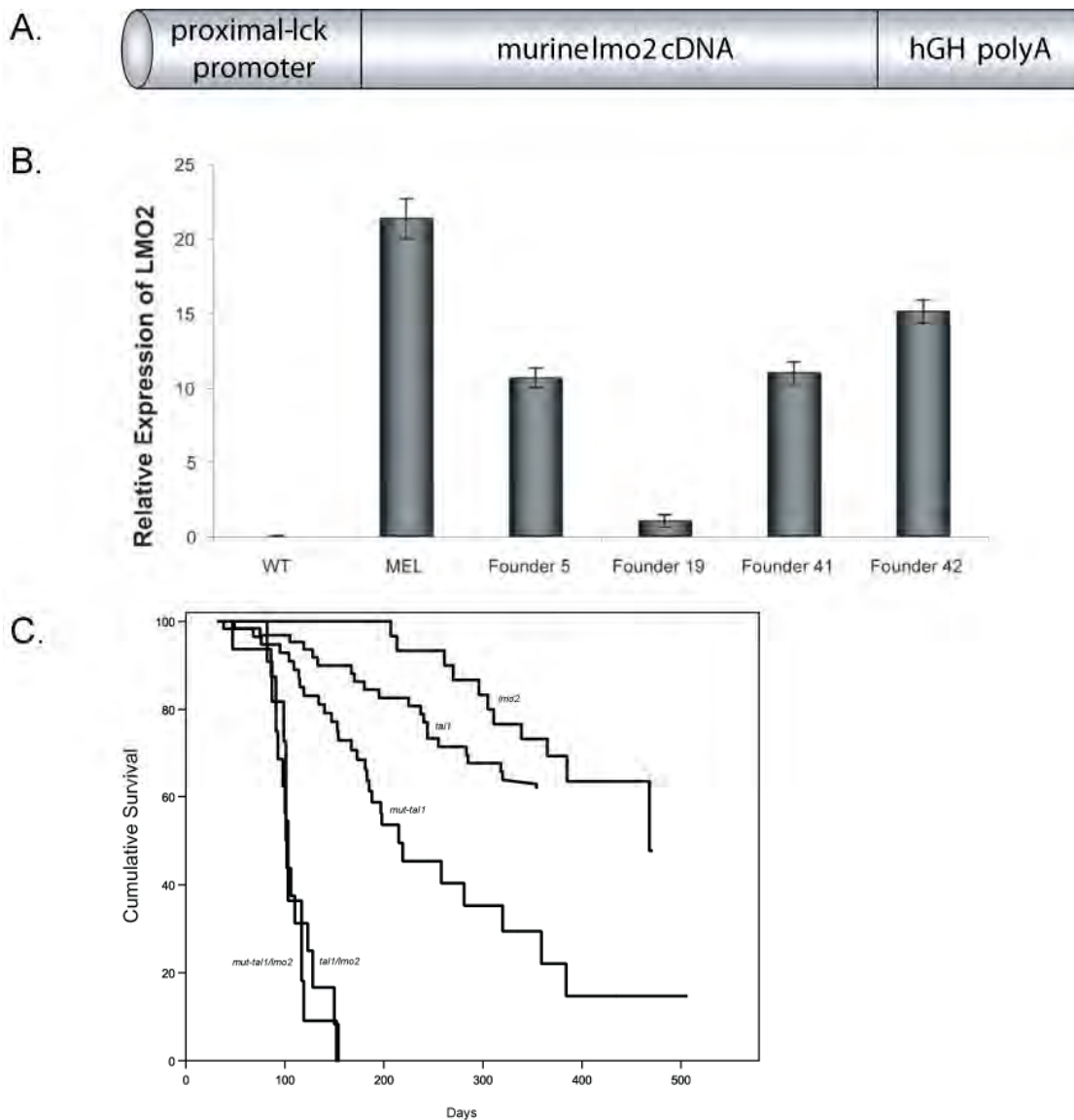


Figure 1-1: The DNA binding properties of TAL1 are not required to cooperate with Lmo2 to cause leukemia in mice. A. Schematic representation of the prox Ick/Lmo2 transgene. The murine Lmo2 cDNA is expressed under control of the proximal Ick promoter and human growth hormone (hGH) splice and poly(A)⁺ addition sequences. B. Expression of the Lmo2 transgene. RNA was isolated from a murine erythroleukemic cell line (MEL) and from thymocytes isolated from wild type (wt) or Lmo2 founder mice designated F5, F19, F41 and F42. Lmo2 mRNA levels were determined using quantitative PCR with β -Actin serving as an internal control. C. Survival curves for the Tal1, mut-TAL1, Lmo2, Tal1/Lmo2 and mut-TAL1/Lmo2 mice. Mice were monitored daily for evidence of disease, upon which the mice were sacrificed and a post-mortem examination performed. The cohort of Tal1/Lmo2 mice consisted of n=15 animals and the cohort of mut-TAL1/Lmo2 mice consisted of n=11 animals. Tal1 and mut-TAL1 survival curves have been published previously (Kelliher et al., 1996; O'Neil et al., 2001).

lines cooperated with Tal1 and the DNA binding mutant to accelerate leukemogenesis in mice ($p < .0001$) (Figure 1C). Surprisingly, Tal1/Lmo2 and mutTAL1/Lmo2 transgenic mouse lines developed disease with nearly identical kinetics (Figure 1C), in spite of the fact that the DNA binding mutant TAL1 transgenic mice develop disease more rapidly than wild type Tal1 transgenic mice (O'Neil et al., 2001). Approximately 30% of Tal1 transgenic mice develop leukemia after a long latency, whereas mutTAL1 mice develop disease with a median survival of 215 days (Kelliher et al., 1996; O'Neil et al., 2001). In contrast, when Lmo2 is co-expressed with Tal1 or mutTAL1, 100% of the mice develop T-ALL-like disease with a median survival of approximately 100 days. No significant difference was observed when Tal1/Lmo2 and mutTAL1/Lmo2 survival curves were compared ($p < 0.581$). Histopathologic examination of tumors isolated from the bitransgenic mice revealed a range of tumor immunophenotypes (Table 1), with tumors predominantly containing DN and DP or CD8SP cells observed in both Tal1/Lmo2 and mutTAL1/Lmo2 mice (Table 1). This study reveals that Tal1/Lmo2 synergy does not require the DNA binding properties of TAL1, suggesting that Lmo2 does not contribute to leukemia by enhancing or altering the transcriptional activity of the Tal1/E47 or HEB heterodimer.

Table 1. Immunophenotype of Tal1/Lmo2 and mut-TAL1/Lmo2 tumors

Animal Number	Genotype	CD3	CD25	DN	CD4	CD8	DP	Phenotype	Survival (Days)
5624	Tal1/Lmo2	31.6	55.7	27.9	0.1	71.2	0.8	DN, CD8-SP	102
5629	Tal1/Lmo2	71.3	0.4	30.9	0.2	68.1	0.7	DN, CD8lo	91
5632	Tal1/Lmo2	94.2	7.7	1.7	0.2	82.6	15.5	CD8-SP	91
5666	Tal1/Lmo2	95.1	95.3	3.3	2.0	2.1	92.6	DP	98
5354	Tal1/Lmo2	29.3	62.9	30.1	0.6	4.3	65.0	DN, DP	47
5338	Tal1/Lmo2	69.5	70.6	25.8	0.1	42.8	31.1	DN, CD8-SP, DP	86
5351	Tal1/Lmo2	23.7	97.9	0.3	0.1	0.5	99.1	DP	106
5313	Tal1/Lmo2	96.2	3.3	0.8	93.4	1.1	4.7	CD4-SP	154
5353	Tal1/Lmo2	27.5	4.6	9.7	80.1	0.4	9.8	CD4-SP	123
6540	mut-TAL1/Lmo2	52.7	13.8	53.7	8.2	10.9	27.1	DN, DP-lo	99
5984	mut-TAL1/Lmo2	50.0	95.5	51.0	1.0	39.5	8.5	DN, CD8-SP	101

Tumors were stained with antibodies to CD3, CD4, CD8 and CD25 and analyzed by flow cytometry. The percentage of stained cells is indicated.

Thymocyte developmental arrest in Tal1/Lmo2 and mutTAL1/Lmo2 mice:

We have shown that thymic expression of Tal1 or the DNA binding mutant results in a decrease in overall thymocyte cellularity due to fewer DP and CD8-positive thymocytes (Kelliher et al., 1996; O'Neil et al., 2001). Expression of Lmo2 under the control of the proximal lck promoter appeared to have no detectable effects on DP thymocyte development (Figure 2B), however Lmo2 expression decreased thymocyte cellularity and skewed DN development; although no increases in the absolute numbers of DN thymocytes was observed (Figure 2A and C). Consistent with published reports (Larson et al., 1996), co-expression of Tal1 and Lmo2 reduces thymic cellularity and blocks DP thymocyte development, resulting in increases in the relative and absolute number of DN thymocyte precursors in preleukemic Tal1/Lmo2 mice (Figure 2B and D). DP thymocyte development was consistently more severely affected in Tal1/Lmo2 mice derived from Lmo2 founder (Fo)19 compared to Fo5 (Figure 2B).

To determine whether the thymocyte developmental block reflects alterations in Tal1-mediated gene expression, we examined thymocyte development in mutTAL1/Lmo2 pre-leukemic mice. We found thymocyte cellularity further reduced in mutTAL1/Lmo2 mice compared to mice that express the TAL1 DNA binding mutant only (Figure 2A). Overall cellularity was similarly reduced in all the Tal1/Lmo2 and mutTAL1/Lmo2 mice examined. Similar to the Tal1/Lmo2 mice, DP thymocyte development was consistently more severely affected in mutTAL1/Lmo2 mice derived from Fo19 compared to founder Fo5 (Figure 2B). Importantly, the DNA binding TAL1 mutant cooperated with Lmo2 to induce similar effects on thymocyte development as wild type TAL1 (Figure 2).

Expression of Lmo2 with Tal1 or mutTAL1 also altered DN thymocyte development, resulting in arrest at the DN3-DN4 precursor stage (Figure 2C and D). In contrast, an E2A or HEB deficiency results in an arrest of DN thymocyte development at the DN1 and DN3 stages, respectively (Bain et al., 1997; Greenbaum and Zhuang, 2002a; Greenbaum and Zhuang, 2002b). These studies demonstrate that Lmo2 does not require the DNA binding properties of TAL1 to perturb thymocyte development. The block in

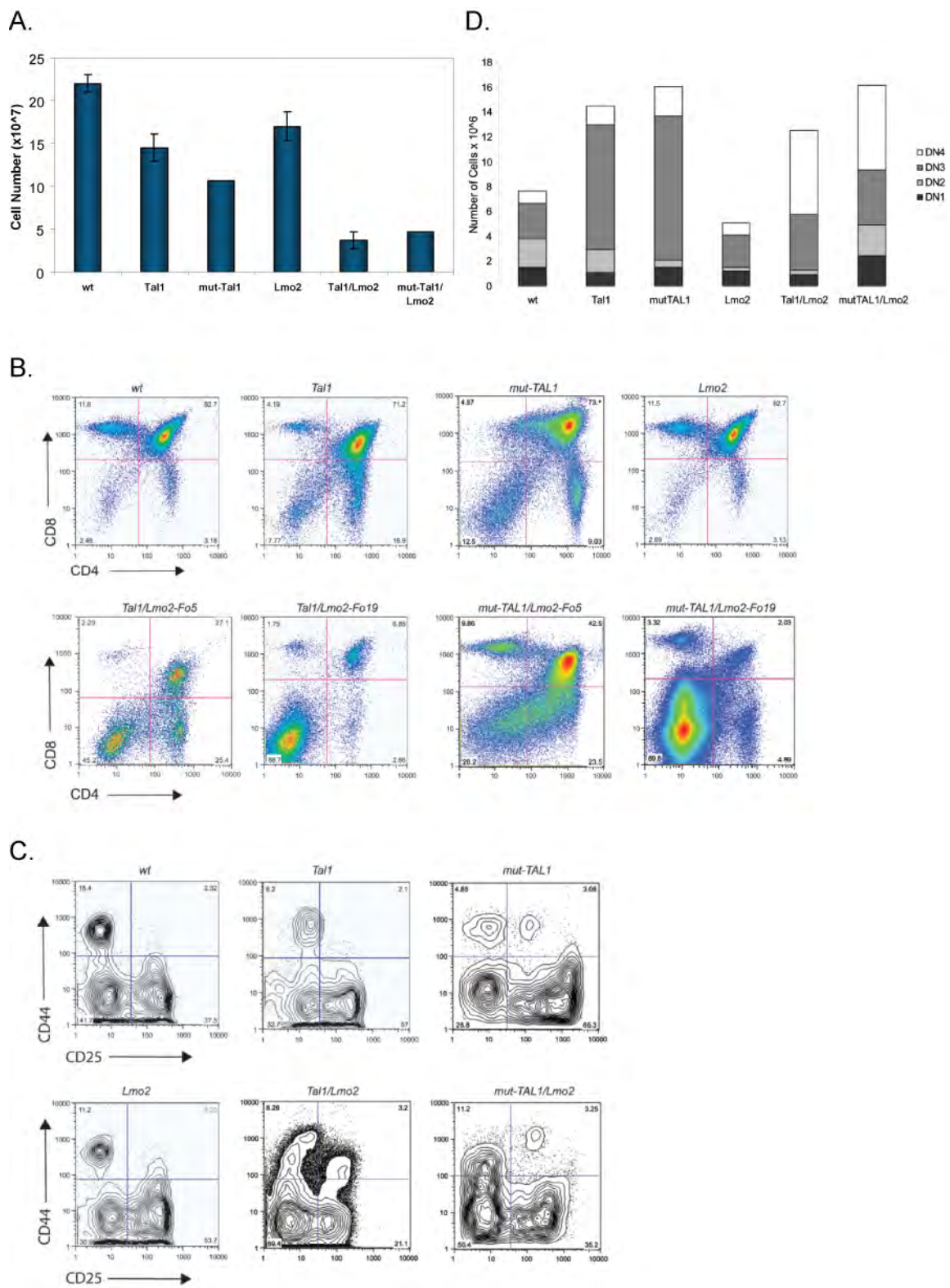


Figure A1-2: A DNA binding mutant of TAL1 cooperates with Lmo2 to perturb thymocyte development. A. Significant decreases in thymic cellularity are observed in Tal1/Lmo2 and mutTAL1/Lmo2 bi-transgenic mice. Total thymocyte numbers from wild type, Tal1, mut-TAL1, Lmo2, Tal1/Lmo2, and mutTAL1/Lmo2 transgenic mice. When compared to wild-type, Lmo2 expression causes a decrease in thymic cellularity ($p=8.0 \times 10^{-6}$) that is exacerbated when either Tal1 or mut-TAL1 is co-expressed ($p=7.8 \times 10^{-15}$ and $p=3.1 \times 10^{-6}$ respectively). B. Coexpression of Lmo2 with either Tal1 or mutTAL1 perturbs thymocyte development. Thymocytes from 4 week old, preleukemic mice were stained with CD4-PE and CD8-FITC and analyzed by flow cytometry. Fourteen Lmo2, 19 Tal1/Lmo2 and 13 mutTAL1/Lmo2 preleukemic mice were analyzed. Representative profiles are shown. C. DN thymocyte development is altered when Lmo2 is co-expressed with either Tal1 or mutTAL1. Thymocytes from 4-week old mice were stained with antibodies for the lineage markers (CD4, CD8, CD3, B220, Mac1, Gr1, and Terr119) and the lineage-negative cells were isolated and stained with CD44-APC and CD25-FITC. Fourteen Lmo2, 19 Tal1/Lmo2 and 13 mutTAL1/Lmo2 preleukemic mice were examined. Representative profiles are shown.

thymocyte development induced by either form of Tal1 is not complete, as DP and SP thymocytes are detected (Figure 2C). Moreover, immunophenotyping of Tal1/Lmo2 and mutTAL1/Lmo2 tumors revealed that tumors from both bitransgenic lines were often heterogeneous, consisting of DN and DP and/or CD8 SP cells (Table 1). For example, although the primary Tal1/Lmo2 tumor 5338 is clonal, it contains DN, DP and CD8-SP cells, suggesting that differentiation proceeds at some level after leukemic transformation.

The expression of E47/HEB-regulated genes is decreased in both Tal1/Lmo2 and mutTAL1/Lmo2 mice.

To understand how Lmo2 can cooperate with a DNA binding mutant of TAL1 and interfere with thymocyte development, we examined the expression of E47/HEB target genes known to regulate thymocyte differentiation. Previously, we found the expression of PreT α , Rag1/2, CD3 CD4, T cell receptor α/β and CD5 repressed in the presence of the Tal1 oncogene (O'Neil et al., 2004). The expression of these thymocyte differentiation genes was further decreased in Tal1/E2A $^{+/-}$ or HEB $^{+/-}$ mice, indicating that Tal1 alters thymocyte development by interfering with the transcriptional activities of the E47/HEB heterodimer (O'Neil et al., 2004). Using real time PCR, we quantified the expression of the immunoglobulin and T cell receptor gene recombinases Rag1 and

Rag2 and the pre-T α chain of the pre-TCR. We found the expression of Rag1 and Rag2 significantly decreased in Tal1 and mutTAL1 preleukemic thymocytes (Figure 3). Slight decreases in Rag1/2 expression were also detected in the Lmo2 transgenic thymus, although no gross perturbations in the thymocyte developmental profile were observed. Co-expression of Tal1 or its DNA binding mutant with Lmo2 resulted in consistent further decreases in Rag1 and Rag2 expression (Figure 3). Similarly, we found preT α expression further repressed in preleukemic Tal1/Lmo2 or mutTAL1/Lmo2 thymocytes than in thymocytes expressing Tal1, mutTAL1 or Lmo2 alone (data not shown). The reduced expression of these E47/HEB-regulated genes does not reflect changes in the relative percentage of DN vs DP stage thymocytes, as significant decreases in Rag1/2 and PreT α gene expression were observed in Tal1 and mutTAL1 animals, where the thymocyte developmental profile is only modestly altered (Figure 2B). These data reveal that in the presence of Tal1 and importantly, a DNA binding mutant of TAL1, Lmo2 expression results in further reductions in E47/HEB transcriptional activity. These findings suggest that Lmo2 binding to Tal1 or mutTAL1/E47 or HEB heterodimers may stabilize the Tal1/E47 or HEB complex, resulting in greater E47/HEB sequestration.

Consistent with our *in vivo* data, the binding affinities for the Tal1/E47 heterodimer have been measured *in vitro* and shown to significantly increase in the presence of Lmo2 (K_A of $\sim 4 \times 10^7$ vs K_A of $\sim 1 \times 10^8$) (Ryan et al., 2008). Therefore, Lmo2 binding appears to increase the affinity of Tal1 for E47 or HEB and this interaction is greater than the affinity of Lmo2 for its LIM domain binding partner, Ldb1 (K_A of 5×10^7) (Ryan et al., 2008). These estimates predict that E protein sequestration may be favored in a setting where Tal1 and Lmo2 are both expressed.

Our findings have therapeutic implications and suggest that interfering with Tal1/E47 or Tal1/Lmo2 binding may release E47/HEB proteins and stimulate leukemic cell differentiation and/or apoptosis. Disrupting a large protein interface such as a helix-loop helix interaction with small molecules may not be feasible. However, in some instances, only a minor part of the protein dimer interfaces contribute to the affinity

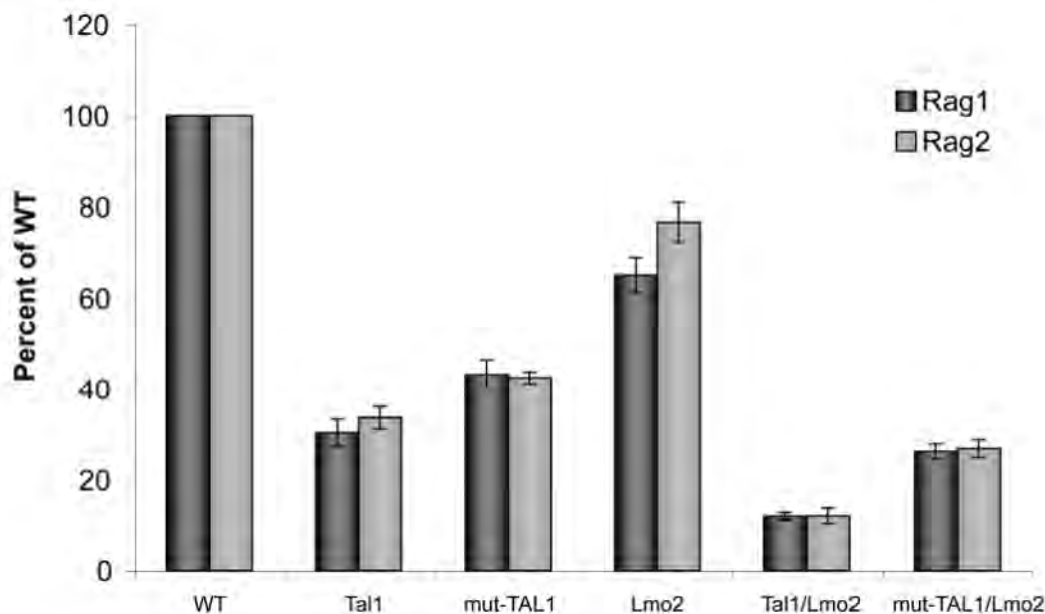


Figure A1-3: Rag1 and Rag2 expression is further repressed in Tal1 and mut-TAL1 transgenic thymocytes when Lmo2 is co-expressed. Thymocytes were isolated from wild type or preleukemic Tal1, mutTAL1, Lmo2, mutTAL1/Lmo2 or Tal/Lmo2 transgenic mice at 4-6 weeks of age. RNA was isolated and Rag1 and Rag2 expression levels quantified by qPCR. The copy number for the gene of interest was normalized to the copy number for β -actin. Data is represented as percent of wild type control.

between proteins (Clackson and Wells, 1995; Kussie et al., 1996; Wells, 1996; Wells and de Vos, 1996). Targeting these regions may be sufficient to inhibit protein:protein interactions. Small molecules have in fact been identified that interfere with the Myc/Max bHLH/LZ interaction and these molecules have been shown to interfere with c-Myc-induced transformation in vitro (Berg et al., 2002). Alternatively, interfering with Lmo2 binding to Tal1 may be sufficient to induce apoptosis of T-ALL cells. Retroviral expression of an anti-Lmo2 single chain Fv antibody fragment or an Lmo2 aptamer have been shown to reduce leukemic growth in transplanted mice (Nam et al., 2008), suggesting that interfering with LMO2 protein interactions may be an effective therapeutic strategy for some relapsed T-ALL patients.

Materials and Methods:

Generation of transgenic mice: Murine Lmo2 cDNA was cloned into p1017, a plasmid containing the proximal Ick promoter and the human growth hormone splice and polyadenylation addition sites. The plasmid DNA was sequenced and digested with SpeI for microinjection into the FVB/N pronuclei. Transgenic founders were identified by Southern blotting and mated with FVB/N mice.

Cell culture and flow cytometry: Mouse T-ALL tumors were minced into a single-cell suspension using frosted slides and cultured in RPMI with 10% fetal bovine serum, 1% glutamine, penicillin/streptomycin, and 50 μ M β -mercaptoethanol at 37°C under 5% CO₂. Thymi from preleukemic mice were gently disrupted with frosted glass slides in order to produce single cell suspensions. The thymocytes were washed with PBS and stained with fluorescent labeled antibodies and subjected to flow cytometry at the FACS facility at the University of Massachusetts Medical Center. For double negative analysis, cells were stained with antibodies for the lineage antibodies, and the lineage negative cells were stained with CD44-APC and CD25-Fitc. Antibodies used in flow cytometry included CD3-PE, CD4-PE, CD4-Cy5 PE, CD8- FITC, CD8-Cy5-PE, CD25-Fitc, CD44-APC, and lineage markers (Pharmingen). Data were analyzed using FlowJo software (Treestar, Inc.). For tumor immunophenotyping, primary tumors cells were stained with either PE or FITC-conjugated anti-mouse Ly-2 (CD8), L3T4 (CD4), CD25, and CD3 antibodies (BD Pharmingen, San Diego, CA, USA), and analyzed by flow cytometry.

Quantitative PCR: RNA was extracted from primary thymocytes using Trizol. cDNA was synthesized using the Superscript first-strand synthesis system (Invitrogen). Rag1, Rag2, and pre-T α expression was assayed using primers as described in reference (Hsu et al., 2003; Huang et al., 2003). To determine target gene expression levels, cDNA was serially diluted and quantified using the SYBR green kit (QIAGEN) gene-specific primers and β -actin specific primers using relative quantification analysis.

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Appendix 2: Targeting the Notch1 pathway in a Mouse T-ALL Model

This chapter represents the contributions of KM Draheim in work submitted as:

Targeting the Notch1 and mTOR pathways in a mouse T-ALL model.

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Abstract:

Gain of function mutations in NOTCH1 are frequently detected in patients with T cell acute lymphoblastic leukemia (T-ALL) and in mouse models of the disease. Treatment of mouse or human T-ALL cell lines with γ -secretase inhibitors (GSI) to inhibit Notch1 activity in vitro results in growth arrest and apoptosis. These studies reveal GSI as potential therapeutic agents in the treatment of T-ALL. To determine whether GSI have anti-leukemic activity in vivo, we treated near end stage *tal1/ink4a/arf+/-* leukemic mice with vehicle or with a GSI developed by Merck Research Laboratories (MRK-003). We found that GSI treatment significantly extended the survival of leukemic mice, when compared to vehicle treated mice. Notch1 target gene expression was repressed and increased numbers of apoptotic cells were detected in the thymic masses isolated from the GSI treated mice. This study demonstrates that Notch1 activity can be successfully inhibited in vivo when administered as a single agent to mice with advanced disease, GSI treatment prolongs survival. This work supports the idea of targeting NOTCH1 in the treatment of T-ALL patients.

Introduction:

T cell acute lymphoblastic leukemia (T-ALL) is associated with the mis-expression of the basic helix-loop-helix transcription factor TAL1/SCL and LIM-domain only proteins LMO1 and LMO2 (Aplan et al., 1991; Aplan et al., 1992; Bash et al., 1995; Bernard et al., 1991; Boehm et al., 1991; Brown et al., 1990; Royer-Pokora et al., 1991). These oncogenes are found mis-expressed in greater than 60% of human T-ALL patients (Bash et al., 1995; Ferrando et al., 2004; Ferrando and Look, 2003). Mouse models of T-ALL recapitulate the disease through ectopic expression of Tal1 in the thymus (Kelliher et al., 1996). These mice develop respiratory distress due to the presence of large thymic masses and have detectable T cell blasts in peripheral blood lymphocytes (PBL), spleen, liver, and kidney (Kelliher et al., 1996; Shank-Calvo et al., 2006). Mis-expression of Tal1 results in perturbed thymocyte development by interfering with the basic-helix-loop-helix (bHLH) heterodimer E47/HEB that regulates the expression of genes critical for thymocyte differentiation including *rag1/2*, *pre-Ta*, *CD4*, *CD3*, *TCRa/b* (Herblot et al.,

2000; O'Neil et al., 2001; O'Neil et al., 2004). Consistent with this finding, loss of the E2A gene that encodes the E47/E12 bHLH protein is associated with human T and B cell leukemias (Mullighan et al., 2007).

Mutations in the Notch 1 receptor have been frequently detected in mouse T-ALL models (Dumortier et al., 2006; Lin et al., 2006; O'Neil et al., 2006; Reschly et al., 2006) and importantly in 54% of T-ALL patients (Grabher et al., 2006; Weng et al., 2004). These mutations cluster in the heterodimerization domains (HD) or result in truncation of PEST regulatory sequences (O'Neil et al., 2006; Weng et al., 2004). Mutations in the HD result in increased susceptibility to cleavage by the gamma-secretase complex, whereas deletion of PEST regulatory sequences results in increased Notch1 stability (Gupta-Rossi et al., 2001; Malecki et al., 2006; Sanchez-Irizarry et al., 2004). Treatment of mouse tall1 leukemic cell lines in vitro with gamma secretase inhibitors (GSI) results in cell cycle arrest and apoptosis, revealing that Notch1 signaling is required for leukemic growth/survival (O'Neil et al., 2006). Notch1-mediated leukemic growth in mouse and human T-ALL cells is mediated in part by the direct transcriptional activation of c-myc (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006). Similarly, Notch1 mediated mammary tumorigenesis in the mouse also appears c-myc dependent (Klinakis et al., 2006). It remains unclear, however, whether c-myc expression is required for Notch1-mediated leukemogenesis or whether other Notch1 target genes contribute.

Although the majority of mouse tall1 leukemic cell lines undergo apoptosis upon GSI treatment in vitro, it is unclear whether Notch1 can be inhibited for extended periods of time in vivo. An additional concern regarding targeting Notch1 in T-ALL is that in contrast to mouse, human T-ALL lines appear relatively GSI resistant in vitro, raising the possibility that GSI alone may not prove effective in the treatment of T-ALL patients. Moreover, whether GSI can be administered in vivo for extended periods of time without associated toxicities remains uncertain.

In this study, we examine the effects of GSI treatment in our mouse T-ALL model. To examine GSI efficacy and to accurately reflect the clinical experience, we treated near end stage leukemic mice and found that GSI treatment extends the survival

of leukemic mice, but is not sufficient to eliminate disease. Collectively, this work supports the idea of targeting NOTCH1 in the treatment of T-ALL.

Results:

The GSI MRK-003 represses Notch1 target gene expression and induces apoptosis of mouse T-ALL cell lines and primary tal1/ink4a/arf^{+/-} tumors.

The prevalence of mutations that result in activated NOTCH1 in T-ALL patients raises the possibility that GSI used to inhibit Notch1 and other GS-dependent substrates in vitro may have anti-leukemic activity in the clinic. GSI have been developed by Merck Research Laboratories, Inc yet their ability to inhibit Notch1-mediated mouse leukemic growth was untested (Lewis et al., 2007; Sparey et al., 2005). To test whether MRK-003 inhibited Notch1, multiple mouse T-ALL cell lines were treated with 1mM or 10mM of MRK-003 and Notch1 target gene expression examined. Decreased levels of *hes1* and *deltex* expression were observed in the MRK-003 treated cultures following treatment for 24 hours (Figure 1A).

We then compared the relative effectiveness of MRK-003 with DAPT, another GSI known to inhibit mouse leukemic growth (O'Neil et al., 2006). Multiple mouse T-ALL cell lines were treated with 1mM MRK-003 or with 1mM of DAPT for three days and cell cycle analysis performed. In all three of the cell lines tested, increases in the percentage of apoptotic cells was observed in MRK-003 treated cultures. In cell line 5151, DAPT treatment failed to induce apoptosis above background levels, whereas 58% apoptotic cells were detected in the MRK-003 treated cultures (Figure 1B). This in vitro data suggested that MRK-003 may be effective at inhibiting Notch1-mediated leukemic growth in vivo. However, tumor adaptation to culture clearly involves the accumulation of additional genetic changes and therefore, in vitro GSI responses using mouse or human T-ALL cell lines may not accurately predict GSI efficacy in vivo.

To test whether MRK-003 might be effective against primary tumors harboring spontaneous notch1 mutations, thymic tumor masses isolated directly from tal1/ink4a/arf^{+/-} mice were treated with vehicle only, with 1mM DAPT or 1 mM MRK-003 for three days. The percentage of apoptotic cells was then determined by Annexin V/PI staining

followed by flow cytometry. As expected, apoptotic cells (32.4%) were observed in the DMSO or vehicle treated cultures over the three day culture period. DAPT treatment, however, increased the percentage of apoptotic cells to approximately 62.2 % (29.8% increase), whereas the MRK-003 treated cultures contained 88.8% Annexin V/PI-positive cells (56.4% increase). Although apoptosis varied among the 3 primary tumors tested in this assay, the trend remained the same.

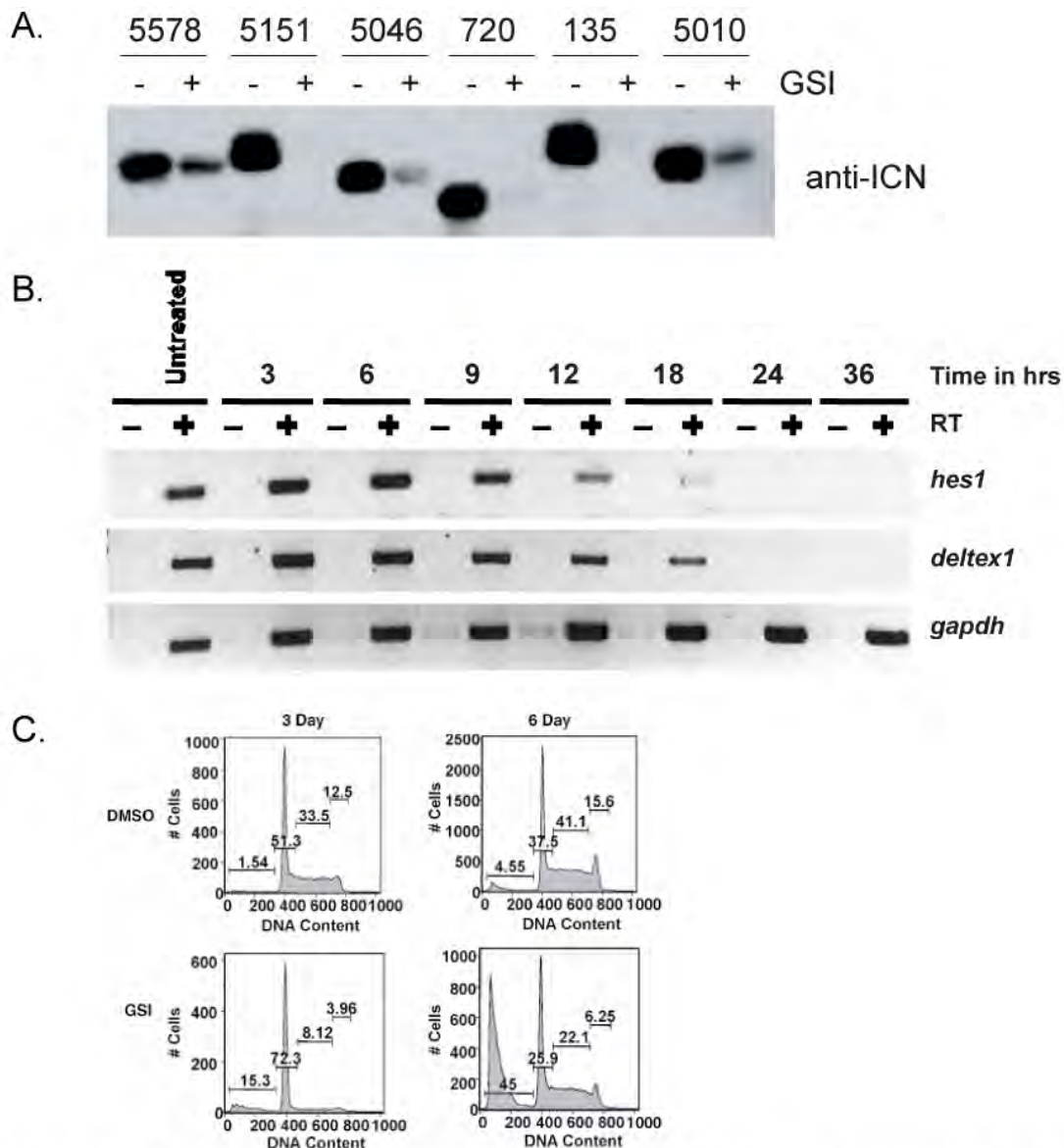


Figure 1. MRK-003 is a potent gamma secretase inhibitor that represses Notch1 target gene expression and induces apoptosis in vitro. (A-B) MRK-003 treatment decreases ICN levels and represses Hes1 and Deltex1 expression. Mouse leukemic cell lines were treated with 1 μ M MRK-003 for the times periods indicated and Hes1 and Deltex1 expression examined using RT-PCR. GAPDH was used as an internal control. (B) MRK-003 treatment of mouse leukemic cell lines results in growth arrest and apoptosis. Three murine leukemic lines (720,135,5151) were treated for 72 hours with 1 μ M DAPT, 1 μ M MRK-003 or 0.01% DMSO. Cells were harvested and stained with PI and DNA content measured by flow cytometry. Three independent tumors were analyzed, one representative experiment is shown. Statistics were analyzed using a Kruskal-Wallis test.

GSI treatment prolongs survival in a mouse T-ALL model

These ex vivo studies indicated that MRK-003 administration to leukemic mice might induce apoptosis in vivo, decrease tumor burden, and increase overall survival. To test this possibility, we treated mice daily with 50 mg/kg, 200 mg/kg, or 1000 mg/kg MRK-003 and determined plasma concentrations of the compound. We found that effective plasma MRK-003 concentrations (1-10 μ M) were achieved in mice treated with all 3 doses (Figure 2A). However, when the MRK-003 compound was given daily at 150 mg/kg, the mice began to develop diarrhea and lose weight (Figure 2C; R1 compared with V). Chronic GSI administration is known to result in gastrointestinal (GI) toxicity due to Notch inhibition in the intestinal epithelium, resulting in gut metaplasia. To assess GSI efficacy on mouse leukemic growth, we adopted an intermittent GSI dosing regimen that achieved effective concentrations of the drug without associated toxicities. Specifically, mice treated with 150 mg/kg MRK-003 for 3 days followed by a 4-day rest period had effective plasma compound levels (Figure 2B), did not lose body weight (Figure 2C, mouse R2), and exhibited limited gut metaplasia. MRK-003 plasma levels peak after administration and effective drug concentrations are detected at 24 hours after treatment. In addition, in a preclinical mouse model, this intermittent dosing schedule was well tolerated, as no evidence of intestinal effects were detected after a 35-day treatment period. In an attempt to accurately reflect the clinical experience, near-end-stage leukemic days followed by a 4-day recovery period. After the 3 days of GSI treatment, plasma compound levels were determined and an EC_{50} of 5 to 10 μ M MRK-003 was achieved (data not shown). Leukemic mice were treated with vehicle or MRK-003 after the 3 days on, 4 days off treatment regimen throughout the duration of the study. We found that GSI treatment resulted in a statistically significant increase in overall survival of leukemic mice compared with vehicle-treated mice (Figure 2D; $P < .005$). The median survival period for GSI-treated mice was 18 days compared with 3 days for the vehicle-treated group. In most cases, responses were evident in the MRK-003-treated mice immediately after the 3-day treatment period, as measured by an increase in physical activity and

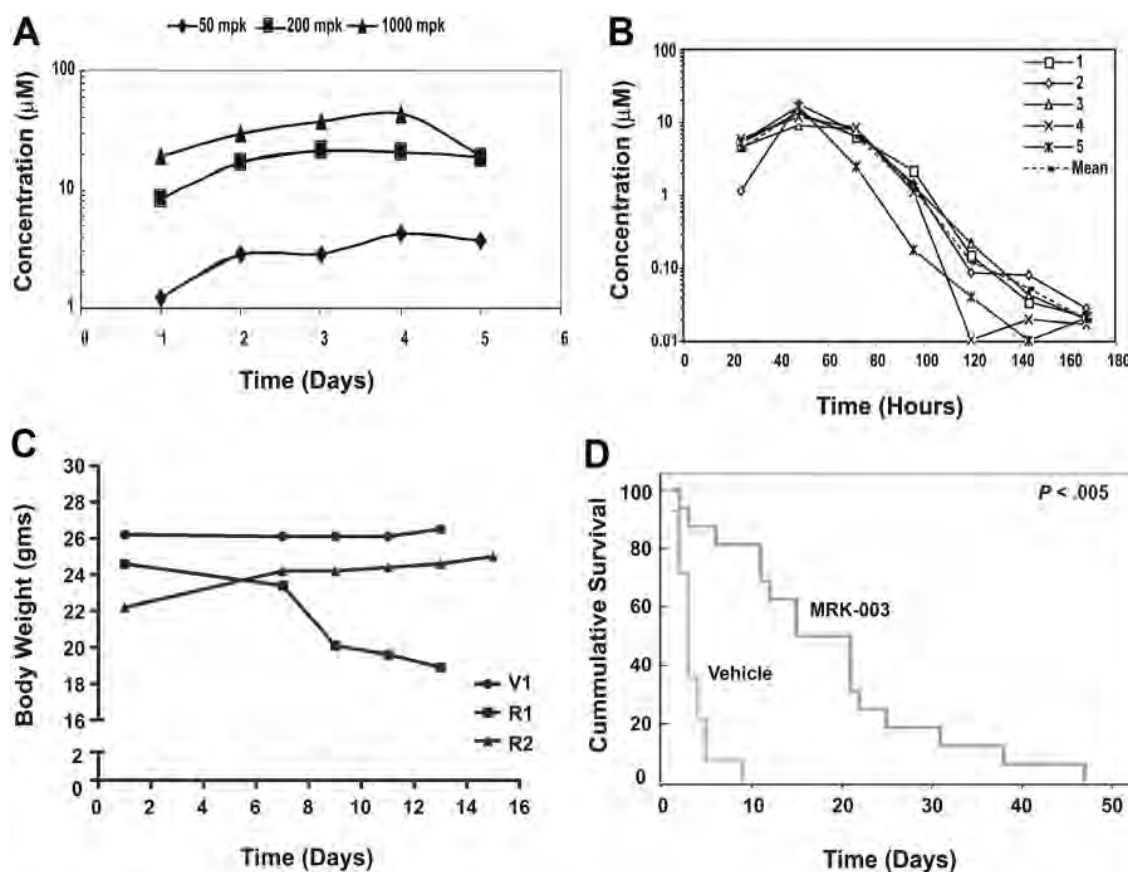


Figure 2. GSI treatment prolongs survival in a mouse T-ALL model. (A) Effective plasma compound levels in MRK-003-treated mice. Compound serum levels were analyzed 1 to 5 days after continuous dosing of MRK-003 treatment. Effective and stable compound levels (1-10 μM) were detected in the serum of treated mice after 12 hours at each drug concentration tested. (B) Plasma compound levels decrease during 4-day rest period. Mice were treated for 3 consecutive days with 150 mg/kg MRK-003. After the first treatment, serum was analyzed for compound levels every 24 hours for the duration of the dosing regimen. (C) Intermittent GSI dosing minimizes "on-target" gastrointestinal toxicity. To define a GSI dosing regimen with limited/no associated toxicity, mice were administered vehicle (V) or 150 mg/kg MRK-003 by oral gavage everyday (R1) or for 3 days followed by a 4-day rest period (R2). Mice were monitored daily for loss of body weight and for evidence of diarrhea. (D) Extended survival in MRK-003-treated leukemic mice. Near-end-stage diseased *Tal1/Ink4a/Arf+/-* mice were treated with 150 mg/kg MRK-003 ($n = 16$ mice) or 0.5% methylcellulose ($n = 14$ mice) orally for 3 days and rested for 4 days until mice were deemed moribund. Median survival for T-ALL mice treated with vehicle is 3 days, and 18 days for GSI treated mice ($P < .005$).

improved rates of respiration. Importantly, body weights are maintained in MRK-003–treated animals after successive cycles of intermittent GSI dosing. This pilot study supports the idea that GSI-associated toxicities may be overcome with intermittent dosing and provides evidence that Notch1 inhibition improves mouse leukemic survival in vivo.

GSI treatment induces apoptosis of leukemic cells in vivo

To determine whether GSI treatment induces apoptosis in vivo, we performed TUNEL staining on thymomas isolated from vehicle- or GSI-treated mice. We detected increases in the percentage of apoptotic tumor cells in mice treated with MRK-003, compared with tumors exposed to vehicle only (Figure 3A-B; $P = .034$ by Wilcoxon rank sum test). However, similar to our in vitro data (Figure 1B and (Sharma et al., 2006)), the in vivo tumor response to GSI was variable. Three of the 4 GSI-treated mice examined exhibited an increase in apoptotic leukemic cells, with 2- to 10-fold increases in TUNEL⁺ tumor cells observed (Figure 3B). An increase in TUNEL⁺ cells was not observed in 1 of 4 tumors from the GSI-treated group (mouse 6448). However, we were unable to detect a Notch1 mutation in this tumor. The reasons for the variable GSI responses both in vitro and in vivo are unclear. One possibility for the variable in vivo responses may be that some tumors require longer treatment periods. For these studies, leukemic mice were treated with vehicle or with GSI for 3 days and then the mice were humanely killed and tumor sections analyzed for the presence of apoptotic cells. It is conceivable that more consistent responses may be observed in leukemic mice treated with multiple GSI doses. Nonetheless, GSI treatment clearly induced apoptosis and extended the survival of leukemic mice.

Transient GSI responses do not reflect development of GSI resistance

One potential reason for variable responses may be that the GSI treated mice develop GSI resistance. To exclude this possibility, T-ALL cell lines were generated from the GSI and vehicle treated mice and their response to GSI quantified in vitro. As expected, truncated Notch1 proteins were detected in both vehicle and GSI treated mice and GSI treatment significantly reduced Notch1 protein levels (Figure 4A). Leukemic

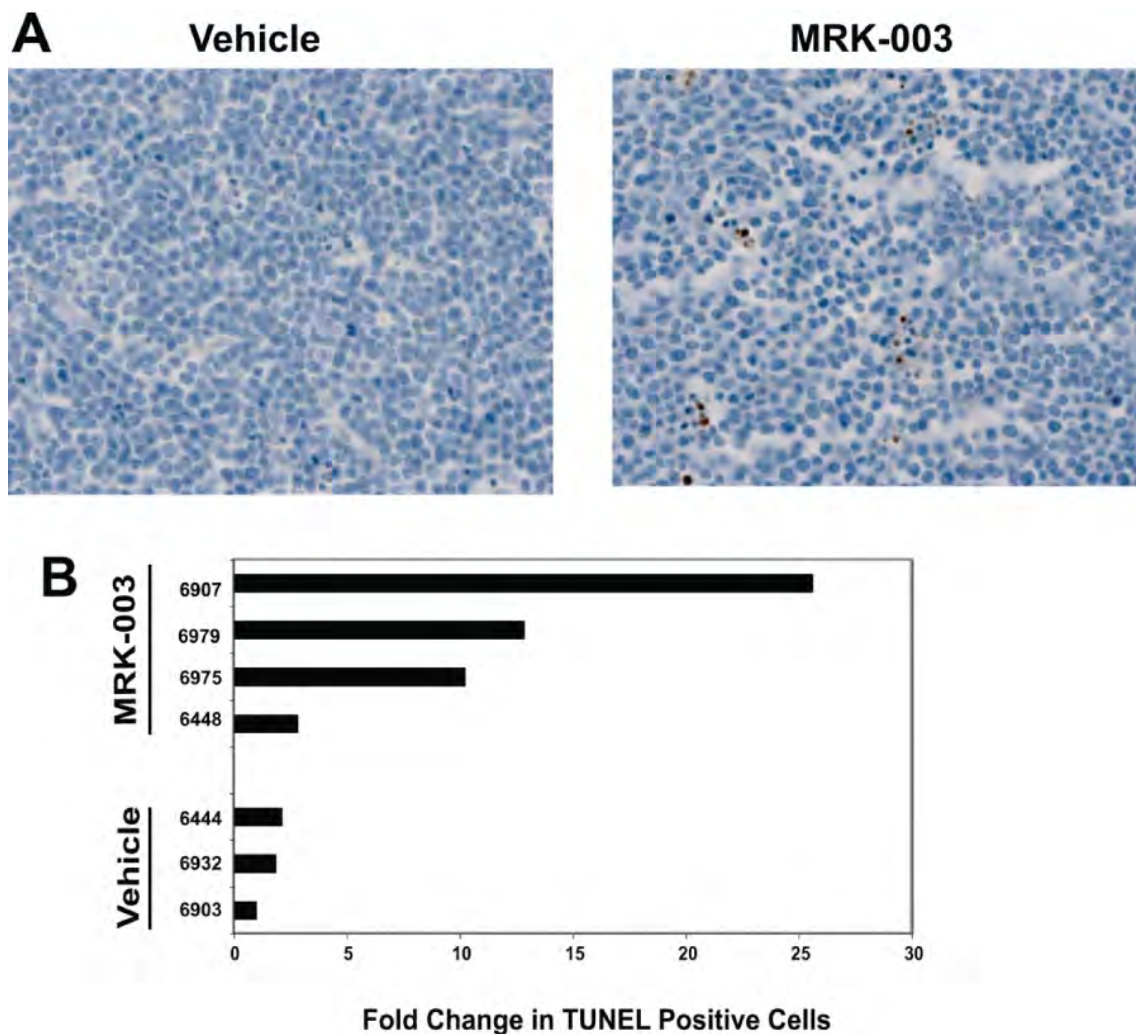


Figure 3. GSI treatment induces apoptosis of leukemic cells in vivo. (A) Leukemic mice were treated with a 150-mg/kg dose of MRK-003 or with vehicle for 3 days. Tumor sections were fixed in 10% buffered formalin and number of apoptotic cells was quantified using TUNEL assay. (B) Bar graph representing the fold change in TUNEL-positive cells compared with vehicle. Ten independent fields/section were counted to obtain the representative value.

cell lines also clearly remained dependent on Notch1 for growth, as G1 arrest and apoptosis was observed upon GSI treatment (Figure 4B). These findings indicate that transient responses to GSI in vivo do not reflect the development of GSI resistance.

Repression of Notch1 target gene expression in GSI treated mice

To further understand why GSI treatment prolonged survival but failed to eliminate disease, we examined Notch1 target gene expression using real time PCR in the vehicle and GSI treated leukemic cohorts. We found *hes1* expression reduced in MRK-003 treated leukemic mice compared to mice that received vehicle only (Figure 5). The levels of *hes1* repression were similar to levels observed when the leukemic cell line 720 is treated with GSI in vitro.

Discussion:

The prevalence of NOTCH1 and FBW7 mutations in human T-ALL (O'Neil et al., 2006; O'Neil et al., 2007; Thompson et al., 2007; Weng et al., 2004) and in mouse T-ALL models (Dumortier et al., 2006; Lin et al., 2006; O'Neil et al., 2006) prompted several laboratories to ask whether leukemic growth remained NOTCH1-dependent. GSI treatment of mouse and human T-ALL cell lines and primary mouse tumors expressing mutated Notch1 proteins (Figure 1C) revealed that sustained Notch1 signals appear required for continued growth and survival in vitro (Lewis et al., 2007; O'Neil et al., 2006; Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2004; Weng et al., 2003). These findings raised the exciting possibility that Notch1 inhibition may prove effective in treating T-ALL patients and led to the opening of a phase 1 clinical trial on 8 relapsed leukemia and lymphoma patients. However, the trial closed due to significant dose limiting toxicities with the critical question regarding GSI efficacy in T-ALL unanswered.

To address this question, we examined the effect of GSI treatment in our mouse T-ALL model where the effects of Notch1 inhibition could be addressed on primary tumors opposed to relapsed disease (and where microenvironmental influences remained intact). Seventy four percent of *tal1/ink4a/arf*^{+/-} transgenic mice develop a T-ALL like disease due to mutations that result in premature truncation of the Notch1 receptor (O'Neil et al., 2006). To determine whether Notch1 can be targeted in vivo, we needed to develop a GSI dosing regimen that minimized the 'on target' gastrointestinal (GI) toxicity associated with Notch1 inhibition. We determined that intermittent GSI dosing

(150mg/kg three days on and four days off) reduced the GI toxicity and then tested whether this regimen had any effect on the survival of leukemic *tal1/ink4a/arf*^{+/-} mice. We found that GSI treated mice

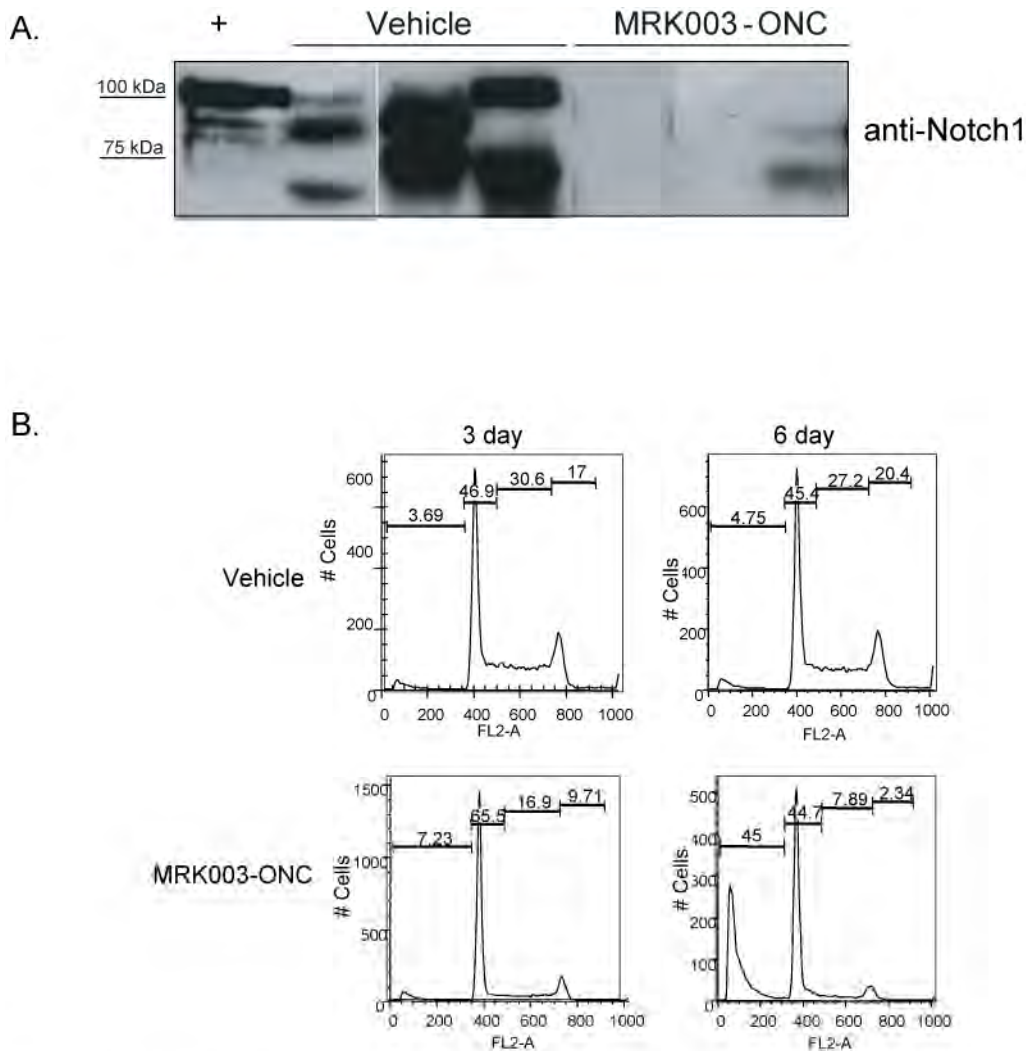


Figure 4. GSI-treated tumors do not appear to develop GSI resistance. Thymomas from MRK-003- or vehicle-treated mice were harvested from the animals and converted to in vitro culture. (A) Leukemic cell lines from vehicle- and GSI-treated mice express high levels of intracellular Notch1 and remain GSI responsive. Leukemic cell lines were treated for 48 hours with 1 μ M MRK-003 or DMSO carrier. Cell lysates were examined for intracellular Notch1 levels by immunoblotting with an anti-Notch1IC Val1744 and anti- β -actin antibodies. (B) Leukemic growth remains Notch1 dependent. Leukemic cell lines, generated from vehicle- and GSI-treated mice, were treated with vehicle or 1 μ M MRK-003 for 3 and 6 days. Cells were then assayed for DNA content by staining with propidium iodide followed by flow cytometry. The figure is a representative experiment using cell line 6838

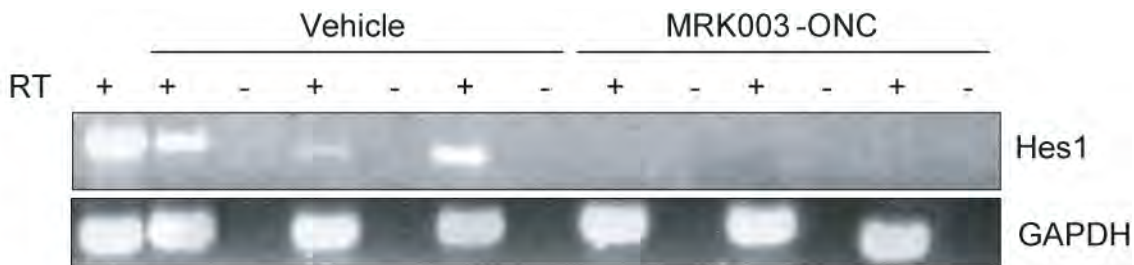


Figure 5. Notch1 target gene expression is repressed in GSI-treated leukemic Tal1/Ink4a/Arf^{+/-} mice. At killing, thymomas were harvested from vehicle- and MRK-003-treated mice and Hes1 expression was quantified using RT-PCR.

survived on average 15 days longer than leukemic mice treated with vehicle only.

Notch1 target gene expression (hes1 and c-myc) was repressed and increased numbers of apoptotic cells were detected in the thymic masses of diseased mice treated with GSI.

These data support the idea that Notch can be successfully targeted *in vivo* and that treatment with GSI as a single agent has efficacy and extended the survival of leukemic mice.

Despite the expression of other T-ALL associated oncogenes such as tal1/scl and lmo1/2 and the loss of tumor suppressors (ink4a/arf, pten), mouse T cell leukemic growth appears preferentially 'addicted' to the Notch1 proliferative signal(s). Although GSI treatment significantly increased the overall survival period, it was not sufficient to cure the mice and 100% of the GSI treated mice eventually succumbed to disease. The transient GSI response was not due to the development of GSI resistance, as all mouse tumors tested remained GSI responsive when re-examined *in vitro*. One possibility is that the transient response to GSI may reflect the intermittent dosing regimen and long term suppression of Notch1 activity may require more frequent GSI administration. Alternatively, chronic GSI treatment may promote reliance on other growth and survival pathways. For example, Gleevec administration to mice with CML-like disease shifts the reliance from activated ABL towards a greater dependence on the IL-7 signaling pathway (Williams et al., 2006). Both studies highlight the importance of examining drug efficacy in mouse models that maintain the appropriate microenvironment.

Although the results from this mouse GSI study are promising, human T-ALL cell lines are far less responsive to GSI treatment in vitro (Lewis et al., 2007; Weng et al., 2004). Even at increased concentrations and extended incubation periods (>10days), little evidence of cell death is observed when human T-ALL cell lines or primary leukemic samples are treated with GSI (Lewis et al., 2007; Palomero et al., 2006; Weng et al., 2004). These findings cast doubt as to whether GSI or other Notch1 inhibitors will be effective in T-ALL patients. Additionally, an effective GSI dose with minimal toxicity may be difficult to achieve in patients. In fact, we found the response of GSI strain dependent, raising the possibility that genetic background may influence patients GSI responses (Pradip Majumder, unpublished data or personal communication).

Materials and Methods:

Mouse T-ALL Cell Lines

Murine leukemic cell lines were cultured in RPMI with 10% FBS, 1% glutamine, Penicillin/ Streptomycin, 50 μ M β -mercaptoethanol at 37°C under 5% CO₂. To inhibit Notch1 signaling, cells were plated at 1x10⁶ in a 10cm dish in the presence of either MRK-003 (Merck Research Laboratories, Inc.). Mock-treated cells were cultured with DMSO at a final concentration of 0.01%.

GSI efficacy studies

A cohort of tal1/ink4a/arf +/- transgenic mice was generated (n= 30) and monitored daily for the onset of leukemia as described previously (Shank-Calvo et al., 2006). Once disease became evident (respiratory distress, inactivity, weight loss) leukemic tal1/ink4a/arf +/- mice were randomly assigned to the vehicle or GSI treatment groups (vehicle cohort n=14, GSI cohort n=16). Leukemic mice were administered either a 150mg/kg dose of freshly prepared MRK-003 (dissolved in 0.5% methylcellulose) or a comparable volume of 0.5% methylcellulose by oral gavage. Mice were treated for three consecutive days followed by a rest period of four days. Mice were euthanized when deemed moribund by a third party blinded to the treatment group. Survival data was plotted using Kaplan-Meier survival curves and statistical analysis performed using SPSS software.

TUNEL staining

Paraffin embedded tumor sections were analyzed for apoptosis using the ApopTag Plus peroxidase TUNEL kit (Chemicon, Temecula, CA). Approximately, 10 fields from each slide were counted and compared to the serial section not treated with the TdT enzyme.

Flow cytometry

Mouse leukemic cells were pelleted by centrifugation for 10 minutes at 2000 rpm, washed in PBS, and resuspended in 70% ice-cold ethanol. Cells were fixed overnight and then stained with propidium iodide. DNA content was analyzed by flow cytometry (FACScan) (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo version 7.0 (Tree Star, Ashland, OR). Following GSI or vehicle treatment for three days, primary thymic masses isolated directly from tal1 transgenic animals were stained with FITC-Annexin V/PI and analyzed by flow cytometry (BD Biosciences, San Jose, CA).

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