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THE FUNCTION OF ERBIN, A SCAFFOLD PROTEIN, AS A TUMOR SUPPRESSOR IN COLON CANCER

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DISSERTATION
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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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Lexington, Kentucky

Director: Dr. Tianyan Gao, Professor of Molecular and Cellular Biochemistry

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ABSTRACT OF DISSERTATION

THE FUNCTION OF ERBIN, A SCAFFOLD PROTEIN, AS A TUMOR
SUPPRESSOR IN COLON CANCER

Erbin belongs to the LAP (leucine-rich repeat and PDZ domain) family of scaffolding proteins that play important roles in orchestrating cell signaling. Here, we show that Erbin functions as a tumor suppressor in colon cancer. Analysis of Erbin expression in patient specimens reveals that Erbin is downregulated at both mRNA and protein levels in tumor tissues. Functionally, knockdown of Erbin disrupts epithelial cell polarity and increases cell proliferation in 3D culture. In addition, silencing Erbin results in an increase in the amplitude and duration of signaling through Akt and RAS/RAF pathways. Moreover, Erbin-loss induces epithelial-mesenchymal transition (EMT), which coincides with a significant increase in cell migration and invasion. Erbin interacts with KSR1 and displaces it from the RAF/MEK/ERK complex to prevent signaling propagation. Furthermore, genetic deletion of Erbin in Apc knockout mice promotes tumorigenesis and significantly reduces survival. Tumor organoids derived from Erbin/Apc double knockout mice have increased tumor initiation potential along with increased Wnt target gene expression as seen by qPCR. Collectively, the studies within this dissertation identify Erbin as a negative regulator of EMT and tumor progression by directly suppressing Akt and RAS/RAF signaling *in vivo*.

KEYWORDS: Polarity, EMT, Cell Motility, ERK signaling, Scaffold protein, and Tumor Suppressor

Payton D. Stevens

December 15, 2017

THE FUNCTION OF ERBIN, A SCAFFOLD PROTEIN, AS A TUMOR
SUPPRESSOR IN COLON CANCER

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December 15, 2017
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For my family.

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CHAPTER 1: INTRODUCTION

1.1 Signaling Activation in Colorectal Cancer

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. Approximately 135,000 new cases and 50,000 deaths are predicted for the year 2017 [1]. According to the United States Centers for Disease Control (CDC), data from 2014 shows the state of Kentucky has the highest rates of CRC incidence in the nation (49.4 persons per 100,000 after age-adjustment), which is a great incentive for myself and the Gao laboratory to study this disease at the University of Kentucky. Additionally, rates of CRC outside of the United States have been dramatically increasing with worldwide incidences currently around 1.2 million annually, drastically increasing the importance for studying this disease [2].

While incidence rates of CRC are increasing, mortality from this disease is predominantly due to metastasis to other organs. The patients diagnosed with early stages of colorectal cancers are treated effectively with surgical resection followed by adjuvant therapy; such as 5-FU, Irinotecan, or Oxaliplatin. Currently, targeted therapies such as Avastin and Cetuximab are reserved for second line therapies, while immunotherapies have thus far shown little promise in CRC. However, while treatments have been shown to be effective for early stage CRC patients, ultimately almost half of all patients diagnosed with CRC will develop disseminated advanced disease which in most cases will be fatal due to poor responses to available treatment options [3]. Understanding the progression of this disease, particularly the mutations involved in late stage metastatic CRC, will allow us to develop necessary treatment options to improve the overall survival of CRC patients.

Over the last thirty years, the risk factors for the development of CRC have been studied at both the population and molecular levels. The development of CRC is

multifactorial, as with many cancer types, involving a combination of environmental factors and genetic predispositions. Several studies have shown that lifestyle choices (many of which are common in the state of Kentucky) such as smoking, alcohol abuse, obesity and physical inactivity, and high fat diets can increase the risk of developing CRC [4, 5]; with other studies showing that diseases such as inflammatory bowel disease can increase CRC risk [6]. Other genetic risk factors include the autosomal-dominant genetic disorder, Familial Adenomatous Polyposis (FAP) which results in the formation of many colon adenomas early in a patient's life, often presenting the late teenage years or twenties [7, 8].

While the risk factors that increase the chances of developing CRC are multifactorial, the genetic mutations that occur as this disease progresses have been extensively investigated. Initially hypothesized by Fearon and Vogelstein in 1990 [9], there seems to be a particular order in which certain genes mutate as CRC initiates and progresses to a more malignant tumor type. This stepwise progression from normal epithelia of the colon to a transformed malignant carcinoma is often accompanied by and a result of chromosomal instability (CIN) as indicated by the frequent occurrence of chromosomal alterations and loss of heterozygosity (LOH) [10, 11]. Alternatively, the CIN pathway of malignant transformation may occasionally be referred to as the "suppressor pathway" to CRC.

The first observed mutation in the CIN pathway that results in the initiation of CRC is most often in the Wnt signaling pathway. This is the same pathway that was also identified to be mutated in a majority of familial FAP cases [12] and has been implicated in CRC that results from obesity and lack of exercise [13]. Several proteins within the Wnt pathway can be mutated and ultimately result in increased signaling. As shown in Figure

1.1, the most common mutations occur in the *APC* gene. Also, for this reason, the most commonly used mouse model to study CRC *in vivo* uses an intestinal specific knockout of *Apc,* resulting in hyperproliferation of the intestinal epithelia and the formation of adenomas.

Under normal, non-stimulated conditions, the APC protein interacts with Axin and β-catenin, along with other members of the destruction complex indirectly, and is responsible for degradation of β-catenin (Figure 1.2) [14]. Upon Wnt activation of the Frizzled receptor the proteins that comprise the destruction complex translocate to the plasma membrane, resulting in β-catenin stabilization and subsequent translocation to the nucleus where transcription is activated [15, 16]. APC mutations result in a loss of function and effectively mimic Wnt stimulated signaling, increasing cytosolic β-catenin and eventually leading to increased translocation of β-catenin to the nucleus and increase in Wnt pathway transcriptional targets such as T-cell factor (Tcf) and lymphoid enhancer factor (Lef). Figure 1.2 shows protein interactions that are responsible for signaling under both basal and Wnt stimulated conditions.

Pathways					
MMR	Wnt	MAPK	TGF	p53	PTEN/Akt
MLH1	APC (>70%)[1]	KRAS (40%)	SMAD2 (5–10%)	P53 (>60%)	PTEN (10%)[*]
MSH2	CTNNB1 (<5%)	NRAS (<5%)	SMAD3 (5%)	BAX (5%)	PIK3CA (15–20%)
MLH3	CMYC (5–10%)	EGFR (5–15%)	SMAD4 (10–15%)	CDK8 (10–15%)	HER2 (<5%)
MSH6	MYB (<5%)	HER2 (<5%)	TGF βIIR (10–15%)[*]		
	CCNE1 (5%)	BRAF (5–10%)	ACVR2 (10%)[*]		
	TCF7L2 (5%)[*]				
	CDK8 (10–15%)				
	FBXW7 (20%)				

Figure 1.1 Frequency for each genetic mutation found in colorectal cancer

Recurrent somatic gene mutations that are found in human CRC, including the percentage of total tumors that harbor each mutation. [1] Frequency observed in all sporadic colon cancers. * associated with mismatch repair (MMR) defects. [17]
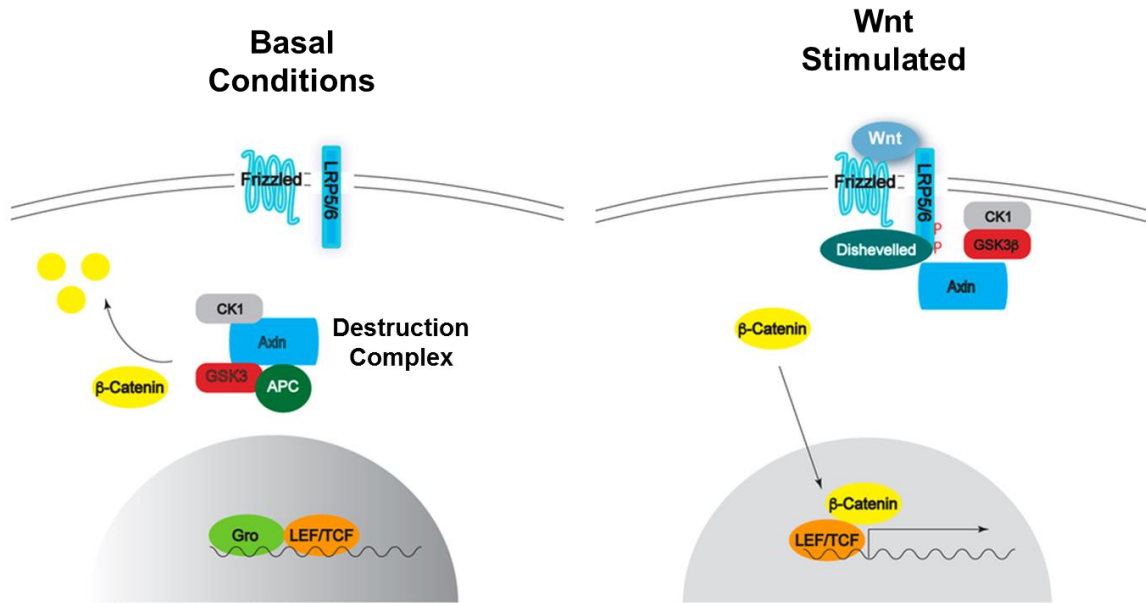
Figure 1.2 General activation of Wnt signaling

APC is a member of the destruction complex as pictured in basal conditions. In this condition β-catenin is phosphorylated and subsequently ubiquitinated and degraded through the proteasome pathway. Upon Wnt stimulation Dishevelled is recruited to the active Frizzled receptors where it facilitates the interaction between the phosphorylated LRP5/6 and Axin [18]. Other proteins that comprise the destruction complex are also translocated to the active receptor and thus are removed and sequestered away from β-catenin [15, 19]. β-catenin is no longer degraded and translocates to the nucleus stimulating expression of Wnt target genes. Adapted from Dunn et al. [20]

More recently, several other proteins have been found to modulate Wnt/APC signaling adding to the complexity of this pathway. The identification of receptors LGR-4 and LGR-5, which are stimulated by the extracellular ligand R-spondin, can significantly increase Wnt signaling upon activation [21]. This activation can be further enhanced by the activation of a co-receptor, LRP6, a protein that is activated through cytoplasmic phosphorylation and results in amplification of Wnt signaling [22]. Understanding the dynamics of how these proteins may alter Wnt signaling in different cancer types is still ongoing.

The functional effects of Wnt signaling activation are well conserved through evolution and Wnt pathway components play an important role in organismal patterning during development [23]. Outside of development, one of the key roles of Wnt signaling is in adult stem cells. This role is particularly important in tissues that have higher rates of self-renewal, which include mesenchymal stem cells in bone marrow, hair follicles, and intestinal stem cells in the crypts [24-26]. However, sporadic CRC formation requires loss of APC function in both alleles [27] and is often observed as an increase in proliferation and the formation of neoplasia within the colon. This increase in tumorigenesis and proliferation has been attributed to an evasion of cell death through the increased activation of Cyclin D1 after APC mutation [28], as well as more complicated mechanisms which include changes in Wnt signaling resulting in alterations to intestinal stem cells and tumor initiating cancer stem cells [29, 30]. Current studies are focused on further determining the molecular mechanism underlying CRC tumor initiation and activation of cancer stem cells.

The next gene that is frequently mutated in the stepwise progression of CRC is the proto-oncogene *KRAS* (Figure 1.1 and 1.5). These mutations, with more than 90%

occurring within exon 12 or 13, lead to further evasion from apoptosis and often coincide with an increase in proliferation [31]. A less common mutation within the same pathway is that of *BRAF*, while mutually exclusive to KRAS mutations, both result in an increase in the mitogen-activated protein kinase (MAPK) (Figure 1.3) [32]. Simultaneous increases in Wnt signaling and MAPK, as well as AMP-activated protein kinase (AMPK), have been observed in CRC with mutated *KRAS* suggesting interactions between these pathways can occur and changes in signaling may alter the prognoses of CRC patients [33, 34]. Understanding the mutations and alterations in protein interactions within the MAPK pathway in CRC are necessary to help understand how this pathway results in increased proliferation and crosstalk between signaling pathways. As MEK inhibitors are currently being tested as second line therapies to treat patients with KRAS mutations, a more in depth knowledge of protein interactions and scaffold proteins involved in this pathway is necessary for more precise treatments.
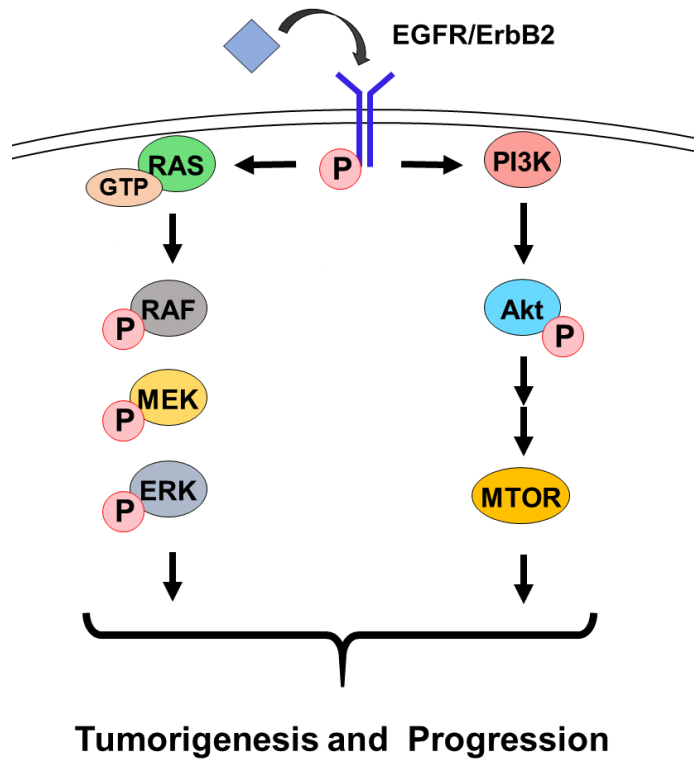
Figure 1.3 Signaling through the PI3K/Akt and ERK/MAPK pathways

A schematic of the signaling that occurs in two of the most mutated pathways in CRC. Following a mutation in the APC signaling pathway, mutations in PI3K and either RAS or RAF, and to a lesser extent at the receptor level, allows the cells to increase transcription for pro-growth and anti-apoptotic proteins.

In addition to activation of Wnt and MAPK pathways, there are several mutations that may further the malignant progression of CRC (Figures 1.1 and 1.5). As with many types of cancer, late stage CRC often acquires a mutation in *TP53* that results in a loss of function in this important tumor suppressor. This mutation results in further avoidance of the apoptotic pathway and thus increases sustained proliferation [35], and often occurs simultaneously with the loss of p53 is LOH of the *18q* chromosome [36]. Additional genes that are contained within this long arm of the 18th chromosome encode for Smad2, Smad4, and Deleted in Colorectal Carcinoma (*DCC*). Additionally PHLPP1, a phosphatase that has been shown to act as a tumor-suppressor in CRC is localized to this region. This phosphatase has been shown to directly dephosphorylate both RAF1 and Akt, inhibiting cell motility and proliferation [37-39]. Importantly, several studies have shown that *18q* LOH is associated with increased metastatic potential and decreased patient survival time [40, 41].

Lastly in the CIN pathway to CRC, mutations can occur in *PI3KCA*, the catalytic subunit of phosphoinositide-3 kinase (PI3K), in tumors with APC mutations (Figures 1.1 and 1.5) [42]. The addition of this mutation leads to upregulation of Akt signaling which increases cell proliferation (Figure 1.3) [43]. Indeed, expression of a constitutively active PI3K in mouse intestinal epithelium results in an increase in tumor number and size [42]. As another potential target for pharmacological inhibition, more studies on the PI3K pathway and its crosstalk with the RAS/RAF pathway, would improve our ability to treat patients that harbor PI3K mutations.

Another aspect to consider when examining the progression of CRC is the microsatellite instability (MSI) status of the tumor. Approximately 15% of CRC have MSI,

which results from a loss of function in DNA mismatch repair (MMR). This DNA proofreading system is responsible for recognizing and repairing single nucleotide mismatches that are not recognized as errors by DNA polymerase. Ultimately, loss of function of these proteins reduces the fidelity at which cells replicate which in turn causes an increase in mutational burden and ultimately results in tumors with greater than a 100-fold increase in mutations [44]. Most CIN pathway CRC tumors are MSI negative, while many tumors that result from genetic predisposition are positive for MSI. In addition to differences in the mutations and type of patients that are MSI positive, MSI status can alter a patient's prognosis. MSI positive CRC tumors are more likely to occur in the proximal colon, have a medullary phenotype as determined by immunohistochemical (IHC) staining, and occur with lymphatic infiltrations of the tumor. Together, these differences result in a longer patient survival time and better prognosis than a patient with a more traditional CIN progression [45]. For these reasons there has been a more recent push to categorize CRC patients into specific sub-categories based upon their unique tumor mutations (Figure 1.4). These subtypes of CRC, called consensus molecular subtypes (CMS), can be used to monitor patient responses and may eventually prove useful for guidance in personalized medicine and individual prognoses [46].

The transformation process from normal epithelial cells to benign hyperplastic growth, to adenoma, and finally to carcinoma results from an accumulation of mutations (Figure 1.5). These mutations typically occur within a particular order and result in a well-established stepwise progression of CRC. Confirming the requirement of this stepwise process *in vitro*, recent publications have used either sh-RNA or CRISPR/Cas9, in mouse or human intestinal stem cells respectively, to decipher the contribution of mutations in

individual genes within the CIN pathway. Normal human intestinal organoids grown in 3D culture were able to transform into tumor organoids after the reduction or mutation of only 4 genes, *APC*, P*53*, *KRAS*, and *SMAD4* [47-49]. Since the most common mutations observed in the CIN process alter cellular signaling events it is desirable to target these pathways in developing future treatments. Additionally, as systemic metastasis of CRC remains the single largest impediment for achieving better overall survival, additional studies and a better understanding of late stage signaling and molecular events leading to cancer metastasis are needed in order to improve the current treatment and prognoses of CRC patients.

CMS1 MSI immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Figure 1.4 Gene expression-based molecular subtypes of colorectal cancer

Consensus molecular subtypes (CMS) are a relatively new concept in identifying various subtypes of CRC based upon the genes that are found to be mutated during tumor molecular sequencing. Additionally, after sub-grouping of the tumors, these CMS can be used to monitor patient response and may prove useful for guidance in personalized medicine [46].
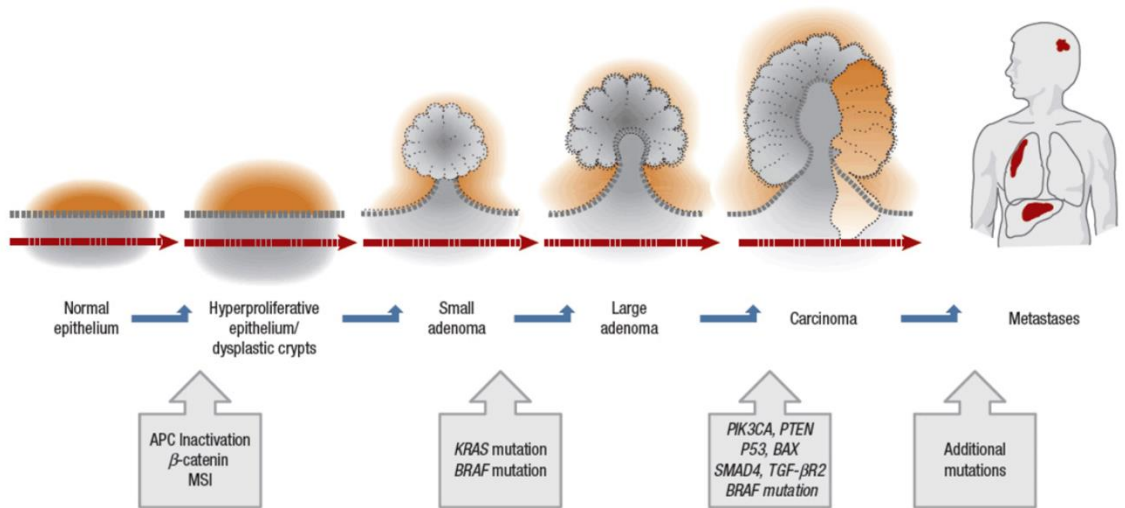
Figure 1.5 Summary of the most frequent mutations occurring in the progression of CRC

Originally proposed by Fearson and Vogelstein, the stepwise progression from normal epithelium to carcinoma has been refined over the past twenty years. We now know that most of all CRC tumors initiate with a Wnt pathway mutation, then gain a mutation in the ERK pathway, followed by additional mutations after the genome is significantly less stable. Image modified from basicmedicalkey.com [50]

1.2 The Role of Scaffold Proteins in Signal Transduction

The majority of the mutations that occur in the CIN pathway in CRC are components of signal transduction pathways that are involved in regulating cell growth. While many of these common oncogenic CRC mutations have been explored for several decades we are only more recently beginning to understand the intricacy of regulating multiple steps within and crosstalk between pathways.

The organization of protein interactions and coordination of signaling are imperative for homeostasis. The proper trafficking and activation of receptors, including the oncogenic receptor tyrosine kinase (RTKs), is necessary to maintain normal epithelial cell signaling and preventing tumorigenesis. Activation of these receptors initiate signaling through pathways that control a variety of transcriptional programs including those that modify proliferation, differentiation, and cell apoptosis [51, 52]. Aberrant activation, occasionally through mutations (Figure 1.1), of these receptors can result in many diseases including cancer. Many different proteins can interact with these receptors, primarily on their cytoplasmic tails that are phosphorylated, including those with Src homology two (SH2) and phosphotyrosine-binding (PTB) domains [53, 54]. Interactions with an assortment of adapter proteins potentially allows a single receptor to alter which downstream pathways are activated depending on which adapter proteins are present or recruited at the time of ligand activation. For example, the association of Grb2, an adaptor that contains a SH2 domain, with the phosphorylated C-terminal tail of an activated RTK can recruit additional proteins such as guanyl nucleotide exchange factor (SOS) to the plasma membrane. Once SOS is in close proximity to RAS, RAS binds GTP and thus facilitates activation of the MAPK pathway [54, 55]. This manner of activating signaling

pathways is conserved from *Caenorhabditis elegans*, with LET-23 activation of RAS, to the ErbB family of receptors in humans. In addition to phosphorylation-mediated transduction of extracellular signals, a family of so called "scaffolding proteins" are involved in facilitating signaling propagation and they are partly responsible for the observed complexity in oncogenic cell signaling.

Scaffolding proteins can alter receptor initiated signaling by changing the location of these receptors, such as helping them localize to the plasma membrane or to a specific domain within that membrane. Additionally, increasing evidence has suggested that the spatial and temporal regulation of signaling pathways require the assembly of tightly controlled signaling complexes that are organized by scaffolding proteins [56]. One example, PICK1, interacts with ErbB2 through a PDZ domain and facilitates ErbB2 receptor clustering, resulting in more efficient signaling activation and receptor internalization [57]. In addition to adaptors and scaffolds that interact at the receptor level, there are also many scaffolding proteins that facilitate protein interactions downstream of the receptors. In the RAS/RAF pathway several pro-signaling scaffolds are known to increase the interactions of signaling proteins at different steps, increasing their ability to phosphorylate the next protein in the cascade. One example is that of Shoc2, a cytosolic protein that is targeted to late endosomes upon EGF-stimulation and positively regulates RAS-mediated signaling [58, 59].

In addition to positive regulation of signaling pathways through scaffold proteins there have been a number of publications that have identified negative regulators. It is believed that these inhibitory proteins may act as a counter balance to their pro-signaling, often proliferation inducing, scaffold counterparts. Sprouty, found in *Drosophila*, and its

mammalian homologue Spred both act to suppress signaling through the ERK pathway [60, 61]. These negative regulating scaffold proteins alter the ability of RTKs to recruit the appropriate adapter proteins [62], and act as negative feedback regulators helping to diminish over-amplified signals.

Certain protein-protein interactions, like those that help to facilitate the role of Shoc2 and Sprouty in altering cell signaling, are required in order for scaffold proteins to effectively promote or inhibit downstream signaling. Many scaffold proteins contain what are referred to as modular interaction domains and each scaffold may contain multiple interacting domains to allow the formation of multi-protein complex. Adding additional complexity, the protein-protein interaction domains within a scaffold protein may have the flexibility for bind several distinct ligands. It is possible for these domains within scaffold proteins to recognize peptide sequences (ie. PDZ, PTB, SH3 domains), other protein domains (ie. PyD, SAM, PB1 domains), or post-translational modifications (ie. SH2, Bromo, WW domains) [63]. Using a combination of these domains allows a cell to precisely modulate an intricate network of protein interactions and adjust these interactions to facilitate a desired downstream event.

1.3 Erbin Distribution and Function in Epithelial Tissues

In addition to Sprouty and Spred, another protein that was found to disrupt the ERK signaling pathway is Erbin. Erbin is a member of the leucine-rich repeat (LRR) and PDZ domain (LAP) protein sequence superfamily. The members of this protein family are evolutionarily conserved, from *Xenopus tropicalis*, to *Drosophila melanogaster*, to *Homo sapien*, and all share somewhat similar domain structures (Figure 1.6). Erbin contains 16 LRRs followed by a single PDZ domain at its C-terminus. LRRs are repeating amino acid sequences, generally 20-29 residues long, which assemble into superhelical structures and form a solenoid protein structure [64, 65]. Additionally, most LRRs are involved in the process of protein-protein interactions and maintain similar overall properties including a horseshoe-like shape and parallel beta-sheets on the concave side that are responsible for protein interactions [66]. The other domain of Erbin, the PDZ domain, is also involved in protein-protein interactions. These domains are about 90 amino acid residues in length which consistently fold into a beta-barrel that is comprised of 6 beta-strands and is most often capped by two alpha helices [67]. Functionally, the PDZ domain interacts with a specific peptide sequence located at the C-terminal tail of other proteins [68]. Together, these domains allow Erbin to interact with several proteins and possibly more than one at a given time.

Other members of the LAP protein family include Scribble, Densin-180, and Lano in mammals [69, 70]. Functionally, LET-413, another member of the LAP protein family which is only expressed in *Caenorhabditis elegans* has been shown to control epithelial cell polarity, while Scribble has been shown to regulate polarity in both *Drosophila melanogaster* and in mammals [71]. Erbin, described as the mammalian homologue to

LET-413 and is structurally very similar to other LAP proteins, may also play a role in maintaining the polarity of epithelial tissues in addition to regulating ERK signaling.
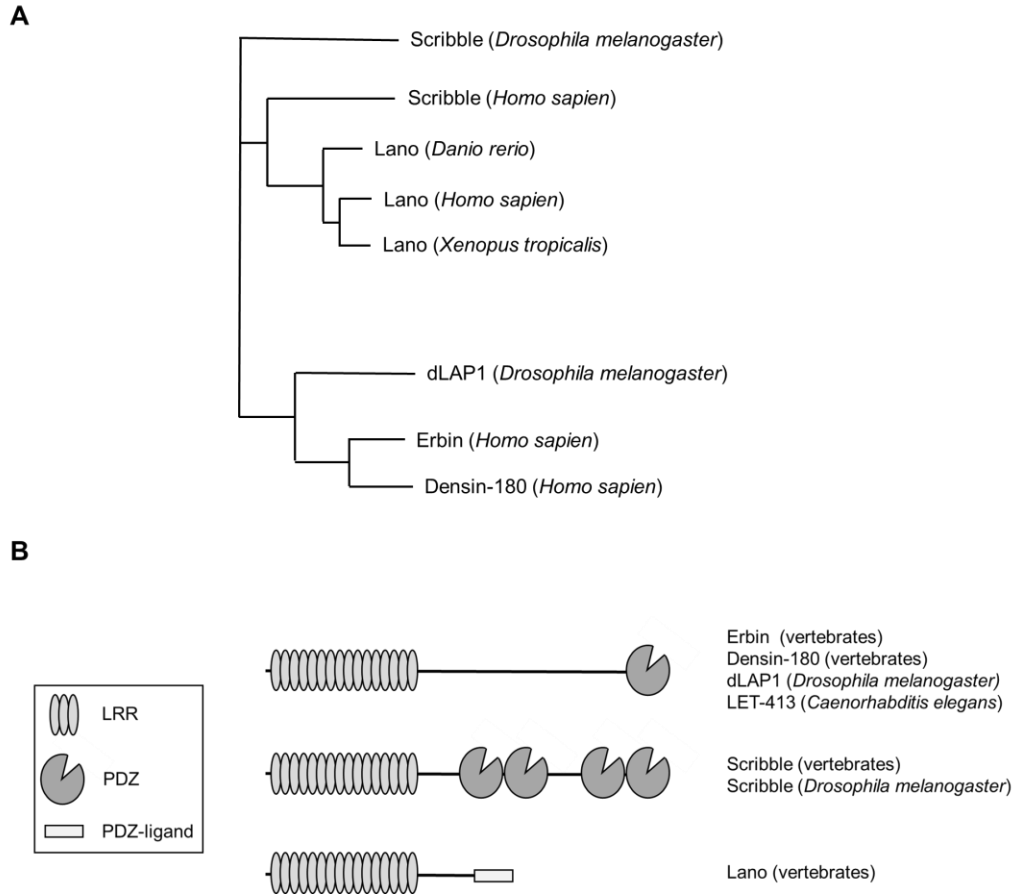
Figure 1.6 Phylogenetic Tree and Domain Structure of LAP Proteins

(A) A phylogenetic tree of LAP proteins, visualizing the divergence and evolutionary history of this proteins superfamily. Distance between proteins approximate sequence similarity. (B) Models that represent the common domain composition of LAP proteins that are used to facilitate protein-protein interactions. Erbin is most similar in identity to Densin-180, dLAP1, and LET-413. Adapted from Santoni et al. [72].

Erbin is located on the long arm of chromosome 5 and has several splicing variants [73]. Erbin mRNA is comprised of 26 exons and alternative splicing can lead to several alterations in the linker region of Erbin (between the LRR and PDZ domain) as well as variants that lack the PDZ domain entirely. Erbin RNA expression peaks during G2/M phase [74] and Erbin was also found to be present in most human and mouse tissues as detected by a 7.2-kb transcript and Western blot analysis detected Erbin in the brain, liver, kidney, spleen, intestine and skeletal muscle [73].

Erbin was first identified by Dr. Jean-Paul Borg's group in 2000 in an attempt to elucidate the protein-protein interactions necessary to organize signaling downstream from the ErbB2 receptor tyrosine kinase [75]. Erbin, specifically the PDZ domain, was found to interact with the amino acids at the C-terminal tail of the ErbB2 receptor, but interestingly not with any other ErbB family members. Conversely, no other PDZ domain interacts with the C-terminal tail of ErbB2, suggesting that this interaction is unique. This was of significance because other PDZ containing proteins can share up to 70% homology with the PDZ domain of Erbin but lack an interaction with ErbB2. Initially the authors in this publication attributed the unique binding affinity to the linker region since this region varies greatly between LAP proteins. However, later studies suggest that Erbin's PDZ domain is in fact structurally unique and could also help specify unique ligands [75]. Following the identification of Erbin as an ErbB2 receptor interacting protein, the authors characterized the functional consequences of this interaction. One of the main findings of this initial publication is that Erbin co-localizes with ErbB2, and is required to localize the ErbB2 receptor to the basolateral membrane of epithelial cells. This localization is necessary for ErbB2 to carry out its normal physiological functions [76].

In addition to this localization, proper orientation on the plasma membrane would be required to receive activation from extracellular ligands, especially with the ErbB2 receptor which is known as an orphan receptor and requires dimerization with another basolaterally localized ErbB family member to signal [77-79]. Furthermore, the authors showed that phosphorylation of ErbB2 upon activation allowed ErbB2 binding with a Src homology and collagen protein (SHC) and drastically reduced Erbin/ErbB2 interaction. Interestingly, this study also showed phosphorylation of an Erbin tyrosine residue, however, no functional experiments were performed in order to explore the consequences of this phosphorylation event [73]. Further proteomic studies have identified more potential Erbin phosphorylation sites through the combination of genetic and peptide-capture approaches. Erbin was identified as a new substrate of AMPK and two possible sites for AMPK phosphorylation were found in Erbin (S915 and S1079); however, no functional changes have been associated with these phosphorylation events [80].

Since the first identification of Erbin, there have been several publications that have helped to clarify how each domain contributes to Erbin's physiological functions. Through the structural comparison of Erbin's PDZ and the first PDZ of ZO-1 it was revealed that the less promiscuous PDZ of Erbin is likely responsible for its more specific protein interactions and strict localization to the adherens junction [81]. Structural studies have found that Erbin contains a class I PDZ domain, but surprisingly it interacts with ErbB2 receptor tyrosine kinase which contains a class II type PDZ ligand. In order to understand how this unique interaction occurs, the Erbin PDZ domain was crystalized bound to a peptide containing the sequence found in ErbB2 ligand. The resulting structure showed that Erbin has a class I PDZ domain that only contains a single alpha-helix (compared to

the two alpha-helices typically present), and also confirmed that phosphorylation of ErbB2 can alter its affinity of binding the PDZ domain of Erbin [82]. Another study confirmed these results using NMR to resolve the structure of the Erbin PDZ domain bound to its ligand. Erbin has a unique binding pocket due to the sidechains responsible for ligand binding and this helps to give the protein its specificity for binding PDZ ligands [75]. Additionally it was noted that like other PDZ ligand containing proteins, Erbin may form oligomers through PDZ-PDZ domain interactions [57].

In addition to studies on the PDZ domain there have also been studies investigating the role of the LRR domain plays in the localization of Erbin. Through the use of truncation and point mutations, it was shown that both the latter part of the LRR domain as well as two cysteine residues (amino acids 14 and 16), which can be palmitoylated, are necessary for the plasma membrane localization of Erbin [83]. However, different groups have separately shown that under certain conditions all of the domains in Erbin can potentially facilitate trafficking of Erbin to the plasma membrane. Different *in vitro* cell systems used in these studies may help explain the observed differences [81, 83, 84].

Since the initial characterization of Erbin, other interacting proteins in addition to ErbB2 have been identified. Several of these proteins are important for maintaining structural integrity of cell-cell junctions, including the interaction with bullous pemphigoid antigen 1 (eBPAG1) which is located at the hemidesmosome in keratinocytes [85]. Additionally at the hemidesmosome, Erbin interacts with the cytoplasmic tail of the β4 integrin. The cytoplasmic tail of the β4 integrin can be phosphorylated, alters intracellular signaling associated with filopodia and lamellae formation in cancer, and has been implicated in increasing invasiveness of colon carcinoma cell lines [86, 87]. However, the

functional implication of the Erbin-β4 integrin interaction has not been investigated. It is interesting to hypothesize that Erbin could simultaneously control the activation of ErbB2/integrin signaling and facilitate structural protein interactions at the cell-cell junction [85].

Other previous studies identified Erbin binding partners through the use of phage display. Several Erbin interacting proteins found are known to localize to the cell-junction [88]. These include three p120-like catenin proteins which bind the PDZ domain of Erbin. Interestingly the C-terminal ligands for two of the three proteins, ARVCF and δ-catenin, match the phage display for the perfect Erbin PDZ binding peptide even more so than that of ErbB2 [84]. Other studies were performed to confirm the interaction between Erbin and the third p120 catenin protein identified p0071, also known as plakophilin-4. p0071 is located at the desmosomes of epithelial cells where it connects the desmosome to the cellular cytoskeleton [89]. Lastly, Erbin was observed to co-localize with β-catenin along the lateral plasma membrane domain and E-cadherin junction [89, 90]. Further confirming this data, Erbin was found to be very abundant (10th most prevalent) at this site of E-cadherin junctions in a gastric cancer cell line [91]. Taken together, in addition to its role in localizing ErbB2 receptors, these data implicate a role of Erbin in facilitating or at least an involvement in several protein-protein interactions that maintain epithelial cell junctions. As a LAP protein this may imply that Erbin may function similarly as LET-413 or Scribble in maintaining epithelial cell structure.

The functional role of Erbin on regulating RAS/RAF signaling was first described in a paper published in 2002 in that Erbin was shown to associate with active RAS, disrupt the RAS/RAF complex, and decrease downstream ERK signaling [92]. This was an

important discovery as it indicated that Erbin may have a direct interaction with signal molecules in addition to its role in restricting protein localization. Furthermore, in a 2006 paper from the same group, Erbin was found to directly inhibit RAS/RAF signaling by replacing Shoc2, a scaffold protein that acts as a positive regulator of the RAS/RAF interaction [93], from the RAS/RAF complex.

More recent studies have since begun to make connections between Erbin's localization, interactions with structural proteins at cell-cell junctions, and ability to control signaling at those sites as well. One example of this was accomplished through studies of desmoglein-1 (DSG1), a desmosomal cadherin protein. Loss of DSG1 expression is linked to several diseases including RASopathies like striate palmoplantar keratoderma (SPPK) where keratinocytes fail to differentiate correctly, and additionally correlates to an increased risk for anal carcinoma [94]. Erbin has been identified as a potential interacting partner that is necessary for DSG1 induced ERK inhibition, which is necessary for controlling epithelial cell differentiation [95]. Mechanistically, over-expression of DSG-1 increases the efficiency of Erbin binding to Shoc2. The increased Erbin/Shoc2 interaction effectively reduces downstream ERK signaling. In keratinocytes, Erbin suppression of MAPK signaling is necessary for correct cell differentiation and patients with SPPK have decreased Erbin-Shoc2 co-localization and increased RAS-Shoc2 co-localization which can possibly explain the RASopathy type of defects observed with this disease [95].

Other studies have explored the physiological functions of Erbin in modulating MAPK signaling in neuronal cells. Several of these studies were performed in Schwann cells, the cells that produce a myelin sheath around neuronal axons helping to insulate peripheral nerves [96]. Loss of Erbin significantly reduced myelination of axons and once

again, the mechanism was linked to an increase in ERK signaling. In addition to the increased ERK signaling, the authors also noted decreased amounts of beta-catenin bound to E-cadherin, which potentially activates Wnt signaling. This could be rescued by treatment with U0126, a MEK inhibitor, suggesting that the increased ERK signaling resulting from loss of Erbin could be linked to increases in Wnt signaling [96].

In addition to Schwann cells, several other studies have elucidated additional roles for Erbin in neurons. Interestingly, Erbin was found to regulate neuronal development and Erbin knockout (KO) mice have impairment of motor coordination [97]. Erbin was found to affect AMPA receptor expression and stability on the surface of excitatory synapses [97], which could be a result of MAPK signaling that results from Neuregulin induced ErbB2 activation. Neuregulin activates ErbB receptors that are located at the postjunctional membranes of synapses. It has been shown that Erbin is necessary for the localization of ErbB2 to the surface of these postjunctional membranes [98], and Erbin stabilizes ErbB2 protein levels in neural cells [99]. Additionally, Erbin null mice could not maintain appropriate levels of ErbB2 necessary for remylenation of axons after injury [100], and loss of Erbin decreased myelination which resulted in decreased nerve conduction velocity [99]. Together, the results obtained from studies of Erbin in neuronal cells suggest that Erbin can stabilize proteins localized at synapses and loss of Erbin leads to pathological alterations as a result of disruption of ErbB2 and MAPK pathways.

More recently, studies began to explore and expand the role of Erbin outside of cell-cell junctions and MAPK associated signaling. An interaction with Smad proteins and a role in TGFβ signaling was discovered through more studies of Erbin in neural cells. Smad2 and 3 both interact with Erbin and not with other LAP proteins, Lano or Scribble

[101]. This was confirmed through a yeast two-hybrid screen, designed to detect proteins binding Smad3 in developing mouse orofacial tissue. Erbin was found to bind to the MH2 domain of Smad3, and to a lesser degree Smad2 [102]. Regions of Erbin, from the linker region, comprised of amino acids from 1004-1280 or 1172-1282, since named the Smad Interacting Domain (SID), were found to be involved in binding Smad proteins. Additionally, through the use of point mutations, located outside of the binding pocket of Erbin's PDZ domain, it was suggested that a slight electrostatic interaction with the exterior of the PDZ domain may also be involved in the binding to Smad3. Binding of Erbin with Smad3 inhibits Smad3/4 interactions from occurring by acting as a sink for Smad3 and reducing Smad induced signaling [101-103].

Within the same pathway it was also found that Erbin interacts with the SMAD anchor for receptor activation (SARA) to regulate TGFβ signaling. SARA binds non-phosphorylated Smad2 and/or Smad3 and recruits them to active receptors where the Smads become activated through phosphorylation. SARA is localized to the plasma membrane and to early endosomes, where it was found to interact with Erbin. The SID of Erbin seems to have affinity for both Smad2/3 and SARA, in which SARA competes with Smads for binding Erbin and interaction of SARA with Erbin blocks Erbin-mediated inhibitory effects on Smad signaling [104]. Disruption of the interaction between Erbin and Smad has been observed in Atopy patients, a disease related to increased allergic reactions or immune responses. Mutations that occur in Erbin impair the formation of the Stat3-Erbin-Smad2/3 complex, which alters the activation of T helper type 2 cytokine expression and results in elevated IgE in the patients with this disease [105].

Furthermore, studies using other biological systems have found that Erbin inhibits ERK signaling in heart tissues and loss of Erbin results in cardiac hypertrophy. In the Erbin KO mouse model, loss of Erbin exacerbated heart failure [106]. Additionally, it has been shown that Erbin interacts with nucleotide-binding oligomerization domain containing protein (Nod2) through the LRR domain in regulating inflammatory responses [107]. Improper activation of the Nod2 leads to Crohn's disease and Blau syndrome, which affect intestinal and skin epithelial cells, respectively. Erbin inhibits the activation of NFκB and cytokine secretion induced by Nod2, and Erbin KO mouse embryos showed increased sensitivity to muramyl dipeptide, a component of bacterial cell wall. Interestingly, Erbin expression is strongly induced by inflammatory stimuli including LPS and TNFα. Collectively, these data suggest Erbin functions as a regulator of inflammation in epithelial tissues [108].

Taken together, these published studies use a variety of systems to explore the structure and function of Erbin. The studies on Erbin have evolved over time, from the initial characterization of Erbin as an ErbB2 binding protein to a protein that is involved in the formation and stability of epithelial cell junctions, and lastly as a protein that can modulate signaling through the RAS/RAF pathway. More recent studies have begun to focus on how Erbin-loss may be implicated in signaling events responsible for several disease states. Additionally, during the past decade a number of publications have begun to examine the role of Erbin in cancer. The functions of Erbin in maintaining epithelial integrity and serving as a negative regulator of oncogenic signaling were of particular interest in these studies.

1.4 The Role of Erbin in Cancer

Although several of the Erbin-interacting proteins are well documented to be involved in oncogenic signaling, there are still relatively few publications investigating Erbin's role in tumor initiation and/or progression. Here, a comprehensive review of the existing publications on the functional role of Erbin in cancer are detailed. Previously published studies, prior to the start of this dissertation research project, were helpful in providing rationales for the dissertation study which focuses on elucidating the role of Erbin in regulating tumorigenesis and CRC progression and the underlying molecular mechanisms.

Many of the first cancer related studies involving Erbin were related to the signaling processes which were more thoroughly explored in neuronal cells. One of the studies investigated Erbin-mediated regulation of Stat3 signaling in cervical cancer. Stat3 signaling in cervical cancer had previously been correlated with a poor prognosis due to increased lymph node metastasis and tumor size, and increased expression and phosphorylation of Stat3 in cancerous and pre-cancer cervical lesions. It was found that in cervical cancer, Erbin regulates Stat3 and TGFβ signaling [105], and that loss of Erbin increases both the phosphorylation and nuclear translocation of Stat3, leading to transcriptional activation of target genes and tumor progression [109].

Subsequently, from the same group, it was shown that Erbin loss allows cervical cancer cells to resist anoikis. Specifically, the authors found that loss of Erbin significantly increased cellular ATP levels and accelerated G1 to S cell cycle progression. The proposed mechanism is through the reduction of cell cycle inhibitors, p27 and p21, after loss of Erbin. Rescue experiments were performed to show that re-expression of p27 or p21 blocked

Erbin-loss induced increase in proliferation [110]. This study provides the initial evidence supporting a tumor suppressor role of Erbin in cervical cancer. Additionally, another study showed that expression levels of Erbin were controlled by the transcription factor c-Myb in a cell cycle dependent manner. The authors also noted that Erbin expression peaked in the G2/M phase and loss of Erbin resulted in the formation of multipolar spindles and ultimately abnormal chromosome division [74]. Together, these data suggest that loss of Erbin would result in both increased proliferation and chromosomal instability, which are both important factors in oncogenic progression.

One of the most important areas of research regarding Erbin's role in cancer is in breast cancer since one of the primary roles of Erbin is to restrict the localization of the ErbB2 receptor. This cancer type is of particular interest because ErbB2-overexpressing breast cancer represents 25-30% of invasive breast cancers. Two studies have focused on this particular type of cancer, but interestingly the two studies resulted in complete opposite findings [111, 112]. The first study suggested that Erbin is a tumor suppressor as Erbin levels were decreased in invasive breast carcinomas. This loss of Erbin coincided with increased phosphorylation of ERK as well as increased Akt phosphorylation. In addition, the authors also showed that knockdown of Erbin increased cell migration. Using the MEK1/2 inhibitor, PD184352, the authors were only able to partially reduce the cell migration observed in Erbin knockdown cells suggesting that the increase in migration results from an increase in signaling via both ERK and Akt pathways [111]. Furthermore, the paper suggested that Erbin-loss confers resistance to trastuzumab, a clinically used antibody targeting the ErbB2 receptor [113, 114]. Interestingly, the use of the Akt inhibitor GDC0941, but not the MEK inhibitor, was able restore trastuzumab sensitivity in MCF-7

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cells stably overexpressing ErbB2 in Erbin knockdown cells [111].

Conversely, using a murine model of ErbB2-driven breast cancer, a second study found that Erbin can act as a tumor promoter [112]. Particularly, knockout of the C-terminal PDZ domain of Erbin *in vivo* decreased the expression of ErbB2 at the protein level but had no effect on ErbB2 mRNA expression. Mechanistically, the presence of Erbin blocked ubiquitination of ErbB2 by enhancing ErbB2/HSP90 interactions. This increased ErbB2 protein stability led to activation of oncogenic signaling downstream of ErbB2 in Erbin mutant mice [112]. Nevertheless, this tumor-promoting effect is likely specific to cancers driven by ErbB2, since deletion of the C-terminal PDZ domain of Erbin had no effect on tumorigenesis in the MMTV-PyVT driven breast cancer model. While these interactions and mechanism of tumor promotion may be unique to this type of breast cancer, recently a review article was published proposing peptide inhibitors of the Erbin/ErbB2 interaction so that HSP90-mediated stabilizing ErbB2 can be disrupted in treating ErbB2-driven breast cancer [115]. However, it remains to be seen whether a mouse model lacking Erbin entirely, not just the PDZ domain of Erbin, would have a tumor inhibitory or promoting effect in an ErbB2 driven tumor model. While truncation of the PDZ domain of Erbin may abolish any direct Erbin-ErbB2 receptor interaction, the N-terminus and LRR of Erbin have also been shown to result in Erbin plasma membrane localization. This plasma membrane localization could result in the truncated Erbin maintaining certain protein interactions, possibly altering ErbB2 signaling indirectly. More studies will be necessary to better understand the complexity of how Erbin alters ErbB2 signaling in different cancer types.

In studies determining the role of Erbin in regulating Wnt signaling, it has been

shown that Erbin can interact with β-catenin in HEK293 cells using immunoprecipitation experiments. The functional effect of this interaction was investigated using a fluorescent reporter system, the TOPflash luciferase reporter plasmid, to measure Wnt signaling activation in the HEK293 and CRC HCT116 cells. Overexpression of Erbin inhibited activation of Wnt target in the reporter assay [116]. While the experiments in this brief publication began to explore the role of Erbin in inhibiting Wnt signaling, there were no experiments performed to explore the physiological consequences of this inhibition.

Furthermore, another study utilized multicellular tumor spheroid models to investigate the role Erbin in the formation of CRC tumor spheroids in suspension. Given the fact that many of Erbin-interaction proteins are at the site of cell-cell junctions, it has been suggested that Erbin could be necessary for maintaining the interactions of junctional proteins in epithelial cells. This study explored the possibility of certain proteins, including LAP proteins such as Erbin, being required for the growth of tumor spheroids using HT29 CRC cells [117]. The cells were grown as traditional 2D monolayer or in suspension for three days. The tumor spheroids were collected from the suspension medium and protein and mRNA levels were compared to the cells grown in 2D. Erbin was one of the most highly upregulated proteins observed in tumor spheroids. Knockdown of Erbin with siRNA dramatically decreased the ability of cells to grow in suspension [117]. Results from this study suggest that loss of Erbin prevents cells from forming the cell-cell junctions required to form multicellular spheroids, and therefore resulted in anoikis. More recently, results from one study suggested that Erbin functions as a tumor promoter in CRC cells by facilitating the interaction between EGFR and ErbB2 receptor with the E3 ligase C-Cbl [118].

In summary, the literature on Erbin-mediated regulation of cell signaling suggests that Erbin negatively controls several pro-growth signaling pathways; including the RAS/RAF, Akt, Smad, and possibly the Wnt pathway. However, the role of Erbin in regulating cell polarity, especially in the context of EMT and CRC progression has not been studied and the role of Erbin in regulating CRC invasion and metastasis remains controversial [118, 119]. My dissertation studies focused on testing the hypothesis that Erbin act as a tumor suppressor in CRC.

1.5 Goals and Hypothesis for Dissertation

The proper establishment of epithelial polarity allows cells to sense and to respond to signals that arise from the microenvironment in a spatiotemporally controlled manner. Therefore, regulation of cell-cell junctions, maintaining cell polarity, and properly controlling cell signaling are vital for halting cancer initiation and progression. Previous studies have demonstrated that loss of cell-polarity, through the disruption of these junctions, is associated with late stage metastatic tumors where cancer cells undergo EMT [120-122]. The function of several well-known oncogenic proteins and tumor suppressors, such as ErbB2, Kras, and Lkb1, were observed to regulate both cell signaling and the integrity of epithelial polarity [121, 123-126].

Given that many other proteins in the LAP family have also been associated with maintenance of epithelial polarity and controlling oncogenic signaling, and after a comprehensive review of the literature it was obvious that a more rigorous study on Erbin's role in these processes in CRC was needed. As previously noted, the development of CRC involves the activation of oncogenic signaling through the Wnt, PI3K/Akt and RAS/RAF pathways in epithelial cells, all of which Erbin has been implicated in regulating and thus makes CRC an ideal disease model to characterize the function of Erbin. Therefore, the primary goal of my dissertation work is to understand the role of Erbin in suppressing the initiation and progression of CRC, with a focus on elucidating the molecular mechanisms by which Erbin regulates epithelial cell polarity and EMT during cancer progression. By employing Erbin knockout mouse models, the functional importance of Erbin was investigated *in vivo*. Together, a combination of *in vitro* and *in vivo* studies as well as CRC patient data were used to better understand how Erbin-loss promotes CRC progression. The

overall goal of my thesis work has been accomplished as a result of the steps detailed in the following Results and Discussion chapters:

1) Characterize the expression of Erbin in CRC patient tumor samples.

2) Determine the effect of Erbin-loss in regulating cell signaling and the underlying molecular mechanism.

3) Define the functional consequences of Erbin-loss in CRC cells.

4) Examine the role of Erbin in tumorigenesis *in vivo* through the use of the Apc/Erbin KO compound mutant mouse model.

5) Determine other possible mechanisms of action that allow Erbin to act as a tumor suppressor in CRC.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Lines and Cell Culturing

Caco2 and SW480 cells obtained from American Type Culture Collection (Manassas, VA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin/streptomycin. LIM2405 obtained from Ludwig Cancer Research Institute were cultured in RPMI-1640 supplemented with 10% FBS, 2mM L-Glutamine, 25mM HEPES and 1% penicillin/streptomycin. Stable Erbin and KSR1 knockdown cells were generated by lentivirus-based shRNAs as previously described. [37, 127, 128]. The shRNA targeting sequences for Erbin are as the following: 5'-GCAGCCAAGTACAACCGTTAA-3' (A1) and 5'- CGGGCTCAAGTTGCATTTGAA-3' (A2). The shRNA targeting sequence for KSR1 is 5'-CAACAAGGAGTGGAATGATTT-3'.

To generate WT and Erbin knockout mouse embryonic fibroblast (MEF) cells, Erbin heterozygous mice were used for breeding and individual embryos at day 13 of gestation were isolated and genotyped. MEF cells were produced from WT and Erbin null embryos by following standard protocols [129]. The primary MEF cells were immortalized using retrovirus-mediated knockdown of p53 using pBabe-puro-shp53 (Addgene).

The Erbin antibody has been reported previously [89]. The phospho-AKT (p-AKT for Ser473), pan-Akt, phospho-RAF1 (p-RAF1 for Ser338), total RAF1, phospho-MEK1/2 (p-MEK for Ser217/221), total MEK1/2, phospho-ERK1/2 (p-ERK for Thr202/Tyr204), total ERK1/2, and E-cadherin antibodies were from Cell Signaling. The vimentin and N-cadherin antibodies were from BD Biosciences. The β-actin and γ-tubulin antibodies were from Sigma-Aldrich.

2.2 Gene Knockdown by shRNA

For stable knockdown of ERBIN or KSR1, lentivirus was produced by combining constructs for packaging (psPAX2), envelope (pDM2G) vectors and targeting shRNA or a non-targeting vector (pLKO.1), at a 4:2:1 ratio (vectors obtained from Sigma Aldrich, St. Louis, MO). Transfection reagent, polyethylenimine (PEI; Polysciences) was mixed with the DNA at 5:1 ratio, DNA to PEI. This was added to 50% confluent HEK 293LTV cells that had been passaged 24 hours prior to transfection. Conditioned, virus containing media was collected 48 and 72 hours after transfection, centrifuged at 2,000 x g for 5 mins and viral supernatant collected. Supernatant was then passed through a 45µm filter to ensure sterility. CRC cell lines were passaged to 50% confluence and viral supernatant, along with 8 µg/ml hexadimethrine bromide (polybrene, Sigma Aldrich), were added to the cells. Viral supernatant was added again 24 hours later, and 48 hours later was replaced with normal growth media. Stable cell line selection was accomplished via puromycin (1-8 µg/ml depending on the cell line) for a minimum of one week. Stable knockdown of the specified protein was observed by collection of cells after selection, followed by Western blot for the protein of interest.

2.3 Cell Growth Assay

Cell lines were plated at a density of 20,000 cells per 12 well plate and allowed to grow for 72 hours. After allowing the cells to proliferate, media is aspirated and wells were washed once with PBS, and then cells were fixed with methanol (500µl) for 10 minutes. Following fixation the methanol is removed and cells are stained with 500µl of crystal violet solution (0.5% Crystal violet and 20% methanol) for 30 minutes. After 30 minutes the crystal violet is removed, the 12 well plate is gently rinsed with water until all excess

stain is removed, and then the plates are air dried. Cells and associated staining is then solubilized in 500μl of 1% SDS in water. The 12 well plate is placed on a shaker at room temperature until all of the stain is dissolved in solution and no cells are visible on the bottom of the plate. 100μl, in triplicate, is removed from each well of the 12 well plate and placed into a 96-well plate followed by reading the absorbance at 570nm (SpectraMR. Dynex Technologies).

2.4 RNA Extraction and QPCR

Total RNA was isolated from mouse tumor organoids using the RNeasy Mini Kit (Qiagen, MD, USA). Equal amounts of RNA were used as templates for the synthesis of cDNA using RT^2 HT First Strand kit (Qiagen). Real-time PCR was performed using mouse *Lgr5-*, *Axin2- Cd44-*, *Ccnd1-*, *Ki67-*, *Alpi-*, *Fabp2-* and *Muc2*-specific probes using StepOne Real-Time PCR system (Applied Biosysems). All values were normalized to the level of β-actin. The overall expression of β-actin mRNA remained unchanged in different treatment groups as determined by the Ct (threshold cycle) values.

2.5 Transwell Migration and Invasion Assay

Cell lines were grown to 50% confluency and were serum starved overnight. The next day, the cells were resuspended in 0.1% bovine serum albumin (BSA), then seeded into the upper chamber of Transwell inserts with an 8 µm pore size membrane (Corning, Tewksbury, MA) at 5x104 cells per insert. For the invasion assay, upper chambers of Transwell inserts were coated with 100μls of Matrigel (BD Biosciences), which was allowed to dry overnight and rehydrated prior to seeding of cells for invasion. The cells were allowed to migrate toward serum-free DMEM containing collagen (15 µg/mL) and Epidermal Growth Factor (EGF) (10 ng/mL) in the lower chamber for 4.5 hr (24 hours for

invasion). At the end of the incubation period, cells remaining in the top chamber were mechanically removed using a cotton swab, and the cells on the bottom of the Transwell inserts were fixed with methanol (10 min), washed once with phosphate buffered saline (PBS), and then stained with 0.5% (w/v) crystal violet in 20% methanol (v/v) (20 min). The stained cells in 4 non-overlapping fields from each insert were counted with an inverted microscope, using the 20X objective (Eclipse TS100, Nikon Instruments, Melville, NY).

2.6 Drug Treatment

Inhibition of signaling pathways using pharmacological inhibition was accomplished through the use of PD98059, a specific MEK1 inhibitor, and MK2206 a specific inhibitor of Akt. Cells for motility assays were starved and simultaneously incubated with the inhibitors, at 10μM and 1μM respectively, overnight prior to Transwell assay. Additionally, the inhibitors were also included in the chemoattractant containing medium for the duration of the assay.

2.7 Single Cell Motility Assay

Control and Erbin knockdown cells were serum starved for 4 hours and plated as single cells onto collagen (15 µg/mL) coated glass bottom culture dishes (MatTek). Cells were stimulated with EGF (10 ng/ml) and the trajectory of moving cells were captured using a Nikon BioStation IM equipped with a CO_2 incubation chamber. Time-lapse phase images were taken every 10 minutes for 6 hours with a 20X objective [37, 130]. The recorded movement of the cells was analyzed using Nikon Elements AR software.

2.8 Western Blotting Analysis

Cells were collected and pelleted for Western blot analysis by placing the 10cm culture dishes containing the cells on ice and rinsing once with cold PBS, followed by the addition of 1ml of cold PBS and scraping cells, transferring them to a 1.5ml centrifuge vial. Cells were quickly pelleted by centrifugation, 3000rcf for 30 seconds. Pellets were then lysed with 100-300 μl cold PPHB lysis buffer (50 mM Na_2HPO_4, 1 mM Sodium Pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton®X-100) with the addition of DTT, benzamidine, leupeptin, PMSF, and sodium orthovanadate (0.5M, 0.5M, 4mg/mL, 0.2mM, and 200μM respectively). Cells were vortexed and lysed for 5 min on ice followed by centrifugation, 18,000rcf for 3 minutes. Supernatant containing soluble proteins were placed in new tubes and protein levels were quantified via Bradford assay followed by normalization. Sample buffer (4X) was added at a ratio of 1:3 and proteins were then separated using 8% SDS-PAGE, transferred and immunoblotted.

2.9 2-D Immunofluorescent Staining

Cells were grown at 37°C in DMEM on glass coverslips. They were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 20 min. All incubations were performed at 22°C. Fixed cells were washed thrice with PBS and then permeabilized with 0.01% Triton®X-100 in PBS for 10 min. Cells were washed thrice with PBS and then incubated with labelling buffer (1% BSA in PBS, pH 7.4) for 30 min. Primary antibodies were diluted in the labelling buffer and incubated with the fixed cells for 1 hr. The cells were washed thrice with PBS and subsequently incubated for 1 hr with either Alexa 594-conjugated anti-rabbit or Alexa 488-conjucated anti-mouse IgG (Invitrogen, Grand Island, NY) diluted in the labelling buffer. The coverslips were washed twice with PBS and once

with distilled water, then mounted on slides. The distributions of the immunodecorated proteins were visualized using an Olympus FlowView FV1000 confocal laser-scanning microscope (Olympus, Center Valley, PA).

2.10 3-D Immunofluorescent Staining

Caco2 cells were grown in a 3D matrix consisting of 50% growth-factor reduced Matrigel (BD Biosciences) and 50% Collagen I (Collagen I, Bovine, Invitrogen, cat.# A10644-01) neutralized with NaOH prior to mixing. Initially, the bottom of a 24 well plate is coated with 200 µl of the Matrigel-Collagen mixture and incubated at 37°C for 30 min. Caco2 cells from normal 2D cell culture are then trypsinized, re-suspended on ice in cell suspension medium (2% FBS and 2% Matrigel in DMEM) at a concentration of 2,500 cells/ml. Then, 500 µl of the cell containing solution is added on top of solidified Matrigel-Collagen mixture in the 24 well plate. Cell suspension medium was changed every 4-5 days, and Caco2 cells were allowed to grow 11 days until acini were formed.

After acinar structures were fully formed they were visualized using phase-contrast imaging. The imaging was done using a Nikon Eclipse Ti-E inverted microscope, and analyzed using the NIS-Elements AR 3.2 software (Nikon). The 3D matrix was partially dissociated by incubating with Collagenase I (30 m at 37°C) and cells were fixed using 4% paraformaldehyde in PBS (30 min at 4°C). All futher incubations were performed at 22°C. The fixed acini and Matrigel solution were pipetted into microcentrifuge tubes, spun down and washed thrice with PBS. Keeping the acini pellet in the microcentrifuge tubes, the cells were permeabilized with 0.01% Triton®X-100 in PBS for 20 min. Cells were spun down and washed thrice with PBS and then incubated with labelling buffer (1% BSA in PBS, pH 7.4) for 1 h. Primary antibodies were diluted in the labelling buffer and incubated with the

fixed cells for 1 hr. The cells were spun down and washed thrice with PBS and subsequently incubated for 1 hr with either Alexa 594-conjugated anti-rabbit or Alexa 488-conjucated anti-mouse IgG (Invitrogen) diluted in the labelling buffer. The acini were spun down and washed thrice with PBS, diluted in distilled water and transferred to a slide. To detect proliferating cells, the cells grown in 3D culture were treated with 5-ethynyl-2´-deoxyuridine (EdU) for 1 h prior to fixation. The EdU positive cells were stained using Click-iT EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific). Images were taken using an Olympus FlowView FV1000 confocal laser-scanning microscope (Olympus).

2.11 Histologic analysis and immunohistochemical (IHC) staining

Mice were euthanized at indicated time-points when showing signs of intestinal neoplasia, such as a hunched stature and rectal bleeding. Intestine segments were opened longitudinally onto filter paper and made into "Swiss-roll" preparations as described previously [37]. For histological analysis, H&E sections were prepared from fixed and paraffin embedded Swiss-roll specimens by following standard techniques. The CRC tissue microarray was created by the Biospecimen Procurement and Translational Pathology Shared Resource Facility of the Markey Cancer Center, which contains 45 pairs of tumor and adjacent normal control tissues collected from CRC patients who had undergone surgery resections at the Markey Cancer Center. For IHC staining, tissue sections were deparaffinized, rehydrated, and treated with hydrogen peroxide. Antigen retrieval was performed using Dako Target Retrieval Solution (DakoCytomation), and IHC staining was performed as previously described [127]. The stained sections were visualized using a Nikon Eclipse 80i upright microscope.

2.12 Mice

All animal procedures were performed by following protocols approved by the University of Kentucky Institutional Animal Care and Use Committee. Erbin knockout mice on C57BL6 background as previously described [99] were maintained by random inter-crossing to sustain a heterogeneous mixed genetic background. To produce animals used in the experiments, $Erbin^{+/-}$ mice were bred with $Apc^{f/f}$ and Villin-cre (Vil-cre) mice (both were obtained from the Jackson Laboratory) on a C57BL6 background. These mice were then inter-crossed to produce three cohorts of animals, including $Apc^{f/+}/Erbin^{+/+}/Vil$-cre (Apc/WT), $Apc^{f/+}/Erbin^{+/-}/Vil$-cre (Apc/Het), and $Apc^{f/+}/Erbin^{-/-}/Vil$-cre (Apc/KO). To monitor survival, these cohorts of mice were followed for up to 6 months.

2.13 Isolation and culture of mouse organoids

Intestinal tumors were isolated from three cohorts of Apc/Erbin compound mutant mice, including Apc/WT, Apc/Het and Apc/KO, cultured in 3D Matrigel. Fresh mouse intestines, containing tumors, are opened longitudinally and multiple tumors are resected while trying to avoid the removal of adjacent and underlying normal tissues. Immediately following resection, the tumors were placed into ice-cold DMEM/F12 supplemented with antibiotics, and transported to the laboratory. Once in the laboratory, using sterile technique, the tumors are washed once with cold PBS and, using a sterile razor, are minced into $3mm^3$ chunks and transferred into a 15 ml sterile tube. The pieces of tumor are wash once more with cold PBS and then centrifuged at 200rcf for 3 minutes. After aspirating the PBS, in order to further break down the tumor pieces to individual cells, 5ml of dissociation buffer (DMEM/F12 + 2.5% FBS, penicillin/streptomycin, 75 U/mL collagenase type IV [Sigma], 125μg/mL dispase type II [Invitrogen] and DNAse) was added to the 15ml tube

and these are incubate for 45 minutes at 37°C with agitation. After the digestion step, the larger tissue fragments are allowed to settle down under normal gravity, and the supernatant is collected into a fresh 50ml Falcon tube, which should contain the single tumor cells. Centrifuge the 50ml tube in order isolated tumor cells at 200rcf for 3 minutes. Wash once with 5ml of PBS and resuspend the cells in 20ml PBS in order to pass through a 70μm cell strainer to once again remove any larger cellular debris. Centrifuge the filtrate at 200rcf for 3 minutes, aspirate PBS, and wash the cells with 5 ml DMEM/F12 medium containing 10% FBS, penicillin and streptomycin in order to remove any remaining digestion buffer. Once again, spin down at 200rcf for 3 minutes, aspirate the wash medium and resuspend the cells in 100μl of 33% Martrigel on ice. Add this tumor cell-Matrigel mixture to a 24-well plate pre-coated with 200μl of solidified, 33% Matrigel. Place this plate in the 37°C incubator to allow the Matrigel to solidify around the cells and then add 500μl of Basal Medium (Advanced DMEM/F12 medium supplemented with penicillin/streptomycin, 1X Glutamax, 1X N-2, 1X B-27, and 1mM N-Acetyl-L-cysteine). Change medium every 2-4 days as needed. Tumor organoids were allowed to grow for 5 days and collected for protein or RNA analysis. For colony formation assays, single cell suspensions of 1,000 cells derived from tumor organoids were seeded into 3D Matrigel as described above. The number of colonies formed after 5 days were counted using an inverted microscope.

Conversely, in order to grow organoids from normal, non-tumorigenic intestinal tissue, a piece of intestine was taken from WT, Erbin Het, and Erbin KO mice. In order to isolate the tissue, fresh mouse intestine was opened longitudinally and chopped into small pieces (3-5 mm^2) and the intestines were washed with cold PBS until the supernatant was

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clear. Ten milliliters of cold digestion buffer (2 mM EDTA, 0.5 mM DTT in PBS) was added and incubated on ice for 30 minutes, then mixed briefly a few times during this process, to start cellular dissociation. To further dissociate the crypts from the normal intestines, the digestion buffer was aspirated followed by the addition of 5 ml of TrypLE Express and digestion at 37°C for 15 minutes. Again, after this time period we allowed the tissue pieces to sediment, and aspirated TrypLE Express, followed by adding cold PBS and vortexing vigorously to dislodge crypts. Individual villi and crypts dissociated from the underlying intestinal wall stroma, and should be suspended in the supernatant. The supernatant was collected and passed through a 70μm strainer to remove the larger villous materials. The supernatant was centrifuged to spin down and pellet the crypts at 200rcf for 3 minutes. Finally, in order to remove any remaining digestion buffers we washed the crypts with 5 ml DMEM/F12 medium containing 10% FBS, penicillin and streptomycin, followed by another centrifugation step at 200rcf for 3min. The medium was aspirated and the crypts were resuspended in 100μl of 33% Matrigel, then we added this crypt-Matrigel mixture to a 24-well plate pre-coated with 200μl of solidified, 50% Matrigel. After the crypts were plated, they were placed in the 37°C incubator to allow the Matrigel to solidify around the crypts. Lastly, to prevent the Matrigel from dehydrating, 500μl of Intestine/Colon Organoid Medium (Advanced DMEM/F12 medium supplemented with penicillin/streptomycin, 1X Glutamax, 1X N-2, 1X B-27, and 1mM N-Acetyl-L-cysteine, supplemented with 100 ng/ml Noggin, 50 ng/ml EGF, and 1 μg/ml R-spondin-1) was added on top of the Matrigel. The media was changed every 2 days of culture.

2.14 Bioinformatic and statistical analysis

In experiments to assess gene or protein expression, rate of cell migration, size and number of cell grown in 3D, and EdU incorporation were summarized using bar graphs and pairwise comparisons between different conditions were carried out using two-sample t-tests. We collaborated with the Markey Cancer Center – Biostatistics and Bioinformatics Shared Resource Facility to determine the relative expression of Erbin gene in human CRC patients. This was accomplished by downloading microarray and patient clinical data from two CRC studies from the Oncomine database. The Cancer Genome Atlas (TCGA) dataset contains 192 adenocarcinoma and 22 normal samples, within which 13 were matched pairs. Expression of Erbin was examined using linear mixed models. The Skrzypczak et al. dataset contains 81 tumors and 24 normal samples. Here, the expression of Erbin was examined using a 2-sample *t* test.

For the Gene Set Enrichment Analysis (GSEA), RNA sequencing data with over 60,000 genes were obtained from the TCGA Colorectal Cancer study. Once again working with the Biostatistics and Bioinformatics Shared Resource Facility, the corresponding gene expression data between ERBIN and the other genes was analyzed to obtain a Spearman correlation value. The genes were then ordered from highest to lowest based on the correlation values. This ranked list was used for the GSEA analysis [131].

CHAPTER 3: RESULTS

3.1 Introduction

Erbin is a member of the leucine-rich repeat (LRR) and PDZ domain (LAP) protein superfamily. It contains multiple protein-protein interaction modules including 16 LRRs followed by a single PDZ domain at the C-terminus. Erbin is known to localize primarily to the adherens junction and plays a role in maintaining the structural integrity of the junction in epithelial cells [85, 88, 89]. The initial discovery of Erbin identified Erbin as an ErbB2/Her2 receptor interacting protein that facilitates the localization of the receptor to the basolateral membrane of epithelial cells [73]. In addition, it has been shown that Erbin attenuates RAF1 activation by disrupting Shoc2-mediated RAS/RAF interaction [92, 93]. Moreover, downregulation of Erbin results in resistance to anoikis in cervical cancer cells via activation of JAK/STAT signaling [109]. However, the role of Erbin in regulating cell polarity, especially in the context of EMT and colorectal cancer (CRC) progression, remains elusive.

It has been well documented that epithelial cells, including those in the gastrointestinal tract, become polarized during the differentiation process [132]. The polarization process, characterized by the formation of specialized junctions between neighboring cells, also results in the segregation of two plasma membrane domains: the apical surface, facing the external medium, and the basolateral surface, connected to adjacent cells and extracellular matrix [133, 134]. The apical and basolateral membranes are segregated by two highly organized junctions, tight junctions and adherens junctions; and the many proteins that compose these junctions are assembled at the site of cell-cell contact [135-137]. Previous studies have demonstrated that loss of cell polarity, through the disruption of these junctions, is associated with late stage metastatic tumors where

cancer cells undergo EMT [120-122]. Therefore, the proper establishment of epithelial polarity allows cells to sense and respond to signals that arise from the microenvironment in a spatiotemporally controlled manner.

By facilitating the assembly of tightly controlled signaling complexes through protein-protein interactions, scaffold proteins are known to play important roles in regulating spatiotemporal responses in cell signaling [56]. One of the best-known examples is the signal propagation in the RAS/RAF pathway, where the step-by-step activation process from RAS to ERK is facilitated by scaffolding proteins [138, 139]. KSR1, a kinase-like protein that lacks enzymatic activity, has been shown to promote cell proliferation and oncogenic potential by enhancing signaling activation through the RAS/RAF pathway [140]. KSR1 binds constitutively to MEK in the cytoplasm in unstimulated cells and translocates to the plasma membrane upon RAS activation [141, 142]. At the plasma membrane, KSR1 facilitates signaling propagation by organizing the formation of RAF/MEK/ERK complex [142, 143]. Although the molecular mechanism by which scaffold proteins positively regulate RAS/RAF signaling has been extensively studied, it remains largely unknown whether scaffolding proteins are involved in signaling termination.

Here, we report the identification of Erbin as a novel tumor suppressor in colon cancer. We show that the mRNA and protein expression of Erbin is markedly decreased in CRC patient specimens. Erbin negatively regulates RAS/RAF signaling by sequestering KSR1 and preventing the formation of KSR1/RAF1 complex. Functionally, knockdown of Erbin results in an increase in cell motility by inducing EMT in colon cancer cells, and

deletion of Erbin gene significantly decreases the lifespan of Apc mutant mice and accelerates the tumor progression.

3.2 Erbin's role as a scaffold protein –

3.2.1 Erbin mRNA expression is significantly downregulated in CRC patient tumor tissue

To determine if Erbin could potentially serve as a tumor suppressor in CRC, we began by examining Erbin mRNA expression (gene symbol: *ERBIN*; previously known as *ERBB2IP*) in CRC patients. We performed bioinformatic analysis of two microarray data sets of human CRC samples. The microarray and patient clinical data of the two studies [24, 25] were downloaded from the Oncomine database. We collaborated with Dr. Chi Wang at the Biostatistics and Bioinformatics Shared Resource Facility of Markey Cancer Center to determine the relative expression of Erbin gene in human CRC patients. The Cancer Genome Atlas (TCGA) dataset contains 192 adenocarcinoma and 22 normal samples, within which 13 were matched pairs. Expression of Erbin was significantly higher in normal samples than in tumors (P<0.001 based on linear mixed models). The Skrzypczak et al. dataset contains 81 tumors and 24 normal samples. Here, the expression of Erbin was also significantly higher in normal samples than in the tumors (P<0.001 based upon a 2-sample *t* test). The mRNA expression levels of Erbin, in both tumor samples and normal tissue samples can be seen in Figure 3.1 A-B.

Further analysis was also performed using the microarray and patient clinical data from the TCGA data set [144]. These patient samples were further divided into groups of normal tissue as well as the adenocarcinoma samples were separated into cancer stages (I-IV). Erbin mRNA levels decreased upon tumor initiation as seen by the decrease in Erbin mRNA in stage 1 tumor tissue when compared to normal tissue (Figure 3.2). However,

while a decrease is observed in stage 1, no further loss of Erbin mRNA is observed as the tumor progresses through stage 2-4.

3.2.2 Erbin is mislocalized in patient tumor tissues as determined by IHC

In addition to examining the mRNA levels, we determined the expression of Erbin protein in matched normal and colon cancer tissues by IHC staining. We obtain a Tissue Microarray (TMA) from the Biospecimen Procurement and Translational Pathology Shared Resource Facility of Markey Cancer Center, which contains 45 normal tissue samples and 45 tumor tissue samples. After IHC staining for Erbin, we noticed the expression of Erbin was along the epithelial cell-cell junction in normal human colon tissues whereas Erbin expression was markedly reduced and mislocalized to cytoplasm in tumor tissues (Figure 3.3 A). The basolateral distribution of Erbin in normal tissues was found to be consistent with those observed previously [3, 26]. In order to quantify these results, the basolateral localization of Erbin was determined for each tissue section on the TMA (scoring was blinded as to remove any bias), and the percentage of samples positive for basolateral membrane localization was graphed. Basolateral membrane localization of Erbin was lost in all CRC tumor tissues examined (Figure 3.3 B and C).

3.2.3 Erbin protein levels are significantly decreased in CRC patient tumor tissue

Furthermore, in addition to looking at Erbin protein localization, we analyzed Erbin protein expression in matched normal and tumor tissues obtained from seven CRC patients using Western blot (Figure 3.4 A). While the calculated molecular weight of Erbin is approximately 150 kDa, Erbin expression was detected as a doublet around 180 kDa on our Western blots. This has been observed previously and reported by several other groups [73, 85]. Consistent with IHC staining results, Erbin protein levels were significantly decreased in tumor tissues compared to normal controls (Figure 3.4 B). These tissues were

collected from seven patients that were at various stages of tumor progression, from T2 to T4a (Figure 3.4 C). The decrease in Erbin protein corresponds with the mRNA data shown in Figure 3.2, suggesting that Erbin expression is decreased upon tumor initiation and is not dependent on tumor stage. Collectively, these data obtained using patient samples provide the first evidence that Erbin may function as tumor suppressor in CRC.
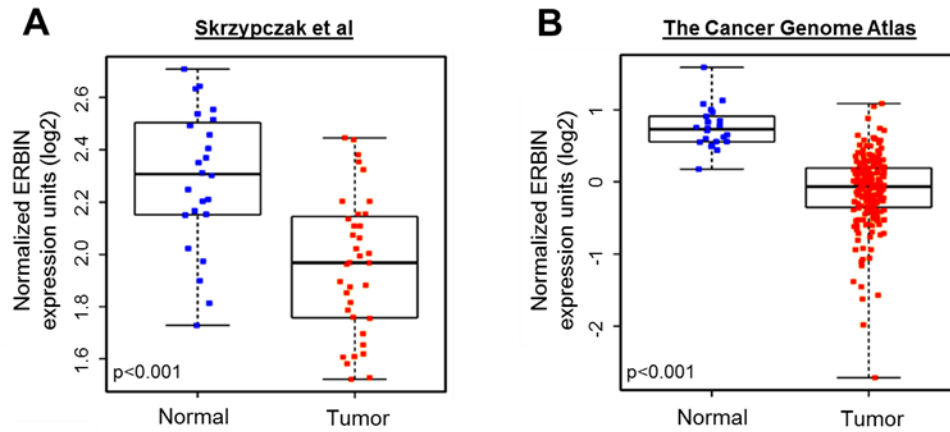
Figure 3.1 Erbin is downregulated in CRC patient samples

(A) and (B), Microarray and patient clinical data from two colorectal cancer studies were downloaded from the Oncomine database. Data from Skrzypczak et al [145](A) contained 24 normal and 36 adenocarcinoma samples; data from TCGA [144] (B) contained 22 normal and 192 adenocarcinoma samples. Two-sample t-tests or linear mixed models were used to compare ERBIN gene expression between adenocarcinoma and normal samples ($p < 0.001$).
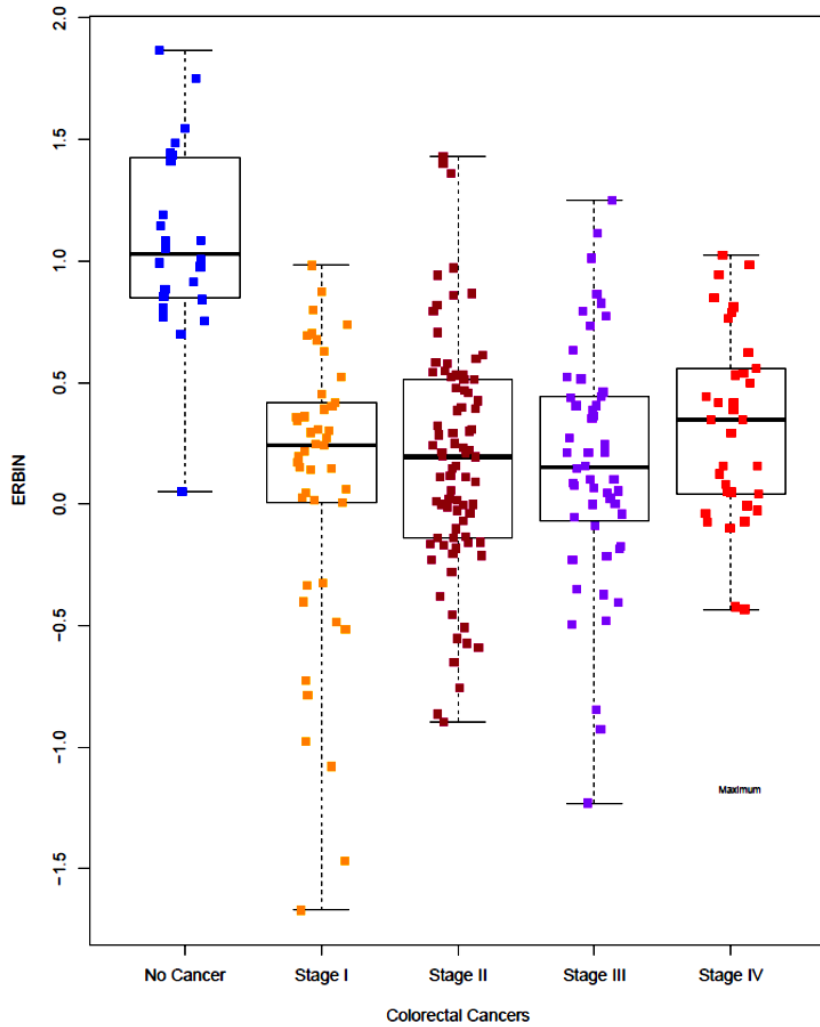
Figure 3.2 Erbin mRNA is downregulated in stage I colorectal cancer

Microarray and patient clinical data from TCGA [144] which contains normal and adenocarcinoma samples were separated into cancer stages (I-IV). Consistent with Figure 3.1, Erbin mRNA levels are decreased in tumor tissue when compared to normal tissue. However, a notable loss of Erbin mRNA occurs in stage I and remain low throughout CRC progression.
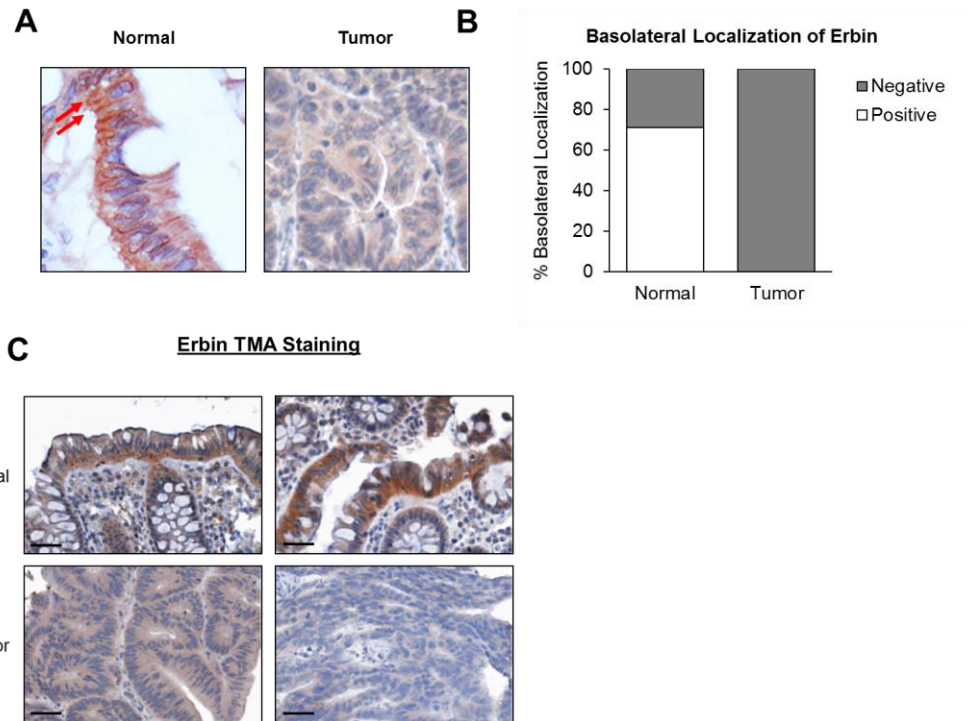
Figure 3.3 Erbin is mislocalized in CRC patient tumor tissue

(A) The expression of Erbin protein was detected in a tissue microarray (TMA) using

IHC. The TMA contains 45 pairs of matched normal and tumor tissues. The basolateral

localization of Erbin was detected in normal colonic epithelial cells as indicated by red

arrows. (B) Bar graph depicts the percentage of basolateral localization of Erbin in both

normal and tumor tissues (n=45). (C) Representative images of IHC staining for Erbin in

CRC TMA. Erbin was localized to the basolateral membranes in normal tissues and

diffuse in tumor tissues. Scale bar, 100μm.

A

pt2885 pt2893 pt2449 pt2782
N T N T N T N T

Erbin
— 250KDa
— 150KDa

1 0.3 1 1 1 0.6 1 0.3

Tubulin
— 50KDa

pt2717 pt2647 pt2769
N T N T N T

Erbin
— 250KDa
— 150KDa

1 0.4 1 0.4 1 0.2

Actin
— 50KDa

B

#

Relative Erbin Expression: 1.2, 1, 0.8, 0.6, 0.4, 0.2, 0

Normal Tumor

C

Patient ID	Gender	Age	Pathology	Staging
pt2885	M	77	Adenocarcinoma	T2, N0, Mx
pt2893	M	51	Adenocarcinoma	T3, N0, Mx
pt2449	F	61	Medullary Carcinoma	T3, N2a, M1a
pt2782	F	73	Adenocarcinoma	T4a, N0, Mx
pt2717	M	81	Adenocarcinoma	T2, N2a, Mx
pt2647	M	78	Adenocarcinoma	T3, N0
pt2769	F	56	Adenocarcinoma	T4a, N1b

Figure 3.4 Erbin protein expression is decreased in CRC patient tumor tissue

(A) Matched normal and tumor tissues from seven CRC patients were analyzed for Erbin expression using Western blot and Erbin antibody [89] (B) The expression of Erbin protein was quantified by normalizing to tubulin or actin. The relative Erbin levels in tumor tissues were compared to Erbin levels in the normal tissues from the same patient. Data represents the mean $\pm$ SEM (# $p<0.0001$). (C) De-identified patient data associated with tissues used for detecting Erbin protein expression by Western blot.

3.2.4 Erbin knockdown in CRC cells alters cell signaling

Previous studies have suggested that Erbin negatively regulates ERK signaling [5, 27]. To determine the function of Erbin in CRC, we silenced Erbin expression using two shRNA lentiviral targeting constructs (A1 and A2) in SW480, LIM2405 and Caco2 colon cancer cell lines. Consistently, knockdown of Erbin resulted in an increase in phosphorylation and activation of both the Akt and MEK/ERK pathways in all three cell lines (Figure 3.5 A). Since both Erbin shRNA targeting constructs had similar effects on silencing Erbin expression and enhancing activation of Akt and MEK/ERK phosphorylation, the subsequent experiments were mostly performed using sh-Erbin-A2 shRNA and key findings were confirmed with sh-Erbin-A1 shRNA. Additionally, using the three CRC cell lines with both sh-Erbin knockdown constructs, the effect of Erbin loss on cell proliferation in normal 2D culture was analyzed. Cells were lifted from a culture plate by trypsin treatment, counted, and an equal number of cells were plated and allowed to grow for 72 hours at which point a crystal violet assay to determine relative cell growth was performed. This assay uses crystal violet to stain the protein of the cells that grew over the 72 hour time period, it is solubilized with 1%SDS solution, and the amount of staining (which should be representative of the total number of cells) is quantified with a plate reader. The results from the crystal violet assay show that loss of Erbin did not have an effect on proliferation (Figure 3.5 B and C).

3.2.5 Loss of Erbin increases amplitude and duration of signaling

To further examine the effect of Erbin-loss on the temporal activation of signaling, stable control and Erbin knockdown SW480 cells were starved for 16 hours and subsequently stimulated with EGF for the indicated time (Figure 3.6 A). Once again we monitored activity of both the Akt and ERK signaling pathways, via Western blotting for

the activating phosphorylation events of each protein. Knockdown of Erbin increased both the amplitude and the duration of signaling through the Akt and several steps of the RAF/MEK/ERK pathways (Figure 3.6 B). Similar results, with increases in amplitude and duration of signaling through both pathways, were obtained in EGF-treated LIM2045 cells (Figure 3.6 C). These results indicate that endogenous Erbin is inhibiting signaling through both of these oncogenic pathways. Increased signaling through these pathways is often associated with phenotypic changes within the cell, and these data led us to make further observations on the functional changes within the Erbin knockdown cells.
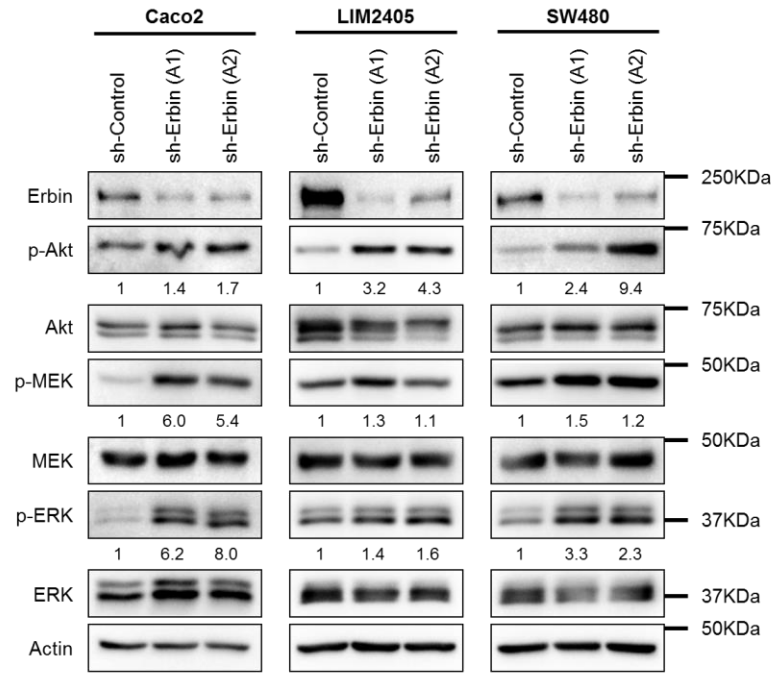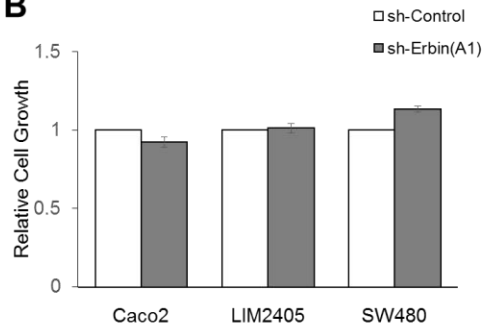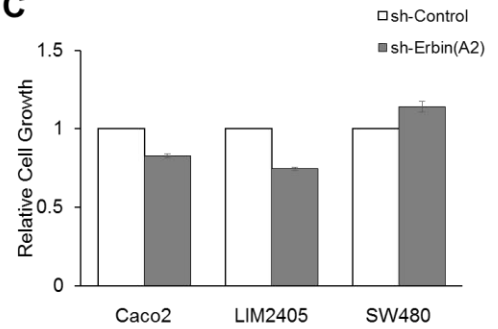
Figure 3.5 Knockdown of Erbin in CRC cells increases Akt and RAS/RAF signaling

(A) Cell lysates prepared from stable control (sh-Control) and Erbin knockdown (sh-Erbin) cells, including Caco2, LIM2405 and SW480, were analyzed for the expression of Erbin and the phosphorylation status of Akt, MEK and ERK using Western blot. Two different shRNA targeting sequences (A1 and A2) were used to silence Erbin in each cell line. The relative phosphorylation of Akt, MEK and ERK was quantified by normalizing ECL signals generated by the phospho-specific antibodies to that of total proteins. (B) and (C) Stable control and Erbin knockdown Caco2, LIM2405, and SW480 cells (generated from sh-Erbin-A1 or sh-Erbin A2 targeting lentivirus) were cultured for 72 hours, and the relative cell growth was determined using crystal violet staining.
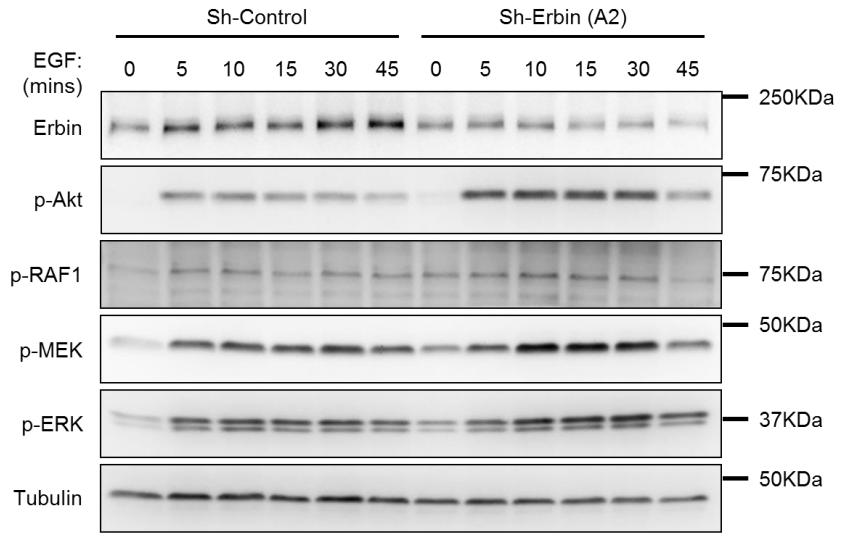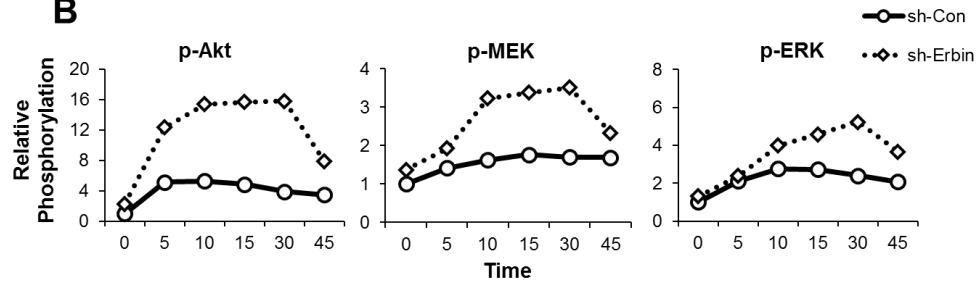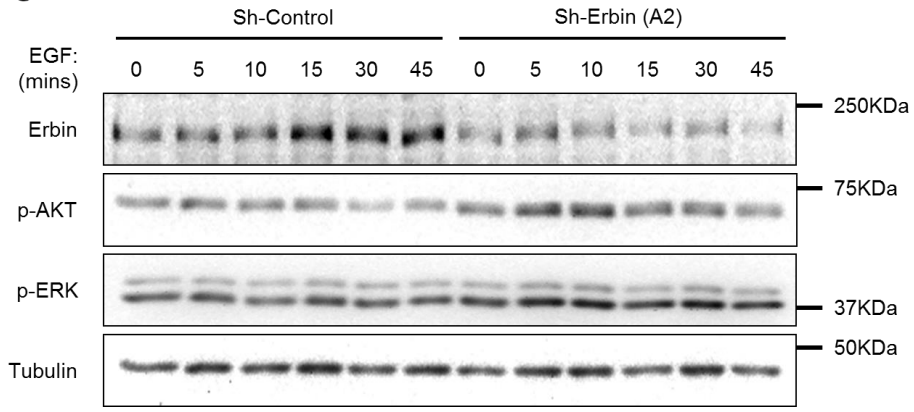
Figure 3.6 Knockdown of Erbin in SW480 cells increases the amplitude and duration of Akt and RAS/RAF signaling

(A) Stable control and Erbin knockdown SW480 cells were stimulated with EGF (10 ng/mL) for the indicated times and activation of signaling molecules were analyzed using Western blot. (B) The relative levels of p-Akt, p-MEK and p-ERK in sh-Control and sh-Erbin SW480 cell lines were quantified by normalizing ECL signals generated by the phospho-specific antibodies to that of total proteins and plotted over the EGF stimulation time course. (C) EGF-induced activation of RAS/RAF signaling in sh-Control and sh-Erbin (A2) LIM2405 cells. Cells were stimulated with EGF (10ng/mL) for the indicated times and activation of signaling molecules were analyzed by Western blot.

3.2.6 Reduction of Erbin results in loss of epithelial cell polarity

We next analyzed functional effects of silencing Erbin by using a 3D cell culture system. This system allows the cells to grow in a more physiologically relevant environment and allows us to determine if the loss of Erbin can alter growth in a 3-dimensional matrix. In our system, Control and Erbin knockdown Caco2 and SW480 cells were seeded into 3D Matrigel and allowed to grow for 10 days to form tumor spheroids. As described in previous studies [28-30], control Caco2 cells were able to form acini-like spherical structures with a single hollow lumen, which consisted of a layer of polarized epithelial cells as indicated by the apical localization of F-actin (Figure 3.7 A). In marked contrast, Erbin depletion altered the acinar structure by inducing the formation of multiple lumens (Figure 3.7 A). Moreover, although not fully polarized, control SW480 cells were able to form tumor spheroids with partially hollowed lumens; however, cell clusters formed by Erbin knockdown SW480 cells lacked lumen structure and exhibited no apical or basolateral differentiation (Figure 3.7 A). Together, these results suggest that Erbin plays an important role in maintaining epithelial polarity.

Furthermore, we found that knockdown of Erbin markedly increased the size of the spheroids in both Caco2 and SW480 cells (Figure 3.7 B). Diameter of the spheroids was measured using phase imaging of the control and knockdown cells, along with Nikon Elements software. To determine if decreased Erbin expression alters cell proliferation in 3D culture, tumor spheroids formed by control and Erbin knockdown SW480 cells were labeled with EdU to mark proliferating cells (Figure 3.7 C). Quantitative results showed that loss of Erbin expression increased cell proliferating (Figure 3.7 D). These results differ from cell growth performed in traditional 2D culture, which showed no difference in cell growth upon loss of Erbin. A more physiologically relevant system, such as growth in 3D

Matrigel, places different stresses upon the cells and thus may require higher Erbin levels

to grow correctly, whereas those stresses are lacking in the 2D culture system.

A

Phase | Actin/DAPI

Caco2
- Sh-Con — 50 um — 10 um
- Sh-Erbin

SW480
- Sh-Con — 50 um — 10 um
- Sh-Erbin

20X | 60X

B

Caco2

Spheroid Size (µm)

*

Sh-Control | Sh-Erbin

SW480

Spheroid Size (µm)

*

Sh-Control | Sh-Erbin

C

Sh-Control | Sh-Erbin

EdU

D

EdU Staining

% EdU Positive

*
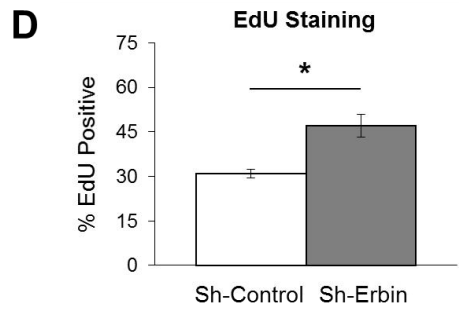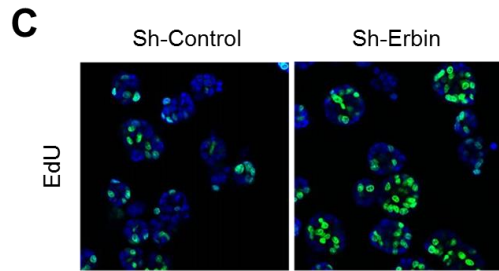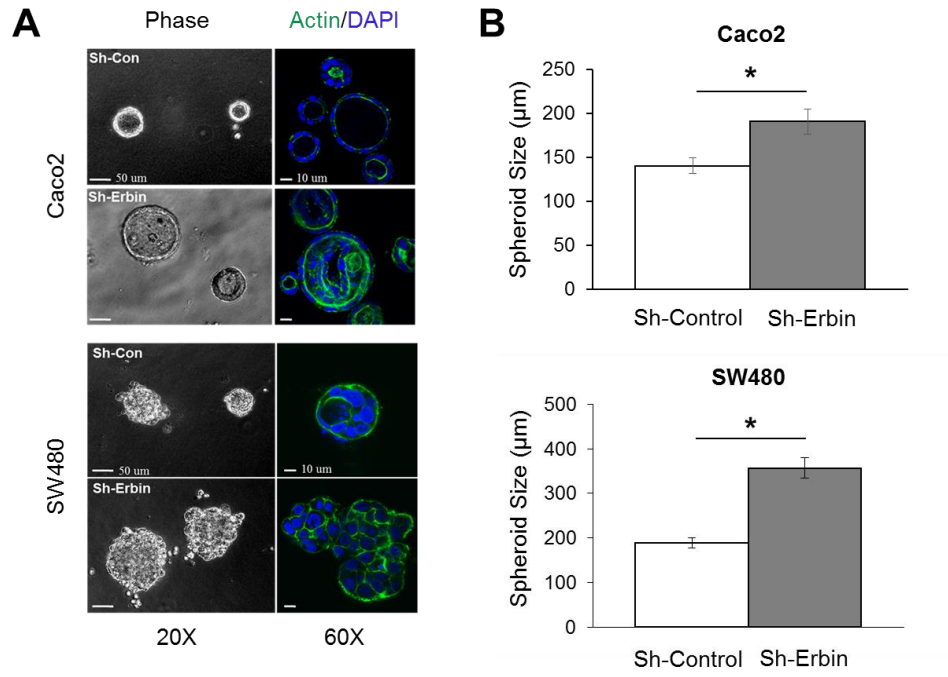
Sh-Control | Sh-Erbin

Figure 3.7 Knockdown of Erbin disrupts epithelial cell polarity and increases cell proliferation

(A) Stable sh-Control and sh-Erbin Caco2 and SW480 cells were cultured for 10 days in 3D Matrigel. IF images were taken from cells stained with Alexa488-phalloidin (green) and DAPI (blue). Phase-contrast images were obtained using a Nikon TE2000 inverted microscope with 10X objective, scale bar, 50µm; and confocal images of stained cells were obtained using an Olympus FlowView FV1000 confocal laser-scanning microscope, scale bar, 10µm. (B) The diameter of 15-35 randomly chosen spheroids of sh-Control and sh-Erbin Caco2 and SW480 cells were measured. Data represents the mean ± SEM (* p<0.05 t-test). (C) Stable sh-Control and sh-Erbin SW480 cells were labeled with EdU to mark proliferating cells. (D) The percentage of EdU-positive cells were quantified and expressed graphically. Data represents mean ± SEM (* p<0.05 t-test).

3.2.7 Knockdown of Erbin results in EMT in CRC cells

To determine the molecular mechanism by which Erbin-loss induces polarity defects and promotes cell proliferation in 3D culture, control and Erbin knockdown SW480 spheroids were collected and analyzed by Western blot. Interestingly, these data indicated that the morphological changes observed in sh-Erbin cells were accompanied by the downregulation of E-cadherin (an epithelial cell marker) and upregulation of vimentin and N-cadherin (markers of fibroblasts/mesenchymal cells), suggesting that Erbin knockdown cells had undergone EMT (Figure 3.8 A). Notably, increases in both Akt and ERK activation were maintained in Erbin knockdown spheroids grown in 3D (Figure 3.8 A). Collectively, these data suggested that downregulation of Erbin disrupts epithelial polarity and induces epithelial to mesenchymal transition (EMT) through increases in Akt- and ERK-mediated oncogenic signaling.

To further confirm the induction of EMT, SW480 cells grown in regular 2D culture were subjected to Western blot analysis. Similarly as the spheroids from 3D culture, Erbin knockdown cells grown in 2D had also undergone EMT as indicated by downregulation of E-cadherin and upregulation of vimentin (Figure 3.8 B).

3.2.8 Loss of Erbin increases cell motility

Since we have observed EMT-like phenotypes in Erbin knockdown colon cancer cells grown in 2D and in 3D, we next determined the role of Erbin in regulating cell motility. Mesenchymal cells are endogenously more motile when compared to epithelial cells, and for this reason the EMT associated with the knockdown of Erbin was hypothesized to also increase cell motility. To monitor single cell motility, time-lapse images of control and Erbin knockdown SW480 and LIM2405 cells were captured and the distances traveled of individual cells were determined using a Nikon BioStation. Erbin

knockdown cells were considerably more motile and average distances traveled by Erbin knockdown cells were significantly increased compared to the control cells (Figure 3.9 A-B). Control cells migrated an average of 100µm and 75µm for the LIM2405 and SW480 cells respectively, while the knockdown of Erbin increased the motility to approximately 160µm for both cell lines. The observed increase in total distance traveled suggests that Erbin-loss can increase cell motility at the single cell level.

In addition, control Erbin knockdown SW480 and LIM2405 cells were subjected to Transwell migration and invasion assays (Figure 3.10 A). We found that both Erbin knockdown SW480, LIM2405, and HT29 cells migrated significantly faster (with approximately a 3-fold, 2-fold, and 5-fold increase in the number of cells migrated respectively) than the control cells (Figure 3.10 B). To ensure that both shRNA constructs conferred an equal increase in cell motility, we silenced Erbin using the two different targeting shRNAs (A1 and A2) and observed comparable effects on promoting migration in SW480 cells (Figure 3.10 C). Furthermore, in an invasion assay, the knockdown of Erbin significantly increased the ability of SW480 cells to invade through Matrigel placed inside of the transwell insert (Figure 3.10 D), thus confirming that loss of Erbin promotes cell migration and invasion as a result of EMT.

3.2.9 Loss of Erbin Increases Cell Motility via Activation of ERK Signaling

Since knockdown of Erbin resulted in increases in signaling through both the Akt and RAS/RAF pathways in CRC cells, we investigated which signaling pathway was functionally connected with the upregulation of cell motility. To this end, pharmacological inhibition of signaling pathways was accomplished through the use of either PD98059, a specific MEK1 inhibitor, or MK2206 a specific inhibitor of Akt. Similarly as with other

Transswell assays, control and Erbin knockdown SW480 cells were starved overnight and simultaneously incubated with either 10μM MEK inhibitor or 1μM Akt inhibitor. Additionally, the inhibitors were also included in the chemoattractant containing medium for the duration of the assay. We observed that inhibition of the ERK pathway completely diminished any increase in motility resultant from the loss of Erbin, while the Akt inhibitor had little to no effect (Figure 3.11).
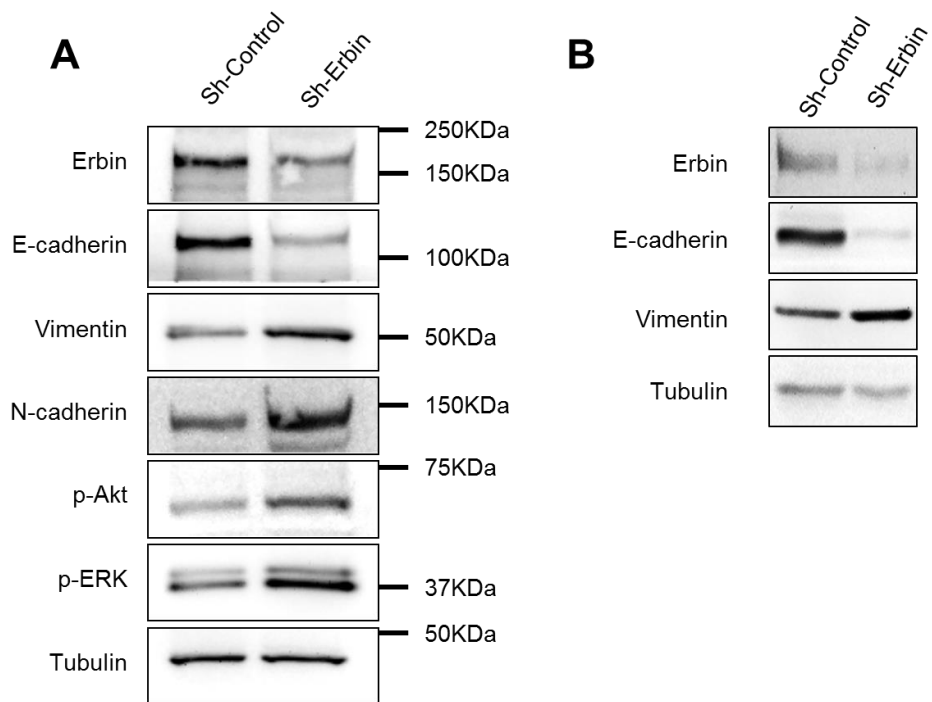
Figure 3.8 Erbin knockdown results in EMT in 2D or 3D culture systems

(A) Stable sh-Control and sh-Erbin SW480 cells were cultured for 10 days in 3D Matrigel and spheroids were collected and analyzed by Western blot for the expression of EMT markers. (B) Stable sh-Control and sh-Erbin SW480 cells grown in 2D culture were analyzed for the expression of Erbin, E-cadherin and Vimentin.
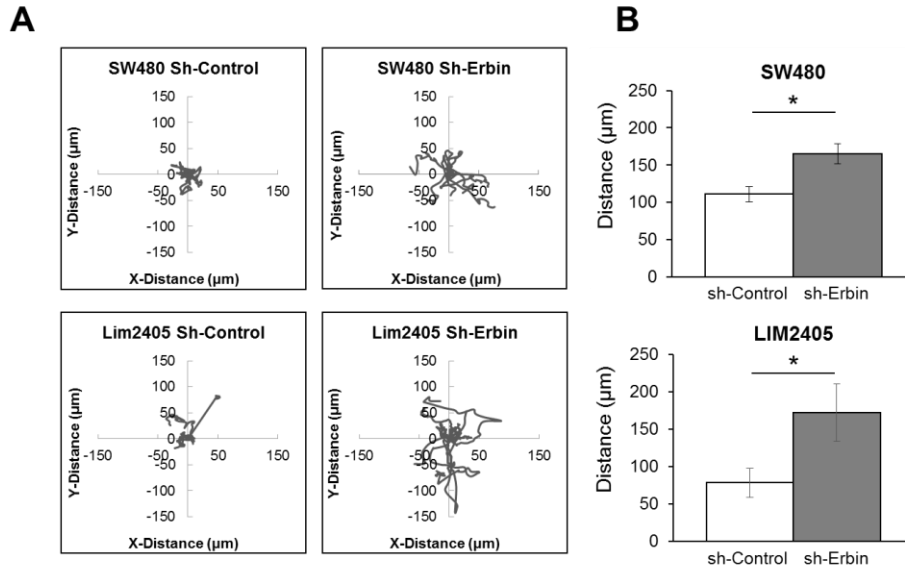
Figure 3.9 Knockdown of Erbin increases cell motility in CRC cells

(A) Migration paths of stable sh-Control and sh-Erbin SW480 and LIM2405 cells were monitored using the Nikon Biostation for 6 hours. The trajectories of 12 randomly chosen cells for each cell line were shown in the graphs. (B) The averaged Path lengths were quantified for sh-Control and sh-Erbin LIM2405 and SW480 cells. Data represents mean $\pm$ SEM (* $p < 0.05$).

A

Upper Compartment

Insert

Porous Membrane

Lower Compartment

+10ng/mL EGF
Attractant

B

SW480

LIM2405

HT29

C

SW480

D

SW480 Invasion
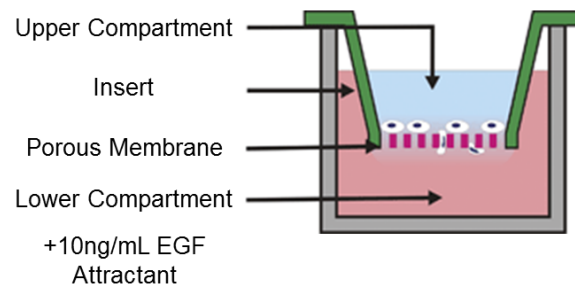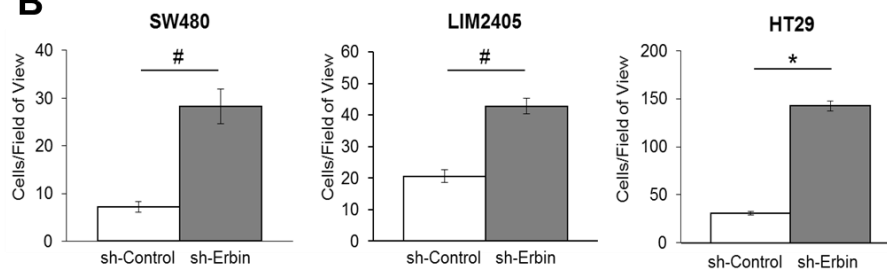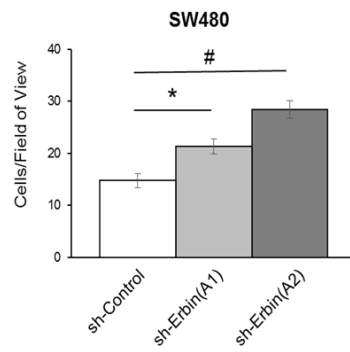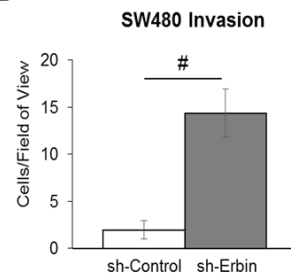
Figure 3.10 Knockdown of Erbin increases CRC cell motility in Transwell assays

(A) CRC cells are placed into the upper chamber of the Transwell and allowed several hours to migrate towards the lower chamber which contains a chemoattractant. The cells that were able to migrate to the lower side of the membrane are then fixed, stained with crystal violet, and counted. Image modified from Keenan and Folch, 2008 [146]. (B) Stable sh-Control and sh-Erbin SW480, LIM2405, and HT29 cells were subjected to Transwell migration assays using collagen and EGF (10ng/mL) as chemoattractants. The number of migrated cells per field of view were counted. Data represents mean $\pm$ SEM (n=4, # $p<0.0001$). (C) Stable sh-Control and sh-Erbin SW480 cells (generating using both A1 and A2 targeting lentivirus) were subjected Transwell migration assays. The number of migrated cells per field of view were counted. Data represents mean $\pm$ SEM (n $=$ 3, * $p<0.05$ and # $p<0.0001$). (D) Stable sh-Control and sh-Erbin SW480 cells were subjected to Transwell invasion assays using 5% FBS as the chemoattractant. The number of invaded cells per field of view were counted. Data represents mean $\pm$ SEM (n=3, # $p<0.0001$).
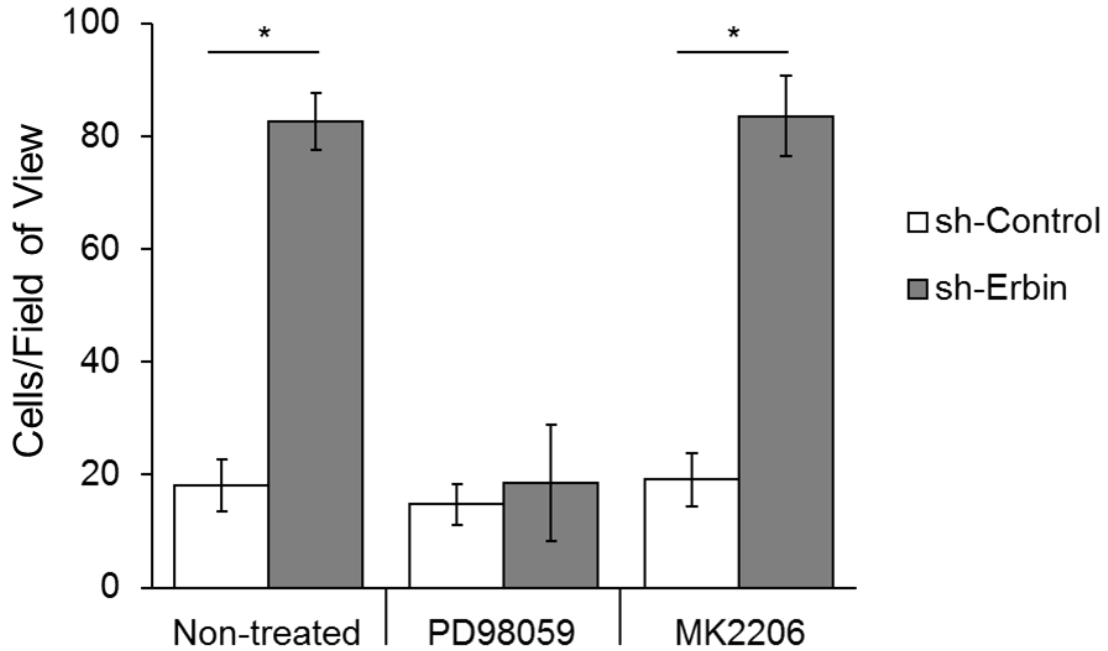
Figure 3.11 ERK/MAPK signaling results in higher CRC motility.

Stable sh-Control and sh-Erbin SW480, LIM2405, and HT29 cells were subjected to Transwell migration assays using collagen and EGF (10ng/mL) as chemoattractants. Treated cell lines were treated with 10μM PD98059 (MEK1 inhibitor) or 1μM MK2206 (Akt inhibitor) overnight and additionally during the 4.5 hours while cells are within the Transwell chamber. The number of migrated cells per field of view were counted. Data represents mean ± SD (*, p<0.05).

3.2.10 Erbin disrupts KSR1 and RAF1 interactions

To define the molecular mechanism underlying Erbin-mediated inhibition of RAF/MEF/ERK signaling activation, we performed experiments examining the possible interactions of Erbin and pro-signaling scaffold proteins within the same pathway. One of the proteins that we identified as a possible interaction partner of Erbin was Kinase Suppressor of Ras 1 (KSR1). KSR1 is known to facilitate the formation of RAF/MEK/ERK complex upon RAS activation [21]. We performed co-immunoprecipitation experiments to determine if Erbin expression affects the interaction between KSR1 and RAF1. 293T cells transfected with Flag-RAF1 in the presence or absence of CFP-KSR1 and Myc-Erbin were immunoprecipitated with the anti-Flag antibody. The overexpressed and endogenous KSR1 were found to pull-down with RAF1 (Figure 3.12 A). However, co-expression of Erbin largely reduced the amount of KSR1 immunoprecipitated with RAF1 (Figure 3.12 A-B). In addition, to determine the interaction between endogenous Erbin and KSR1 directly, co-immunoprecipitation experiments were performed in LIM2405 cells using antibodies against Erbin. Indeed, we found that endogenous KSR1 was co-immunoprecipitated with Erbin (Figure 3.12 C).

3.2.11 Erbin displaces KSR1 from the signaling complex and inhibits signaling

To gain additional insight into the functional effect of Erbin-KSR1 interaction, we examined if Erbin may disrupt KSR1/RAF1 complex. Co-immunoprecipittion experiments were performed using control and Erbin knockdown CRC cells as shown in Figure 3.13 A. A graphic representation of known KSR1 interacting proteins is shown in Figure 3.13 B. Results from these co-immunoprecipitation experiments demonstrated that the formation of KSR1-RAF1 complex was increased in both Erbin knockdown Caco2 and LIM2405 cells (Figure 3.14 A). Furthermore, we found that Erbin-loss induced an increase in ERK

activation that was abolished in cells where KSR1 was also silenced (Figure 3.14 B). The

effect of silencing Erbin, KSR1 or in combination on ERK signaling was analyzed; and the

results showed that Erbin knockdown induced increase in ERK activation was eliminated

when KSR1 was depleted as well, thus confirming the functional interplay between Erbin

and KSR1 in regulating RAF/MEK/ERK signaling. Collectively, these results suggest that

Erbin functions to prevent KSR1 from forming a signaling complex with RAF1 and inhibits

signaling activation downstream of RAF1.
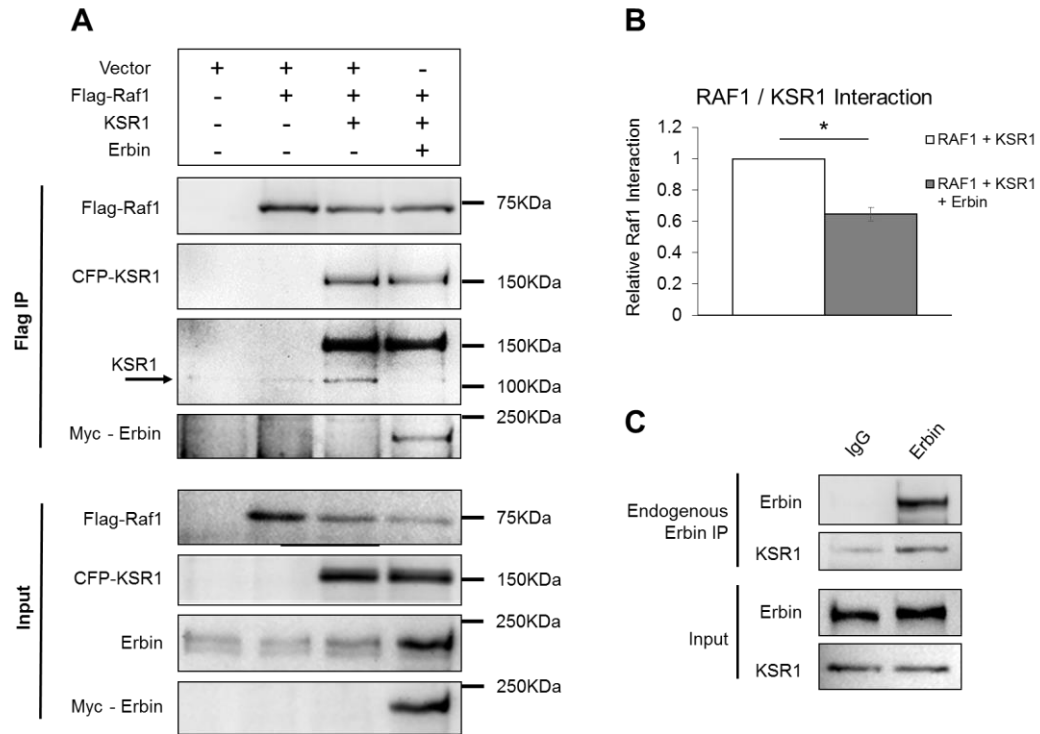
Figure 3.12 Erbin disrupts the interaction between KSR1 and RAF1

(A) 293T cells transfected with Flag-RAF1 and CFP-KSR1 in the presence or absence of Myc-Erbin co-expression were lysed and immunoprecipitated with Flag-affinity beads. The presence of KSR1 and Erbin in the immunoprecipitates were detected by Western blot. The arrow indicates the endogenous KSR1 co-immunoprecipitated by Flag-RAF1. (B) The relative amount of KSR1 co-immunoprecipitated with RAF1 in the presence or absence of Myc-Erbin co-expression was quantified and expressed graphically (n=3, * p<0.05). (C) Erbin interacts with endogenous KSR1. Endogenous Erbin was immunoprecipitated from LIM2405 cell lysates and the presence of KSR1 in the immunoprecipitates was detected by Western blot.
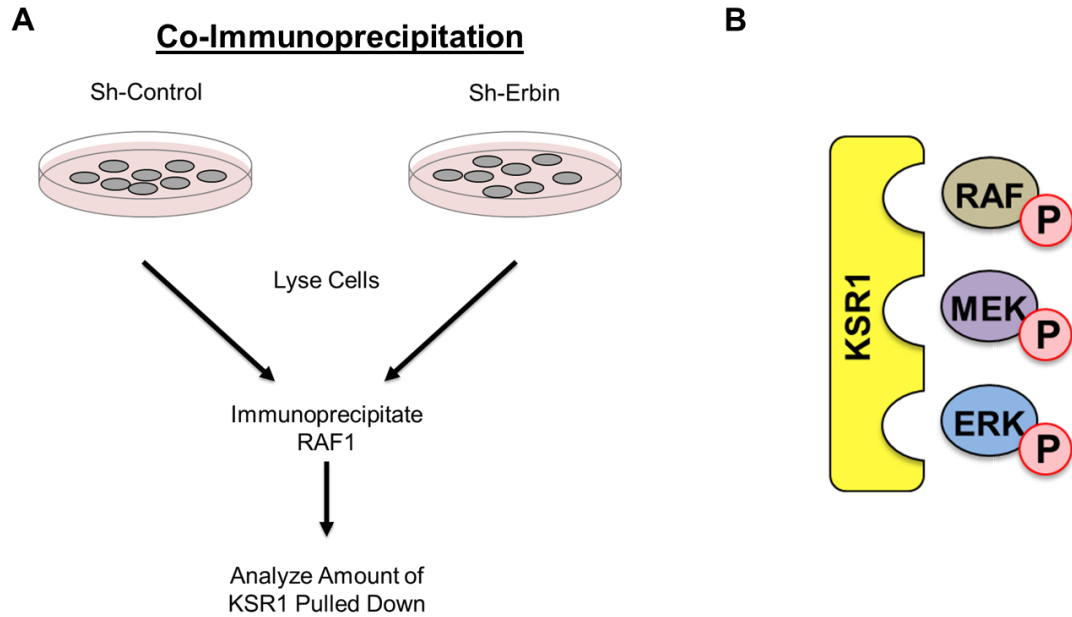
Figure 3.13 KSR1 and RAF1 immunoprecipitation schematic

(A) Stable control or Erbin knockdown CRC cells were lysed and RAF1 is immunoprecipitated from the cells. The levels of co-immunoprecipitated KSR1 were then determined, with the varying levels of Erbin. (B) A graphic representation of the interaction that occurs between KSR1 and members of the RAS/RAF pathway. The presence of KSR1 facilitates the phosphorylation cascade to enhance signaling activation.
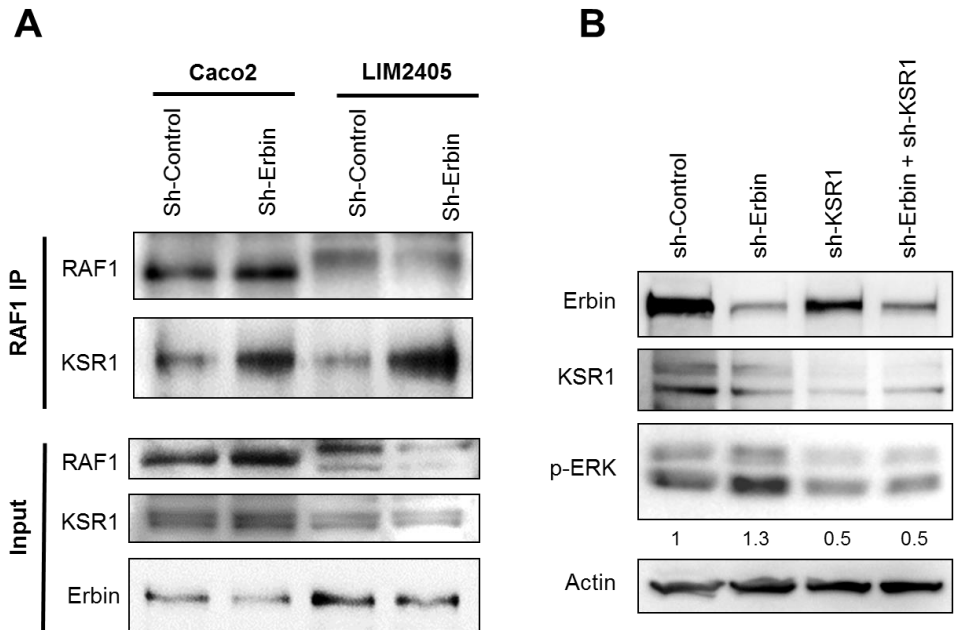
Figure 3.14 Erbin displaces KSR1 from the positive RAF1 signaling complex

(A) Endogenous RAF1 was immunoprecipitated from sh-Control and sh-Erbin Caco2 and LIM2405 cells. The levels of KSR1 co-immunoprecipitated with RAF1 were detected by Western blot. (B) The expression of KSR1 and Erbin were silenced individually or in combination in SW480 cells using corresponding shRNA lentivirus. Cell lysates were analyzed for the phosphorylation status of ERK. The relative levels of ERK phosphorylation in different cells were quantified by normalizing ECL signals generated by p-ERK to that of total proteins.

3.2.12 Summary of In Vitro Studies

Collectively, results from our in vitro studies suggest that Erbin plays an important role in inhibiting EMT and oncogenic signaling in CRC cells.

- The Erbin expression levels, including both mRNA and protein, are decreased in patient tumor tissues when compared to the levels in normal colon tissue.

- Knockdown of Erbin increases signaling through both the Akt and RAS/RAF pathways in CRC cell lines.

- The loss of Erbin results in loss of epithelial polarity, increased cellular motility and EMT.

- Erbin disrupts the function of a pro-signaling scaffold protein, KSR1, and reduces the activation of oncogenic signaling cascade in cancer cells.

3.2.13 Erbin Knockout reduces lifespan of Apc mice

To determine the effect of Erbin-loss on tumorigenesis of CRC *in vivo*, we crossed Erbin-null mice with the Apc$^{f/f}$/Vil-cre mouse model [31] to investigate the susceptibility of mice deficient in Erbin to Apc-driven intestinal adenomas. Erbin knockout (KO) mouse models have been used in previous studies to investigate the role of Erbin in inflammatory responses and cardiac hypertrophy [32, 33]. However, no reports have been made that the knockout of Erbin alone results in the development of spontaneous tumors. To understand the role of Erbin on tumorigenesis, studies have paired the Erbin KO mouse model with a tissue specific tumor-initiating mutation, such as mutant Kras-driven skin tumorigenesis model [147]. For this reason, we chose to cross the Erbin KO mouse with the well-established Apc$^{f/f}$/Vil-cre mouse model and monitor the initiation and progression of the intestinal tumors. The active promoter of Villin, an intestinal specific protein, drives the

transcription of the Cre-recombinase in the intestines, which leads to the removal of the floxed Apc gene. This loss of tumor suppressor Apc is very similar to the mutations that occur in human CRC initiation and leads to upregulation of Wnt signaling in the intestinal epithelia, eventually leading to hyperproliferation and formation of adenomas.

To specifically assess the role of Erbin in tumor initiation and progression in intestinal epithelium, we crossed Erbin KO mice onto the Apc mutant background to generate the following three cohorts of mice: $Apc^{f/+}$/Vil-Cre/$Erbin^{+/+}$, $Apc^{f/+}$/Vil-Cre/$Erbin^{+/-}$ and $Apc^{f/+}$/Vil-Cre/$Erbin^{-/-}$ (Apc/WT, Apc/Het, and Apc/KO, respectively). Survival studies showed that knockout of a single allele of Erbin was sufficient to markedly accelerate the tumorigenesis process, resulting in a significantly shorter lifespan when compared to Apc/WT mice (Figure 3.15). Knockout of both alleles of Erbin further accelerated this process and significantly decreased survival (Figure 3.15). The mean survival times for the Apc/WT, Apc/Het, and Apc/KO were over 180 days, 132 days, and 77 days respectively. In addition to recording the survival times for each cohort of mice, we also made swissrolls from the intestines. This included opening up the intestines longitudinally and rolling the intestine from the distal end towards proximal, creating several layers of intestine, which were then paraffin embedded (Figure 3.16 A). Histopathological analysis of the swissrolls revealed that adenomas were detected in both intestine and colon regions in all three cohorts of mice. While the mice from the different cohorts had significant changes in survival time, the cohorts had an equal tumor number and tumor burden when the mice presented with "end of life" symptoms (hunched stature and rectal bleeding). However, when Apc/WT mice were sacrificed at time points that corresponded to average lifespan of Apc/Het and Apc/KO mice (4.5 and 2 month,

respectively), it was apparent that the tumor burdens were increased in Apc/Het and Apc/KO mice compared to Apc/WT mice (Figure 3.16 B). The difference in tumor burdens was particularly clear in Apc/KO mice, in that more than half of mice in this cohort reached maximum tumor burdens at 2 month when no tumors were detected in Apc/WT mice (Figure 3.16 B).
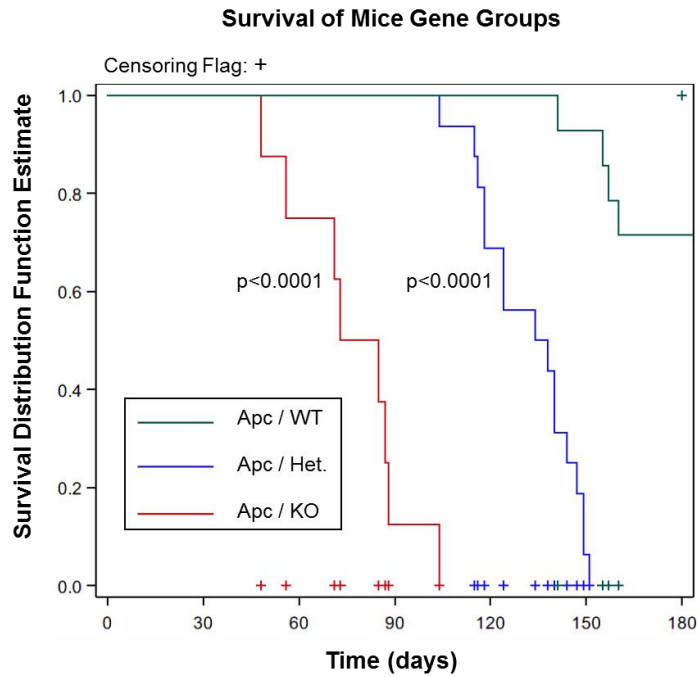
Figure 3.15 Erbin deletion decreases survival in Apc mutant mice

Erbin-loss significantly reduces the lifespan of Apc mutant mice. Kaplan-Meier curve shows the survival distribution of three cohorts of mice: $Apc^{f/+}$/Vil-Cre/$Erbin^{+/+}$, $Apc^{f/+}$/Vil-Cre/$Erbin^{+/-}$ and $Apc^{f/+}$/Vil-Cre/$Erbin^{-/-}$ (Apc/WT, Apc/Het, and Apc/KO, respectively). Numbers of mice in the three cohorts are: Apc/WT (n=14), Apc/Het (n=16) and Apc/KO (n=8). Statistical significance (determined by Log Rank test) is given for comparisons between Apc/WT and Apc/Het (p<0.0001) and Apc/WT and Apc/KO (p<0.0001).
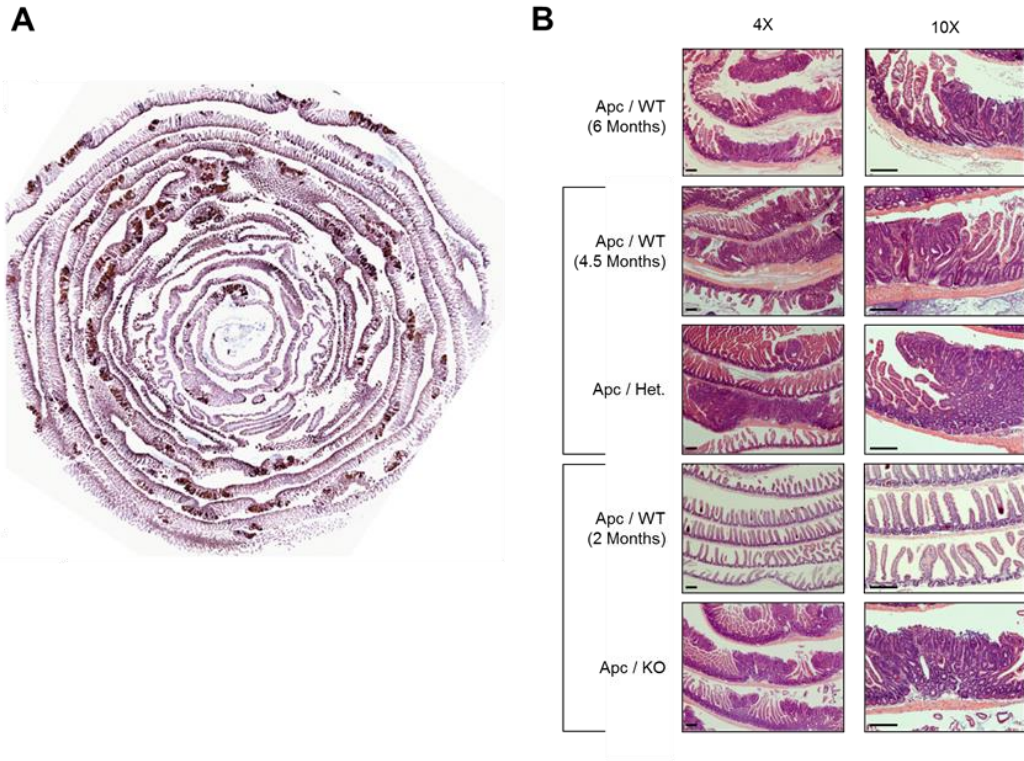
Figure 3.16 Erbin deletion decreases survival and increases tumorigenesis in Apc mutant mice

(A) A representative image of a "swissroll" made from mouse intestines. Modified from

Colnot et al. 2004 [148] (B) Histology analysis of intestinal adenomas of Apc/WT and age-

matched Apc/WT and Apc/Het mice (at 4.5 months) as well as Apc/WT and Apc/KO mice

(at 2 months). Scale bar, 200μm.

3.2.14 Knockout of Erbin increases cell signaling in both tumor organoids and MEF cells

In order to further explore the mechanisms for the observed increase in tumorigenesis, tumor cells isolated from Apc/WT and Apc/KO mice were allowed to grow into tumor organoids in 3D Matrigel. This culture system lacks several medium additives necessary for the growth of normal epithelial tissue, and therefore selects for only growing the tumors within the mouse intestines. These organoids are comprised of adenoma cells which form cystic structure without budding (Figure 3.17 A). To determine if Erbin inhibits signaling in mouse adenomas, protein lysates prepared from tumor organoids were subjected to Western blot analysis. Both Akt and ERK phosphorylation were markedly increased in Erbin KO organoids (Figure 3.17 B). Moreover, consistent with results obtained in CRC cells, we found that the amplitude and duration of EGF-stimulated Akt and RAF/MEK/ERK signaling were largely increased in Erbin KO MEF cells (Figure 3.17 C). Together, these data showed that Erbin-loss promotes the activation of Akt and RAF/MEK/ERK signaling and tumorigenesis in mouse models of CRC.

3.2.15 Normal epithelial cells are not significantly different in Erbin KO mice

Since the knockout of Erbin alone did not result in the development of spontaneous tumors we were interested in determining whether there were differences in normal intestinal growth and differentiation. Intestinal tissue from Erbin WT, heterozygous, and KO mice were collected, and these tissues were then made into swissrolls and IHC stained for various protein markers for proliferation and cell lineages. We compared the proliferation and differentiation of normal intestinal epithelial cells in WT, heterozygous and Erbin KO mice and found no difference in the number of proliferating cells and differentiated cells of different cell linages (Figure 3.18).

3.2.16 Erbin Knockout does not alter gene expression in normal intestinal organoids

In addition to collecting normal epithelial tissue for IHC staining, we also grew these cells in 3D culture. We were able to propagate these cells and collect them for analysis via qPCR, further analyzing any differences in stemness, differentiation, or proliferation. Consistent with the lack of changes observed IHC staining, normal intestinal tissues were cultured in Matrigel no significant differences were observed in the resulting organoid shape or size (Figure 3.19 A). Similarly, the expression of genes associated with normal intestinal stem and differentiated cells remained unchanged in intestinal organoids derived from Erbin heterozygous and homozygous KO mice compared to WT mice (Figure 3.19 B).

3.2.17 Erbin Knockout significantly increases the stemness in tumor organoids

We next analyzed the tumor initiation capacity of mouse tumor organoids using the colony formation assay. Apc/WT and Apc/KO organoids were dissociated into single cells and re-seeded into Matrigel. The number of tumor organoids formed was counted after 5 days in culture. Knockout of Erbin resulted in a two-fold increase in organoid formation (Figure 3.20 A). Since increased tumor initiation capacity is often associated with cancer stem cells, our results suggest that Erbin-loss may enhance stem-like properties in tumor organoids.

To test the hypothesis that Erbin may influence the stemness of tumor derived organoids, we determined the profile of stem cell gene expression using quantitative RT-PCR analysis. Apc/WT and Apc/KO organoids were grown in 3D culture for 5 days and then were collected and analyzed for the expression of genes that are typically associated with cancer stem cells (eg. Lgr5, Axin2, and Cd44), intestinal cell differentiation (eg. Alpi, Fabp2, and Muc2), and cell proliferation (eg. Ccnd1 and Ki67). The tumor organoids

derived from Apc/KO mice expressed higher levels of genes associated with stem cells and cell proliferation, which coincided with decreased expression of genes associated with differentiated intestinal epithelial cells (Figure 3.20 B). These data would suggest that the loss of Erbin is resulting in an increase in adenoma cell stemness, and these changes are likely the underlying cause for the observed increase in the organoid tumor-initiation assay and the earlier occurrence of tumorigenesis in the Apc/KO mice. Additionally, we also made similar observations in tumor organoids derived from the Apc/Het mice, where the expression of genes associated with stem cells and cell proliferation were significantly increased and intestinal differentiation decreased (Figure 3.20 C), confirming that haplodeficiency of Erbin is sufficient to promote tumorigenesis.
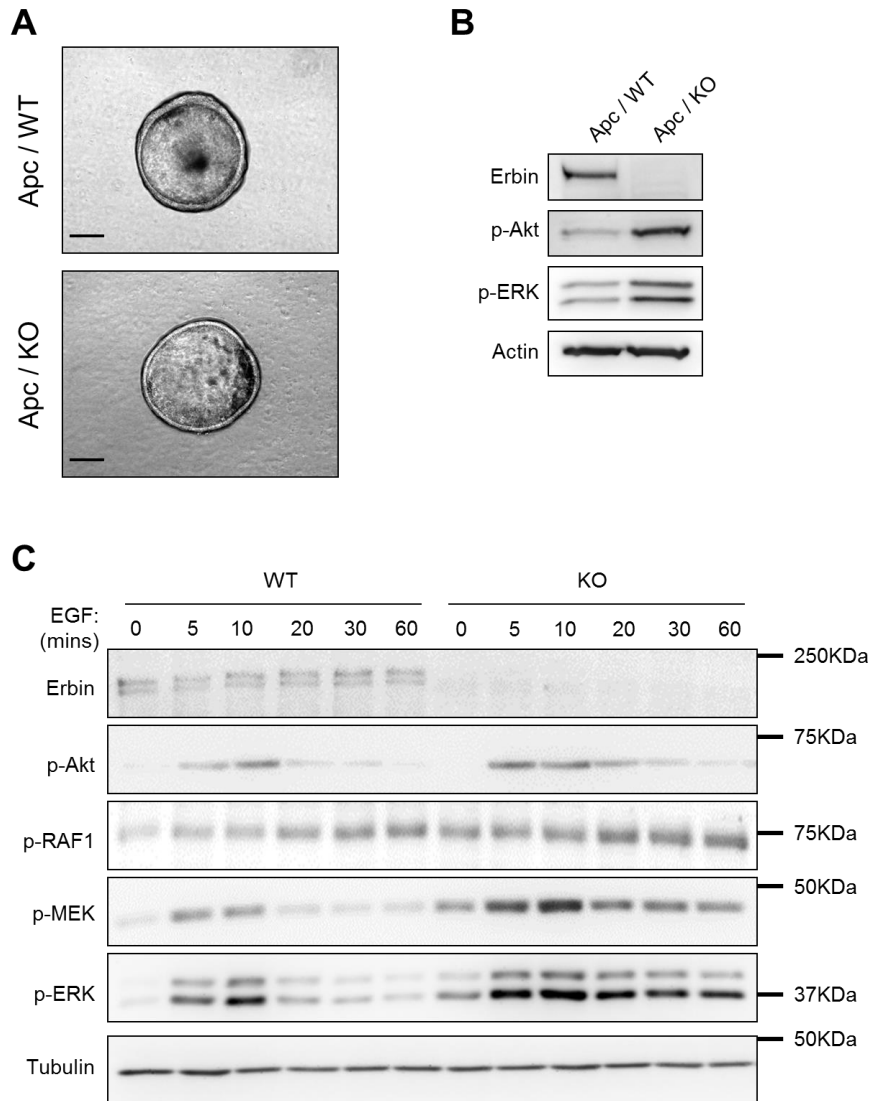
Figure 3.17 Erbin deletion activates Akt and RAS/RAF signaling in Apc mutant mice

(A) Tumor organoids were prepared from tumor tissues of Apc/WT and Apc/KO mice and grown in 3D Matrigel. Scale bar, 200μm. (B) Cell lysates were prepared from tumor organoids as shown in (A) and analyzed for the phosphorylation status of Akt and Erk by Western blot. (C) MEF cells generated from WT and Erbin KO mice were stimulated with EGF (10ng/mL) for the indicated times and analyzed for the activation of Akt and RAS/RAF signaling by Western blot.
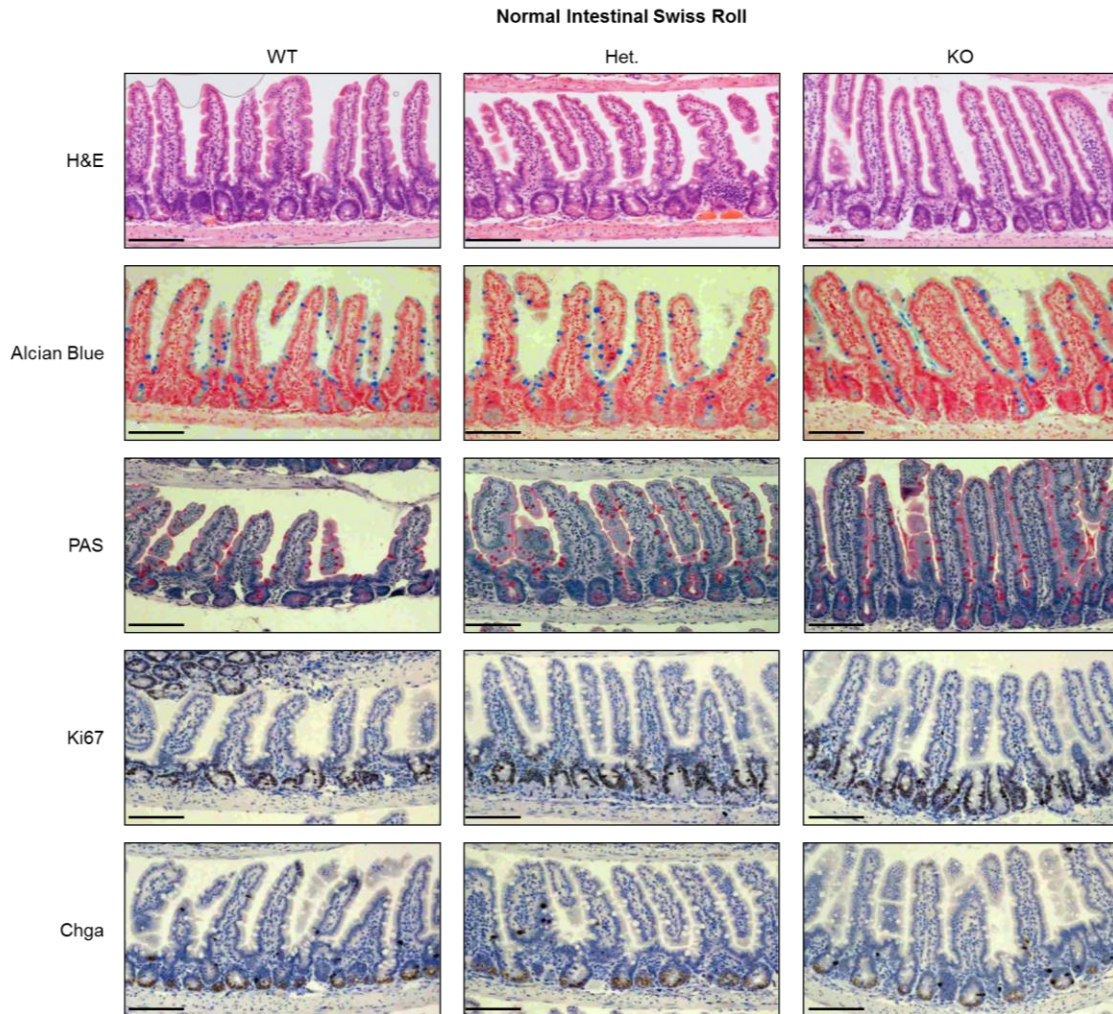
Figure 3.18 Knockout of Erbin has no significant effect on the proliferation and differentiation of normal intestinal epithelial cells

Swiss rolls of intestinal tissues from WT, Erbin$^{+/-}$ (Het) and Erbin$^{-/-}$ (KO) mice were analyzed for the appearance of normal crypt-villus structure using H&E staining. Alcian blue and PAS staining were used to identify goblet cells and Paneth cells; whereas Ki67 and chromogranin A IHC staining detects proliferating cells and enteroendocrine cells, respectively. Scale bar, 200μm.
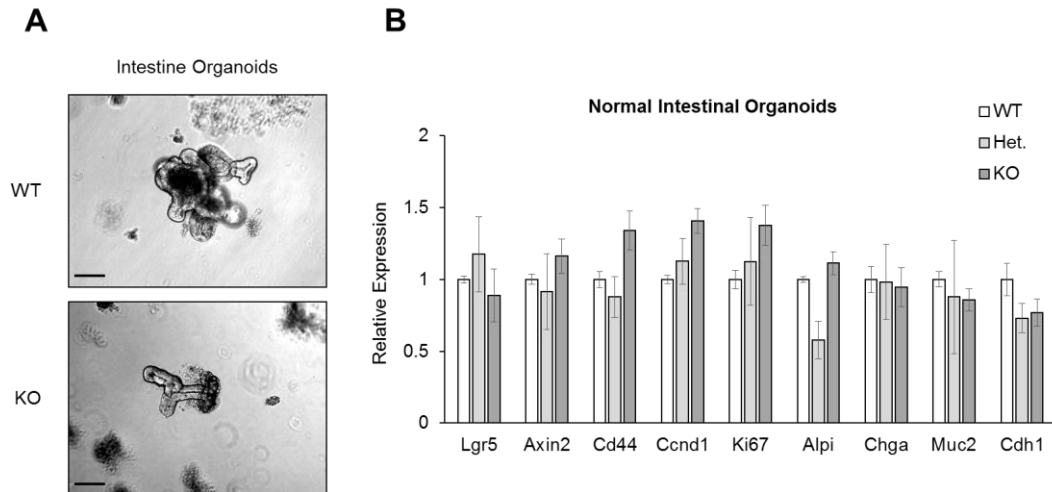
Figure 3.19 Loss of Erbin does not significantly alter the expression of genes associated with intestinal stem cells and differentiation in normal intestinal organoids

(A) Representative phase contrast images of intestinal organoids derived WT and Erbin$^{-/-}$ (KO) mice grown in 3D Matrigel for 5 days. Scale bar, 200μm. (B) Intestinal organoids from WT, Erbin$^{+/-}$ (Het) and Erbin$^{-/-}$ (KO) mice were grown in 3D Matrigel and collected after 5 days. Total RNA were prepared and used for qRT-PCR analysis for the expression of genes associated with intestinal stem and progenitor cells (Lgr5, Axin2 and Cd44), cell proliferation (Ccnd1 and Ki67) and intestinal cell differentiation (Alpi, Chga, Muc2 and Cdh1). Data represents mean ± SEM, n=3.

A

Single Cell Colony Formation

B

Tumor Organoids

C
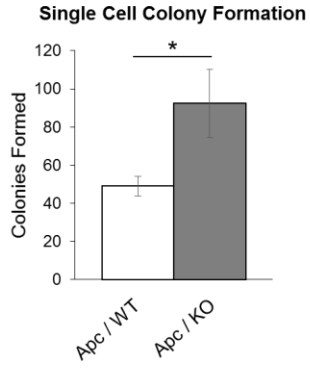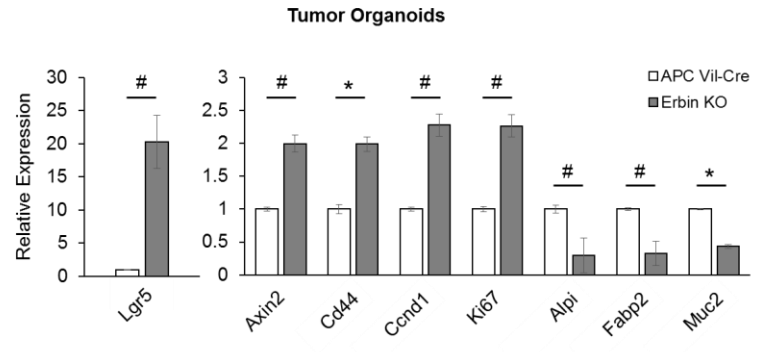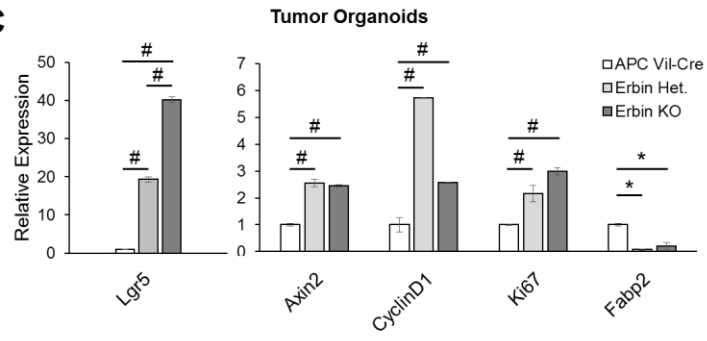
Tumor Organoids

Figure 3.20 Loss of Erbin enhances cancer stem cell properties in tumor organoids

(A) Tumor organoids derived from Apc/WT and Apc/KO mice were dissociated into single cells and 1,000 cells were reseeded in 3D Matrigel. The number of cells that were able to successfully form colonies were quantified. Data represents mean ± SEM (n=3, * p<0.05). (B) Tumor organoids derived from Apc/WT and Apc/KO mice grown in 3D Matrigel for 72 hours were collected and analyzed for the expression of genes associated with cancer stem cells (Lgr5, Axin2 and Cd44), cell proliferation (Ccnd1 and Ki67) and intestinal differentiation (Alpi, Fabp2 and Muc2) using quantitative RT-PCR. (C) Tumor organoids derived from three cohorts of mice (Apc/WT, Apc/Het and Apc/KO) were grown in 3D culture for 72 hours, collected, and analyzed using qRT-PCR for the expression of genes associated with cancer stem cells (Lgr5 and Axin2), cell proliferation (Ccnd1, Ki67) and intestinal cell differentiation (Fabp2). Data represents mean ± SEM (n = 4, * p<0.05 and # p<0.0001).

3.2.18 Gene Set Enrichment Analysis confirms Erbin's role in colorectal cancer

Since cancer stem cell associated genes, such as Lgr5 and Cd44, are targets of Wnt signaling, our data suggested that Erbin-loss results in activation of the Wnt pathway in Apc mutant tumors. Furthermore, through a collaboration with the Markey Cancer Center – Biostatistics and Bioinformatics Shared Resource Facility, we analyzed if Erbin expression is associated with cancer-related biological pathways by analyzing gene expression data from the Cancer Genome Atlas (TCGA) CRC RNA-seq dataset. Gene Set Enrichment Analysis (GSEA), several example gene sets that were analyzed, and the resulting distribution of association is shown in Figure 3.21 A. The actual results from the GSEA showed Erbin expression associated with several pathways, including a positive association with the E-cadherin adherens junction (AJ) pathway and Erbin negatively associated with Wnt signaling (Figure 3.21 B). These data support our findings that loss of Erbin promotes the disruption of epithelial polarity by reducing E-cadherin expression and enhances cancer stem cell property by increasing signaling through the Wnt pathway. Taken together, our results showed that genetic deletion of Erbin promotes tumor formation and progression in vivo upon activation of Akt and ERK signaling. Loss of Erbin enhances the stem-like characteristics in tumor organoids and potentiates tumor initiation.

A

Gene Set Examples:

E-cadherin Junction
Metabolism of RNA
Translation
Synthesis of DNA
Proteasome
Wnt Signaling
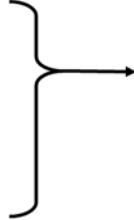
Correlation with Gene of Interest

Positive
Correlation

Negative
Correlation
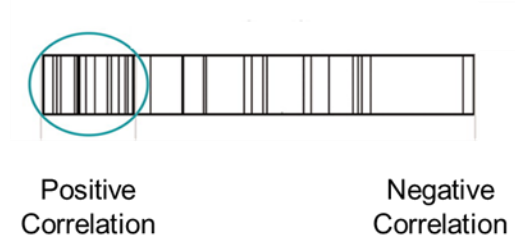
B
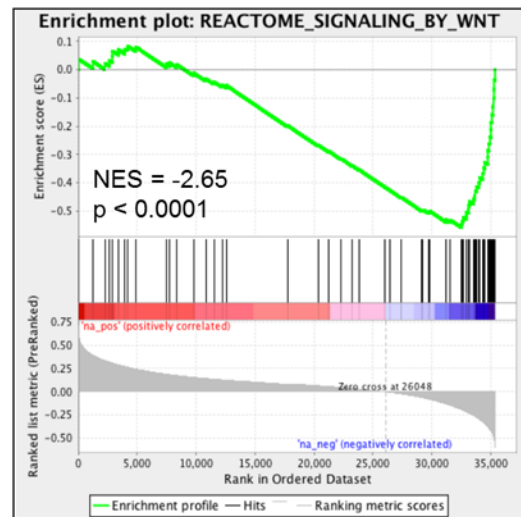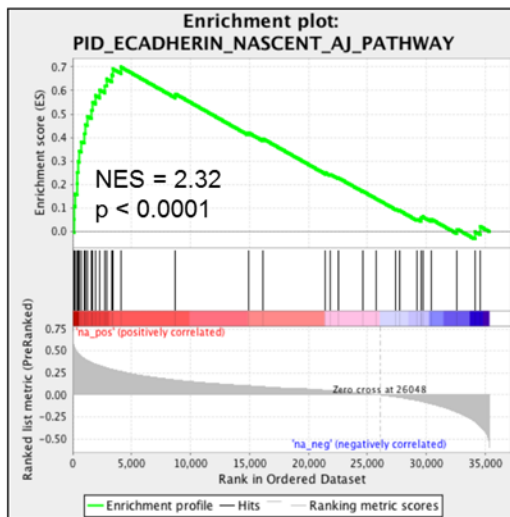
Figure 3.21 Gene Set Enrichment Analysis confirms Erbin's role in control epithelial junctions and Wnt signaling

(A) An example of several gene sets that correlate the expression of Erbin in CRC patients by analyzing RNA-seq data obtained from the TCGA database. The genes in each gene set are analyzed and determined to have a positive or negative association with Erbin, which can be quantified for each gene set to determine if Erbin expression is correlated to any signaling or physiological pathways (B) Gene Set Enrichment Analysis was performed and the enrichment plots showed significant correlation with the E-cadherin pathway (NES = 2.32, FDR <0.0001) and Wnt signaling (NES = –2.65, FDR <0.0001) with Erbin expression in CRC patients.

In summary, the collection of data provides strong evidence confirming the role of Erbin as a tumor suppressor in CRC. Previous data had shown that Erbin could interact with many proteins at cell-cell junctions and alter ERK signaling, which resulted in some physiological consequences in normal cell differentiation. However, while Erbin's role in cancer has not been as thoroughly explored, with some publications concluding that Erbin may be a tumor promoter, the data collected in my dissertation studies would help confirm a tumor suppressor role. By playing an important role in maintaining epithelial cell polarity, Erbin functions as a negative scaffold to directly inhibit oncogenic signaling at multiple steps, thus effectively inhibiting tumorigenesis and progression (Figure 3.22). Mechanistically, these data have shown that Erbin can alter KSR1/RAF1 interactions and suppress ERK signaling, strengthening the idea that Erbin is a negative-regulating scaffold protein.
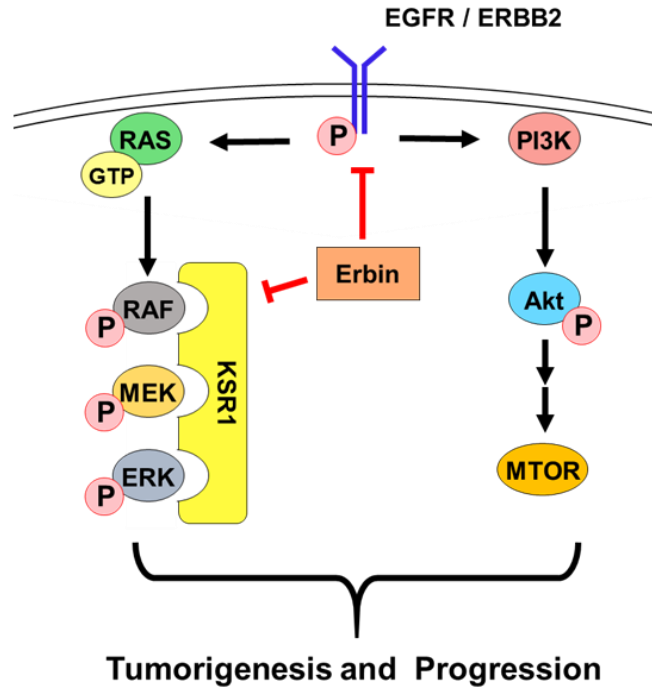
Figure 3.22 Erbin inhibits tumorigenesis and progression of CRC

A diagram showing the mechanism underlying Erbin-mediated tumor suppressor function in CRC. Our study demonstrates that loss of Erbin expression promotes tumor progression as a result of increased RAS/RAF and Akt signaling. Basolateral expression of Erbin in normal epithelial cells prevents hyperactivation of RAS/RAF signaling by sequestering KSR1 from binding RAF1. In addition, Erbin may inhibit Akt activation by suppressing ERBB2 function.

3.3 Other roles for Erbin in regulating cell signaling

3.3.1 Erbin alters ErbB2/EGFR signaling

In addition to examining Erbin-mediated inhibition of RAF/MEK/ERK signaling through a KSR1-dependent mechanism, we also conducted experiments to test the hypothesis that Erbin-loss induced polarity defect may activate EGFR signaling directly. EGFR and EGF-like ligands are known to be over-expressed or mutated in many cancers including CRC, as well as breast, prostate, kidney, ovary, brain, pancreas, bladder, and lung [149, 150]. Stimulation of EGFR primarily leads to the activation of RAS/RAF signaling that is known to mediate multiple processes involved in tumor progression, including angiogenesis, invasion, migration, proliferation, and evasion of apoptosis [151].

Erbin directly interacts with ErbB2, this interaction is required for basolateral localization of ErbB2, and ErbB2 is a preferred signaling partner of other ErbB family receptors including EGFR [73]. EGFR-ErbB2 heterodimers enhance signaling due to evasion of receptor endocytosis and degradation [152]. Additionally, previous research studies have shown that the loss of polarity can lead to aberrant activation of receptor tyrosine kinases (RTK) due to disruption of asymmetrical distribution of the receptors [153]. In the case of EGFR, it is well established that the expression and localization of EGFR is highly restricted to the basolateral plasma membrane in polarized epithelial cells [154]. The basolateral distribution of the receptors is not only responsible for proper activation but also for the degradation and appropriate quenching of the signal. Following EGFR stimulation, the receptor undergoes ligand-induced receptor endocytosis initialized by the receptors diffusing laterally through the membrane to localize within clathrin-coated pits [155]. Redistribution to coated pits is necessary to internalize multiple activated receptors in one vesicle, and once multiple EGFRs are located within that pit, they are

rapidly internalized [156, 157]. The results of this process are either the eventual

degradation of the receptor and the associated growth factor in the lysosome, or the

recycling of the receptor back to the plasma membrane where it can be activated once again

[158]. This process can therefore lead to the attenuation of signaling by removing actively

signaling receptors and degrading them, as well as reducing the total number of receptors

available for subsequent ligand activation on the cell surface [159, 160].

Mechanistically, after receptor stimulation, EGFR cytoplasmic tail tyrosines are

phosphorylated and it is through the interactions between these auto-phosphorylation sites

and a variety of adapter proteins that allow for specificity in the cellular response (Figure

3.23).
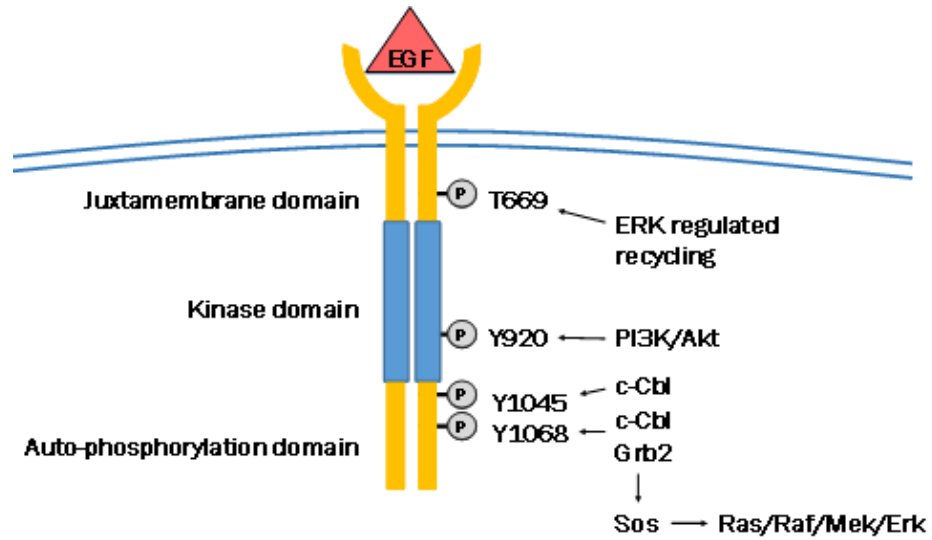
Figure 3.23 EGFR phosphorylation sites

The multiple phosphorylation sites on the cytoplasmic tail of the EGF Receptor are involved in the recruitment and activation of many different adapter proteins, which lead to activation of various downstream pathways.

Two examples of physiological processes that are dependent on the recruitment of these adapter/interacting proteins after EGFR stimulation include the activation of the RAS/RAF and Akt pathways which requires the recruitment of Grb-2. Also, for degradation or recycling of EGFR to occur, a recruitment of the E3-ligase, c-Cbl, to the activated receptor is required. Additionally, it is not clear whether altered receptor distribution as the result of disruption of apical-basal polarity may contribute to the hyperactivation of EGFR signaling in CRC.

3.3.2 The role of Erbin in EGFR expression, internalization and degradation
To date, it is not known whether Erbin is involved in regulating EGFR. Therefore to begin to determine if the disruption of polarity resulting from the loss of Erbin has an effect on EGFR signaling, we investigated the time course of EGFR phosphorylation and internalization using CRC cell lines, including SW480, Caco2, LIM2405, and DLD1, as well as MEF cells. The expression of Erbin was silenced using lentivirus-mediated RNAi in CRC cell lines. Control and Erbin knockdown cells were treated EGF for 0-45 minutes. Similarly, WT and Erbin KO MEF cells were used with EGF stimulation. Here, we monitored EGFR activity, via Western blot for phosphorylation at several sites on the cytoplasmic tail. Unexpectedly the phosphorylation of EGFR at the Y1068 site (Figure 3.24) was markedly reduced in the Erbin knockdown cells. Notably, the altered EGFR phosphorylation site is involved in the recruitment of c-Cbl (Figure 3.23). In addition to observing phosphorylation differences at the EGFR level, in all CRC cell lines with reduced levels of Erbin we also noted decreased total protein levels of EGFR, ErbB2, and ErbB3 (ErbB4 was undetectable in CRC cells). Contrarily, the Erbin KO MEF cells had dramatically increased levels of EGFR and phosphorylation at the Y1068 site (Figure 3.25). Further experiments are required to fully understand the differences in signaling, but

could possibly be due to the presence/absence of certain adapter proteins in the different cell systems.

To further analyze if altered EGFR phosphorylation in Erbin knockdown cells affects the internalization of EGFR, we monitored the time course of EGFR endocytosis upon treating cells with Alexa 488-labeled EGF using immunofluorescence imaging. Interestingly, as shown in Figure 3.26, abundance of fluorescent-labeled EGF was found on the cell surface after treating cells for 4 minutes in Erbin knockdown cells indicating decreased endocytosis of EGFR. In addition, the knockdown cells showed attenuated degradation of the green fluorescent signal suggesting a slower rate of EGFR trafficking to the lysosome.

In summary, the disruption of epithelial polarity as a result of Erbin downregulation may alter the time course of EGFR activation. Despite differential effects were observed on EGFR phosphorylation upon Erbin depletion in different cell lines, Erbin-loss consistently activates RAS/RAF signaling downstream of EGFR. However, future studies are needed to further determine the molecular mechanism underlying Erbin-mediated regulation of EGFR at the receptor level.
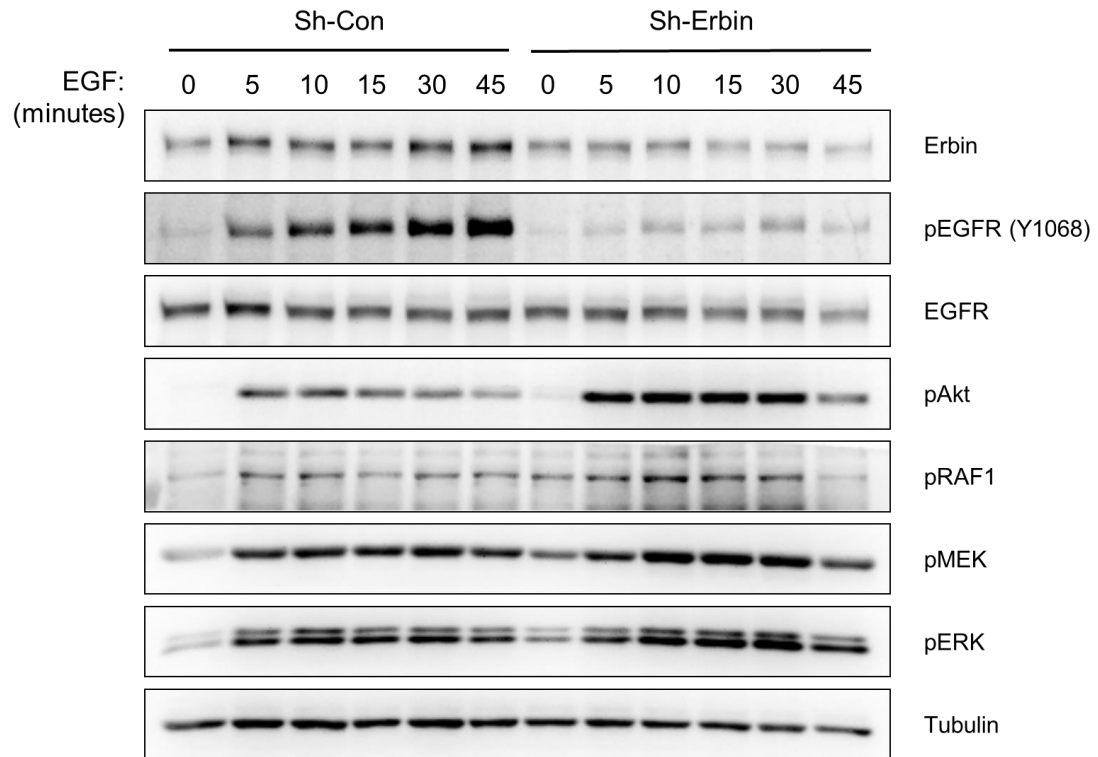
Figure 3.24 Knockdown of Erbin in SW480 decreases EGFR phosphorylation

Stable sh-Control and sh-Erbin SW480 cells were stimulated with EGF (10 ng/ml) for the indicated times and activation of signaling pathways and EGFR phosphorylation (Y1068) were analyzed via Western blotting. While Erbin knockdown cells have decreased phosphorylation of EGFR, downstream proteins such as ERK and Akt both show increased and prolonged phosphorylation and activation.
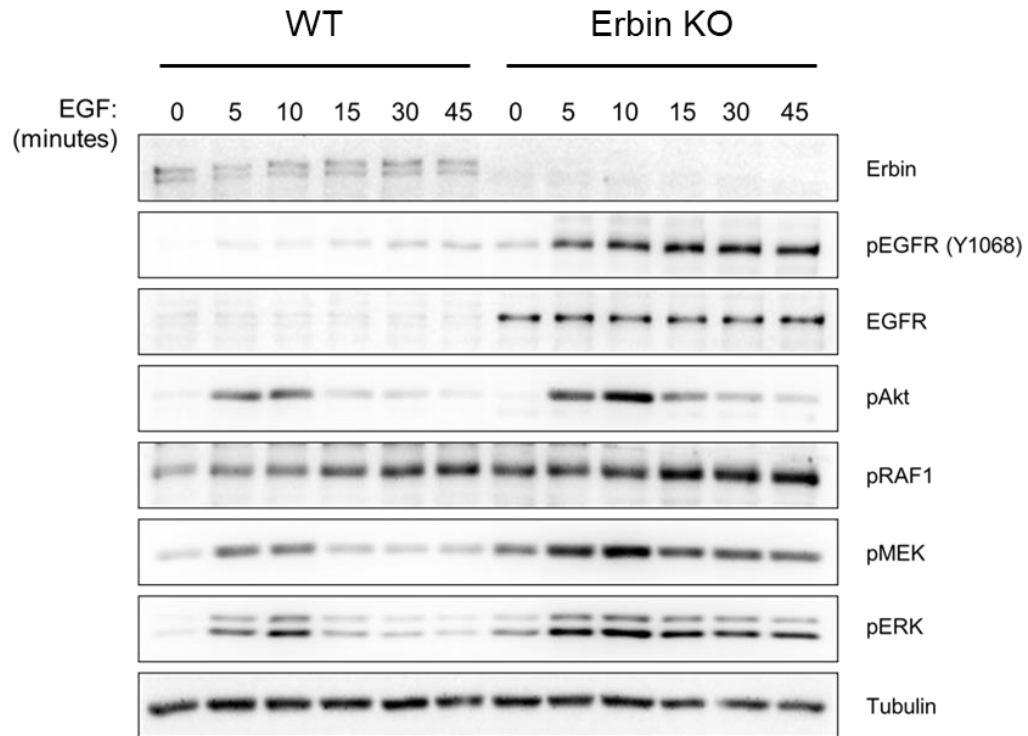
Figure 3.25 Knockdown of Erbin increases EGFR levels and phosphorylation in MEF cells

Wildtype and Erbin KO MEF cells were stimulated with EGF (10 ng/ml) for the indicated times and activation of signaling pathways and EGFR phosphorylation (Y1068) were analyzed via Western blotting. Interestingly, Erbin KO cells have increased total levels and phosphorylation of EGFR, which is not observed in CRC cells with reduced Erbin levels. However, regardless of EGFR phosphorylation, downstream proteins such as ERK and Akt both show similar increases in phosphorylation and activation.
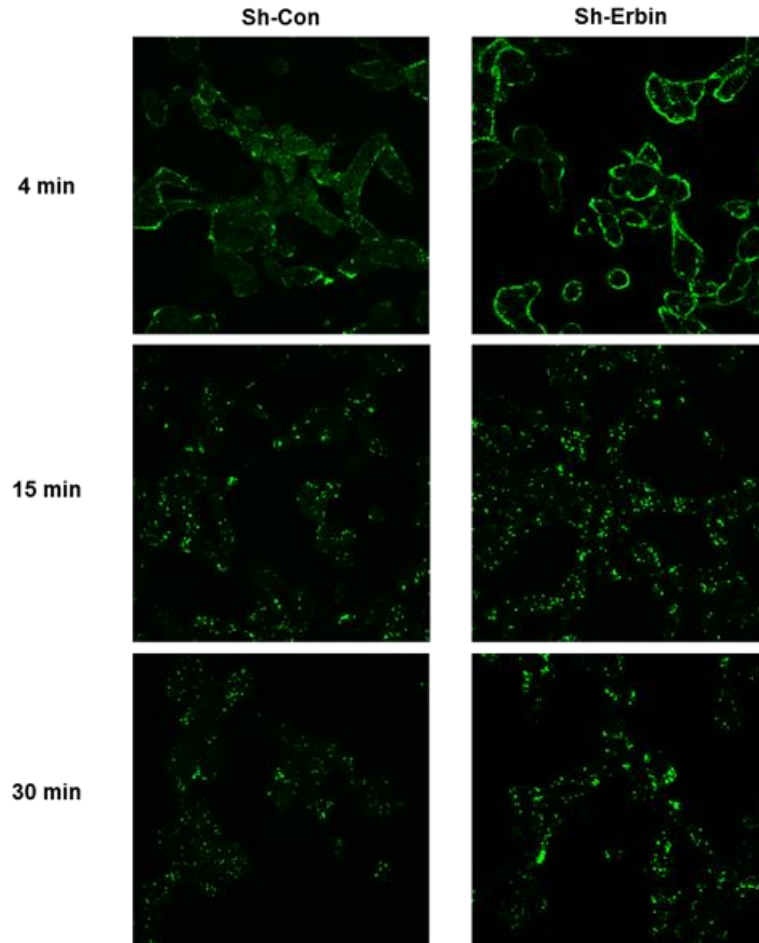
Figure 3.26 Knockdown of Erbin alters EGFR distribution and internalization in SW480 cells

The expression pattern of EGFR in sh-Control and sh-Erbin SW480 cells at 4, 15, and 30 minutes after the cells were stimulated with Alexa-488 labeled EGF. Increases in fluorescent EGF bound at 4 minutes may be the result of more EGFR localized to the plasma membrane of Erbin knockdown cells. Also, slower degradation of EGF signal as observed at 30 minutes may indicate prolonged EGF-induced signaling in Erbin knockdown cells.

CHAPTER 4: DISCUSSION

4.1 The Role of Erbin as a Tumor Suppressor in CRC

Loss of epithelial polarity is a hallmark of advanced malignant tumors. Emerging evidence supports the notion that disruption of polarity promotes EMT and the malignant progression of cancer [15, 35, 36]. Several oncogenes and tumor suppressors (such as ErbB2, Kras, and Lkb1) are known for their ability to regulate cell signaling as well as the integrity of epithelial polarity [30, 37, 38]. In this study, combining *in vitro* and *in vivo* analyses, we identify Erbin as a tumor suppressor in CRC. Analysis of gene expression datasets revealed that Erbin mRNA expression in patient tumor tissue is significantly downregulated. Additionally, using Western blotting and immunohistochemical staining of patient tissues, we demonstrated that Erbin protein expression is decreased, and mislocalized, in the tumor specimens. Functionally, the knockdown of Erbin in CRC cells results in the disruption of epithelial cell polarity as observed through the formation of multiple lumen cell clusters with aberrant distribution of apical and basolateral proteins in 3D culture. The loss of polarity is accompanied by an increase in cell proliferation as shown by increased spheroid size and the number of EdU positive cells per 3D tumor sphere. Moreover, knockdown of Erbin increases the amplitude and duration of signaling through both the Akt and RAF/MEK/ERK signaling cascades, inducing EMT in CRC cell lines. Consecutively, we observed significant increases in cell motility as measured by Transwell migration assays as well as with time-lapse live cell imaging and determined this motility to be associated with increased signaling through the MAPK. Using co-immunoprecipitation experiments, we identified KSR1, a positive regulator of ERK signaling, as an interacting protein of Erbin. We showed that Erbin functions by displacing KSR1 from the RAF-MEK-ERK complex thus preventing signal transduction through the

ERK pathway. Furthermore, through the development of the Apc-Erbin knockout double mutant mice, we investigated the impact of Erbin-loss on intestinal tumorigenesis *in vivo*. The loss of Erbin expression in Apc mutant mice results in a significantly shorter lifespan, with a stepwise reduction in survival as each allele of Erbin is lost. To understand the mechanism of the increased tumorigenesis after Erbin knockout, tumor organoids from those mice were used to confirm increased Akt, RAS/RAF signaling, and interestingly the expansion of the stem-like tumor initiating cell population. Taken together, these results suggest that Erbin functions as a negative regulator of both EMT and cell motility by directly suppressing Akt and ERK signaling. Additionally, these data suggest that loss of Erbin expression promotes Wnt signaling and tumor formation *in vivo*.

Previous studies have suggested that Erbin inhibits the activation of ERK by disrupting upstream Shoc2-mediated RAS/RAF interaction [92, 93]. However, Shoc2 is primarily localized to the cytoplasm and actively facilitates RAS/RAF signaling within endosomal compartments [59]. It remains an open question how Erbin, a protein that is primarily localized to the basolateral membrane, interferes with Shoc2-dependent activation of RAS/RAF signaling within the endosome. In our study, we show that Erbin decreases RAF/MEK/ERK signaling through directly competing with KSR1. KSR1 is known to translocate to the plasma membrane upon RAS activation [21, 22]. While results from our study and others demonstrate that Erbin is localized at the basolateral membrane, it is possible that Erbin could be localizing to multiple sub-domains of the plasma membrane and this could help explain how Erbin could have multiple inhibitory roles, such as with ErbB2, Shoc2, and KSR1. However, specifically for the KSR1 interaction, being in close proximity with receptor tyrosine kinases (such as EGFR and ErbB2) and the site

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of RAS activation, the presence of Erbin likely blocks the access of KSR1 to RAS-bound RAF and reduces KSR1-RAF interaction (Figure 4.1). In our study, in addition to increased ERK signaling, the knockdown of Erbin induces activation of Akt in colon cancer cells and mouse tumor organoids. Pharmacological Akt inhibition was unable to decrease cell motility; however, we have not fully investigated the implications of increased Akt signaling after Erbin knockdown. Future studies would be necessary to determine if the loss of polarity, increased proliferation, or changes to Wnt signaling are downstream of Akt. The literature regarding signaling changes within the Akt pathway, specifically after Erbin loss, is inconsistent and seems to be dependent upon the cell type or system in which the study was performed [5, 34]. The differential effect of Erbin-loss on Akt activation may be due to protein interactions that are cell type specific.

In cells where silencing Erbin induces loss of epithelial polarity and EMT (such as in colon cancer cells), it may lead to mislocalization of receptor tyrosine kinases and aberrant activation of both Akt and ERK signaling downstream. The role of Erbin in receptor trafficking is vastly under studied. Erbin could inhibit signaling by altering ErbB family member protein trafficking or endocytosis, increasing degradation or resulting in fewer interactions with pro-signaling scaffolds (ie. Shoc2 and KSR1). Endogenous Erbin protein is mostly localized to the plasma membrane [85], where it directly interacts with ErbB2 and this interaction is required for the receptors basolateral localization [73] and ErbB2 is a preferred signaling partner of other ErbB family receptors including EGFR. Previous data has shown that EGFR-ErbB2 heterodimers exhibit increased signaling due reduced receptor endocytosis and degradation [152]. Therefore, since Erbin can alter ErbB2 localization, through heterodimer interactions Erbin could also alter the localization

of all ErbB family members. Through interactions with the C-terminal tail of ErB2 Erbin could change the signaling from these receptors whether that is through physically restricting the receptors to a sub-domain of the plasma membrane or by altering adapter protein interactions. In our studies we observed an EMT phenotype and a loss of polarity in the CRC cell lines, which could lead to the loss of proper receptor localization and aberrant activation of signaling downstream of receptors (Figure 4.2).
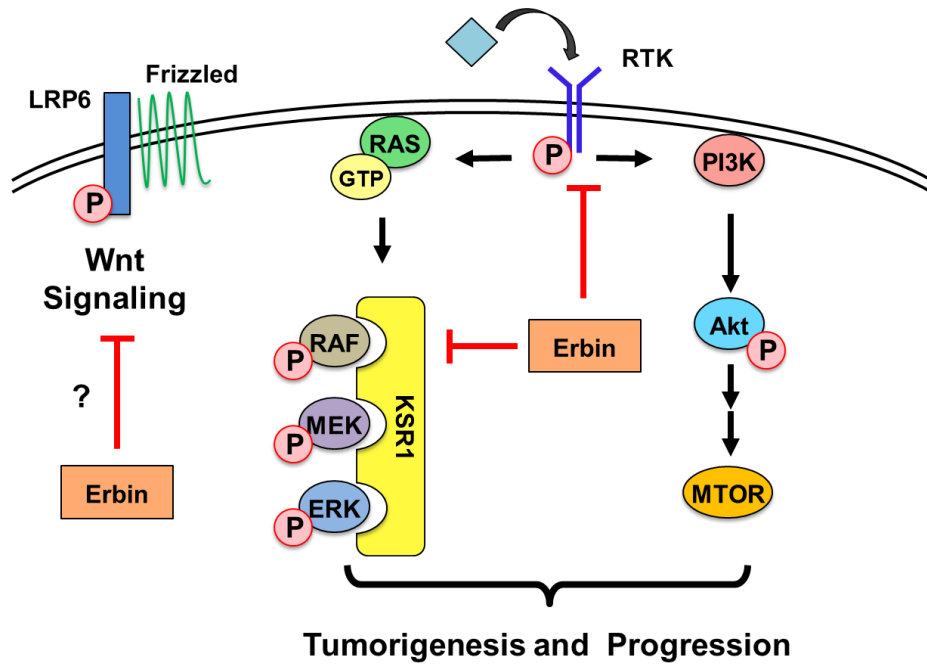
Figure 4.1 Erbin is a negative signaling scaffold protein in multiple pathways

While Erbin was previously known to interact with the ErbB2 receptor, our data have shown that Erbin can also inhibit the RAS/RAF pathway through inhibition of KSR1 facilitated signaling. Additionally, Erbin was shown to reduce transcription levels of Wnt target genes. Further studies will be necessary to determine exactly where in the Wnt signaling pathway Erbin is contributing to this regulation.
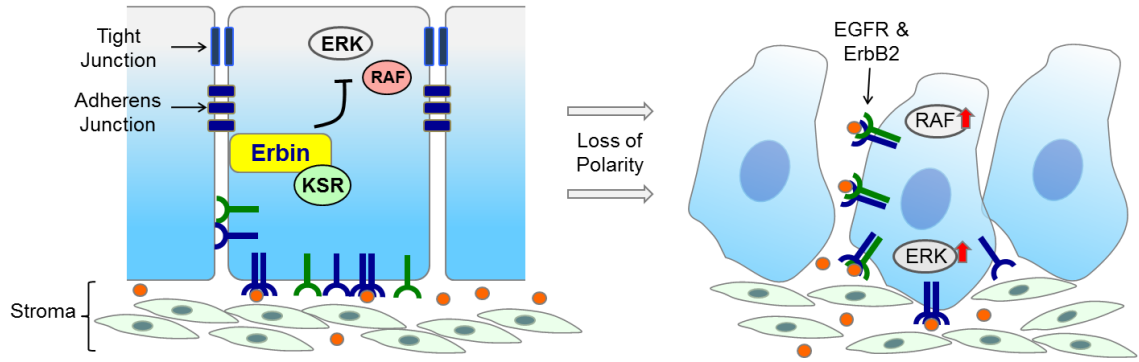
Figure 4.2 Loss of polarity induces malignant transformation of epithelial cells

Once polarized, two adjacent intestine epithelial cells are connected by the tight and adherens junctions which separate the membrane into apical and basolateral domains. Erbin, ErbB2, and EGFR are basolateral membrane associated proteins. In polarized cells, only the receptors localized on the basolateral membrane are in contact with ligands in the microenvironment of stroma. When polarity is lost, cells undergo EMT and EGFR/ErbB2 receptors can become mislocalized and hyperactivated.

In our study, we find that Erbin-loss alters the time course of EGFR phosphorylation, and receptor trafficking and degradation. Our results in Figure 3.24 show that while there are reduced total levels of EGFR/ErbB2/ErbB3 in whole cell lysates there are also alterations in EGFR phosphorylation that could result changes to EGFR/adapter protein interactions. Additionally, in Figure 3.26, we show that loss of Erbin in SW480 cells results in more EGF ligand binding at the cell surface. The results that total EGFR levels may be lower in CRC cells, while cell surface EGFR levels are higher and more sustained after EGF stimulation, suggests that Erbin may play a role in recruiting certain adapter proteins to the receptors and effecting receptor trafficking or sorting after endocytosis. However, knowing that the MEF cells had a very different response to the loss of Erbin (increased EGFR protein and phosphorylation levels) could lead us to conclude that the role of Erbin at the plasma membrane may depend upon the presence of a cellular junction or other interacting partners. These conflicting results, and possibility that other proteins are involved in the regulation, only make the role of Erbin in controlling RTK signaling more fascinating and more studies will be necessary to conclude if this process is involved in tumor progression and EMT.

Intriguingly, knockout of Erbin in Apc mutant mice only resulted in an increase in tumorigenesis and are not sufficient to induce EMT phenotype as the expression of E-cadherin remains unchanged in Apc/KO tumor tissues and organoids compared to Apc/WT (as seen by qPCR, data not shown). Additionally, as further evidence that Erbin KO is insufficient to induce an EMT phenotype, loss of Erbin in Apc tumor organoids does not result in a loss of polarity in 3D culture (Figure 3.17 A). Thus, additional oncogenic alterations, such as PIK3CA and KRAS mutations presented in human colon cancer cells,

are likely needed to fully engage the EMT program in Erbin deficient tumor cells. Studies using CRC cell lines with mutant KRAS, such as DLD1, along with DLD1 cells in which WT KRAS has been reintroduced would be useful to determine if mutational status could affect interactions between Erbin and its pro-signaling interacting partners, resulting in different signaling events.

While the protein interactions and mechanistic roles for Erbin are relatively consistent, the role of Erbin in cancer has been controversial. A number of studies have shown that Erbin negatively regulates Akt and ERK signaling, as well as cell proliferation and survival in different types of cancer cells [7, 40], thus acting as a tumor suppressor. However, other studies have indicated that Erbin-loss increases tumorigenesis [34, 41], resulting in the conflict within the field. Results from our study have provided several lines of evidence supporting the tumor suppressor function of Erbin in CRC: i) analysis of human CRC gene expression datasets with large sample sizes indicates that Erbin mRNA expression is significantly downregulated in CRC patients; ii) Erbin protein expression is decreased in CRC patient specimens by Western blot and IHC analyses; iii) knockdown of Erbin disrupts epithelial polarity and promotes EMT to increase cell motility; iv) knockout of Erbin in Apc mutant mice promotes tumor progression and significantly reduces survival; and v) tumor organoids derived from Erbin KO mice have increased cancer-stem cell properties and Wnt signaling. Our findings are also corroborated by the GSEA analysis in which Erbin expression is found to be associated with increased E-cadherin junctions and decreased Wnt signaling. Interestingly, we find that knockout of Erbin further increases the expression of Wnt target genes in Apc mutant tumors, which already have increased levels of Wnt signaling when compared to normal epithelia. While loss of

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polarity in epithelial cells often results in decreased E-cadherin levels (as we observed *in vitro*), which could in turn result in less β-catenin sequestered to cell junctions and increased Wnt signaling; we observed increased Wnt signaling in Apc/Erbin KO organoids which maintain E-cadherin levels and an epithelial cell structure. Therefore we suspect that the increase in Wnt signaling after Erbin loss is not related to EMT. It has been shown recently that oncogenic KRAS promotes Wnt signaling through ERK-mediated phosphorylation of LRP6 [42]. Future studies are needed to determine if activation of ERK is required to mediate Erbin-dependent regulation of Wnt signaling in CRC. Alternatively, a straight forward study of the E-cadherin/Erbin/β-catenin complex could be performed to determine if Erbin is required for the formation or stability of this interaction. Using the Apc/KO organoids as an example, in an *in vivo* model it may be possible to maintain relatively normal levels E-cadherin after loss of Erbin, but it may be possible to simultaneously decrease interactions between β-catenin and E-cadherin if Erbin is responsible for facilitating that protein-protein interaction. Decreased β-catenin and E-cadherin interactions would result in an increase in transcription of Wnt target genes, as well as an increase in "stemness" within the cell.

In summary, our study has uncovered a pivotal role of Erbin in maintaining epithelial cell polarity and suppressing EMT in CRC. In addition to regulating polarity, Erbin disrupts the pro-signaling scaffold protein KSR1 and effectively reduces ERK signaling, which we have linked to increased cell motility in CRC. By developing novel *in vivo* mouse models and tumor organoid systems, we demonstrate that Erbin exerts its tumor suppressor function by negatively regulating both the Akt and RAF/MEK/ERK signaling pathways and that Erbin-loss can promote Wnt signaling and tumor initiation. Here, the

identification of the functional interplay between Erbin-KSR1 highlights the importance of

scaffolding proteins in providing the spatiotemporal control of cell signals.

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

The characterization of Erbin loss in CRC during my dissertation research, including the phenotypic, mechanistic, and *in vivo* data, answered several questions about the tumor suppressor role of Erbin. However, these data have also raised several more important mechanistic questions.

One of the most pertinent questions raised is, how does loss of Erbin increase signaling in the Wnt pathway *in vivo*? It will be important to study this phenomenon and I believe that the use of the tumor organoid system that we developed will provide an excellent method to examine this. Future studies would involve inhibiting either the ERK or Akt pathways in the Apc/WT and Apc/KO organoids, then observing expression levels of Wnt target genes via qPCR. If either of the Erbin regulated pathways are responsible for the observed increase in Wnt signaling, inhibition of that particular pathway would decrease Wnt target gene expression levels. Conversely, if neither of the signaling pathways are implicated in signaling cross-talk with the Wnt pathway, Erbin may alter protein localization within the Wnt pathway itself. As discussed, Erbin could affect the E-cadherin/β-catenin interaction, or it may be possible that Erbin interacts with other Wnt-pathway proteins at the plasma membrane. We have observed that Erbin can potentially alter EGFR trafficking and endocytosis. Similarly, when Wnt interacts with its receptor, Frizzled, at the membrane, the entire Wnt signaling complex is internalized. It may be plausible that Erbin is generally involved in the recruitment of proteins necessary for receptor endocytosis and the quenching of signaling.

Another question that has been raised during the production of these data is, how is Akt activated in CRC after Erbin loss? As noted, this effect seems to be cell type dependent

since not all studies observe increases in Akt phosphorylation when Erbin levels are reduced. However, it has been shown in ErbB2 over-expressing NIH3T3 cells that ErbB2 receptor signaling to the PI3K/Akt pathway requires the involvement of the α6-β4 integrin [161, 162]. It may be possible that the differential effect of Erbin on the Akt pathway could be explained by varying levels of integrin expression between cell types. Exploring this possibility could be accomplished through immunoprecipitations of the ErbB2/Integrin interactions, with and without Erbin. Alternatively, to determine if Erbin interacts with the integrin in CRC, co-immunoprecipitation of Erbin with over-expressed α6-β4 integrin with and without the long cytoplasmic tail of β4, while simultaneously monitoring Akt phosphorylation, may elucidate a mechanism of action. Lastly, monitoring Akt phosphorylation and transiently knocking down α6-β4 integrin in sh-Erbin CRC cell lines could also be explored. If the integrin is involved in the necessary signal transduction to the Akt pathway, increases in Akt activation would be lost after the co-knockdown. Additionally, outside of Akt regulation, Erbin could affect cell motility through interactions with the α6-β4 integrin, either by altering protein stability or localization.

Although not addressed in my studies, another potential mechanism that may contribute to increased tumorigenesis in Erbin knockout mice is that Erbin has been shown to play a role in regulating inflammation. It is well known that chronic inflammation can lead to increased risk for CRC and it has been suggested that Erbin loss of function can cause chronic inflammation [105, 108]. Paired with my observations that Erbin loss results in increased *in vivo* tumorigenesis, it would be interesting to determine if increased inflammation in the Apc/KO mouse model was partially responsible for tumor initiation. The observed increase in spleen size of the Erbin KO mice may support this idea.

Future studies are necessary to determine a more comprehensive understanding of Erbin's role in receptor trafficking in CRC. Signaling molecules, such as EGFR, ErbB2, Shoc2, and KSR1, are localized in close proximity upon receptor activation. By impinging on signal transduction at multiple levels, Erbin could function as an effective negative regulator of the entire oncogenic signaling cascade. Given the changes in EGFR protein expression and phosphorylation in Erbin knockdown CRC cells, studying the interactions between EGFR or ErbB2 receptors and their adapter proteins may provide a better understanding for how Erbin is altering several downstream signaling pathways at the receptor level. Use of cell surface biotinylation and immunoprecipitation of receptors, with and without Erbin, could be used to understand these processes and also whether Erbin could alter trafficking of other RTKs. Additionally, examining the phosphorylation of juxtamembrane T669 of EGFR, which has been shown to be phosphorylated by ERK and implicated in inducing EGFR recycling, in Erbin knockdown cells may provide additional information on whether the recycling of EGFR is altered by Erbin-loss.

Finally, since many of the pathways that Erbin interacts with are mutated in CRC development and progression, a more thorough understanding of how Erbin interacts with these mutant proteins is needed. Erbin loss may alter the protein interaction networks and possibly how oncogenic proteins such as KRAS interact with downstream partners. Observations of increased BRAF protein levels after Erbin knockdown in several CRC cell lines may suggest that the loss of this scaffold protein could alter protein stability due to changes in protein-protein interactions. With this in mind, many of the proteins within the Erbin signaling network are currently targeted with cancer therapeutics. Understanding the

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protein interactions and consequences of Erbin loss may allow us to explore the potential

of using Erbin as a diagnostic marker for developing personalized treatment strategies.

APPENDICES

Appendix: List of Acronyms and Abbreviations

18q	Long arm of 18th chromosome
2D	Traditional 2-dimensional cell culture
3D	3-dimensional cell culture
AJ	Adherens Junction
Akt	Protein Kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli
ARVCF	Armadillo repeat protein deleted in velo-cardio-facial syndrome
BSA	Bovine Serum Albumin
CFP	Cyan Fluorescent Protein
CIN	Chromosomal Instability pathway
CRC	Colorectal Cancer
DCC	Deleted in Colon Cancer
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DSG1	Desmoglein-1
eBPAG1	Bullous pemphigoid antigen 1
ECL	Enhanced chemiluminescence
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor (ErbB1)
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular signal-regulated kinase
FAP	Familial adenomatous polyposis
FBS	Fetal Bovine Serum
Flag	Synthetic peptide tag – peptide sequence: DYKDDDDK
G1	Gap 1 phase of cell cycle
G2	Gap 2 phase of cell cycle
GDC0941	Pictilsib, Akt inhibitor
Grb2	Growth factor receptor-bound protein 2
GSEA	Gene Set Enrichment Analysis
GTP	Guanosine triphosphate
H&E	Hematoxylin and eosin stain
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HSP90	Heat shock protein 90
IgE	Immunoglobulin E
IHC	Immunohistochemical Staining
kb	Kilobase
kDa	Kilodalton
KO	Knockout of a gene

KSR1	Kinase suppressor of Ras1
LAP	LRR and PDZ containing protein
Lef	Lymphoid enhancer factor
LGR-4/5	Leucine rich repeat containing G protein-coupled receptor 4 or 5
LKB1	Liver kinase B1
LOH	Loss of Heterozygosity
LPS	Lipopolysaccharides
LRP6	Low-density lipoprotein receptor-related protein 6
LRR	Leucine Rich Repeats
M	Mitotic phase of cell cycle
MAPK	Mitogen-activated protein kinases
MEF	Mouse Embryonic Fibroblast cells
MH2	MAD homology 2
MK2206	Akt Inhibitor
MMR	Mismatch repair
mRNA	Messenger RNA
MSI	Microsatellite Instability
Myc	Synthetic peptide tag – peptide sequence: EQKLISEEDL
NFκB	Nuclear factor κB
NMR	Nuclear magnetic resonance
Nod2	Nucleotide-binding oligomerization domain-containing protein 2
p21	Cyclin-dependent kinase inhibitor 1
p27	Cyclin-dependent kinase inhibitor 1B
PAS	Periodic acid-Schiff
PBS	Phosphate Buffered Saline
PD184352	MEK1/2 inhibitor
PD98059	MEK1 inhibitor
PEI	Polyethylenimine
PICK1	Protein interacting with C kinase - 1
PDZ	PSD94/Dlg1/ZO-1 domain
PTB	Phosphotyrosine-binding domain
qPCR	Reverse transcriptase quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNA-seq	RNA sequencing for quantity of RNA in a biological sample
RTK	Receptor Tyrosine Kinase
S	Synthesis phase of cell cycle
SARA	Smad anchor for receptor activation
SEM	Standard Error of the Mean
SH2	Src homology 2 domain
SH3	Src homology 3 domain
SHC	Src homology and collagen
ShRNA	Short hairpin RNA
SOS	Son of Sevenless
SPPK	Striate palmoplantar keratoderma
Stat3	Signal transducer and activator of transcription 3

Tcf	T-cell Factor
TCGA	The Cancer Genome Atlas
TGFβ	Transforming growth factor beta
TMA	Tissue microarray
TNFα	Tumor necrosis factor alpha
TP53	Gene for Tumor protein p53
U0126	MEK1/2 inhibitor
Vil-Cre	Cre recombinase driven by the villin 1 promoter
WT	Wildtype
ZO-1	Zonula occludens-1

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Publications:

Stevens PD, Wen YA, Xiong X, Zayseva YY, Li A, Wang C, Farmer T, Inagaki M, Marchetto S, Borg JP, Gao T. Erbin suppresses colorectal cancer progression by antagonizing KSR1-mediated RAS/RAF signaling. Submitted to Cancer Research. November 2017.

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Oral Presentations:

May, 2017   "Erbin Inhibits Colon Cancer Progression by Antagonizing KSR1-
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              Selected Student Speaker
              Markey Cancer Center Annual Research Day, University of Kentucky

Dec, 2016   "My Path to a Research Career"

Selected Speaker
Biomedical Career Seminar, Markey Cancer Center Career Training in
Oncology Program, University of Kentucky

Oct, 2016   "The Role of Erin in Colon Cancer"
Student Seminar Presentation
Department of Molecular and Cellular Biochemistry, University of
Kentucky

May, 2016   "The Tumor Suppressor Function of Erbin in Colon Cancer"
Selected Student Speaker
Department of Molecular and Cellular Biochemistry, Annual Retreat,
University of Kentucky

Apr, 2016   "High-Fat Diet Induced PPAR-delta Activity Increases Stemness"
Student Seminar Presentation
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Apr, 2015   "Erbin-mediated Regulation of Colon Cancer"
Student Seminar Presentation
Department of Molecular and Cellular Biochemistry, University of
Kentucky