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Loss Of Rap1gap: A Driver In The Progression From Dcis Of The Breast To Idc

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LOSS OF RAP1GAP: A DRIVER IN THE PROGRESSION FROM DCIS OF THE BREAST TO IDC

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DEDICATION

This dissertation is dedicated to all those that are affected by cancer and to all the little girls, stutterers and future trail blazers who will influence research for the better.

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BM Basement Membrane

CT Cycle Threshold

DCIS Ductal Carcinoma *In situ*

DIC Differential Interference Contrast

DQ Dye Quenched

ECM Extracellular Matrix

EMT Epithelial to Mesenchymal Transition

ER Estrogen Receptor

ERK Extracellular Regulated Kinase

GAP GTPase Activating Protein

GEF Guanosine nucleotide exchange factor

GFP Green Fluorescent Protein

GTP Guanosine Triphosphate

HER2 human epidermal growth factor receptor 2

IDC Invasive Ductal Cancer

IHC Immunohistochemistry

LOH Loss of Heterozygosity

MAPK Mitogen Activated Protein Kinase

MEK MAPK/ERK1/2 KINASE

NGS Next Generation Sequencing

PBS Phosphate Buffered Saline

PR Progesterone Receptor

qPCR Quantitative polymerase chain reaction

rBM Reconstituted Basement Membrane

SEER Surveillance Epidemiology and End Results

CHAPTER 1: INTRODUCTION

1.1 DCIS (Ductal Carcinoma *In situ*)

Mortality from breast cancer has declined for the past two decades, and this decline (Siegel, Naishadham et al. 2012) was thought due to both introduction of screening programs in the 1980s, resulting in earlier diagnosis and intervention (Kopans 2011, Puliti and Zappa 2012), and the development and optimization of chemotherapy (Early Breast Cancer Trialists' Collaborative, Peto et al. 2012). Meta-analysis of 11 randomized clinical trials with 13 years follow-up estimated reduction of 20% of cancer in women invited for screening (Independent 2012).

DCIS (Ductal Carcinoma *in situ*) accounts for 15%-25% of newly diagnosed breast cancer cases in the United States (Polyak 2010). DCIS, by definition, is a non-invasive cancer. It is characterized by the accumulation of abnormal cells in the mammary duct without invasion into the basement membrane and into the stroma (Burstein, Polyak et al. 2004). Unlike the dramatic improvement in the detection of DCIS, the molecular mechanisms that govern the progression to invasive phenotype are shrouded in mystery. Until 1980, DCIS represented less than 1% of breast cancer (Van Cleef, Altintas et al. 2014). Apparent incidence has increased, in part, due to the increasing use of mammography screens and improved imaging technologies (Kuerer, Albarracin et al. 2009). This means that more DCIS lesions are detected earlier and more frequently due to improvements in imaging technology (i.e., mammograms).

Overdiagnosis is a direct result of mammography and is manifested by the dramatic increase in DCIS (Gotzsche and Nielsen 2011, Gotzsche, Jorgensen et al. 2012). Overdiagnosis refers to a detection of subclinical disease that, if left untreated,

would not cause symptoms or death. The National Institutes of Health Office of Medical Application Research commissioned a review on the incidence, treatment and outcomes of DCIS (Virnig, Tuttle et al. 2010). The results led to the conclusion that the incidence of DCIS has risen from 1.87 per 100,000 in 1973 to 3.25 in 100,000 in 2004, with the increase mostly accounted for by the introduction of screening mammography (Virnig, Tuttle et al. 2010). Recently, Welch et al. used 1975-2012 data from the SEER (Surveillance, Epidemiology and End Results) database to calculate the tumor-size distribution and size specific incidence of breast cancer among women 40 years and older (Welch, Prorok et al. 2016). They concluded that after the introduction of screening, the proportion of detected breast tumors that were small (invasive tumors measuring <2 cm or *in situ* tumors) increased from 36% to 68%. The proportion of detected tumors that were large (invasive tumors measuring ≥ 2 cm) was decreased from 64% to 32%. The authors concluded that although the rate of detection of large tumors fell after the introduction of screening, this distribution was primarily the result of additional detection of small tumors. They add that women are more likely to have breast cancer overdiagnosed than have earlier detection of a tumor that was destined to grow in size.

Some DCIS, if left untreated, will rapidly progress to invasive cancer and/or recur following treatment whereas others will remain indolent (not grow or progress to an invasive disease that may require therapeutic intervention). Thus, once DCIS is detected, treatment is frequently offered to women even though the majority present with an indolent form of DCIS (Sanger, Engels et al. 2014), resulting in overtreatment. Overdiagnosis leads to overtreatment that may not benefit the patient and can

potentially harm her because of overdiagnosed disease. The “harm” is the cost to a woman’s mental (anxiety) and physical well-being (i.e., side effects from unnecessary treatment). A consequence of overdiagnosis is that in some cases, women have their cancer treated by surgery, radiation therapy or medication, but neither the woman nor the physician can know whether this cancer would be the one that could possibly lead to death or one that would have remained undetected for the rest of the woman’s life (Independent 2012). An indolent DCIS, if left alone, is less likely to threaten the patient’s health within her lifetime and therefore does not require treatment (Kaur, Mao et al. 2013). The current recommended treatment for DCIS is wide excision lumpectomy, with or without radiation, mastectomy or hormone therapy after surgery (http://www.breastcancer.org). Out of 496,488 women diagnosed with DCIS, 33.4% had a single mastectomy and 7.0% had a contralateral prophylactic mastectomy (removal of the opposite healthy breast) (Wong, Freedman et al. 2016).

The issue of overdiagnosis and overtreatment has led to the launching of clinical trials that address the management of low risk DCIS. For example, LORD (Management of Low-risk DCIS), sponsored by the European Organization for Research and Treatment of cancer, is a randomized, international, multicenter phase III trial that aims to determine whether screen-detected low-grade DCIS can safely be managed by an active surveillance strategy or conventional treatment (radiation) over a 10 year period. The primary outcome is an ipsilateral recurrence (defined by location on the same side as primary lesion) and survival without invasive breast cancer (https://clinicaltrials.gov/ct2/show/NCT02492607?term=DCIS). This new trial is just beginning to accrue patients and so results are not available. Another recent project,

named WISDOM (Women Informed to Screen Depending on Measures Risk) aims to investigate whether a an individualized (tailored to the individual patient; based on family history) approach to breast cancer screening is as safe and effective as annual mammograms (J. Clin Oncol 34, 2016 suppl; abstr e 13035). Therefore, it is important to distinguish between which lesions will progress to invasive breast cancer, and to illuminate the pathways that drive the progression to IDC (invasive ductal cancer). Additional clinical trials address the efficacy of current treatment options, such as the sole employment of wide-excision without radiation therapy for treatment of small DCIS of grade 1-2 (https://clinicaltrials.gov/ct2/show/NCT00165256).

DCIS is heterogeneous, expressing a variety of markers such ER (Estrogen Receptor), PR (Progesterone Receptor), HER2/ERBB2 (Human Epidermal Growth Factor Receptor), etc. To address this heterogeneity, multiple clinical trials have been designed to assess the treatment of DCIS, tailored to the expression of these markers. For example, clinical trials evaluating the treatment of HER2/EGFR expressing DCIS (of high grade) with lapatinib were designed to test the inclusion of lapatinib treatment as an interval between biopsy diagnosis and surgery. This study was terminated due to low accrual (https://clinicaltrials.gov/ct2/show/NCT00555152?term=DCIS&rank=21).

Meta-analyses have also been performed on multiple studies focusing on DCIS. Analyses were performed by the Cochrane Breast Cancer Group in 2013 on four randomized controlled trials totaling of 3925 women. They compared treatment with breast conserving surgery alone *vs.* breast conserving surgery with the addition of radiation therapy. The results suggested that the addition of radiation therapy reduced the risk of recurrence of either DCIS or breast cancer in the treated breast by 51%, with

no long-term toxicity from radiotherapy (Goodwin, Parker et al. 2013). Another review, published in 2012 by the same group, examined whether women taking tamoxifen lived longer compared to those who did not take hormone therapy after local excision. The findings were based on two large studies with 3375 participants. The review states that even though administration of tamoxifen after local excision of DCIS reduced the risk of DCIS recurrence, it did not reduce the risk of overall mortality (Staley, McCallum et al. 2012). The results of other clinical trials suggest that treatment of DCIS with adjuvant tamoxifen may be beneficial to DCIS patients. A NSABP (National Surgical Adjuvant Breast and Bowel Project) B24 study was performed to retrospectively evaluate the benefits of adjuvant tamoxifen in patients with DCIS after lumpectomy and radiation (Allred, Anderson et al. 2012). The results of the study suggest that treatment of DCIS with tamoxifen after lumpectomy and radiation conferred benefit to patients with ER+ DCIS. Patients with ER-positive DCIS treated with tamoxifen *(vs* placebo) showed significant decrease in subsequent breast cancer at 10 years. No significant benefit was observed in ER- DCIS. One thousand seven hundred and ninety-nine patients were recruited. The absolute risk of ipsilateral or contralateral recurrence was reduced from 19 per 1000 women to 6 per 1000 women. These data suggest that adding tamoxifen post-lumpectomy/radiation may benefit patients.

Previous expression profiling studies have demonstrated that very few genes are differentially expressed between DCIS and invasive breast cancer of similar grade (Ma, Salunga et al. 2003, Porter, Lahti-Domenici et al. 2003, Chin, de Solorzano et al. 2004, Sgroi 2010). A few include COX2, Ki67 and pRB (Polyak 2010). Our previous RNAseq studies also found down-regulation of DST, HSRA1 and TGFBI in the progression to

DCIS (Kaur, Mao et al. 2012), which is in agreement with other studies (Calaf, Echiburu-Chau et al. 2008, Lee, Stewart et al. 2012, Wang, Eckert et al. 2012). Molecular screening approaches have been developed, and they are useful in making informed decisions about post-surgery adjuvant treatments or recurrence (Rudloff, Jacks et al. 2010). A subset of markers from the 21 gene Oncotype DX® test can predict the likelihood of progression (Solin, Gray et al. 2013). A recent pooled analysis of four prospective studies regarding the application of Oncotype DX to ER+ HER2- patients has been performed. The conclusion was that employment of this test led to the decrease in chemotherapy recommendation rate by 11% (from 55% to 44%). The highest change in chemotherapy recommendation rates were in patients who would have been originally recommended for chemotherapy for grade 2 tumors (Albanell, Svedman et al. 2016). The Oncotype DX DCIS Score –short form estimates the risk of ipsilateral breast recurrences in patients with DCIS treated with breast conserving surgery without adjuvant radiation therapy. Recent studies were also designed to evaluate the cost effectiveness of using the Oncotype DX DCIS score in the treatment of women diagnosed with DCIS. The authors concluded that even though usage of the DCIS score lowered the proportion of women undergoing radiation therapy, incorporation of the DCIS score was not cost effective (Raldow, Sher et al. 2016). The authors emphasize the importance of engaging patients in the complex and controversial decision making process.

In the traditional breast cancer progression model, invasive breast cancers were thought to rise through a stepwise linear progression from normal breast epithelium to hyperplasia to atypical hyperplasia to ductal carcinoma *in situ* to invasive breast cancer,

resulting from progressive accumulation of genetic mutations in epithelial tissue. This progression model has been supported by human clinical and epidemiological data and by molecular clonality studies addressing the relationships between *in-situ* and invasive areas of the same tumor and DCIS and local invasion (Polyak 2010). Other models of breast cancer progression are proposed. For example, the non-linear or branched progression models propose that DCIS would be a precursor to IDC, but different grades of DCIS would progress to IDCs of the same grade (Wellings and Jensen 1973).

Another model of progression proposes that DCIS and IDC could develop from the same progenitor cell and progress independently (Sontag and Axelrod 2005). Some evidence suggests that DCIS may even regress. Histological studies performed on high grade DCIS show evidence of regression. This is possibly due to factors such as fibrosis, chronic inflammation, and the presence of periductal T cells that elicit a targeted immune response to certain lesions (Wasserman and Parra-Herran 2015). The authors also suggest that an immune response is likely to eradicate those tumors with high immunogenicity, thus selecting for less immunogenic clones.

Analysis of clinical specimens has revealed several features of DCIS that are associated with progression to invasive cancer (Knudsen, Ertel et al. 2012, Lee, Stewart et al. 2012). Gene profiling studies from several groups comparing DCIS to invasive breast cancer have shown that gene expression programs associated with EMT (epithelial to mesenchymal transition) are associated with invasive breast cancer (Knudsen, Ertel et al. 2012, Lee, Stewart et al. 2012, Witkiewicz, Cox et al. 2014). One conclusion from these results is that DCIS undergoing EMT would have the propensity to progress in invasive disease. Cell-cell junctions and basal attachment to the

membrane are lost during the process of EMT and are associated with invasive disease (Witkiewicz, Cox et al. 2014).

Interestingly, other reports also indicate that epigenetic alterations and tumor environment play a role in DCIS progression. Analysis of bulk and micro-dissected tissues has suggested that genes differentially expressed between DCIS and IDC are enriched for changes in processes related to the tumor microenvironment (Lee, Stewart et al. 2012, Vargas, McCart Reed et al. 2012). Additional reports have provided evidence that epigenetic changes (Widschwendter and Jones 2002, Fleischer, Frigessi et al. 2014) contribute to DCIS progression, and may be mediated by myoepithelial cells (Hu, Yao et al. 2008).

It is important to mention that breast cancer has been characterized into 5 different subtypes, with distinct molecular features. Sorlie *et al* reported a distinctive "molecular portrait" of breast cancer using 456 cDNA clones (Perou, Sorlie et al. 2000, Sorlie, Perou et al. 2001). The tumors were classified into five intrinsic subtypes with distinct clinical outcomes, i.e., luminal A, luminal B, HER2-overexpression, basal-like and normal like tumors (Sorlie, Tibshirani et al. 2003). These subtypes have been validated by IHC (immunohistochemistry) with some exceptions (Parker, Mullins et al. 2009). Luminal A is characterized as ER (Estrogen Receptor)/PR (Progesterone Receptor) positive. The luminal B subtype is characterized as ER or PR positive, and HER2 (Human Epidermal Growth factor receptor 2) positive. HER2 overexpressing types are characterized by HER2 enrichment, while being ER/PR negative. Basal like breast cancer is characterized as triple negative. These subtypes have been associated with different prognoses. Patients with luminal A tumors have the best prognosis and

patients with basal like breast cancer have the worst prognosis. Patients can be separated for treatment options based on their subtypes (Hon, Singh et al. 2016). This classification relates to the types of patient-derived breast cancer cell lines used in this dissertation. For example, MCF-7 and T47-D are invasive breast cancer cell lines of luminal A subtype; they express both ER and PR without HER2 amplification (Neve, Chin et al. 2006, Prat, Parker et al. 2010). MDA-MB-231, BT-549 and Hs578t are triple negative basal breast cancer cell lines (Neve, Chin et al. 2006, Prat, Parker et al. 2010).

Heterogeneity is also observed in invasive and pre-invasive DCIS with similar variation in clinicopathological features such as histological grade, ER/PR expression, HER2/ERBB2 status (Livasy, Perou et al. 2007, Clark, Warwick et al. 2011). This is particularly important, since reports have proposed that molecular features (gene signatures) associated with disease progression are unique to intrinsic subtypes (Lesurf, Aure et al. 2016). For example, invasive gene signatures for the Luminal A subtype was enriched for immune and cell cycle processes. Luminal B subtypes contained invasive signature enriched for immune-related responses, cell metabolism and cell cycle. The invasive signature for the HER2 expressing subtype was enriched for cell adhesion, ECM-receptor interaction and cell motility (Lesurf, Aure et al. 2016). These results of this study point to the hypothesis that each specific subtype undergoes a distinct evolutionary course of disease progression from pre-invasive to invasive stage.

Deregulation of tumor suppressors has been documented in the progression of DCIS to invasive breast cancer. Rb (retinoblastoma) is a key negative regulator of proliferation and connects multiple signaling pathways to cell cycle machinery (Cobrinik

2005, Knudsen and Knudsen 2008). Studies have indicated that the disruption of the Rb tumor suppressor pathway is significantly associated with recurrence and disease progression of DCIS in multiple independent cohorts. The analysis of the cohorts included direct staining and use of surrogate markers such as p16ink4a and Ki67 (Gauthier, Berman et al. 2007, Ertel, Dean et al. 2010, Kerlikowske, Molinaro et al. 2010, Knudsen, Pajak et al. 2012). However, Rb disruption alone may not be sufficient and might require overexpression of an oncogene, such as ErbB2. Studies indicate that ErbB2 over expression is frequently found in DCIS; one study documented ErbB2 overexpression in more than half of the DCIS cases analyzed (Allred, Clark et al. 1992, Hoque, Sneige et al. 2002). Studies have implicated the cooperation of loss of the Rb pathway and ErbB2 over-expression in specifically driving DCIS progression by deregulation of mammary cell proliferation, acinar morphology (Witkiewicz, Cox et al. 2014). This is one instance in which loss of tumor suppression and over-expression of oncogenes together drive the progression of DCIS to invasive breast cancer.

1.2 MCF10 Progression Series

The MCF10 progression series has been employed for this dissertation research. The model of the MCF10 family of cell lines was developed by investigators at the Michigan Cancer Foundation, now known as Karmanos Cancer Institute. The basis of the model is the human cell line of MCF10A, which was isolated from breast tissue, showing very mild, hyperplastic changes and extensive fibrocystic phenotype. This breast tissue was from a 36-year-old woman who was in good health with no evidence of malignancy (Miller, Soule et al. 1993). MCF10A is one of the series of cell lines that was established by the spontaneous immortalization of the original, mortal cultures.

These cells, though immortalized, grow in a confined monolayer with contact inhibition. The transformed MCF10.Neo T cell line was then derived from the MCF10A after transfection with mutated T24 *H-ras*. When injected into immunodeficient nude mice, the MCF10.Neo T cells develop into a series of cell lines, including MCF10.AT1 and MCF10.DCIS (Miller, Soule et al. 1993, Dawson, Wolman et al. 1996). MCF10.Neo T cells form small flat nodules (Dawson, Wolman et al. 1996). MCF10.CA1d cell line is one of five tumorigenic cell lines derived from xenografts after trocar transplantation of organoids. The organoids were derived from transplantation of MCF10AT1K.cl2 cells into mice. The other cell lines are MCF10.CA1a, MCF10.CA1b and MCF10.CA1c (Santner, Dawson et al. 2001). MCF10.CA1d cells have been employed in this project.

1.3 Next Generation Sequencing and Rap1Gap

High throughput gene expression analysis has been used to identify genes that may be up or down-regulated throughout the premalignant and malignant progression of breast cancer. Previously, NGS (next generation sequencing) was performed on RNA samples obtained from the Mattingly and Sloane laboratories from cells grown on rBM (reconstituted basement membrane). The purpose was to identify a panel of genes that could be involved in development of DCIS. Three different models of DCIS were used (MCF10.DCIS, SUM 102 and SUM 225) in comparison to MCF10A (representing non-transformed breast epithelium). SUM 102 was derived from a surgical resection of a DCIS with micro-invasion and SUM 225 was derived from a chest wall recurrence in a patient originally diagnosed with DCIS (Ethier, Mahacek et al. 1993, Ethier 1996). Data analysis was performed by the Krawetz laboratory. By comparing the number of reads on each gene in three DCIS cell lines with that in the control MCF10A cells, the

expression of 157 genes was found to be significantly altered (fold change > 4) with 63 being increased in expression in all three DCIS cell lines. Fig. 1.1 is a Venn diagram of results from mRNA-Seq analysis of three models of DCIS compared to MCF-10A, which have been published (Kaur, Mao et al. 2012). The 63 significantly increased genes common to all three DCIS cell lines included Rap1Gap, a negative regulator of Rap1 GTPase activating protein. Genomatix analysis was used to further analyze the mRNA seq results to gain insights into the common frameworks in the promoter regions of the 63 upregulated genes that were found in all three models of DCIS. From a total of 82,703 modules queried in the entire human genome, 244 promoter loci were found to be associated with these 63 up-regulated genes. Enrichment analysis (represented in Table 1) showed that the common framework (predicted cluster of regulating transcription factors) RXRF-ZF02-ZF02-PLAG-HDBP is highly enriched [336-fold] in our data-set, present in the promoters of RAP1GAP, SPRY4 and PDGFB genes (Kaur, Mao et al. 2012).

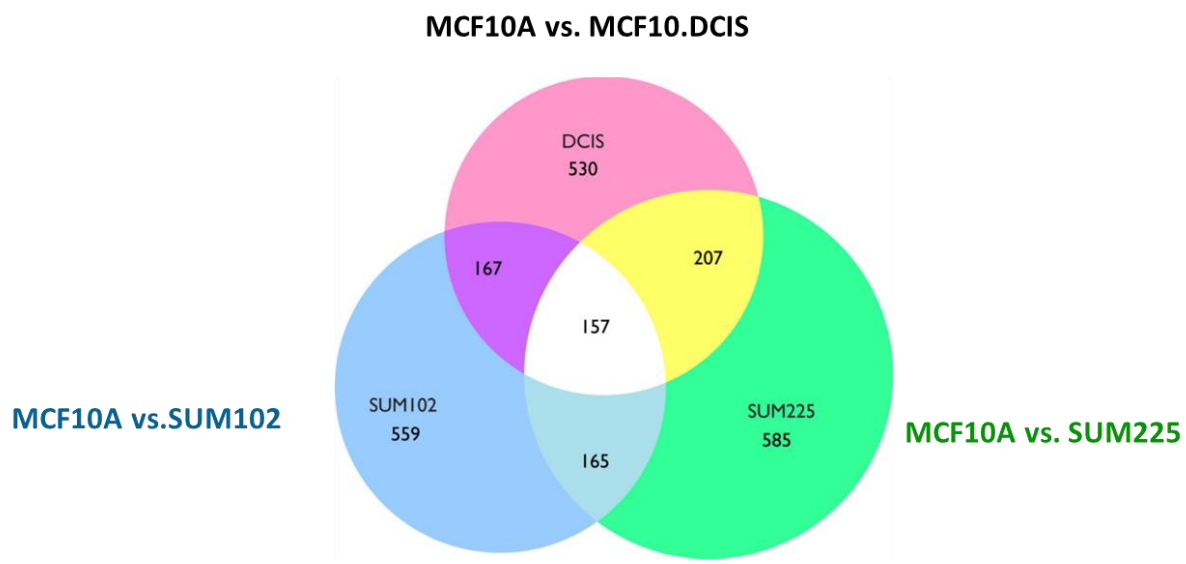


Figure 1.1: Venn diagram showing the results of microarray analysis of the three models of DCIS: MCF10.DCIS, SUM 102 and SUM 225.

There are a total of 157 genes that are consistently differentially expressed in MCF10A *vs*. DCIS models.

Biological Process	Common Framework	# framework in 244 promoters	# framework in human promoters	Enrichment
RNA metabolic process	CTCF-KLFS-ZF02-PLAG-ETSF	3	30	33.64
	ZF02-SP1F-KLFS-GLIF	3	28	36.04
	ZF02-EGRF-KLFS-GLIF	3	50	20.18
Regulation of biological processes	KLFS-EGRF-SP1F-CTCF	5	266	6.32
	SP1F-ZF02-EGRF	11	2545	1.45
Communication	RXRF-ZF02-ZF02-PLAG-HDBP	3	3	336.36
	CTCF-EGRF-SP1F-KLFS	3	48	21.02
	CTCF-ZF02-SP1F-KLFS	3	51	19.79
Regulation of macromolecule metabolic process	CFCF-EGRF-SP1F	20	5131	1.31

Table 1: **Common framework as analyzed by the Genomatix tool**.

The numbers highlighted in yellow represent the significantly enriched promoter elements for the 63 upregulated genes. Marked in red, the RXRF-ZF02-PLAG-HDBP framework was highly enriched on the Rap1Gap promoter (with an enrichment of 336).

1.4 Rap1Gap

Rap1Gap is a member of a family of GTPase activating proteins (GAPs) that facilitate GTP hydrolysis of Rap1 GTPases (Raaijmakers and Bos 2009). The *Rap1Gap* gene, which lies at 1p36-p35 in the human, is conserved in many species, including *Drosophila melanogaster, Caenorhabditis elegans, Anopheles gambia*, *Mus musculus, Rattus norvegicus* and *Homo sapiens* (from: http://www.ncbi.nlm.nih.gov/homologene?term=RAP1GAP). Rap1Gap protein is expressed in a variety of normal human tissues. Rap1Gap is abundantly expressed in the cerebral cortex, adrenal gland, salivary gland, lung, liver and gall bladder. (http://www.proteinatlas.org/ENSG00000076864-RAP1GAP/tissue). Rap1Gap protein is also expressed in thyroid follicular cells (Tsygankova, Prendergast et al. 2007), pancreatic ductal cells (Zhang, Chenwei et al. 2006) and colonic epithelial cells (Tsygankova, Ma et al. 2010).

Rap1Gap is involved in a variety of cellular processes. Some of these processes are proliferation, adhesion and morphogenesis (Altschuler and Ribeiro-Neto 1998, Asha, de Ruiter et al. 1999, Zhang, Chenwei et al. 2006). Introduction of Rap1Gap has been shown to cause delay in G1-S transition (Zhang, Mitra et al. 2006). In addition to its role in cell proliferation, Rap1Gap is involved in integrin-mediated cell adhesion (Caron, Self et al. 2000, Reedquist, Ross et al. 2000). In thyroid cancer cell lines, silencing of Rap1Gap alters the distribution of E-cadherin, β catenin and p120 catenin by reducing their accumulation at adherens junctions (Tsygankova, Ma et al. 2010).

1.4.1 Rap1Gap in Cancer

Rap1Gap has been investigated in epithelial solid tumors such as melanoma, prostate, pancreatic cancer, squamous cell carcinoma and thyroid cancer (Weiss, Biwer et al. 1997, Zhang, Chenwei et al. 2006, Tsygankova, Prendergast et al. 2007, Bailey, Kelly et al. 2009, Zheng, Gao et al. 2009, Zuo, Gandhi et al. 2010). qPCR studies in pancreatic cancer show that while Rap1Gap was expressed in normal pancreatic tissue, it was not present in pancreatic adenocarcinomas. The authors suggest that inactivation of Rap1Gap may not be critical to early pancreatic cancer development (Zhang, Chenwei et al. 2006) and may be involved in later stages of tumor progression (Zhang, Chenwei et al. 2006). Similar studies in prostate cancer implicate Rap1 activation in the promotion of prostate cancer metastasis. In addition, increase in Rap1 activity (or decrease in Rap1Gap) was observed in invasive prostate cancer cell lines, such as DU-145, PC3 and PC3-M cell lines and that active Rap enhances migration and invasion via activation of integrins (Bailey, Kelly et al. 2009). Other work on Rap1Gap in melanoma, thyroid and squamous cell carcinoma shows that Rap1Gap loss increased cell proliferation, survival, migration and invasion and that loss of Rap1Gap occurs through epigenetic silencing and LOH (loss of heterozygosity) (Zheng, Gao et al. 2009, Zuo, Gandhi et al. 2010, Banerjee, Mani et al. 2011). LOH is a common genetic event in many cancer types and is defined as one of the early observations of a change in polymorphic markers from a heterozygous state in the germline to an apparently homozygous state of DNA in cancer cells (Ryland, Doyle et al. 2015). Epigenetic gene silencing refers to non-mutational gene inactivation that can be faithfully propagated from precursor cells to clones of daughter cells. The addition of methyl groups to

cytosine residues in CpG dinucleotides in DNA is a mechanism through which epigenetic silencing is achieved (Tycko 2000).

1.4.2 Rap1Gap: A Potential Tumor Suppressor

Research suggests that since Rap1Gap is frequently lost in several tumor types, it may function as a tumor suppressor (Tsygankova, Feshchenko et al. 2004). For example, LOH of the *Rap1Gap* gene is present in a significant number of pancreatic tumors (Zhang, Chenwei et al. 2006). Loss of Rap1Gap function promotes growth, survival and invasion of pancreatic cancer cells *in vitro* and *in vivo* (Zhang, Chenwei et al. 2006). In addition, studies on primary thyroid tumor cell lines show that Rap1Gap expression is lost at a higher frequency in more aggressive tumor types via promoter hypermethylation and LOH (Zuo, Gandhi et al. 2010). Studies in prostate cancer indicate that activation of Rap1 induced prostate cancer cell migration and invasion and enhanced the rate and incidence in mouse xenograft models. Re-expression of Rap1Gap in aggressive prostate cancer cells impaired migration and invasion (Bailey, Kelly et al. 2009). In squamous cell carcinoma of head and neck, Rap1Gap is shown to inhibit proliferation (Zhang, Mitra et al. 2006), invasion (Mitra, Goto et al. 2008) and progression though the cell cycle. *In-vivo* studies indicate that human squamous cell carcinoma cells transfected with Rap1Gap produced significantly smaller tumors in mice (Zhang, Mitra et al. 2006). In leukemia, Rap1Gap also has been shown to promote apoptosis (Qiu, Qi et al. 2012). Taken together, in multiple models, Rap1Gap is lost (epigenetic silencing or LOH) in aggressive cancer cell lines and tumors. Re-expression of Rap1Gap inhibits cell proliferation and passage through the cell cycle. Experiments

in pancreatic cancer *in-vivo* mouse models indicate that introduction of Rap1Gap inhibits tumor formation, tumor size and metastasis (Zhang, Chenwei et al. 2006).

Tumor suppressors serve as transducers of anti-proliferative signals and inhibit cell cycle progression. Tumor suppressors also exert their effects through induction of apoptosis and end mitotic differentiation (Weinberg 1991). An important characteristic of tumor suppressor genes is that they are lost in many cancer types, such as melanoma, breast, small cell lung cancer, bladder cancer, retinoblastoma, colorectal cancer etc. (Klein 1987, Kaden, Gadi et al. 1989, Kaden, Bardwell et al. 1989). Rap1Gap possesses the majority of these well-studied characteristics, which suggests that Rap1Gap may function as a tumor suppressor.

1.5 Rap1Gap mRNA transcripts are highly expressed in MCF10.DCIS

The previous NGS results and Genomatix analysis revealed increased Rap1Gap transcript levels in MCF10.DCIS compared to MCF10A, thus indicating that Rap1Gap could be as a pivotal factor in the development of DCIS (Kaur, Mao et al. 2013). In order to validate the mRNA seq analysis, qPCR (quantitative polymerase chain reaction) was performed on mRNA extracted from the MCF10 cell series grown in 2D. The MCF-7 cell line served as the appropriate positive control for initial characterization of Rap1Gap. This information was obtained from abstract #3840 presented at the 102nd AACR meeting in Orlando Florida, which indicated that Rap1Gap is highly expressed in MCF-7 cells. This work was done by the Meinkoth laboratory, focused on Rap1Gap in thyroid colon cancer cells. As seen in Figure 1.2, mRNA expression levels in MCF10.DCIS are high compared to MCF10A. However, interestingly, mRNA expression in SUM 102 cells is low, which is does not agree with the original mRNA seq

analysis. A reason for this discrepancy might be due to the passage number of SUM 102 cells used in the initial mRNA seq analysis *vs* qPCR study. A repeat of the qPCR study is necessary to confirm the results. Table 2 depicts the mean CT (Comparative Threshold) values of Rap1Gap and 18S in the MCF10 progression series.

A.

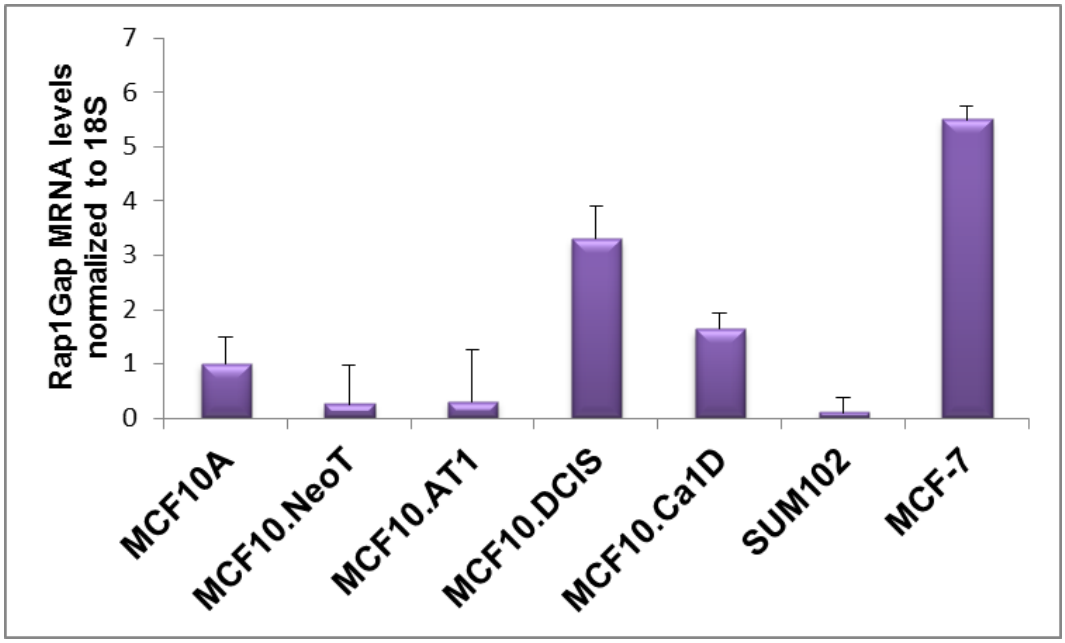


Table 2. Mean CT Values of Rap1Gap and 18S

	MCF10A	Neo T	AT1	DCIS	CA1d	SUM 102	MCF-7
Rap1Gap	30.36	32.17	32.416	28.71	29.74	33.82	27.54
18S	15.67	16.18	15.04	15.04	15.04	15.04	15.04

Figure 1.2: Validation of transcriptome data via qPCR of Rap1Gap in MCF10 series.

A. Results of Rap1Gap qPCR analysis of mRNA transcripts from the MCF10 series, SUM 102 and MCF-7 cultured in 2D. 18S was used to normalize Rap1Gap mRNA levels. Table 2 shows mean CT values of Rap1Gap and18S in the MCF10 progression series.

1.6 Rap1

Rap1 GTPase was first discovered by Kitayama and colleagues in 1989 as a gene product that normalized a malignant phenotype of *K-ras* transformed fibroblasts (Kitayama, Sugimoto et al. 1989). It was discovered later that Rap1 antagonized the activity of Ras by competing for Raf1, a serine-threonine kinase (Bos 1998). Loss of function mutations in Rap1 is lethal at the larval stage in *Drosophila melanogaster* (Asha, de Ruiter et al. 1999).

Rap1 has two isoforms- Rap1a and Rap1b. Rap1 binds either to GTP or GDP, and the transition between the two states represents a molecular switch (Takai, Sasaki et al. 2001). Endogenous Rap1 is activated through stimulation of various transmembrane receptors such as receptor tyrosine kinases, heterotrimeric G-protein coupled receptors, cytokine receptors and cell-cell adhesion molecules. These indirect upstream activators of Rap1 were discovered after the development of the Rap1 activity assay, via using Ral GDS-RBD fusion proteins (Franke, Akkerman et al. 1997). Since Rap is located downstream of so many pathways, it is not surprising that a plethora of Rap1 “on“ and “off” switches are in place to fine-tune diverse signaling pathways that are mediated by Rap1. Figure 1.3 is a depiction of the fine-tuned diverse signaling of Rap1.

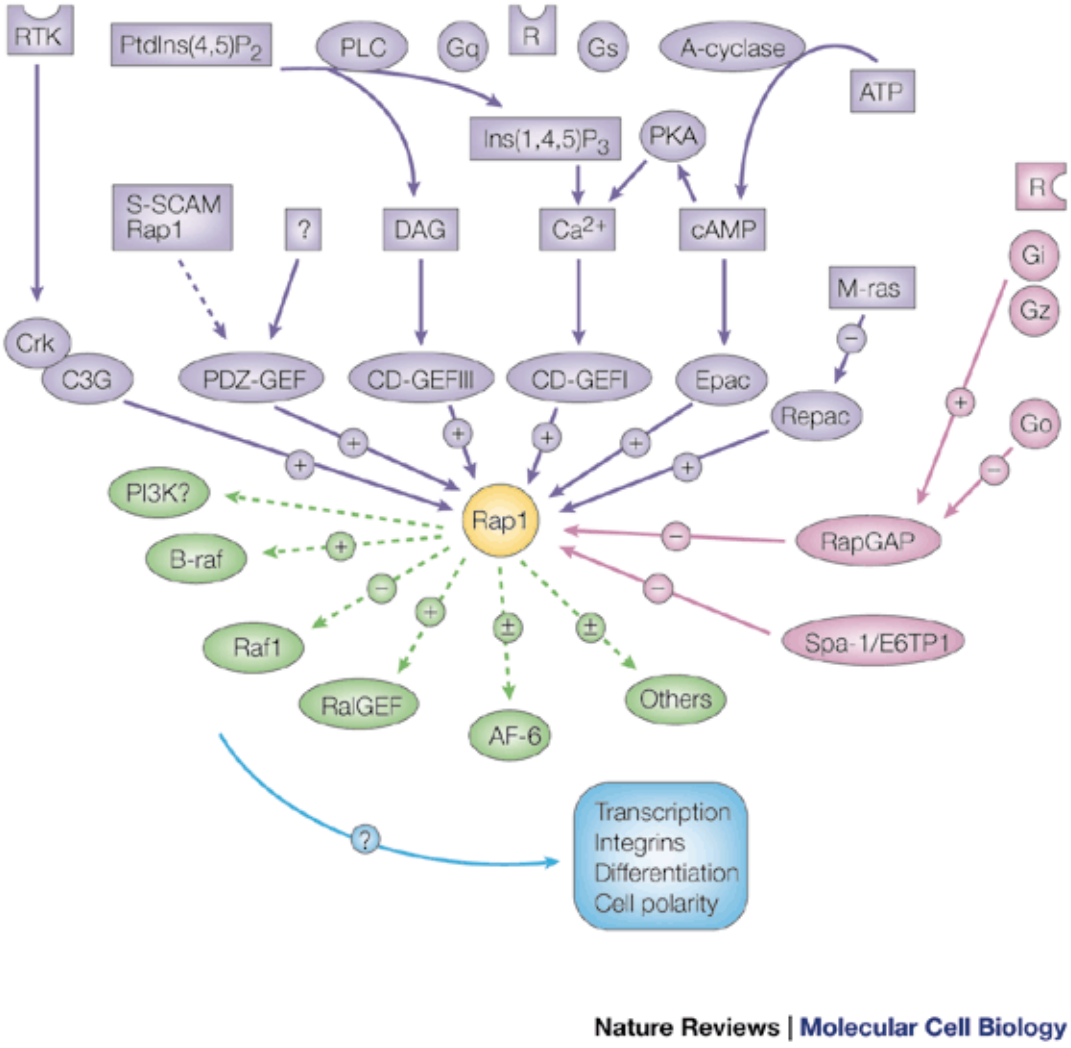


Figure 1.3: Fine-tuned regulation of Rap1.
Image taken with permission from: *Nature Reviews Molecular Cell Biology* **2**, 369-377 (May 2001)

1.6.1“ON”-The activators of Rap1- the GEFs

GEFS, which are guanine nucleotide exchange factors, facilitate the release of GDP (guanosine diphosphate) allowing Rap1 to bind to GTP. C3G (Crk SH3-domain binding guanine nucleotide releasing factor) was the first Rap1 specific GEF to be identified. It specifically mediates Rap1 activation induced by receptor tyrosine kinases such as those for PDGF and IFNα, the T-cell receptor complex, and ephrin kinase (Gotoh, Hattori et al. 1995).

Activation of Rap1 through cAMP was initially thought to indicate the possible involvement of protein kinase A, a target of cAMP. This observation eventually led to the identification of Epac1 (exchange protein directly activated by cAMP). Epac1 is activated *in vitro* and *in vivo* by direct binding of cAMP (de Rooij, Zwartkruis et al. 1998, Kawasaki, Springett et al. 1998). In addition to C3G and Epac1, two other types of GEFs have been characterized; these are the CD-GEFs and the PDZ GEFs (Gloerich and Bos 2011).

1.6.2 “OFF”- The deactivators of Rap1: the GAPs

Two families of Rap1-specific GAPs sharing a catalytic GAP-related domain (GRD) have been identified. These are the RapGaps and the SPA-1 family of proteins (Bos, de Rooij et al. 2001). GAPs enhance the intrinsic GTPase activity of Rap1 to hydrolyze bound GTP to GDP. Proteins bearing the GRD are conserved in *C. elegans* and *D. melanogaster*, extending to mammals. Figure 1.4 shows the different GAPs and their domains. Rap1Gap was the first Rap1 specific GAP to be isolated (Polakis, Rubinfeld et al. 1991).

1.6.3 The Roles of Rap1

Rap1 has been shown to be activated in response to various growth factors, cytokines and chemokines that act on receptor tyrosine kinases and G-protein couple receptors (Bos, de Rooij et al. 2001).

Initial studies following the discovery of Rap1 showed that a dominant active Rap1 mutant (Rap1V12) attenuated Ras-mediated ERK activation, probably via competitive interference with c-Raf activation by Ras (Cook, Rubinfeld et al. 1993, Hu, Kariya et al. 1997). Subsequent studies have indicated that the MAPK-related signaling pathways controlled by Ras and Rap1 may be spatially and temporally separated (Mochizuki, Ohba et al. 1999, Zhu, Qin et al. 2002, Ohba, Kurokawa et al. 2003).

Rap1 plays an important role in the regulation of cell-cell adhesion. Functional studies on Rap1 in testes of *Drosophila melanogaster* have shown that Rap1 signaling regulates morphogenic processes through the proper positioning of adherens junctions (Knox and Brown 2002, Wang, Singh et al. 2006). These results were corroborated by studies of Rap1 in the control of endothelial barrier function in endothelial cells (Cullere, Shaw et al. 2005, Fukuhara, Sakurai et al. 2005). Research has shown that while the activity of Rap1 plays a role in the organization of epithelial polarity in normal human breast epithelial cells, increased aberrant activation of Rap1, which implies lack of Rap1Gap control, can lead to tumor formation and progression to malignancy (Itoh, Nelson et al. 2007).

In addition to cell adhesion, Rap1 plays a major role in various integrin-mediated biological processes, such as immunological synapse formation, macrophage phagocytosis, chemokine-induced adhesion and transmigration of leukocytes, lymphocyte and dendritic cell homing to peripheral organs, platelet adhesion and

aggregation, as well as adhesion of several distinct cell lines to various ECM proteins such as fibronectin, fibrinogen, collagen and laminin. This is in line with studies that show that In normal and malignant conditions, the functional coordination of E-cadherin and the integrins is essential for maintenance of cellular architecture and facilitation of dissemination into the adjacent area of the stroma, especially during ductal branching in mammary gland development (Wiseman and Werb 2002).

Rap1 regulates recycling, avidity and affinity of integrins that are associated with the actin cytoskeleton (Retta, Balzac et al. 2006). In human cell lines, Rap1 controls T-cell receptor, CD31 and CD98 induced activation of $\alpha_1\beta_2$ (Katagiri, Hattori et al. 2000, Reedquist, Ross et al. 2000, Suga, Katagiri et al. 2001, Katagiri, Hattori et al. 2002, Sebzda, Bracke et al. 2002), but is also required for Mn^{+2} or activating antibody-induced $\alpha_1\beta_2$-mediated adhesion (de Bruyn, Rangarajan et al. 2002). Thus, Rap1 controls integrin-mediated cellular functions by modulating inside-out activation processes. Rap1 is suggested to regulate integrin activation either directly through their polarized spatial distribution or through the effect of cytoskeleton dynamics (Caron 2003, Katagiri, Maeda et al. 2003, Bos 2005).

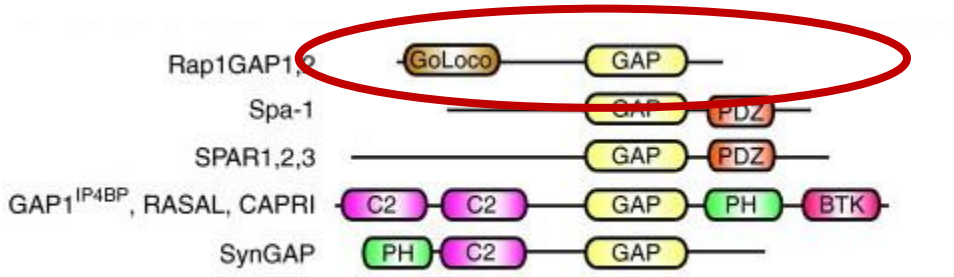


Figure 1.4: Family of Gaps.

Depicted here is a family of the GAPs, some of which are regulators of Rap1. Circled in red is Rap1Gap, which is of interest in this dissertation. The GoLoco domain, which contains the conserved Asp-Gln-Arg triad, is located at the N terminus. This domain, which plays a direct role in GDP binding, is conserved throughout multiple species of the animal kingdom. Image taken with permission from: Golrich, M. and Bos. J.L. 2011. Trends Cell Biol. 2011 Oct;21(10):615-23.

1.7 Regulation of E-cadherin

E-cadherin is a principal effector of cell-cell adhesion and is a critical determinant of tissue architecture and function in both developing and adult organisms (Wheelock and Johnson 2003). Therefore, orchestration of E-cadherin functions plays an essential role in physiological and pathological processes. The cadherins, the hallmark of a fully differentiated epithelium (Wong and Gumbiner 2003), are single-pass trans-membrane glycoproteins characterized by a variable number of extra-cellular cadherin domains that mediate cell-cell adhesion in all solid tissues. In epithelia, E-cadherin is an essential component of the adherens junctions, which are specialized calcium-dependent adhesive structures required for formation and maintenance of stable cell-cell adhesion (Wheelock and Johnson 2003). β-catenin interacts directly with the cadherin cytoplasmic tail and α-catenin binds to both β-catenin and actin, connecting the cadherin-catenin complex to the actin cytoskeleton (Balzac, Avolio et al. 2005).

E-cadherin has also been shown to behave as a tumor suppressor by inhibiting human mammary and prostate tumor cell invasion (Wong and Gumbiner 2003). In addition, a direct role of E-cadherin in the suppression of invasion has been demonstrated by the reversion of undifferentiated, invasive cells to a differentiated phenotype after the transfection of E-cadherin cDNA in cell culture models (Doki, Shiozaki et al. 1993). Studies have shown that the strong adhesive properties of E-cadherin prevent cells from dissociating from other cells and migrating into the adjacent microenvironment. This was shown by using E-cadherin blocking antibodies, which increased cell invasion (Frixen, Behrens et al. 1991). However, other studies have shown that suppression of tumor invasion by E-cadherin would be independent of

adhesion itself, and that adhesion suppressive mechanism of tumor invasion is neither necessary nor sufficient (Wong and Gumbiner 2003) for invasion. Several immunohistochemical studies have reported a strong correlation between E-cadherin loss and the initiation and progression of tumors (Berx, Becker et al. 1998). This down-regulation is generally due to transcriptional repression; loss of E-cadherin function by genetic inactivation or epigenetic silencing is a common characteristic of invasive carcinomas (Lombaerts, van Wezel et al. 2006).

1.8 The role of cytoskeletal remodeling in invasion

The cytoskeleton plays a pivotal role in the various aspects of cell physiology-including mitosis, cell division, volume control and cell polarity. It also receives, integrates and transmits both intracellular and extracellular signals. Cytoskeletal networks are composed of microtubules, actin and septins, which act as signaling processors received from the membrane (Bezanilla, Gladfelter et al. 2015). Actin and vimentin will be of focus in this dissertation, since actin and vimentin re-organization play an important role in the process of invasion.

1.8.1 Actin

Actin belongs to a family of global multi-functional proteins that form microfilaments. It participates in many important cellular processes such as muscle contraction, cell motility, cell division, polarization and cytokinesis (Doherty and McMahon 2008). Actin defines the “skeleton” of the cell and is responsible for the shape of the cell (Mattila, Batista et al. 2016) and is spatio-temporally regulated. It also has emerged as a key player in cellular signaling and is remodeled in order to provide force

and support for the cellular structures and serve as tracks for vesicle or organelle movement.

In epithelial cells, actin cortical rings are present and in tight association with cell-cell adhesions (Haynes, Srivastava et al. 2011). They are located at the inner surface of the baso-lateral side (Hofer, Jons et al. 1998). During the process of invasion, cells may acquire EMT (epithelial to mesenchymal transition)-like characteristics. These include the dissolution of cortical rings and re-organization of actin into parallel bundles or stress fibers and loss of epithelial cell polarity (Haynes, Srivastava et al. 2011).

1.8.2 Vimentin

Vimentin is a type III intermediate filament (IF) protein and is ubiquitously expressed in normal mesenchymal cells. Like actin, it plays a role in the maintenance of cellular integrity and provides resistance against stress (Satelli and Li 2011) and regulates adhesion by controlling the function of integrins (Ivaska, Pallari et al. 2007).

In the context of cancer, increased vimentin expression is observed in a variety of epithelial cancers and has been strongly correlated with increased tumor growth, invasion and poor prognosis (Satelli and Li 2011). Vimentin is also known as a canonical marker of EMT (epithelial to mesenchymal transition), which takes place in both embryogenesis and metastasis (Hay 2005); expression levels correlate with mesenchymal cell shape and motility (Mendez, Kojima et al. 2010). Studies in breast cancer show that vimentin expression is elevated in several aggressive breast cancer cell lines (Gilles, Polette et al. 2003) and is strongly correlated with increased migration and invasion of breast cancer cells (Gilles, Polette et al. 2003, Korsching, Packeisen et

al. 2005). Other studies have documented over expression of vimentin in breast cancer tissues (Kokkinos, Wafai et al. 2007).

1.9 3D Overlay Culture: An overview

The mammary epithelium is organized into two layers: the luminal epithelial cells that make milk proteins, and the highly contractile basal, or myoepithelial, cells surrounding the luminal cells. The myoepithelial cells contact the basal lamina, and regulate the function and polarity of the apical cells. Much evidence supports the idea that adhesion to the basal lamina helps to establish cellular polarity (Barcellos-Hoff, Aggeler et al. 1989, Bissell and Ram 1989). Cell–matrix adhesion regulates mammary cell structure and function in culture models. For example, inhibition of mammary epithelial cell contact with the BM (basement membrane) alters morphology, inhibits, survival, decreases proliferation, and inhibits differentiation (Barcellos-Hoff, Aggeler et al. 1989). Culturing cells on a reconstituted BM matrix or on laminin restores many of these functions (Streuli, Schmidhauser et al. 1995). Interaction of mammary cells with the ECM (extra cellular matrix) is mediated by integrins, and luminal epithelium expresses the α2β1, α3β1 and α6β1 heterodimers (Prince, Klinowska et al. 2002).

Given the significant body of evidence regarding the influence of the ECM on the structure and function of mammary epithelial cells, it makes sense to utilize 3D overlay culture in this project. It is more likely to recapitulate the *in vivo* milieu. In addition to interrogation of the effects of the ECM on structure, function and differentiation of epithelial cells, there are other important applications that will be highlighted at this time. Culture of cells on an ECM yields more physiologically relevant information about signaling pathways and drug efficacy.

The most relevant method of culture of mammary epithelial cells in 3D in rBM (reconstituted basement membrane) was developed in the Brugge and Bissell laboratories(Debnath, Muthuswamy et al. 2003, Lee, Kenny et al. 2007). A depiction of the 3D culture method utilized in this dissertation work is depicted in Figure 1.5. Cells grown on a matrix are relatively resistant to chemotherapeutic drugs (Weaver, Lelievre et al. 2002) and studies from our laboratory have found that 3D overlay culture reveals a critical sensitivity of Ras driven breast cancer models to MEK (MAPK/ERK kinase) inhibitors that was absent in a 2D microenvironment (Li, Chow et al. 2010). Additionally, the cross-talk between some signaling pathways is not apparent in 2D- and it is their growth in a 3D environment that unveils previously unknown dynamics (Wang, Weaver et al. 1998). Proof of this cross talk is observed in the more pronounced phenotypic differences between non-transformed and malignant epithelial cells cultured on rBM. This would be due to the interplay between multiple pathways governing epithelial cell polarity and organization of the structures (Petersen, Ronnov-Jessen et al. 1992, Debnath and Brugge 2005, Lee, Kenny et al. 2007). This phenomenon is observed in this project. Evidence of differences in Src signaling in 2D and 3D environments will be addressed in Chapter 5.

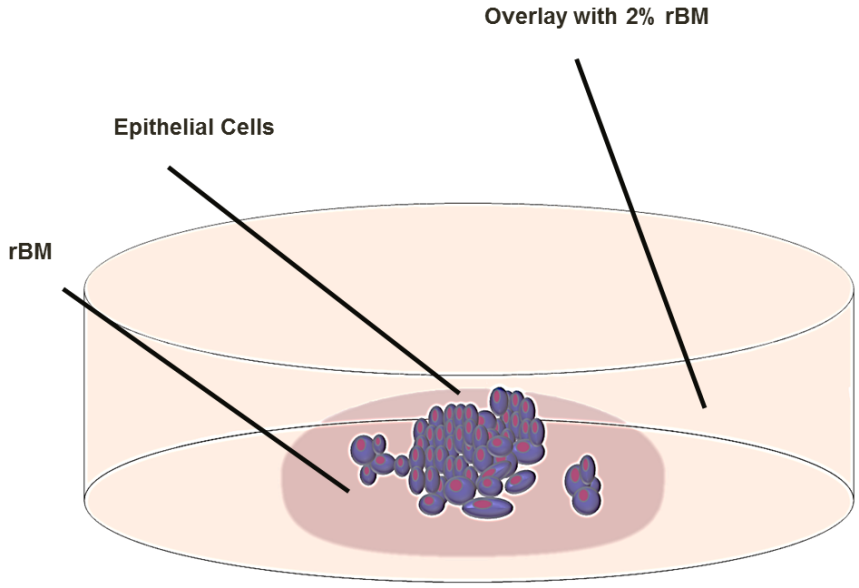


Figure 1.5: 3D Overlay Culture: A method that more accurately recapitulates *in vivo* microenvironment.

Epithelial cells are plated in rBM (reconstituted basement membrane) and an overlay added to embed the cells in a 3D matrix. When placed in a 3D environment, cells exhibit differences in organization of epithelial structures, gene expression profiles and protein expression. The 3D structures represented here simulate transformed epithelial cells (MCF10.DCIS).

1.10 ERK/MAPK

The MAPK (mitogen activated protein kinase) pathway is widely studied. The classical MAPK pathway consists of RAS, RAF, MEK and ERK. It relays a cascade of signals which regulate the processes of proliferation, differentiation, growth and migration (Dhillon, Hagan et al. 2007). Ligand mediated activation of receptor mediated kinases triggers guanosine triphosphate (GTP) loading of Ras GTPase, which then recruits Raf kinases to the plasma membrane for activation. Raf then activates MEK1/2, which in turn activates ERK1/2 (Cargnello and Roux 2011). ERK1/2 activation has been observed in a wide variety of cancers and is closely associated with cancer development, migration, invasion and metastasis (Park, Jung et al. 2011).

CHAPTER 2: METHODS

2.1 Cell Culture: 2D and 3D

MCF10A series of cells were maintained as monolayers in 2D culture at 37°C, 5% CO_2 in growth medium: DMEM/F12 (without phenol red) containing 5% horse serum, 20 ng/ml epidermal growth factor, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 50 U/ml penicillin, and 50 µg/ml streptomycin. For 3D culture, a trypsinized single cell suspension in a 4-ml volume of the assay medium (DMEM/F12 containing 2% horse serum, 5 ng/ml EGF, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, 50 U/ml penicillin, 50 µg/ml streptomycin, and supplemented with 2% (v/v) of Cultrex) were carefully loaded on top of the rBM, and will be cultured at 37°C and 5% CO_2, with the medium being changed every 4 days. 3D structures were fixed or harvested after 8 days of growth.

2.2 Retroviral Infection for shRNA Knockdown

Targeted cell lines for Rap1Gap knockdown were MCF10.DCIS, MCF10.Ca1D, and T47-D breast cancer cells. HEK293T cells were maintained at 37°C in DMEM containing 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and supplemented with 4 mM l-glutamine, and 1 mM sodium pyruvate until 90% confluent. For transfection, the medium was changed to omit the antibiotics. Then, the cells were triply transfected with equal amounts of the retrovirus construct, pVPack-GP plasmid, and pVPack-VSV-G plasmid using Lipofectamine 2000 reagent according to the manufacturer's instructions. The medium was changed 6 hours after transfection, and the cells were cultured at 37°C for 16 hours when extensive expression of the reporter dsRed proteins could be monitored by fluorescent microscopy. The medium was changed and the cells were cultured at 30°C for 24 hours for the production of retroviruses. The virus broth was

pipetted and centrifuged for 5 minutes at 200*g* to remove cell debris. Fresh medium was added and the HEK293 cells were continued to be cultured for 24 hours at 30°C to harvest a second batch of virus broth.

The supernatant broth containing viruses was supplemented with polybrene at a final concentration of 12 µg/ml by the addition of a 4 mg/ml polybrene stock solution. Cells were trypsinized, centrifuged, and resuspended in growth medium to form single cell suspensions at a concentration of 2 x 10^6 cells/ml. The cell suspensions were mixed (3:1 v/v ratio) with virus mixture, plated onto tissue culture dishes at low starting confluence (typically ~5%), and cultured at 37°C. The next day, the medium was changed, and the cells were subjected to a second round of infection using the second batch of viruses as described above. On the third day, the medium was changed again, and the culture continued. Virus transduction efficiency was checked using confocal fluorescent microscopy. The infection efficiency was ≥ 90% based on fluorescence.

2.3 Transient Re-expression of GFP-Rap1Gap

The gold standard control for knockdown experiments is re-expression of the protein of interest. Originally, the plan was to virally infect the DCIS Rap1Gap shRNA cell lines with GFP-Rap1Gap. Many attempts were made to clone GFP-Rap1Gap into various vectors. Restriction digests and ligation experiments were repeated, and sizes of the fragments were confirmed to confirm proper ligation. Repeated transformation experiments in competent bacterial cells yielded 2-3 colonies. Restriction digests of plasmid DNA isolated from the colonies did not confirm the presence of the full insert. Different competent cells and different ligation reagents were also used to troubleshoot.

After 8 months of trial and error, the procedure had to be abandoned and we decided to proceed with the transient transfection.

Targeted cell lines for transient overexpression of GFP-Rap1Gap were MCF10A, DCIS.Rap1Gap shRNA and SUM 102. Target cell lines were cultured until 80% confluent on 35mm dishes. On the day of transfection, media were changed and cells were given antibiotic free medium. Plasmid DNA and Lipofectamine 2000 were mixed in Optimem and allowed to incubate at room temperature for 5 mins. Both the solutions were mixed together and incubated for 20 mins at room temperature. The mixture was then pipetted and added to the cells. Cells were incubated at 37^{o} C for 6 h. The medium were changed and fluorescence was monitored after 48 h.

2.4 Immunofluorescence

Cells growing on rBM were cultured in 35mm dishes. To fix structures, cultures were briefly washed with pre-warmed PBS and incubated in 4% paraformaldehyde, pH 7.5 in PBS for 30 minutes at room temperature. The fixation was quenched by three washes with 0.75% glycine in PBS. Then, the cellular structures were permeabilized with 0.5% Triton X-100 (v/v) in PBS for 10 minutes and blocked with a 1-hour incubation in immunofluorescence (IF) buffer: 130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH2PO4, 0.1% BSA (v/v), 0.2% Triton X-100 (v/v), and 0.05% Tween-20 (v/v), pH 7.5. Structures were incubated at 4°C with primary antibody in IF buffer overnight in a humidified chamber. After washing three times with IF buffer, the 3D structures were further incubated with the fluorescent conjugated secondary antibodies together with other fluorescent cellular staining reagents (e.g., DAPI, Alexa Fluor-568 phalloidin or Alexa Fluor-488) at room temperature for 2 hours. After three washes with IF buffer, the 3D

structures were imaged with a confocal microscope (780; Zeiss, Göttingen, Germany) as described previously (Yang and Mattingly 2006).

2.5 3D Culture Experiments

Growth of cells in 3D rBM overlay culture (Cultrex, from Trevigen) was assayed by measurement of volume of structures formed. Fixed cells were imaged with an LSM 780 confocal microscope (Carl Zeiss GmbH, Jena, Germany) using 10x or 63x objective dipping lenses. Tile-scan mode was used to obtain a 2x2 or 4x4 phase contrast tile image.

2.6 Morphometric Analysis of Outgrowths of 3D Structures

Differential interference contrast (DIC) images of three random fields at 20x magnification were used to measure the outgrowths. Outgrowths were defined as small thorn like structures emanating from the center of the main structure. Two blinded individuals counted the outgrowths in the images captured at 20x magnification in accordance with the following parameters: outgrowths >0.5 mm in length and width were counted. Connections and branches in between the structures were not counted. Statistical analysis was performed by using one-way ANOVA by GraphPad Prism software.

2.7 Cell Proliferation Assays

Ten-thousand MCF10.DCIS-dsRed control and MCF10.DCIS-Rap1Gap shRNA cells were plated in 60-mm dishes. At 48, 96 and 144 h time points, attached cells were trypsinized and centrifuged at 1000 *g*. After resuspension, cells were counted with a hemocytometer via trypan blue exclusion, a common method used to check cell viability and facilitate counting. Live cells have intact membranes that prevent trypan blue dye

from entering. Results were recorded as cell number. Experiments were repeated three times under same conditions.

2.8 Lysate Preparation for Immunoblotting

To obtain sufficient material for immunblotting from 3D rBM cultures, the overlay culture process was performed on 60-mm culture dishes. The cultures were briefly washed with ice-cold PBS (phosphate buffered saline). 3D structures were then scraped in ice-cold PBS + 5 mM EDTA supplemented with protease and phosphatase inhibitors and rocked on ice for two hours. 3D structures were centrifuged for 3 min at 1000 *g*, resuspended and rocked on ice for 30 mins to further dissolve the Cultrex. After one wash with PBS, the cells were solubilized in a buffer designed for both lysis: 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM sodium pyrophosphate, 2 mM EDTA, 1% (v/v) Nonidet P40, 1% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 0.005% (w/v) bromphenol blue, and supplemented with protease inhibitor mixtures according to the manufacturer's instructions. The cell lysates were subjected to brief sonication and heated in 100°C for 5 min. Thirty micrograms of protein were loaded onto 8-12% SDS-polyacrylamide gels for electrophoresis, ran at 90 V for 2 h. The proteins from the gel were transferred onto nitrocellulose membrane, blocked with 2% milk solution, and probed for specific target proteins with corresponding antibodies.

2.9 Preparation of Cytosolic and Nuclear Fractions

Cells were scraped into extraction buffer containing 10 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1% NP40 (v/v) and protease inhibitor cocktail and lysed by using a 26 gauge needle. The lysate was centrifuged for 5 min at 1000 x *g*

to yield supernatant (cytosolic fraction) and pellet (nuclear fraction and any unbroken cells) which was re-suspended in RIPA buffer and sonicated.

2.10 Rap1 Activity Assay

Rap1 activity assays were performed according to the instructions of the manufacturer (Millipore). Whole cell lysates were prepared by incubating the cells in ice-cold lysis buffer (50 mmol/L Tris-HCl (pH 7.4), 0.5 mol/L NaCl, 1% NP40, 2.5 mmol/L $MgCl_2$, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 10% glycerol). Lysates were centrifuged at 14,000 × *g* for 5 minutes at 4°C. The supernatant was removed and assayed for protein concentration using the Bio-Rad protein assay.

Thirty microliters of Ral GDS-RBD agarose were added to each tube containing 0.5 mL cell extract and rotated for 45 minutes at 4°C. Pellet beads were collected by centrifugation (10 seconds, 14,000 × *g*) and washed thrice with lysis buffer. The beads were resuspended in 20 µL of 2× Laemmli buffer followed by boiling for 5 minutes. Samples were run on 15% gel, transferred, and blotted for Rap1. Light exposures of immunoblot bands for the activity assay and other immunoblots were selected to that the bands were not saturated. The blots were quantified using densitometry via Multi Gauge© software.

2.11 Invasion Assays

Thirty thousand cells of MCF10.DCIS and MCF10.DCIS Rap1Gap shRNA were seeded in serum-free media on BD (Franklin Lakes, NJ, USA) cell culture inserts (8 µm pore size) pre-coated with Cultrex® (2 mg/ml) and allowed to invade toward media supplemented with 5% horse serum for 24h. Invading cells were stained and mounted on slides using the Dif-Quik kit (Thermofisher). Cells were visualized using a Zeiss

Axiovert microscope, and were counted using ImageJ software. Data were collected from at least three independent experiments performed in triplicate.

2.12 Live-cell Proteolysis Assays

35-mm petri dishes were coated with 50 µl of rBM containing 25 mg/ml of DQ-collagen IV (2.5%) (Invitrogen Life Technologies) and placed in a 37 °C incubator for 15 min to solidify. One thousand cells were plated on top of the rBM and incubated at 37 °C for 30–60 min until they attached. The overlay was added to the cells and cultured for 8 days. Overlay is defined as assay media with lower concentration of the same ingredients as phenol red free maintenance media, with 2% horse serum and 2% Cultrex® (v/v). Phenol red free media is preferred since phenol red interferes with fluorescence detection. Degradation products of DQ collagen IV (green) were imaged with a Zeiss LSM 780 META NLO confocal microscope at 488 nm using a 20x and a 63x water immersion objectives. Z- stack images were captured and used to make 3D reconstructions of the spheroids using Volocity 4.2.0 software (Perkin Elmer, Waltham, MA). Using the Z-stack, the extent to which the cells invaded into the rBM was assessed by the presence of fluorescent cleavage products.

CHAPTER 3: RESULTS

3.1. Rap1Gap protein is highly expressed in MCF10.DCIS in both 2D and 3D conditions

Previous studies aimed to determine specific gene expression changes common to three models of DCIS in comparison to MCF10A cells as a model of normal human mammary epithelium. mRNA seq results showed that Rap1Gap was up-regulated in all three models of DCIS, compared to MCF10A cells. Multiple studies have focused on loss of Rap1Gap in acquisition of invasive phenotype in a variety of tissue types. This dissertation is focused on the loss of Rap1Gap in breast cancer.

Assessment of Rap1Gap protein expression in the MCF10 progression series was performed in order to validate the initial transcriptome analysis (Fig 1.1). The immunoblots in Figure 3.1 are of Rap1Gap protein expression in the MCF10 progression series in lysates prepared from 3D and 2D environments. The immunoblot in Panel A is of Rap1Gap levels from lysates prepared from cells grown in 3D. MCF10A cells express low levels of Rap1Gap; MCF10.DCIS expressed higher levels, and MCF10.CA1d, a model of invasive breast cancer, expressed an intermediate level. The immunoblot in panel B (Rap1Gap levels in lysates harvested from 2D culture) depicts a similar trend. Rap1Gap protein levels in MCF10.Neo T cells grown in 3D are significantly higher than lysates grown in 2D environment, but the reason for this difference is not known. For initial standardization of the antibody for Rap1Gap, lysates of MCF-7 cells were used as a positive control for immunoblotting [see published AACR abstract (#3840, 2011) from Prof. Judy Meinkoth], Thus, Rap1Gap protein levels are low in MCF10A cells, increased in MCF10.DCIS and reduced in MCF10.CA1d cells.

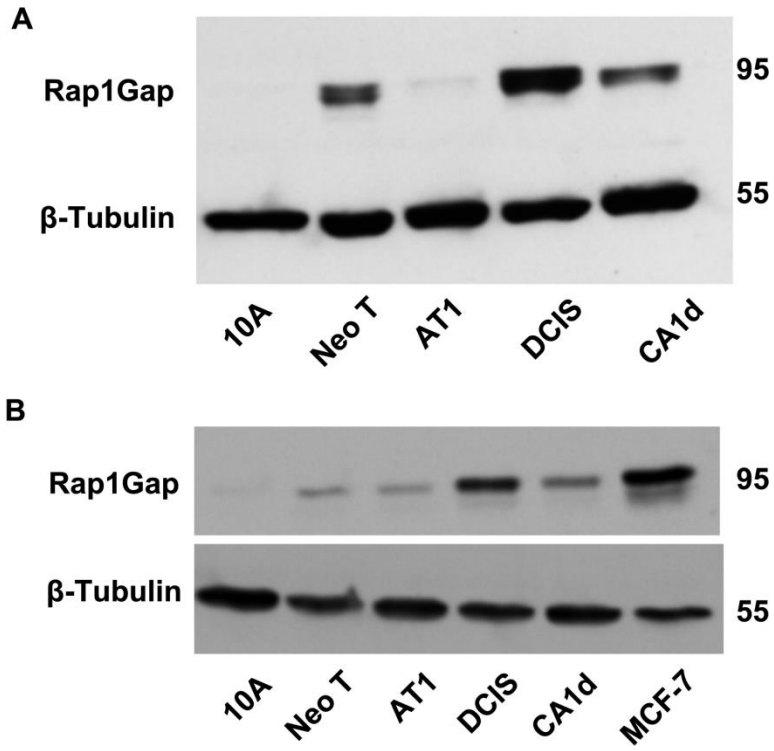


Figure 3.1: Rap1Gap protein is highly expressed in MCF10.DCIS, with reduced expression in MCF10.Ca1D, a model of human invasive breast cancer.

A. Lysates were prepared after 8 days of culture in rBM. Immunoblotting was performed using a polyclonal antibody against Rap1Gap. The molecular weight of Rap1Gap is 95 kDa. β-tubulin was used as a loading control. **B.** Lysates prepared from cells grown in 2D after 3 days in culture. MCF-7 cells were used as a positive control. β-tubulin served as a loading control. Results are representative of 3 independent experiments.

3.2 Rap1Gap is abundantly expressed in luminal hormone responsive cell lines yet weakly expressed in mesenchymal and metastatic breast cancer cell lines

As mentioned previously, multiple studies have demonstrated loss of Rap1Gap as a characteristic of acquisition of invasive phenotype. For example Rap1Gap is lost in aggressive pancreatic and thyroid cancers (Zhang, Chenwei et al. 2006, Zuo, Gandhi et al. 2010). Basal breast cancer cell- lines express characteristics that are reflective of invasive metastatic phenotype (Perou, Jeffrey et al. 1999, Chung, Bernard et al. 2002). Rap1Gap is also highly expressed in differentiated epithelial tissues (Zhang, Mitra et al. 2006, Tsygankova, Ma et al. 2010). Luminal breast cancer cell lines, which are distinct from basal breast cancer cell lines, preferentially express genes that are associated with a differentiated phenotype (Beck, Zerler et al. 2001, Feldman, Sementchenko et al. 2003, Charafe-Jauffret, Ginestier et al. 2006). Hence, we hypothesized that Rap1Gap may be lost in invasive, metastatic breast cancer cell lines and highly expressed in luminal breast cancer cell lines.

In Figure 3.2, the results show that Rap1Gap is expressed at higher levels in luminal cell lines (MCF-7 and T47-D), compared to lower expression in the basal breast cancer cell lines (MDA-MB-231, Hs578t and BT549). In the two DCIS lines derived from patients (SUM 225 and SUM 102), Rap1Gap levels are strikingly different. SUM 225, a cell line expressing luminal markers (Behbod, Kittrell et al. 2009) and derived from a chest wall recurrence abundantly expresses Rap1Gap; SUM 102 cells of basal type derived from a DCIS with micro-invasion (Ethier, Mahacek et al. 1993, Ethier 1996), expresses lower levels of Rap1Gap, which is in agreement with the qPCR data in Figure 1.2. The stark differences in Rap1Gap expression in SUM 225 and SUM 102

potentially address the heterogeneity seen in DCIS patients. These potential differences might be due to transcriptional and/or translational regulation. For example, qPCR analysis of Rap1Gap reveals low Rap1Gap transcripts in SUM 102 cells, potentially leading to low protein levels. SUM 225 cells express higher levels of Rap1Gap, both at the mRNA and protein levels. This might be due to either concomitant synthesis of more protein or post-translational stabilization of existing protein.

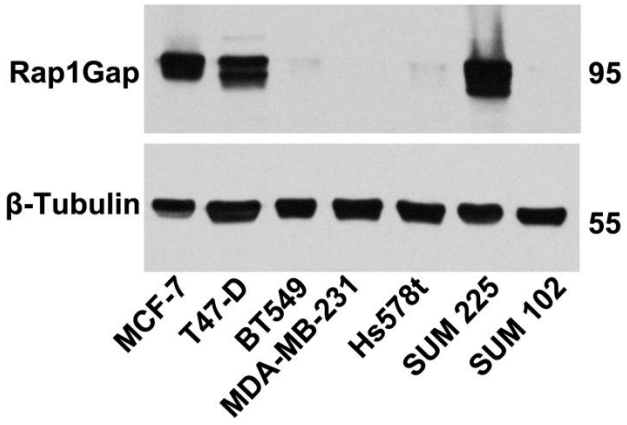


Figure 3.2: Rap1Gap expression in breast cancer cell lines.

Lysates were prepared from 3D cultures grown for 8 days on rBM, subject to SDS-PAGE and immunoblotting using anti-Rap1Gap antibodies. β-tubulin is a loading control. Results are representative of three independent experiments. Luminal cell lines are MCF-7 and T47-D; BT549, MDA-MB-231 and Hs578t are basal cell lines; SUM 225 and SUM 102 are DCIS cell lines.

3.3. Rap1Gap is found in the nuclear and cytosolic fractions in the MCF10 series

Rap1Gap is known to play a role in the maintenance of adherens junctions and cell-ECM attachments (Tsygankova, Ma et al. 2010) characterizing it as a cytosolic protein. Staining of Rap1Gap in colon and thyroid tissue samples (http://www.proteinatlas.org/ENSG00000076864-RAP1GAP/tissue) also indicate that Rap1Gap is located in the cytosol. Images from Protein Atlas were reviewed and confirmed that Rap1Gap is present in the cytosol. The database mentioned above did not mention evidence of Rap1Gap nuclear staining. Further analysis by a trained pathologist is necessary to confirm the presence of Rap1Gap in the nucleus. To determine the localization of Rap1Gap in the MCF10 series, cell fractionation experiments were performed on lysates prepared from cells grown in 2D cultures. Interestingly, as seen in Figure 3.3, Rap1Gap is found in both cytosolic and nuclear fractions.

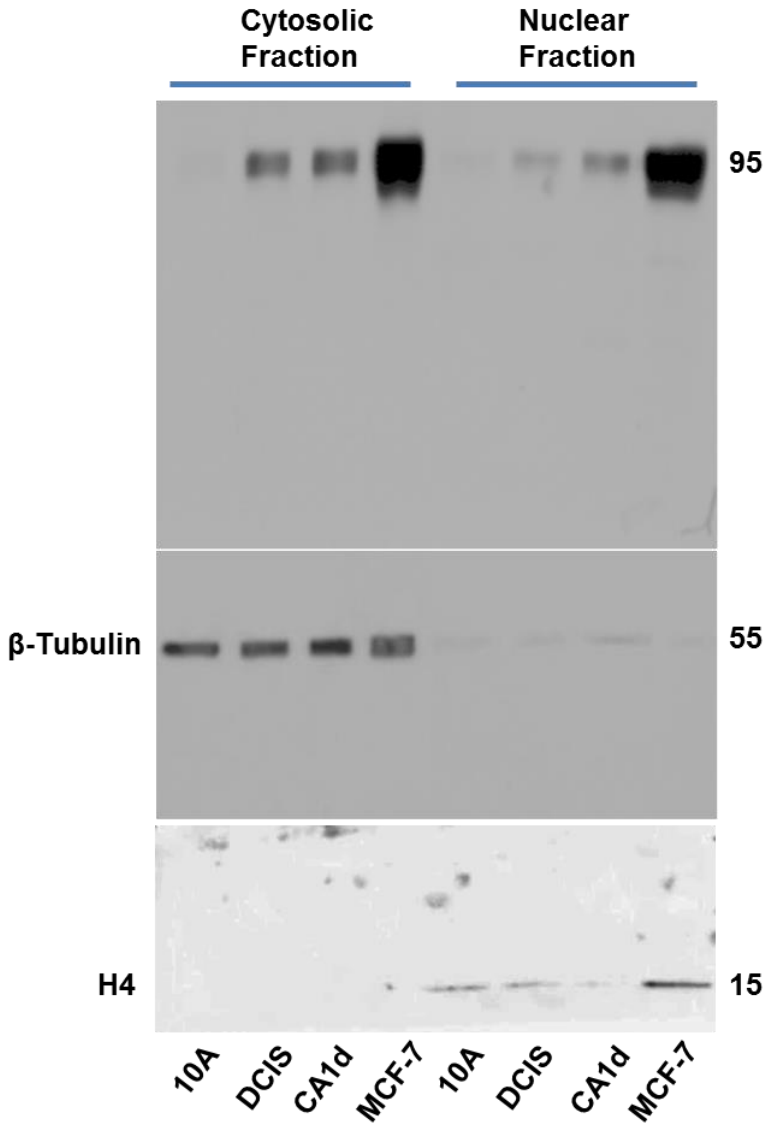


Figure 3.3: Rap1Gap is found in the cytosolic and nuclear fractions.

Cells were grown in 2D for 4 days. Cytosolic and nuclear fractions were processed according to protocol described in the methods. Both fractions were subject to SDS-PAGE and immunoblotted for Rap1Gap, tubulin and H4 (Histone 4). MCF-7 serves as a positive control. β-Tubulin and H4 were used as controls for loading and to show proper separation of cytosol and nuclear compartments of the lysate respectively.

3.4 Rap1Gap knock down in MCF10.DCIS transforms cell morphology from epithelial to fibroblastic phenotype

Previously, it was observed that Rap1Gap was significantly up-regulated in MCF10.DCIS and is reduced in MCF10.CA1D cells. Breast cancer cell lines with mesenchymal characteristics express very low levels of Rap1Gap. Hence, we hypothesized that knocking down Rap1Gap would confer mesenchymal characteristics in DCIS. This would include transformation to spindle shaped cells and invasive phenotype. Lentiviral knockdown experiments with two separate shRNA sequences; (TTGGTGTGTGAAGACGTCA (kd1) and TCTTCTCACTCAAGTACGA (kd2) were performed.

Panel A in Figure 3.4 shows MCF10.DCIS cells infected with pSIREN dsRed lentiviral control (Con) or shRNA 1 (kd1) and 2 (kd2). DCIS cells have an epithelial phenotype, with wide cell bodies and some cell-cell contacts. Knockdown of Rap1Gap in DCIS transforms cellular shape to long and spindle like, with less frequent cell-cell contacts. Panel B is an immunoblot showing robust knockdown of Rap1Gap with two separate shRNA sequences.

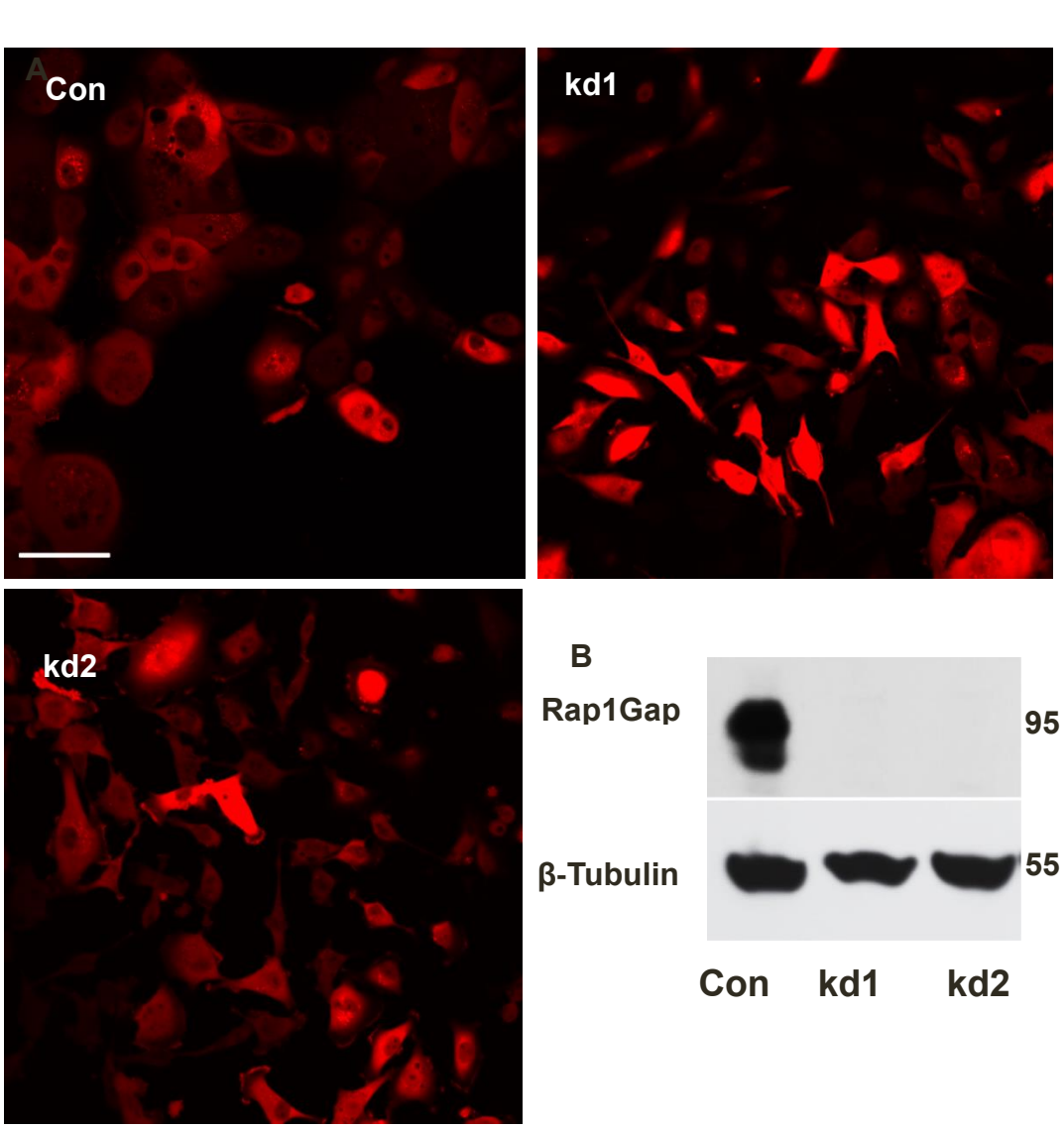


Figure 3.4: Rap1Gap silencing in MCF10.DCIS transforms cell morphology from epithelial to fibroblastic phenotype.

MCF10.DCIS cells infected with pSIREN dsRed control (Con) or Rap1Gap shRNA (kd1 and kd2) and were cultured for 3 days in 2D. **A.** Live Images were taken at 20x magnification. Scale bar, 50 µm. **B.** Immunoblot of Rap1Gap knockdown in MCF10.DCIS cells with two separate shRNA sequences, compared to the lentiviral control.

3.5 Silencing of Rap1Gap in MCF0. DCIS leads to increase in cell proliferation

Previous studies have shown that Rap1Gap controls cell proliferation (Wang, Zhang et al. 2014) and may have tumor suppressor properties. Therefore, in the context of breast cancer, Rap1Gap may regulate cell proliferation. We hypothesized that that silencing of Rap1Gap may cause increased proliferation. Ten thousand cells were plated in triplicate for termination at 48, 96 and 144 h time points.. Here, we show that DCIS cells devoid of Rap1Gap proliferate at a higher rate. As seen in Figure 3.6, at 48 h time point, there is little difference in cell number in the control line *vs.* the DCIS cells transduced with the Rap1Gap shRNA. By 96 h (4 days), the differences in increase in proliferation are more evident. At 6 days, in both knockdown lines, mean cell numbers culminate at 140,000 cells vs. the control line, which reached 40,000 cells when the experiment was terminated. We speculate that there is little difference in the cell proliferation between the control cells vs. the DCIS shRNA cells when plated at a low density. As cells continue proliferating, the increased density of cells might increase the proliferation rate of the cells with Rap1Gap knockdown, which is reflected at 96 and 144 h time points. Loss of contact inhibition is less likely to be factor, since the cell density did not reach >100% confluency after 6 days of culture. Thus, silencing of Rap1Gap in MCF10.DCIS causes increased cell proliferation.

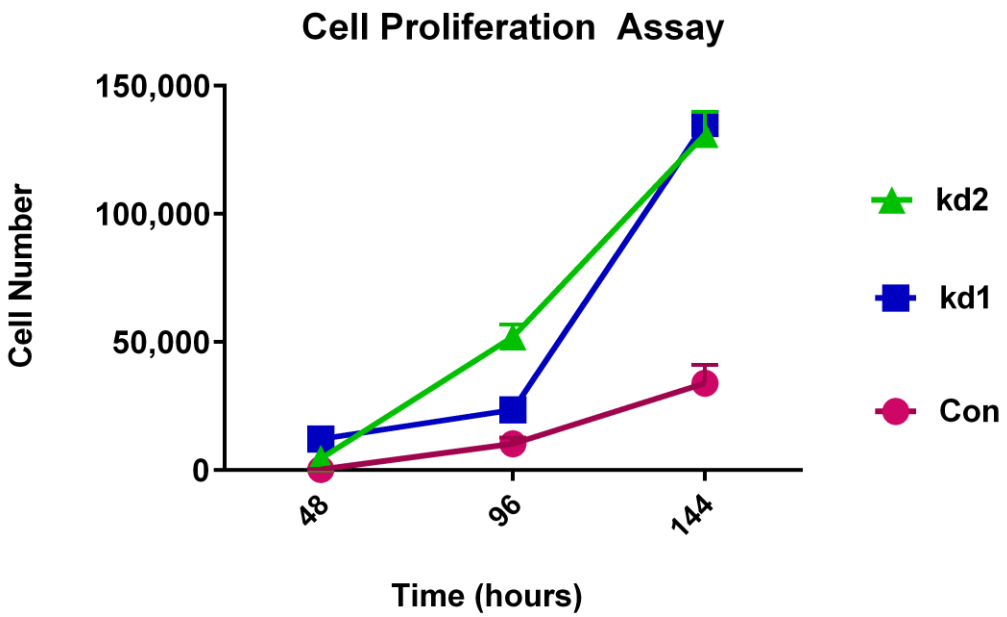


Figure 3.5: Rap1Gap silencing in MCF10.DCIS leads to increase in cell proliferation.

Ten thousand cells were plated and allowed to grow for 48, 96 and 144 h in 2D conditions. Cell numbers were determined by trypan blue exclusion and manual counting on a hemocytometer. Data are the mean SEM of triplicate samples and are representative of three independent experiments.

3.6 Rap1Gap silencing leads to the appearance of invasive outgrowths.

In a 2D environment, MCF10.DCIS cells have an epithelial phenotype, characterized by wide cell bodies. Under the same conditions, MCF10.DCIS Rap1Gap shRNA cells acquire a spindle shaped morphology (Figure 3.4). When grown in rBM, MCF10.DCIS, cells form dense dysplastic structures, reminiscent of DCIS in patients. We hypothesized that MCF10.DCIS Rap1Gap shRNA cells, when grown in rBM would exhibit the appearance of outgrowths, a characteristic of invasive phenotype. MCF10.DCIS lentiviral control and MCF10.DCIS shRNA cells were grown in 3D for 8 days and cells were imaged live. We show that the morphology of 3D structures of MCF10.DCIS Rap1Gap shRNA is strikingly different. These structures are no longer dense, and form multicellular invasive outgrowths. Additionally, these structures are much bigger and appear more disorganized. Figures 3.6.1 and 3.6.2 show the morphology of DCIS Rap1Gap shRNA cells at 20x and 63x magnification. Figure 3.6.3 panel A shows representative 4x4 DIC images used to quantify the outgrowths. Panel B is the results of the outgrowth analysis. The purpose of the quantification was to ensure that the appearance of outgrowths is an effect of Rap1Gap silencing. Some outgrowths were observed in the control. The p value of 0.0012 means that the differences in the number of invasive outgrowths are significant. Thus silencing of Rap1Gap in MCF10.DCIS induces the appearance of outgrowths, a characteristic of invasive phenotype.

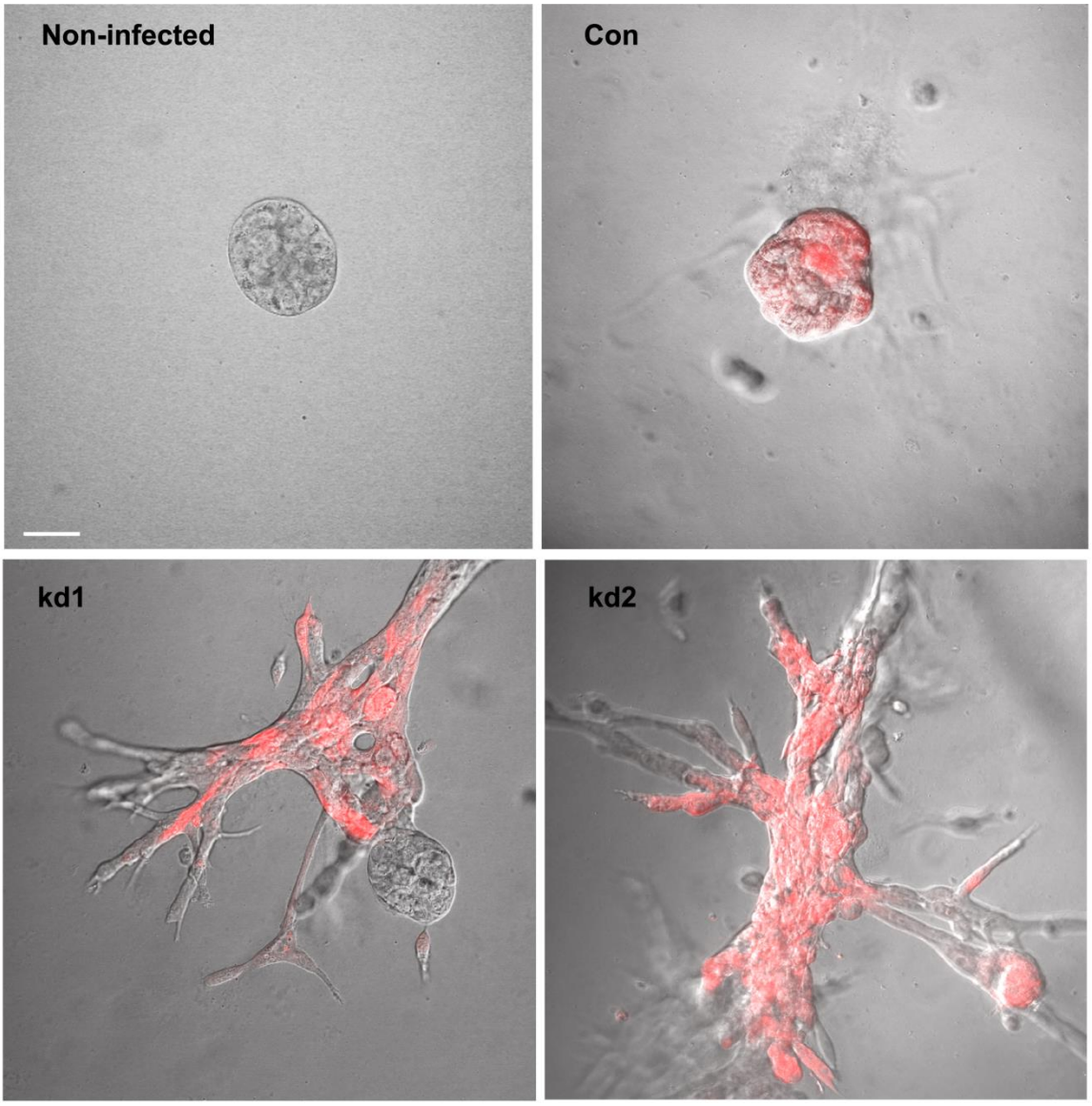


Figure 3.6.1: Rap1Gap silencing leads to appearance of invasive outgrowths.

Cells were grown in 3D for 8 days and imaged live. DIC (Differential Interference Contrast) images with fluorescent overlay of non-infected MCF10.DCIS, lentiviral control, kd1, kd2. Images were taken at 20x magnification. Scale bar, 50 µm. Images are representative of at least three independent experiments.

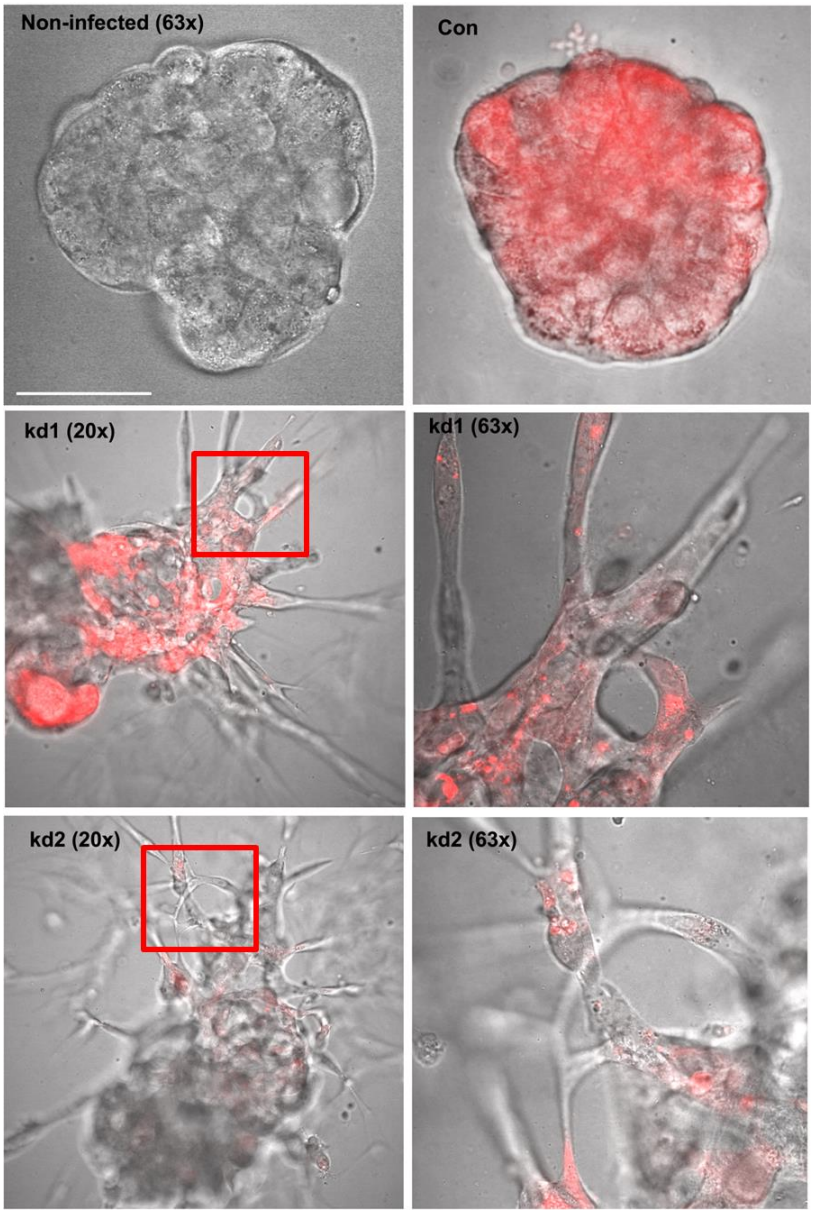


Figure 3.6.2: Rap1Gap silencing leads to appearance of invasive outgrowths.

Cells were cultured in 3D for 8 days to allow growth of structures. Structures were imaged live. DIC images (top) are side-by-side comparisons of non-infected DCIS vs. lentiviral control at 63x magnification. The images in the middle and bottom rows represent 20x and 63x of same structures side by side. Red squares of outgrowths on the left show regions of interest and are magnified at 63x magnification on the right. Scale bar, 50 µm.

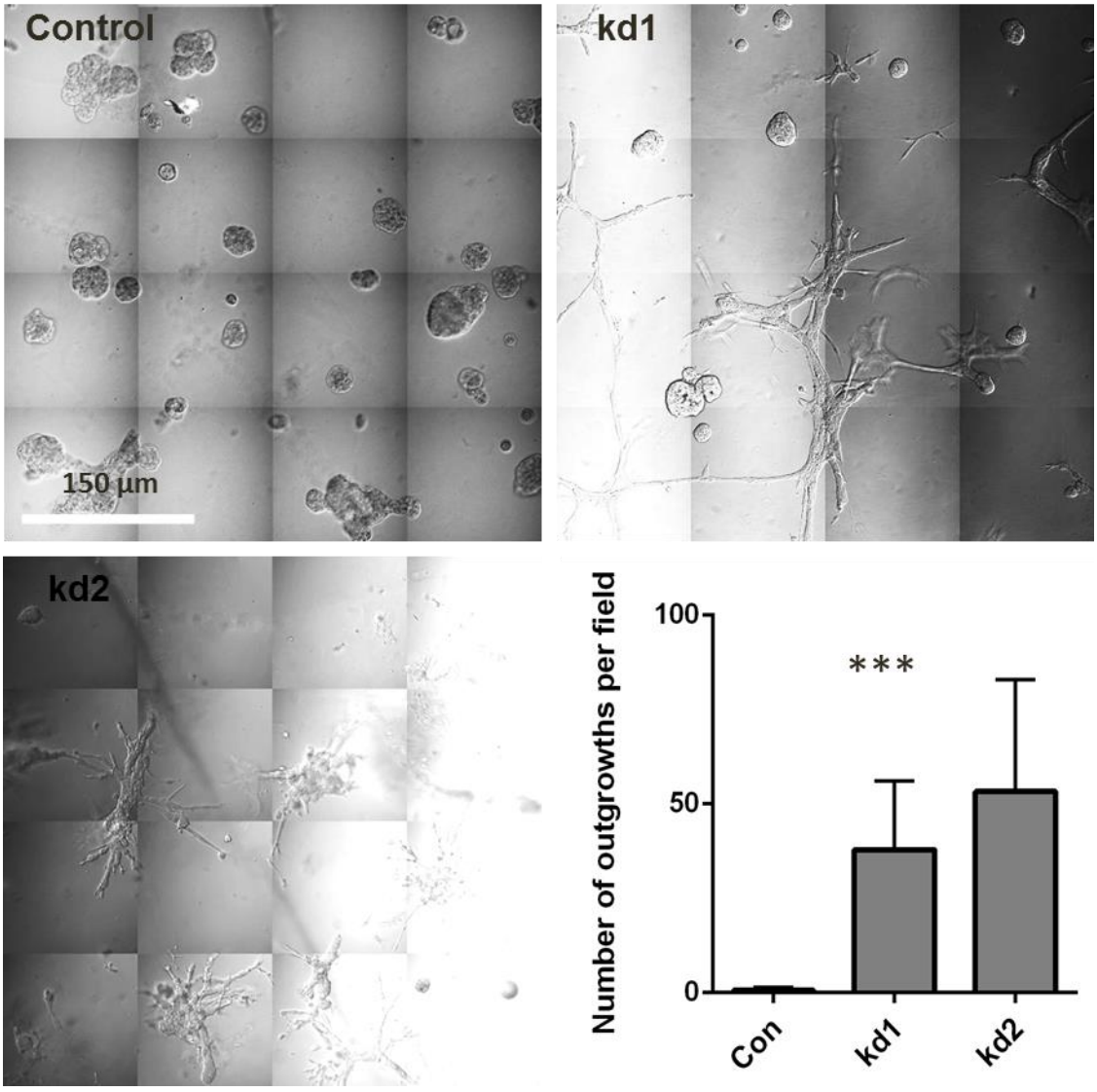


Figure 3.6.3: Analysis of Outgrowths.

After culture in rBM for 8 days, DIC images of live 3D structures, consisting of 16 contiguous fields were taken at 20x magnification. The bar graph represents number of outgrowths per field (mean ± SEM from 3 independent experiments). Outgrowths were counted by two blinded individuals according to parameters described in the results. Statistical analysis was performed using one-way ANOVA.

3.7 Lentiviral silencing of Rap1Gap in MCF10.DCIS leads to reduction of E- cadherin protein levels and breakdown of adherens junctions

E-cadherin is an important component of adherens junctions, which in some cases are lost during the transition to an invasive phenotype (Thompson, Torri et al. 1994). One characteristic of invasive breast cancer is reduction in E-cadherin and breakdown of adherens junctions. We hypothesized that reduction in E-cadherin and concomitant breakdown of adherens junctions may be a consequence of Rap1Gap knockdown, via Rap1 deregulation (Balzac, Avolio et al. 2005). In a 2D environment, DCIS cells containing the Rap1Gap shRNA plasmid acquire a spindle shaped morphology compared to the lentiviral control (Figure 3.4).

Cells were grown in 3D for 8 days, fixed and stained for E-cadherin in order to visualize adherens junctions. To confirm reduction in E-cadherin protein levels, immunoblotting was performed. As seen in Figure 3.7 panel A, adherens junctions are visible in the lentiviral control (red, stained for E-cadherin). The immunoblot in Panel B shows robust expression of E-cadherin in the control, with significant reduction in the knockdown lines (kd1 and kd2). Two exposures of E-cadherin are shown to emphasize that this protein is reduced, not lost. GAPDH was used as a loading control to confirm equal protein loading. Thus, lentiviral silencing of Rap1Gap leads to reduction of E-cadherin levels and breakdown of adherens junctions.

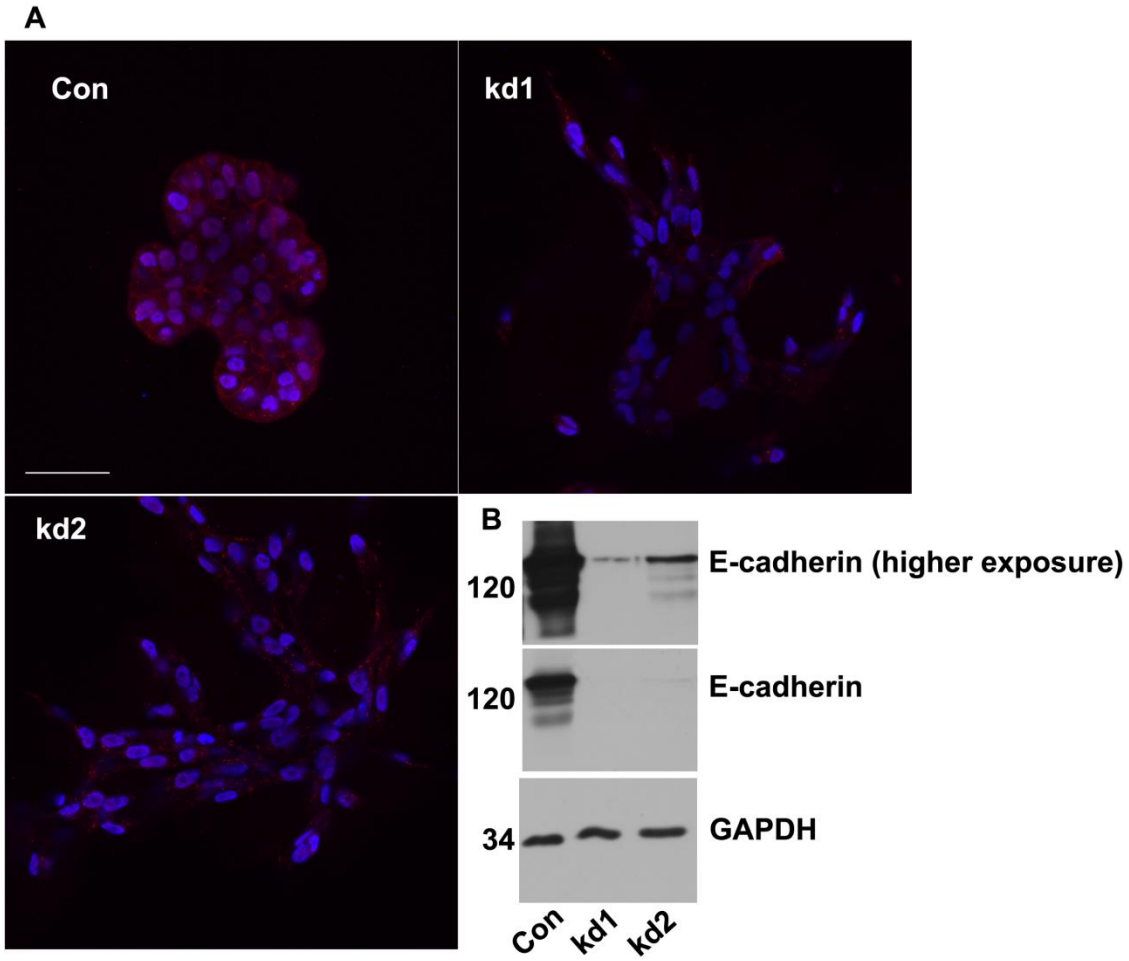


Figure 3.7: Rap1Gap lentiviral silencing leads to reduction of E-cadherin protein levels and breakdown of adherens junctions.

A. Cells were cultured in 3D for 8 days and fixed. DCIS lentiviral control and DCIS Rap1Gap shRNA cells were grown on rBM for 8 days and fixed. Images are four contiguous fields, taken at equatorial position. Red (E-cadherin) is Far Red, pseudo-colored. DAPI represents fluorescent detection of DNA. Images were taken at 63x magnification. Scale bar, 150 µm. **B**. Cells were grown in 3D for 8 days and harvested. Lysates were subjected to SDS-PAGE and immunoblotting for E-cadherin and GAPDH (loading control). Images are representative of three independent experiments.

3.8 Lentiviral silencing of Rap1Gap in DCIS leads to cytoskeletal remodeling and vimentin reorganization

Remodeling of the cytoskeleton is a significant part of the invasion process and is carefully orchestrated (Yamaguchi and Condeelis 2007, Mouneimne, Hansen et al. 2012). We had observed the appearance of invasive outgrowths in DCIS-Rap1Gap shRNA cells (Figure 3.6.2, red squares), and thus we hypothesized that they would indicate a significant change in the organization of the actin cytoskeleton. Cells were grown in rBM for 8 days, then fixed and structures were stained for F-actin. As seen in Figure 3.8.1, fluorescent probe detection of filamentous actin reveals significant changes in actin cytoskeleton reorganization. Cortical rings, which are typically present in MCF10.DCIS, are not present in majority of the structures with the Rap1Gap shRNA (Figure 3.8.1, Figure 3.8.2).

Vimentin is a well–studied member of the family of the intermediate proteins. It has been well established that vimentin plays a role in the process of invasion. Hence, vimentin reorganization may be part of the cytoskeletal reorganization. Cells were seeded in 3D for 8 days, fixed, and stained with vimentin antibody. As observed in Figure 3.8.3, in the lentiviral control cells, vimentin is apparently closely associated with F-actin. However, it appears that brighter staining of vimentin was observed in the center of the structures in the kd2. The brighter staining in the DCIS Rap1Gap shRNA cells might signify either higher expression in certain areas of the structure or more concentration of the protein in the center of the structure. Thus, silencing of Rap1Gap in MCF10.DCIS causes F-actin and vimentin organization.

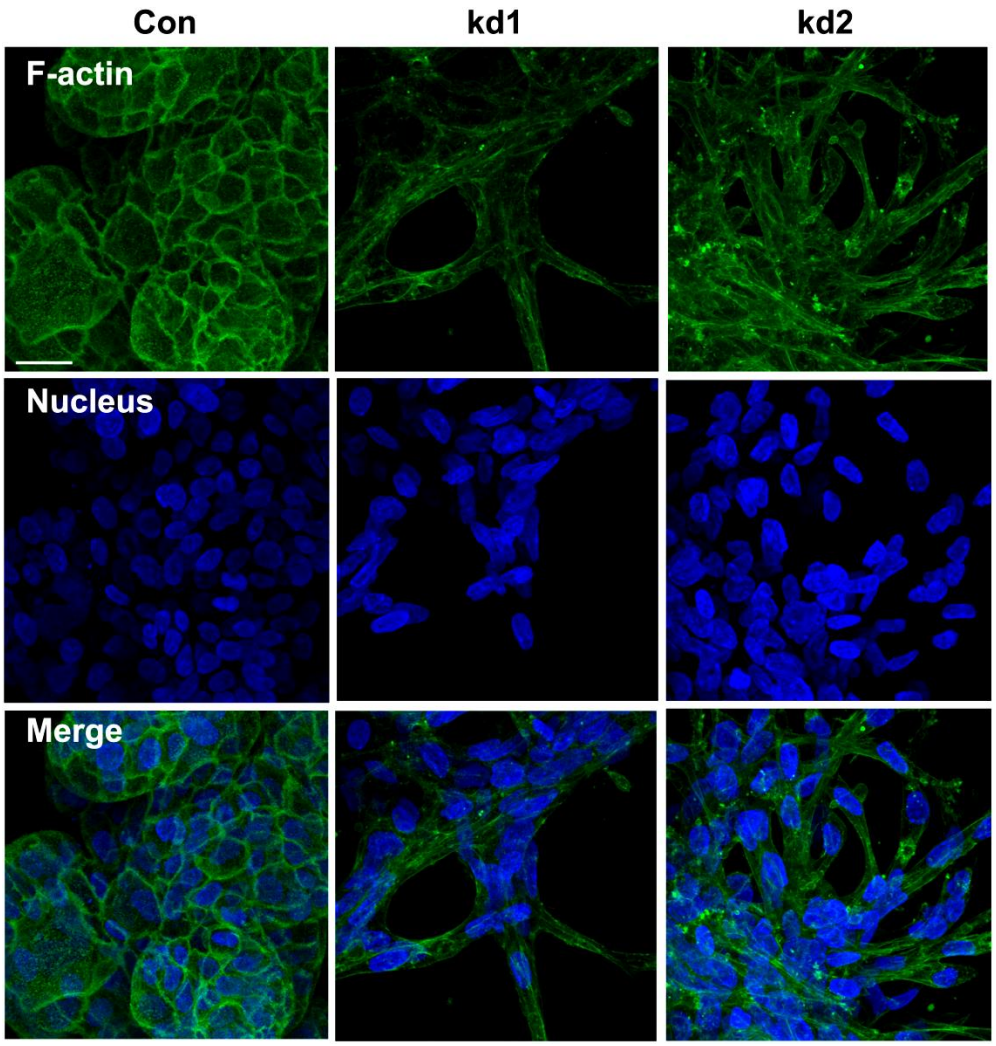


Figure 3.8.1: Lentiviral silencing of Rap1Gap in DCIS leads to cytoskeletal remodeling.

Cells were grown in rBM for 8 days and fixed. Fluorescent probe detection of filamentous actin (F-actin, green) and nuclei (blue) in the lentiviral control and kd1 and kd2 lines that have silenced Rap1Gap expression. Images, taken at 63x magnification, are collapsed z stacks of overlaid green and blue channels. Images are representative of at least 3 independent experiments. Scale bar, 50 μm.

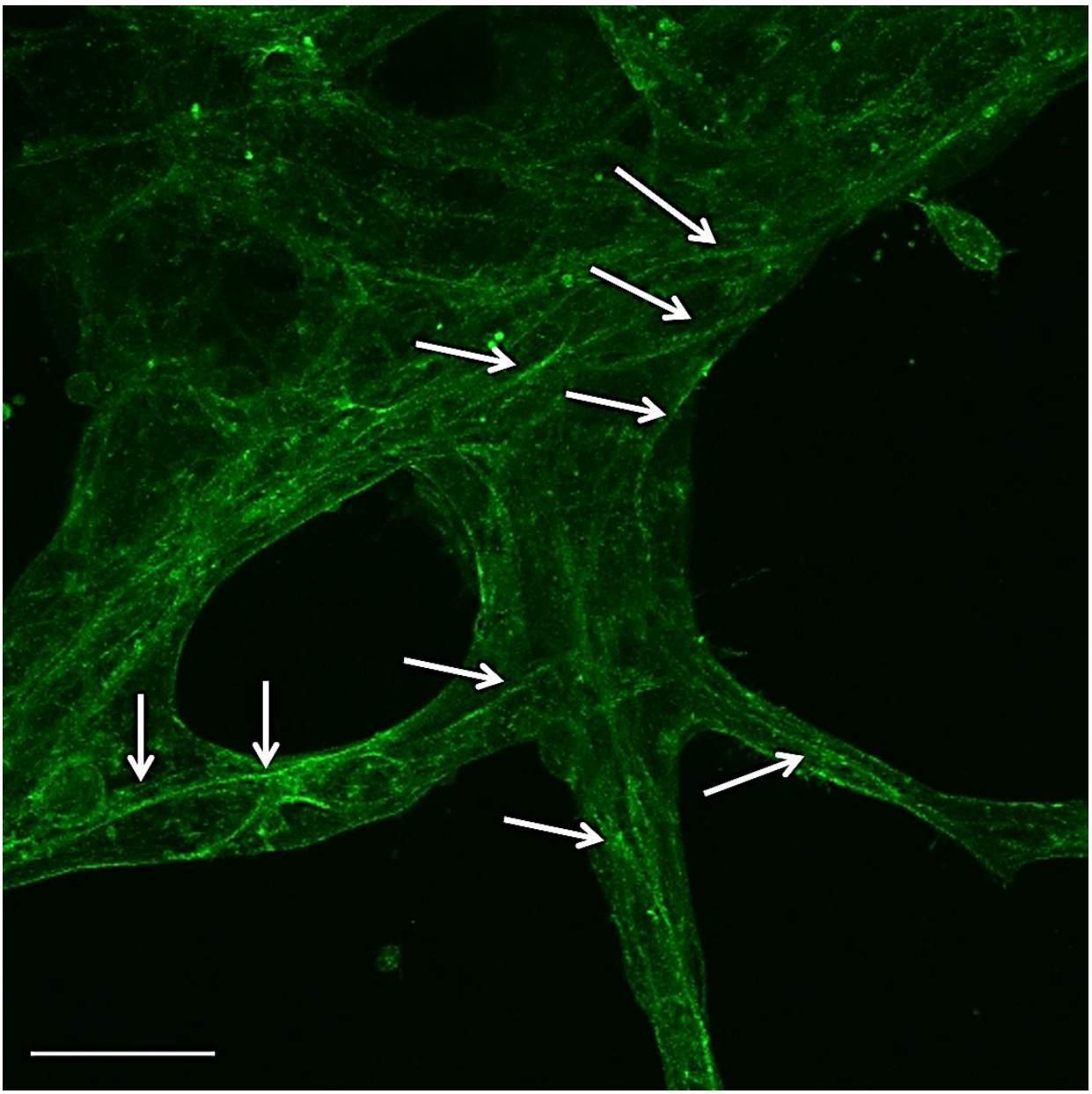


Figure 3.8.2: Lentiviral silencing of Rap1Gap in DCIS leads to cytoskeletal remodeling.

Fluorescent probe detection of filamentous actin (F-actin, green). Image, taken at 63x magnification is an expanded image of the kd1, (middle image, top panel of Figure 3.8.1) to emphasize the presence of F-actin stress fibers. The white arrows point to the stress fibers in the outgrowths. Size bar represents 50 μm.

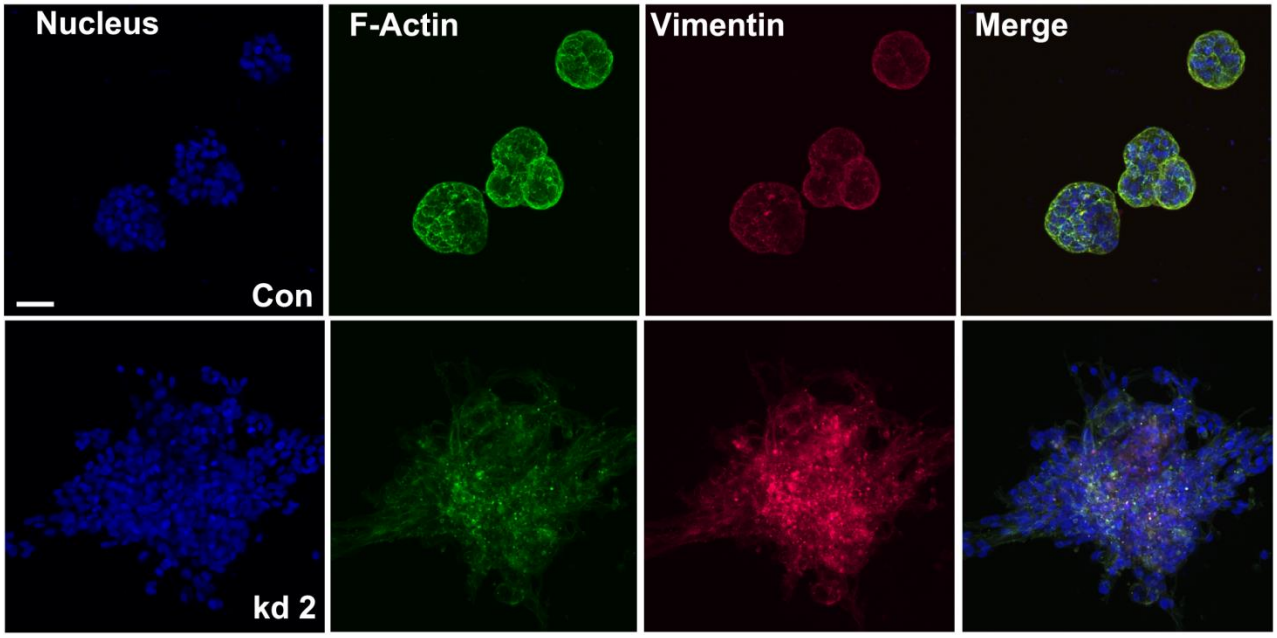


Figure 3.8.3: Rap1Gap lentiviral silencing in DCIS leads to vimentin reorganization.

3D structures were fixed after 8 days of culture. Then, the structures were stained for filamentous actin (green), vimentin (magenta) and nuclei (blue). Images, taken at 20x magnification, are collapsed z stacks. Size bar represents 50 µm. Images are representative of 2 independent experiments (total of 4 images).

3.9 Silencing of Rap1Gap in MCF10.DCIS leads to enhanced invasion

Cytoskeleton remodeling is a significant part of the invasion process and is carefully orchestrated (Yamaguchi and Condeelis 2007, Mouneimne, Hansen et al. 2012). The appearance of invasive outgrowths in DCIS-Rap1Gap shRNA cells were observed, and we hypothesized that they would indicate a significant change in the organization of the actin cytoskeleton, leading to invasion.

Invasion assays were carried out. MCF10.DCIS control and MCF10.DCIS Rap1Gap shRNA cells were serum starved overnight. Thirty thousand cells were seeded and allowed to invade through the membrane for 24 h. Cells were fixed and stained blue. Figure 3.9 shows dramatic increase in invasion by the DCIS knockdown cells through the membrane when compared to the control. The graph in panel B depicts mean number of invaded cells per field. Statistical analysis was done using one-way ANOVA, $p \leq 0.05$.

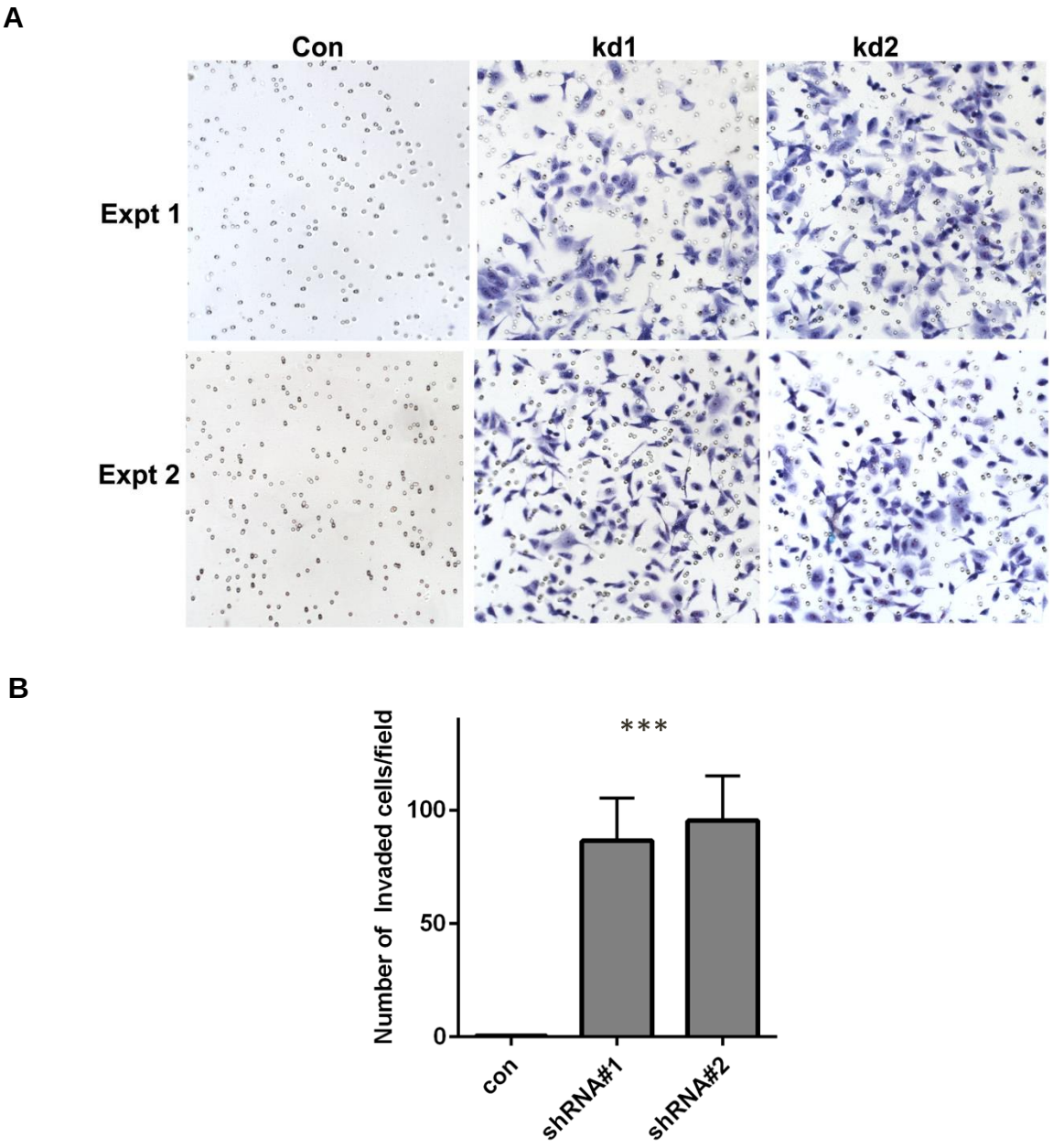


Figure 3.9: Silencing of Rap1Gap in MCF10.DCIS leads to enhanced invasion.

A. Cells were allowed to invade through an 8 um pore membrane for 24 h and fixed and stained blue. Images, taken at 20x magnification are representative of 15 images per triplicate, per condition, per experiment. **B.** Bar graph represents mean number of invaded cells. Error bars represent standard error of the mean. Data set is the mean of 4 independent experiments. Statistical analysis was done using one way ANOVA.

3.10 Lentiviral silencing of Rap1Gap leads to increased collagen IV degradation

Silencing of Rap1Gap resulted in the progression of DCIS to an invasive phenotype. This was characterized by the appearance of multicellular outgrowths (Figure 3.6), significant remodeling of actin cytoskeleton and vimentin (Figure 3.8), and increase in invasion (Figure 3.9). We hypothesized that invasive structures may exhibit an increase in DQ collagen degradation.

Cells were grown in 3D for 8 days in rBM supplemented with 2.5% DQ collagen and imaged live. The results in Figure 3.10.1 indicate that the multicellular structures formed by the DCIS Rap1Gap shRNA cells exhibit an increase in collagen IV degradation (brighter green fluorescence) compared to the lentiviral control. Collagen IV degradation appears to be either contained within the cells (as seen in kd2 line) or occurs pericellularly and dispersed farther across the 3D landscape. This observed degradation of collagen IV could be due to the activity of proteases, since they are involved in the malignant progression and play a role in aggressive phenotype (Moin, Sameni et al. 2012). More studies are needed to further test this hypothesis.

To further analyze the effects of lentiviral silencing on proteolytic degradation, 3D reconstructions of z stacks (Figure 3.10.2) were made to show depth of collagen IV degradation. More detailed investigation of green fluorescence of the structures (63x magnification, collapsed z stacks in Figure 3.10.3) indicates that proteolysis can be pericellular or within the cell. Thus, lentiviral silencing of Rap1Gap results in increased proteolysis.

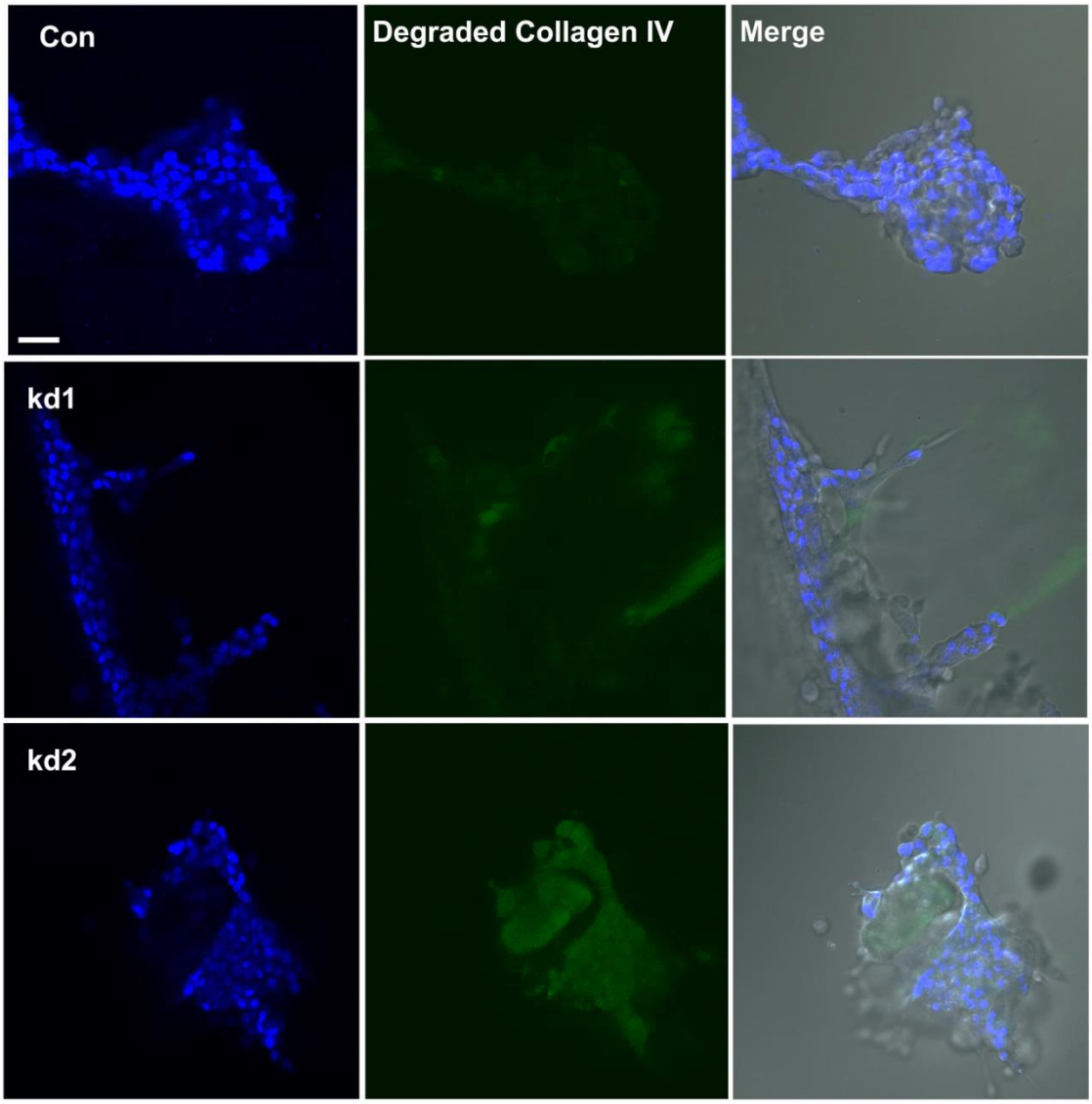


Figure 3.10.1: Silencing of Rap1Gap leads to increased collagen IV degradation.

Cells were cultured for 8 days in rBM supplemented with DQ collagen. Structures were imaged live to detect presence of cleavage products (green fluorescence). Blue represents nuclei. Images, taken at 20x magnification, were taken at equatorial position. Scale bar, 50 µm.

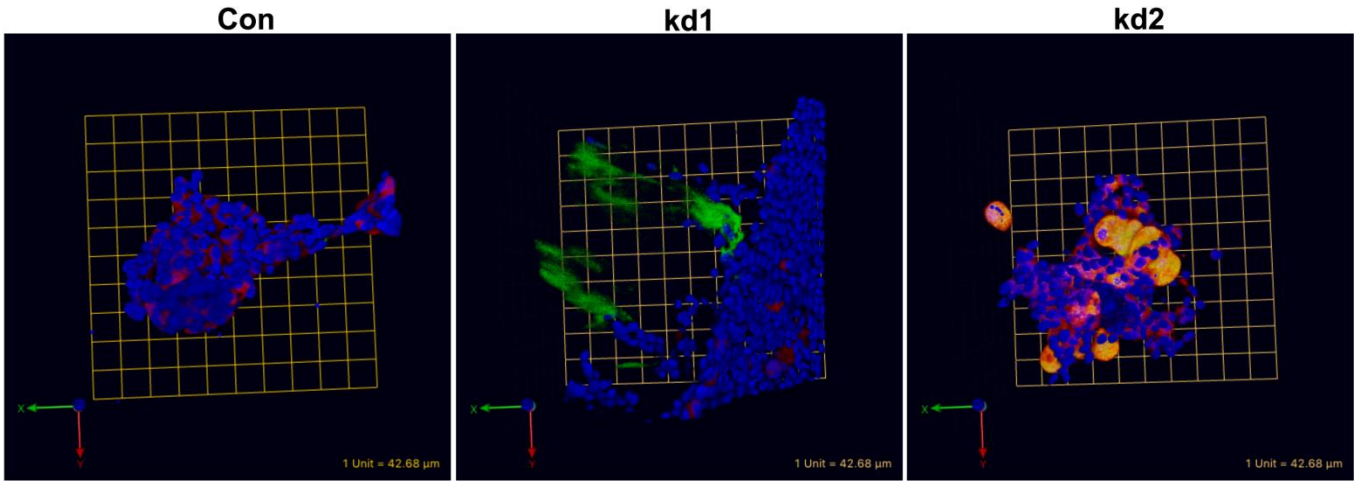


Figure 3.10.2: Silencing of Rap1Gap leads to increased collagen IV degradation.

The panels in this figure are 3D reconstruction of z-stacks of the images in Figure 3.10.1. The red is dsRed, a reporter protein for the lentiviral transduction. Blue is Höechst stain of the nuclei of live cells. Green fluorescence is the result of cleavage of DQ collagen IV. The yellow color observed in kd2 is co-localization of the red reporter (dsRed) and green fluorescence as a result of proteolysis. Images are representative of three independent experiments.

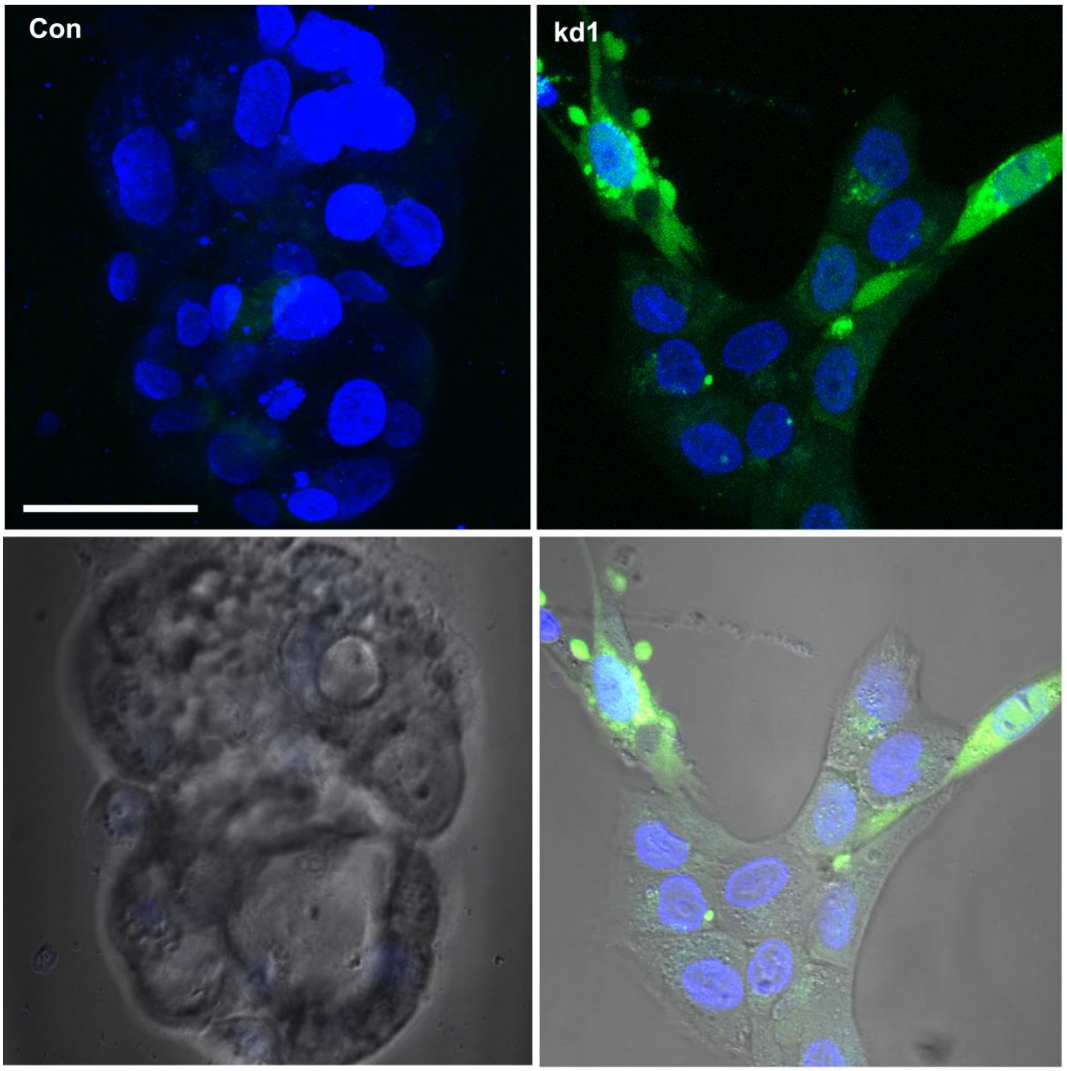


Figure 3.10.3: Silencing of Rap1Gap leads to increased collagen IV degradation- a closer look.

Cells were cultured for 8 days in rBM supplemented with DQ collagen. Structures were imaged live to detect presence of cleavage products (green fluorescence). Blue is nuclear stain of live cells. Images, taken at 63x magnification are collapsed Z stacks (top panel). The bottom panels are DIC images overlaid with green and blue, and are same structures taken at equatorial position. Scale bar, 50 µm.

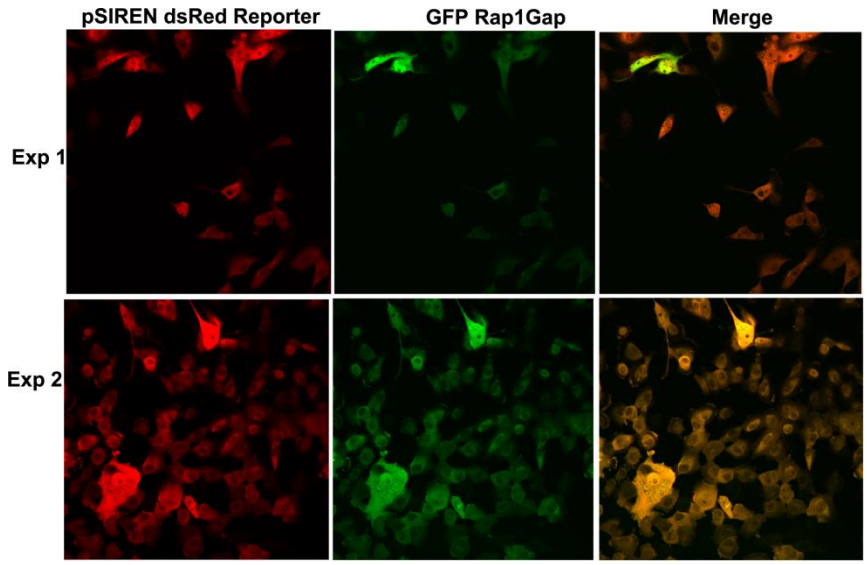
3.11 Rap1Gap re-expression in MCF10.DCIS Rap1Gap shRNA cells reverses invasive phenotype

To further test the role of Rap1Gap in the transition to an invasive phenotype, we performed rescue experiments. Rap1Gap was re-expressed in the DCIS Rap1Gap kd2 line (which had demonstrated a stronger phenotype in the previous assays: see Figures 3.6.1, 3.6.3 and 3.9). GFP was transfected into DCIS Rap1Gap kd2 shRNA cells. Cells were allowed to grow in 2D conditions for 3-5 days before imaging and preparation of lysates. Transfected cells were also trypsinized and seeded directly on rBM, to be cultured with overlay for 8 days before fixing and staining for F-actin.

Panel A in Figure 3.11.1 shows successful transient transfection of GFP-Rap1Gap into the kd2 line by assay of the reporter's green fluorescence. Panel B shows evidence of robust over-expression of GFP-Rap1Gap compared to the GFP-control by immunoblotting for Rap1Gap. Figure 3.11.2 displays fluorescent detection of F-Actin in DCIS Rap1Gap shRNA cells housing the Rap1Gap over-expression. The multicellular invasive outgrowths, characteristic of cells with silenced Rap1Gap expression, were blocked, restoring the dense dysplastic structure and F-actin cortical rings that are found in MCF10.DCIS.

The overlay DIC image in Figure 3.11.3 is a tiled image of 4 contiguous fields stitched together to assess the effect of the rescue experiment on other structures in the area. This is evidence that the rescue experiment, though transient, has consistent effects over time even though not all cells within a structure retain the GFP reporter protein. Thus, re-introduction of Rap1Gap DCIS Rap1Gap shRNA cells results in reversion of invasive phenotype, characterized by re-appearance of cortical rings.

A



B

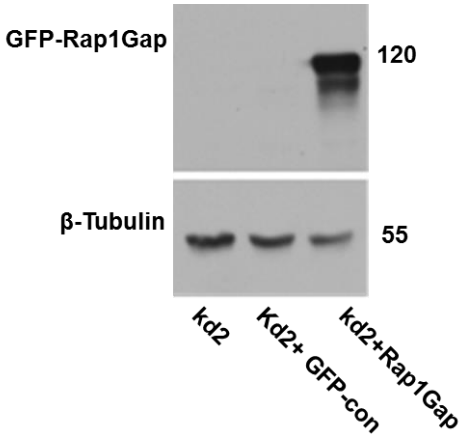
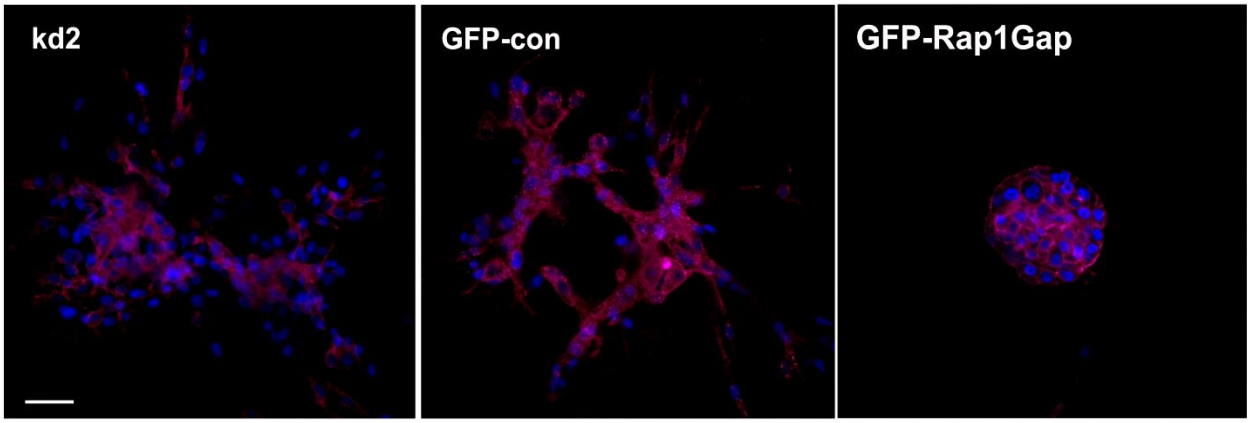


Figure 3.11.1: Rap1Gap Re-expression in MCF10.DCIS shRNA.

A. Images are of MCF10.DCIS cells, previously infected with Rap1Gap shRNA2 (kd2) transfected with the GFP-Rap1Gap over expression plasmid. Images shown are at 20x magnification of live cells monitored for detection of dsRed (red) and GFP (green). Images are representative of two separate transfection experiments that were viewed 3-5 days post transfection. **B.** Parallel lysates from cultures described in panel **A** were prepared and subject to SDS-PAGE and immunoblotting using Rap1Gap and β-tubulin antibodies. β-tubulin serves as a loading control. Images are representative of at least three independent experiments.

A.



B

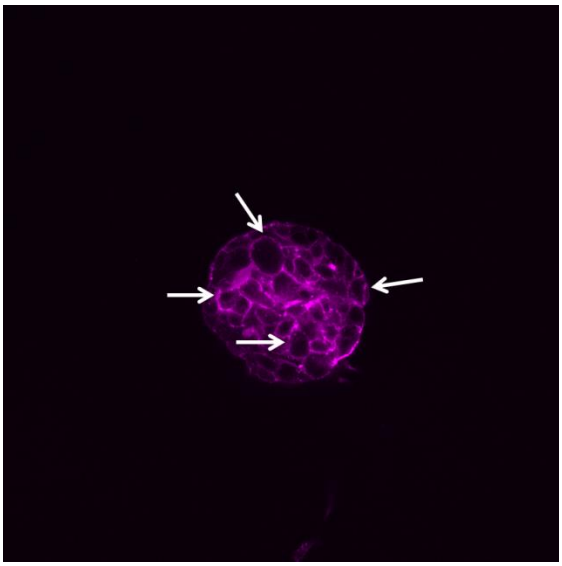


Figure 3.11.2: Rap1Gap re-expression in MCF10.DCIS Rap1Gap shRNA cells reverses invasive phenotype.

A. Cells were cultured in rBM for 8 days. The structures were fixed and stained for F-actin (magenta) and nuclei (blue). Size bar represents 50 μm. These images, taken at 20x magnification, are representative of 3 independent experiments**. B**. Expanded single image (20x magnification) of DCIS Rap1Gap shRNA kd cells transfected with GFP-Rap1Gap from panel A. Only the magenta signal is shown to emphasize presence of actin cortical rings (arrows).

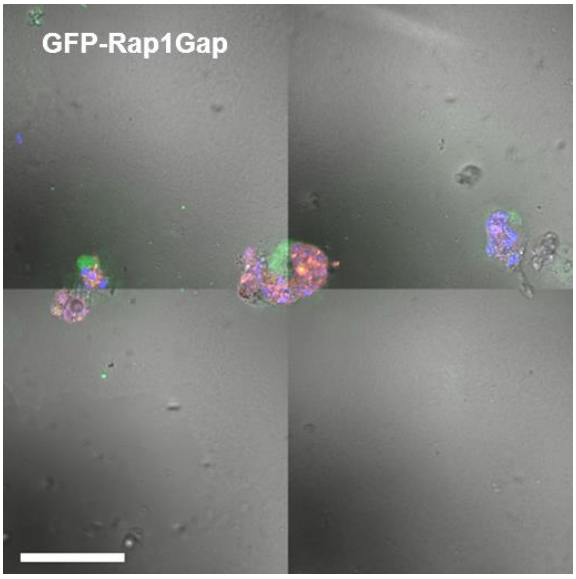


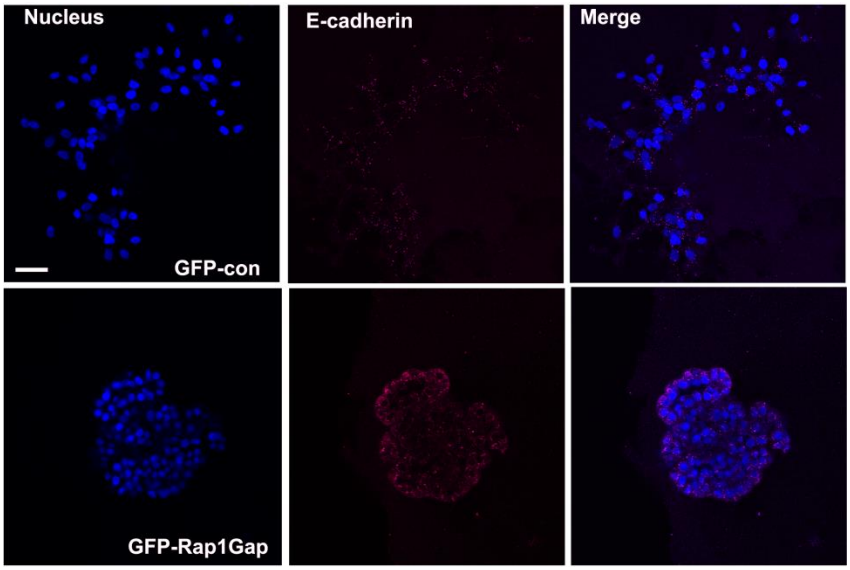
Figure 3.11.3: Rap1Gap re-expression in 3D culture.

Cells were grown on rBM for 8 days. Structures were fixed and stained for F-actin (magenta) and nuclei (blue). Fluorescent detection of dsRed (red) and GFP (green) was also performed. Images are 4 contiguous fields stitched together to cover a wider field. Size bar is 150 μm.

3.11.1 Re-expression of Rap1Gap in MCF10.DCIS Rap1Gap shRNA cells partially restores E-cadherin levels and adherens junctions

The previous results (Figure 3.11.2) have indicated that Rap1Gap re-expression leads to restoration of the dense and compact phenotype and actin cortical rings that are characteristic of MCF10.DCIS cells in rBM culture. We have also shown that lentiviral silencing of Rap1Gap leads to marked decrease in E-cadherin and breakdown of adherens junctions (Figure 3.7). We therefore wanted to investigate whether E-cadherin is re-expressed upon re-introduction of GFP-Rap1Gap. Transfected cells were seeded in 3D and allowed to grow for 8 days. Structures were fixed, processed and stained for E-cadherin. As seen in Figure 3.11.4 panel A, and Figure 3.11.5, re-introduction of Rap1Gap in the kd2 line partially restores adherens junctions. The immunoblot in Figure 3.11.4 panel B shows increase in E-cadherin protein levels following re-expression of Rap1Gap toward the level in control MCF10.DCIS cells. Re-expression of Rap1Gap in MCF10.DCIS Rap1Gap shRNA cells results in partial restoration of E-cadherin levels and adherens junctions.

A



B

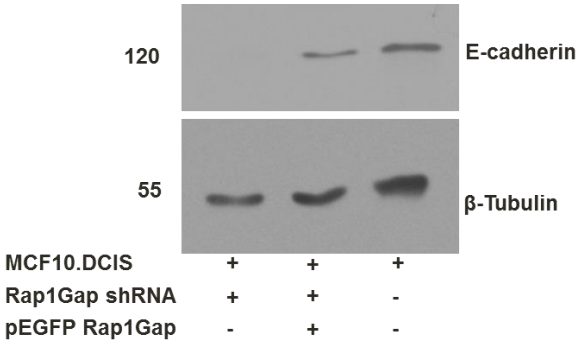


Figure 3.11.4: Re-expression of Rap1Gap in MCF10.DCIS Rap1Gap shRNA cells partially restores E-cadherin levels and adherens junctions.

A. Cells were cultured in 3D for 8 days. Structures were then fixed and stained for E-cadherin (magenta) and nuclei (blue). Images taken at 20x magnification. Scale bar represents 50 μm. **B.** GFP-Rap1Gap transfected cells were seeded in rBM for 8 days. Lysates were prepared and subjected to SDS-PAGE and immunoblotting for Rap1Gap and β-tubulin (loading control).

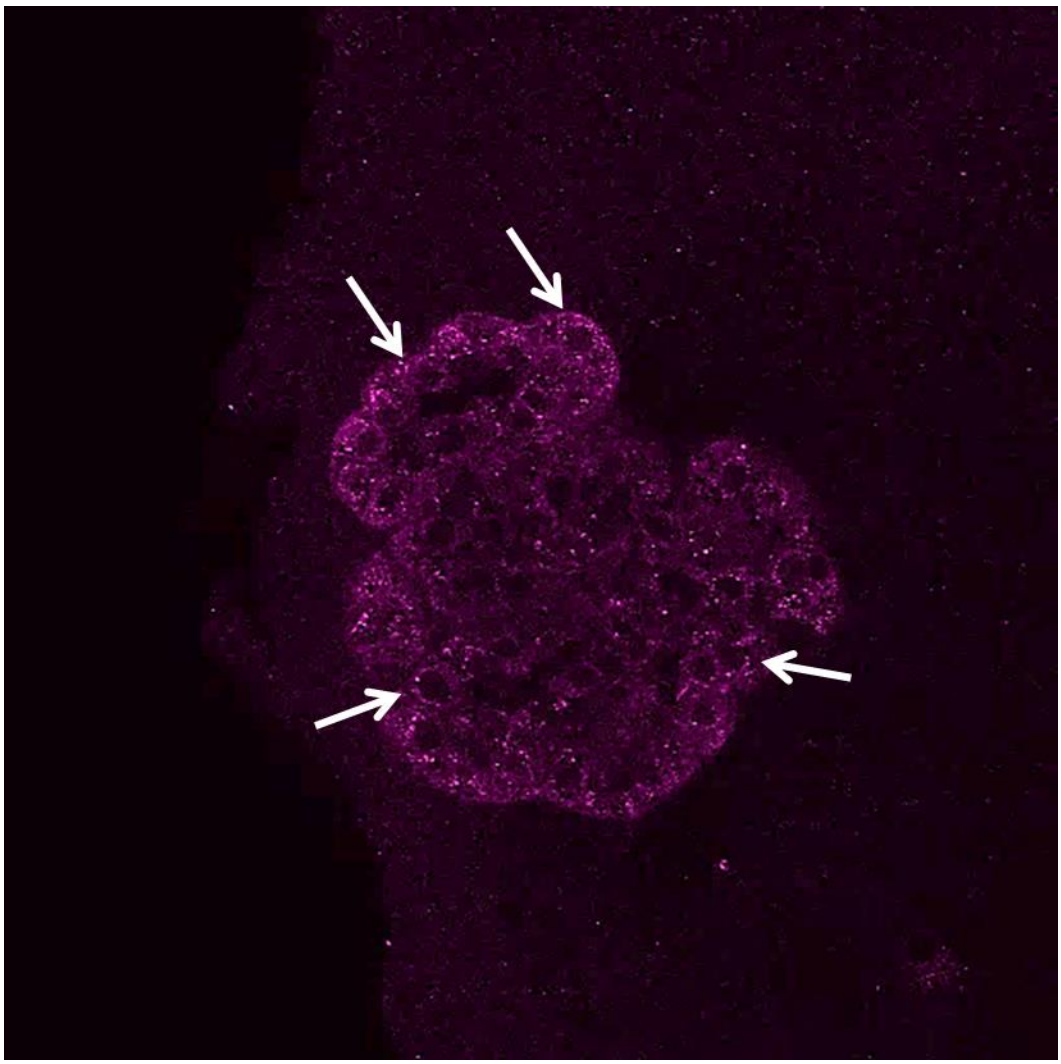


Figure 3.11.5: Re-expression of Rap1Gap in MCF10.DCIS Rap1Gap shRNA cells partially restores E-cadherin levels and localization.

Shown in magenta, fluorescent probe detection of E-cadherin in DCIS Rap1Gap shRNA cells transfected with GFP-Rap1Gap that had been cultured in 3D for 8 days. Image is a single and expanded version of the previous image in Figure 3.11.4 (middle image, bottom row, A) to emphasize the localization of E-cadherin. Arrows point to localization of E-cadherin.

3.12 Lentiviral silencing of Rap1Gap in MCF10.DCIS causes an increase in Rap1 activity

Given that Rap1Gap is a negative regulator of Rap1, we reasoned that knockdown of Rap1Gap would result in an increase in Rap1 activity. Cells were grown in 2D for 5 days or until 70% confluency and serum starved overnight. Rap1 activity assays were performed to detect active Rap1. As seen in Figure 3.12, modest Rap1 activity is observed in the control. Rap1 activity was increased in both of the DCIS Rap1Gap shRNA lines (kd1 and kd2) and MCF10.CA1d. In addition, re-expression of GFP-Rap1Gap caused a reduction in Rap1 activity. Panel B shows densitometry analysis of active Rap1. We show that while silencing of Rap1Gap in MCF10.DCIS increases Rap1 activity, re-expression of Rap1Gap reduces Rap1 activity.

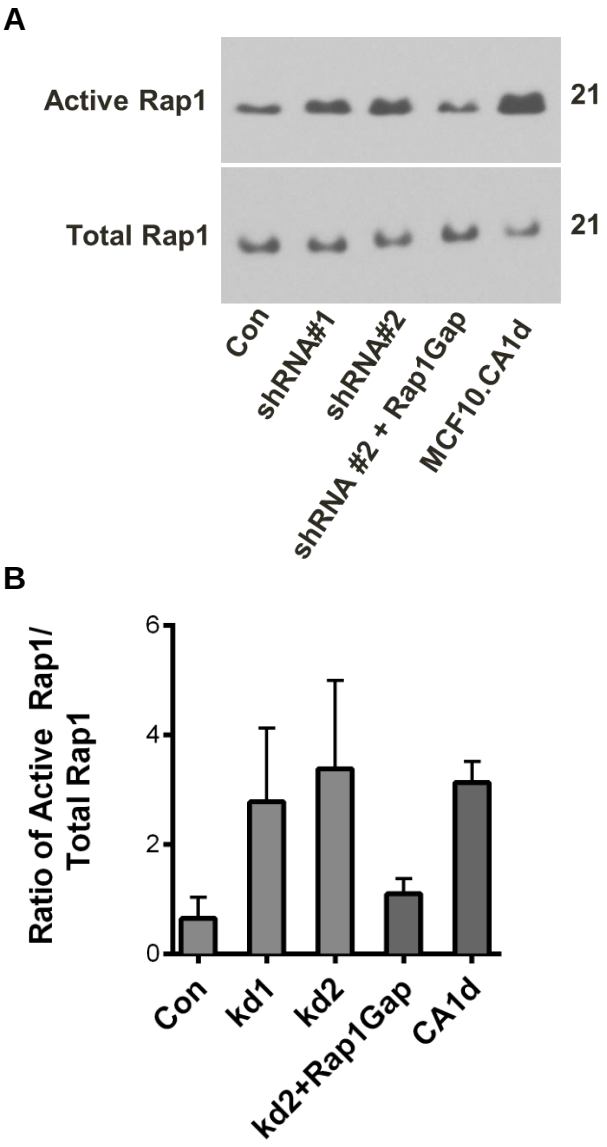


Figure 3.12: Lentiviral silencing of Rap1Gap in MCF10.DCIS causes an increase in Rap1 activity.

A. Cells were grown until 70% confluency and serum starved overnight. Total Rap1 was used to verify even loading of lysate used in the actity assay. **B.** Densitometric scans of active Rap1 and total Rap1. Ratio of Rap1 GTP vs total Rap1. Error bars represent standard deviation. Data taken from two separate experiments.

3.13 Rap1Gap silencing in MCF10.DCIS causes increase in ERK1/2 MAPK activation

Previous immunohistochemical studies show increased MAPK/ ERK1/2 signaling in breast cancers compared to normal tissue via IHC (Sivaraman, Wang et al. 1997), and that primary tumors of node positive patients have relatively higher ERK1/2 activation (Mueller, Flury et al. 2000). Hence, we hypothesized that Rap1Gap silencing may result inERK1/2 MAPK activation.

Cells were grown in rBM for 8 days and lysates were harvested, subjected to SDS-PAGE and immunoblotting ERK1/2. The results in Figure 3.12, panel A, show increased phosphorylation of ERK1/2 in MCF10.DCIS cells with silenced Rap1Gap expression (lines kd1 and kd2) grown in 3D. An even more robust increase in ERK1/2 phosphorylation was observed in MCF10.Ca1D cells. Panel B is box-whisker plot of ERK1/2 activation in lysates harvested from 3D conditions. One-way ANOVA statistical analysis was performed with a p value = 0.0006. Thus, silencing of Rap1Gap in MCF10.DCIS results in increase in ERK1/2 activation.

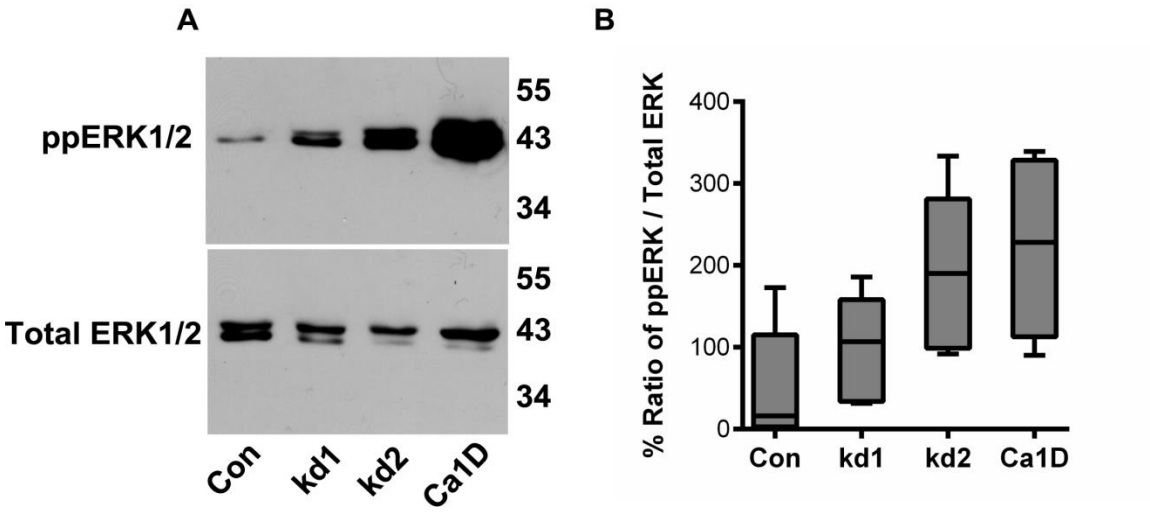


Figure 3.13: Rap1Gap knockdown in MCF10.DCIS causes increase in ERK1/2 MAPK activation.

A. Lysates were prepared from cells were grown in rBM for 8 days and subjected to SDS PAGE and immunoblotting. The blot was first probed for ppERK1/2 and then stripped and reprobed for total ERK1/2. **B.** Box whisker plot shows densitometry % ratio of phosphorylated ERK1/2 vs. Total ERK1/2 from immunoblots from three separate experiments.

CHAPTER 4: DISCUSSION

4.1 NGS, qPCR validation, and immunoblotting of Rap1Gap

This dissertation project aimed to delineate the role of Rap1Gap in the progression to invasive breast cancer by using the MCF10 progression series, in the context of 3D. Previous data from NGS studies revealed Rap1Gap was consistently up-regulated in MCF10.DCIS and two other DCIS lines- SUM 225 (derived from a chest wall recurrence) and SUM 102, an extensive primary DCIS with micro-invasion (Kaur, Mao et al. 2013), compared to MCF10A cells, a model of non-transformed breast epithelium. Further mining of the NGS data revealed significantly enriched common frameworks (as referenced in Table 1), with a 336 fold enrichment. This suggests that up-regulation of Rap1Gap is a pivotal part of the DCIS signature.

Validation of NGS results via qPCR show that Rap1Gap transcripts are low in MCF10A and are increased in MCF10.DCIS and SUM 225 cells. However, Rap1Gap transcripts are low in SUM 102 cells, which is not in agreement with the initial transcriptome analysis. There are several explanations for such a difference. One could be that while NGS was performed on RNA of cells grown in 3D, the qPCR analysis was performed on RNA from cells grown in 2D. This is not surprising, as several instances of differences in gene and protein expression in 2D and 3D are documented. For example, in several epithelial ovarian cancer cell lines, β-catenin and BRCA1 were upregulated in 2D *vs* 3D environment (Lee, Mhawech-Fauceglia et al. 2013). Higher expression of cleaved caspase-3 is observed in 3D structures compared to 2D monolayer culture (Lee, Mhawech-Fauceglia et al. 2013). Additional examples such as differences in drug sensitivity, differentiation, etc. in the breast cancer models are

mentioned in section 1.7. A potential technical difference worthy of mention is the passage number of SUM 102 cells used in both the mRNA seq and qPCR analysis might be different, resulting in the difference in the results. Another technical difference is the probes/primers used in the mRNA seq and qPCR analysis, which would target different exons of the same gene.

In lysates prepared from cells grown in 3D, Rap1Gap protein levels are also low. This implies post-transcriptional regulation of Rap1Gap. One possible explanation is that in the breast, Rap1Gap might be subject to proteasome degradation (Wang, Zhang et al. 2014).

Immunoblotting results show that MCF0A cells express little Rap1Gap, whereas it is increased in MCF10.DCIS. Compared to lysates extracted from cells grown in 2D, Rap1Gap levels in MCF10.Neo T cells are significantly higher. This is interesting, since MCF10.Neo T cells are a model of transformed epithelial cells. MCF10.Neo T cells were transformed by transfection with mutated H-Ras (Dawson, Wolman et al. 1996). A possible explanation is that increase in Rap1Gap might initially antagonize transformation initiated by Ras. This would be consistent with models where Rap1 is an antagonist of activated Ras (Tsygankova, Prendergast et al. 2007) and the data that decrease and increase of Rap1 Gap levels increases and decreases Rap1 activity levels, respectively (Figure 3.12). Down regulation of Rap1Gap leads to activation of ERK1/2/MAPK (Figure 3.13), which may correlate with invasive phenotype (Tsygankova, Prendergast et al. 2007). Rap1Gap levels are reduced in MCF10.CA1D cells (Figure 3.1). These findings led us to hypothesize that reduction of Rap1Gap would play a role in the progression to invasive breast cancer.

Interestingly, rather than overexpression of Rap1Gap, there has been interest in loss of Rap1Gap in cancer. For example, studies show that down-regulation of Rap1Gap has been linked to invasion and EMT in multiple cancers (Zhang, Chenwei et al. 2006, Zhang, Mitra et al. 2006, Zheng, Gao et al. 2009, Tsygankova, Ma et al. 2010, Zuo, Gandhi et al. 2010). There is one published study that mentions mutations of unknown significance have been observed in breast cancer (Sjoblom, Jones et al. 2006). We show that the loss of Rap1Gap may act as a switch in the progression to invasive breast cancer. I show that down-regulation of Rap1Gap via lentiviral silencing leads to increased Rap1 and ERK1/2 MAPK activation. Cells acquire an invasive phenotype, characterized by extensive cytoskeletal remodeling and enhanced invasion.

4.2 Rap1Gap is decreased in invasive breast cancer cell lines

Protein expression of Rap1Gap is increased in DCIS and decreased in invasive breast cancer cell lines. Some invasive breast cancer cell lines (i.e., MDA-MB-231, BT549, and Hs578t) exhibit a mesenchymal phenotype in 2D and stellate phenotype in 3D. We hypothesized that MCF10.DCIS cells, which exhibit an epithelial phenotype characterized by wide cellular bodies and some adherens junctions, would acquire a fibroblastic phenotype when Rap1Gap is silenced. Cells acquired a spindle shape (Figure 3.4), with observed reduction in E-cadherin as a result of lentiviral silencing (immunofluorescence images and immunoblot in Figure 3.7). In a 3D environment, MCF10.DCIS cells form compact dysplastic structures that model DCIS *in vivo*. Adherens junctions (visualized as the localization of E-cadherin, a major component of adherens junctions) and cortical rings (F-actin) are visible in these structures when assayed by immunofluorescence. The MCF10.DCIS cells transduced with Rap1Gap

shRNA acquire a starkly contrasting phenotype. The majority of the structures with the Rap1Gap shRNA form long invasive outgrowths, signifying reorganization of F-actin that enables cell elongation and directional motility (Thiery and Sleeman 2006, Yilmaz and Christofori 2009, Yilmaz and Christofori 2010). Thus, lentiviral silencing of Rap1Gap in DCIS leads to at least two phenotypic changes: reduction of E-cadherin and F-actin re-organization.

Such an array of phenotypic changes would be consistent with the characteristics of EMT. There are three types of EMT. Type 1 EMT occurs in implantation, embryogenesis and organ development; Type II occurs in tissue regeneration and organ fibrosis; Type III is reported in cancer (Kalluri and Weinberg 2009). In this discussion, the focus is on type III EMT, which is most germane to cancer. EMT is described as a complex, transient and reversible process, characterized by the loss of epithelial characteristics (such as cell–cell attachments, adhesion, and apical–basal polarity) and the gain of mesenchymal characteristics (such as increased motility, invasive properties, and a spindle-like morphology) (De Craene and Berx 2013). Studies have suggested that loss of functional E-cadherin contributes to EMT, where epithelial cells exhibit loss of cell-cell adhesion, increased motility and invasiveness (Thompson, Torri et al. 1994). Depletion of Rap1Gap in DCIS caused a reduction in E-cadherin and dissolution of adherens junctions.

4.3 Silencing of Rap1Gap in MCF10.DCIS leads to cytoskeletal remodeling

In addition to alterations in adherens junctions, MCF10.DCIS Rap1Gap shRNA cells exhibit invasive outgrowths and distinct cytoskeletal remodeling. Epithelial cells possess apico-basal polarity which helps anchor them to the basement membrane;

changes from apico-basal polarity to front-rear polarity occur during EMT (Lamouille, Xu et al. 2014). Since the outgrowths appear flattened (as depicted in the 3D reconstructions), it is possible that the transition to front-rear polarity is occurring, and could be further investigated.

Vimentin has been widely reported as a marker of EMT (Mendez, Kojima et al. 2010), and in breast cancer, vimentin is found to be highly expressed in high-grade ductal carcinomas (Liu, Zhang et al. 2013). Vimentin cannot be used as an EMT marker in this project. MCF10.DCIS cells already abundantly express vimentin. Increase in vimentin expression as a result of silencing of Rap1Gap in MCF10.DCIS was not confirmed. In addition to F-actin reorganization (immunofluorescence images, Figure 3.8.2), we show reorganization of vimentin occurs as a result of Rap1Gap silencing (immunofluorescence images, Figure 3.8.3). Here, we emphasize that in these studies, vimentin is re-organized in DCIS Rap1Gap shRNA cells. In the future, EMT could be confirmed by evaluating the emergence of quintessential EMT markers such as N-cadherin, Twist, Snail or Zeb (Sanchez-Tillo, Liu et al. 2012) via immunoblotting or other methods.

4.4 Silencing of Rap1Gap leads to enhanced invasion in MCF10.DCIS

Given the observed cascade of phenotypic changes that have been elicited by the silencing of Rap1Gap in MCF10.DCIS, we hypothesized that the 3D structures formed by DCIS Rap1Gap shRNA cells may be invasive. The results from invasion assays performed (Figure 3.9, panel A) show a significant increase in invasion when Rap1Gap is silenced. One observation is that there was a very small number of invading MCF10.DCIS cells. This phenomenon is in agreement with previous work, which shows evidence of some invasion by MCF10.DCIS, both *in vivo* and *in vitro* (Miller, Santner et al. 2000, Li, Mullins et al. 2008). The number of DCIS Rap1Gap shRNA cells that invaded was significantly higher than the control. The invasion assay was performed in a semi-3D environment, with a coating of 2 mg/ml Cultrex®. The results of the invasion assay indicate that the cells are motile and are able to move through the Cultrex® and the pores of the membrane.

4.5 Silencing of Rap1Gap in MCF10.DCIS leads to increased collagen IV degradation

We have shown that re-organization of F-actin and dissolution of adherens junctions is associated with the invasion process of MCF10.DCIS cells when Rap1Gap is silenced. This finding led us to question another interesting aspect of the invasion process- remodeling by proteases. Given the stark phenotypic changes acquired by Rap1Gap silencing in MCF10.DCIS cells, and given the observed enhanced invasion capability, we hypothesized that increased degradation of type IV collagen would play a role in invasion, and that silencing of Rap1Gap in MCF10.DCIS would lead to increased proteolysis.

In the breast, the basement membrane (BM) is the main ECM that interacts with the luminal and myoepithelial cells of the breast duct. It is primarily made up of entactin, proteoglycans, laminins and collagen type IV (Oskarsson 2013). We showed that Rap1Gap silencing in MCF10.DCIS cells enhanced intracellular degradation of collagen type IV (Figure 3.10.1, Figure 3.10.2 and Figure 3.10.3). Many proteases are involved in the remodeling of the ECM. In breast cancer; for example, the participation of cysteine cathepsins has been reported (Sameni, Moin et al. 2000) (Curino, Engelholm et al. 2005). The type of protease involved here is yet to be confirmed and characterized, and is a potential focus of future projects. Some studies have shown that Rap1Gap plays a role in metastasis and extravasation via inside-out signaling of the integrins, particularly β-1 integrin (Kato, Liao et al. 2012) in melanoma. This is particularly intriguing because independent studies have elegantly reported that β-1 integrin promotes collagen type IV degradation in breast and prostate cancers cells, and that reduction of β-1 integrin expression and function reduced degradation of dye quenched collagen IV and invasion (Sameni, Dosescu et al. 2008). Even though the proteolysis assay was performed over a period of 8 days, it complements the results of the invasion assay. We hypothesize that the cells secrete proteases to move through the rBM.

4.6 Re-expression of Rap1Gap leads to reversal of invasive phenotype

The cascade of phenotypic changes that silencing of Rap1Gap has elicited in MCF10.DCIS has been addressed. As a control, to confirm whether these changes are due to reduction in Rap1Gap, rescue experiments were performed. We transiently re-introduced GFP-Rap1Gap into the DCIS Rap1Gap shRNA#2 (kd2) cells (Figure 3.11.2). Re-introduction of Rap1Gap inhibits the formation of invasive outgrowths. The

structures formed are round and compact, and the F-actin cortical rings are present. Additionally, we also show that E-cadherin levels increased and adherens junctions are partially restored (immunofluorescence images and immunoblot in Figure 3.11.4).

4.7 Rap1Gap limits Rap1 and ERK1/2 MAPK activation

The results in Figure 3.12 show that DCIS cells transduced with Rap1GapshRNAs exhibit higher Rap1 activation, and that Rap1 activation is reduced following re-expression of Rap1Gap. There is also a significant increase in ERK1/2 MAPK activation in the knockdown cells grown in 3D compared to the control (Figure 3.13). ERK1/2 MAPK signaling is stronger in the shRNA#2 (kd2) compared to Rap1Gap shRNA#1 (kd1). Previous studies from our laboratory have confirmed the presence of activated H-Ras in MCF10.DCIS (Li and Mattingly 2008). This is expected because MCF10.DCIS is isogenic to the MCF10.NeoT cell line (see section 1.2 for more information), which harbors the constitutively active T24-H-Ras (Dawson, Wolman et al. 1996). Since H-Ras is already constitutively active in MCF10.DCIS, the assumption is that DCIS Rap1Gap shRNA cells also harbor constitutively active H-Ras. Since ERK1/2 is further activated in the DCIS Rap1Gap shRNA cells, we conclude that Rap1 and Rap1Gap might participate in downstream signaling, between H-Ras and ERK1/2. Rap1Gap and Rap1 may regulate ERK1/2 by antagonizing signals relayed by H-Ras.

4.8 Alternative mechanisms

Other mechanisms have been proposed to delineate the role of Rap1Gap in cancer progression. For example, studies show that overexpression of Rap1Gap in pancreatic cancer cells lines (Zhang, Chenwei et al. 2006) resulted in inhibition of FAK activation and cell spreading without changes in ERK1/2/MAPK phosphorylation. On the

contrary, studies in melanoma show that Rap1Gap over-expression leads to reduced ERK/MAPK phosphorylation (Zheng, Gao et al. 2009). Previous studies in different cancers have reported that blockage or RNAi of ERK1/2 inhibits migration and invasion in prostate and human osteocarcinoma cells respectively. (Si, Peng et al. 2012, Li, Li et al. 2013). In the context of ductal breast cancer, ERK1/2 also mediates invasive breast ductal cancer cell migration and invasion (Ma, Lan et al. 2012). Our findings are in agreement with these reports. Other studies have also shown that Rap1 could exert its effects through Src and FAK. For example, depletion of Rap1Gap in colon cancer cells induces increase in Src and FAK activation (Tsygankova, Ma et al. 2010). In addition to the regulation of E-cadherin, inside-out signaling via the integrins, which link the ECM to the actin skeleton at focal adhesion sites, is also regulated by Rap1, via Src activity (Hynes 2002). We investigated Src phosphorylation profiles in MCF10.DCIS vs MCF10.DCIS shRNA cells, which are discussed in Chapter 5.

Rap1 may regulate recycling, avidity and affinity of the integrins that are associated with the actin cytoskeleton, including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_L\beta_2$, $\alpha_{IIb}\beta_3$ (Bos, de Bruyn et al. 2003, Caron 2003, Dustin, Bivona et al. 2004, Bos 2005). Studies in pancreatic cancer cells support the role of Rap1Gap in the regulation of integrin activation (Zhang, Chenwei et al. 2006). Thus, Rap1Gap plays a pivotal role in integrating cell-cell adhesion and ECM (extracellular matrix) attachment in both normal and malignant conditions.

4.9 Other GAPs, Rap1Gap2

Previous studies have shown that various GAPs, related to Rap1Gap, are implicated in proliferative disorders that are similar to cancer. For example, mice

deficient in *Spa-1* develop a spectrum of myeloid disorders that resemble chronic myeloid leukemia (Ishida, Kometani et al. 2003). Another member of Rap1Gap family, E6TP1 (Singh, Gao et al. 2003), contributes to cervical cancer and other cancers associated with chronic human papilloma virus infection. It is targeted for ubiquitin mediated degradation by viral E67 oncoprotein.

The biological functions of Rap1Gap2 are not widely studied. Studies on Rap1Gap2 have involved elucidating its potential role in platelet aggregation (Hoffmeister, Riha et al. 2008, Neumuller, Hoffmeister et al. 2009), axonal outgrowths in olfactory sensory neurons (Sadrian, Cheng et al. 2012) and sex specific association of asthma incidence in Latina females (Myers, Scott et al. 2014). In the context of cancer, IHC analysis shows Rap1Gap2 has been shown to be moderately or highly expressed in thyroid cancer, lobular and ductal carcinomas. (http://www.proteinatlas.org/ENSG00000132359-RAP1GAP2/cancer).

In summary, lentiviral shRNA silencing of Rap1Gap in MCF10.DCIS induced increase in cell proliferation, Rap1 and ERK1/2/MAPK activity, appearance of outgrowths, extensive cytoskeletal reorganization, increase in proteolytic degradation of collagen IV and acquisition of invasiveness. Re-expression of Rap1Gap in MCF10.DCIS-Rap1GapshRNA cells reduced Rap1 activity and suppressed the development of invasive outgrowths in 3D structures. Thus, reduction of Rap1Gap in DCIS may act as a switch to progression to an aggressive phenotype via deregulated Rap1 and ERK1/2/MAPK activation.

This is the first mechanistic study of Rap1Gap in breast cancer. We have developed a model in which over-expression of Rap1Gap may be linked to pre-

malignant progression of breast cancer, whereas subsequent reduction in Rap1Gap may act as a switch to invasive phenotype. As depicted in the model in Figure 4, Rap1Gap protein levels are highest at DCIS stage. Upregulation of Rap1Gap protein in DCIS correlates with E-cadherin delocalization and leads to decreased Rap1 and MAPK activation. Down-regulation of Rap1Gap is proposed to be a molecular switch for progression to invasive ductal carcinoma. Loss of Rap1Gap leads to increase in Rap1 and ERK1/2 /MAPK activation, weakening of cell-cell junctions, cytoskeletal remodeling and invasion. Subsequently, increased cell motility and invasion will ensue. Re-expression of Rap1Gap causes reversal of the invasive phenotype, which includes reappearance of cortical rings, partial restoration of E-cadherin expression and localization consistent with adherens junctions.

Despite the improvement in detecting DCIS, our understanding of the molecular evolution in DCIS is scarce. The results obtained from this study may lead to diagnostic tools that can be used to discern the difference between DCIS lesions that remain indolent and those that are likely to progress.

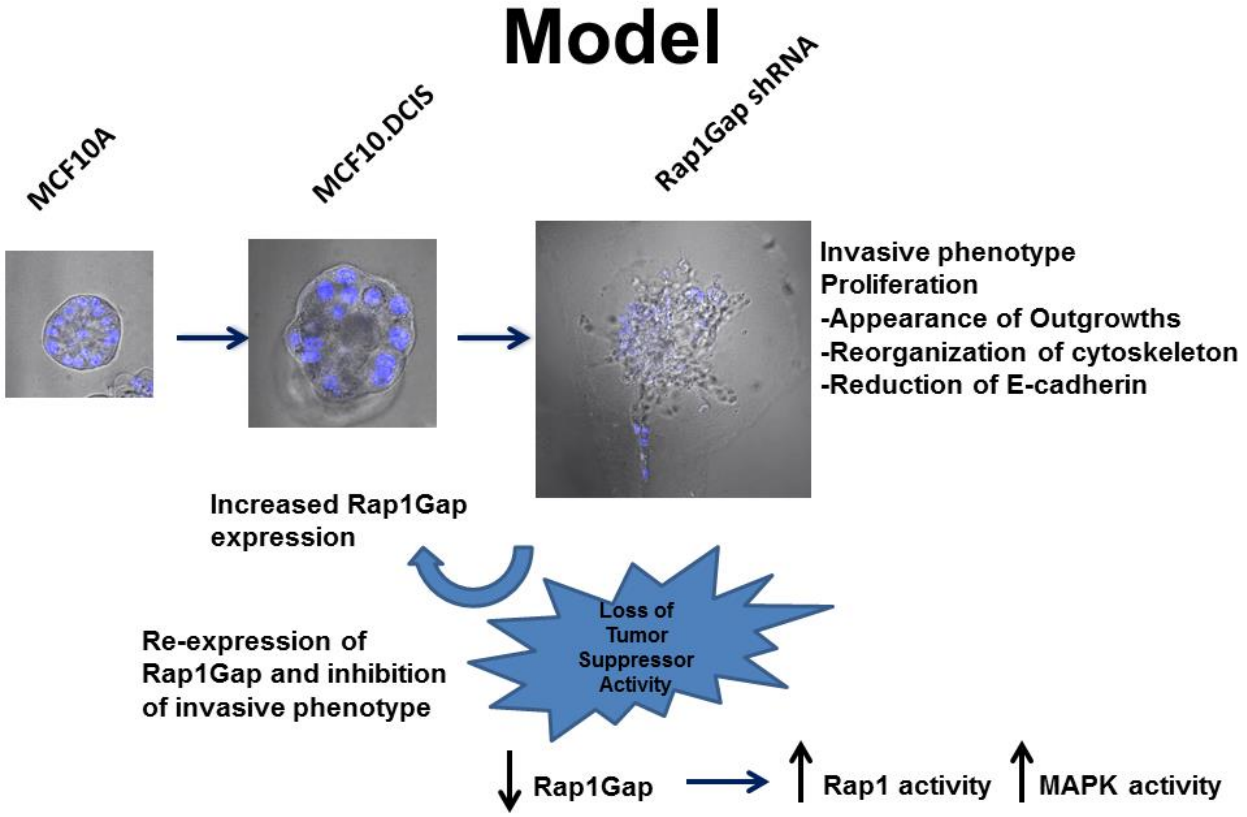


Figure 4: Model. Rap1Gap protein levels culminate at DCIS stage. The over expression of Rap1Gap protein in DCIS leads to E-cadherin delocalization, decrease in Rap1 and ERK1/2 activity. Downregulation of Rap1Gap is a molecular switch for progression to invasive ductal carcinoma, occurring through dramatic increase in Rap1 and ERK1/2 activation. Invasive phenotype, characterized by appearance of multicellular outgrowths, weakening of adherens junctions, extensive cytoskeletal remodeling ensues. Re-expression of Rap1Gap inhibits manifestation of the invasive phenotype via decrease in Rap1 activity and inhibition of appearance of invasive outgrowths.

CHAPTER 5: OTHER DIRECTIONS

5.1 The original hypothesis- Rap1Gap and Src

The original hypothesis of this dissertation was that loss of Rap1Gap, leading to activation of Rap1 and Src kinase, drives the transformation of DCIS to invasive carcinoma via deregulation of epithelial junctions and cell matrix attachments.

5.1.1 Src Kinase

Src is a non-receptor protein tyrosine kinase that transduces signals that are involved in the control of a variety of cellular processes such as proliferation, differentiation, motility, and adhesion (Gelman 2011). Src was initially identified by Peyton Rous in 1911 as the transforming agent in chicken sarcomas. Since then, a whole family of Src kinases has been discovered. Src family kinases play a critical role in cell adhesion, invasion, proliferation, survival, and angiogenesis during tumor development (Lutz, Esser et al. 1998). Overexpression or high activation of Src kinase occurs frequently in tumor tissues and they are central mediators in multiple signaling pathways that are important in oncogenesis (Lutz, Esser et al. 1998). Activation of Src occurs as a result of disruption of the negative regulatory processes that normally suppress Src activity (Bjorge, Jakymiw et al. 2000). In epithelial cells, activation of Rap1 is associated with increased Src kinase activity (Retta, Balzac et al. 2006). Disruption of adherens junction and enhanced cell matrix adhesion are associated with increased or over activated Src (Bailey, Kelly et al. 2009). In epithelial cells, as depicted in Figure 5.1, strong activation of Rap1 by E-cadherin is associated with and controlled by Src.

In addition to the regulation of E-cadherin, inside-out signaling via the integrins, which link the ECM to the actin skeleton at focal adhesion sites, is also regulated by Rap1, via

Src kinase activity (Hynes 2002). Specifically, Rap1 may regulate recycling, avidity and affinity of the integrins that are associated with the actin cytoskeleton, including $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_L\beta_2$, $\alpha_{IIb}\beta_3$ (Bos, de Bruyn et al. 2003, Caron 2003, Dustin, Bivona et al. 2004, Bos 2005). Studies in pancreatic cancer cells support the role of Rap1Gap in the regulation of integrin activation (Zhang, Chenwei et al. 2006). Additionally, studies in colon cancer cells highlight the downstream of action of Rap1Gap to Src; in Rap1Gap depleted cells, Src activity was increased (Tsygankova, Ma et al. 2010). Thus, these studies lead me to hypothesize that Rap1Gap may play a pivotal role in integrating cell-cell adhesion and ECM attachment in both normal and malignant conditions via Src, in the realm of breast cancer.

Rationale: Rap1Gap and Src together play a role in the maintenance of adherens junctions

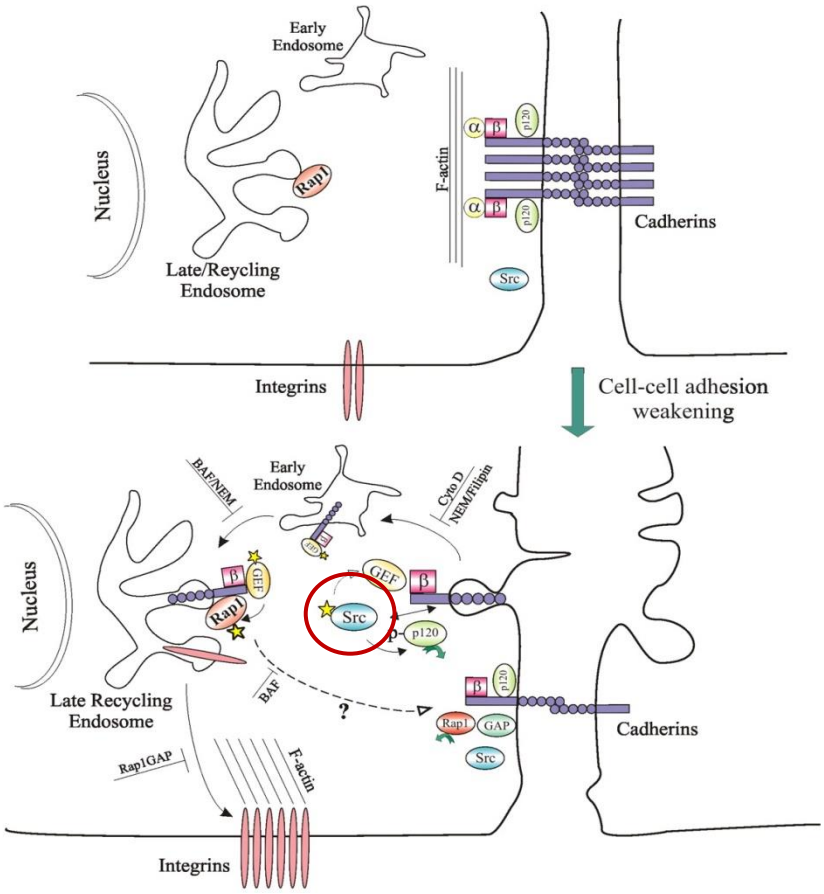


Figure 5.1: Pictorial depiction of Rap1 and Src and their role in the maintenance of adherens junctions in epithelial cells.

Rap1 is diffusely distributed into the cytoplasm. Activation of Rap1, associated with Src (circled in red) activation leads to weakening of cell-cell junctions. E-cadherin, β-catenin and p120 catenin localization are altered, leading to weakening of the adherens junctions. Image taken with permission from Balzac, F., Avolio, M., Degani,S., Kaverina, I. Torti,M,. Silengo, L, J. Small, V., Retta, S. F. J Cell Sci 2005;118:4765-4783.

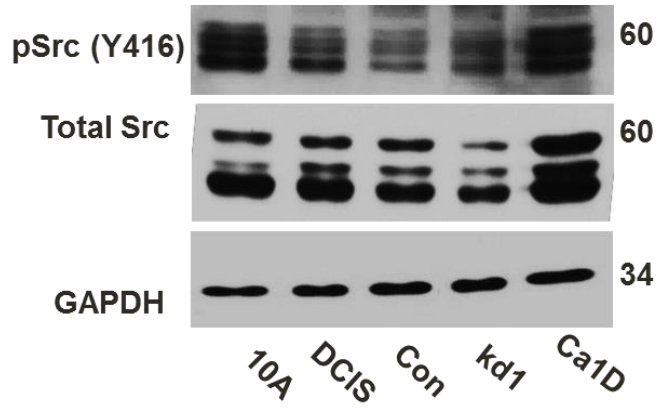
5.1.2 Src activation profiles are dramatically different in 2D *vs.* 3D microenvironments

Based on previous reports, we hypothesized that Rap1Gap down regulation leads to Src activation and acts as an impetus for the acquisition of invasive phenotype in DCIS. On the contrary, the results show the opposite of this original hypothesis. Figure 5.2 shows immunoblots of Src phosphorylation profiles in the MCF10 series – in 2D vs. 3D conditions. Phosphorylation of Src at tyrosine-416 has been linked to its activation (Harvey, Hehir et al. 1989). Here, in panel 2A, in lysates prepared in 2D conditions, we show that Src is phosphorylated in MCF10A and MCF10.Ca1d cells. However, there is little apparent difference in Src phosphorylation in one of the DCIS Rap1Gap shRNA lines (kd1) compared to the MCF10.DCIS control.

Figure 5.2 panel B shows a dramatic change in Src phosphorylation in the MCF10 series in lysates harvested from 3D cultures. MCF10A cells exhibit robust phosphorylated Src. DCIS Rap1Gap shRNA #1 (kd1) and DCIS Rap1Gap shRNA #2 (kd2) have lower Src phosphorylation profiles, with a slightly higher Src phosphorylation in kd1. There are other notable instances of similar dose-dependent responses between the kd1 and kd2 in other experiments in this dissertation. In lysates grown in 3D, Src phosphorylation is higher in the lentiviral control compared to the parental line. This might be due to an artifact of the viral transduction. This does not change the conclusion that Src phosphorylation is dramatically reduced in the DCIS cells with the Rap1Gap shRNA. The differences in Src activation in 3D vs. 2D environments is striking. The data here reveal down-regulation of Src phosphorylation by Rap1Gap silencing that is not evident in a 2D microenvironment.

The conclusion drawn from this experiment is that in the MCF10 progression series, Rap1Gap might not work through Src in the process of invasion. There is one other study done on human alveolar basal epithelial cells (A549 cells) that shows that cell spreading and focal adhesion formation can be directed by Rap1 without Src involvement (Ross, Spanjaard et al. 2012).

A



B

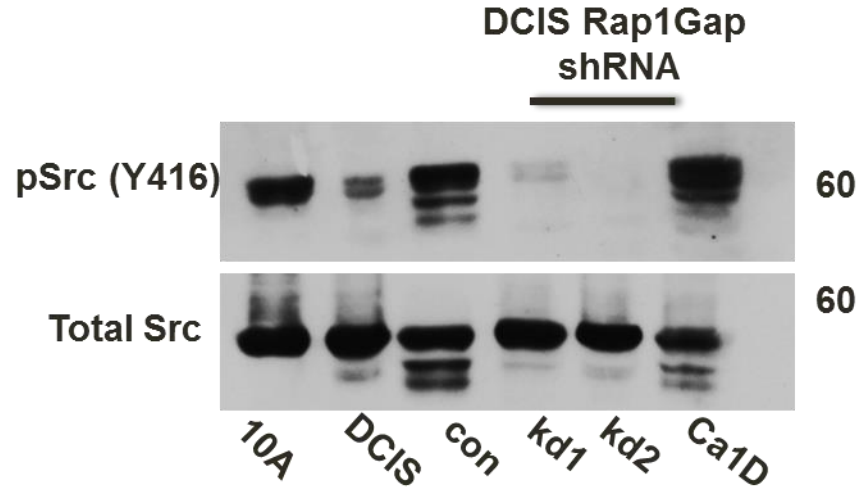


Figure 5.2: MCF10 series cells grown in 2D and 3D conditions reveal stark differences in Src activation.

A. Lysates prepared from cells grown in 2D conditions and subjected to SDS PAGE and immunoblotting and detection of perch and total Src. GAPDH was used as a loading control. **B**. Cells were allowed to grow in 3D for 8 days and lysates were prepared and subjected to SDS-PAGE. Immunoblot shows detection of pSrc and total Src. Results are representative of three experiments.

5.2 Rap1Gap over-expression in SUM 102 cells

A significant portion of this work revolves around the employment of MCF10.DCIS as model to demonstrate the potential role of Rap1Gap in the progression of DCIS. We propounded the idea that depletion of Rap1Gap unleashes the acquisition of invasive phenotype. As mentioned earlier in this dissertation, SUM 102 cells are characterized as a DCIS with micro-invasion (Barnabas and Cohen 2013). We have shown that this basal cell line expresses reduced levels of Rap1Gap (Figure 3.2). Since SUM 102 is a DCIS with micro-invasion, we reasoned that over-expression of Rap1Gap would induce morphological and underlying molecular changes that are reminiscent of a non-invasive DCIS.

The immunoblot results in Figure 5.3 show that robust Rap1Gap over-expression was achieved. When the cells were monitored post transfection, we observed that the many of the cells were not viable, and therefore more sensitive to transfection with plasmid. This might be explained by the effects of over-expression of Rap1Gap on cell viability and proliferation. Due to the sensitivity of these cells to transfection with Rap1Gap, it was not practical to further pursue this part of the project. Further investigation of the effect of Rap1Gap over-expression on the viability and potential inhibition of SUM 102 cell proliferation would be needed.

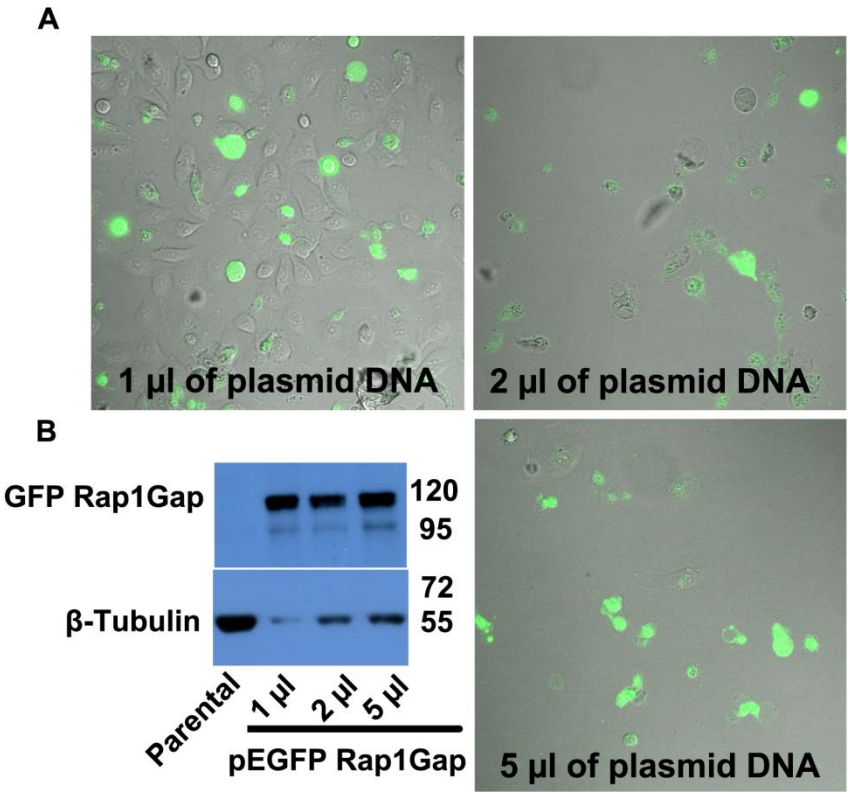


Figure 5.3: Rap1Gap overexpression in SUM 102 cells.

A. SUM 102 cells were transfected with GFP Rap1Gap as indicated. DIC images of live cells were taken 3-4 days post transfection. The overlaid green fluorescence shows GFP reporter of Rap1Gap over-expression. **B.** After 5-7 days of transfection, lysates were prepared and subject to SDS-PAGE. Immunoblot of Rap1Gap over-expression in SUM 102 cells. β-tubulin was used as a loading control.

5.3 Rap1Gap over-expression in MCF10A cells

As described earlier, both at the mRNA and protein levels, Rap1Gap was observed to be upregulated in MCF10.DCIS compared to MCF10A cells, a model of non-transformed mammary gland epithelium. We have presented data that support the hypothesis that Rap1Gap, when lost, manifests in an invasive phenotype in DCIS. Additionally, as noted in other studies, Rap1Gap may act as a tumor suppressor (Zhang, Chenwei et al. 2006). Therefore, we aimed to address the question whether the observed over-expression of Rap1Gap in DCIS drives the development to DCIS from a non-transformed state or if it is a tumor suppressive response to the development of DCIS. Hence, we decided to transiently overexpress Rap1Gap in MCF10A cells, a model of normal breast epithelium.

The DIC images in Figure 5.4, panel A show that the MCF10A cells transfected with GFP control form structures that resemble non-transformed MCF10A cells. Panel B shows an immunoblot of efficient over-expression of Rap1Gap in MCF10A cells. The MCF10A cells transfected with GFP-Rap1Gap from structures that appear essentially similar to the control MCF10A though slightly bigger. They are not reminiscent of MCF10.DCIS, which are dense and dysplastic. Unlike MCF10A cells, which form hollow lumens when grown in rBM, dysplastic structures are characterized by disorganized nuclei and inability to form lumens. The interpretation of these images does not allow us to conclude that Rap1Gap overexpression drives the progression towards DCIS. It is possible that the up-regulation of Rap1Gap is more likely a response to the development of DCIS; i.e. a potential suppressor of progression to DCIS stage.

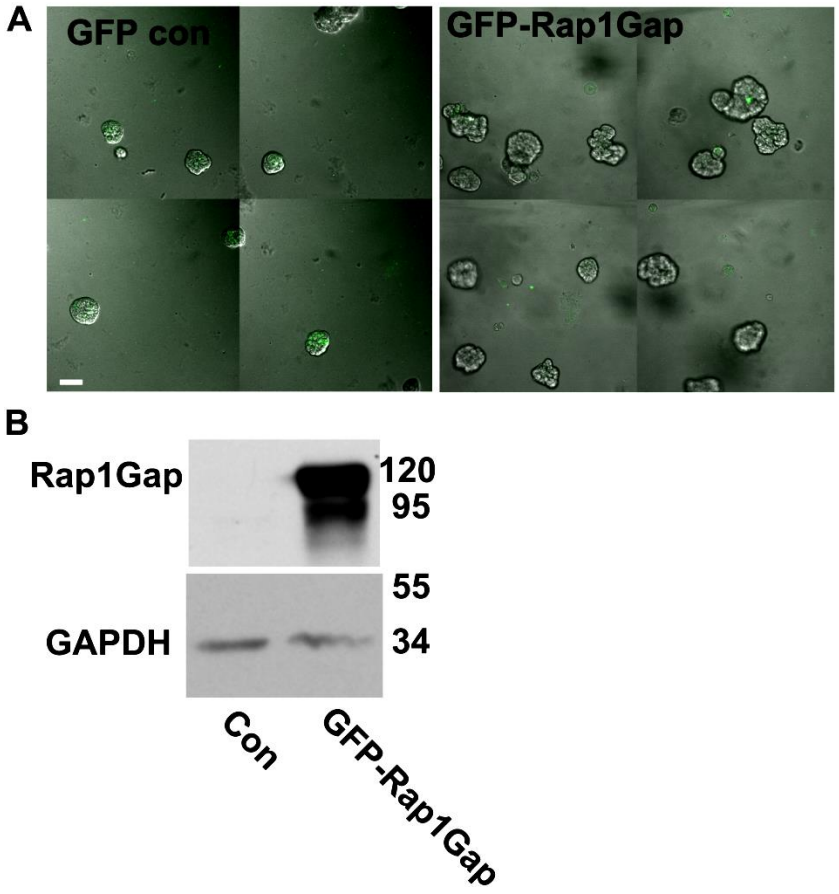


Figure 5.4: Rap1Gap overexpression in MCF10A cells.

A. Five days after transfection, single cell suspensions were seeded in 3D and allowed to grow for 8 days. Contiguous DIC 2x2 stitched images were taken to assay a wider field of cells. Scale bar, 50 µm. **B.** Cells were transfected with GFP Rap1Gap and lysates were made and subjected to SDS-PAGE and immunoblotting. GAPDH was used a loading control.

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ABSTRACT

LOSS OF RAP1GAP: A DRIVER IN THE PROGRESSION FROM DCIS OF THE BREAST TO IDC

by

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The purpose of this study is to determine the role of Rap1Gap in the progression of DCIS (ductal carcinoma *in situ*) to IDC (invasive ductal cancer). We employed an in-vitro three-dimensional (3D) overlay model that provides a physiologically relevant microenvironment to study mechanisms of malignant progression. Previous studies from this laboratory aimed to determine specific gene expression changes common to three models of DCIS- MCF10.DCIS, SUM 102 and SUM 225 in comparison to MCF10A cells, a model of non-transformed human mammary epithelium. The expression of 295 genes was found to be significantly altered, with 63 being increased in expression in all three DCIS cell lines.

The mRNA-Seq results were further mined by Genomatix analysis to gain an insight into common frameworks in promoter regions of these 63 up-regulated genes. 244 promoter loci were found to be associated with these 63 up-regulated genes. Enrichment analysis showed that the common framework RXRF-ZF02-ZF02-PLAG-

HDBP is highly enriched [336-fold], being present in the promoters of RAP1GAP, SPRY4 and PDGFB genes.

Rap1Gap is a GTPase-activating protein (i.e., an inactivator) for the small GTPase, Rap1. It is known to be involved in regulation of cell adhesion and has been previously studied in pancreatic and thyroid cancers, where a decrease in its expression has been associated with malignant progression. Immunoblotting results show that in cells grown in 3D, Rap1Gap levels in MCF10.CA1d cells are reduced compared to those in MCF10.DCIS cells. Lentiviral shRNA silencing of Rap1Gap in DCIS induced an increase in Rap1 and MAPK activity, as determined by Rap1 activity assay and immunblotting for phosphorylated ERK1/2. Confocal immunofluorescence (staining of F-actin) of 3D structures and invasion assays reveal appearance of multicellular outgrowths, extensive cytoskeletal organization and increase in invasion. Concomitantly, increase in collagen IV degradation was observed. Lentiviral silencing of Rap1Gap also resulted in an increase in proliferation. Re-expression of Rap1Gap in DCIS-Rap1GapshRNA cells reduced Rap1 activity and suppressed the development of invasive outgrowths in 3D structures. Additionally, adherens junctions and E-cadherin levels were partially restored. Thus, we conclude that reduction of Rap1Gap in DCIS acts as a switch to progression to an invasive phenotype via deregulated Rap1 and MAPK activation.

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