

# Mechanisms of Ect2 Regulation in Cytokinesis and Oncogenesis

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## **Abstract**

### **DANIELLE R. COOK: Mechanisms of Ect2 Regulation in Cytokinesis and Oncogenesis (Under the direction of Channing Der)**

Ect2 is a member of the human Dbl family of guanine nucleotide exchange factors and are activators of Rho family small GTPases (RhoA, Rac1 and Cdc42). Ect2 was identified originally as an oncogene. However, the truncated and activated Ect2 variant identified was due to a DNA manipulation artifact and to date has not been found in human cancers. Ect2 has also been shown to be essential for mammalian cell cytokinesis in established cell culture models. My studies involve further evaluation of Ect2 in normal and neoplastic cell biology. First, to further evaluate a role for Ect2 in cytokinesis, we evaluated the consequences of an Ect2 deficiency in normal cells. Our studies showed that an Ect2 deficiency in primary mouse embryo fibroblasts (MEFs) caused a defect in cytokinesis, migration, and altered cell morphology in vitro. In vivo loss of Ect2 in a mouse caused embryonic lethality. Thus, Ect2 is essential for normal development; a characteristic that has only been found in two other Dbl family RhoGEFs of 25 members evaluated. Second, in a gene array analysis of colorectal cancer (CRC), we identified *ECT2* transcriptional overexpression and further analysis of oncomine database also confirmed the transcriptional overexpression of Ect but was not due gene amplification. We then found an

increase in Ect2 protein expression in primary and metastatic CRC tumors and cell lines. Depletion of endogenous Ect2 by shRNA in CRC cell lines caused a reduction in anchorage-independent growth and Matrigel invasion without a corresponding defect in cytokinesis. Analyses of Ect2 protein expression in a CRC tumor microarray (N=149) found Ect2 protein overexpression in tumor tissue, but surprisingly, the ratio of cytoplasmic to nuclear Ect2 correlated with improved patient survival. This finding contrasted with earlier studies that suggested that the mislocalization of the normally nuclear restricted Ect2 to the cytoplasm was important for Ect2 to function as an oncogene.

With this unexpected finding, I then utilizing my validated MEF and CRC cell line models to assess the structural and functional requirements for Ect2 support of normal cell cytokinesis and cancer cell growth. Ect2 is a large multidomain protein. In addition to tandem Dbl homology RhoGEF catalytic and pleckstrin homology regulatory domains found in all Dbl RhoGEFs (>70 human members), Ect2 contains N-terminal tandem BRCT domains not found in any other RhoGEFs and two nuclear localization sequences that promote Ect2 nuclear localization. Using missense mutants of full length Ect2 with impaired domain/motif function, I found that the BRCT domains are dispensable for Ect2-dependent CRC growth but necessary for normal cell cytokinesis. In contrast, disruption of the Ect2 nuclear localization sequences abolished the ability of Ect2 to support both normal cell cytokinesis and CRC tumor cell growth. The former observation was unexpected since there is no nuclear envelope during cytokinesis. The latter finding was also surprising, contrasting with previous

findings in lung cancer, but consistent with our findings in CRC tumor tissue microarray. In particular, we found that the cytoplasmic localized, constitutively activated Ect2 that potently causes growth transformation of NIH 3T3 mouse fibroblasts was deleterious for CRC growth. In summary, my studies may resolve a paradox in the field, to explain why the originally truncated and activated Ect2 identified in mouse fibroblasts have not been found in human cancer.

## **Dedication**

I dedicate this work to my family for always supporting and loving me. My family gives me resolution to pursue my dreams and for that, I am forever grateful.

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and I feel lucky to have had a supportive division. Each of my committee members also played vital roles during my transition by meeting with me and giving me advice. Thank you Dr. Deshmukh, Dr. Hanh, Dr. Graves, and Dr. Jarstfer! I am forever grateful for everyone who helped me during that transition and I am happy to say that it all worked out for the best.

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## Abbreviations

APC- Adenomatous polyposis coli or Anaphase promoting complex

BCS- Bovine calf serum

BRCA- Breast-cancer associated protein

BRCT- BRCA1 C-terminal

BSA-Bovine serum albumin

CRC- Colorectal Cancer

D-box-Destruction box (found in most APC substrates)

Dbl- Diffuse B-cell lymphoma

DH- Dbl homology

DMEM- Dulbecco's modified eagle's medium

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

DOCK- Dedicator of cytokinesis

Ect2- Epithelial cell transforming sequence 2

FBS- Fetal bovine serum

FL- Full-length

FRET- Fluorescent or Förster resonance energy transfer

GAP-GTPase activating proteins

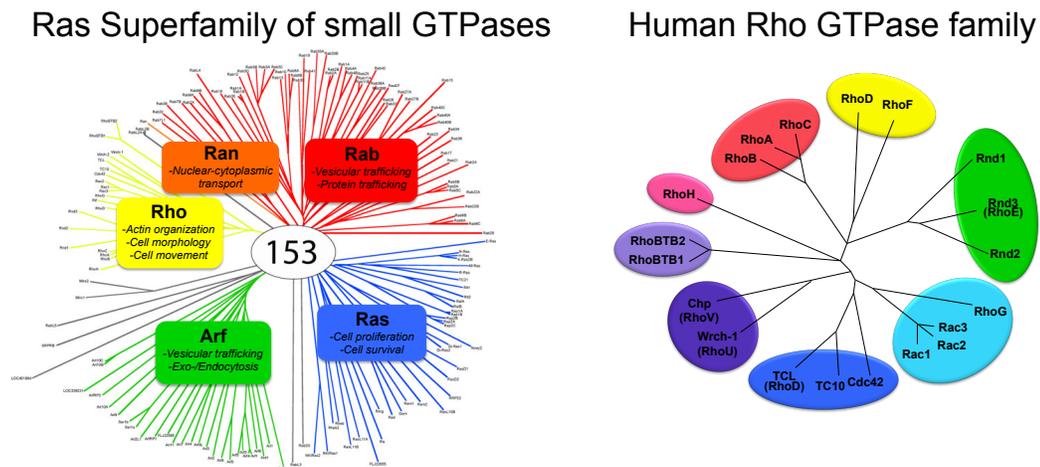
GDI- Guanine nucleotide-dissociation inhibitors

GDP-Guanine di-phosphate  
GEF-Guanine exchange factors  
GFP- Green fluorescent protein  
GTP- Guanine tri-phosphate  
GTPases- Guanosine triphosphatases  
HA- Hemagglutinin  
KDa- Kilodalton  
MEF- Mouse embryonic fibroblasts  
MTT- 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide  
NLS- Nuclear localization sequence  
OD- Optical density  
PBS- Phosphate buffered saline  
PH- Pleckstrin homology  
Ras- Rat Sarcoma  
Rho- Ras-homologous  
RNA- Ribonucleic acid  
RNAi- RNA interference  
SDS- Sodium dodecyl sulfate  
shRNA- Short hairpin RNA  
TBST-Tris buffered saline with tween 20  
WT- Wild type

## Chapter 1 Introduction

### Superfamily of Ras GTPases

The RAS gene was originally identified in two different cancer causing virus originally discovered in rat, hence the name Ras (rat sarcoma) [1] and is the founding member of the Ras Superfamily of small GTPases containing over 150 members [2, 3]. Members of this superfamily are divided into five major branches based on sequence and functional similarities: Ras, Rho, Rab, Ran, and Arf. (Figure 1-1) However, variations in structure, post-translational modifications, subcellular locations, and effectors proteins allow GTPases to function as modulators of complex and diverse range of cellular processes [4].

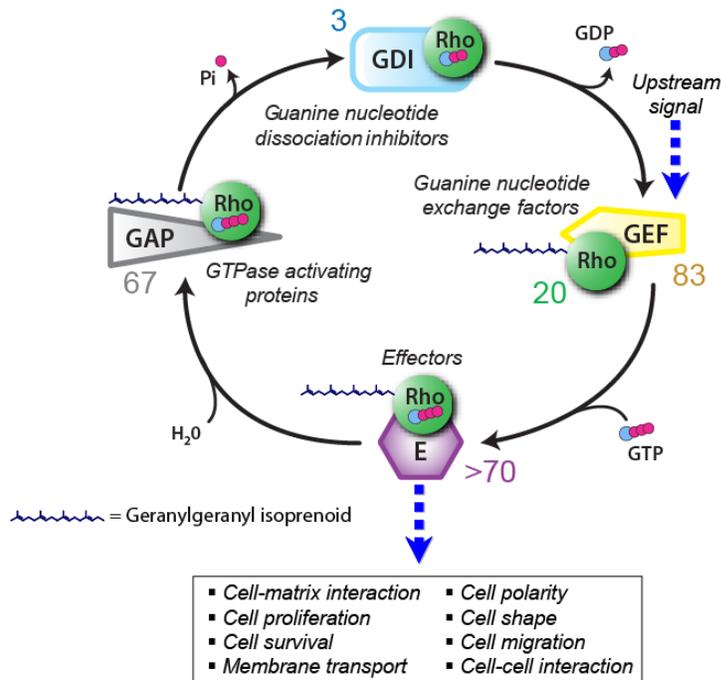


**Figure 1-1 Small GTPase**

Left panel: Ras Superfamily Right panel: Human Rho GTPases (20 members; RhoA, Rac1, and Cdc42 are characterized the best)

## ***Rho GTPases***

Rho family proteins (20 human members) comprise a major branch of the Ras superfamily of small GTPases, with RhoA, Rac1 and Cdc42 the most extensively studied and characterized. (Figure 1-1) Rho GTPases specifically regulate actin organization, cell motility, polarity, growth, survival and gene transcription [5]. Rho GTPases are binary switches that cycle between an active GTP-bound and an inactive GDP-bound state [6]. Rho-specific guanine nucleotide exchange factors (RhoGEFs) accelerate the intrinsic exchange activity of Rho GTPases to stimulate formation of Rho-GTP. Rho-specific GTPase activating proteins (RhoGAPs) stimulate the intrinsic hydrolysis activity of Rho GTPases, resulting in the inactive GDP-bound state [7]. Rho-specific guanine nucleotide-dissociation inhibitors (GDIs) comprise a third class of regulatory proteins. RhoGDIs bind Rho GTPases in a GDP bound state and also extract and sequester Rho GTPases from the cell membrane where Rho GTPases can be activated [8]. (Figure 1-2)



**Figure 1-2 Rho GTPase Signaling [9]**

***The discovery of RhoGEFs: diverse in numbers and structure***

The first RhoGEF was identified initially as an oncogene in mammalian cells. Using the NIH 3T3 mouse fibroblast focus formation assay that led to the discovery of mutant Ras in human cancer, analysis of genomic DNA isolated from a human diffuse B-cell lymphoma resulted in the discovery of the *DBL* oncogene that encoded an N-terminally truncated protein [10]. Dbl was subsequently shown to catalyze the exchange activity of Cdc42 [11]. Additional NIH 3T3 focus formation and related assays identified Dbl-related proteins, in particular Vav and Ect2. That RhoGEFs were discovered initially as oncoproteins provided the first suggestion that Rho GTPases may also have a function in oncogenesis.

Dbl and the Dbl-related proteins all share a ~200 amino acid catalytic Dbl homology (DH; also called RhoGEF) and an immediately adjacent regulatory ~100 amino acid pleckstrin homology (PH) domain [12]. Additional RhoGEFs with this tandem DH-PH domain structure were identified by genetic and biochemical approaches and by in silico database searches. There are 72 human Dbl family RhoGEFs, with conserved orthologs found in all vertebrate species and in invertebrates, including *Drosophila*, *C. elegans*, *S. cerevisiae* and *S. pombe*. Beyond their shared DH-PH domains, Dbl RhoGEFs diverge significantly in their flanking N- and C-terminal sequences, which commonly contain a diversity protein-protein interaction domains and motifs involved in regulating intrinsic RhoGEF catalytic activity, determining subcellular localization or facilitating complex formation with other proteins. (Figure 1-3)

Humans also possess a second structurally distinct but biochemically similar class of RhoGEFs, the dedicator of cytokinesis or DOCK family (11 human members) [13]. DOCK RhoGEFs are characterized by conserved Dock-homology region-2 (DHR2) catalytic domain with no sequence homology to DH domains. Although plant species possess Rho-like GTPases (Rho of plants; Rop) [14], and are regulated by RhoGAPs homologous to mammalian RhoGAPs [15], they lack either Dbl or DOCK RhoGEFs and instead RopGEFs possess a structurally distinct plant-specific Rop nucleotide exchanger (PRONE) catalytic domain [16]. Finally, although there are no Rho orthologs in prokaryotes, pathogenic bacteria possess effector proteins that regulate the function of their

mammalian host Rho GTPases, in particular, proteins that mimic the function of mammalian RhoGEFs [17, 18].

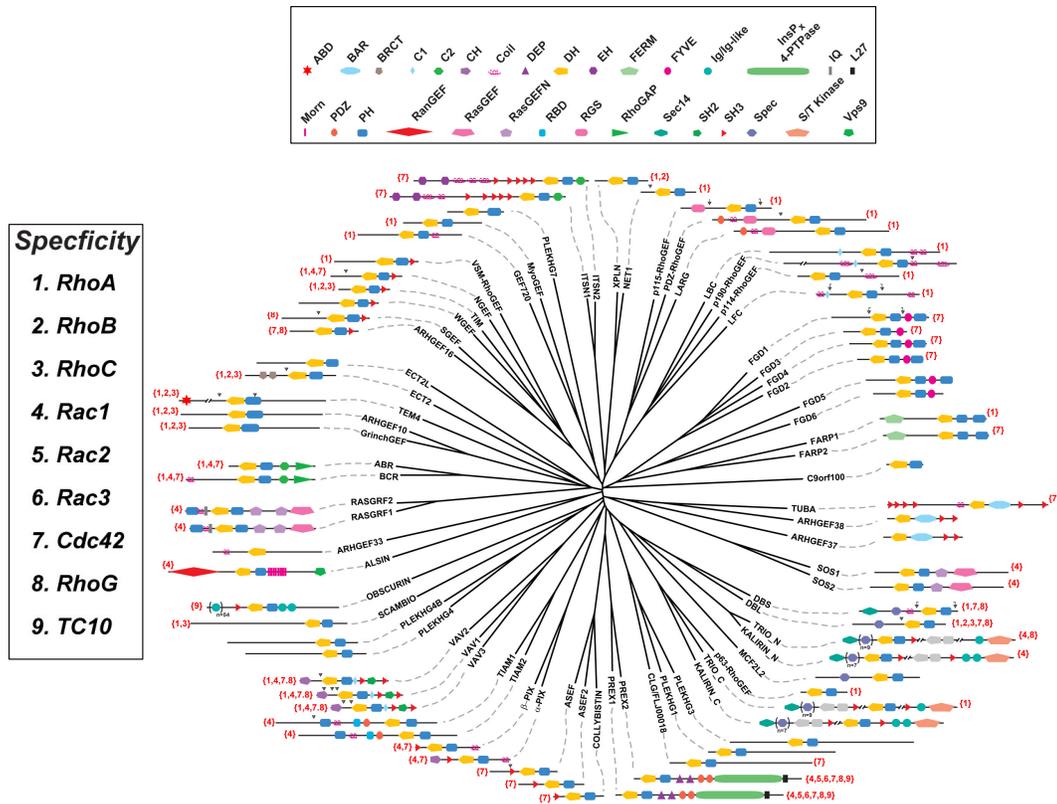
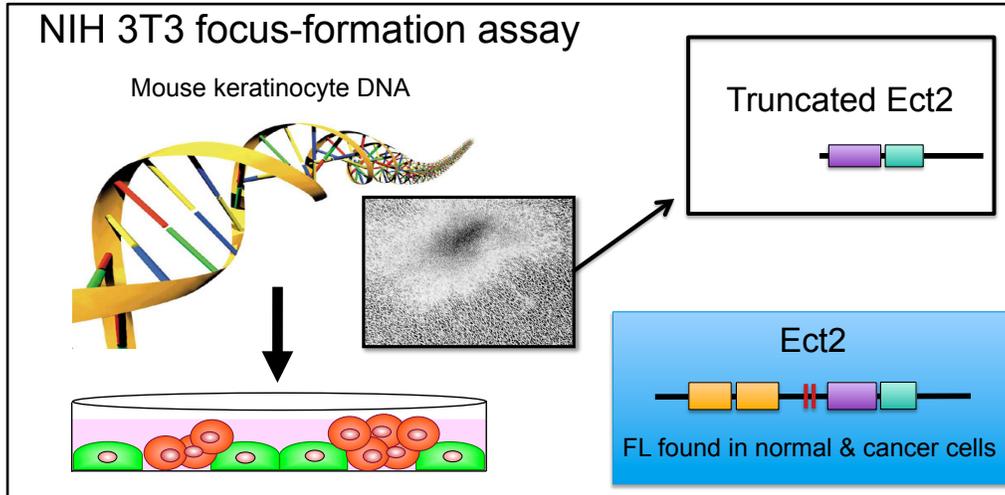


Figure 1-3 Dbl family RhoGEFs

**Ect2: Epithelial Cell Transforming Sequence 2**

In 1993, human Ect2 was originally discovered as an oncogene that transformed NIH 3T3 cells [19]. However, the Ect2 protein found to transform fibroblast was a truncated version of the full-length protein that is not found in nature. (Figure 1-4) The best-characterized function of Ect2 is for its role in normal cell cytokinesis, however there are studies supporting a non cell-cycle function in cancer.

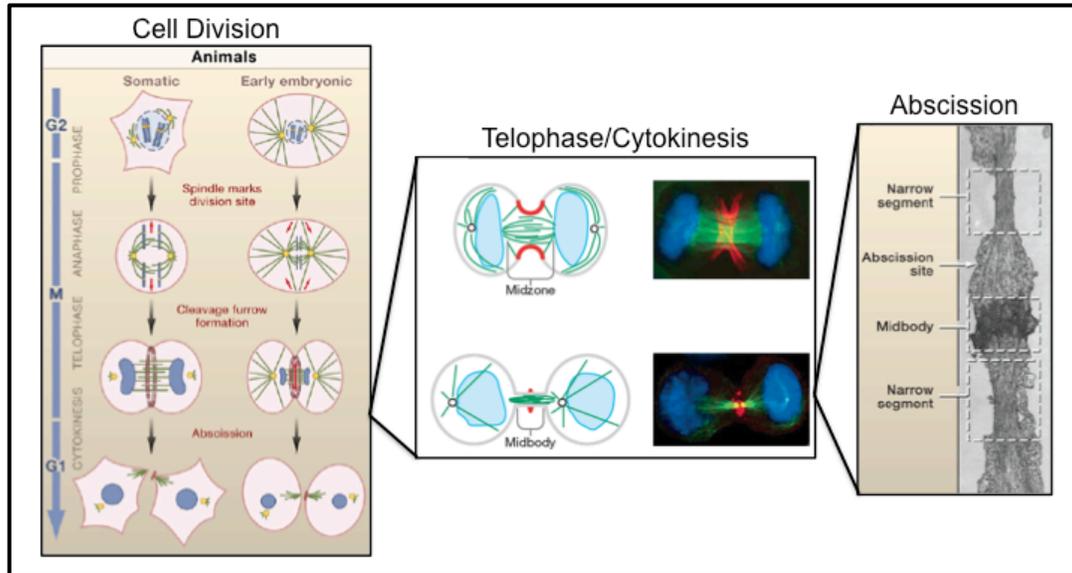


**Figure 1-4 Discovery of Ect2 in NIH 3T3**

The transforming Ect2 was truncated and constitutively activated protein not found in nature or cancer [19]

### ***Ect2 in Cytokinesis***

Cytokinesis is the physical division of one cell into two and is the final step in mitosis or meiosis, a cell division process that is tightly regulated by many proteins and is essential in proliferation or development respectively. (Figure 1-5) Cytokinesis requires coordinated actions of the cytoskeleton, membrane systems, and other cell cycle machinery, which are precisely controlled spatially and temporally. During late anaphase the actomyosin ring is formed and then starts to ingress during telophase giving rise to the cleavage furrow and by late telophase the cleavage furrow is fully ingressed forming an intracellular bridge, called the midbody [20]. SNARE-dependent membrane fusion steps promote the abscission event needed to complete cytokinesis [21]. Cytoskeleton rearrangements are required during the formation of the actomyosin ring and then ingression until abscission is complete is dependent on Rho GTPase localization and activity [22-26].



**Figure 1-5 Cytokinesis**[20, 21, 23]

Before the human Ect2 was characterized, mutations in Pebble (Pbl), the *Drosophila* ortholog of Ect2, caused a block in cytokinesis [27, 28]. After Ect2 was discovered, functional studies of Pbl in *Drosophila* established a critical role in cytokinesis. At the onset of cytokinesis, Pbl is associated with the cleavage furrow where it activates Rho1 to initiate assembly of the contractile ring and promote cytokinesis [29]. Similarly, Let-21 the *C. elegans* ortholog of Ect2 is required for formation of the cleavage furrow [30]. Further evidence of Ect2 function in cytokinesis was demonstrated by the interference of mammalian Ect2 function through the expression of a dominant negative ECT2 allele, microinjection of an Ect2 antibody, or Ect2 RNAi-mediated knockdown all lead to accumulation of multinucleated cells indicative of a failure to undergo cytokinesis [31, 32]. Furthermore, studies have shown that the role of Ect2 in cytokinesis is to activate RhoA signaling pathways as well as recruit other proteins such as

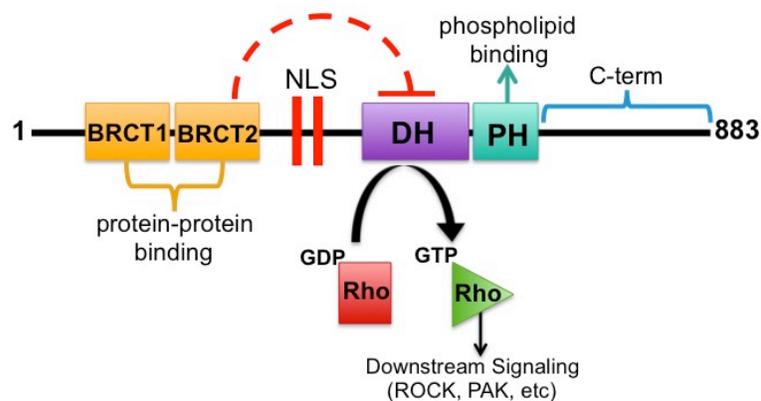
RacGAP1; knockdown of either RhoA or RacGAP lead to multinucleation [23, 33, 34].

### ***Ect2 Domain Structure***

Ect2 is multi-domain protein with unique features not found in other RhoGEFs. (Figure 1-6) For example, the N-terminus of Ect2 contains a pair of tandem BRCA1 C-terminal (BRCT) repeats. BRCT domains are found in a large superfamily of ~40 orthologous proteins that have functions in cell cycle checkpoints and/or in the DNA damage response [35]. BRCT domains can promote heterodimerization with other BRCT-containing proteins or recognize phosphorylated peptides. There is evidence suggesting that the second but not the first BRCT domain of Ect2 may function as auto-inhibitor of the DH domain [31, 36]. These data support a model where protein binding or modification of Ect2 relieves BRCT auto-inhibition, leading to reversible activation of Ect2 RhoGEF function. There is evidence that BRCT domains found in tandem can cooperate to provide sequence-specific binding of phosphorylated peptides [37]. For example, the tandem BRCTs domains have a positive role to promote Ect2 localization to the midbody during cytokinesis: the BRCT domains bind to Plk1-phosphorylated MgcRacGAP31 (RacGAP1), a protein that, like Ect2, is both located to the midbody during cytokinesis and required for its successful completion [26].

Another rare feature of Ect2 is that it contains two nuclear localization sequences (NLS) and is located in the cell's nucleus during interphase. Net1 is the only other RhoGEF found in the nucleus and was found recently that Net1 is

dispensable for mouse development (Jeffrey Frost, personal communication). The N-terminal truncated forms of Ect2 lacking the NLS motifs are able to transform NIH 3T3 as well as full-length Ect2 containing missense mutation that abolish NLS function, suggesting that mislocalization to the cytoplasm is important for Ect2 to drive growth transformation. Furthermore, cytoplasmic Ect2 has been linked to Rac1 activation and cellular transformation [38].



**Figure 1-6 Ect2 multi-domain RhoGEF**

The C-terminus of Ect2 comprises the DH-PH domains contained in all Dbl family RhoGEFs. While it is known that the DH domain is required for GEF activity the function of the PH domain remains to be investigated [12]. Furthermore, there remains considerable confusion regarding which Rho GTPases are targets of Ect2 and whether distinct Rho GTPases facilitate Ect2 control of cytokinesis versus growth transformation. Most studies have attributed Ect2 regulation of cytokinesis to its activation of RhoA [23, 24, 39-42], whereas transformation of lung tumor cells by full length (FL) Ect2 has been linked to Rac1 activation [38]. However, for transformation of NIH 3T3 mouse fibroblast

cells, all three Rho GTPases were necessary for transformation by an N-terminally truncated Ect2 ( $\Delta$ N-Ect2) [36, 43]. Usually RhoGEF substrate specificity is dictated solely by the DH domain [12], however the GTPase specificity of Vav, was influenced by its flanking sequences [44]. However, for Ect2 it is unknown if the DH domain alone or flanking sequences determine GTPases specificity.

Following the tandem DH-PH domains, the C-terminus is composed of highly conserved sequence of approximately 363 amino acids that lacks any known domains or motifs. (Figure 1-7) However, this C-terminal sequence of Ect2 was found to be required for  $\Delta$ N-Ect2 to cause growth transformation of NIH 3T3 mouse fibroblasts and was shown to alter the Rho GTPases activated in vivo [43]. Recently, TEK- and D-box (destruction box) domains have been described in the C-terminus and shown to be necessary for full length Ect2 degradation following mitosis by an ubiquitin ligase, APC/Cdh1 (anaphase promoting complex) [45, 46]. Destruction box (D-box) is a conserved motif, RXXL, found in a majority of APC substrates [47], while a TEK-box motif is found in ubiquitin and several human APC substrates; it was identified to play a role in substrate binding and poly-ubiquitination [48].

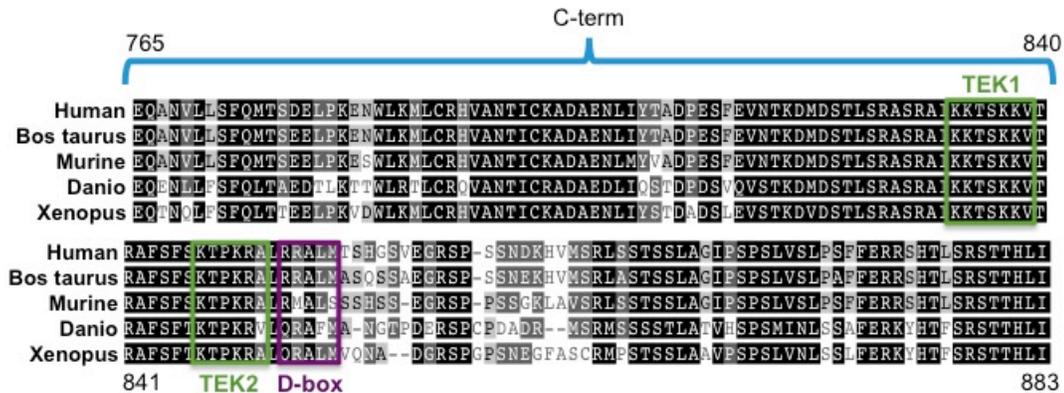


Figure 1-7 Ect2 C-terminus

### ***RhoGEFs and development***

The combined number of human Dbl and DOCK RhoGEFs greatly exceed the number of Rho GTPases, suggesting apparent redundancy in RhoGEF function. This is particularly striking for RhoA, where at least 25 Dbl RhoGEFs can activate this single GTPase. With six of 20 human Rho GTPases constitutively active and not believed to be regulated by RhoGEFs [49], the apparent redundancy in RhoGEFs is even more significant.

One approach to evaluate the apparent redundancy of RhoGEFs has been the generation of mice deficient in one or more RhoGEFs, which additionally addresses their involvement in development. The role of at least 24 Dbl family RhoGEFs in mouse development have been evaluated, with only four (Sos1, Trio, AKAP13 and Ect2) found to be essential for mouse development [50-53]. However, since these proteins possess other functions independent of their RhoGEF functions, it is not clear that the embryonic lethality seen is due to the loss of the RhoGEF function. Sos1 and Trio both have non-RhoGEF catalytic activities: Sos1 is also a RasGEF and Trio contains two distinct DH-PH RhoGEF

domains and a serine/threonine kinase domain. AKAP13 is a scaffolding protein with additional activities that include association with the regulatory subunit of protein kinase A to spatially regulate protein kinase A substrate utilization, protein kinase C and D isoforms, and interaction with heterotrimeric G alpha subunits. In contrast Ect2 has no other known catalytic function aside from its RhoGEF activity suggesting that Ect2 is an essential RhoGEF among a highly redundant family of proteins. In my studies, I determined that the requirement for Ect2 in development is likely due to its critical role in cytokinesis [53].

### ***Ect2 in development***

Recently we showed that *Ect2*-deficient mice are not viable [53]. Whereas heterozygous *Ect2*<sup>+/-</sup> mice showed normal development and lifespan, no *Ect2*<sup>-/-</sup> embryos were found at birth or as early as embryonic day 8.5, suggesting a requirement for Ect2 for either preimplantation or early postimplantation development. We further characterized the defect in vitro with isolated blastocysts at E3.5 and identified homozygous *Ect2*<sup>-/-</sup> blastocysts displayed abnormal outgrowth, indicating that Ect2 is required for peri-implantation development.

The best-characterized normal function of Ect2 is its role in cytokinesis. Even though there are at least 25 other RhoGEFs that can active RhoA, Ect2 has been shown to regulate RhoA during cytokinesis, the final step in meiosis and mitosis. Therefore, it is likely that Ect2 is required for development because of its key role in cell division. Consistent with this hypothesis, a deficiency in MgcRacGAP (RacGAP1), which facilitates Ect2 localization during cell division,

also produces a similar embryonic lethality phenotype [54].

### ***RhoGEFs and cancer***

The three Ras proteins are the founding members of the Ras superfamily of small GTPases and they comprise the most commonly mutated oncoproteins in cancer. However, with the exception of the recently described mutational activation of Rac1 in melanoma [55, 56], Rho (Ras homologs) GTPases are not frequently mutated in human cancer. Nevertheless, there is substantial experimental evidence that aberrant Rho GTPase function can contribute to cancer cell proliferation, invasion and metastasis [57]. Rather than direct mutational activation, Rho GTPases are instead activated by indirect mechanisms in cancer. These mechanisms include altered location by GDIs, increased RhoGEF and/or decreased RhoGAP activity, and altered gene expression of Rho GTPases, RhoGEFs, RhoGAPs, and RhoGDI expression. Other mechanisms include alternative gene splicing such as Rac1b [58].

Although Dbl and other RhoGEFs identified as oncoproteins were found to be activated by deletion of sequences, most commonly by deletion of sequences N-terminal to the DH domain, these altered and constitutively

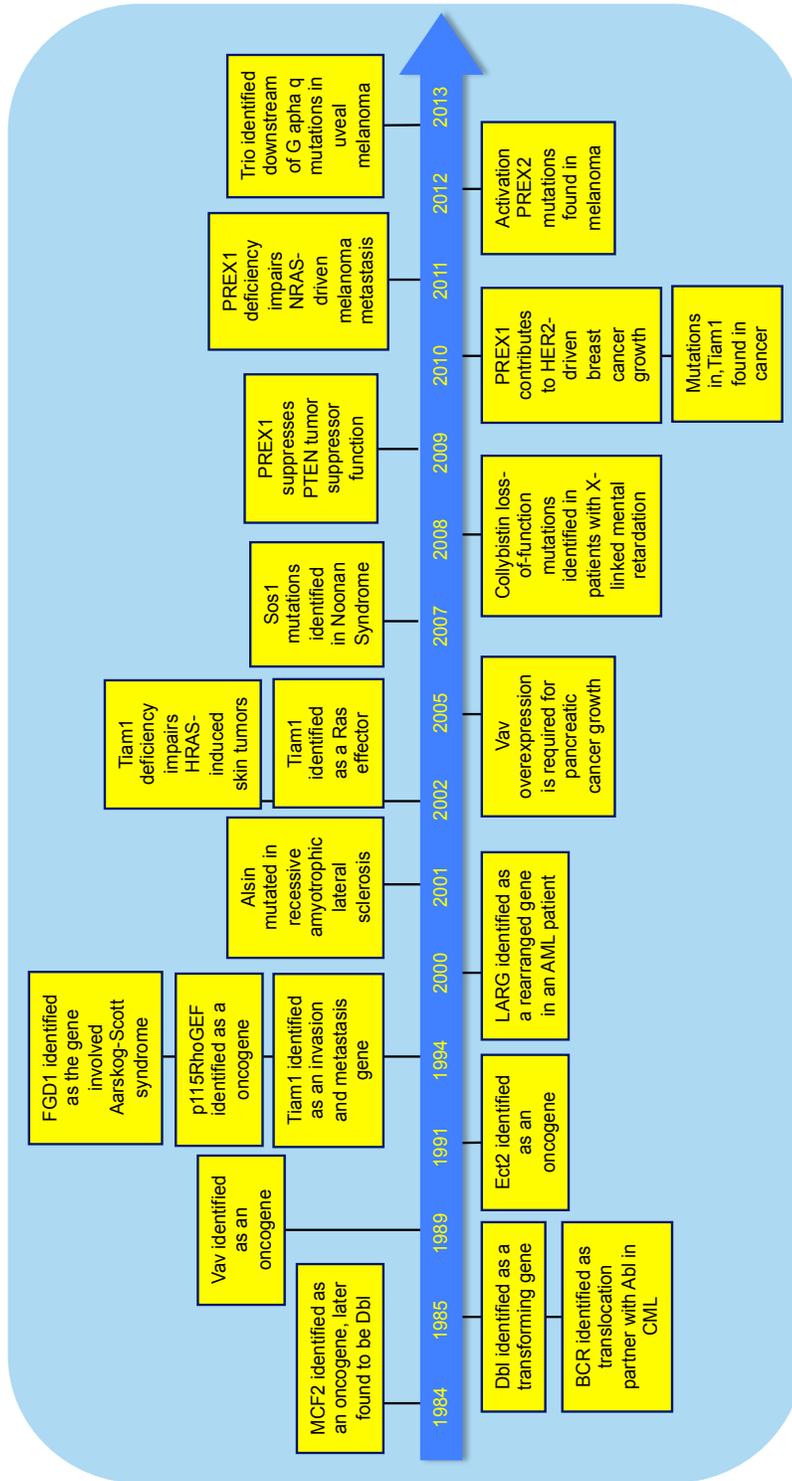


Figure 1-8 RhoGEF history

activated RhoGEFs were not present in the original tumor DNA and instead arose artifactually during the DNA isolation/transfection procedure [59]. Similarly, other Dbl RhoGEFs identified as oncogenes also possessed rearranged sequences, most commonly N-terminal deletion of sequences upstream of the DH domain, which arose during in vitro DNA manipulation/or the transfection process. Surprisingly, despite their potent transforming activities when assayed in NIH 3T3 mouse fibroblasts, such activated Dbl RhoGEFs have not been identified in cancers. Instead, mechanisms that lead to the deregulated expression and/or activation of full length RhoGEFs have been identified. In this section, we summarize the findings with the Dbl RhoGEFs with the strongest evidence for their involvement in cancer growth. (Figure 1-8)

### ***Ect2 in Cancer***

Ect2 gene and/or protein overexpression has been described in glioblastoma [60-62], non-small cell lung cancer (NSCLC) [38], lung and esophageal [63], pancreatic [44] and oral cancer [64].

The *ECT2* gene is located in a region of the chromosome 3q26 which is a region that is frequently altered in human tumors [65]. It was found that Ect2 overexpression was caused by gene amplification in non-small cell lung cancer (NSCLC) and this also correlated with copy number of the PKC $\zeta$  gene (*PRKCI*) [38]. Regulation of tumor growth and invasion of non-small cell lung cancer was through mislocalization of Ect2 to the cytoplasm where it could bind to PKC $\zeta$ -Par6 to activate Rac1 [38]. Interestingly, the role of Ect2 in NSCLC was independent of Ect2 normal function in cytokinesis. Depletion of Ect2 expression in NSCLC

did not result in any proliferation or multi-nucleation defects that are associated with Ect2 loss in normal cells [53]. Furthermore, this study was the first description of a mislocalization and activation of Rac1 mechanism mediated by Ect2. In my studies, I have addressed a role for altered mislocalization of Ect2 to the cytoplasm in driving CRC growth.

Another study showed that Ect2 expression correlated with poor prognosis of glioma patients and that in vitro Ect2 was important for proliferation, migration, and invasion of glioma cells [61]. However, the depletion of Ect2 expression in glioma tumor cell lines showed an increase in multinucleation, characteristic of a defect in cytokinesis. Therefore, the decrease in proliferation, migration and invasion could be caused by the inability for cells to complete mitosis. Another study found that Ect2 was overexpressed in glioblastoma and showed that Ect2 was required to mediate the invasive behavior in glioblastoma in vitro by siRNA knockdown [62].

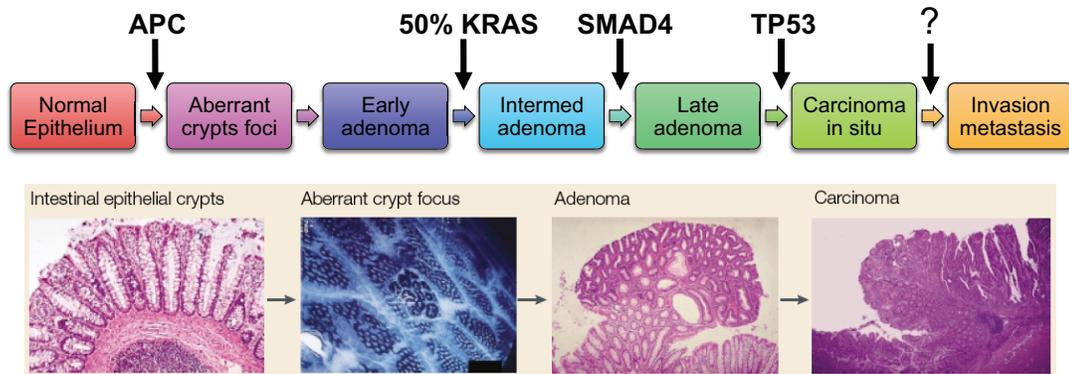
To search for novel biomarkers that may be improved diagnosis, prognosis, and therapeutic intervention of CRC, a recent meta-analysis of publicly available data from GenBank identified *ECT2* as one of nine genes significantly elevated levels in CRC tissues compared to normal tissues [66]. However, whether Ect2 protein expression is elevated and is a driver for CRC growth was not determined. Independently, our microarray gene expression studies identified *ECT2* overexpression in CRC. CRC is the number two cause of cancer deaths for men and women in the US, with 50,830 deaths expected in 2013 [67]. Hence, there remains a dire need for new therapies for this deadly

cancer. In my dissertation research, I have evaluated the role of Ect2 in normal and neoplastic growth, with a focus on the role and mechanism by which aberrant Ect2 expression may drive CRC growth.

### ***Colorectal Cancer (CRC)***

Colorectal cancer (CRC) is a major cause of cancer morbidity and mortality, with ~150,000 U.S. residents diagnosed annually with CRC, and approximately one-third of CRC patients die from the disease [67]. The lifetime risk of CRC (from 2005-2009) in the United States is ~5%, and the average age at diagnosis is 69 years [68]. Risk factors for CRC development include, age, diet, and inherited and/or somatic mutations [69].

The prevailing model for CRC tumorigenesis based on the accumulation of genetic mutations, each defining a different step in the adenoma–carcinoma sequence [70](Figure 1-9). In this sequence, the first hit that associated with adenomatous polyps is mutation and/or loss of the *APC* gene. *K-ras* mutations are found in ~50% of colorectal cancers and are thought to be relatively early events that correlate histologically with early to late adenomas. There is evidence to suggest that *TP53* (p53) mutations occur more frequently in high-grade dysplastic polyps; these mutations are associated the transition from adenoma to carcinoma [71]. Further mutations in *TGF-βIIIR*, *SMAD4*, *BAX*, and/or mutations in mismatch repair genes (*e.g. hMLH1*, *hMSH2*) have all been identified factors in the development and progression of CRC [70, 72].



**Figure 1-9 Genetic model of colorectal cancer**

Colorectal cancer progresses through accumulation of genetic mutations [70, 73].

### ***Specific Aims***

Based on the importance of Rho GTPases activity in cellular behavior; I sought to determine Ect2 specific effect in normal and tumor biology. I used structural and functional full-length Ect2 mutants to determine critical Ect2 elements required for cytokinesis and oncogene.

The next two chapters of my dissertation address my original three aims. Aim I, determine the role of Ect2 overexpression in the invasive and metastatic growth properties of colorectal cancer. (Chapter 3) Aim II, determine the structural and functional requirements of Ect2 activity in normal cell cytokinesis. (Chapter 2 & 3) Aim III, determine the structural and functional requirements of Ect2 that confer an oncogenic phenotype. (Chapter 3) I conclude my dissertations with final thoughts on my studies and future directions. (Chapter 4)

## **Chapter 2 Characterization of *Ect2* in development and normal cell function<sup>1</sup>**

### **Overview**

*Ect2* is a member of the human Dbl family of guanine nucleotide exchange factors (RhoGEFs) that serve as activators of Rho family small GTPases. Although *Ect2* is one of at least 25 RhoGEFs that can activate the RhoA small GTPase, cell culture studies using established cell lines determined that *Ect2* is essential for mammalian cell cytokinesis and proliferation. To address the function of *Ect2* in normal mammalian development, we performed gene targeting to generate *Ect2* knockout mice. The heterozygous *Ect2*<sup>+/-</sup> mice showed normal development and lifespan, indicating that *Ect2* haploinsufficiency was not deleterious for development or growth. In contrast, *Ect2*<sup>-/-</sup> embryos were not found at birth or post-implantation stages. *Ect2*<sup>-/-</sup> blastocysts were recovered at embryonic day 3.5 but did not give rise to viable outgrowths in culture, indicating that *Ect2* is required for peri-implantation development. To further assess the importance of *Ect2* in normal cell physiology, we isolated primary fibroblasts from *Ect2*<sup>fl/fl</sup> embryos (MEFs) and ablated *Ect2* using adenoviral delivery of Cre recombinase. We observed a significant increase in multinucleated cells and accumulation of cells in G2/M phase, consistent with a role for *Ect2* in

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<sup>1</sup> Chapter adapted from published paper: "The *ect2* rho Guanine nucleotide exchange factor is essential for early mouse development and normal cell cytokinesis and migration" Cook, D. R., Solski, P. A., Bultman, S. J., Kauselmann, G., Schoor, M., Kuehn, R., Friedman, L. S., Cowley, D. O., Van Dyke, T., Yeh, J. J., Johnson, L., Der, C. J. *Genes and Cancer*, 2011

cytokinesis. *Ect2* deficiency also caused enlargement of the cytoplasm and impaired cell migration. Finally, although Ect2-dependent activation of RhoA has been implicated in cytokinesis, Ect2 can also activate Rac1 and Cdc42 to cause growth transformation. Surprisingly, ectopic expression of constitutively activated RhoA, Rac1, or Cdc42, known substrates of Ect2, failed to phenocopy Ect2 and did not rescue the defect in cytokinesis caused by loss of *Ect2*. In summary, our results establish the unique role of *Ect2* in development and normal cell proliferation.

### ***Introduction***

Rho family small GTPases are regulators of diverse cellular processes that include cell proliferation and survival, actin organization and cell shape, polarity and movement, and endocytosis and exocytosis [6, 49]. There are 20 human Rho GTPases, with RhoA, Rac1 and Cdc42 the best-characterized members. Rho GTPases function as GDP-GTP regulated binary switches that are activated in response to extracellular stimuli. Activated Rho GTPases in turn associate with effectors that then stimulate cytoplasmic signaling networks [74]. Rho-specific guanine nucleotide exchange factors (RhoGEFs) promote GDP-GTP exchange and formation of active Rho-GTP [12], whereas Rho-specific GTPase activating proteins (RhoGAPs) accelerate hydrolysis of the bound GTP to stimulate formation of inactive Rho-GDP [75].

There are 83 human RhoGEFs, with Dbl family RhoGEFs comprising the largest group (69 members) [9, 12]. Dbl family RhoGEFs are characterized by a conserved Dbl homology (DH) catalytic domain and an adjacent C-terminal

pleckstrin homology (PH) regulatory domain. The DH domain may be highly or broadly specific for a subset of Rho GTPases. For example, Tiam1 is a specific activator of Rac, Asef is specific for Cdc42, whereas Vav is broadly active and can activate RhoA, Rac, Cdc42 and RhoG.

The significantly greater number of RhoGEFs relative to their GTPase substrates suggests highly redundant mechanisms for Rho GTPase activation. This apparent redundancy in activators is particularly striking for RhoA, where there are at least 25 Dbl RhoGEFs that can activate this Rho GTPase [12]. However, the multiple RhoGEFs for one Rho GTPase provides mechanisms where a specific Rho GTPase can be activated by divergent signals, leading to distinct spatio-temporal patterns of activation and signaling. This divergence in RhoGEF regulation and function is reflected by the otherwise highly divergent sequences that flank the DH-PH domains [74]. RhoGEFs are generally large proteins (>1000 amino acids), and often contain several domains involved in their localization, association with other proteins, regulation of GEF catalytic activity and Rho GTPase effector selectivity. For example, among all human RhoGEFs, only Ect2 contains tandem BRCT (BRCA1 C-terminal) domains that are normally found in proteins (e.g., BRCA1, TP53BP1) involved in DNA damage signaling, repair and coordination of cell cycle checkpoints [37, 76]. The BRCT domains may serve to regulate the RhoGEF catalytic activity and additionally can interact with phosphorylated proteins that dictate Ect2 subcellular localization [31, 77, 78].

Ect2 is also only one of two Dbl family RhoGEFs, aside from Net1 [79], that exhibits a nuclear localization in interphase cells [32]. Since Rho GTPases are membrane-associated and/or cytoplasmic, it has been proposed that this nuclear localization is an inactive repository for Ect2, since disruption of nuclear localization activates Ect2 transforming activity [36]. However, a recent study found that nuclear Ect2 maybe active [80], and thus, whether nuclear Ect2 has a function during interphase and distinct from cytokinesis remains unresolved. The best-known function of Ect2 involves its requirement for cytokinesis *in vitro* [65, 81]. In early mitosis (prometaphase) when the nuclear envelope is dissolved, Ect2 is released into the cytoplasm. As cells enter anaphase, Ect2 is recruited to the central spindle by the central spindlin protein complex comprised of Cyk4/MgcRacGAP and MKLP1/CHO1. The central spindle facilitates contractile ring formation, leading to cleavage furrow ingression and midbody formation. Ect2 is recruited to the midbody where Ect2 then activates RhoA to facilitate midbody abscission and completion of cytokinesis. Ectopic expression of a noncatalytic fragment of Ect2 that includes the BRCT domains caused inhibition of U-2 OS human osteosarcoma [32] or adenovirus-transformed HEK 293T human kidney neuronal-like [82] to complete cytokinesis, resulting in the accumulation of multinucleated cells. Similarly, microinjection of anti-Ect2 antibody into HeLa human cervical carcinoma cells also inhibited cytokinesis [32]. Subsequent studies using transient RNA interference suppression of Ect2 expression in HEK 293T [82] or HeLa [31, 83] cells also demonstrated a

requirement for Ect2 in cytokinesis *in vitro*. No assessment of mammalian Ect2 function in normal cells *in vitro* or *in vivo* has been described.

Although Ect2 was identified originally as an activated oncoprotein that was activated by truncation and loss of N-terminal sequences that include the BRCT domains and nuclear localization signals [19], no such truncated proteins have been detected in human cancers. Instead, recent studies have identified aberrant overexpression and mislocalization of full length Ect2 to the cytoplasm in glioblastoma and lung cancer tumor tissue and cell lines [38, 60, 62]. These studies used RNA interference to suppress Ect2 expression, which caused impaired lung tumor cell anchorage-independent growth and Matrigel invasion *in vitro* and reduced tumorigenic growth *in vivo*. Interestingly, in lung tumor cells, Ect2 suppression did not impair cytokinesis, indicating that Ect2 function in oncogenesis is distinct from that in cytokinesis [38]. Further support for this possibility was provided by the observation that constitutively activated Rac1 could rescue the loss of endogenous Ect2 and restore lung tumor cell growth [38]. This finding contrasts with previous studies that found that RhoA is the substrate critical for Ect2-dependent cytokinesis [65, 81].

Previous studies of mammalian Ect2 function have been done in established cell line studies *in vitro*. To evaluate the function of Ect2 *in vivo*, in normal cells and in the context of heterogeneous tissue, we generated both floxed (conditional) and  $\Delta$ floxed (constitutive) knockout mice to assess the consequences of Ect2 loss in development and normal cell proliferation. Constitutive loss of *Ect2* causes embryonic lethality, with defective growth at the

late blastocyst stage. Utilizing mouse embryo fibroblasts derived from *Ect2*<sup>fl/fl</sup> conditional mice, we determined that loss of *Ect2* completely impaired cell proliferation and migration *in vitro*, causing accumulation of cells in G2/M phase and formation of enlarged multi-nucleated cells. Surprisingly, expression of activated Rho GTPases failed to rescue the loss of *Ect2* to restore cell proliferation or migration. Our observations provide further evidence for the highly unique function of this RhoGEF in normal cell physiology.

### **Methods and Materials**

**Vector construction:** The targeting vector was based on a 5.3 kb genomic fragment from the *ect2* gene encompassing exons 8 to 12 and surrounding sequences. This fragment, obtained from the C57Bl/6J RP23 BAC library, was modified by inserting the distal loxP site into intron 8 and the proximal loxP site including an FRT-flanked neomycin resistance gene into intron 7. A thymidine kinase (TK) cassette was inserted at the 3' end of the genomic fragment.

**Embryonic stem (ES) cell culture:** The quality tested C57BL/6NTac ES cell line was grown on a mitotically inactivated feeder layer comprised of MEFs in Dulbecco's modified eagle (DMEM) high glucose medium supplemented with 20% fetal bovine serum (FBS; PAN Biotech GmbH) and 1200 U/ml leukemia inhibitory factor (Millipore; ESG 1107). One x 10<sup>7</sup> ES cells and 30 µg of linearized DNA targeting vector were electroporated (Biorad Gene Pulser) at 240 V and 500 µF. Positive selection with G418 (200 µg/ml) started on day two and counterselection with gancyclovir (2 µM) started on day five after electroporation.

Resistant ES cell colonies with a distinct morphology were isolated on day eight after transfection and expanded in 96 well plates. Correctly recombined ES cell clones were identified by Southern blot analysis using external and internal probes and were frozen in liquid nitrogen.

**Generation of mice:** The animal study protocol was approved according to the German Animal Welfare Act (§ 8 (1) TierSchG) by the local authority. Mice were kept in the animal facility at TaconicArtemis GmbH in microisolator cages (Techniplast Sealsave). Feed and water were available *ad libitum*. Light cycles were on a 12:12 h light:dark cycle with the light phasing starting at 06:00 h. Temperature and relative humidity were maintained between 21 to 23°C and 45 to 65%, respectively. After administration of hormones, superovulated BALB/c females were mated with BALB/c males. Blastocysts were isolated from the uterus at dpc 3.5. For microinjection, blastocysts were placed in a drop of DMEM supplemented with 15% FBS under mineral oil. A flat tip, piezo actuated microinjection-pipette with an internal diameter of 12 - 15 µm was used to inject 10 to 15 targeted C57BL/6NTac ES cells into each blastocyst. After recovery, eight injected blastocysts were transferred to each uterine horn of 2.5 days post coitum, pseudopregnant NMRI females. Chimerism was measured in chimeras (G0) by coat color contribution of ES cells to the BALB/c host (black/white). Highly chimeric mice were bred to i) C57BL/6-Tg(CAG-Flpe)2Arte females mutant for the presence of the Flp recombinase gene or to ii) C57BL/6-Gt(ROSA)26Sor<sup>tm16(Cre)Arte</sup> females mutant for the presence of the Cre recombinase gene. This allowed detection of germline transmission by the

presence of black, strain C57BL/6, offspring (G1) and creation of i) selection marker deleted conditional (floxed) knockout mice or ii) constitutive knockout mice by Flp- or Cre-mediated removal, in one breeding step. Chimeras bred to iii) C57BL/6 wild-type mice resulted in germline offspring with selection marker in the *Ect2* genomic locus described as targeted mice.

**Genotyping of mice by PCR:** Genomic DNA was extracted from 1 to 2 mm long tail tips using the NucleoSpin Tissue kit (Macherey-Nagel). Genomic DNA (2  $\mu$ l) was analyzed by PCR protocol 1 in a final volume of 50  $\mu$ l in the presence of 2.0 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 100 nM of each primer, and 2 U Taq DNA polymerase (Invitrogen) with the following primers: 1 = (5'-GCACTCCAATTATGAAGCCAGAATGG-3'), 2 = (5'-CAATATGTTGGGTAGAGAGATGGC-3') and 3 = (5'-TCCTCCGGGTGGACCAGAG-3') detecting the presence of the wild-type allele (335 bp), targeted (413 bp), the conditional allele (413 bp) and the constitutive allele (498 bp). Following a denaturing step at 95°C for 5 min, 35 cycles of PCR were performed, each consisting of a denaturing step at 95°C for 30 s, followed by an annealing phase at 60°C for 30 s and an elongation step at 72°C for 1 min. PCR was finished by a 10 min extension step at 72°C. Amplified products were analyzed using 2% standard TAE agarose gels. A second PCR protocol for genotyping the *Ect<sup>fl/fl</sup>* mice used two primers: 3 = (5'-TCTGATCCACGTGATCATAG-3'), and 4 = (5'-CTGGCTT CATAATTGGAGTGC-3'). A 420 bp wild-type fragment or a 520 bp mutant fragment was amplified with these two primers. For genotyping the blastocysts, a nested PCR protocol was

used. The outgrowths were scraped from the well with a micropipette tip and transferred to a PCR tube. The tubes were spun down and the supernatant was removed. The cells were resuspended in 5  $\mu$ l of 400ng/ $\mu$ l Proteinase K/17  $\mu$ M SDS and overlaid with mineral oil. The tubes were incubated for 1 h at 50°C, and then denatured at 99°C for 30 min. For the first PCR reaction, 45  $\mu$ l of a PCR mix containing 25 pmol of external primers (5'-CCCTCCAGGTTGAGAACTGCTACTAAG-3' and 5'-GCAGGCTGAGAGCAAGCCAGGAGA-3'). After amplification, 1  $\mu$ l of this first PCR reaction was used for a second round of PCR reactions, which used PCR protocol 1 as described above.

**Blastocyst outgrowth analyses:** Timed breedings of *ect2*<sup>+/-</sup> breeding pairs were set up and at E3.5, blastocysts were flushed from the uterine tract from the females. Blastocysts were placed in 20  $\mu$ l of ES cell medium (DMEM, 15% FCS, 0.01%  $\beta$ -mercaptoethanol) in Nunc dish microwells (Nalgene, Palo Alto, CA) and grown under mineral oil in 5% CO<sup>2</sup> incubator at 37°C. After seven days, the cultures were evaluated and scored for degree of outgrowth, photographed, and then genotyped by PCR.

**Mouse embryonic fibroblasts cultures:** *Ect2*<sup>fl/fl</sup> mice were crossed and at E15.5 days embryos were isolated from the female. Embryos were rinsed with PBS, and the head and dark red organs were removed from the culture. The tissue was minced and resuspended in a solution of PBS/trypsin/EDTA and incubated with gentle shaking at 37°C for 30 min. The cell/tissue solution was gently centrifuged and the supernatant was removed and the loose pellet was

resuspended in DMEM supplemented with 10% FCS. This solution was then filtered through a cell filter and then plated onto 150 mm tissue culture plates and grown in a 5% CO<sup>2</sup> incubator at 37°C. After 18 h, the medium was changed to fresh growth medium. MEFs were immortalized by trypsinizing the cells every three days at replating on a 100 mm tissue culture plate at a density for 1 X 10<sup>3</sup> cells/ dish. For knocking out Ect2 expression in the *Ect2<sup>fl/fl</sup>* MEFs, cells were infected with recombinant adenovirus expressing Cre recombinase (Ad-Cre) with a green fluorescent protein expressing control adenovirus (Ad-GFP) used to assess Cre-specific activities (Gene Transfer Vector Core, University of Iowa).

**Constructs:** An expression vector for human Ect2 was generated using a human Ect2 cDNA sequence from an expression vector kindly provided by Dr. A. P. Fields (Mayo Clinic, Rochester, MN), which was subcloned into the FUGW lentiviral expression vector [84], a gift from Dr. Bryan Roth (University of North Carolina at Chapel Hill, Chapel Hill, NC). Mammalian expression vectors of wild-type human Rho GTPases [RhoA, Rac1 and Cdc42] were constructed by ligation of cDNA sequences into a pBabe-puro retrovirus expression vector [85] that introduces an N-terminal HA epitope tag. The constitutively active [RhoA(Q63L), Rac1(Q61L) and Cdc42(Q61L)] and fast-cycling Rho GTPases mutants [RhoA(F30L), Rac1(F28L) and Cdc42(F28L)] were created using QuikChange site-directed mutagenesis kit (Stratagene).

**Western blot:** To evaluate protein expression, four days after Cre infection, the MEFs were lysed, and analyzed by SDS-polyacrylamide gel electrophoresis and western blot analysis, with anti-Ect2 antibody (Santa Cruz

Biotechnology), anti-HA (Covance) and anti- $\beta$ -actin (Sigma) as a loading control. Additionally, we utilized a second Ect2 polyclonal antibody generated against KLH-conjugated linear peptide corresponding to C-terminal sequences in human Ect2 (Millipore).

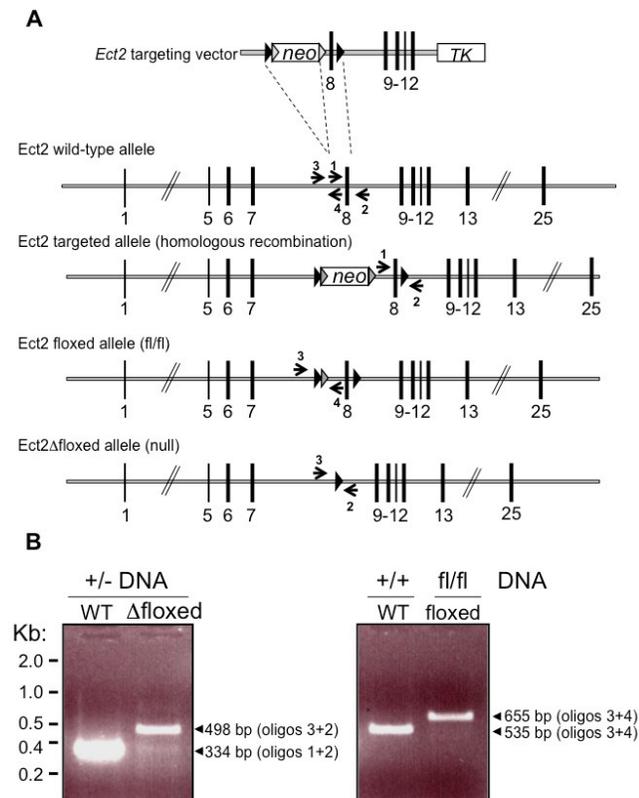
**Immunofluorescence:** To visualize nuclei content, three days after Ad-Cre-infection the MEFs were plated onto coverslips. Twenty-four h after attachment, coverslips were rinsed with PBS, and fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. The cells were stained for 5 min with 5 ng/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS, washed 2-3 times with PBS and then mounted onto slides. The cells were visually analyzed for nuclei content on an Olympus BX61 upright fluorescence microscope. Images collected using Velocity (Perkin Elmer). To visualize polymerized F-actin, cells were stained with Alexa Fluor® 647 phalloidin (Invitrogen).

**Flow cytometry:** Cells were trypsinized, washed in PBS and then fixed in 70% ethanol and then stored at -20°C. The day prior to analysis the cells were washed in PBS and then stained with 0.5 ml of propidium iodine staining solution (1% Triton-X-100, 1 mg/ml PI, 100 mg/ml of RNase A) overnight at 4°C. Data were collected on Beckman Coulter CyAn analyzer and ModFit (Becton Dickinson) was used for cell cycle analysis.

## **Results**

**Generation of Ect2 mutant mice.** In order to evaluate the function of Ect2 in mammalian development we generated a conditional null mutation of the

mouse *Ect2* locus by gene targeting. Our targeting strategy focused on exon 8, which encodes the N-terminal tandem BRCT domains, by flanking it with *loxP* sites (Figure 2-1A). Cre-mediated deletion resulted in a  $\Delta$ floxed allele lacking exon 8 that is a null mutation based on the complete loss of Ect2 protein expression as we describe below. Figure 2-1A shows a schematic diagram of the wild-type *Ect2* allele, the targeting vector, the targeted allele after homologous recombination, and the resulting floxed (conditional) and  $\Delta$ floxed (conditional null) alleles.



**Figure 2-1 Targeted disruption of the mouse *Ect2* gene produces a null mutation**

(A) Schematic diagram of the targeting vector, the wild-type allele, and the resulting *Ect2* floxed allele (fl/fl) after FLP mediated deletion and the *Ect2* $\Delta$ floxed (null) allele after Cre mediated deletion. Numbered, black bold vertical lines represent *Ect2* exons and horizontal gray bar represents flanking intron sequences. *LoxP* and FRT sites are indicated by black and grey triangles, respectively. PCR primers for genotype analysis are indicated by the arrows. (B)

Genotypic analysis of wild-type (+/+), heterozygous  $\Delta$ floxed (+/-), mutant (null; mut) and floxed (fl/fl) DNA by PCR. Primer pair 1 and 2 produced a 334 bp wild-type fragment. Primer pair 3 and 2 produced a 498 bp mutant fragment. Primer pair 3 and 4 produced a 535 bp fragment from the wild-type allele, and a 655 bp fragment from the floxed allele. Abbreviations used are: DNA isolated from heterozygous constitutive ( $\Delta$ floxed) mice, +/- DNA; DNA isolated from wild-type mice, +/+ DNA; DNA isolated from homozygous conditional (floxed) mice, fl/fl DNA; detection of the constitutive allele,  $\Delta$ floxed; detection of the conditional allele, floxed; detection of the wild-type allele, WT. (Data provided by Kausemann, Schoor, Kuehn, Friedman)

***Peri-implantation lethality of Ect2 mutant embryos.*** A ubiquitous Cre driver was used to generate constitutive null heterozygotes. Intercrosses of these  $Ect2^{+/-}$  mice failed to produce any homozygous mutants at birth among 224 offspring, suggesting that  $Ect2^{-/-}$  is embryonic lethal (Table 2-1). Therefore, we performed  $Ect2^{+/-}$  intercrosses as timed matings and genotyped embryos at progressively earlier stages of development. No  $Ect2^{-/-}$  embryos were obtained at embryonic days (E) 15.5, 13.5, or 8.5 (Table 2-1). These results suggested that Ect2 is required for either pre-implantation or early post-implantation development.

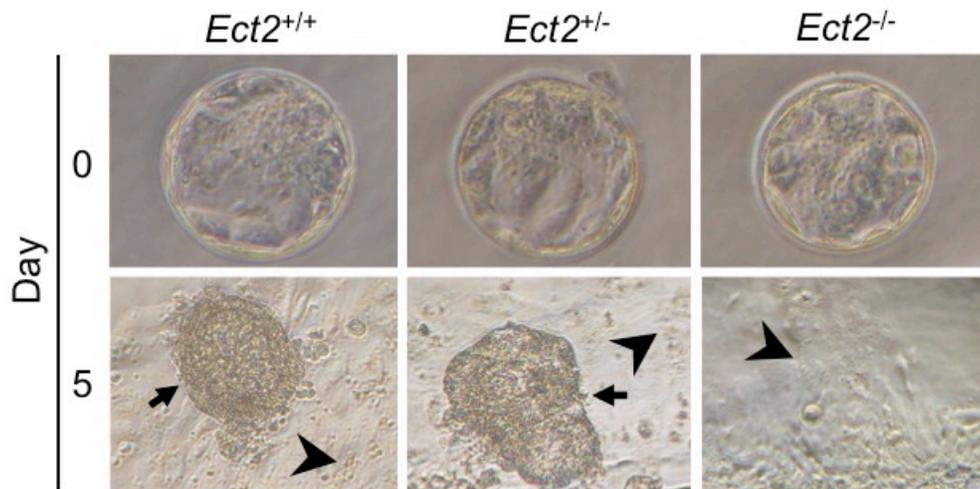
**Table 2-1. Genotypes of Weanlings and Embryos from Ect2 +/- Intercrosses**  
(Data provided by Solski, Bultman, Cowley, and Van Dyke)

Stage	Genotype		
	+/+	+/-	-/-
At weaning	92	132	0
Embryos (13.5-15.5)	10	16	0
Embryos (8.5) <sup>a</sup>	5	5	0

<sup>a</sup> Examination of maternal uteri exhibited empty deciduas, suggesting peri-implantation lethality.

To investigate the peri-implantation stage in more detail, we performed additional  $Ect2^{+/-}$  intercrosses, isolated blastocysts at E3.5, and analyzed their outgrowth *ex vivo* [86]. Homozygous  $Ect2^{-/-}$  blastocysts had a morphologically

normal trophectoderm (TE) and inner cell mass (ICM) (Figure 2-2). We cultured 125 blastocysts for five days and then scored the resulting outgrowths and determined their genotypes. After this culture period, 84% of wild-type outgrowths and 92% of *Ect2*<sup>+/-</sup> outgrowths were scored as normal based on the presence of both TE and extensive ICMs (Figure 2-2, Table 2-2). In contrast, no *Ect2*<sup>-/-</sup> outgrowths were scored as normal. Instead, 14% of homozygous blastocysts failed to hatch from their zona pellucida and the remaining 86% were scored as abnormal based on the ICM not being viable. These results indicate *Ect2* is required for peri-implantation development.



**Figure 2-2 *Ect2*<sup>-/-</sup> blastocysts display abnormal growth *in vitro***

Photographs of wild-type (+/+), heterozygous (+/-), and homozygous null (-/-) blastocyst at time of isolation at E3.5 (Day 0) and after being cultured for 5 days (Day 5). Arrows point to inner cell mass, which is missing in *Ect2*<sup>-/-</sup> blastocysts. Arrowheads point to trophectoderm. (Data provided by Solski, Bultman, Cowley, and Van Dyke)

**Table 2-2 Phenotypes and Genotype of Blastocyst Outgrowth Derived from *Ect2* intercrosses**

(Data provided by Solski, Bultman, Cowley, and Van Dyke)

	+/+	+/-	-/-	ND
Normal <sup>a</sup>	27	58	0	12
Abnormal <sup>b</sup>	3	2	6	6
Unhatched	2	3	1	5
Total	32	63	7	23

ND, not determined.

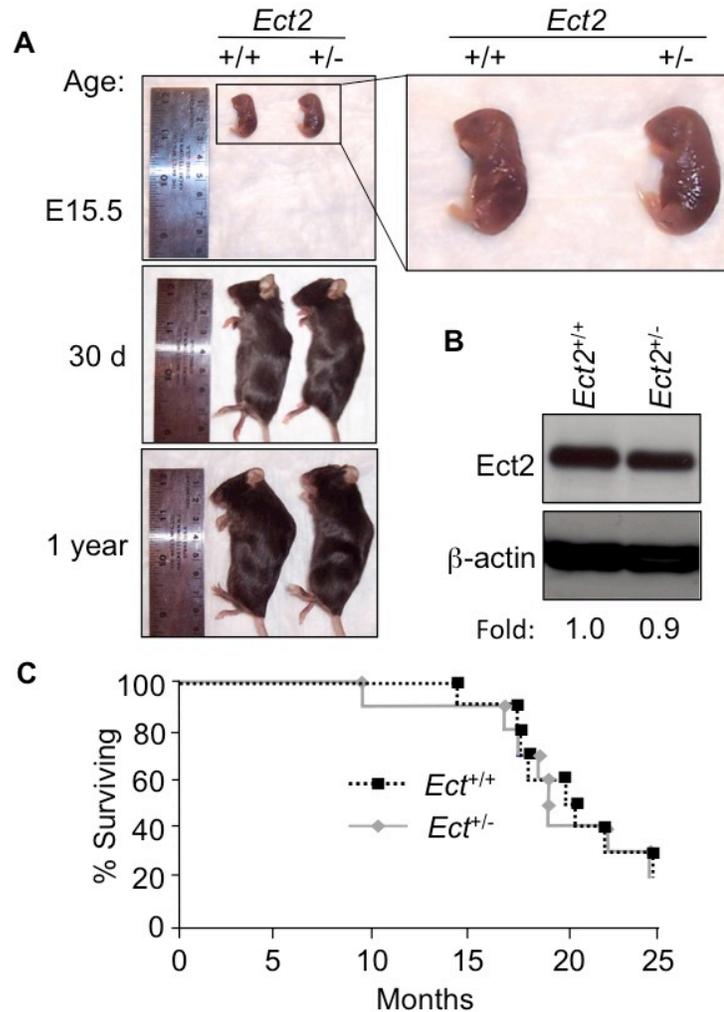
<sup>a</sup>Survival and good outgrowth for trophectoderm and inner cell mass.

<sup>b</sup>Hatched, but inner cell mass failed to grow

**Normal development of *Ect2*<sup>+/-</sup> mice.** Since *Ect2*<sup>-/-</sup> mice were embryonic lethal, we monitored *Ect2*<sup>+/-</sup> mice for haploinsufficiency. We compared *Ect2*<sup>+/-</sup> mice to their wild-type littermates at several stages of development. No changes in weight, gross appearance, or histology were found in *Ect2*<sup>+/-</sup> compared to wild-type littermates at E15.5, 30 days, and one year (Figure 2-3A and data not shown). The lifespan of *Ect2*<sup>+/-</sup> mutant mice were then followed longitudinally. No differences in survival were observed between *Ect2*<sup>+/-</sup> mice and their wild-type littermates (Figure 2-3B).

Given that we did not identify phenotypic differences between *Ect2*<sup>+/-</sup> mice and their wild-type littermates, we examined whether the reduced gene dosage of *Ect2*<sup>+/-</sup> heterozygotes resulted in a corresponding decreases in the level of Ect2 protein expression. We isolated mouse embryo fibroblasts (MEFs) at E15.5 from both *Ect2*<sup>+/+</sup> and *Ect2*<sup>+/-</sup> mice, and examined Ect2 protein levels (Figure 2-3C). We found no significant differences in Ect2 protein expression between

*Ect2*<sup>+/-</sup> and *Ect2*<sup>+/+</sup> MEFs, suggesting that *Ect2*<sup>+/-</sup> mice had equivalent levels of Ect2 protein compared to their wild-type littermates.

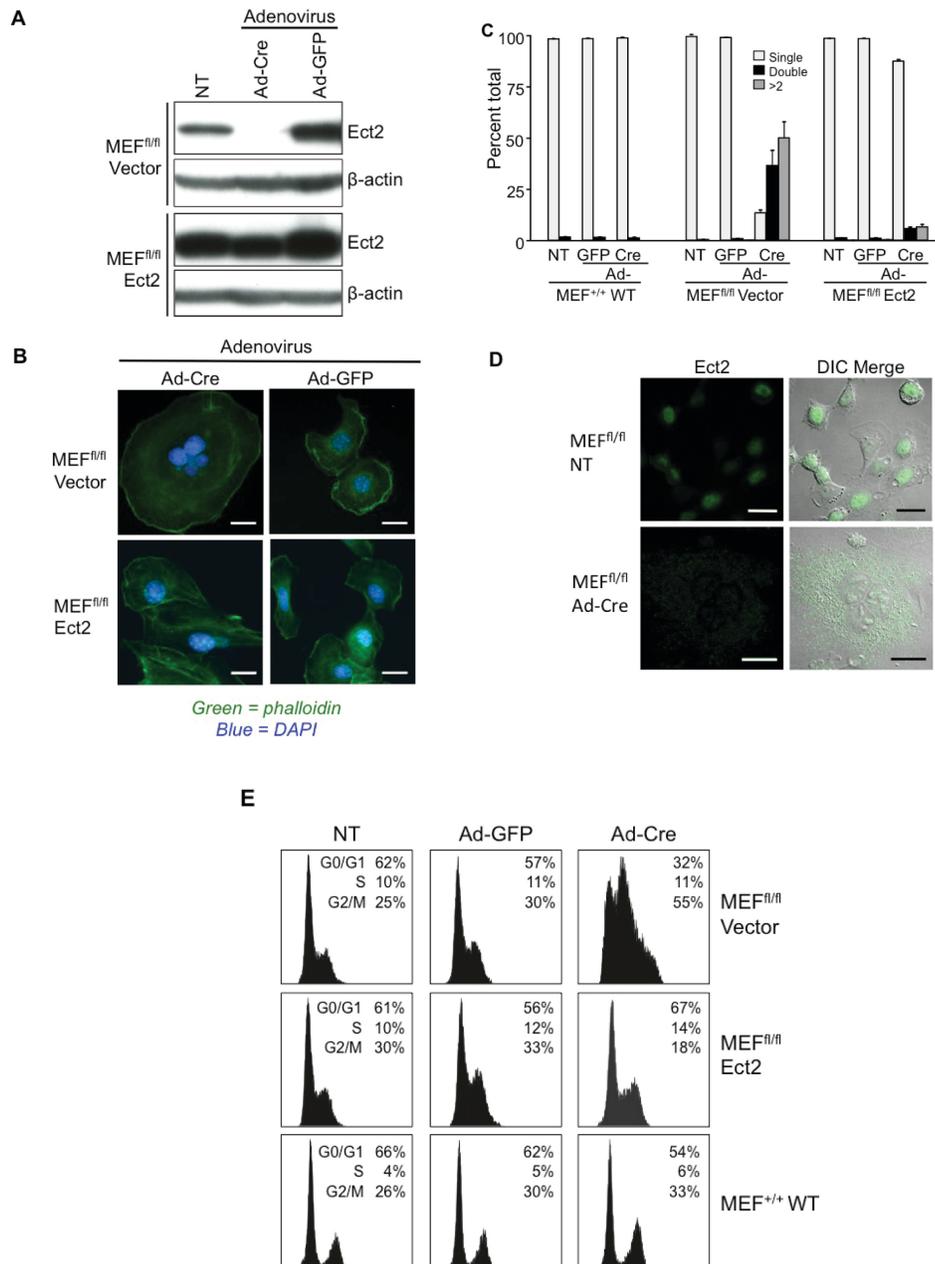


**Figure 2-3 *Ect2*<sup>+/-</sup> mice exhibit normal development and survival**

(A) *Ect2*<sup>+/-</sup> mice are similar in size and appearance as *Ect2*<sup>+/+</sup> mice at E15.5, 30 days, and 1 year. (B) Ect2 protein expression in wild-type and *ect2*<sup>+/-</sup> MEFs. Cell lysates from the indicated cells were blotted with anti-Ect2 antibody and anti-β-actin to verify equivalent total protein loading. Densitometry scanning was used to quantitative the level of Ect2 expression, which were then normalized to the level of β-actin and then normalized to +/+ MEFs, with fold difference shown. (C) *Ect2*<sup>+/-</sup> mice have a similar survival age as *Ect2*<sup>+/+</sup> mice. Mice were monitored for 25 months. (Data provided by Solski, Bultman, Cowley, Van Dyke, and Yeh)

***Ect2* deficiency results in multinucleated cells with altered morphology and impaired migration.** As *Ect2* deficiency was embryonic lethal, we isolated and immortalized MEFs from *Ect2<sup>fl/fl</sup>* and *Ect2<sup>+/+</sup>* mice. These cells were then infected with recombinant adenovirus expressing Cre (Ad-Cre) to excise exon 8 of *Ect2*. An infection efficiency of 90-100% was achieved and this resulted in a complete loss of *Ect2* expression (Figure 2-4A). Ad-Cre-infected *Ect2<sup>fl/fl</sup>* MEFs were much larger in size than their wild-type counterparts and appeared to have abundant nuclei and cytoplasm at 72 h post-infection. To examine this observation further, we sparsely plated Ad-Cre-infected MEFs on coverslips 24 h post-infection and allowed the cells to grow for 72 h. Using fluorescent microscopy, we found that Ad-Cre-infected *Ect2<sup>fl/fl</sup>* MEFs were large and multinucleated. Both *Ect2<sup>fl/fl</sup>* and *Ect2<sup>+/+</sup>* MEFs appeared normal when infected with a control recombinant adenovirus expressing green fluorescent protein (Ad-GFP; Figure 2-4B and data not shown). We quantitated the difference in nucleation and found that Ad-Cre-infected MEFs which are *Ect2*-deficient had a significantly higher percentage of multinucleated cells (~85%) compared to wild-type MEFs (<3%), suggesting that loss of *Ect2* expression causes a defect in mitosis (Figure 2-4C). To further assess *Ect2* expression in *Ect2<sup>fl/fl</sup>* MEFs infected with Ad-Cre, immunofluorescence of *Ect2* was performed and the *Ect2<sup>fl/fl</sup>* MEFs infected with Ad-Cre showed a loss of specific *Ect2* staining whereas the non-treated control showed clear *Ect2* nuclear staining as expected (Figure 2-4D).

The increase in multinucleation associated with loss of Ect2 expression was also quantified by cell cycle analysis, and the *Ect2<sup>fl/fl</sup>* MEFs showed a large increase in cells in G2/M phase when infected with Ad-Cre as compared to the Ad-GFP control virus and untreated MEFs (Figure 2-4E). Furthermore, Ad-Cre-infection had no effect on wild-type MEFs indicating the specificity of the phenotype. Finally, the defect in cytokinesis could be rescued by infection with the FUGW lentivirus expression vector that encodes full length wild type human Ect2, demonstrating that impaired cytokinesis was due to loss of Ect2 expression (Figure 2-4A-E).



**Figure 2-4 Loss of Ect2 expression results in an accumulation of multinucleated cells**

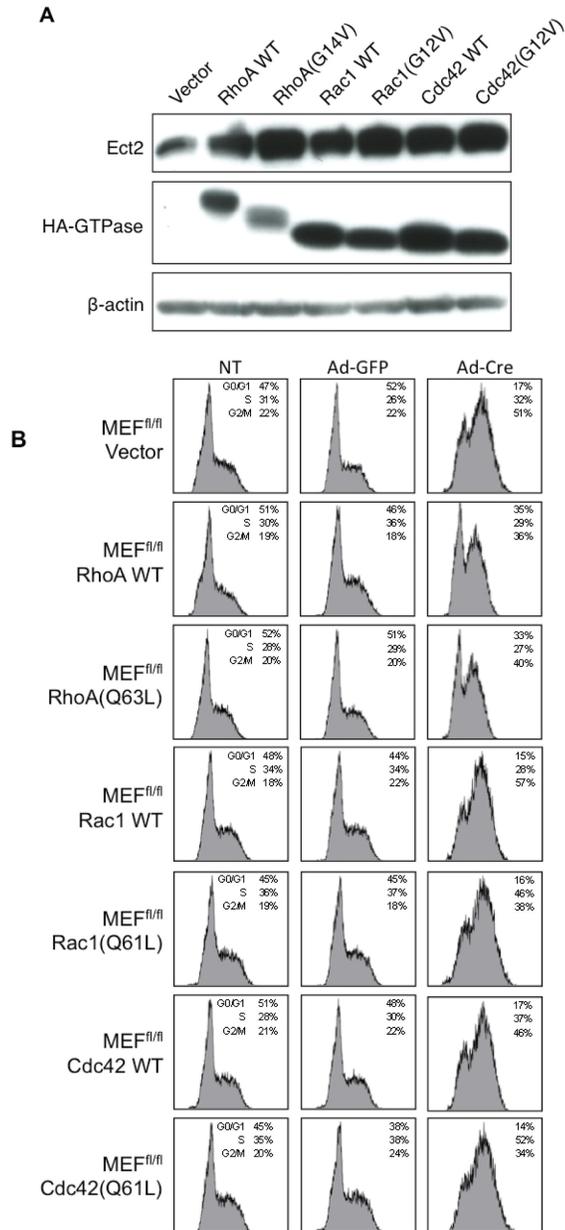
(A) Loss of Ect2 expression in Ad-Cre-infected MEFs. Western blot analysis with anti-Ect2 antibody that recognizes the C-terminus of Ect2 was done to verify loss of Ect2 protein expression upon Cre-mediated excision of Ect2. Blot analysis with anti- $\beta$ -actin antibody was done to verify equivalent total protein. NT, no treatment; Ad-Cre, infected with Cre adenovirus; Ad-GFP, infected with control vector GFP adenovirus. (B) Fluorescent images of Ect2<sup>fl/fl</sup> MEFs ectopically expressing FUGW vector or expressing Ect2 and infected with Ad-GFP control vector or Ad-Cre adenovirus. Scale bar is 20  $\mu$ m. Nuclei were stained with DAPI (blue) and F-actin with phalloidin (green) (C) Quantification of nuclei per cell in Ect2<sup>+/+</sup>, Ect2<sup>fl/fl</sup> vector, Ect2<sup>fl/fl</sup> MEFs with

no treatment (NT), adenovirus GFP (Ad-GFP), or adenovirus Cre (Ad-Cre). Cells were stained with a nuclear specific dye DAPI. Results are representative from one of three experiments. Nuclei per cell were counted for approximately 100 cells per field and five fields per condition. Standard error of the mean (SEM) is shown and were determined by the standard deviation divided by the square root of  $n = 5$  different fields. (D) Immunofluorescence of Ect2 expression in Ect2<sup>fl/fl</sup> MEFs that were not treated (NT) or infected with Cre adenovirus (Ad-Cre). Green is endogenous Ect2 expression detected using an anti-Ect2 antibody. (E) Cell cycle profiles by flow cytometry of analysis of Ect2<sup>+/+</sup>, Ect2<sup>fl/fl</sup> vector (FUGW), Ect2<sup>fl/fl</sup> Ect2 (FUGW-Ect2). Abbreviations used are: MEFs with no treatment ,NT; control virus,Ad-GFP; Cre recombinase expressing virus, Ad-Cre.

Since Ect2 is an activator of Rho GTPases and Rho GTPases regulate actin reorganization, we also evaluated the consequences of Ect2 loss on actin organization. Phalloidin staining of control and Ad-Cre-infected MEFs showed similar low levels of actin stress fibers, but there was a reduction in membrane ruffling (Figure 2-4B). Since Rho GTPases can regulate cell attachment and migration we also utilized time-lapse microscopy to monitor cell motility. Control Ad-GFP-infected MEFs exhibited high motility, whereas Cre-mediated loss of Ect2 caused a striking near complete loss of this activity.

***Rho GTPases cannot rescue the multinucleated phenotype caused by loss of Ect2.*** There is considerable evidence that suggests that the requirement for Ect2 during cytokinesis is for activation of RhoA at the central spindle and midbody to complete mitosis [65, 81]. However, there is also evidence that Ect2 activation of Cdc42 but not Rac1 is important for mitosis [83]. In contrast, a recent study determined that ectopic expression of constitutively activated Rac1 but not RhoA (or Cdc42) that rescued the loss of endogenous Ect2 to restore lung carcinoma cell anchorage-independent growth and invasion [38]. Thus, the specific Rho GTPase important for the role of Ect2 in cytokinesis remains to be elucidated.

To determine if a specific activated Rho GTPase could rescue the cytokinesis defect caused by the ablation of Ect2 expression, hemagglutinin (HA) epitope-tagged wild-type or constitutively active, GTPase-deficient mutants [RhoA(Q63L), Rac1(Q61L) and Cdc42(Q61L)] were each stably expressed in *Ect2<sup>fl/fl</sup>* MEFs prior to Cre expression (Figure 2-5A). Surprisingly, expression of wild-type or constitutively active GTPases failed to rescue the multinucleation phenotype associated with the loss of Ect2 expression (Figure 2-5B). Since fast cycling mutants with enhanced intrinsic nucleotide exchange activities may better model GTPase activation by a RhoGEF [87], we also ectopically expressed RhoA(F30L), Rac1(F28L) or Cdc42(F28L) in Ect2-depleted MEFs but no rescue of cytokinesis was seen. These negative results contrast with the ability of constitutively activated Rac1(Q61L) to rescue loss of cytoplasmic mislocalized Ect2 in lung cancer cells [38], and may indicate the requirement for a more precise temporal and spatial control of GTPase activity during normal cell cytokinesis.



**Figure 2-5 Rho GTPases did not rescue the defect cytokinesis caused by loss of Ect2**

(A) Western blot analysis of *Ect2*<sup>fl/fl</sup> MEFs ectopically expressing HA-tagged RhoA, Rac1, and Cdc42 wild-type (WT) and constitutively active mutants of RhoA (Q63L point mutation) or Rac1 and Cdc42 (Q61L). (B) Cell cycle profiles by flow cytometry of analysis of *Ect2*<sup>fl/fl</sup> MEFs ectopically expressing HA-tagged RhoA, Rac1, and Cdc42 wild-type (WT) and constitutively active GTPases with no treatment (NT), control virus (Ad-GFP) or Cre infection (Ad-Cre).

## **Discussion**

Despite the fact that Ect2 is one of at least 25 RhoGEFs that activate RhoA, observations made in cell culture studies and in *Drosophila* [29] and *C. elegans* [88] suggest an essential and unique role for this RhoGEF in cytokinesis and cell growth. To evaluate a function for Ect2 in normal mammalian cell physiology, we generated knockout mice. We found that an Ect2 deficiency caused early embryonic lethality and a complete loss of proliferation *in vitro* that was associated with impaired cytokinesis, altered cell morphology and suppressed cell migration. We conclude that Ect2 function is unique among all 83 mammalian RhoGEFs in its essential requirement for cytokinesis.

Our studies provide the first analyses of mammalian Ect2 function in normal cells *in vitro* and *in vivo*. Previously, Ect2 function *in vivo* was evaluated in *Drosophila* and *C. elegans* invertebrate species. In *Drosophila*, loss of function of the Ect2 ortholog, *pebble*, resulted in embryonic lethality [29]. Homozygous *pebble* deficient mutant embryos at the end of embryogenesis contained fewer and larger cells with enlarged nuclei. The first 13 rapid syncytial nuclear divisions proceeded normally in *pebble* deficient embryos. Following cellularization, the postblastoderm nuclear divisions occurred (mitoses 14, 15 and 16), but cytokinesis was not observed, with multinucleated cells seen at cycle 15 mitoses. In *C. elegans*, RNA interference of *Ect-2* caused sterility and embryonic lethality, in addition to reduced migration of embryonic P-cells [88]. However, there is evidence for a second mammalian Dbl family RhoGEF, GEF-H1, in regulation of cytokinesis[22] and no GEF-H1 orthologs are found in *Drosophila* or *C. elegans*.

Additionally, Ect2-independent cytokinesis has been described in some human cell types,[89]. Therefore, we anticipated the possibility that Ect2 may not be required for normal cell cytokinesis *in vivo*. However, our finding of embryonic lethality secondary to Ect2 deficiency is consistent with the essential role for this RhoGEF in normal cell cytokinesis. As Ect2 activates Rho GTPases that can be activated by other RhoGEFs, Ect2 must regulate Rho GTPases in a highly unique spatial and/or temporal pattern of activation that cannot be facilitated accurately by any other RhoGEF.

Similar to our observations with an *Ect2* deficiency, a *MgcRacGAP* deficiency also results in pre-implantation lethality [54]. *MgcRacGAP* is a component of central spindlin and facilitates Ect2 recruitment to the central spindle. siRNA depletion of *MgcRacGAP* phenocopies Ect2 loss and results in a cytokinesis defect *in vitro* [23, 25, 26, 90]. At E3.0-3.5, the *MgcRacGAP* deficiency caused formation of binucleated blastomeres, suggesting that *MgcRacGAP* is required for normal mitosis and cytokinesis in the pre-implantation embryo. All homozygous mutant blastocysts failed to grow out *in vitro*. The similar developmental consequences of *Ect2* or *MgcRacGAP* loss suggest that the *Ect2* deficiency-induced embryonic lethality is a consequence of the role of Ect2 in cytokinesis.

A recent study addressed a role for Ect2 in mouse oocyte meiosis *in vitro* and found that Ect2 depletion caused a block in the extrusion of the first polar body [91]. This observation suggests a much earlier requirement for Ect2 in development than suggested from our studies. This is likely due to the presence

of maternal Ect2 in the oocytes derived from *Ect2*<sup>-/+</sup> intercrosses. The requirement for Ect2 in meiosis was found to be associated with RhoA and RhoA activation of its effector, the ROCK serine/threonine kinase.

In a majority of previous studies addressing the consequence of a specific RhoGEF deficiency for normal mouse development, while specific cellular defects have been identified, generally normal development and viability were retained [12]. These observations are consistent with the redundant functions of RhoGEFs, with 83 mammalian RhoGEFs regulating 14 Rho GTPases (six Rho GTPases are constitutively active and not believed to be regulated by RhoGEFs) [49]. While two Dbl RhoGEFs have been found to be essential for mouse development, because they possess additional non-RhoGEF catalytic functions, it is not clear that this is due to the loss of their RhoGEF activities. For example, a *Sos1* deficiency leads to embryonic lethality [50, 92], but this is most likely due to the fact that *Sos1* is also a RasGEF and key regulator of Ras activation by receptor tyrosine kinases. Deficiency in another Dbl RhoGEF, Trio, also causes embryonic lethality, with *Trio*<sup>-/-</sup> embryos dying between E15.5 and birth, or shortly thereafter [51]. However, Trio is multifunctional protein possessing two distinct DH-PH tandem domains, one active on RhoA and the second on Rac1, and additionally a protein serine/threonine kinase domain. Thus, whether the essential function of Trio in development is its two RhoGEF functions is not clear. In contrast to *Sos1* and Trio, Ect2 has no other known catalytic function aside from its RhoGEF activity, although Ect2-interacting proteins may facilitate Ect2-dependent, non RhoGEF-associated functions important for cytokinesis.

The role of Ect2 in cytokinesis has been attributed to recruitment of RhoA and localized RhoA activation at the central spindle complex [26, 39]. Additionally, our analyses of the isolated DH-PH domain fragment of Ect2 identified activity for RhoA, the related RhoB and RhoC isoforms, but not for eight other Rho GTPase tested *in vitro*, including Rac1, Rac2 and Rac3 (data not shown). However, one study found that Ect2 was required for Cdc42 activation during mitosis [83] Furthermore, we and others found that constitutively activated N-terminally truncated Ect2 can activate RhoA, Rac1 and Cdc42 *in vivo* [43]. To determine the key Rho GTPase important for Ect2 regulation of cytokinesis, we determined if ectopic expression of constitutively activated RhoA, Rac1 or Cdc42 could rescue the loss of endogenous Ect2 and restore proper cytokinesis. This approach was utilized recently to show that constitutively activated Rac1 alone could restore Ect2-dependent lung tumor cell growth [38]. Therefore, we were surprised that no one activated Rho GTPase was able to rescue the block in cytokinesis caused by Ect2 depletion. One possible explanation for this is that Ect2 may need to activate multiple Rho GTPases concurrently. Interestingly, consistent with our findings, activated RhoA orthologs did not rescue the Ect2 deficiency-induced cytokinesis defect in *Drosophila* or *C. elegans* [88]. Another possible explanation may be that Ect2 activation of RhoA during cytokinesis is highly regulated spatially and temporally. Therefore constitutively activated RhoA may not accurately mimic this more precise regulation. Our ongoing evaluation of recombinant Ect2 Rho GTPase specificity found activity for only RhoA and related isoforms. Therefore, we favor the second possibility and our future

studies will address the importance of Ect2 subcellular localization in supporting cytokinesis.

In addition to a cytokinesis defect, we also observed a defect in MEF cell morphology and migration. Ect2 loss in MEFs resulted in enlarged and flattened cells with increased cytoplasmic content. Whereas wild-type MEFs are highly motile, Ect2 loss resulted in a near complete loss of movement. Similar Ect2 deficiency-associated defects were seen in normal cell migration in *C. elegans* and *Drosophila* [88, 93], and in tumor cell migration and invasion [38, 62]. However, these defects were associated with Rac and not RhoA activation. We observed that the Ect2 depletion-associated loss of MEF cell motility was associated with decreased membrane ruffling activity, an activity also associated with Rac activity.

In summary, our studies provide the first analysis of Ect2 function in normal cells *in vitro* and *in vivo*, and we show that, despite the existence of multiple RhoGEFs, Ect2 plays a unique role in development and progression through mitosis. Using our conditional mouse model, our ongoing studies will assess the consequences of Ect2 deficiency in adult tissue homeostasis and in tumor progression and maintenance. Furthermore, using MEFs with conditional Ect2 loss, where we can rescue the cytokinesis defect by ectopic wild type Ect2 expression, we will perform detailed structure-function analyses of Ect2 to delineate what aspects of Ect2 are essential for its unique role in maintenance of normal cell cytokinesis.

### ***Chapter 3 Aberrant Ect2 expression and subcellular localization in colorectal cancer<sup>2</sup>***

#### **Overview**

Ect2 is an activator of the RhoA small GTPase, is nuclear restricted in interphase cells and essential for normal cell cytokinesis. Ect2 was identified originally as potent oncoprotein when expressed in NIH 3T3 mouse fibroblasts. However, the mechanism of activation, N-terminal truncation and cytoplasmic mislocalization (designated  $\Delta$ N-Ect2) was due to in vitro DNA manipulation and truncated Ect2 proteins have not been found in human cancer. We identified elevated full length Ect2 protein levels in CRC tumors and cell lines. Our immunohistochemistry analyses of a CRC tumor tissue microarray detected both cytoplasmic and nuclear Ect2, consistent with cytoplasmic mislocalization as a mechanism for Ect2 activation. However, unexpectedly, the ratio of cytoplasmic to nuclear expression correlated with improved patient survival. To address the importance of subcellular localization in CRC, we first determined that suppression of Ect2 expression impaired CRC cell line anchorage-independent growth and Matrigel invasion. Surprisingly, we found that ectopic expression of Ect2 variants with impaired nuclear localization did not rescue loss of endogenous Ect2 to restore anchorage-independent growth. Furthermore, the

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<sup>2</sup> Chapter adapted from manuscript: "Aberrant Ect2 expression, subcellular localization and function in colorectal cancer" Cook, D.R., Kang, M., Martin, T.D., Galnako, J.A., Tran, J., Elizabeth, R.D., Statford, J.K., Temitope, K.O., Yeh, J., Robert, S.S., Der, C.J.

cytoplasmic and constitutively activated  $\Delta$ N-Ect2 mutant strongly impaired CRC growth. We conclude that nuclear Ect2 is important to support CRC growth, whereas cytoplasmic Ect2 activity is deleterious for CRC growth and may explain why truncated Ect2 proteins have not been found in human cancers. Finally, we determined that Ect2 nuclear localization is also essential to support normal cell cytokinesis. In summary, our results emphasize the critical role of precise subcellular localization in dictating Ect2 function in normal and neoplastic cells.

### ***Introduction***

Rho family small GTPases function as key signaling nodes that are activated by diverse extracellular stimuli that act on receptor tyrosine kinases, G protein-coupled receptors, integrins and other cell surface receptors. Once activated Rho GTPases engage downstream effectors that regulate cytoplasmic signaling networks that control actin organization, cell cycle progression and gene expression [6]. Therefore, it is not surprising that the aberrant activation of Rho GTPases has been implicated in cancer, neurological and developmental disorders and other human diseases [57, 94, 95]. However, in contrast to the related Ras small GTPases which are mutated in cancer and developmental disorders, Rho GTPases are infrequently mutated in disease. Instead, Rho GTPase activities are disrupted by indirect mechanisms [9].

The human Rho GTPase family is comprised of 20 members, with RhoA, Rac1 and Cdc42 the best-studied [49]. Rho GTPases function as GTP-GDP regulated binary on-off switches. Extracellular stimuli activate Rho-selective guanine nucleotide exchange factors (RhoGEFs) to promote GDP-GTP

exchange and formation of active Rho-GTP. Active Rho-GTP then binds multiple effectors, stimulating a cascade of cytoplasmic signaling networks. When the stimulus is terminated, Rho-selective GTPase activating proteins (RhoGAPs) accelerate the intrinsic GTP hydrolysis activity of Rho GTPases, returning the protein to the inactive GDP-bound state. Aberrant activation of RhoGEFs or loss-of-function of RhoGAPs has been found in cancer, leading to persistent formation of the GTP-bound protein and stimulus-independent activation of effector signaling [9]. For example, we previously showed that the Rac-selective RhoGEF Tiam1 is an effector of mutationally activated Ras [96]. That mice deficient in Tiam1 or Rac1 show impaired mutant *RAS*-induced tumor formation demonstrates the important driver function of Rac activation in cancer development [97-99]. Similarly, we recently found that the Rac-selective RhoGEF P-Rex1 was upregulated by ERK mitogen-activated protein kinase signaling in melanoma and P-Rex1 deficient mice showed greatly reduced incidence of metastatic melanoma formation in an *NRAS/INK4A*-driven mouse model of melanoma [100]. A genome sequencing study identified frequent mutational activation of the related P-Rex2 protein in melanoma [101].

Dbl family proteins comprise the largest family of RhoGEFs (>70 human members) and their initial discovery as activated oncogenes provided the first evidence that aberrant Rho GTPase activation may drive cancer growth [12]. For example, Dbl, Ect2 and Vav RhoGEFs were detected in NIH 3T3 mouse fibroblast focus formation assays [10, 19, 102]. Their mechanism of activation involved N-terminal deletion of sequences upstream of their catalytic Dbl

homology domain. However, these activation events were determined to occur as a consequence of in vitro DNA manipulation rather than bona fide genetic events in cancer cells. Rather surprisingly, despite their potent transforming activities in NIH 3T3 cells, truncated RhoGEFs have not been found in human cancers [9]. Paradoxically, when implicated in cancer growth, only full length (FL) RhoGEFs have been found. Furthermore, while Ect2 is normally sequestered in the nucleus of interphase cells, they found that Ect2 was mislocalized and found in the cytoplasm due to phosphorylation by protein kinase C iota [38]. This mechanism of activation is functionally analogous to the N-terminally truncated and activated Ect2 (designated  $\Delta$ N-Ect2) discovered initially in NIH 3T3 focus formation assays, where nuclear localization sequences (NLS) were lost in the truncated protein [19]. That disruption of the NLS motifs in FL Ect2 created a transforming protein when expressed in NIH 3T3 cells provided strong evidence that Ect2 oncogenic function is unmasked by mislocalization from the nucleus to the cytoplasm [36].

We identified *ECT2* as a gene elevated in expression in human primary colorectal cancer (CRC). We determined that Ect2 protein levels were elevated in CRC tumor tissue and cell lines and that suppression of Ect2 expression impaired CRC cell line anchorage-independent growth and Matrigel invasion. Consistent with previous studies of Ect2 expression in other cancers, we also found Ect2 in the cytoplasm and nucleus. However, our immunohistochemistry staining of a CRC tumor microarray, unexpectedly, the ratio of cytoplasmic to nuclear Ect2 was associated with improved survival, suggesting that nuclear Ect2

may be important for cancer development and cytoplasmic Ect2 may be deleterious for cancer growth. To address this possibility, we determined that nuclear Ect2 was important to support CRC anchorage-independent growth and conversely that cytoplasmic, constitutively activated Ect2 was detrimental to CRC growth. Finally, we determined that CRC growth dependency on Ect2 was distinct from the role of Ect2 in cytokinesis, and unexpectedly, despite the absence of the nuclear envelope in cells undergoing cytokinesis, Ect2 support of cytokinesis required intact NLS motifs. In summary, our studies demonstrate a critical role for proper Ect2 subcellular localization in normal and neoplastic cell biology.

### ***Methods and Materials***

**Gene array analysis:** Gene array analysis was performed by RNA isolation and hybridization using Agilent human whole genome 4×44 K DNA microarrays (Agilent Technologies). RNA was extracted from macrodissected snap-frozen tumor samples using Allprep Kits (Qiagen) and quantified using nanodrop spectrophotometry (ThermoScientific). RNA quality was assessed with the use of the Bioanalyzer 2100 (Agilent Technologies); similar quality RNA was selected for hybridization using RNA integrity number and by inspection of the 18S and 28S ribosomal RNA. One microgram of RNA was used as a template for DNA preparations and hybridized to Agilent 4×44 K whole human genome arrays (Agilent Technologies). cDNA was labeled with Cy5-dUTP and a reference control (Stratagene) was labeled with Cy3-dUTP using the Agilent (Agilent Technologies) low RNA input linear amplification kit and hybridized overnight at

65°C to Agilent 4×44 K whole human genome arrays (Agilent Technologies). Finally, arrays were washed and scanned using an Agilent scanner (Agilent Technologies).

**Oncomine Analyses:** Sample data includes age, histology, microsatellite status, TNM stage, KRAS mutation status, sex, stage, and others. This dataset is a combination of Colon Adenocarcinoma [COAD] and Rectum Adenocarcinoma [READ] data from the TCGA data portal and consists of Level 2 (processed) data. Sample data includes age, histology, microsatellite status, TNM stage, KRAS and BRAF mutation status, sex, stage, and others. This dataset is a combination of Colon Adenocarcinoma [COAD] and Rectum Adenocarcinoma [READ] data from the TCGA data portal and consists of Level 3 data (segmented using CBS). The resulting segments were mapped to RefSeq gene coordinates as provided by UCSC (UCSC refGene, July 2009; hg18, NCBI 36.1, March 2006). The samples were originally run on the Affymetrix SNP6 platform.

**Tumor samples and construction of tissue microarrays (TMAs):**

Cancer Care Outcomes Research and Surveillance Consortium (CanCORS) is a population-based cohort study. The study design of CanCORS has been described previously [103]. North Carolina CanCORS site enrolled newly diagnosed cases of CRC in North Carolina, encompassing both rural and urban areas. In addition to information collected by patient surveys and medical record review, the University of North Carolina site collected tissue blocks on consenting subjects. The study was approved by the human subjects committees at all participating institutions. TMAs were constructed from formalin-fixed,

paraffin-embedded colorectal tissues. Each microarray block included duplicate or triplicate cores of CRC and adjacent normal tissues from each patient. 29 TMAs that included 441 patients were selected for staining.

**Immunohistochemistry (IHC) for ECT2:** To validate the anti-Ect2 polyclonal rabbit antibody (Millipore) for IHC, we utilized CRC cell lines and tissue. Positive controls included a colon cancer tissue and HT29 cells. HT29 cells with *ECT2* gene knockdown were used as negative controls. TMAs, positive and negative control slides were stained using anti-Ect2 antibody at 1:350 dilution using Bond Antibody diluent (Leica Microsystems Inc.). Antigen was heat retrieved in citrate buffer, pH 6, for 30 min. The remainder of the staining was carried out using the Bond Polymer Refine Detection kit with the Bond Autostainer (Leica Microsystems Inc.) for the following times: primary antibody 6 h, post-primary 8 min, polymer 8 min, peroxide block 5 min, 3,3-diaminobenzidine 10 min, hematoxylin 7 min, and bluing 5 min.

**Scoring of TMAs:** Prior to scoring, crypts and colorectal epithelium in each core on the TMAs were manually annotated to remove the lamina propria and non-epithelial cells. Only the annotated areas were scored. Scoring for Ect2 expression was carried out using Cytoplasm algorithm in Imagescope (Aperio Inc.). Aperio Imagescope algorithms have been used for tumor tissue slide and TMA scoring and demonstrated good concordance with visual scoring in previous studies [104-106]. Further, the scoring was confirmed on a random sample of cores by visual inspection. Any cores with less than 300 epithelial cells, missing from falling off in the staining process, exhausted from prior sectioning, or cores

with staining artifact were excluded from analysis, which yielded 146 patients for analyses. Ect2 protein expression was measured by H-scores for nuclear or cytoplasmic staining (Cytoplasm Algorithm User's Guide from Aperio). Briefly, H-score is an intensity score derived from the average intensity of the staining of the corresponding area (cytoplasm or nucleus) or, in other words, cellular average. For example, there are three intensity thresholds (1+, weak, 2+, moderate and 3+, intense), and H-score equals sum of (% of cells with 1+ staining)+2\*(% of cells with 2+ staining)+3\*(% of cells with 3+ staining). This would yield a range of scores from minimum of 0 and maximum score of 300, where 300 would represent 100% of the cells are 3+ in intensity. In this study, H-score and Ect2 scores represent the same, and these words were used interchangeably.

**Statistical analysis:** ECT2 protein expressions were compared between tumor and normal tissues or between nuclear and cytoplasmic areas using paired t-test. Ect2 tumor scores were divided into tertiles, with cutoffs determined by Ect2 scores in normal tissues. To determine the association between patient characteristics and Ect2 tumor score tertiles, Kruskal-Wallis test was used for 2 category variables, Spearman correlation for variables with more than 2 categories (differentiation), and Wilcoxon rank test for continuous variable (overall survival). Cox proportional hazards models were used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for mortality associated with continuous ECT2 scores. Multivariate models were fit to adjust for age (in 10 year increments), stage (American Joint Committee on Cancer/ Union

Internationale Contre le Cancer stages 1, 2, 3, or 4), grade (well, moderate or poorly differentiated), location of CRC (proximal or distal), and chemotherapy (yes or no). All analyses were performed using SAS Version 9.2 (SAS Institute, Cary, NC).

**Cell lines and plasmids:** CRC cell lines were obtained from ATCC and maintained in either DMEM-H or RPMI-1640 supplemented with 10% fetal calf serum, and frozen down to maintain limited passage history. Lentivirus vectors with shRNA sequences targeting human *ECT2* and an expression vector for an shRNA resistant *Ect2* cDNA expression vector kindly provided by Alan Fields (Mayo Clinic, Rochester, MN). The shRNA-resistant *ECT2* cDNA sequence was used as a template for introduction of missense mutations or generation of truncations by the QuikChange site-directed mutagenesis kit (Stratagene). The wild type or mutant *ECT2* cDNA sequences were subcloned into the pCDH EF1 IRES puro lentiviral vector (Systems Biosciences) to encode an N-terminal hemagglutinin (HA) epitope tag. Immortalized mouse embryo fibroblasts (MEFs) derived from mouse embryos that harbor a conditional *Ect2* floxed allele (*Ect2<sup>fl/fl</sup>*) [53]. Cre-mediated deletion results in a  $\Delta$ floxed allele lacking exon 8 that is a null mutation based on the complete loss of *Ect2* protein expression. Recombinant adenovirus expressing Cre recombinase (Ad-Cre) or green fluorescent protein (Ad-GFP) to control for effects due to adenovirus infection were used to induce loss of *Ect2* expression (Gene Transfer Vector Core, University of Iowa).

**NIH 3T3 transformation Assays:** NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% Colorado

calf serum (Colorado Serum Company). Cells were transfected using lipofectamine 2000 (Invitrogen) in 6-well plates. Approximately 14-days post-transfection, the plates were stained with crystal violet, and the appearance of foci of transformed cells was quantified by visual inspection.

**Matrigel invasion assays:** Real-time invasion assays were performed on the xCELLigence system (Roche). Similar to traditional Boyden chamber a CIM-Plate 16 (Roche) was used where the top of the trans-well was coated with Matrigel (BD Bioscience) and Matrigel and allowed to gel at 37°C, 5% CO<sub>2</sub> for two h. After two h, cells were plated over the Matrigel in serum-free growth medium and complete growth medium supplemented with 10% fetal calf serum was added to the bottom of the transwell as a chemoattractant. Invasive cells will migrate through the Matrigel then through the micropores of the CIM-Plate 16. These migrating cells are detected by the electronic sensing microelectrodes, producing changes in impedance, reported as cell index values. The xCELLigence system was set to collect impedance data every two min for at least 40 h [107, 108].

**Anchorage-independent growth assays:** Anchorage-independent growth assays consisted of suspending cells in soft agar as described previously [109]. Briefly, cells were resuspended in growth medium containing ~0.3% bacto agar (BD Biosciences) and plated at 20,000 cells per well over a 0.6% bacto agar layer in 6-well plates. Cells were maintained at 37°C for 2-4 weeks. After this time, viable colonies were stained with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide viability stain for ~30 min at 37°C. Then

number of viable colonies of was quantified by counting the number of colonies in three wells per condition. Results are expressed as mean  $\pm$  S.D.

**MTT proliferation assays:** Cells were plated in 96-well plate at 1,000 cells/well and allowed to grow at 37°C, 5% CO<sub>2</sub>, for 24-120 h. To assess growth MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) was dissolved in PBS at 5 mg/ml and 20  $\mu$ L of MTT solution was added to each well. The plates were incubated at 37°C, 5% CO<sub>2</sub> for two h. After two h, the medium and MTT solution is removed and then the formazan product produced by living cells is dissolved by adding 100  $\mu$ L of DMSO. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on ELx800 universal microplate reader (Bio-Tek instruments) at 570 nm.

**Flow Cytometry:** Flow cytometry studies were performed on a Beckman-Coulter (Dako) CyAn ADP. The analyzer is equipped forward and side scatter and 9 colors of fluorescence using 405 nm, 488 nm and 635 nm excitations.

**Cell Cycle:** DNA content measurement identifies G<sub>0</sub>/G<sub>1</sub>-, S- and G<sub>2</sub>/M-phases of the cell cycle by propidium iodide staining. Cells were trypsinized, washed in PBS and then fixed in 70% ethanol and then stored at -20°C. The day prior to analysis the cells were washed in PBS and then stained with 0.5 ml of propidium iodide staining solution (1% Triton-X-100, 1 mg/ml PI, 100 mg/ml of RNase A) overnight at 4°C. Data were collected on Beckman Coulter CyAn analyzer at at The UNC flow cytometry core facility and ModFit (Becton Dickinson) was used for cell cycle analysis.

**Western blot analyses:** To evaluate protein expression cell lysates were resolved by SDS-polyacrylamide gel electrophoresis followed by western blot analysis with antibodies that recognize Ect2 (Millipore), HA (Covance),  $\beta$ -actin (Sigma), lamin A/C (Cell Signaling), and HSP 90 (Upstate).

**Ect2 RhoGEF activity assays:** The bacterially expressed glutathione S-transferase fusion protein containing the nucleotide-free mutant of RhoA [GST-RhoA(G17A)] was used as an affinity reagent to isolate active Ect2 expressed in cells were done as we have described in detail previously [110-112]. Briefly, Ect2 expressing cell lysates were normalized for equivalent total cellular protein and then incubated with 30  $\mu$ g of purified GST-tagged RhoA(17A) bound to glutathione-sepharose beads for 60 min at 4°C. Samples were then washed with lysis buffer and processed for SDS-PAGE.

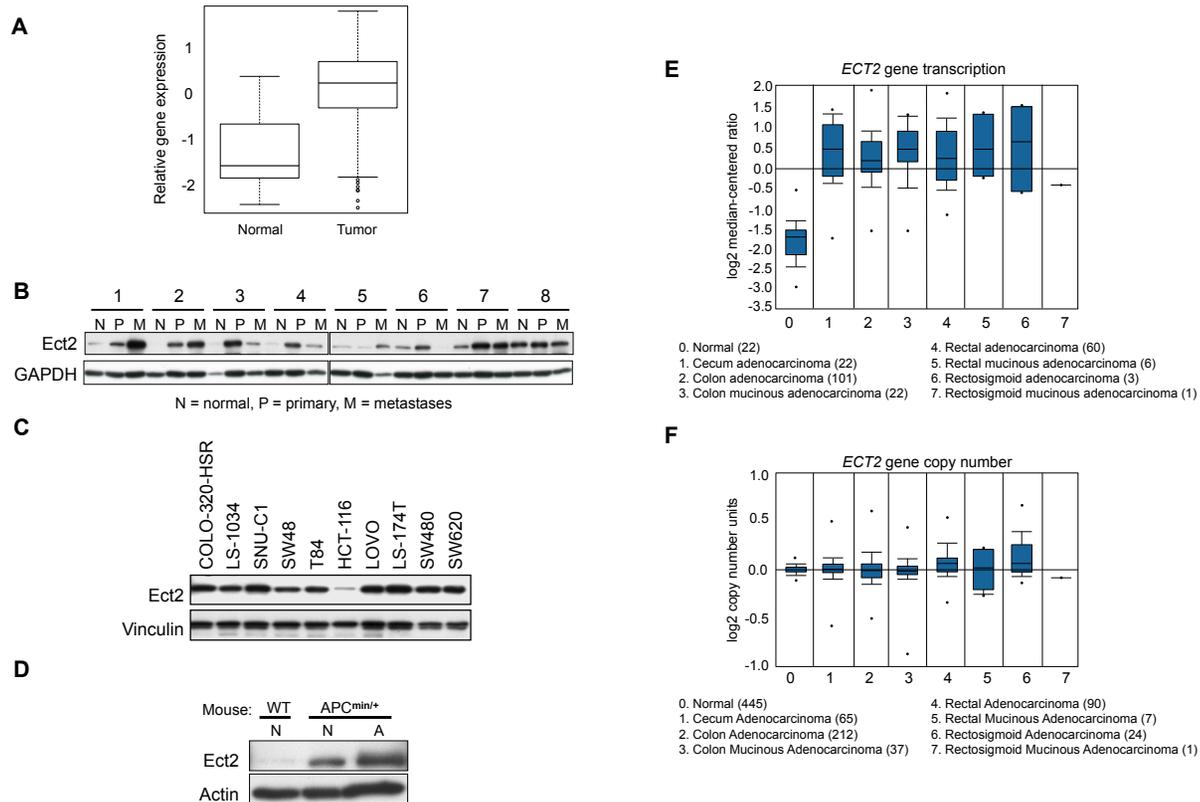
**Immunofluorescence:** Twenty-four h after attachment, coverslips fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. The cells were stained for 5 min with 5 ng/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS, washed 2-3 times with PBS and mounted with FluorSave (Calbiochem). The cells were visually analyzed for nuclei content on an Olympus BX61 upright fluorescence microscope. Images collected using Velocity (Perkin Elmer). To visualize polymerized F-actin, cells were stained with Alexa Fluor® 647 phalloidin (Invitrogen).

## **Results**

***Increased Ect2 gene and protein expression in CRC.*** To identify genes deregulated in expression in CRC, we performed gene microarray analyses on a

set of primary CRC and unmatched nontumor tissue. One gene found upregulated was *ECT2* (Figure 3-1A). To determine whether Ect2 protein expression is elevated in CRC tumors, we performed blot analyses on a panel of matched normal, primary and metastatic CRC tissue. Increased Ect2 protein that comigrated with FL Ect2 was seen in six of eight primary CRC tumors when compared with normal (Figure 3-1B), with variable results seen with metastatic tumors. We also found high Ect2 protein expression in a majority of CRC cell lines (Figure 3-1C). Finally, adenoma tissue from the APC/Min mouse also exhibited elevated FL Ect2 protein levels (Figure 3-1D). When considered with *APC* mutation as the first genetic alteration in CRC tumor progression [69], and the consistent increase seen in primary CRC tumors, this

Figure 1



### Figure 3-1. Increased *Ect2* RNA and protein expression in colorectal cancer

A. Box plot of *Ect2* mRNA expression from a microarray of 47 normal colon samples compared to 184 tumor samples. \*\*\*P-value = 4.784417e-16

B. *Ect2* protein expression in normal, primary, and metastatic tissues from 8 CRC patients. C. Elevated *Ect2* protein expression in panel of CRC cell lines. D. Increased *Ect2* protein in APC<sup>min</sup> mouse adenoma. E. *Ect2* mRNA levels in 215 colorectal adenocarcinoma and 22 paired normal colorectal tissue samples were analyzed F. No significant *ECT2* gene amplification in CRC. Data compiled from 436 CRC adenocarcinoma and 351 paired normal blood and 94 paired normal CRC tissue samples were analyzed.

suggests that increased Ect2 expression is an early event in CRC tumor progression.

Our identification of *ECT2* by gene array analyses suggested that increased Ect2 protein expression was due to increased *ECT2* gene transcription. To further address this possibility, we utilized OncoPrint analyses and found increased *ECT2* expression in both colon and rectal adenoma and adenocarcinoma from five published gene microarray datasets [113-117] as well as from the TCGA database (Figure 3-1E). In particular, the TCGA data set comprised of RNA data from 215 CRC adenocarcinoma and 22 paired normal CRC tissue and genomic DNA copy number data from 436 CRC and 351 paired normal blood and 94 paired normal colorectal tissue samples indicate that increased *ECT2* expression is not due to gene amplification (<http://tcga-data.nci.nih.gov/tcga/>) (Figure 3-1F). This result contrasts with lung cancer, where increased *ECT2* gene expression was attributed to *ECT2* gene amplification [38].

***Increased ratio of Ect2 cytoplasmic-to-nuclear staining correlates with improved CRC patient survival.*** In addition to overexpression, the deregulated subcellular localization of normally nuclear Ect2 has also been described as a mechanism for causing aberrant Ect2 function in cancer [36, 38]. To determine if cytoplasmic mislocalization is associated with Ect2 in CRC, after validating an anti-Ect2 antibody for immunohistochemistry (IHC) analyses, we performed Ect2 expression analyses on a CRC tumor microarray. The results are presented for 146 patients that had Ect2 scores for normal, tumor or both as well

as information on overall survival and clinicopathologic characteristics. Shown in Figure 3-2A are representative stained tumor sections. Tumors displayed a range of staining, with the moderate to high levels seen in tumor but not adjacent nontumor tissue.

The overall Ect2 score (sum of nuclear and cytoplasmic scores) was higher in the tumor compared to the adjacent normal tissue (87.9 versus 72.5, respectively), but the difference was not statistically significant ( $p=0.09$ ). Nuclear staining was more prevalent than cytoplasmic staining ( $51.1 \pm 3.6$ ; average of tumor and normal nuclear scores  $\pm$  standard error) versus  $29.1 \pm 2.6$ , respectively) ( $p < 0.001$ ). We also evaluated nuclear and cytoplasmic Ect2 protein expressions separately to determine the localization within each region. The tumor had higher Ect2 expression than the normal, for both cytoplasmic and nuclear sites ( $p=0.13$  nuclear,  $p=0.06$  cytoplasmic) (Figure 3-2B). When the ratio of Ect2 cytoplasmic score over Ect2 nuclear score was compared between tumor and normal, the ratio was significantly higher in the tumor tissues ( $p=0.01$ ) (Figure 3-2C).

Next we assessed the relationship between tertiles of Ect2 expression (low, medium and high) and clinicopathologic characteristics. There was a significant association between nuclear Ect2 expression and tumor grade. In particular, well-differentiated tumors tended to have low Ect2 nuclear expression, but as the grade increased to poorly differentiated tumors, Ect2 nuclear expression was increased ( $p=0.04$  for trend) (Table 3-1). Males tended to have higher cytoplasmic to nuclear Ect2 score ratios compared to females who were more likely to have lower Ect2 ratios (Table 3-2). Tumors located in the distal

colon and rectum also had higher Ect2 ratios than proximal tumors, which tended to have low Ect2 ratios (Table 3-2). No statistically significant associations were found between cytoplasmic Ect2 scores and evaluated clinicopathologic characteristics. Furthermore, there were no statistically significant associations between nuclear Ect2 expression alone or cytoplasmic Ect2 expression alone and overall survival. However, we observed that Ect2 cytoplasmic to nuclear ratio showed a positive relationship. Overall survival improved with increasing cytoplasmic to nuclear expression ratio ( $p=0.001$ ), suggesting that the site of localization of Ect2 either in the nucleus or cytoplasm may be a better determinant of CRC prognosis (Figure 3-2D).

**Table 3-1 Associations between patient characteristics and tertiles of ECT2 scores for nuclear expression**

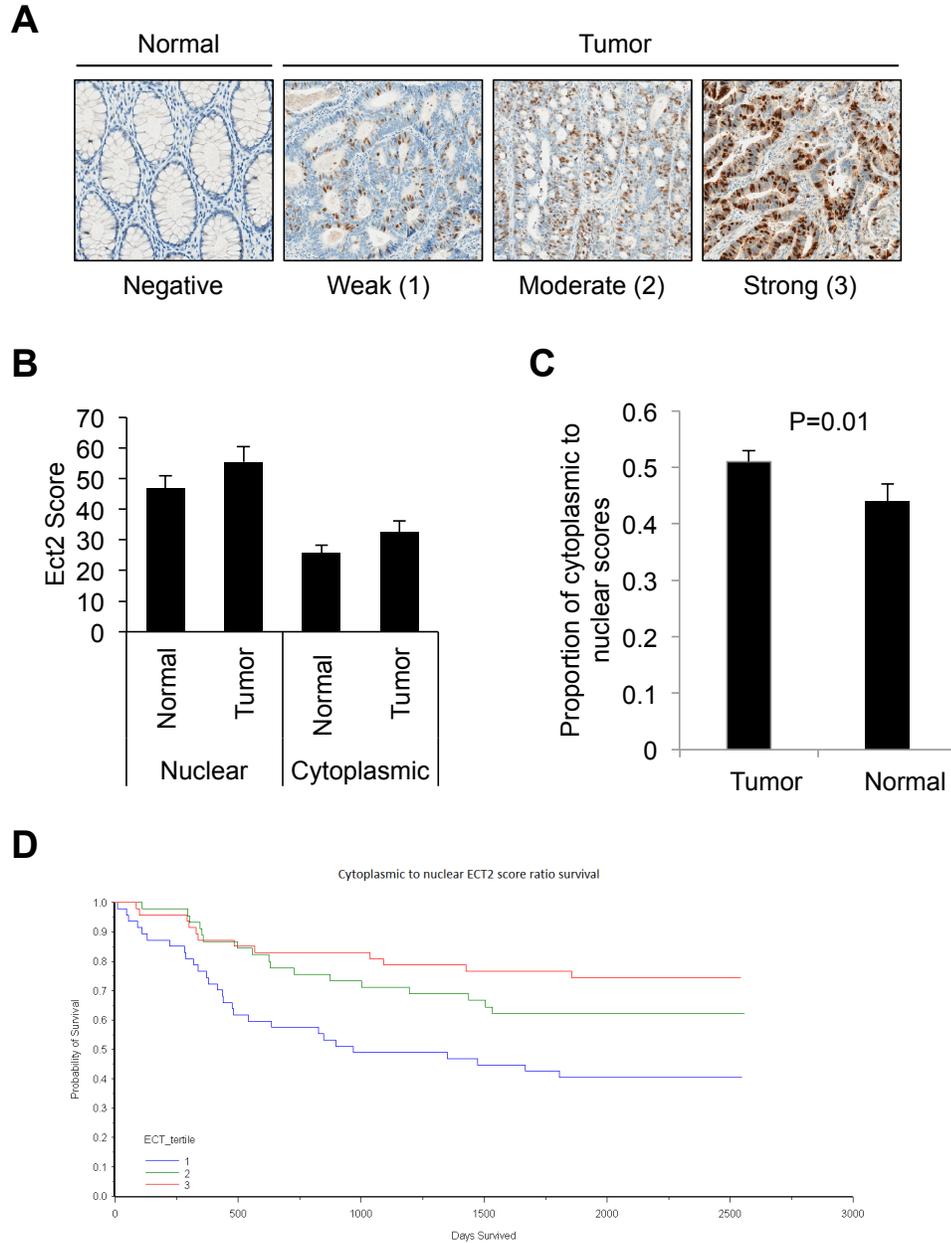
	N	ECT2 low	ECT2 medium	ECT2 high	P value
Age, mean (SE)	139	66.1 (1.6)	67.0 (2.0)	65.1 (2.1)	0.81
Gender, n (%)					
Male	77	24 (31)	26 (34)	27 (35)	0.54
Female	62	23 (37)	19 (31)	20 (32)	
Race, n (%)					
White	107	34 (32)	34 (32)	39 (36)	0.38
Black	29	11 (38)	10 (34)	8 (28)	
Stage, n (%)					
1+2	50	17 (34)	11 (22)	22 (44)	0.76
3+4	58	16 (28)	23 (40)	19 (33)	
Differentiation, n (%)					
Well	6	4 (66)	1 (17)	1 (17)	0.04
Mod	81	26 (32)	25 (31)	30 (37)	
Poor	18	3 (17)	6 (33)	9 (50)	
Location, n (%)					
Proximal	46	15 (33)	14 (30)	17 (37)	0.62
Distal	56	18 (32)	22 (39)	16 (29)	

To address if Ect2 expression correlates to patient characteristics leading to an increase in mortality, we employed Cox proportional hazards models. In the univariate model, a higher Ect2 cytoplasmic to nuclear ratio was significantly

associated with a lower risk of death in a piecewise manner: as the ratio increased by 0.5 increments, the risk of death decreased by 40%. However, increasing age (by 10 year increments), higher tumor stage than lower stages, and poor tumor grade were associated with increased risk of death. Even after adjustment for age, stage, differentiation, location of tumor and chemotherapy, a higher cytoplasmic to nuclear Ect2 ratio still demonstrated a reduced risk of dying that was statistically significant, suggesting that a high proportion of cytoplasmic Ect2 expression to nuclear expression may be a good prognostic marker in CRC.

**Table 3-2 Associations between patient characteristics and tertiles of ECT2 scores for the ratio of cytosolic to nuclear expression**

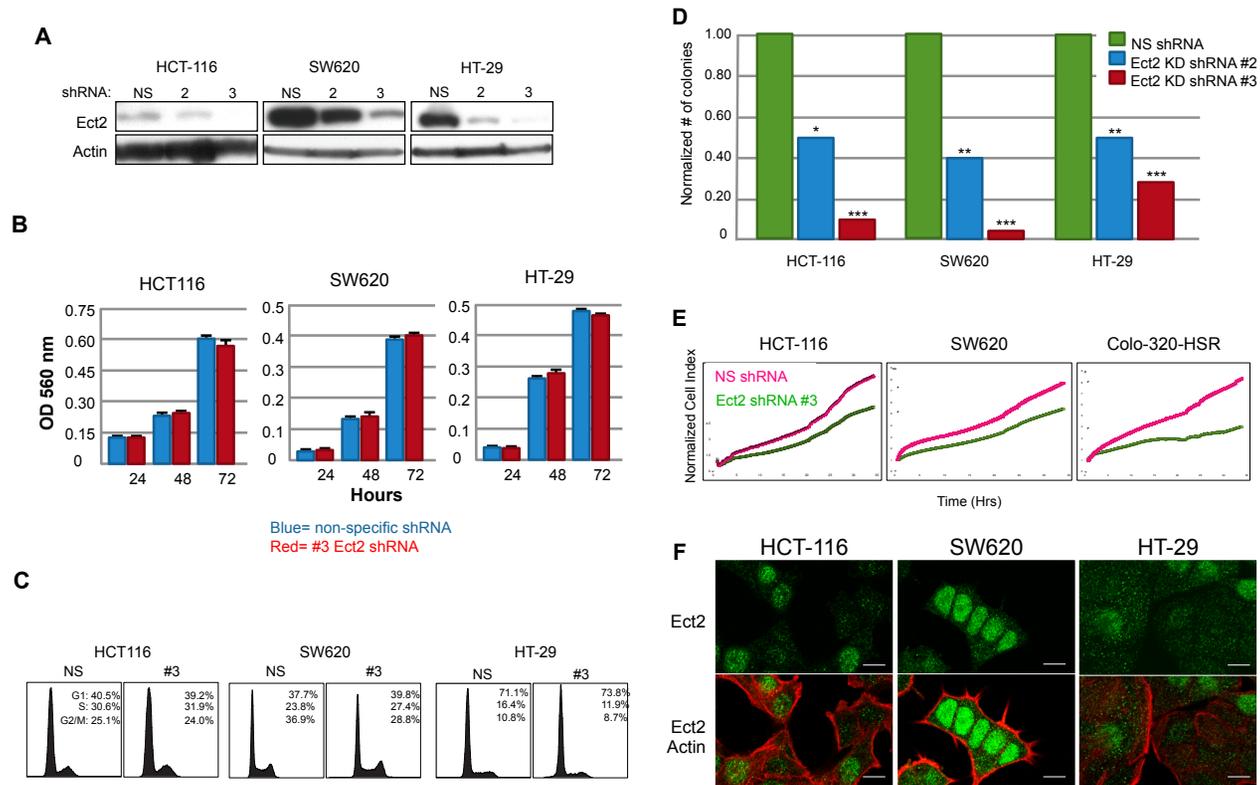
Characteristic	N	ECT2 low	ECT2 medium	ECT2 high	P value
Age, mean (SE)	139	67.8 (1.9)	67.0 (1.9)	63.5 (1.9)	0.25
Gender, n (%)					
Male	77	21 (27)	26 (34)	30 (39)	0.06
Female	62	26 (42)	19 (31)	17 (27)	
Race, n (%)					
White	107	39 (36)	34 (32)	34 (32)	0.29
Black	29	8 (28)	9 (31)	12 (41)	
Stage, n (%)					
1+2	50	12 (24)	16 (32)	22 (44)	0.77
3+4	58	14 (24)	21 (36)	23 (40)	
Differentiation, n (%)					
Well	3	0 (0)	2 (33)	4 (67)	0.48
Mod	57	22 (27)	27 (33)	32 (40)	
Poor	14	4 (22)	7 (39)	7 (39)	
Location, n (%)					
Proximal	46	19 (41)	15 (33)	12 (26)	0.07
Distal	56	15 (27)	18 (32)	23 (41)	



**Figure 3-2 Increased ratio of cytosolic to nuclear Ect2 correlates with overall survival**

A. Increased Ect2 cytoplasmic and nuclear staining in CRC tumor. Representative examples of Ect2 staining by IHC for adjacent nontumor colorectal tissue and colorectal tumor tissues with weak, moderate, or strong intensity of Ect2 staining. Shown are 5x magnification in Aperio Imagescope. B. Ect2 expression in tumor cell cytoplasm and nuclear compartments. Ect2 score comparison between normal and tumor by nuclear and cytoplasmic locations. (P-value=0.13 nuclear, P-value=0.06 cytoplasmic) C. Increased cytoplasmic-to-nuclear Ect2 staining in CRC tumor tissue. The ratio of Ect2 cytoplasmic score over ECT2 nuclear score was compared between tumor and normal, the ratio was significantly higher in the tumor tissues (P-value=0.01) D. Increased cytoplasmic-to-nuclear Ect2 staining correlates with improved CRC patient survival. Kaplan-Meier overall survival curve for the ratio of cytosolic to nuclear Ect2 (P-value=0.001)

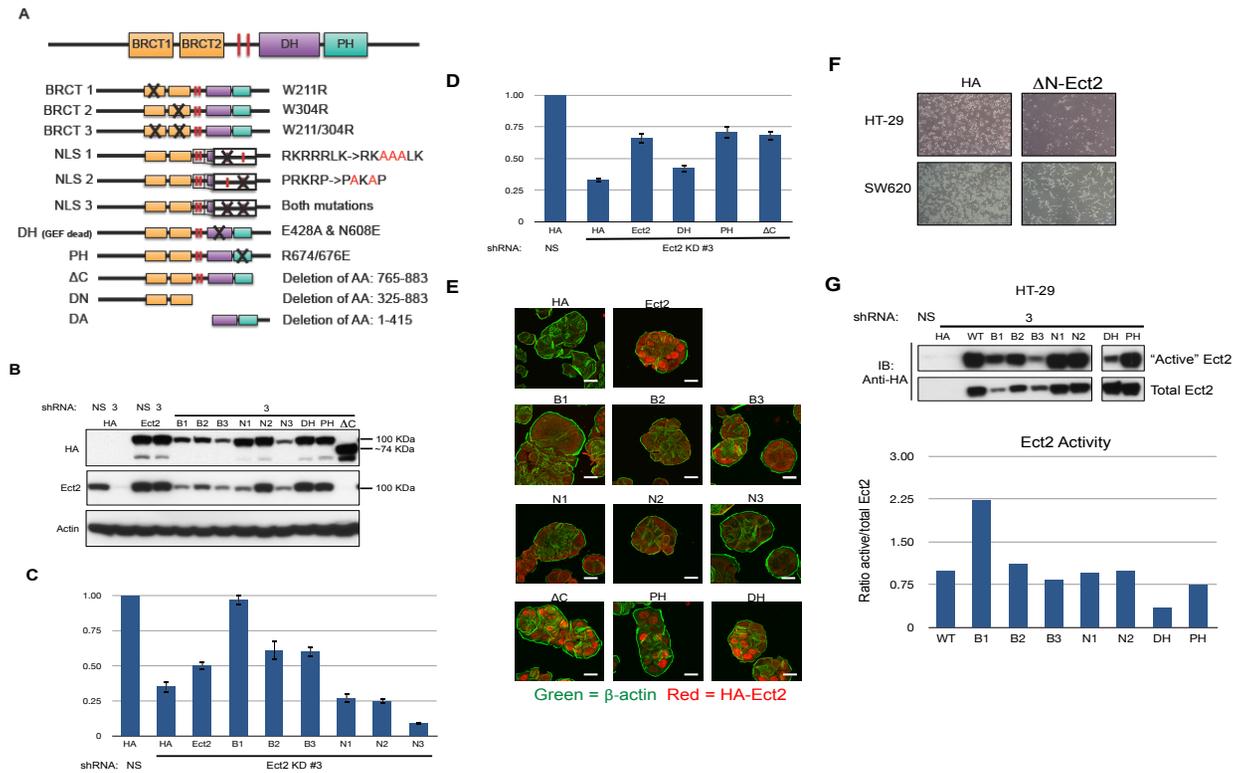
***Ect2 promotes CRC anchorage-independent growth and Matrigel invasion.*** To address a role for cytoplasmic and nuclear Ect2 in CRC biology, we first determined if Ect2 contributes to the aberrant growth properties of CRC cell lines. Utilizing two previously validated lentivirus-based *ECT2* shRNA vectors [38], we established CRC cell lines with stable suppression of Ect2 protein expression (Figure 3-3A). Suppression of Ect2 expression did not significantly perturb anchorage-dependent cell proliferation (Figure 3-3B) or alter cell cycle progression (Figure 3-3C). However, a significant reduction in anchorage-independent growth as determined by reduced colony formation in soft agar (Figure 3-3D) or invasion through Matrigel (Figure 3-3E) was seen. Thus, similar to lung cancer cells [38], endogenous Ect2 expression functions as a driver of CRC tumor cell growth and invasion independent of its role in regulation of cytokinesis.



### Figure 3-3 Ect2 is required for CRC cell line anchorage-independent growth and Matrigel invasion

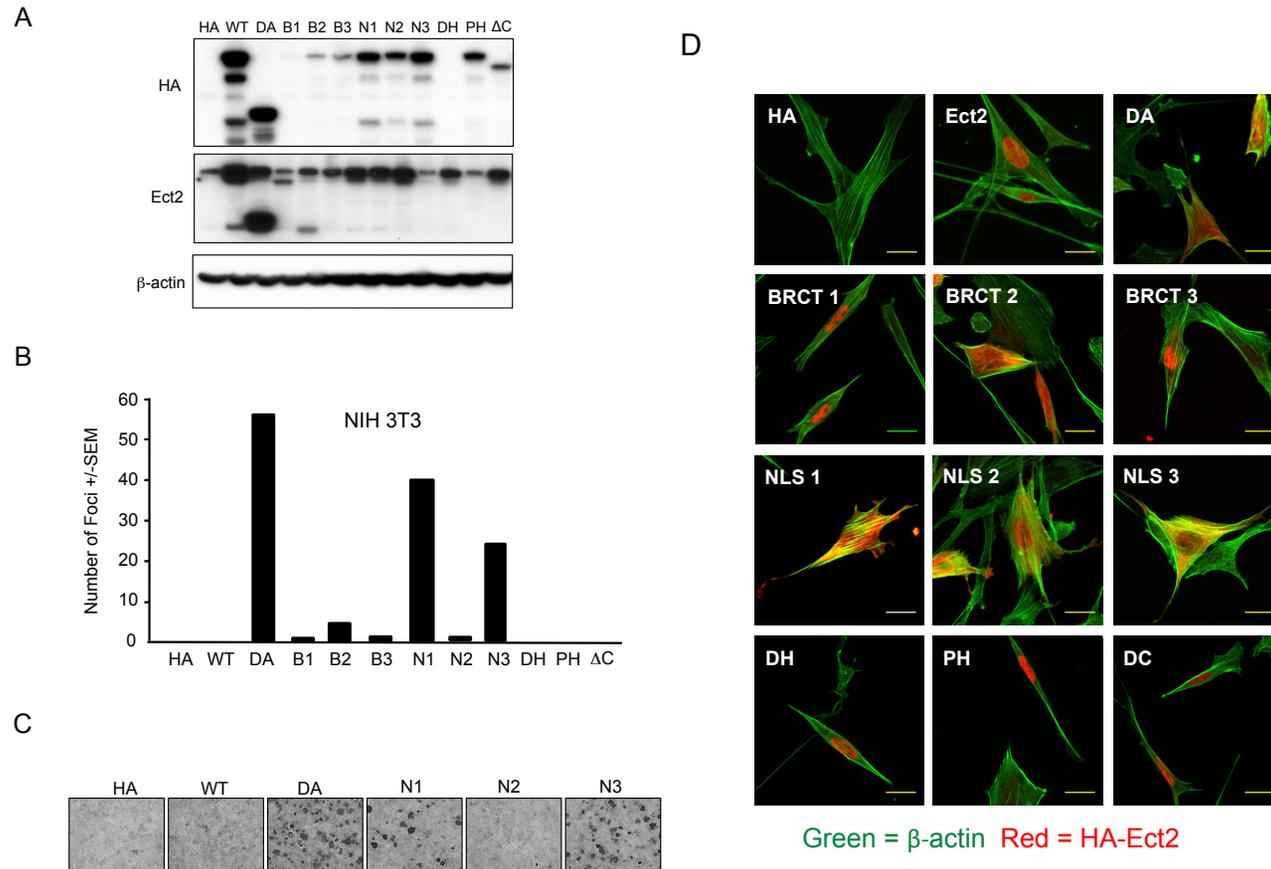
A. Western blot of the indicated CRC cell lines stably-infected with shRNA sequences targeting two different *ECT2* sequences and non-targeting (NS) shRNA negative control. B. An MTT viability assay was used to monitor CRC cell line proliferation. C. Cell cycle analysis was done by DNA-content staining with propidium iodide (PI) of the indicated CRC cell lines. Data shown are representative of three independent experiments. D. Suppression of Ect2 expression reduces CRC cell line anchorage-independent growth. Normalized graph of anchorage-independent growth as a function of Ect2 expression in CRC cell lines (n=3, P-value: \* P<0.05, \*\*P<0.01, \*\*\* P<0.001) E. Suppression of Ect2 expression reduces invasion in vitro. Real-time analyses of invasion (xCELLigence, Roche) of CRC cells through Matrigel. F. Immunofluorescence of endogenous Ect2 in green and actin in phalloidin; scale bars are 20  $\mu$ m

Whereas in a recent analyses in lung cancer found that cytoplasmic Ect2 was important for driving lung cancer growth [38], our IHC analyses found that increased nuclear Ect2 was associated with CRC progression and that cytoplasmic Ect2 may have a protective role. To address a role for nuclear localization in Ect2 support of CRC growth, we generated a set of HA epitope tagged structural/functional mutants of full length Ect2 (Figure 3-4A). We then utilized HT-29 cells to evaluate the structural requirements for Ect2 support of anchorage-independent growth. Suppression of endogenous Ect2 reduced colony formation and ectopic expression of wild type FL Ect2 at levels comparable or higher than endogenous restored colony formation (Figure 3-4B and C). In contrast, expression of a putative catalytic mutant with DH domain missense mutations did not restore colony formation, supporting the RhoGEF function of Ect2 in driving cancer growth. We next evaluated versions of full-length variants of Ect2 with missense mutation of one (N1 or N2) or both (N3) NLS motifs. In contrast to endogenous Ect2 (Figure 3-2F) or ectopic expression of HA-Ect2 (Figure 3-4D), the NLS mutant proteins showed loss of nuclear staining and instead exhibited strong cytoplasmic staining (Figure 3-4D). In contrast to either ectopic WT or BRCT mutants of Ect2 that rescue colony formation, none of the NLS mutants where able to restore colony formation. These results suggest that the nuclear localization of Ect2 is required to support CRC growth.



### Figure 3-4 Nuclear Ect2 is required to support anchorage-independent growth

A. Diagram of Ect2 structural mutants showing missense or deletion mutations. B. Western blot of cells with stable shRNA suppression of endogenous Ect2 and ectopic expression of HA epitope-tagged Ect2 mutants in HT-29 cells. C, D. Analysis of CRC anchorage-independent growth. Representative experiment of BRCT and NLS Ect2 mutants (panel C) and then DH, PH and  $\Delta$ C Ect2 mutants (panel D) compared to vector (HA) and WT Ect2 rescue. E. Immunofluorescence of HA-tagged Ect2 mutants in HT-29, where red is anti-HA and green is phalloidin; scale bars are 20  $\mu$ m. F. Shown are phase-contrast bright field CRC cells infected with a lentiviral expression vector encoding  $\Delta$ N-Ect2. G. Equivalent activation state of cytoplasmic and nuclear Ect2. Pulldown analyses using the nucleotide-free RhoA mutant that binds preferentially to activated RhoGEFs.



**Figure 3-5 Cytoplasmic Ect2 causes morphologic and growth transformation of NIH 3T3 mouse fibroblasts.**

A. Ectopic expression of WT and mutant Ect2 in NIH 3T3 cells. Western blot analysis of NIH 3T3 expressing HA-tagged Ect2 mutants B, C. NLS mutants of Ect2 causes transformation of NIH 3T3 cells. Quantitation of focus forming activity (panel B) with photographs of representative fields of NIH 3T3 cultures (panel C). D. Immunofluorescence of HA epitope-tagged Ect2 mutants in NIH 3T3, where red is anti-HA and green is phalloidin; scale 20  $\mu$ m.

We also assessed the role of other sequences and domains in Ect2-driven CRC growth. We previously found that the C-terminus of Ect2 was essential for the ability of  $\Delta$ N-Ect2 to transform NIH 3T3 cells and deletion of this C-terminal sequence from FL Ect2 caused a partial reduction in its ability to restore colony formation (Figure 3-4B). We also found previously that mutation of the PH domain did not impair  $\Delta$ N-Ect2 transforming activity and similar mutation of the PH domain did not reproducibly reduce the ability of FL Ect2 to restore colony formation. Thus, in contrast to other RhoGEFs, the PH domain does not appear to be critical for DH domain function. Finally, we also assessed the roles of the BRCT domains. Previous studies found that the BRCT domains can serve as autoinhibitory domains by associating with the DH-PH domains and preventing effective GEF catalytic activity [31]. Additionally, the BRCT domains may interact with phosphorylated proteins and regulate Ect2 recruitment to the central spindle complex during cytokinesis [77, 78]. Missense mutation of the conserved tryptophan (W) residue was shown previously to disrupt the structural integrity and function of the BRCT domain [31]. Interestingly, we found that the single B1 or B2 or tandem B3 BRCT mutant proteins were also mislocalized from the nucleus and showed enhanced cytoplasmic staining (Figure 3-4D). Despite mislocalization and reduced expression of the BRCT domain mutants (B1, B2, & B3), these mutants were still able to rescue colony formation (Figure 3-4C).

Altered subcellular localization from the nucleus to the cytoplasm may alter the intrinsic RhoGEF activity of Ect2 and contribute to the reduced ability of the NLS mutants. We previously described the use of dominant negative

nucleotide-free mutants of Rho GTPases that form nonproductive complex with RhoGEFs, as an affinity reagent to monitor the RhoGEF catalytic activity of cell expressed proteins [110-112]. Using this approach, we first validated that the recombinant GST-RhoA 17A fusion protein preferentially recognized wild type Ect2 relative to the GEF-dead DH domain mutant protein (Figure 3-4D). In contrast, both the N1 and N2 mutants were efficiently precipitated at a level comparable to wild type Ect2. Thus, the mislocalization of the NLS mutants did not impair their intrinsic RhoGEF catalytic function and the reduced colony formation activity may then be due to activation of different subcellular pools of Rho GTPases.

***Cytoplasmic localization is associated with Ect2 transformation of NIH 3T3 fibroblasts.*** Whereas the cytoplasmic, constitutively activated  $\Delta$ N-Ect2 truncated protein exhibits potent transforming activity when assayed in NIH 3T3 fibroblasts, no truncated proteins have been described in human cancers. Thus, we speculated that perhaps  $\Delta$ N-Ect2 may not be effective in supporting CRC growth. For these analyses we established NIH 3T3 cells stably expressing FL Ect2 wild type and mutant proteins (Figure 3-5A). We first verified that under our experimental conditions that  $\Delta$ N-Ect2 is transforming and cytoplasmic mislocalized in NIH 3T3 cells (Figure 3-5B and C). Surprisingly, when we utilized a lentivirus-based cDNA expression vector (puromycin resistant) encoding  $\Delta$ N-Ect2, we failed to isolate puromycin-resistant stable populations of HT-29 cells that ectopically expressed this truncated protein. The cells that arose showed altered cellular morphology, elongated and poorly adherent, suggesting an

induction of apoptosis (Figure 3-4E). However, we were unable to verify the basis for this growth suppression since there was insufficient viable cells to perform a detailed analysis of the mechanism of growth inhibition.

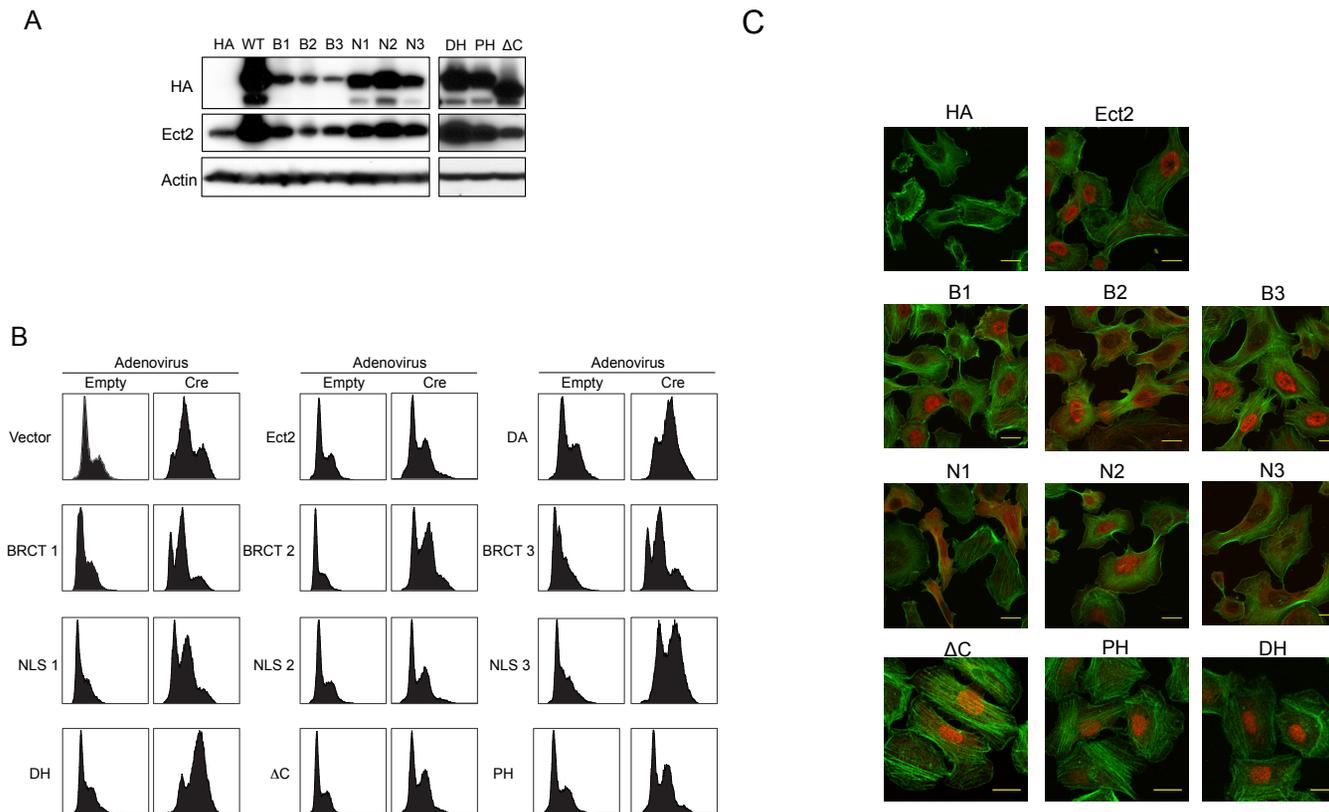
We also evaluated the transforming activity of full length Ect2 structural/functional mutants. The transforming activity of the NLS mutants correlated with Ect2 mislocalization, with N1 and N3 mutants having increased cytosolic Ect2 and focus forming activity compared to N2 (Figure 3-5A and C). Unfortunately, while the BRCT mutants are also mislocalized (Figure 3-5D) to the cytosol these mutants did not express at the same levels of the NLS mutants and therefore the transformation ability is not comparable. However, some foci do arise from the BRCT mutants suggesting that these proteins may have transforming activity.

***Ect2 support of CRC growth is independent of its role in cytokinesis.***

Our finding that shRNA suppression of Ect2 did not reduce CRC anchorage-dependent proliferation (Figure 3-2D) suggests that the impaired anchorage-independent growth and invasion was not due simply to a defect in cytokinesis. Consistent with this possibility, our flow cytometry analysis did not find increased that is consistent with impaired cytokinesis (Figure 3-2E). This result contrasts with our previous finding that Ect2 depletion in mouse embryo fibroblasts (MEFs) caused significant accumulation of cells in G2/M. Taken together, these results suggest that Ect2 function in cytokinesis is mechanistically distinct from Ect2 supported cancer growth. To address this question, we assessed the ability of

different Ect2 structural mutants to rescue the loss of endogenous Ect2 to support cytokinesis in MEFs.

In contrast to anchorage independent growth in CRC cells, where the BRCT domains are dispensable for growth, cytokinesis requires intact BRCT domains. (Figure 3-6A and B) The Ect2 BRCT mutants expressed in MEFs also display mislocalization similar to CRC cells and NIH 3T3 fibroblasts (Figure 3-6C). Additionally, the NLS mutants are also mislocalized and cannot rescue cytokinesis (Figure 3-6A-C). As expected, the GEF dead mutant (DH) cannot support cytokinesis Ect2, consistent with the requirement of Ect2 GEF activity for cytokinesis. Surprisingly, the C-terminal truncation and the PH domain missense mutant were able to rescue cytokinesis. Both of these mutants are properly localized to the cells nucleus during interphase and are able to bind dominate negative nucleotide-free RhoA, suggesting other functions of these domains are not necessary for normal function. Finally, we found that ectopic expression of the constitutively activated  $\Delta$ N-Ect2 truncated protein, as we saw with NIH 3T3 cells, was well tolerated by the *Ect2*<sup>fl/fl</sup> MEFs prior to Cre-mediated loss of Ect2. However, this variant did not support cytokinesis, most likely due to the loss of the NLS motifs and its cytosolic localization.



**Figure 3-6 Nuclear localization is essential for Ect2 to support normal cell cytokinesis in mouse embryo fibroblasts**

A. Western blot of MEFs expressing HA-tagged Ect2 mutants. B. NLS and BRCT mutants fail to restore cytokinesis in cells deficient in endogenous Ect2 expression. Representative cell-cycle graphs, by DNA content with propidium iodide of MEFs expressing HA-tagged Ect2 mutants then treated with either empty (control) or Cre (excise endogenous Ect2) adenovirus. C. Immunofluorescence of HA-tagged Ect2 mutants in MEFs, where red is anti-HA and green is phalloidin; scale bar is 20  $\mu$ m.

## ***Discussion***

Conventional chemotherapy with cytotoxic drugs remains the foundation for first-line treatment of metastatic CRC, with limited advances in the development of signal transduction-targeted therapies for this cancer. To identify new molecular determinants for CRC diagnosis and therapy, we performed a microarray study and identified elevated *ECT2* gene transcription in primary CRC. Since Ect2 was identified originally as an activated oncogene, we determined if Ect2 protein expression was altered and served a drive role in CRC. In particular, since previous studies in other cancers suggested that mislocalization of the normally nuclear restricted Ect2 into cytoplasm may facilitate aberrant Ect2 function [36, 38], we additionally addressed the role and function of nuclear and cytoplasmic Ect2 in CRC growth. We determined that Ect2 protein levels are altered in both expression levels and subcellular localization in CRC tumors and cell lines and we established a role for Ect2 in CRC cell line growth and invasion in vitro that is distinct from the involvement of Ect2 in cytokinesis. Unexpectedly, our IHC analyses of CRC tumors found that the cytoplasmic to nuclear ratio correlated with better patient survival. Consistent with this observation, we found that nuclear Ect2 is critical for driving CRC growth whereas cytoplasmic Ect2 activation was deleterious for CRC cell viability. Our findings also provide an explanation for why the originally discovered truncated and oncogenic Ect2 variants identified in mouse fibroblast transformation assays have not been found in human cancers.

Our determination that Ect2 is overexpressed in CRC, and that suppression of Ect2 expression impaired CRC cell line growth and invasion in vitro, expand the spectrum of human cancers where Ect2 may serve a driver function. Previously, Ect2 protein overexpression has been described for glioblastoma, lung and esophageal cancer [38, 61-63]. Our identification of *ECT2* RNA overexpression by microarray analyses and our OncoPrint evaluation of existing microarray databases indicate that the increased levels of Ect2 protein is driven by increased gene transcription. Our OncoPrint analyses of existing database information support our suggestion that this is caused by an increase in gene transcription, but not by the gene amplification which the causes Ect2 overexpression in lung cancer [38]. A future direction of our studies will be to identify a mechanism for aberrant *ECT2* gene overexpression in CRC. Our finding that Ect2 protein levels were elevated in adenoma tissue from the APC min mouse, together with OncoPrint data that also showed increased *ECT2* in patient adenoma tissue, argue that *ECT2* upregulation likely occurs early in tumor progression.

Ect2 was identified originally as an N-terminally truncated and constitutively activated protein ( $\Delta$ N-Ect2), in the NIH 3T3 focus formation assays that discovered mutationally activated Ras proteins in human cancers [19]. A key basis for the transforming activity of  $\Delta$ N-Ect2 was loss of NLS motifs, rendering the normally nuclear restricted Ect2 mislocalized into the cytoplasm. While no equivalent Ect2 truncation mutants have been described in human cancers, a recent study found that phosphorylation of full length Ect2 may force cytoplasmic

localization that may be critical for a role for Ect2 in driving lung cancer growth. To assess whether a mislocalization mechanism was also important for Ect2 overexpression in CRC, we performed a detailed analysis of nuclear and cytoplasmic Ect2 in a CRC tumor tissue microarray. Although not significant, we observed an increase in Ect2 expression in both nuclear and cytoplasmic compartments compared to normal adjacent tissue. However, surprisingly, our analyses found that the ratio of cytoplasmic to nuclear Ect2 in CRC compared to normal adjacent tissue is statically significant ( $p < 0.001$ ) and an increased ratio correlates with increased overall survival. Our observations suggest that nuclear Ect2 is important for supporting CRC progression and/or growth, whereas cytoplasmic Ect2 may be beneficial for patient survival. This proposal contrasts with a recent study that speculated that cytoplasmic Ect2 was an important driver of lung cancer growth [38], suggesting cancer type differences in Ect2 driven cancer growth. In summary, our results emphasize the importance of assessing both Ect2 subcellular localization as well as overexpression in evaluating an association between aberrant Ect2 expresison and clinicopathologic characteristics.

Since the localization data from our IHC analyses was unexpected, we utilized CRC cell lines that we established to be Ect2-dependent for anchorage-independent growth to evaluate a role for nuclear and cytoplasmic Ect2 in CRC growth. We utilized various structure/function Ect2 mutants to address the mechanism of Ect2 function in CRC compared to NIH 3T3 transformation and normal cell cytokinesis. Importantly, our studies are consistence our observation

that cytosolic Ect2 may be beneficial since the cytoplasm mislocalized Ect2 mutants (N1-3) did not support CRC anchorage independent growth (Figure 3-5C). However, BRCT Ect2 mutants were also cytoplasmic mislocalized but still had nuclear Ect2 suggesting that it is nuclear Ect2 that is required for growth. This observation also highlights the importance of the ratio of cytosolic to nuclear Ect2, suggesting that it is the relative proportion of Ect2 in these cellular compartments that determines growth. We also found that the transforming variant of Ect2 ( $\Delta$ N-Ect2) cannot support CRC cell growth but instead it was highly deleterious to CRC growth in vitro. Thus, our mechanistic studies are consistent with our findings with CRC patient tumors, and support our hypothesis that cytoplasmic Ect2 is detrimental for cancer growth and also addresses why a constitutively activated truncated protein Ect2 has not been found in cancer. A future question will be to assess mechanisms that cause Ect2 mislocalization to the cytoplasm that may then be exploited pharmacologically to convert Ect2 from an oncogene into a tumor suppressor. Another issue will be determine whether Ect2 activates the same Rho family small GTPases in the cytoplasm and nucleus and whether the effectors activated by these GTPases are distinct.

Similar with observations in lung cancer [38], we demonstrate that loss of Ect2 in CRC cell lines does not cause a defect in cell proliferation, suggesting that the role of Ect2 in cancer is distinct with its role in normal cell cytokinesis. To address this question specifically we used Ect2 structure/function mutants to identify what domains are required for cytokinesis. We found that unlike CRC cell lines, MEFs required intact BRCT domains. Furthermore, the NLS mutants did

not support cytokinesis suggesting that proper localization is also required for Ect2 function in cytokinesis. This result was unexpected, since the nuclear envelope is absent during cytokinesis [24]. Perhaps nuclear sequestration of Ect2 is needed as the nuclear envelope reforms and cells complete cytokinesis and exit from mitosis. As expected, we showed that the GEF-dead Ect2 mutant DH does not support cytokinesis, demonstrating that Rho GTPase regulation is the basis for Ect2 function in cytokinesis. This is consistent with previous studies implicating RhoA activation in Ect2-dependent cytokinesis [81]. In contrast, we found that mutation of the PH domain did not impair Ect2 support of cytokinesis, an unexpected finding since the PH domain is typically essential for proper DH domain function for other Dbl family RhoGEFs. Another surprising result was our determination that the C-terminal sequence following the tandem DH-PH domain is also dispensable for cytokinesis. Previously, we showed that this C-terminal sequence was necessary for  $\Delta$ N-Ect2 transformation of NIH 3T3 cells, by dictating RhoGEF specificity in vitro [43]. More recently it was shown that Ect2 protein levels during mitosis was regulated through APC/Cdh1 interaction with the C-terminus [46]. However, our results suggest that while the C-terminus of Ect2 may have functional roles it is not necessary to complete cell cycle. Normal cell cytokinesis is a highly regulated cell program that requires appropriate temporal and spatial signaling. Thus as expected, most of the Ect2 structure/function mutants cannot support proper cytokinesis.

In summary, our studies provide further evidence that Ect2, a protein essential for normal cell cytokinesis, when overexpressed and mislocalized in

cancer, can acquire a driver role in cancer by a mechanism independent of regulation of cytokinesis. Of the more than 70 Dbl family RhoGEFs, Ect2 together with the Vav and P-Rex RhoGEF isoforms comprise a small handful with significant roles in cancer. Of these RhoGEFs, whereas P-Rex and Vav are mainly activators of Rac, Ect2 is primarily an activator of RhoA. Thus, in addition to being only one of two Dbl RhoGEFs with nuclear localization and possessing domains not found on any other RhoGEF, Ect2 may also have an unusual role in human carcinogenesis.

## ***Chapter 4 Final thoughts and future directions***

### ***Overview***

My dissertation research studies have addressed the roles and mechanisms by which the Ect2 RhoGEF regulates normal cell cytokinesis and tumor cell growth. The key findings from my studies are that (1) Ect2 is essential for development, most likely due to its critical role in normal cell cytokinesis, (2) Ect2 overexpression is a driver of CRC growth, (3) Ect2 may serve distinct nuclear and cytoplasmic functions in CRC growth, and finally, (4) the structural requirements for Ect2 are distinct for growth transformation of mouse fibroblasts and CRC, and for regulation of normal cell cytokinesis. My studies may also provide an explanation for a major paradox in the field, why the potentially activated Dbl RhoGEFs identified initially as truncated proteins in NIH 3T3 transformation assays have not been found in human cancers. Below I discuss some key future questions and directions that have been prompted by my findings.

### ***Determining Nuclear Versus Cytosolic Ect2 Function***

The data from our recent structure-function studies of Ect2 in colorectal cancer suggests that nuclear and cytosolic Ect2 have different functions. Furthermore, the function of Ect2 in these individual compartments may be antagonistic and consistent with our TMA data from patients where a higher ratio of cytosolic compared to nuclear Ect2 correlated with increased overall survival.

To further validate our findings that cytosolic Ect2 could be negatively regulating anchorage-independent growth we could use nuclear transporter inhibitors, leptomycin A and/or B, in hopes that these are able to block the ability of Ect2 to become cytosolic. Leptomycin A and B are both potent inhibitors of nuclear export while the exact target of A is unknown B specifically blocks CRM1 (chromosomal region maintenance)/exportin 1 [118-120]. If these inhibitors are able to block cytosolic Ect2 we will investigate anchorage-independent growth in the presence of nuclear transport inhibitors. If nuclear localization is the critical site for Ect2 driven CRC growth, then forced nuclear retention may actually enhance CRC growth. However, there since these inhibitors also block the transport of other proteins there could be significant off-target effects.

The basis for cytoplasmic mislocalization is not clear. One simple explanation may be that mere overexpression saturates nuclear interactions, resulting in cytoplasmic "leakage". One approach to evaluate this will be to use an inducible expression system where the levels of Ect2 expression could be titrated. There are many systems that would be amenable to such manipulation, for example the doxycycline-TET or the cumate inducible system by Systems Bioscience. In our current system, overexpression of WT Ect2 leads to an increase in cytosolic Ect2 and therefore may be the reason for both variable and reduced rescue in soft agar. In an inducible system we should be able to have a range of Ect2 expression such that the WT rescue could have no cytosolic Ect2

up to an expression level where there is additional Ect2 in the cytoplasm allowing us to see the effect of mislocalized Ect2 in anchorage-independent growth.

Another approach to evaluate the role of subcellular localization can be to generate an Ect2 variant that is more strongly nuclear sequestered. To accomplish this, we could tag Ect2 with an additional stronger nuclear localization sequence (NLS) such as the SV40 large T-antigen sequence. However, if the mislocalization of Ect2 to the cytoplasm is dependent on the expression level, it suggests that it is excess of protein that is causing the mislocalization, then it is possible that this approach may not work.

If Ect2 has distinct biological roles in the cytoplasm and nucleus, one logical basis for this is that each pool will activate a spatially distinct pool of RhoA, that then utilizes spatially distinct effectors. The impact of subcellular localization in determining effector utilization is seen in situations where Ras family GTPase subcellular localization is controlled by phosphorylation, leading to interactions with different effectors [6, 80, 121]. Currently there are limited methods that allow one to look at GTPase activity cellular compartments. However, genetically encoded biosensors would allow us investigate protein activity in live cells. Specifically, Dr. Kaus Hahn and colleagues have developed FRET (fluorescent or Förster resonance energy transfer) based biosensors of Rho GTPase [122, 123]. These biosensors allow one to investigate GTPase activity real-time with spatial resolution in live cells, allowing us to determine the location of GTPase activity to better understand differences between nuclear versus cytosolic Ect2 downstream activity.

Although my pull down analyses with dominant negative RhoA suggests that Ect2 is equally active in the nucleus and cytoplasm, whether this approach accurately measures true catalytic activity is not known. Another possibility would be to design a FRET biosensor for Ect2. While there are no published reports of RhoGEF biosensors we know of groups taking this approach and while having an Ect2 biosensor would be a nice tool it would likely take considerable time to optimize. The ability for a biosensor to work depends on many factors, for instance FRET between fluorescent proteins depends on the relative dipole orientation of each fluorescence protein, for efficient energy exchange the dipoles need to be perpendicular to each other. The distance between the fluorescent proteins need to be less than 10 nm, for FRET to occur, and many biosensors require a linker region between the fluorescent protein and the protein of interest that usually needs to be optimized. Finally, since there is no known structure or models of Ect2 folding, no other RhoGEF biosensors to model from, designing an Ect2 biosensor would probably be time consuming but not impossible to achieve.

Finally, as stated above, it is likely that Ect2 will interact with distinct proteins when in the nucleus and cytoplasm. This is particularly relevant since the BRCT domains can act as protein-protein interaction domains. To address this possibility, we could use a proteomic approach in tandem with a signaling pathway screen to identify nuclear from cytosolic Ect2 function in CRC. We will use mass spectrometry to identify novel binding partners of Ect2 in cytosolic versus nuclear cellular compartments by immunoprecipitating endogenous Ect2 from these compartments. Use of Ect2 domain/deletion mutants can then be

used to map the site of interaction of these proteins. This would not only be the first complete proteomic analysis of Ect2 but also the first step in identifying proteins important in Ect2 function in nuclear or cytosolic compartments. To complement this approach we would do a screen for changes in pathways upon Ect2 induction. There are a variety of techniques or screens we could use to address changes in signaling pathways. We would do RNA sequencing, which provides quantifiable changes in all RNA: non-coding, coding and micro RNA [124]. Another approach we would employ is proteome phospho-arrays by R&D systems where we can look at changes in phosphorylation status of receptor tyrosine kinases (RTK) and other intracellular kinases by western blot. Identifying proteins that interact with Ect2 along with signaling pathways that change upon loss or activation of Ect2 will allow for a more detailed insight into Ect2 function.

### ***Inhibitors of Ect2-mediated exchange***

Our studies have validated Ect2 as a driver in CRC. However, RhoGEFs are not classically druggable proteins. However, druggability is based more on success, and protein kinases were once considered undruggable. There is currently limited evidence that perhaps RhoGEFs are druggable. We propose studies to identify small molecule inhibitors of Ect2, a Rho guanine because its importance in cancer is well validated: we showed Ect2 gene and/or protein overexpression in colon while there are other reports of its importance in lung, brain and pancreatic cancers. We have shown that Ect2 plays a role in tumor cell anchorage-independent growth and Matrigel invasion. If successful, our

establishment of the tractability of Ect2 as a target for inhibitors should support efforts to develop inhibitors against other GEFs involved in cancer.

Our approach is similar to an already validated screen using a fluorescent polarization guanine nucleotide-binding assay [125]. The bases of this screen is GTP binding of Rho GTPases and fluorescent anisotropy. When Rho GTPases are in the inactive form, Rho is bound guanine diphosphate (GDP) but upon the binding of the Dbl homology/pleckstrin homology (DH/PH) domain of a RhoGEF (such as Ect2), a conformational change occurs in the switch regions of Rho, allowing the higher concentrated nucleotide GTP bind to Rho. We will use a fluorescently labeled nonhydrolysable form of the guanine nucleotide, GTP $\gamma$ S [126]. As a result, when Rho is bound to fluorescent GTP the rotation of the fluorescent signal is slowed and the polarized light will remain polarized while unbound fluorescent GTP rotates fast and will depolarize the light [127, 128].

Potential mechanisms of inhibition of Ect2 stimulated Rho nucleotide binding, could include allosteric inhibition by drug binding to Rho, allosteric inhibition by drug binding to Ect2, competitive inhibition at the site of interaction between Ect2 and Rho, or finally competitive inhibition nucleotide binding to Rho.

However, in contrast this the previous screen we will use both full-length and dominate active Ect2 protein and screen against activation of RhoA, Rac1, and Cdc42 GTPases since Ect2 has be implicated to active all of these GTPases. We expect some percentage of full-length Ect2 protein to be able to active GTPases based on our pervious pull-down results (Figure 3-4). However, since Ect2 is auto-inhibited [40] we will also screen using the active proportion of the

protein. Furthermore, we may identify unique inhibitors of Rho GTPases, which would allow us to further interrogate the individual pathways in Ect2 function.

To validate any promising hits from the initial high throughput fluorescence-based screen, we will utilize a secondary GEF assay which monitors the incorporation of <sup>3</sup>H-GTP into Rho [129]. The advantages of this secondary screen are one, there is no tag on the nucleotide (e.g., BODIPY-TR) that could potentially interfere with inhibitor binding, and two, it does not rely on a fluorescence signal which can sometimes be modulated by the specific chemical properties of the small molecule such as a cyclic structure. Together, the application of both assays will improve our chances to identify molecules that will be effective in our subsequent functional assays.

Next, putative Ect2 inhibitor hits will counterscreen against other RhoGEFs that are selective for RhoA (Larg), Rac1(Tiam1, P-Rex1) and Cdc42 (Asef) by using similar exchange assays. Those inhibitors that are selective for Ect2 but not the other RhoGEFs, will then evaluate in cell-based assays.

We will determine if the Ect2 specific inhibitors can block dominate-active Ect2 driven transformation of NIH3T3 by formation of foci compared to WT Ect2, and Dbl as a positive control. We will test the ability of the Ect2 specific inhibitors to impair anchorage-independent growth of CRC cell lines compared to Ect2 knockdown cells. We will also assess the ability of the inhibitors to block invasion on CRC cell lines through Matrigel. Our longer-term goal will be to develop Ect2 inhibitors for evaluation in mouse models of colon cancer, in particular, in patient-derived tumor xenograft models.

### ***Mechanisms of Ect2-driven motility and invasion***

Our previous findings demonstrate that Ect2 has a role in CRC cell invasiveness and there is evidence that Rho GTPases regulate different aspects of cell motility in cancer [57]. Additionally, we described a defect in the motility of MEFs upon Cre treatment resulting in the loss of Ect2; these cells exhibit a severe defect in random single cell migration [53]. Pebble, the *Drosophila* homolog of Ect2, is required for mesoderm migration via activation of RHO1. This activity was independent of the role of Pebble/Ect2 in cytokinesis [130]. Therefore it would be reasonable to investigate Ect2 function in cell motility and cancer cell invasiveness.

To address the mechanism of motility by Ect2 we can start with the analysis of our different Ect2 mutants produced previously (Figure 3-4). Using real-time invasion assays along with single cell motility assays of these Ect2 mutants should elucidate the structural requirements for Ect2 function in motility and/or invasion. We predict that if Ect2 has a direct role in motility it would require its RhoGEF function, however, the location of Ect2 activity and its downstream activity would differ from that of Ect2 in cytokinesis. We predict that the RhoGEF function is critical for the role of Ect2 in motility due to the importance of Rho GTPase signaling in both migration and invasion. The subcellular localization of Ect2 may also play a role in the ability of Ect2 to promote motility. Therefore, the previous analysis of Ect2 function in the nucleus compared to cytosol will also assist in clarifying the mechanism of Ect2 function in motility and invasion.

### ***Ect2 Mouse Model of Colorectal Cancer***

To address the role of Ect2 in a mouse model of colorectal cancer we need to first know if Ect2 is required for intestinal development, crypt formation, and maintenance. We propose crossing our *Ect2<sup>fl/fl</sup>* mouse containing a loxP site-flanked Ect2 sequence with a villin-Cre mouse to selectively ablate Ect2 in the entire intestine. The villin-Cre (VC) mouse is a well-characterized model where the promoter from the *villin* gene drives the expression of Cre recombinase in 100% of epithelial cells of the small and large intestines [131]. This would provide the first in vivo evaluation of Ect2 function in differentiated mammalian tissue.

However, we may find that Ect2 is essential for intestinal development or maintenance of colonic tissue causing an inability to use the VC model to determine if Ect2 has a role in a mouse model of CRC. To overcome this potential limitation, we can use the VC-ERT mouse strain, which encodes an estrogen receptor-Cre fusion protein and therefore expresses a tamoxifen inducible form of villin-Cre [132]. In this model, no deletion of Ect2 would occur until the VC-ERT; *Ect2<sup>fl/fl</sup>* adult mice are treated by intraperitoneal injection of tamoxifen.

Next we will use either the VC/*Ect2<sup>fl/fl</sup>* or VC-ERT/*Ect2<sup>fl/fl</sup>* mouse to cross with the with the *APC<sup>min/+</sup>* mouse to determine if Ect2 loss impairs colon tumorigenesis. The *APC<sup>min/+</sup>* mouse carries a truncation mutation at codon 850 of the *APC* gene that causes multiple intestinal neoplasia (Min), typically resulting in ~30 polyps in the small intestine per animal [133, 134]. This mouse model has been useful to identify proteins and mechanisms involved in colorectal cancer

tumorigenesis [73]. Additionally, our preliminary analyses found that Ect2 is overexpressed in adenomas from this model (Figure 3-1), indicating that it will be an ideal model for our studies investigating Ect2 in colorectal cancer.

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