The Importance of ERBB Receptor Tyrosine Kinase Signaling in Colorectal Cancer — Implications for EGFR-Targeted Therapies

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ABSTRACT

Ming Yu: The Importance of ERBB Receptor Tyrosine Kinase Signaling in Colorectal Cancer— Implications for EGFR-Targeted Therapies

(Under the direction of Dr. David W. Threadgill)

Colorectal cancer (CRC) is one of the leading causes of cancer-related death in the United States. Our current understanding of the molecular pathways associated with this malignancy has led to the development of novel molecule targeted therapies, exemplified by small molecule inhibitors and monoclonal antibodies targeting the epidermal growth factor receptor (EGFR/ERBB1). EGFR is a member of the ERBB family of receptor tyrosine kinases consisting of EGFR(ERBB1), ERBB2, ERBB3 and ERBB4. They are transmembrane receptors to elicit cellular signaling pathways in response to extracellular stimuli. Upon ligand binding, ERBB family receptors dimerize to phosphorylate the cytoplasmic kinase domain, resulting in activation of complex downstream signaling cascades, among which the RAS/MEK/MAPK pathway delivers pro-proliferative signals and the PI3K/ATK/mTOR cascade act as a pro-survival pathway. The ERBB family members play a pivotal role in many aspects of cellular biology. As such, misregulation or dysfunction of ERBB receptors has been implicated in many disease states, in particular cancers of epithelial tissues, making the ERBB pathways valuable targets for pharmacological inhibition in cancer treatment. For EGFR-targeted therapies, although preclinical and early clinical studies presented encouraging results, the large-scale clinical trials clearly demonstrate that the majority of patients do not respond. This discrepancy

demonstrates that little is known about the mechanisms underlying tumor response to EGFR-targeted therapies. In this study, by using Apc^{Min} mouse models of familial CRC, we generated mice with *Egfr* deletion exclusively in the intestinal epithelium and demonstrated that although EGFR signaling is critical for establishment of most intestinal tumor, tumors can arise independent of EGFR activity. Moreover, we identified gene expression signatures of EGFR-independent tumors and provided evidence for ERBB3 activity in mediating compensatory pathways. Consequently, we further established the importance of ERBB3 pathway during intestinal tumorigenesis with both Apc^{Min} mouse models of familial CRC and azoxymethane (AOM) model of sporadic CRC. Finally, by utilizing mice harboring a hypomorphic *Egfr* allele on four different strain backgrounds, we demonstrated the strong background modulation of tumor response to EGFR inhibition. These studies may advance understanding of ERBB biology during intestinal tumorigenesis and help design better therapies in combination with EGFR-targeted agents.

To my parents Yiwen Yu and Zhiping Liu

To my husband Yun

To my son ChenChen

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List of Abbreviations

ACF	aberrant crypt foci
AOM	azoxymethane
APC	adenomatous polyposis coli
Areg	amphiregulin
ATP	adenosine 5'-diphosphate
Btc	betacellulin
CRC	colorectal cancer
Dtr	diptheria toxin receptor
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Epgn	epigen
Ereg	epiregulin
FAP	Familial adenomatous polyposis
FGF	Fibroblast growth factor
GI	gastrointestinal
HE	hematoxylin and eosin
HNPCC	hereditary non-polyposis colorectal cancer
IGFR	insulin growth factor receptor
IHC	immunohistochemistry
kD	kilodalton
mAb	monoclonal antibody

MAPK	mitogen-activated protein kinase
MEK	mitogen-activated and extracellular signal regulated kinase kinase
Min	multiple intestinal neoplasia
Mom	modifier of Min
mTOR	mammalian target of rapamycin
Nrg	neuregulin
NSCLC	non small cell lung cancer
PI3K	phosphatidylinositol 3-kinase
QTL	quantitative trait analysis
RTK	receptor tyrosine kinase
SAM	significance analysis of microarray
Tgfa	transforming growth factor alpha
TGFBR	transforming growth factor beta
TKI	Tyrosine kinase inhibitor
wa2	waved-2

Chapter 1 General introduction

1.1 Introduction to colorectal cancer

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies in industrialized countries. The incidence of CRC in the US during 2007 will be approximately 153,760 cases (Jemal et al., 2007). Over the past decade, CRC death rates have decreased slightly due to progress made in early detection, diagnosis and treatment (Figure 1-1). Nonetheless, CRC remains the second leading cause of cancer-related deaths in the U.S., accounting for 52,180 deaths in 2007 (Jemal et al., 2007).

The risk factors associated with CRC can be categorized into non-genetic and genetic risk factors. Among the non-genetic factors are age (by the age 70, about 50% of the Western population will develop adenomatous polyps), a Western style diet (high fat/low fiber), a sedentary life style, obesity and incidences of inflammatory bowel disease. Although several genes that contribute to familial cases of CRC are known, only a few susceptibility genes have been identified for sporadic CRC; the fact that individuals with a family history of CRC are more likely to develop CRC strongly indicates the existence of genetic risk factors.

1.1.1 Genetic predisposition to CRC

CRC cases with known causative mutations, classified as 'familial CRC', account for about 20% of CRC. The most common inherited forms of CRC are familial adenomatous polyposis (FAP) and hereditary non-polyposis CRC (HNPCC). The roles of genetic defects underlying these two conditions have been well characterized. In contrast, our understanding of low-penetrance mutations that contribute to the remaining familial CRC and the majority of CRC that arise sporadically is still evolving. Identifying these

low-penetrance mutations would help to identify genetically predisposed individuals with an elevated risk for CRC in order for early preventive or therapeutic intervention.

The Familial adenomatous polyposis (FAP).

FAP accounts for less than 1% of CRC cases. FAP patients develop hundreds to thousands of polyps as early as adolescence making these patients at a high risk for developing CRC. The molecular mechanism causing FAP is a germline mutation in the *APC* (adenomatous polyposis coli) gene (Groden et al., 1991; Nishisho et al., 1991). The *APC* gene is a tumor suppressor gene in that it is a negative regulator of the WNT-signaling pathway through its role in the destruction of β-catenin (Munemitsu et al., 1995; Rubinfeld et al., 1995). In cells loosing the remaining wildtype *APC,* β-catenin is no longer degraded, causing its accumulation and translocation into the nucleus where it functions as a transcription co-activator with the T-cell factor (TCF) and lymphoid enhancer factor (LEF) family of transcription factors (Behrens et al., 1996; Molenaar et al., 1996). In *APC* mutant cells, the β-catenin/TCF/LEF complex activates the transcription of genes that are involved in cellular transformation, including $p16^{INK4a}$*, c-MYC*, and *cyclin D1* (Korinek et al., 1997; Morin et al., 1997). In *APC* mutant colonocytes, excess cytosolic β-catenin also forms complexes with E-cadherin, leading to enhanced cell-cell adhesion. These tight cellular junctions likely slow the migration of colonocytes to the tip of villus where they normally undergo anoikis. Thus *APC* mutant colonocytes linger within the crypts, contributing to the broadening of the proliferative zone (Nathke et al., 1996; Wong et al., 1996). With the accumulation of other mutations, such as in *KRAS*, CRC is initiated (Dlugosz et al., 1997).

Hereditary non-polyposis colorectal cancer (HNPCC)

HNPCC, also known as Lynch Syndrome, predisposes individuals to CRC in addition to cancers in other organs such as stomach, ovaries, small bowel, endometrium, uroepithelial epithelium, and the brain (Lynch et al., 1977). HNPCC accounts for 3 to 6% of all CRC cases (Lynch and de la Chapelle, 1999; Peltomaki et al., 2001). Affected individuals develop one or several polyps. These polyps have a higher frequency of progressing to malignancy because of germline mutations in DNA mismatch repair (MMR) genes including *MSH2, MLH1, PMS1, PMS2*, which underlie 90% of Lynch Syndrome cases. Deficiency in the MMR system leads to a high frequency of DNA replication errors and changes in short repeated DNA sequences, a condition known as microsatelite instability.

The sporadic CRC

The majority of CRC cases are believed to arise sporadically. However, genetic factors still contribute to sporadic CRC. Several low-penetrance alleles in sporadic CRC have been identified, including the I1307K mutation in the *APC* gene, the *TGFBR1*6Ala* allele, *HRAS1*VNTR* and *BLM*Ash* (Kondo and Issa, 2004). Risk factors such as age and diet could modulate the onset of CRC in these genetically predisposed individuals, possibly through epigenetic changes.

1.1.2 Molecular complexity of CRC

Understanding the molecular complexity of CRC is required to improve prevention and treatment. The development of CRC is generally thought of as a well defined sequence of events involving a complex series of genetic and epigenetic alterations, resulting in distinct pathological changes - from aberrant crypt focus (ACF) or hyperplasia to benign adenoma, carcinoma and finally metastasis (Kinzler and Vogelstein, 1996)(Figure 1-2).

This cumulative multi-stage process usually occurs over many years. In individuals with inherited mutations, the time course of initiation and/or progression to malignancy can be significantly shorter. For example, FAP patients have a much higher rate of initiation from normal epithelium to benign polyps, although the progression rate to carcinoma is not altered. Conversely, in HNPCC patients the initiation step appears unchanged compared to sporadic cancers, but once initiated the progression from polyps into malignancy is much sooner.

Recently, gene expression profiling of 100 human CRC adenomas revealed that human colon cancers exhibit gene expression patterns strikingly similar to those of embryonic colon development (Kaiser et al., 2007), highlighting the notion that cancer and normal embryonic development share common gene regulatory networks.

Distinct from classical genetic alterations, epigenetic abnormalities also contribute to CRC carcinogenesis. Abnormal genome-wide hypomethylation is particularly frequent in CRC, associated with over-expression of proto-oncogenes (Issa, 2000; Kondo and Issa, 2004). In contrast to genome-wide hypomethylation, focal hypermethylation can result in transcriptional silencing of tumor suppressor genes (Feinberg and Tycko, 2004; Greger et al., 1989). Indeed, promoter hypermethylation in CRC have been reported for important tumor suppressor genes including *APC*, *LKB1*, $p16^{INK4a}$, *hMLH1,* and *TIMP3* (Kondo and Issa, 2004).

1.1.3 Mouse models of human CRC

Over the last two decades, many mouse models have been generated that accurately recapitulate aspects of the underlying molecular pathogenesis as well as the cellular and tissue distribution of human CRC. Mice harboring individual causative mutations are

frequently intercrossed to more closely mimic the accumulation of genetic mutations occurring during malignant transformation in human CRC. These mouse models have been utilized as an important experimental tool for studying CRC.

Mouse models for hereditary CRC

***Apc^{Min}*.** FAP patients, harboring a germ-line mutation in *APC*, develop hundreds of intestinal adenomas. A nonsense mutation in the orthologous gene in mice is referred to as the *multiple intestinal neoplasia* allele of *Apc* (*Apc^{Min}*) (Moser et al., 1990). *Apc^{Min}* mice develop tens to hundreds of intestinal tumors, depending on the mouse strain harboring the mutation (Dietrich et al., 1993; Shoemaker et al., 1998). Several genetic modifier loci including *Mom1* have been identified. These modifiers affect both tumor latency and multiplicity (Dietrich et al., 1993; Moser et al., 1992). Despite the fact that FAP patients primarily have colonic lesions while the *Apc^{Min}* mice develop predominantly intestinal tumors, the *Apc^{Min}* mouse model best recapitulates the molecular alternations occurring during CRC initiation, thus it is extensively used for testing CRC prevention and therapy.

Mutations in MMR genes. Germline mutations in the mismatch repair (MMR) genes, including *MLH1*, *MSH2*, *PMS1*, and *PMS2,* cause Lynch syndrome in humans. Mice with targeted mutations in each of the known MMR genes have been generated (Prolla et al., 1998). Deficiency in MMR genes leads to microsatelite instability in these mice. While mice that are haploinsufficient for a MMR gene do not develop noticeable phenotypes, nullizygous mice display a reduced life span due to spontaneous tumorigenesis usually in lymph nodes, but relatively few in the intestinal tract. These mice, however, are genetically susceptible to CRC upon exposure to mutagens like N-methyl-N-nitrosourea (Qin et al., 2000). MMR deficiency also accelerates intestinal tumorigenesis in

Apc^{Min} mice (Reitmair et al., 1996).

Mutations in TGFB signaling. Transforming growth factor beta (TGFB), exerts growth inhibitory effects in epithelial cells. The TGFB signal acts through the TGFB1/TGFB2 serine/threonin receptor complex. A series of related intracellular SMAD proteins, SMAD2/3/4, are responsible for transmitting a signal from the activated TGFB receptors into nucleus. Mutations in *TGFB* signaling contribute to both familial and sporadic CRC. About 50% of juvenile polyposis, a less common form of hereditary CRC, is caused by *SMAD4* mutations. Mice lacking *Tgfbr2* are embryonically lethal, precluding its use to test the role of *Tgfbr2* in HNPCC (Oshima et al., 1996). Additionally, mice nullizygous for *Tgfbr1* die early due to massive multi-organ inflammatory disease (Kulkarni et al., 1993; Shull et al., 1992), demonstrating the immuno-regulatory nature of TGFB. When *Tgfbr1*$^{-/-}$ is transferred onto an immuno-deficient *Rag2*$^{-/-}$ background, the compound mutant *Tgfbr1*$^{-/-}$ *Rag2*$^{-/-}$ mice live to adulthood and rapidly develop colonic carcinoma (Engle et al., 1999).

The targeted null mutation in *Smad4* is embryonic lethal (Sirard et al., 1998). However, mice harboring one copy of a targeted null allele of *Smad4* do not develop intestinal polyps, but do show enhanced tumorigenesis in combination with heterozygosity for *Apc*^{Min} (Takaku et al., 1998). Interestingly, although *SMAD3* mutations have not been detected in human CRC, mice deficient in *Smad3* on a 129Sv/J background display colonic cancers similar to late stage human CRC, including adenocarcinoma and metastatic disease (Zhu et al., 1998). Moreover, when *Smad3*-deficient mice are bred to an *Apc*^{min} background, the compound genetic defects exhibit a synergistic effect on tumorigenesis, particularly in the distal part of colon (Sodir et al., 2006). It is of note that the reported

carcinomas developed in *Smad3* deficient mice are highly influenced by the endogenous gut microflora in susceptible strains (Maggio-Price et al., 2006), highlighting the notion that inflammation plays an important role in carcinogenesis, especially through TGFB signaling.

Azoxymethane (AOM) models for sporadic CRC. AOM is a procarcinogen that can be metabolized *in vivo* into a colon-specific carcinogen. Administration of AOM in mice produces adenocarcinoma *in situ* within the colon, mimicking cancer distribution in human CRC. More importantly, AOM-induced tumors in mice exhibit remarkable similarity in initiation, progression, distribution and histology with sporadic CRC in humans (Wang et al., 1998). Also, mutations that occur in sporadic CRC, including *Apc, Kras2, Tgfb1* and *β-catenin*, have been detected in AOM-induced tumors (Shivapurkar et al., 1994; Vivona et al., 1993). Thus, this model has been used extensively in studying sporadic CRC.

Cross-species and cross-model gene expression comparisons of 100 human colorectal cancer samples and 39 colonic tumors from Apc^{Min}, AOM-induced, $Smad3^{-/-}$, $Tgfr1^{-/-}Rag2^{-/-}$ mouse models have revealed the molecular characteristics for each model (Kaiser et al., 2007). Unsupervised clustering separated the four mouse models into two distinct classes based on their global gene expression patterns: Apc^{Min} and AOM-induced vs. $Smad3^{-/-}$and $Tgfr1^{-/-}$ $Rag2^{-/-}$ tumors. Nuclear β-catenin-positive Apc^{Min} /AOM tumors display up-regulation of genes associated with increased canonical WNT signaling. In contrast, $Smad3^{-/-}$ and $Tgfr1^{-/-}$ $Rag2^{-/-}$ tumors lack activation of canonical WNT signaling, but they exhibit up-regulation of genes associated with inflammatory responses. Furthermore, this comprehensive, comparative molecular analysis has revealed that the

four mouse models of CRC are similar to human CRC tumors, although *Apc*^{Min}/AOM are more similar than the *Smad3*$^{-/-}$ and *Tgfr1*$^{-/-}$ *Rag2*$^{-/-}$ tumors at a transcriptional level. Thus, *Apc*^{Min} and AOM-induced models are perhaps more relevant to human CRC.

1.1.4 Management of CRC

Over the past decade, remarkable progress has been made in the management of CRC. Consequently, treatment of CRC has evolved into multi-modality therapeutic approaches to both primary and metastatic malignancies. For patients who have resectable CRC, surgery is the treatment of choice. However, up to a third of patients have locally advanced or metastatic forms of CRC at the time of diagnosis, which precludes a surgical cure. Even in patients that undergo apparently curative resection, many still develop advanced, non-resectable CRC that requires systemic treatment. The cornerstone of systemic treatment for CRC is 5-fluorouracil (5-FU), which was developed in 1957. It is a fluorinated pyrimidine that acts primarily through inhibition of thymidylate synthetase, the rate-limiting enzyme in pyrimidine nucleotide synthesis. Chemotherapy with 5-FU improves median survival from five to 11 months compared to the best supportive care (Varadhachary and Hoff, 2005). For the subsequent 40 years, chemotherapy with 5-FU was the only option for patients with non-resectable CRC. In the past decade, two chemotherapy drugs, irinotecan and oxaliplatin, have been included in the standard care regimen for improved survival of patients with metastatic CRC. Irinotecan is a topoisomerase I inhibitor, and oxaliplatin is a platinum-based chemotherapy drug. Today, the median survival time of patients with CRC has doubled from 10 months with 5-FU alone to 20 months with 5-FU plus these newer cytotoxic agents.

Despite the improvement using these cytotoxic chemotherapeutic drugs for CRC, many patients develop a progressive disease that is resistant to chemotherapy. In these 'chemo-refractory' patients, no effective chemotherapeutic treatment is available. Also, the severe chemotherapy-related cytotoxicities, although rare, affect patient's life quality. Fortunately, advances in understanding tumor biology have revolutionized drug design. A number of molecule-targeted drugs are now being evaluated in clinical trials and some have reached the market. These drugs specifically target altered pathways in tumor cells, potentially achieving anti-tumor efficacy with lower toxicity resulting in higher tolerance. Examples are therapies targeting the epidermal growth factor receptor (EGFR). Anti-EGFR therapies have achieved clinical activity in about 10% of patients with metastatic CRC that are resistant to chemotherapy (Chong and Cunningham, 2005), with limited toxicities. Combinations of newer targeted agents with traditional chemotherapies are being evaluated currently in clinical trials.

1.2 The ERBB family of receptor tyrosine kinases and colorectal cancer

1.2.1 Introduction to the ERBB family of receptor tyrosine kinases

The ERBB family of receptor tyrosine kinases consist of four closely related members: the epidermal growth factor receptor (EGFR or ERBB1), ERBB2, ERBB3 and ERBB4 (Gullick and Srinivasan, 1998) (Figure 1-3A). They are ligand-induced receptor tyrosine kinases composed of three essential domains: the extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic domain with intrinsic tyrosine kinase activity (Wells, 1999) . Activation of ERBB receptors is controlled spatially and temporarily by the stimulation of EGF-related ligands, in an autocrine or paracrine manner (Beerli and Hynes, 1996). Upon ligand binding, they undergo homo- or heterodimerization

(Weiss et al., 1997). The activated receptors recruit and phosphorylate downstream effecter proteins activating a cascade of intracellular signaling pathways. Depending on the ligand, the receptor dimers formed, the pathways being recruited, and concurrent molecular events within the cell, the biological output can regulate many cellular activities including proliferation, migration, adhesion, differentiation and survival (Olayioye et al., 2001; Salomon et al., 1995) (Figure 1-3B). The activated receptor complexes are endocytosed and are either directed to endosomal compartments for degradation (Levkowitz et al., 1998; Waterman et al., 1998), or recycled back to the cell surface (Waterman et al., 1998).

The complexity of signaling by ERBB receptors stems from their interdependence and complementation. Each receptor has a unique spectrum of ligand specificity. Of the four ERBB members, ERBB2 and ERBB3 are functionally non-autonomous: ERBB2 does not have a recognized ligand, while ERBB3 lacks intrinsic kinase activity. ERBB2 is a preferred binding partner for other ERBBs. The EGFR and ERBB2 heterodimer not only displays a reduced internalization rate, prolonged signaling power and biological potency (Lenferink et al., 1998), but also has an expanded signal diversity (Graus-Porta et al., 1997). ERBB3 is the only member in the family that contains multiple PI3K binding sites. Once trans-phosphorylated, ERBB3 can bind directly to PI3K and activate the PI3K-AKT pathway. Although functionally incomplete on their own, the heterodimer between ERBB2 and ERBB3 is not only completely functional, but has the most potency of the ERBB family with respect to signaling.

Activated ERBB family members stimulate a variety of intracellular signaling pathways, of which the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)-AKT pathways are of most significance (Yarden and

Sliwkowski, 2001). Additionally, the signal transducer and activator of transcription (STAT), protein kinase C and phospholipase D pathways (Citri et al., 2003) are also important and are known to mediate anti-apoptotic signaling in various cell types (Garcia, 2001). Examples of important effecter proteins include the intracellular tyrosine kinase SRC (Biscardi et al., 1999; Tice et al., 1999) and the mammalian target of rapamycin (mTOR), a serine-threonine kinase activated downstream of PI3K-AKT (Bjornsti and Houghton, 2004).

To add another layer of complexity, the ERBB family members are also involved in a broader signaling network by active 'cross-talk' with other receptor classes. For example, the G-protein coupled receptor protein (GPCR) can transactivate EGFR by increasing the availability of EGF-related ligands (Carpenter, 2000; Prenzel et al., 1999). Another important example is the binding of growth hormone to its receptor can also lead to EGFR phosphorylation through Janus tyrosine kinase (JNK) (Yamauchi et al., 1998). These examples demonstrate that exposure of cells to non-EGF related peptides can also activate EGFR-mediated signaling pathways.

1.2.2 ERBB family members in intestinal development and homeostasis

Normal signaling through ERBB family members is essential in almost every tissue of the body, including skin, brain, lungs and the GI tract. This ERBB-mediated signaling is required during embryonic development and is then used throughout life for the maturation and maintenance of most organ systems (Miettinen et al., 1995; Sibilia et al., 1998; Threadgill et al., 1995). Among the four ERBB members, EGFR and its cognate ligands are most relevant in intestinal development. It has been shown that EGF stimulation leads to accelerated lung and intestinal maturation before birth and in newborn animals (Vinter-

Jensen, 1999). Additionally, it was demonstrated that EGFR hyperstimulation in TGFA overexpressing transgenic mice (Acra et al., 1998) or injection of EGF in adult mice (Dyduch, 1990), leads to increased intestinal epithelial proliferation and an increase in the dimension of the crypt/villi unit. Interestingly, although EGFR deficiency in mice caused defects in proliferation and differentiation in epithelial compartments of the lungs and the skin, no abnormalities were observed in the intestinal epithelium in mutant pups until weaning (Sibilia and Wagner, 1995; Threadgill et al., 1995), suggesting the presence of compensatory pathways supporting intestinal epithelium development in surviving *Egfr*$^{-/-}$ pups.

Signaling through EGFR is critical for intestinal adaptation following small bowel resection. Elevated post-resection EGFR expression was detected within the crypt and muscularis compartments in the small bowel (Knott et al., 2003). EGFR regulates intestinal adaptation to resection by mediating enterocyte proliferation, a process that requires MAPK-dependent *p21*$^{Waf1/Cip1}$ expression (Sheng et al., 2006). In addition, EGFR activation prevents resection-induced enterocyte apoptosis by modulating the proapoptotic protein BAX in a p38MAPK-dependent manner(Sheng et al., 2007). Consistent with these observations, EGF administration in mice, associated with increased EGFR mRNA and protein, augments intestinal adaptation following resection (Helmrath et al., 1998). In contrast, adaptation following resection is impaired in mice with reduced EGFR kinase activity (Helmrath et al., 1997).

1.2.3 Deregulation of ERBB family member signaling in CRC

Overexpression of one or more ERBB receptors has been found in the majority of carcinomas, with amplification of ERBB2 in 20-30% of metastatic breast cancers.

Abnormal activation of the ERBB family members has been documented in many other types of human epithelial cancers, including lung, skin, breast, prostate gland, head/neck and colorectal cancers. In CRC, the role of EGFR has been extensively studied and EGFR has been viewed as an attractive drug target for CRC treatment. Other ERBB family members, particularly ERBB2 and ERBB3, have been also implicated in CRC. The role of ERBB4 has not yet been elucidated. The advances in understanding of ERBB biology together with the growing knowledge of the complex functional interactions among ERBB members should allow the design of better ERBB-targeted drugs.

EGFR

EGFR over-expression has been detected in 70-80% of metastatic CRC (Messa et al., 1998; Porebska et al., 2000). CRC tumors with aberrant EGFR signaling present with advanced disease stages and poor prognosis (Mayer et al., 1993). Blocking EGFR in colon cancer cell lines achieved anti-tumor activity by inhibiting EGFR-mediated signaling pathways including PI3K-AKT, MAPK and SRC, resulting in increased cell cycle arrest, decreased proliferation and increased apoptosis (Moyer et al., 1997; Wu et al., 1995; Wu et al., 1996). In addition, a synergistic anti-tumor effect was observed when an EGFR blockade was combined with the chemotherapeutic agent irinotecan in colon cancer cells grown as xenografts in nude mice (Prewett et al., 2002). These combined therapies effectively controlled tumor growth and led to extensive tumor necrosis, decreased tumor cell proliferation, increased tumor cell apoptosis, and a marked decrease in tumor vasculature. These results suggest that an EGFR blockade could overcome cellular resistance to irinotecan, and this combined therapy approach might be effective for patients with chemo-refractory CRC.

ERBB2

Similar to EGFR, ERBB2 is also frequently overexpressed in CRC when compared to normal adjacent colon tissue, though the actual frequency varies with detection technology (Gill et al., 2002; Nathason et al., 2003). It also has been demonstrated that ERBB2 stimulates proliferation in colon epithelial cells (Nowak et al., 1997); inhibition of ERBB2 causes regression of EGFR-dependent cancer cells grown as xenografts in athymic mice (Kuwada et al., 2004). A recent study reported somatic mutations in the ERBB2 kinase domain detected in gastric, breast and colorectal carcinomas from Asian patients (Lee et al., 2006). However, anti-ERBB2 therapies have been employed primarily in breast cancers, which have the greatest degree of overexpression of ERBB2 compared to other common epithelial tumors; few studies have examined the therapeutic potential of ERBB2 inhibition in CRC. Since EGFR and ERBB2 are preferred heterodimerization partners and deliver a strong mitogenic signal, targeting ERBB2 and EGFR simultaneously in colon cancers might be a more effective approach than targeting a single receptor. In *in vitro* studies, inhibition of EGFR by small molecule inhibitors led to increased activation of ERBB2 in colon cancer cells, suggesting the compensatory regulation within ERBB family members in response to EGFR inhibition (Learn et al., 2006). Targeting both the EGFR and ERBB2 had additive effects on cell proliferation inhibition, apoptosis induction and tumor inhibition, demonstrating a cooperation of EGFR and ERBB2 in colon cancer cells (Kuwada et al., 2004). In 2007, the US Food and Drug Administration (FDA) approved the dual EGFR/ERBB2 inhibitor lapatinib (Tykerb, GSK) to be used in combination with capectabine (Xeloda), for patients with advanced metastatic breast cancer that is ERBB2 positive. Its efficacy in CRC warrants further study.

ERBB3

Overexpression of ERBB3, often accompanied with EGFR or ERBB2 overexpression, has been frequently detected in a variety of cancers, including breast (Naidu et al., 1998), gastric (Kobayashi et al., 2003), ovarian (Rajkumar et al., 1996), pancreatic (Friess et al., 1999) and colorectal cancers (Ciardiello et al., 1991; Maurer et al., 1998). Indeed, the ERBB3/PI3K/AKT axis contributes to gefitinib sensitivity in NSCLC cells (Engelman et al., 2005). Recently, it was demonstrated that ERBB3-dependent activation of PI3K/AKT, driven by amplification of the MET proto-oncogene, underlies the acquired resistance to gefitinib in a subset of NSCLC patients (Engelman et al., 2007). Additionally, ERBB3-dependent signaling, through ERBB2-ERBB3 heterodimers, has been shown to contribute to the enhanced invasiveness of mammary tumor cells (Xue et al., 2006). Altogether, it has become increasing clear that in cancers driven by EGFR or ERBB2 signaling, as seen in breast cancer and NSCLC cells, ERBB3 mainly functions as a signaling partner /substrate for EGFR or ERBB2 and mediates resistance to inhibitors of EGFR and ERBB2 in cancer cells. To date, little is known about the role of ERBB3 in colorectal cancer.

Cancer research over the past decades has uncovered some of the crucial downstream pathways mediated through ERBB family members in CRC cells. In an over-simplistic way, ERBB signals can be roughly divided into two categories: the pro-proliferative axis consisting of the RAS-RAF-MEK (mitogen-activated and extracellular signal regulated kinase kinase)-MAPK pathway and the pro-survival axis comprising the PI3K-mTOR-AKT pathway. Although these pathways are probably controlled by many RTK outputs, aberrant signaling in ERBB family members results in alterations in activity

of these downstream effecters, accounting for increased proliferation, increased protein synthesis and decreased apoptosis in cancer cells.

1.2.4 Therapeutic targeting of ERBB family members in CRC

In vivo pharmaceutical studies that target EGFR in the Apc^{Min} mouse model of CRC have produced conflicting results. In one study, EGFR inhibition by small molecule kinase inhibitors EKI-785 and EKB-569 achieved a significant reduction in polyp number (Torrance et al., 2000). In contrast, Ritland et al. observed no effect on polyp number by using CFPQA, an irreversible EGFR kinase inhibitor (Ritland et al., 2000). The first genetic evidence to confirm the validity of EGFR as a drug target for CRC came from Apc^{Min} tumors formed on an $Egfr^{wa2}$ background (Roberts et al., 2002); the receptor encoded by $Egfr^{wa2}$ has an impaired kinase activity due to a Val743 -> Gly substitution in the kinase domain (Luetteke et al., 1994). The reduced EGFR activity caused by the $Egfr^{wa2}$ allele dramatically inhibited tumor multiplicity by up to 90%. Interestingly, the Apc^{Min} tumors that did arise in $Egfr^{wa2}$ homozygous mice were indistinguishable in size, expansion, and pathological progression compared to those tumors arising in mice with wildtype activity levels of EGFR, suggesting that some tumors can grow independently of EGFR.

Based on promising preclinical results, EGFR-targeted therapies are now in advanced clinical development and several have been licensed to treat cancer patients in combination with other therapies (Hynes and Lane, 2005). Indeed, two classes of agents targeting EGFR, small molecule tyrosine kinase inhibitors (TKIs) that target the tyrosine kinase domain and monoclonal antibodies (mAb) that target the extracellular ligand-binding domain, have received regulatory approval as cancer treatments. Since the ligand-

receptor interaction is the first step in initiating further signaling cascades, blocking the ligand-binding domain with mAb inhibits ERBB signaling and results in receptor internalization. In addition, some mAbs also elicit host immune responses, referred to as 'antibody-dependent cell-mediated cytotoxicity', to kill tumor cells bound with the antibody. Alternatively, ERBB TKIs inhibit ERBB kinase activity by binding to the intracellular kinase domain and preventing ATP binding.

Cetuximab (Erbitux, Imclone) was the first chimeric human/mouse IgG1 mAb targeting EGFR that received approval from the FDA for patients with metastasized CRC who failed to respond to conventional chemotherapy. Subsequently, panitumumab (Vectibix, Amgen), a fully human IgG2 anti-EGFR mAb, was approved for patients with CRC that progressed following standard chemotherapy. Both cetuximab and panitumumab demonstrated a positive effect in about 10% of patients with chemotherapy-refractory metastatic CRC (Cunningham et al., 2004; Hecht, 2004; Saltz, 2004). It should be noted that although EGFR expression was initially the criteria to select patients who would potentially benefit from cetuximab, it was subsequently found not to correlate with patient response to the drug. Interestingly, accumulating evidence suggests that the development of a skin rash, a common side effect with EGFR mAb, could be a good indicator of favorable response and survival (Galizia et al., 2007). However, the molecular mechanisms underlying these clinical responses to EGFR-targeted therapy have not been completely elucidated.

As opposed to EGFR mAb, the performance of EGFR TKIs in CRC treatment has been less consistent. Gefitinib (Iressa, AstraZeneca), an orally dosed EGFR TKI, was first approved for marketing in 2003 to treat NSCLC. Although Gefitinib exhibited anti-tumor

activity against human CRC cell lines growing as xenografts (Bianco et al., 2002), large-scale multinational phase III clinical trials demonstrated no survival benefits when gefitinib was used in combination with chemotherapy (Gatzemeier et al., 2004; Giaccone et al., 2004; Herbst et al., 2004). However, another reversible TKI, Erlotinib (Tarceva; OSI) has demonstrated its efficacy and safety in patients with metastatic CRC in a phase II trial (Townsley et al., 2006). Ongoing clinical trials are further evaluating its use as a single agent or in combination with conventional chemotherapy in CRC treatment. Due to the interdependence within different ERBBs, newer generations of TKIs are being developed to target multiple ERBB family members, such as dual- or pan-ERBB inhibitors. In particular, lapatinib (Tykerb, GSK), the dual EGFR/ERBB2 inhibitor, has been approved for use against ERBB2-positive breast cancers.

An understanding of the molecular basis for sensitivity or resistance to EGFR inhibition would help predict how tumor cells respond to the activities of anti-EGFR agents. So far, the presence of somatic mutations in *EGFR* that may be associated with resistance to EGFR-targeted therapy has only been identified in a sub-population of non-small cell lung cancer patients (Paez et al., 2004; Pao et al., 2004). Moreover, the presence of *KRAS* mutations, arising frequently in smokers, has been linked with resistance to EGFR inhibitors in lung cancer patients (Pao et al., 2005). In other types of cancers such as CRC, little is known about the molecular mechanisms underlying response to EGFR-targeted therapies. It is likely that many factors are involved, including a lack of relevance of the EGFR signaling pathways to tumor growth, inherent molecular heterogeneity of tumors, and/or genetic heterogeneity of patients. Using the Apc^{Min} mouse model of CRC and the hypomorphic $Egfr^{wa2}$ allele, previous studies have shown that a subset of intestinal

polyps arise on a background with reduced EGFR activity (Roberts et al., 2002). These polyps develop despite a greatly reduced level of active receptor, and the size, expansion, and pathological progression of these polyps appear identical to those with wildtype EGFR. These results support the concept that CRC can be EGFR independent, i.e. tumors do not respond to EGFR inhibition due to lack of relevance of EGFR-mediated signaling or compensatory activation of other pathways for tumor growth.

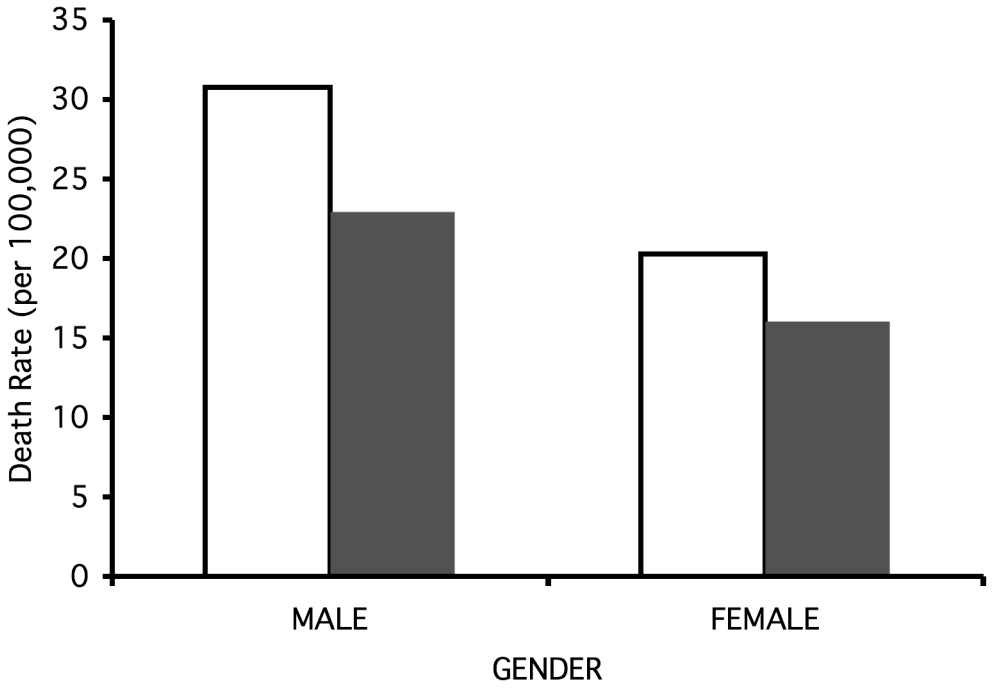


Figure 1-1 The colorectal cancer death rates in the United States, 1990 to 2003.

The source of data is from Jemal et al., 2007.White bar represents Year 1993 and white bar represnts Year 2003.

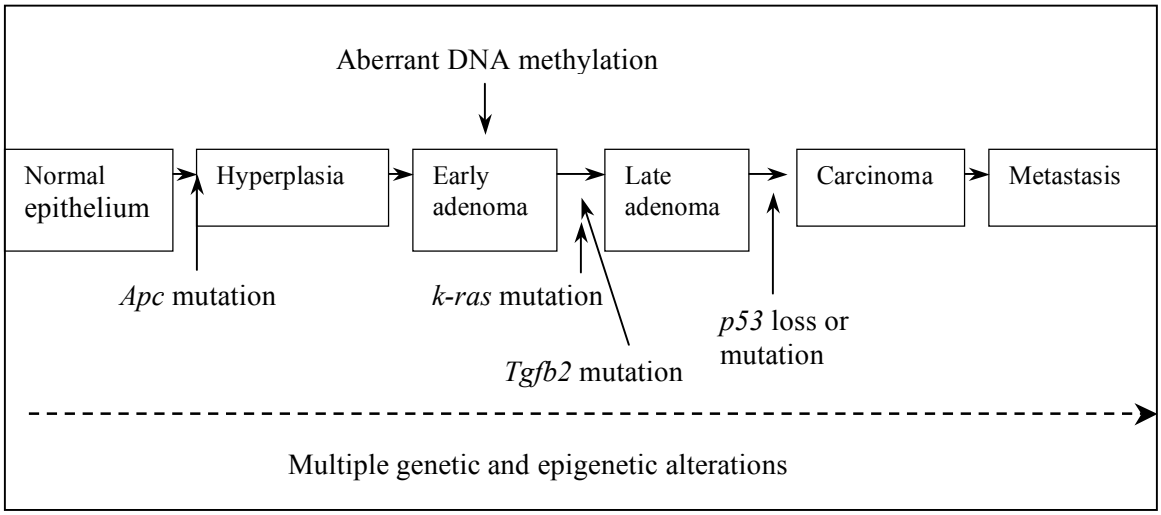


Figure 1-2 The molecular pathogenesis of colorectal cancer (adapted from (Kinzler and Vogelstein, 1996)).

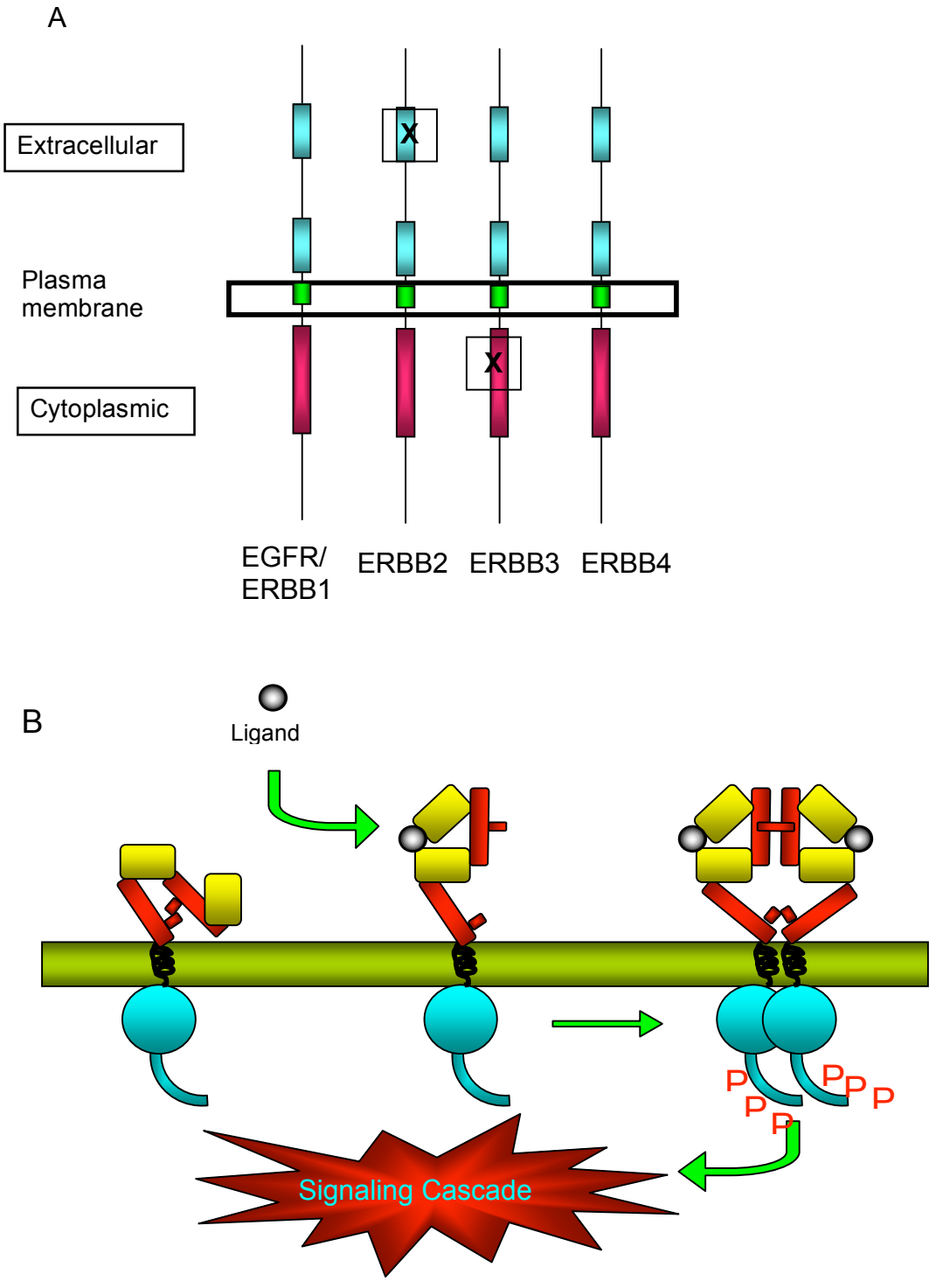


Figure 1-3 Conserved molecular structure and signaling cascades of the ERBB family members.

Chapter 2 Identification of EGFR-independent signatures in *Apc*^{Min} tumors

Abstract

The epidermal growth factor receptor (EGFR) has been intensely pursued as a therapeutic target for colorectal cancer (CRC) due to its aberrant activity in tumor tissues. However, large-scale clinical trials have achieved limited success, suggesting greater complexity of EGFR biology than previous anticipated. Using the Apc^{Min} mouse model of CRC, we previously showed that a subset of intestinal polyps arise on a background with reduced EGFR activity; the size, expansion, and pathological progression of these polyps appear EGFR-independent. Therefore, we hypothesize that although normal EGFR signaling is critical for establishment of most intestinal tumors, tumors can grow independent of EGFR activity. To test this hypothesis, we generated mice with cre-mediated intestinal epithelia-specific *Egfr* deletion. BrdU incorporation studies indicate that inactivation of EGFR does not decrease proliferation in the intestinal epithelium. We observed a 57.5% reduction in total polyp number in 3-month-old $Egfr^{tm1Dwt/tm1Dwt}$, Vil-Cre, Apc^{Min} mice compared to wild-type *Egfr* littermates (46.2 ± 25.7, versus 108.8 ± 61.2; $p_{(one-sided)} = 0.006$). Interestingly, polyps forming in $Egfr^{tm1Dwt/tm1Dwt}$, Vil-Cre mice were slightly larger than those forming in the controls (1.08 ± 0.57 mm versus 1.04 ± 0.56 mm; $p_{(one-sided)} = 0.04$), suggesting that absence of EGFR signaling does not affect the growth of tumors. Microarray gene expression profiles of these EGFR-independent tumors were analyzed by Significance Analysis of Microarray software and revealed distinctive molecular features that partition tumors based on EGFR status. These signatures are marked by up-regulation of components in cell cycle regulations and RAS-MAPK pathway, correlating well with dramatic increase in p42/44 MAPK activity and proliferation index seen in EGFR-independent tumors. Meanwhile, the protein level of ERBB2 and ERBB3 is elevated in

these tumors, correlating with increased Akt phosphorylation and impaired caspase-3 activity. These studies define a subset of tumors that grow independent of EGFR and demonstrate that this tumor subtype is likely to be constitutive of EGFR wild type tumors. Thus, the identification of molecular characteristics of EGFR-independent tumors provides a novel mechanism of tumor resistance to EGFR inhibition, and might help to identify which tumors will respond to this therapy and provide compelling rational to explore the combinatorial therapies of EGFR inhibitors with another drug that target RAS-MAPK pathway.

2.1 Introduction

The epidermal growth factor receptor (EGFR) is the prototypical ligand-induced receptor tyrosine kinase (RTK) that belongs to the highly conserved ERBB family (Yarden and Sliwkowski, 2001). It was the first RTK to be targeted for anti-cancer therapies (Masui et al., 1984; Sato et al., 1983; Zirvi, 1983). Upon ligand binding, EGFR homo- or hetero-dimerizes with other ERBB family members (Weiss et al., 1997). Activated EGFR then initiates a complex signal transduction network, which includes the Ras/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)-AKT pathways among others (Yarden and Sliwkowski, 2001).

Accumulated evidence implicates the importance of EGFR in the development of human epithelial neoplasms (Hynes and Lane, 2005). This receptor was initially found to be overexpressed in squamous cell carcinomas and glioblastomas (Derynck, 1992; Libermann et al., 1985). Overexpression or hyperactivation of EGFR has been found to be associated with tumor progression and poor prognosis in a variety of epithelial malignancies such as lung, breast, head and neck and gastrointestinal tract cancers (Salomon et al., 1995; Nicholson et al., 2001; Sharma et al., 2007). Due to these aberrant alterations in EGFR-mediated signaling pathways EGFR was proposed as a plausible target for selective anti-cancer therapy. Subsequently, numerous EGFR-targeting agents have been developed for cancer treatment. These agents have achieved anti-tumor effects in cancer cell lines and xenografts in nude mice by inhibiting EGFR phosphorylation and down-regulating EGFR-mediated signaling pathways, including PI3K-AKT, MAPK and SRC. The down-regulation of these pathways via EGFR inhibition results in increased cell

cycle arrest, decreased cell proliferation and increased apoptosis (Moyer et al., 1997; Wu et al., 1995; Wu et al., 1996). Due to the promising results from preclinical and early clinical trials, two classes of EGFR-targeting agents, small molecule tyrosine kinase inhibitors that target the tyrosine kinase domain and anti-EGFR monoclonal antibodies (mAb) that target the extracellular ligand-domain of EGFR, received regulatory approval as cancer treatments..However, the large-scale multinational phase III clinical trials demonstrated no survival benefits for patients with metastatic colorectal cancer when the TKI gefitinib (Iressa, AstraZeneca) were used in combination with chemotherapy (Gatzemeier et al., 2004; Giaccone et al., 2004; Herbst and Sandler, 2004). In addition, the chimeric IgG1 antibody cetuximab (Erbitux, IMC-C225; ImClone) and human IgG2 antibody panitumumab demonstrated a positive effect in only 10% of patients with chemotherapy-refractory metastatic CRC (Cunningham et al., 2004; Hecht, 2004; Saltz, 2004). The molecular mechanisms underlying these clinical responses to EGFR-targeted therapy have not been elucidated.

An understanding of the molecular basis for sensitivity or resistance to EGFR inhibition is essential to improve response rates and to identify those cancers likely to respond to anti-EGFR therapies. The presence of somatic mutations in the *EGFR* associated with sensitivity to EGFR-targeted therapies has only been detected in a sub-population of patients non-small cell lung cancer (PMID: 15118125 and 15118073) (Paez et al., 2004; Pao et al., 2004). Moreover, the presence of *KrRAS* mutations, arising frequently in smokers, has been linked with resistance to EGFR inhibitors in lung cancers (Pao et al., 2005). In other types of cancers such as CRC, little is known about the molecular mechanisms underlying the response to EGFR-targeted therapies. It is likely that

many factors including a lack of dependency on the EGFR signaling pathway by CRCs, inherent molecular heterogeneity of cancers, and/or genetic heterogeneity of patients are involved. Using the *Apc*^{Min} mouse model of CRC and the hypomorphic *Egfr*^{wa2} allele, we previously showed that polyp development is greatly reduced, but that a subset of intestinal polyps still arise on a background with reduced EGFR activity (Roberts et al., 2002; Torrance et al., 2000). The growth and pathological progression of polyps with impaired EGFR signaling appear identical to those with wild-type levels of EGFR. These results suggest that CRCs can arise independently of EGFR.

To test the hypothesis that tumors can grow independently of EGFR, we generated *Apc*^{Min} mice specifically lacking EGFR in the intestinal epithelium using a conditional *Egfr* allele (*Egfr*^{tm1Dwt}). In the present study, we demonstrate that *Apc*^{Min} mice deficient for EGFR in the intestinal epithelium have an equivalent reduction in the number of intestinal adenomas as observed with the *Egfr*^{wa2} allele on the *Apc*^{Min} background. Similarly, the size of the tumors that grow in the absence of EGFR is not impaired. Using these genetically distinct tumors, we show that tumors have an EGFR-dependent gene expression signature that reflects the activation status of compensatory pathways regulating cell proliferation and apoptosis. Our results define a subset of tumors that grow independently of EGFR and a putative biomarker for response to EGFR inhibitor therapy.

2.2 Materials and Methods

Mice. C57BL/6J (B6)- *Apc*^{Min} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Cre transgenic mice, B6;D2-Tg(Vil-cre)20Syr (MMHCC, 01XE7) were obtained from NCI-Frederick and maintained on C57BL/6J background as hemizygous.

Mice were fed Purina Mills Lab Diet 5058 under specific-pathogen-free conditions in an American Association for the Accreditation of Lab Animal Care–approved facility. Mice were euthanized by CO_2 asphyxiation for tissue collection.

Genotyping. Mice were genotyped for the *Apc*^{Min} allele as previously described (Roberts RB et al., 2002). Cre transgenic mice were determined using PCR with cre-S1, 5'-gtgatgaggttcgcaagaac-3' and cre-AS1, 5'-agcattgctgtcacttggtc-3' primers which brings a 278-bp PCR product. Mice were genotyped for the *Egfr*^{tm1Dmt} allele as previously described (Lee et al., 2007).

Tissue collection. The small intestine and colon were removed from each mouse. The small intestine was cut into thirds. Each segment were gently flushed with PBS to remove fecal material, cut longitudinally, and splayed flat. Representative tumors were scored before cutting in half under the dissecting microscope. One half were used for RNA extraction and array experiment; another half were fixed in 10% neutral buffered formalin at 4 °C overnight for histological analysis, or snap-frozen for use in cryo-sectioning.

Macroadenoma counts. The tumor number and diameter were obtained for the entire length of the small intestine and colon, with a dissecting microscope and in-scope micrometer at 5x magnification. The smallest tumors that can be counted are approximately 0.3 mm in diameter. Tumor scoring was performed without knowledge of genotype by the investigator. Changes in tumor growth rate were recorded grossly as tumor size. In addition to tumor size, tumors were carefully scored based on number and location along the gastro-intestinal (GI) tract.

Histology and Immunohistochemistry: Intestinal tissues or colon samples were rolled into a jellyroll before fixing in 10% neutral buffered formalin. The processed tissues were

embedded in paraffin and sectioned (7 μm). Every 50 μm, sections were taken and stained with H&E.

Immunohistochemical procedures were performed as described (Park et al., 2005). Colon tumors were rapidly dissected, fixed in 4% paraformaldehyde, and embedded in paraffin before cutting ten μm thick sections. Antigen-retrieval was performed by boiling for 20 min in citrate buffer, pH 6.0. Sections were treated with 0.3% hydrogen peroxide in PBS for 30 min, washed in PBS, blocked in PBS plus 3% goat serum and 0.1% Triton X-100, and then incubated with primary antibodies and HRP-conjugated goat anti-rabbit secondary antibody (Sigma, St. Louis, MO). Antigen-antibody complexes were detected with DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol.

Microarray experiments. Total RNA was isolated from individual tumors or normal epithelial tissue using Trizol (Invitrogen) according to the manufacturer's protocol. Isolated RNA was quantified using OD260 nm measurements in a DU 800 spectrophotometer (Beckman Coulter). RNA sample integrity and concentration was verified using the RNA Nano 6000 Chip Assay on a BioAnalyzer 2100 (Agilent).

RNA labeling was carried out using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Two hundred and fifty nanograms of RNA isolated from cell lines or 250 ng reference RNA was incubated with 0.7 μL T7 promoter primer at 65°C for 10 min. For the mouse arrays, a total RNA reference pool isolated from C57BL/6J embryos was used. The reactions were incubated on ice for 5 min. cDNA was synthesized using 5 × First Strand Buffer, 0.1 M dithiothreitol, dNTP mix, RNase OUT, and MMLV-RT at 40°C for 120 min and the reaction stopped at 65°C for 15 min. The reactions were

placed on ice for 5 min, tap spun, and 1mM of cyanine 3-CTP or cyanine 5-CTP (Perkin Elmer) was added to the reactions for the reference and cell lines, respectively. Fluorescent cRNA was synthesized using 4 × transcription buffer, 0.1 M dithiothreitol, NTP mix, 50% PEG, RNase OUT, inorganic pyrophosphatase, and T7 RNA polymerase at 40°C for 120 min. The labeled cRNA was purified using an RNeasy Kit (Qiagen). cRNA was quantified using a DU 800 spectrophotometer (Beckman-Coulter), and 1 μg of labeled reference and 1 μg of labeled sample were combined with 30 μL control target solution, 9 μL 25 × fragmentation buffer, and 225 μL 2 × hybridization buffer before loading onto hybridization chambers containing the appropriate microarray. Agilent 21K mouse oligo arrays (Agilent) were used for tumor and normal tissue samples. The slides were incubated in a Rotisserie hybridization oven at 60°C for 17 h before the microarrays were washed and scanned using a microarray scanner (Agilent).

Data analysis. Data from the microarrays were extracted using Feature Extraction Software (Agilent) and uploaded into GeneSpring (Agilent). The data were normalized first by Lowess and then centered around the 'untreated' samples before filtering using the default minimum expression level of 0.1. Filtered gene lists were exported for statistical analysis using Significance Analysis of Microarrays (SAM) software (Stanford). Lists of differentially expressed genes for each tumor genotype were generated using a two-class unpaired comparison at a false discovery rate (FDR) of 5 %.

Quantitative real time PCR (qRT-PCR). Genes with significant changes in expression between *wa2* tumors and *wt* tumors, based on ANOVA analysis, were confirmed by qRT-PCR. cDNA were synthesized from total RNA from each tumor using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PCR reactions were set up in

96-well MicroAmp Reaction Plates (Applied Biosystems), using cDNA template in Taqman Universal PCR Master Mix and 6-FAM labeled primer-probe sets for genes of interest (Applied Biosystems). Reactions were run on a Strategene MX3000P machine with analysis software. Threshold cycles (Ct) for each target gene will be determined by an in-program algorithm, assigning a fluorescence baseline based on readings prior to exponential amplification. Analysis of relative gene expression data will be calculated using the ΔΔCt method (Livak and Schmittgen, 2001), with appropriate endogenous controls.

Western blotting. An extract of harvested tissues was prepared by homogenization in 10 volumes (10 ml/g) of homogenization buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin, 1 μg/ml of aprotinin). The concentration of cleared lysate was measured by Bradford assay (Biorad) and equal amounts of protein lysate were loaded onto a 6% acrylamide gel, electrophoresed, and transferred to a polyvinylidenedifluoride membrane (Bio-Rad). The membrane was incubated in blocking solution containing 5% nonfat dried milk in TBST (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature and subsequently incubated with anti (Transduction Laboratories), anti EGFR #1001 (Santa Cruz), anti phosphorylated p42/44 MAPK and anti phosphorylated Akt (cell signaling technology) and anti Akt antibody (Cell signaling) per ml in TBS (10 mM Tris-HCl [pH 7.4], 150 mM NaCl) at 4°C overnight. Following incubation with primary antibody, the membrane was washed four times in TBST and then incubated in blocking solution containing goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase for 1 hr at room temperature. The membrane was further washed four times in

TBST, and specific protein complexes were visualized with the enhanced chemiluminescence system (Amersham-Pharmacia).

Statistics. The nonparametric Mann–Whitney U test was used to analyze tumor data. Student *t* test was used to analyze other comparisons. One-sided P values are given.

2.3 Results

2.3.1 EGFR is dispensable for normal proliferation and histological organization of the intestinal epithelium.

A constitutional deletion of *Egfr* results is pre- or post-natal lethality in mice depending upon the strain (Sibilia and Wagner, 1995; Threadgill et al., 1995; Strunk et al., 2004). To overcome this limitation, a conditional knockout allele for *Egfr* was generated ($Egfr^{tm1Dwt}$) by flanking exon 3 with loxP sites (Lee T-C, manuscript in preparation). This allele was used to generate mice with the *Egfr* gene exclusively deleted in the intestinal epithelium by crossing $Egfr^{tm1Dwt}$ mice with mice carrying Tg(Vil-Cre)20Syr that expresses CRE from the Villin promoter in the small intestine and colon. The resulting *Vil-Cre,* $Egfr^{tm1Dwt}$ mice did not exhibit overt phenotypes when compared to their *Egfr* wildtype littermates (Figure 2-1A). Moreover, these mice displayed a normal epithelium structure (data not shown).

To evaluate the requirement of EGFR signaling for proliferation of the intestinal epithelium, proliferating cells were analyzed using BrdU incorporation at three months of age. Unexpectedly, *Vil-Cre,* $Egfr^{tm1Dwt}$ mice had a slight increase in the number of BrdU-positive nuclei when compared to their *Egfr* wildtype littermates, although this difference

was not statistically significant (Figure 2-1B). These results show that deletion of *Egfr* in this intestinal epithelium does not affect basal cell proliferation rates.

2.3.2 Intestinal adenoma in the Apc^{Min} model can arise independently of EGFR activity

Using the Apc^{Min} mouse model of human colorectal cancer, we and others previously showed that a subset of intestinal polyps arise in the context of reduced or pharmacologically inhibited EGFR activity (Roberts et al., 2002; Torrance et al., 2000): the size, growth and pathological progression of polyps with reduced EGFR activity may be due to stochastic variation in residual EGFR activity or may be EGFR-independent. To distinguish these possibilities, we generated Apc^{Min} mice with intestinal epithelium deficient for EGFR (Figure 2-2A).

At three months of age, tissue-specific deletion of *EGFR* (*Vil-Cre,* $Egfr^{tm1Dwt}$, Apc^{Min}) displayed a 57.5% reduction in total polyp number compared to *Egfr* wildtype littermates (46.2 ± 25.7, versus 108.8 ± 61.2; $P_{(one\text{-}sided)} = 0.006$) (Figure 2-2B). This EGFR-dependent reduction in the number of small intestinal polyps was observed in the proximal and middle regions of small intestine (Figure 2-2C). No difference was observed in polyp number between *Vil-Cre,* $Egfr^{tm1Dwt/+}$ and $Egfr^{+/+}$ mice. Similar to the original $Egfr^{wa2}$ studies, the size of polyps that developed in *Vil-Cre,* $Egfr^{tm1Dwt}$ mice were not smaller than those in wildtype *Egfr* mice, and were even slightly larger than polyps that developed in corresponding littermate controls (1.08 ± 0.57 mm versus 1.04 ± 0.56 mm; $P_{(one\text{-}sided)} = 0.04$) mirroring the slight elevation in basal epithelium rates in the absence of EGFR (Figure 2-2D). Additionally, histological analysis of polyps from *Vil-Cre,*

Egfr^{tm1Dwt}, *Apc*^{Min} and *Egfr*$^{+}$, *Apc*^{Min} mice did not reveal any difference in morphology or pathological progression (data not shown).

To confirm that the *Egfr*^{tm1Dwt} allele underwent CRE-mediated recombination in polyps derived from intestinal epithelium of *Vil-Cre, Egfr*^{tm1Dwt}, *Apc*^{Min} mice, DNA was isolated from epithelial cells that had been dissected from individual polyps by laser-capture-microdissection (LCM). PCR genotyping confirmed that only the recombined *Egfr*^{tm1Dwt} allele (*Egfr*$^{\Delta}$) was detectable in individual *Apc*^{Min} polyps from *Vil-Cre, Egfr*^{tm1Dwt} mice (Figure 2-2E). The *Egfr*$^{\Delta}$ allele is functionally null, since western blot analysis showed that tumors from *Vil-Cre*, *Egfr*^{tm1Dwt}, *Apc*^{Min} mice produce no detectable EGFR protein (Figure 2-2F), while a normal 170 kD EGFR protein can be detected in corresponding *Egfr* wildtype control tumors.

2.3.3 EGFR-independent tumors have a distinct gene expression signature

To determine whether EGFR-independent tumors have a distinct gene expression signature, we analyzed RNA from epithelia from wildtype mice, tumors from *Apc*^{Min} mice with wildtype *Egfr*, and tumors from *Apc*^{Min} mice with either reduced EGFR activity (*Egfr*^{wa2}) or deficient for EGFR (*Vil-Cre, Egfr*^{tm1Dwt}). Normalization of the data to the average of the wildtype, non- *Apc*^{Min} epithelial samples yielded 17,990 differentially expressed genes. Hierarchical clustering with the 17,990 genes using a correlation-based centroid-linkage algorithm demonstrated a significant distinction between tumors and normal epithelial tissue (Figure 2-3A). Interestingly, tumors with wildtype *Egfr* were highly heterogeneous in their global gene expression patterns and did not cluster by *Egfr* genotype.

Significance Analysis of Microarray (SAM) was used to identify differences between *Egfr* wildtype and mutant/deficient Apc^{Min} tumors. This analysis revealed that 103 genes display statistically significant changes in their mean expression level based upon *Egfr* genotype, with a false discovery rate of 10%. Hierarchical clustering of the 103 genes partitioned the tumors samples into two primary groups, those with wildtype *Egfr* and those that are deficient or mutant for *Egfr* (Figure 2-3B).

Based on the SAM analysis, the top genes out of these 103 genes that were differentially expressed based upon *Egfr* genotype were also subjected to hierarchical clustering (Figure 2-3C). Interestingly, three genes in the RAS-MAPK pathways (*Arhgap4*, *Arhgef1, Map4k2*) were significantly upregulated in EGFR-independent tumors, suggesting that these tumors have elevated levels of components in RAS-MAPK pathway, potentially compensating for the loss of the upstream EGFR signal. Additionally, genes that are known to play an important role in cell cycle progression (*E2f2, Ccnd3* and *Cdc6)* show higher levels of expression in EGFR-deficient tumors.

2.3.4 EGFR-independent tumors increased MAPK activity and proliferation

To confirm the gene expression profiling results, the activity of p42/44 MAPK was evaluated by immunohistochemistry (IHC). Tumors from Apc^{Min} mice labeled with the anti-p42/44 MAPK antibody displayed p42/44 MAPK immuno-reactivity in the nucleus and cytoplasm of adenomas as well as the proliferative compartment of normal crypts. In tumors from $Egfr^{+}$, Apc^{Min} mice, the p42/44 MAPK immuno-reactivity was limited to a few cells localized within the tumor (Figure 2-4 A-B). In contrast, tumors from the *Vil-Cre,* $Egfr^{tm1Dwt}$, Apc^{Min} mice showed strong p42/44 MAPK immunoreactivity throughout the tumor (Figure 2-4 C-D). In agreement with the increase in p42/44 MAPK activity by

IHC, the proliferation index quantified by Ki67 IHC showed that the numbers of proliferating cells were significantly increased in tumors in *Vil-Cre, Egfr*^{tm1Dwt}*, Apc*^{Min} mice (Figure 2-5 A-E), consistent with basal differences in proliferation measured by analysis of BrdU incorporation.

2.3.5 Reorganization in ERBB family function and elevated anti-apoptotic signaling in EGFR-independent tumors

To investigate whether lack of EGFR in tumors would lead to altered protein level of other ERBB family members such as ERBB2 and ERBB3, western blot analysis was performed on protein samples extracted from individual tumors from the *Vil-Cre, Egfr*^{tm1Dwt}*, Apc*^{Min} mice and littermate controls (Figure 2-6). Whereas no detectable EGFR protein was present in tumors from the *Vil-Cre, Egfr*^{tm1Dwt}*, Apc*^{Min} mice, the protein level of ERBB2 and ERBB3 was elevated compared to tumors from EGFR wildtype control mice. The AKT-kinase is known to lie downstream of ERBB3 and regulates an anti-apoptotic pathway in epithelial cells (Song et al., 2005). Activation of AKT-kinase as detected by phospho-AKT antibody was increased in EGFR-deficient tumors. Consequently, activity of caspase-3, a critical executioner of apoptosis, was dramatically decreased in EGFR-deficient tumors as detected by cleaved caspase-3 antibody. These results indicate that absence of EGFR results in elevated ERBB2 and ERBB3 protein levels, which may contribute to the increased phospho-AKT level and decreased caspase-3 activity.

2.3.6 EGFR ligands show elevated expression in EGFR-independent tumors

To investigate the dynamics in expression of EGFR ligands in EGFR-independent tumors, real-time PCR analysis was performed on tumors with different *Egfr* genotypes. Tumors lacking EGFR showed an overall elevation in EGFR ligand mRNA level (Figure

2-7). In particular, the *Ereg* (epiregulin), *Epgn* (epigen), and *Tgfa* (transforming growth factor-alpha) were expressed at significantly higher levels in tumors from *Vil-Cre*, $Egfr^{tm1Dwt}$, Apc^{Min} mice than those with wildtype *Egfr*, whereas *Btc* (betacellulin), *Egf* (epidermal growth factor), *Dtr* (diphtheria toxin receptor/heparin-binding Egf), *Areg* (amphiregulin) were expressed at levels similar to those in the wildtype *Egfr* tumors.

2.4 Discussion

The fact that majority of colon cancer patients do not respond to anti-EGFR therapeutics, despite promising pre-clinical data, is a major hindrance to the success of these agents. Previous reports using genetic and pharmacological inhibition to reduce but not eliminate EGFR activity were inconclusive as to whether colon tumors can arise and grow independently of EGFR activity since the studies could not distinguish EGFR-independence from variable residual EGFR activity. In the current studies, we took advantage of a conditional knockout allele of *Egfr* to prove that a subset of colon tumors does arise independently of EGFR signaling. Our genetic approach demonstrates that despite having far fewer polyps than Apc^{Min} mice with a widetype *Egfr*, Apc^{Min} mice with intestinal-epithelia specific *Egfr* deletion do develop polyps and these polyps grow in an EGFR-independent manner. The absence of EGFR in these polyps exerts little or no suppression on their growth and may even enhance tumor growth. Therefore, some tumors are likely not to respond to EGFR inhibition since they do not rely on EGFR for survival or proliferation. In contrast, targeting EGFR would be most effective for those cancers that are dependent upon EGFR signaling. Although mechanistically different, diffferential response to EGFR inhibition is well documented in NSCLC where patients harboring

EGFR activating mutations exhibit dramatic clinical responses to Gefitinib (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004).

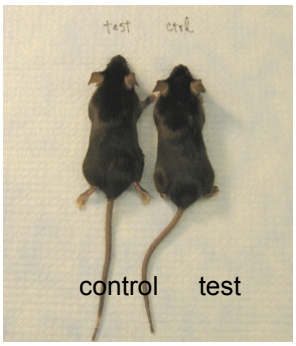
EGFR-independent polyps must rely on alternative signaling pathways for their growth and survival. However, given the diversity and complexity of the EGFR signaling network, it is not likely that the level of EGFR expression itself is an appropriate marker for EGFR-independence. More likely, a host of molecular alterations contribute to growth in the absence of EGFR, including changes in the level of activating ligands, ERBB family members or effectors of downstream signaling cascades. To generate a molecular biomarker for EGFR-status, we utilized global gene expression analysis. Although less targeted, expression profiling can identify at the transcriptional level differences in gene expression associated with EGFR-status and potentially identify compensatory pathways responsible for EGFR-independent tumor growth. When expression profiles from Apc^{Min} tumors with epithelial deletion of *Egfr* were compared by hierarchical clustering with those tumors containing wildtype *Egfr*, two out of seven *Egfr* wildtype tumors clustered among the tumors with epithelial deletion of EGFR. One explanation for this result is that a subset of tumors in the *Egfr* wildtype tumor population share more similarity in global gene expression patterns with *Egfr* mutant tumors because the EGFR status is constitutional and present before selection by loss or inhibition of EGFR. Subsequently SAM was used to identify a list of genes that show statistically significant differential gene expression between EGFR-dependent and independent tumors. Among the top upregulated genes were *E2f2*, *Ccnd3 and Cdc6*, important positive regulators of cell cycle progression. Of particular interest is that several components in the RAS-MAPK pathway were upregulated in the EGFR-independent tumors, including *Arhgap4, Map4k2* and *Arhgef1*. IHC analysis

revealed a dramatic increase in p42/44 MAPK activity in EGFR-independent tumors, supporting functional upregulation of the MAPK pathway. Consistent with activation of the MAPK proliferation pathway, the level of Ki-67 staining was elevated indicating a higher proliferation index in the *Egfr* mutant tumors. This observation was also consistent with the fact that EGFR-independnet tumors are slightly larger than those with normal EGFR activity. Taken together, the discovery of elevated RAS-MAPK pathway components and MAPK activity suggests that an alternative transmembrane signal compensates for loss of EGFR in EGFR-independent tumors. Constitutive activation of RAS/MAPK is proposed to be the molecular basis for acquired resistance to gefitinib in ERBB2-overexpressing human gastric cancer cell lines derived from liver metastasis (Yokoyama et al., 2006). Another recent study in metastatic CRC patients has shown that patients with dysregulated MAPK pathway, via an activating *Kras* mutation, are more resistant to Cetuximab treatment (Khambata-Ford et al., 2007).

Considering that activating *Kras* mutations have not been reported in Apc^{Min} tumors and the intimate cross-talk within EGFR/ERBB family members, it remains to be determined what the up-stream transmembrane signal is that compensates for loss of EGFR and activation of the downstream MAPK pathway. The elevated protein level of ERBB2 and ERBB3 in EGFR-independent tumors strongly indicates that the ERBB2/3 heterodimer may mediate compensatory pathways. Indeed, the ERBB2/3 heterodimer could also contribute to EGFR-independent tumor growth by mediating pro-survival signals, as phospho-AKT level is increased in EGFR-independent tumors while the caspase-3 activity is dramatically impaired. Consistent with this notion, ERBB3 has been shown to mediate sensitivity to EGFR inhibition in pancreatic and colorectal cancer cells

(Buck et al., 2006). Our findings may have important clinical implications for CRC patients who do not respond to EGFR-targeted therapies. Out results suggest that EGFR inhibition, either by small molecular inhibitors or by monoclonal antibodies, may be ineffective in the subset of EGFR-independent tumors that exhibit activation of the MAPK pathway. Also, ERBB3 could be used as a biomarker to predict clinical responsiveness of patients to anti-EGFR therapy. Furthermore, combining inhibitors to important downstream effecters such as MAPK or AKT with anti-EGFR therapeutics should be considered to achieve better efficacy.

A



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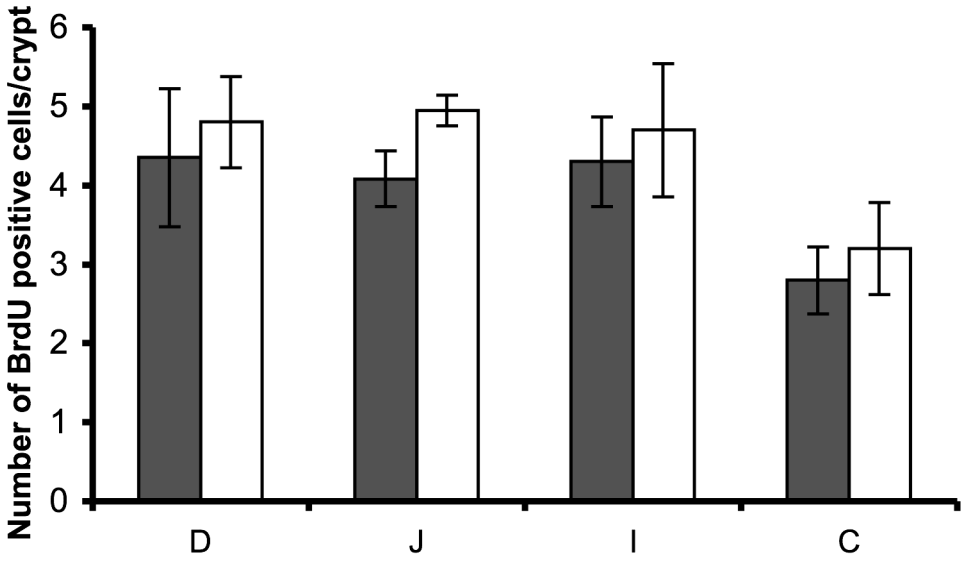
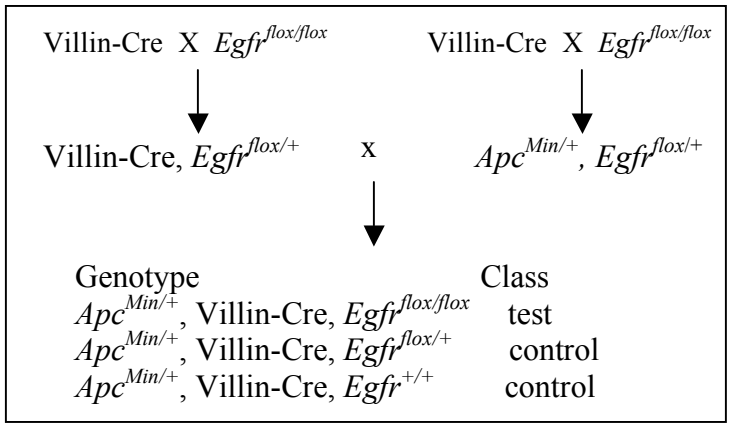


Figure 2-1 Inactivation of Egfr in the intestinal epithelium does not decrease the proliferation of the epithelial cells.

Figure 2-1. Inactivation of Egfr in the intestinal epithelium does not decrease the proliferation of the epithelial cells. (A) An example of a three-month-old *Vil-cre*, *Egfr*^{tm1Dwt} mouse showing no overt phenotypes compared to its control littermate. Control: Egfr^{+} mouse; test: *Vil-cre*, *Egfr*^{tm1Dwt} mouse (B) The number of BrdU positive cells/crypt does not alter in the small and large intestines of three-month-old *Vil-cre*, *Egfr*^{tm1Dwt} mice compared to its control littermate. The black bar represents the control animals; the white bar represents the *Vil-cre*, *Egfr*^{tm1Dwt} mice. D: duodenum; J: jejum; I: iliem; C: colon. Error bar: ± SD.

A


Villin-Cre X Egfr flox/flox
Villin-Cre X Egfr flox/flox
Villin-Cre, Egfr flox/+
x
Apc Min/+, Egfr flox/+
Genotype
Class
Apc Min/+, Villin-Cre, Egfr flox/flox
test
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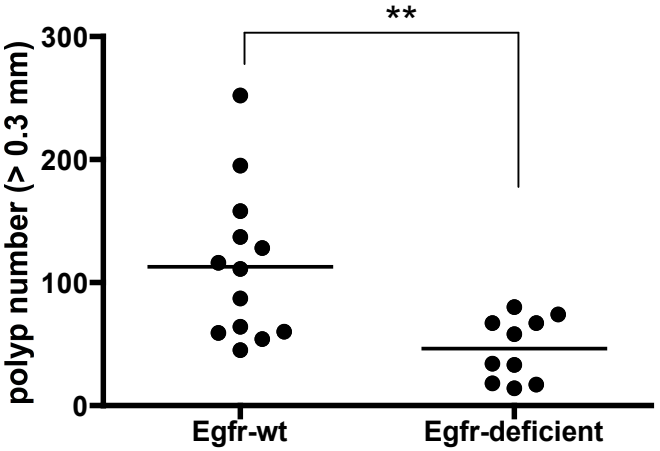
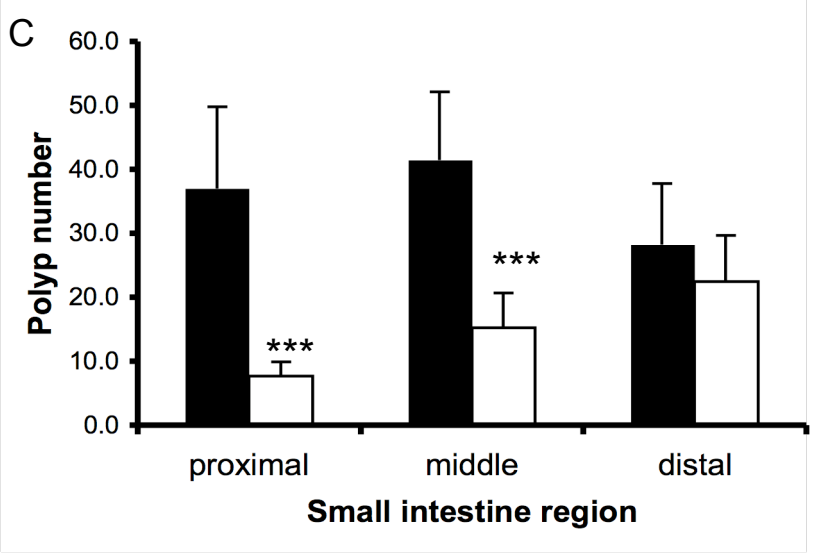
control

B


**
300
200
100
0
polyp number (> 0.3 mm)
Egfr-wt
Egfr-deficient

C


60.0
50.0
40.0
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proximal
middle
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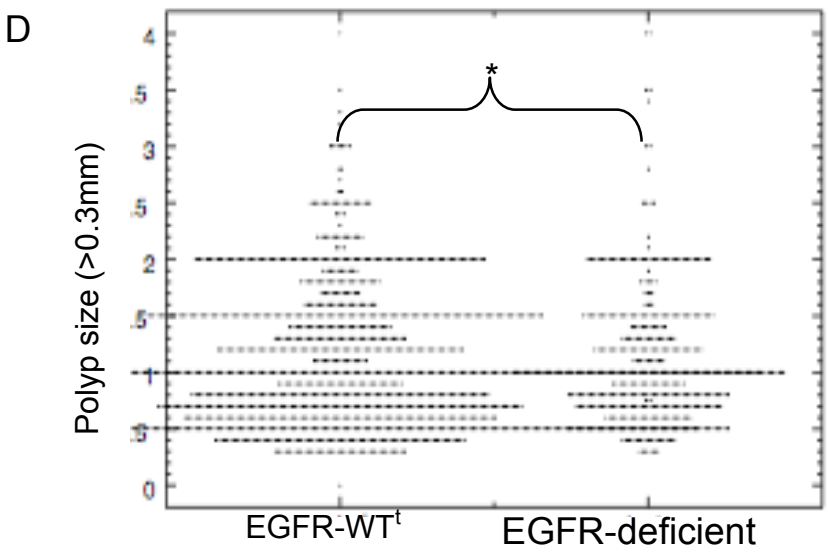
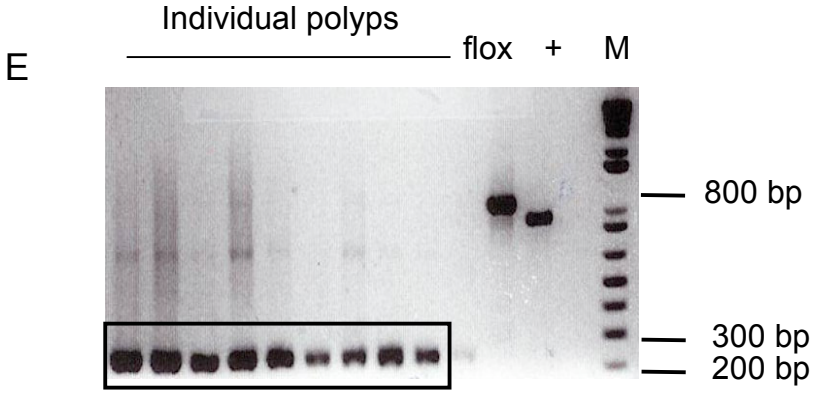
Small intestine region





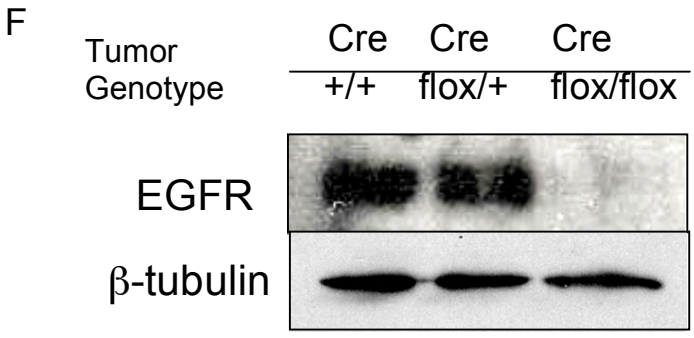
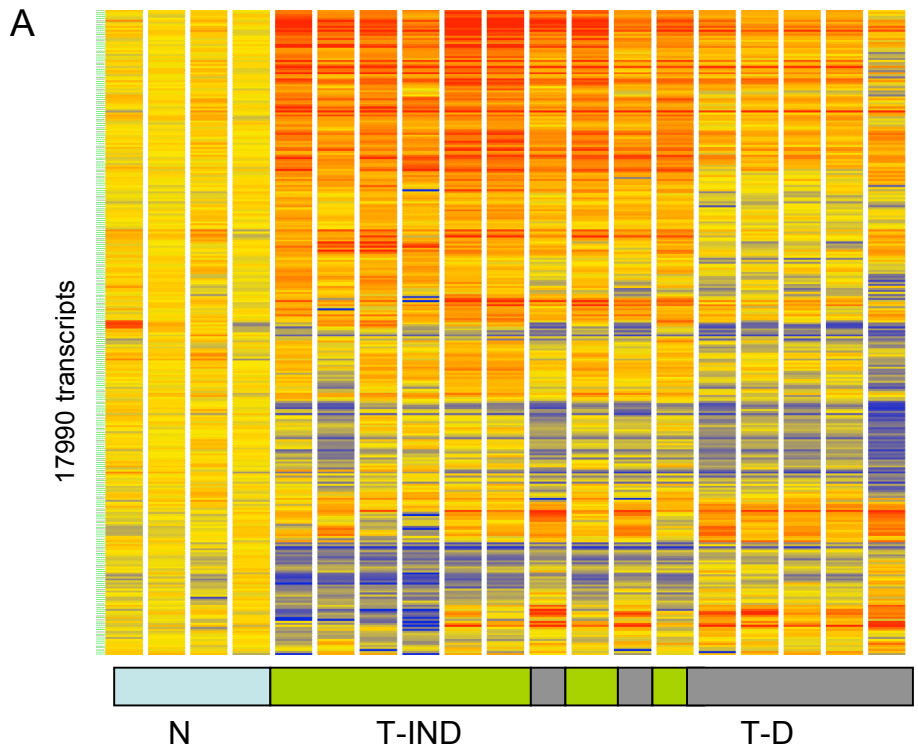


Figure 2-2 EGFR-independent intestinal adenoma growth in *Apc*^{Min} mice

Figure 2-2. EGFR-independent intestinal adenoma growth in Apc^{Min} mice. A. Breeding scheme used to generate intestine epithelia-specific *Egfr* deletion in Apc^{Min} mice. (B) Macroadenoma multiplicity in Apc^{Min} mice of different *Egfr* genotype. Each dot represents the polyp number in each 3-month-old mice, (n_{wt}=14, $n_{deficient}$=10). (C) The reduction of multiplicity along the small intestinal tract. (D) Macroadenoma size in Apc^{Min} mice of different *Egfr* genotype. Each point in represents the size of individual polyps. (E) PCR genotyping of representative Apc^{Min} tumors. PCR amplicons display the generation of the $Egfr^{\Delta}$ allele in tumors by Villin-cre-mediated excision of $Egfr^{tm1Dwt}$. The sizes of the PCR products for the $Egfr^{\Delta}$, $Egfr^{+}$ and $Egfr^{tm1Dwt}$ alleles are 234 bp, 800 bp and 1024 bp, respectively. M, 1-kb DNA ladder (F) Western blot analysis of EGFR protein levels in individual tumors of different genotypes. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Mann-Whitney U test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T-IND
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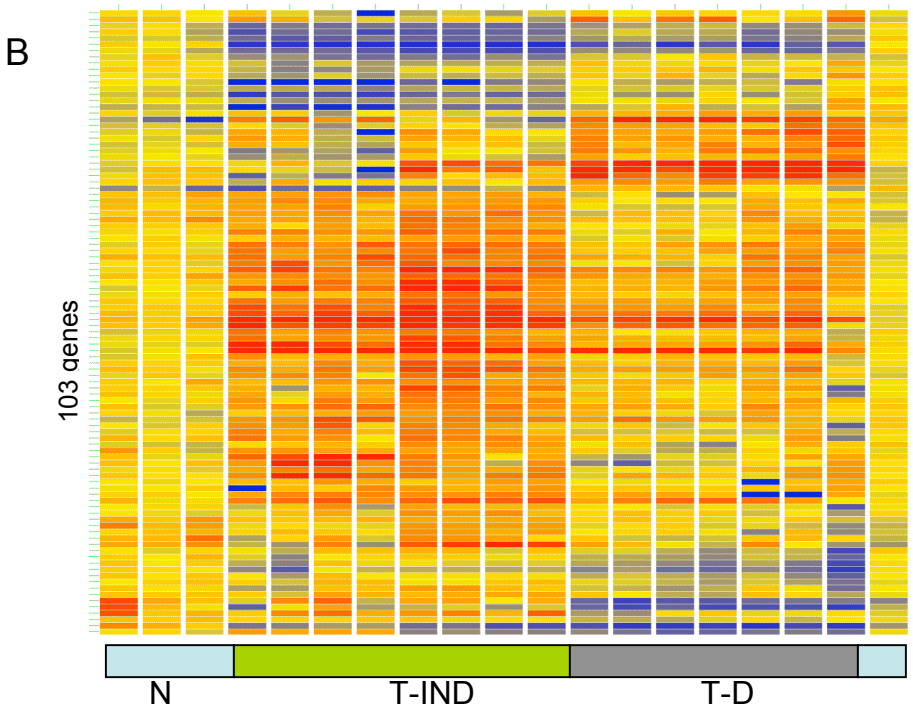
B
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N
T-IND
T-D

C

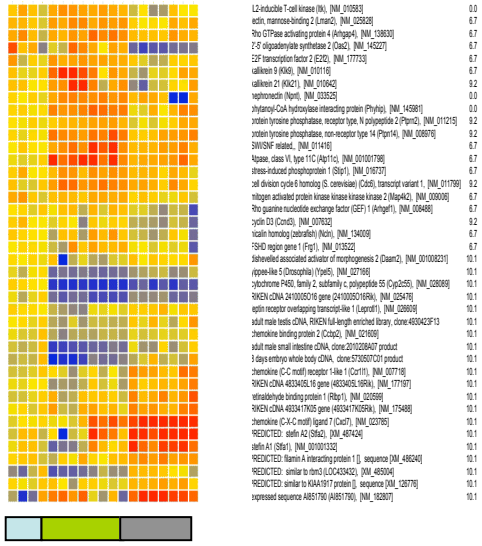


Figure 2-3 Hierarchical clustering of gene expression patterns from *Apc*^{Min} tumors

Figure 2-3. Hierarchical clustering of gene expression patterns from Apc^{Min} tumors. A. Global gene expression patterns analyzed by clustering 17,990 transcripts. Hierarchical clustering of (B) the 103 genes identified by SAM that are differentially expressed between *Egfr* mutant and *Egfr* wildtype tumors and (C) the 40 most differentially expressed genes. Blue block (N): samples of intestinal epithelia from EGFR wildtype mice; green block (T-IND): samples of EGFR-independent intestinal tumors; grey block (T-D): samples of EGFR-dependent intestinal tumors.

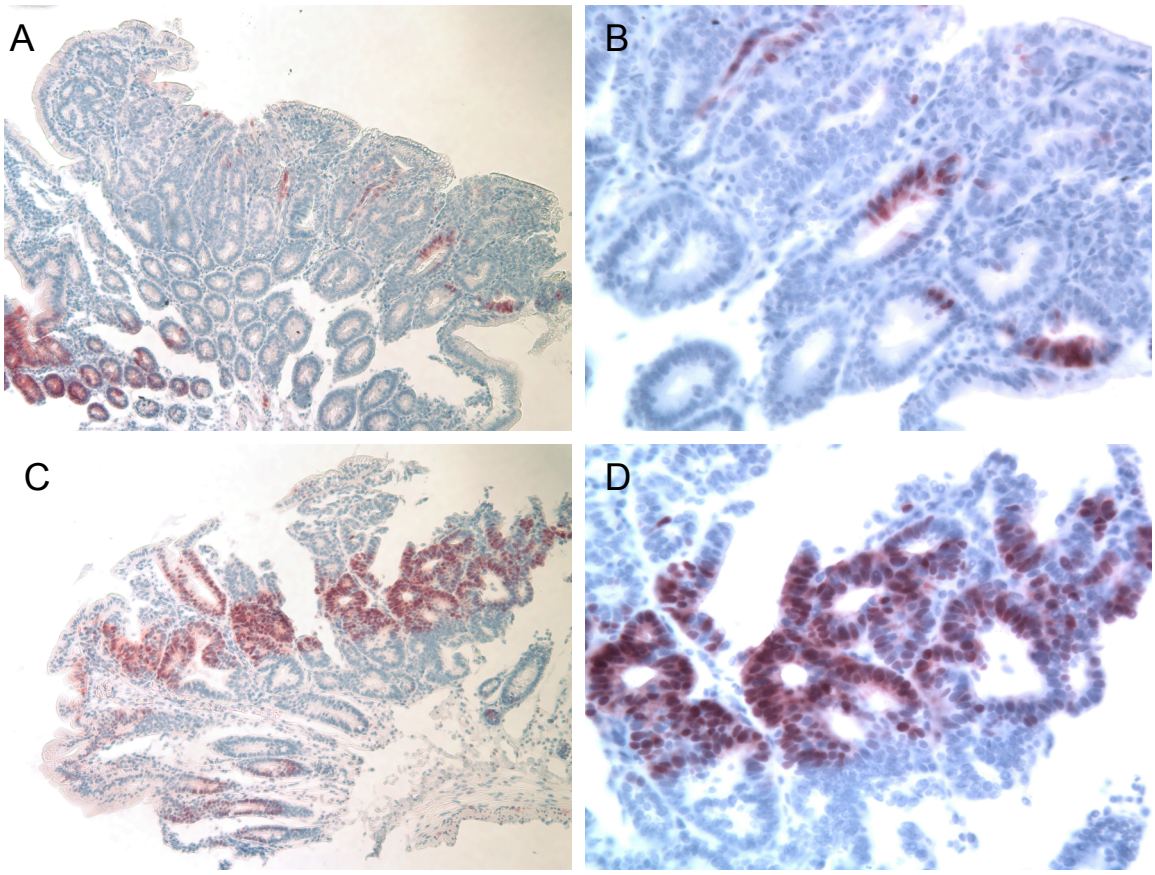


Figure 2-4 IHC analysis of MAPK activity in EGFR-independent *Apc*^{Min} tumors

Figure 2-4 IHC analysis of MAPK activity in EGFR-independent *Apc*^{Min} tumors. Representative immunoreactivity of phospho-42/44 MAPK of *Apc*^{Min} adenomas with wildtype Egfr at (A) 100x and (B) 400x showing variable staining. Polyps from *Villin-cre*, *Egfr*^{tm1Dwt}, *Apc*^{Min} mice display an increased level of phospho 42/44 MAPK immunoreactivity at (C) 100x and (D) 400x.

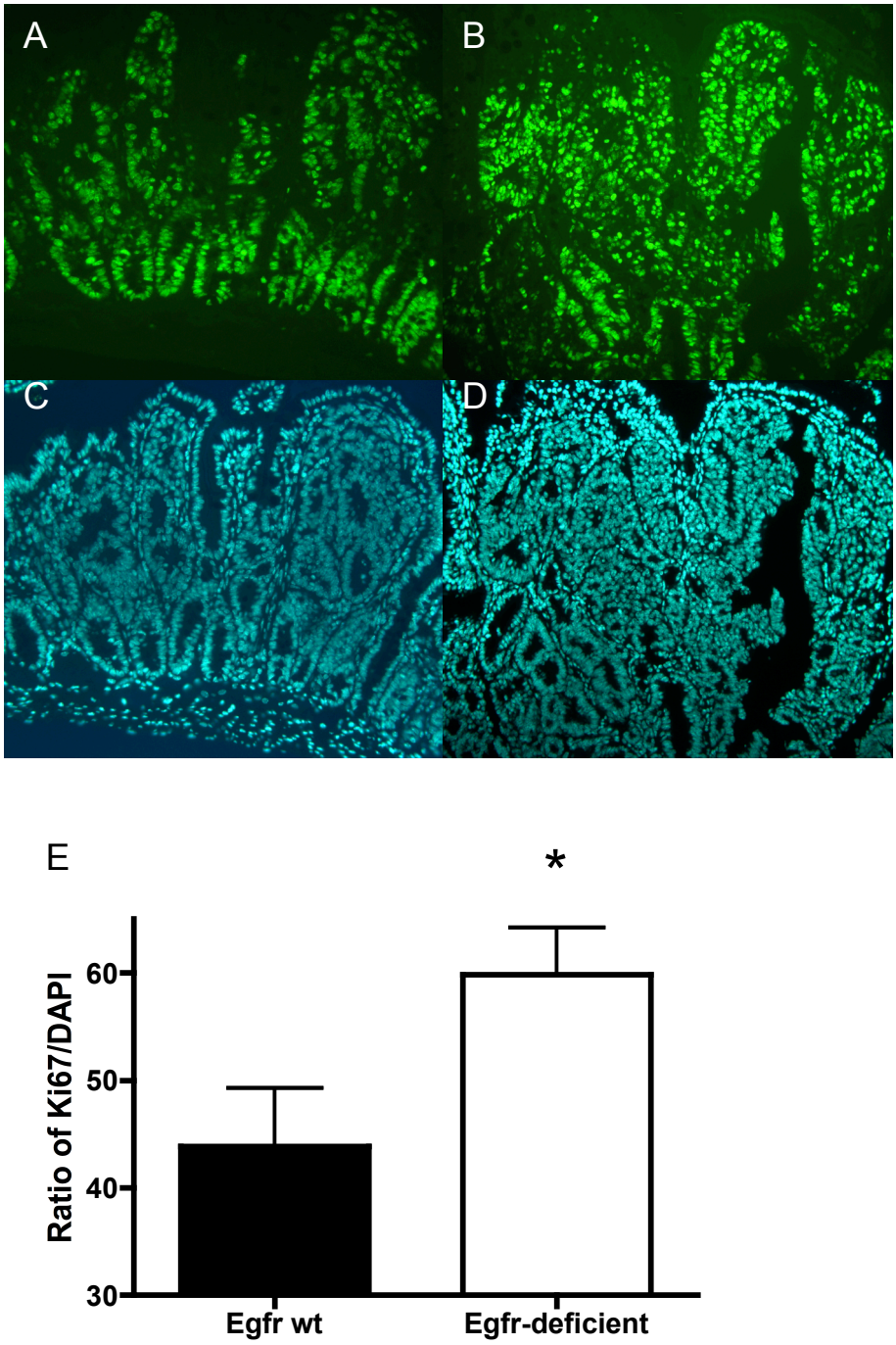


Figure 2-5 Analysis of proliferation index in EGFR-independent *Apc*^{Min} tumors

Figure 2-5 Analysis of proliferation index in EGFR-independent *Apc*^{Min} tumors. Anti-Ki67 immunofluorescence (green) in a representative polyp section from a 3-month-old *Villin-cre, Egfr*^{tm1Dwt}*, Apc*^{min} (A) and *Egfr* wildtype control (B) mice. DAPI (blue) was used for nuclear contrast (C-D). 200x magnification. E. Quantification of proliferation index. *, $P < 0.05$, student t test.

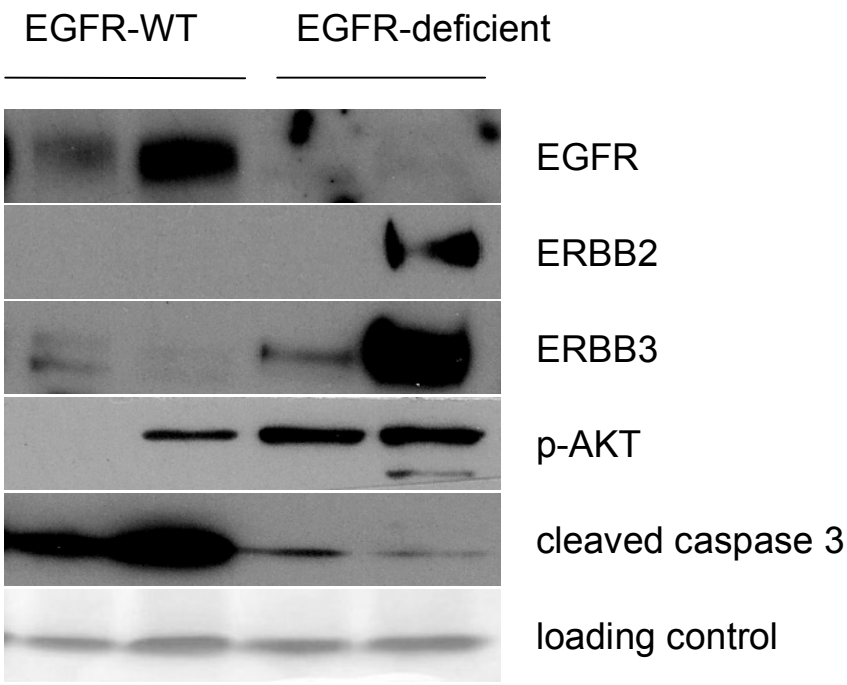


Figure 2-6 Reorganization in ERBB family function and elevated anti-apoptotic signaling in EGFR-independent tumors.

Figure 2-6 Reorganization in ERBB family function and elevated anti-apoptotic signaling in EGFR-independent tumors. Representative western blots showing level of total EGFR, total ERBB2 and ERBB3, phospho-AKT, cleaved caspase3 and loading control of two individual EGFR wildtype and EGFR-independent tumors.

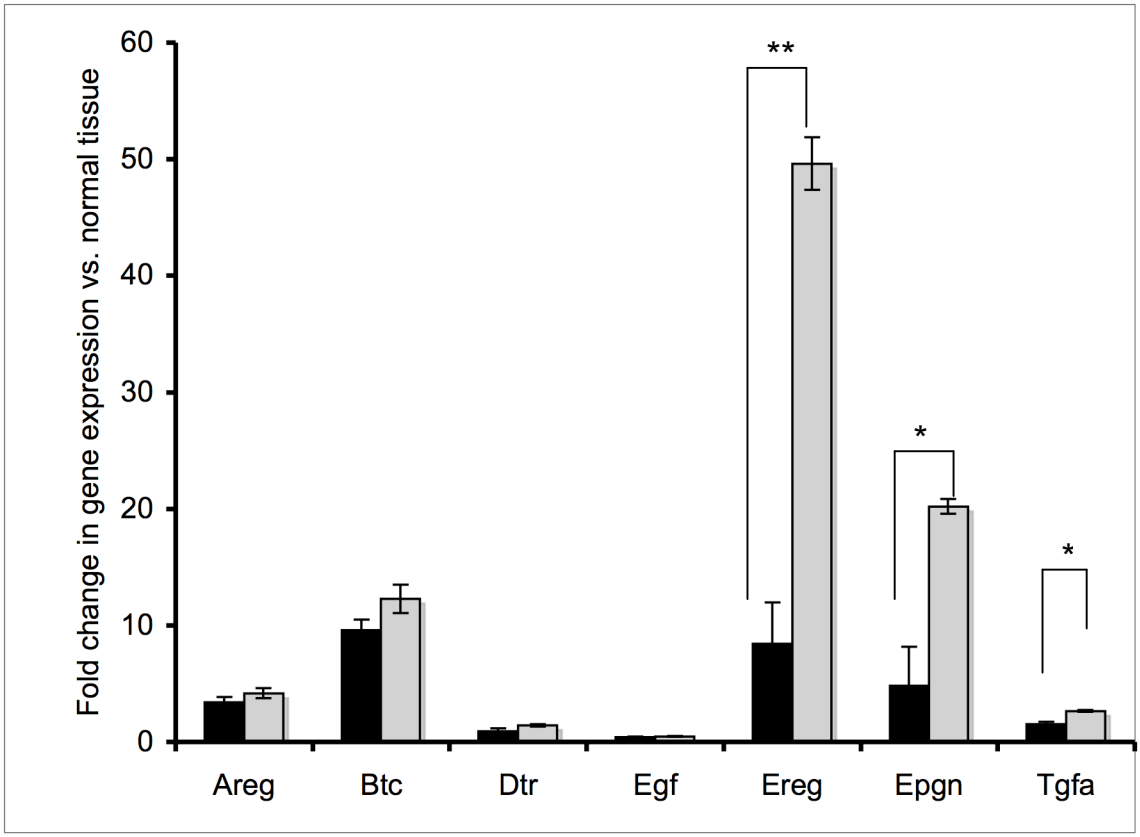


Figure 2-7 EGFR ligands gene expression in EGFR-independent *Apc*^{Min} tumors

Figure 2-7 EGFR ligands gene expression in EGFR-independent Apc^{Min} tumors. Quantitative real time PCR analysis of the expression of EGFR ligands amphiregulin (*Areg*), epigen (*Epgn*), epiregulin (*Ereg*), betacellulin (*Btc*), diphtheria toxin receptor (*Dtr*), epidermal growth factor (*Egf*) and transforming growth factor-alpha (*Tgfa*). Bars, SE. Significantly different gene expression in Egfr mutant versus *Egfr* wildtype tumors.**= $P < 0.001$; *= $P < 0.05$, student t test. Black bar: *Egfr*+ Apc^{Min} tumors; grey bar: EGFR-independent Apc^{Min} tumors

Chapter 3 The importance of ERBB3 signaling during intestinal tumorigenesis

Abstract

The role of ERBB3 during intestinal tumorigenesis has been under-appreciated, probably due to its defective kinase activity. In this study, we generated mice with CRE-mediated intestinal epithelia-specific *Erbb3* deletion to examine the impact of ERBB deficiency on the Apc^{Min} mouse model of Familial Adenomatous Polyposis (FAP). We observed a 88.5% reduction in total polyp number in 3-month-old Apc^{Min}, *Vil-Cre*, $Erbb3^{flox\Delta}$ mice compared to wild-type *Erbb3* littermates (10.6 ± 6.8, versus 91.9 ± 76.0; $p_{(one\text{-}sided)} < 0.00001$). Perhaps more importantly, polyps forming in *Vil-Cre*, $Erbb3^{flox\Delta}$ mice were significantly smaller than those forming in the controls (0.62 ± 0.48 mm versus 0.94 ± 0.45 mm; $p_{(one\text{-}sided)} < 0.0001$), suggesting that normal levels of ERBB3 signaling is essential for tumor growth in Apc^{Min} mice. While the proliferation rate as measured by Ki67 staining in ERBB3-deficient polyps was comparable to polyps with wild-type ERBB3, an increase in TUNEL-positive cells were observed in polyps from Apc^{Min}, *Vil-Cre, Erbb3*$^{flox\Delta}$ mice, indicating that ERBB3-dependent signaling prevents apoptosis in Apc^{Min} polyps. Consistently, the ERBB3-deficient polyps display reduced immunoreactivity for phosphorylation of S6, a downstream mediator of the PI3K/AKT/mTOR pathway, but the phosphorylation level of p42/44 MAPK was comparable to polyps with wildtype ERBB3 activity. In azoxymethane (AOM) model of sporadic colorectal cancer, a significant decrease in tumor susceptibility was observed in ERBB3-deficient mice ($p_{(one\text{-}sided)} < 0.05$), compared to the wild-type ERBB3 controls. However, there was no difference in the size of AOM-induced colonic tumors between two groups (3.65 ± 1.6 vs. 3.58 ± 1.1; p = 0.92). These results suggest that ERBB3 also contributes to colonic tumors induced by the carcinogen AOM, but probably through a different mechanism depending on tumor location. Taken

together, this study reveals the importance of ERBB3-mediated PI3K/AKT/mTOR in intestinal tumorigenesis, thus provides a valuable target for therapeutic intervention.

3.1 Introduction

ERBB3 belongs to the ERBB family of receptor tyrosine kinases (RTK), which includes EGFR (epidermal growth factor receptor – ERBB1), ERBB2 and ERBB4 (reviewed in (Yarden and Sliwkowski, 2001)). ERBB3 shares structural domains with other ERBB family members, consisting of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. Unlike other ERBB receptors, ERBB3 lacks intrinsic kinase activity and cannot auto-phosphorylate (Guy et al., 1994). Upon binding to a ligand, ERBB3 can be trans-activated on cytoplasmic tyrosine residues by forming heterodimers with other ERBB family members, of which ERBB2 is the preferred partner (Yarden, 2001) Tyrosine-phosphorylated ERBB3 becomes a docking site for downstream adaptor proteins, leading to subsequent activation of intracellular signaling cascades. Most notably, tyrosine-phosphorylated ERBB3 has the highest binding affinity for PI3K among ERBB receptors due to the nine binding docking sites for the p85 subunit of PI3K (Soltoff et al., 1994; Kim et al., 1994). Consequently, activation of ERBB3 frequently results in strong activation of the AKT signaling, a critical oncogenic stimulus whose aberrant activity is implicated in a wide range of cancers (reviewed in (Luo et al., 2003)).

The ERBB family members play an important role in cancer biology. In particular, EGFR and ERBB2 have been actively pursued as anti-cancer targets due to their aberrant activation in many human malignancies (reviewed in (Hynes and Lane, 2005)). In contrast, the function of ERBB3 has been less appreciated due to its defective kinase activity. Nonetheless, accumulating evidence has implicated that ERBB3 plays a critical role in

cancer. Overexpression of ERBB3 often accompanies EGFR or ERBB2 overexpression and has been frequently detected in a variety of cancers, including breast cancers (Naidu et al., 1998), colorectal cancer (Ciardiello et al., 1991; Maurer et al., 1998), gastric cancer (Kobayashi et al., 2003), ovarian cancer (Rajkumar et al., 1996) and pancreatic cancer (Friess et al., 1999). In ERBB2-driven tumors, ERBB3 functions as an intimate signaling partner that promotes the transforming potency of ERBB2, usually by activating the PI3K/AKT pathway (Holbro et al., 2003; Siegel et al., 1999; Soltoff et al., 1994). Recently it was discovered that ERBB3 couples EGFR to the PI3K/AKT pathway in non-small cell lung cancer (NSCLC) cells that are sensitive to the EGFR inhibitor like gefitinib (Engelman et al., 2005). Consistent with a role in EGFR blockade resistance, ERBB3-dependent activation of PI3K/AKT, driven by amplification of the MET proto-oncogene, underlies the acquired resistance to inhibitors of EGFR in a subset of NSCLC patients (Engelman et al., 2007). Additionally, ERBB3-dependent signaling, through ERBB2-ERBB3 heterodimers, has been shown to contribute to the enhanced invasiveness of mammary tumor cells (Xue et al., 2006). Altogether, it has become increasing clear that in cancers driven by EGFR or ERBB2 signaling, as seen in breast cancer and NSCLC, ERBB3 mainly functions as a signaling partner /substrate of EGFR or ERBB2 and mediates resistance to inhibitors of EGFR and ERBB2 in cancer cells. Very little, however, is known about the role of ERBB3 in other cancers like those developing in the colon.

Mice with constitutional deficiency of ERBB3 die perinatally due to profound neuronal and cardiac defects (Erickson et al., 1997; Riethmacher et al., 1997). To investigate the role of ERBB3-dependent signaling during intestinal tumorigenesis, we generated a conditional *Erbb3* allele and ablated ERBB3 specifically in the intestinal epithelium. We

show that ERBB3-dependent signaling has a critical role in tumor development in two mouse models of human colon cancer, the *Apc*^{min} mouse model of spontaneous intestinal tumorigenesis and the azoxymethane (AOM) mouse model of sporadic intestinal (colonic) tumorigenesis. In both mouse models, ERBB3 deficiency dramatically reduced tumor multiplicity. Unlike EGFR (Roberts et al., 2002), ERBB3-dependent signaling pathways regulate intestinal tumor growth; ERBB3-deficient *Apc*^{Min} polyps display a significant reduction in size that is due to increased tumor cell apoptosis. Furthermore, we demonstrate that ERBB3 contributes to tumor survival via the AKT pathway since the phosphorylation of ribosomal protein S6, a downstream target of the PI3K/AKT/mTOR pathway, is dramatically reduced. Overall, these results establish the importance of ERBB3-dependent signaling pathway in intestinal tumorigenesis.

3.2 Materials and methods

C57BL/6J (B6)-*Apc*^{Min} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Cre transgenic mice, B6;D2-Tg(Vil-cre)20Syr (MMHCC, 01XE7) were obtained from NCI-Frederick and maintained on C57BL/6J background as a hemizygous. Mice were fed Purina Mills Lab Diet 5058 under specific-pathogen-free conditions in an American Association for the Accreditation of Lab Animal Care–approved facility. Mice were euthanized by CO_2 asphyxiation for tissue collection.

Genotyping. Mice with *Erbb3*$^{\Delta}$ allele or *Erbb3*^{flox} allele were crossed to Cre transgenic lines to get *Erbb3*$^{\Delta}$/+.*Cre*^{Tg}/+ or *Erbb3*^{flox}/+.*Cre*^{Tg}/+ mice. These mice were further backcrossed to *Erbb3*$^{\Delta}$/+ or *Erbb3*^{flox}/+ mice to get conditionally targeted *Erbb3* (*Erbb3*$^{flox\Delta}$) mice. The genotype of each mouse was determined by PCR using mErbb3-S1 and mErbb3-AS1 for *Erbb3*. These primers give a 354-bp for the endogenous *Erbb3* allele, a 235-bp for the

Erbb3$^{\Delta}$ allele, a 488-bp for the *Erbb3*^{flox} allele, and 193-bp PCR product specific for the *Erbb3*$^{flox\Delta}$ allele, respectively. Mice were genotyped for the *Apc*^{Min} allele as previously described (Roberts RB et al., 2002). Cre transgenic mice were determined using PCR with cre-S1, 5'-gtgatgaggttcgcaagaac-3' and cre-AS1, 5'-agcattgctgtcacttggtc-3' primers which brings a 278-bp PCR product.

Tissue collection. The small intestine and colon were removed from each mouse. The small intestine was cut into thirds. Each segment were gently flushed with PBS to remove fecal material, cut longitudinally, and splayed flat. Representative tumors were scored before cutting in half under the dissecting microscope. One half were used for RNA extraction and array experiment; another half were fixed in 10% neutral buffered formalin at 4 °C overnight for histological analysis, or snap-frozen for use in cryo-sectioning.

Macroadenoma counts. The tumor number and diameter were obtained for the entire length of the small intestine and colon, with a dissecting microscope and in-scope micrometer at 5x magnification. The smallest tumors that can be counted are approximately 0.3 mm in diameter. Tumor scoring was performed without knowledge of genotype by the investigator. Changes in tumor growth rate were recorded grossly as tumor size. In addition to tumor size, tumors were carefully scored based on number and location along the gastro-intestinal (GI) tract.

Histology and Immunohistochemistry: Intestinal tissues or colon samples were rolled into a jelly-roll before fixing in 10% neutral buffered formalin. The processed tissues were embedded in paraffin and sectioned (7 μm). Every 50 μm, sections were taken and stained with H&E.

Immunohistochemical procedures were performed as described (Park et al., 2005). Colon tumors were rapidly dissected, fixed in 4% paraformaldehyde, and embedded in paraffin before cutting ten μm thick sections. Antigen-retrieval was performed by boiling for 20 min in citrate buffer, pH 6.0. Sections were treated with 0.3% hydrogen peroxide in PBS for 30 min, washed in PBS, blocked in PBS plus 3% goat serum and 0.1% Triton X-100, and then incubated with primary antibodies and HRP-conjugated goat anti-rabbit secondary antibody (Sigma, St. Louis, MO). Antigen-antibody complexes were detected with DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. The primary antibodies are anti-beta-catenin (Santa Cruz), rabbit polyclonal phospho-S6 Ribosomal Protein (Ser235/236) antibody (Cell Signaling #2211), polyclonal rabbit phospho-p44/42 MAP Kinase (Thr202/Tyr204) antibody (Cell Signaling, Danvers, Massachusetts).

Apoptosis and proliferation. The apoptotic cells were detected using an ApopTag *In Situ* Apoptosis Detection Kit (Chemicon). The assay was performed according to the manufacturer's manual. After deparaffinization, the tissues sections were incubated in proteinase K for 15 min. at room temperature. The sections were then incubated with terminal deoxynucleotidyl transferase (TdT) enzyme at 37oC for one hour, washed in three changes of PBS and incubated with anti-digoxignenin conjugate in a humidified chamber at room temperature for 30 minutes. The color was developed by incubating the sections with peroxidase substrate and then counterstained with haematoxylin for 30 seconds. For detection of proliferative cells, Ki-67 antibody (1: 50; Novocastra) was used. The assay was performed following the manufacturer's protocols. The scoring of apoptotic and proliferative cells was done at x 400. A positive control slide of rat mammary glands provided by the manufacturer

was used as positive control for the *In Situ* apoptosis detection assay. For the Ki-67 staining, small intestinal crypt cells were used as an internal positive control.

Statistics. The nonparametric Mann–Whitney U test was used to analyze tumor counts. One-sided P values are given.

3.3 Results

3.3.1 Apc^{Min} intestinal tumor development is dependent upon ERBB3

To examine the potential role of ERBB3 signaling during intestinal tumorigenesis, we generated Apc^{Min} mice with wildtype or intestinal epithelium specific deletion of ERBB3 (Figure 3-1A). At three months of age, all Apc^{Min} mice examined (n=16 ERBB3 wildtype, n= 14 ERBB3 deficient) developed visible polyps (> 0.3mm in diameter) in the small intestine regardless of *Erbb3* genotype. However, the number of macroadenomas in the small intestines of Apc^{Min}, *Vil-cre,* $Erbb3^{flox\Delta}$ mice was reduced dramatically compared with that in Apc^{Min} controls (10.6 ± 6.8 vs. 91.9 ± 76.0; $p < 0.00001$; Figure 3-1B). This ERBB3-dependent reduction in the number of small intestine polyps was observed in all regions of the small intestine, with the greatest effect in the proximal and middle part of the small intestine (Figure 3-1C). Whereas half of the Apc^{Min} control mice developed at least one colon polyp, no colon polyps were observed in any of the Apc^{Min}, *Vil-cre,* $Erbb3^{flox\Delta}$ mice (Figure 3-1D). Altogether, these results demonstrate that epithelial-specific ERBB3 signaling is important during intestinal tumorigenesis in Apc^{Min} mice.

To assess the importance of ERBB3 on tumor growth, the size of the residual polyps in Apc^{Min}, *Vil-cre,* $Erbb3^{flox\Delta}$ mice was compared with those in Apc^{Min} control mice (Figure 3-1E). Polyps forming in Apc^{Min} mice lacking intestinal epithelial expression of ERBB3 were significantly smaller than those forming in Apc^{Min} control mice (average size 0.62 ± 0.48 vs.

0.94 ± 0.45; p< 0.0001; Figure 3-1E), suggesting that normal levels of ERBB3 signaling is essential for tumor growth in *Apc*^{Min} mice. While only 60 % of the polyps in age-matched control mice were small, 87.0 % of the polyps in *Apc*^{Min}, *Vil-cre, Erbb3*$^{flox\Delta}$ mice were less than 1mm in diameter (Figure 3-1F). Similarly, the fraction of middle-sized polyps between 1 and 2 mm in diameter was also reduced in *Apc*^{Min}, *Vil-cre, Erbb3*$^{flox\Delta}$ mice compared to age-matched controls (9.3 % vs. 38.2 %). Interestingly, a few large intestinal polyps (greater than 2 mm in diameter) were present on the ERBB3-deficient background, suggesting that their growth might be ERBB3-independent. Histological analysis of size-matched polyps did not reveal morphological differences related to *Erbb3* genotype.

3.3.2 ERBB3 signaling prevents apoptosis in *Apc*^{Min} polyps

The proliferative and apoptotic rates within *Apc*^{Min} tumors was measured to determine the cellular mechanism responsible for reduced tumor size in the absence of ERBB3 in the intestinal epithelium. Staining with the proliferation marker Ki67 showed the proliferating cells in normal intestinal tissue are confined to the proliferative zone of the crypts, which was expanded in tumors, identified by nuclear accumulation of β-catenin, in *Apc*^{Min} mice irrespective of *Erbb3* genotype (Figure 3-2 A-F). Conversely, the apoptotic rate of tumors as measured by TUNEL was significantly different based upon *Erbb3* genotype. An increase in the number of TUNEL-positive cells was observed in tumors from *Apc*^{Min}, *Vil-cre, Erbb3*$^{flox\Delta}$ mice compared tumors from *Apc*^{Min} control mice (Figure 3-2 G-J). These results suggest that reduced tumor growth caused by epithelial deletion of *Erbb3* is due to an elevated level of cell death, indicating ERBB3 provides a survival signal for intestinal tumor cells.

3.3.3 ERBB3-dependent signaling is mediated by mTOR/S6.

To investigate the molecular mechanism of ERBB3-dependent tumor cell survival, the activity of downstream signaling effectors in size-matched polyps from *Apc*^{Min}, *Vil-cre, Erbb3*^{floxΔ} and *Apc*^{Min} control mice was examined (Figure 3-3). In *Apc*^{Min} control mice, strong cytoplasmic immunoreactivity in non-transformed normal epithelial cells surrounding polyps as well as within polyps was observed for phosphorylation of ribosomal protein S6 (pS6), a downstream mediator of the PI3K/AKT/mTOR pathway (Figure 3-3A). However, in size-matched polyps from *Apc*^{Min}, *Vil-cre, Erbb3*^{floxΔ} mice, the pS6 immunoreactivity was significantly reduced (Figure 3-3B). In contrast, immunoreactivity for p42/44 MAPK, which is associated with proliferation signal in epithelial cells, was comparable between *Erbb3* genotypes (Figure 3-3 C-D) . These results demonstrate that while signaling through p42/44 MAPK is not effected by ERBB3 deletion, signaling via S6 is significantly reduced in the absence of ERBB3.

3.3.4 ERBB3 is required in a subset of colonic tumors.

To confirm results obtained using the *Apc*^{Min} model, which predominantly develops small intestinal tumors, the dependency on ERBB3 signaling was also investigated in the azoxymethane (AOM) model. Similar to the *Apc*^{Min} model, a significant decrease in tumor multiplicity was observed in the absence of ERBB3 in the intestinal epithelium (Figure 3-4). ERBB3 wildtype mice were highly susceptible to AOM treatment: 73% of AOM treated mice developed one or more colonic polyp, with an average of 4.7 polyps per mouse. Conversely, only 30% of *Vil-cre, Erbb3*^{floxΔ} mice treated with AOM developed colonic polyps, with an average of 2 polyps per mouse ($p < 0.05$). In contrast to the size-effect on residual polyps observed in the *Apc*^{Min} mouse model, there is no significant difference in the size of AOM-induced colonic tumors between two groups (3.65 ± 1.6 vs. 3.58 ± 1.1; p =

0.92). These findings suggest that ERBB3 signaling contributes to intestinal and colonic tumors irrespective of the model, but a different mechanism may mediate ERBB3-independent tumor growth depending on tumor location.

3.4 Discussion

Although ERBB3 lacks intrinsic kinase activity, circumstantial evidence has accumulated suggesting that activation of ERBB3-dependent pathways can modulate tumor phenotypes (Kobayashi et al., 2003; Xue et al., 2006). By generating an intestinal-specific deletion of *Erbb3*, we were able to examine intestinal polyp development in a genetic environment deficient for ERBB3 activity. In the Apc^{Min} mouse model of familial adenomatous polyposis (FAP), ERBB3 deficiency had a profound effect on polyp number, reducing the mean tumor number by 88.5%. Furthermore, there was a complete absence of polyps in the colons from Apc^{Min}, *Vil-Cre*, $Erbb3^{flox\Delta}$ mice. This robust anti-tumor activity of *Erbb3* deletion was confirmed in the AOM mouse model of colon cancer. ERBB3-deficiency significantly reduced the average size of remaining Apc^{Min} polyps. Such size-reduction effect has not been observed when EGFR activity was reduced (Roberts et al., 2002), highlighting the unique role of ERBB3-dependent signaling in regulating tumor growth. To our knowledge, we provide the first direct evidence that the kinase-dead ERBB3 is pivotal for regulating tumor growth *in vivo*.

While the extent of proliferation in Apc^{Min} polyps lacking ERBB3 was comparable in those with wildtype ERBB3, a higher number of apoptotic cells were detected in the ERBB3-deficient polyps from Apc^{Min} mice, demonstrating an important role for ERBB3 in tumor cell survival. Consistent with a lack of effect on cell proliferation, ERBB3-deficient polyps, have normal levels of p42/44 MAPK activation, which is the predominant mitogenic signal. In

contrast, p70S6 kinase (S6K) activity, assayed by ribosomal protein S6 phosphorylation, is reduced in ERBB3-deficient polyps. S6K is a target of the mammalian target of rapamycin (mTOR). The mTOR/S6K pathway is associated with cell survival through regulation of cell cycle arrest and apoptosis (reviewed in (Asnaghi et al., 2004)). Therefore, our results indicate that ERBB3 signaling contributes to tumor growth by prevention of apoptosis via activation of mTOR/S6K. ERBB3 can couple EGFR to the PI3K/AKT upon growth factor stimulation (Kim et al., 1994; Soltoff et al., 1994) and PI3K/AKT lies upstream of mTOR/S6K. Therefore, the requirement of the ERBB3 signaling pathway in intestinal tumor progression could result from its unique role of linking EGFR signaling to PI3K/AKT, thus activating the mTOR/S6K pathway to promote cell survival. EGFR also activate PI3K/AKT through association with the adaptor protein GAB1 in Apc^{Min} polyps (Moran et al., 2004). It is possible that PI3K/AKT is activated by EGFR via two mechanisms, association with GAB1 and coupling with ERBB3. Our results suggest that ERBB3 activation of PI3K/AKT is the major mechanism in Apc^{Min} polyps.

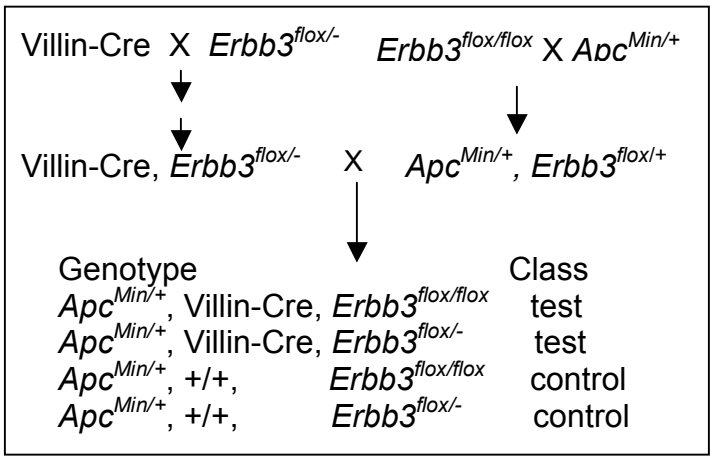
Unlike the small intestinal polyps in the Apc^{Min} model, epithelial-specific deletion of ERBB3 in the AOM did not result in tumor size reduction, although the tumors were much fewer in number. This difference could be due in part to the fact that Apc^{Min} mice develop polyps by loss of APC, while in the AOM model, tumors are induced by stabilization of β-catenin. However, recent gene expression profiling shows that these two models are highly similar (Kaiser et al., 2007), suggesting that the difference in the route of tumor initiation in the Apc^{Min} and AOM models likely does not contribute to molecular differences resulting in ERBB3 sensitivity. An alternative possibility is that a subset of colonic tumors can grow independently of ERBB3, similar to what we have previously observed for EGFR (Roberts et

al., 2002). Since *Apc*^{min} mice lacking ERBB3 in the intestinal epithelium did not develop colonic tumors, they either lack the ERBB3-independent class or this class is infrequent in the *Apc*^{Min} model as opposed to the AOM model.

In this study, we observed a profound ERBB3-dependnent reduction in polyp multiplicity and size. This robust anti-tumor activity of targeting ERBB3 may result from its unique link to PI3K/AKT and its downstream effecter mTOR. Furthermore, as ERBB3 partners with EGFR/ERBB2 and delivers essential signals, a lack of ERBB3 would abolish EGFR/ERBB3 and ERBB2/3 heteromiders simultaneously, which may contribute to the anti-tumor effects. Consequently, targeting ERBB3 and disrupting heterodimer formation, or using antibodies that inhibit ERBB3 heterodimerization with other ERBBs, may be more efficient than targeting individual receptors. Our findings also suggest that inhibition of the mTOR/S6 pathway, the major downstream effecter of ERBB3-dependent signaling, may be effective in treating intestinal cancers.

Our study highlights the importance of regulators of intestinal tumor progression that are dependent on the ERBB3 signaling pathway. It will be important to determine whether ERBB3-dependent signaling also contributes to tumorigenesis in other cancers such as breast cancer, NSCLC and prostate cancer, where PI3K/AKT is strongly implicated. For this purpose, the conditional ERBB3 targeted allele used in this study would be an ideal tool and our findings illustrate the value of using mouse models to study human diseases.

A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Erbb3flox/flox X Apc Min/+
Villin-Cre, Erbb3flox/-
X
Apc Min/+, Erbb3flox/+
Genotype
Class
Apc Min/+, Villin-Cre, Erbb3flox/flox test
Apc Min/+, Villin-Cre, Erbb3flox/- test
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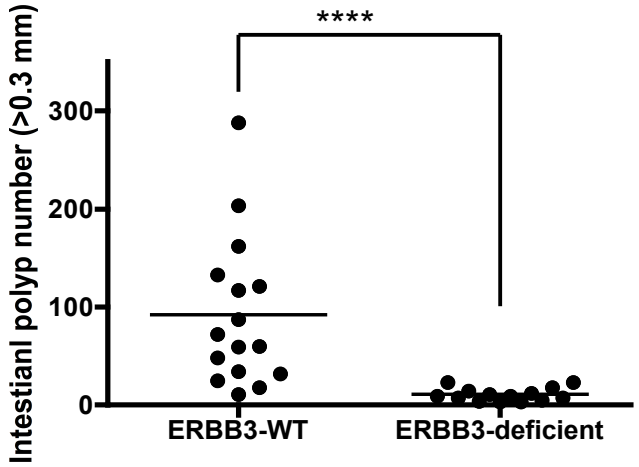
Apc Min/+, +/+, Erbb3flox/- control

B


****
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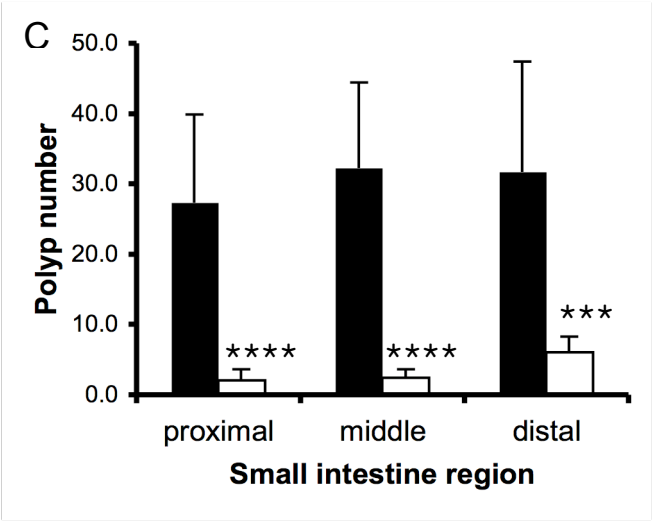
200
100
0
ERBB3-WT
ERBB3-deficient

C


Polyp number
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40.0
30.0
20.0
10.0
0.0
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proximal
middle
distal
Small intestine region

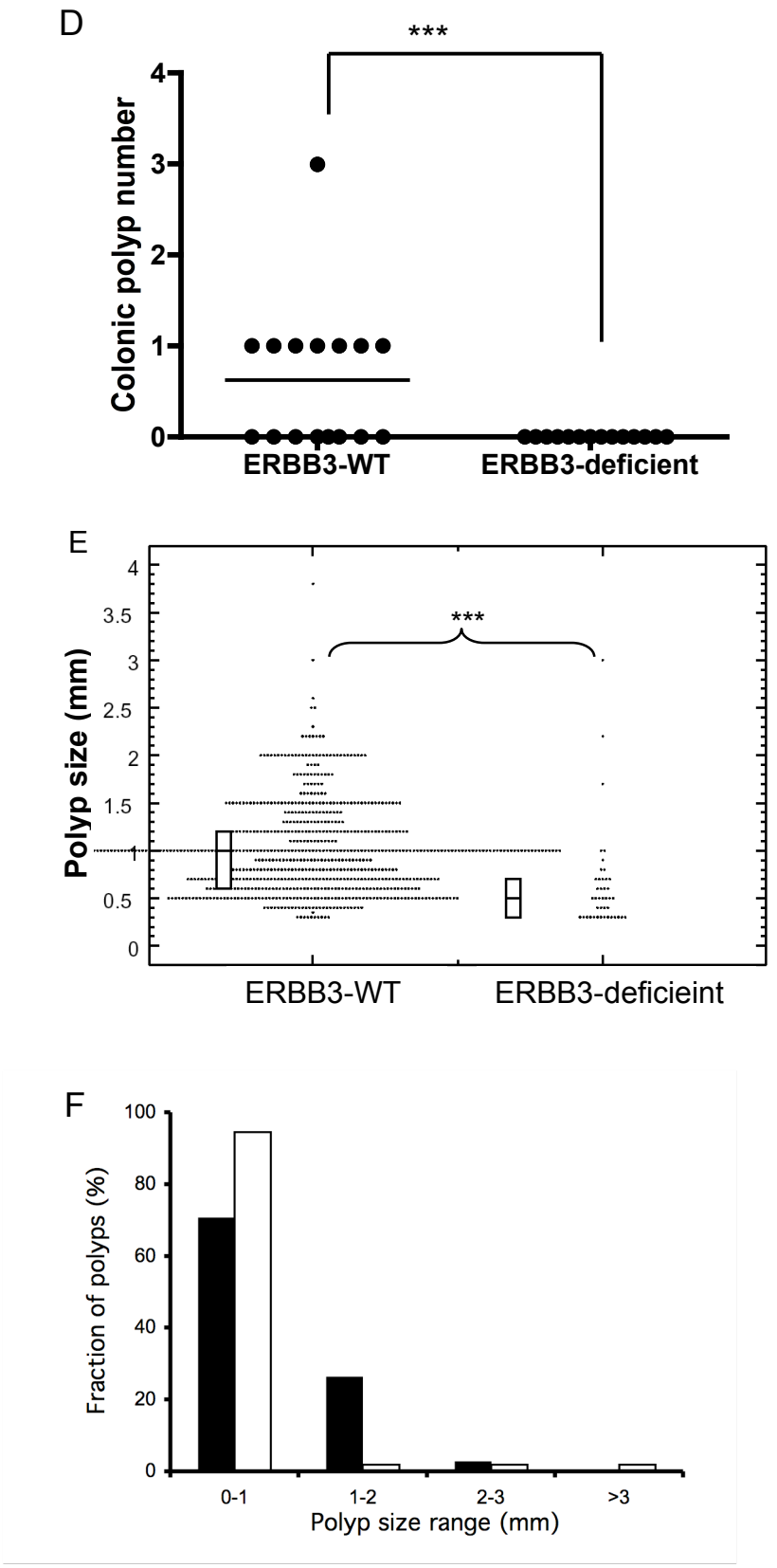

Figure 3-1 Effects of ERBB3-deficiency on *Apc*^{Min} tumor development

Figure 3-1 Effects of ERBB3-deficiency on *Apc*^{Min} tumor development. (A) Breeding scheme to generate intestine epithelia-specific *Erbb3* deletion. (B) Intestinal macroadenoma multiplicity in *Apc*^{Min} mice. Each dot represents polyp number in single 3-month-old *Erbb3* wt (n= 16) and mutant (n =14) mice. (C) Frequency of intestinal polyps stratified by small intestine region. (D) Colonic polyp multiplicity in *Apc*^{Min} mice. (E) Intestinal macroadenoma size analysis. Each dot represents a single polyp. (F) Size range of tumors in *Apc*^{Min} mice. ***, $P < 0.0001$; ****, $P < 0.00001$; Mann-Whitney U test.

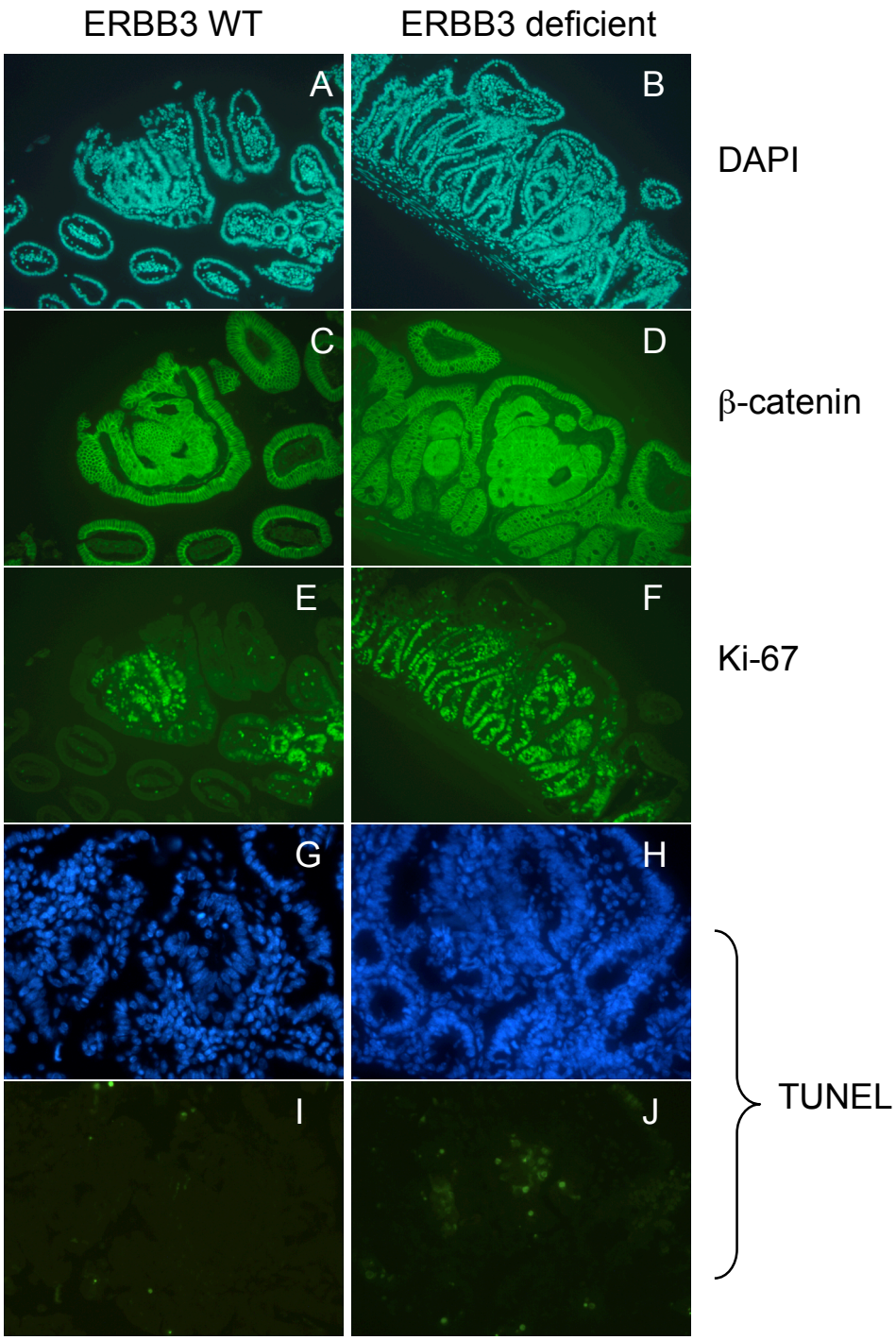

Figure 3-2 Proliferation and apoptosis in *Apc*^{Min} polyps from 3-month-old ERBB3 wildtype and deficient mice

Figure 3-2 Proliferation and apoptosis in Apc^{Min} polyps from ERBB3 wildtype and deficient mice (A-B) Diamidino-phenylindole (DAPI) staining, (C-D) beta-catenin immunostaining (E-F) Ki-67 immunostaining and (G-H) TUNEL labeling with DAPI counter-staining of size matched intestinal polyps from 3-month-old ERBB3 wildtype and ERBB3 deficient mice.

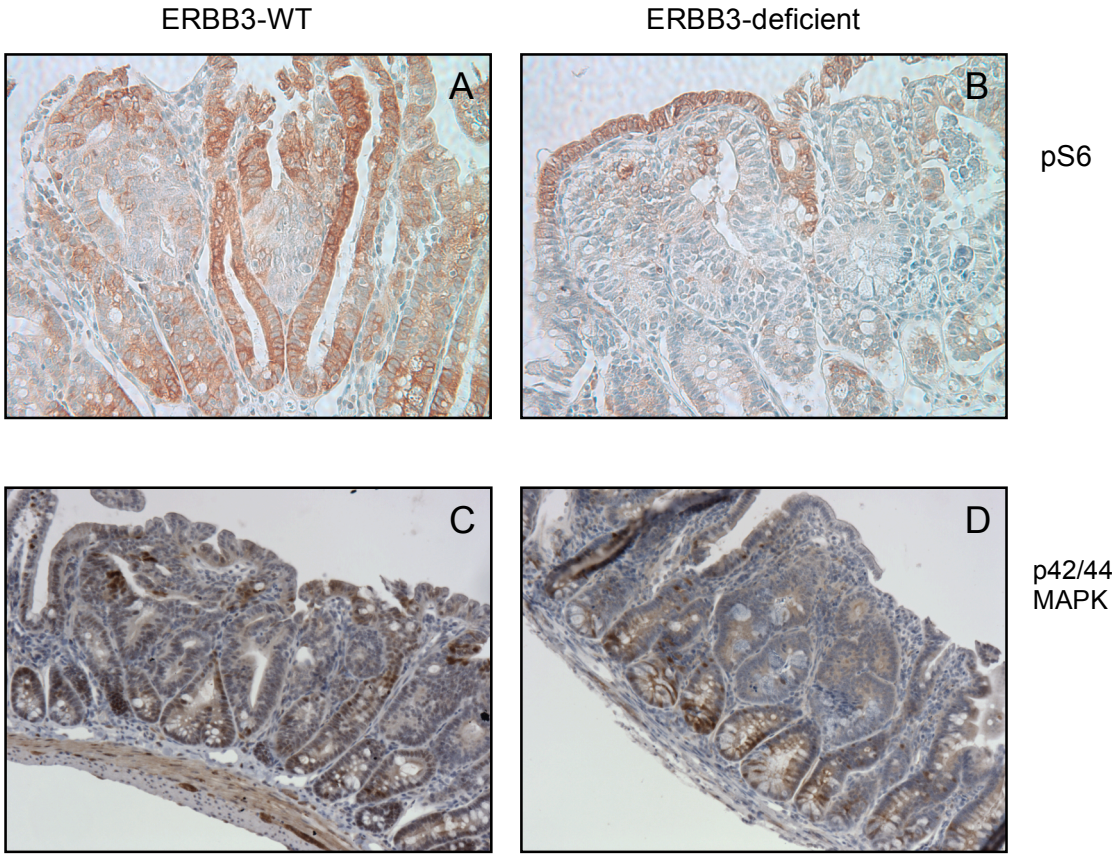

Figure 3-3 Pathway analysis in ERBB3-deficient *Apc*^{Min} polyps

Figure 3-3 Pathway analysis in ERBB3-deficient *Apc*^{Min} polyps. (A-B) Immunostaining of phosphorylated ribosomal protein S6 (pS6) in intestinal polyps from 3-month-old *Erbb3* wildtype and deficient mice. (C-D) Immunostaining of p42/44 MAPK in intestinal polyps from 3-month-old *Erbb3* wildtype and deficient mice.

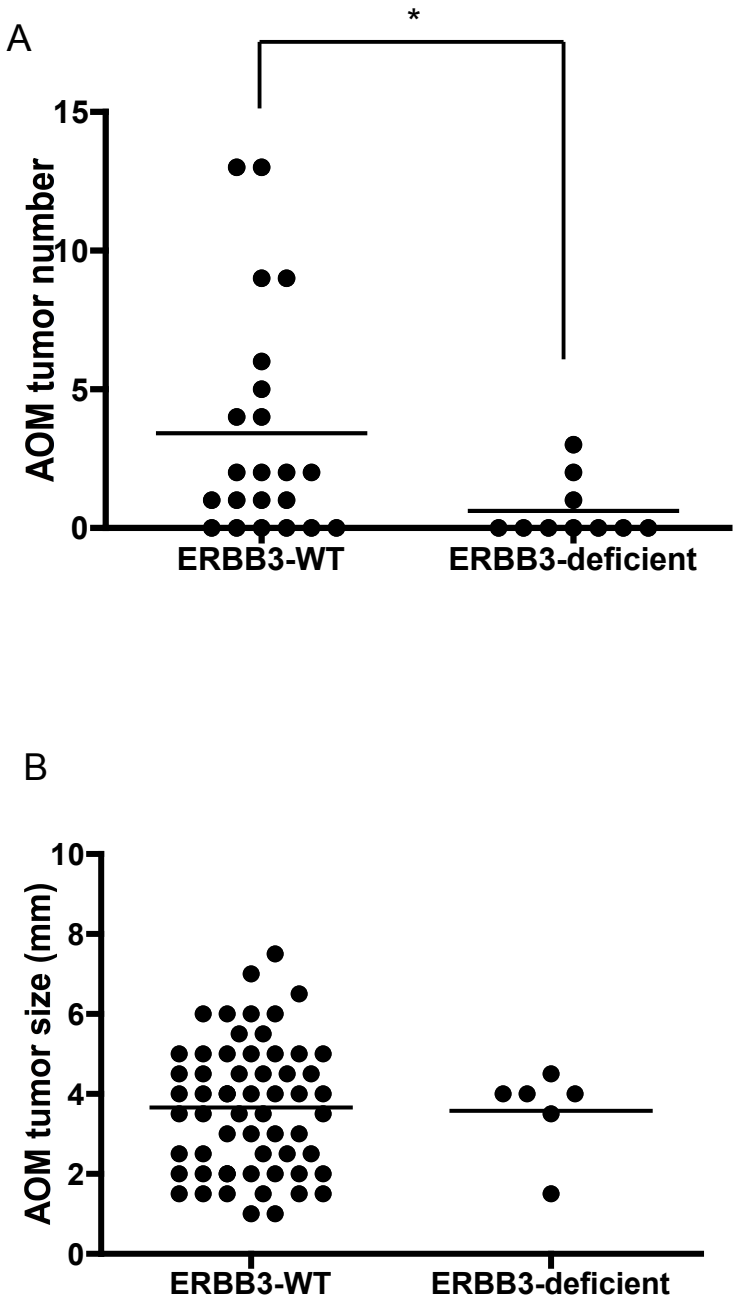

Figure 3-4 Effect of ERBB3-deficiency on AOM-induced colonic tumor development

Figure 3-4 Effect of ERBB3-deficiency on AOM-induced colonic tumor development. Colonic polyp multiplicity (A) and size (B) in azoxymethane-treated *Erbb3* wildtype (n=22) and mutant (n=10) mice. *, $P < 0.05$; Mann-Whitney U test.

Chapter 4 Genetic background effect on *Apc*^{Min} tumor response to EGFR inhibition

Abstract

The *Apc*^{Min} polyps forming in the hypomorphic *Egfr*^{wa2} mice appear to grow independently of EGFR (Roberts et al., 2002). To examine the effects of genetic background on EGFR-independent tumor growth, we generated F1 hybrids by intercrossing *Apc*^{Min} allele on C57BL/6J with *Egfr*^{wa2} allele on four congenic inbred strains: B6, BTBR/J, A/J and 129S1/SvImJ (129). There was significant strain-specific variation in tumor multiplicity and size, suggesting that BTBR/J, A/J and 129 contain modifiers that reduce tumor number and size. More importantly, the reduction in tumor number in *Apc*^{Min}, *Egfr*$^{wa2/wa2}$ mice relative to *Apc*^{Min} mice carrying a wild-type *Egfr* allele varied with different genetic background. While the B6.A F1 and B6.129 F1 backgrounds exhibited 70% reduction in EGFR-dependent tumor growth, the C57BL/6J and B6.BTBR F1 backgrounds supported 50% of EGFR-independent tumor growth. These results have implication for understanding the genetic heterogeneity in tumor response to EGFR-targeted therapies in human patients.

4.1 Introduction

Studies into the factors contributing to colorectal cancer (CRC) have demonstrated that genetics greatly affects an individual's susceptibility to this disease (reviewed in (Kinzler and Vogelstein, 1996)). Familial adenomatous polyposis (FAP), resulting from a germline mutation in the adenomatous polyposis coli (*APC)* tumor suppressor gene (Fearnhead et al., 2001), is one of the most well characterized familial causes of CRC. Individuals with FAP develop hundreds to thousands of colonic polyps and have a high risk of developing CRC if left untreated. Variation in polyp number is thought to be modulated by the site of the mutation in the *APC* gene, environmental factors, and the effects of unknown genetic modifiers (Crabtree et al., 2002). Understanding the genetic complexity underlying susceptibility to CRC should lead to better prevention, diagnosis and treatment.

A nonsense mutation at codon 850 in the mouse homolog of the *APC* gene, referred to as the *multiple intestinal neoplasia* allele of *Apc* (Apc^{Min}), predisposes mice to intestinal tumorigenesis (Moser et al., 1990; Su et al., 1992). Despite the fact that the colon is primarily affected in FAP patients as opposed to the small intestine in Apc^{Min} mice, the Apc^{Min} model recapitulates the molecular initiating events occurring in FAP patients and has been used extensively as a model of human CRC. The number of polyps that arise in Apc^{Min} mice is greatly influenced by genetic background (Dietrich et al., 1993; Moser et al., 1992). Quantitative trait analysis (QTL) identified 'modifier of *Min*' 1 (*Mom1*) on chromosome 4 as a major modifier of the Apc^{Min} phenotype (Dietrich et al., 1993); *Mom1* affects both tumor multiplicity and size in a semi-dominant fashion (Gould et al., 1996).

Inbred strains carrying the *Mom1* sensitive allele (*Mom1*^{s}), including C57BL/6J (B6), BTBR/J (BTBR), A/J and 129S1/SvImJ (129), are more susceptible to polyp formation caused by the *Apc*^{Min} mutation, whereas inbred strains carrying the *Mom1* resistant allele (*Mom1*^{r}), including AKR/J, BALB/cJ, C3H/HeJ and SWR/J, are relatively resistant to polyp formation. The *Mom1* locus is estimated to account for about 50% of the genetic variation in polyp number among genetically different *Apc*$^{Min/+}$ mice (Dietrich et al., 1993), indicating that other modifiers also exist. Since the discovery of *Mom1*, at least four additional *Mom* loci have been identified by QTL analysis (reviewed in Table 1). Along with the identification of *Mom* loci, other non-polymorphic genetic modifiers have been recognized by transferring mutations of interest onto a *Apc*$^{Min/+}$ background (reviewed in Table 1). Modifier genes identified using the *Apc*^{Min} mouse model should provide insights into the hereditary factors that influence CRC susceptibility and severity in humans.

EGFR-targeted therapies, such as small-molecule-inhibitors and monoclonal antibodies, are now approved for use in metastatic CRC patients or are in advanced clinical trials for other uses. These clinical trials clearly demonstrate that the responses of CRC patients to EGFR-targeted therapies exhibit extensive variation, with some patients responding well, while the majority failing to display any benefit. With the exception of lung cancer, where an activating mutation in EGFR is associated with a dramatic response to anti-EGFR therapy, the mechanisms underlying the response to EGFR inhibitors is currently unknown. A important observation in clinical studies is the lack of association between expression of EGFR as determined by immunohistochemistry, and clinical benefit from anti-EGFR therapy. Interestingly, there is a striking correlation between the development of a skin rash, the prototypical toxicity of EGFR inhibitors, and therapeutic

response (Cohen et al., 2003; Janne, 2003). The underlying mechanistic explanations are currently unknown.

A possible reason for the varied outcomes may be due to the pharmacological properties of EGFR-inhibiting drugs, with higher plasma concentrations corresponding to better patient response. However, the actual relationship between plasma drug level and clinical outcome has not been established in clinical studies. It is more likely that genetic differences among individuals contribute to the different responses observed with anti-EGFR drugs. Gene expression profiling of 100 human colorectal adenomas has revealed that human colon tumors exhibit extensive molecular heterogeneity (Kaiser et al., 2007). These results suggest that although the development of CRC undergoes similar morphological changes (Kinzler and Vogelstein, 1996) and tumors share similar histopathological characteristics, the genetic and genomic events leading to malignancy are distinct for each tumor. Therefore, we hypothesized that genetic heterogeneity affects the response to anti-cancer therapeutic agents, and that genetic modifiers may be a source of the variability among patient responses to EGFR-targeted drugs.

To genetically model the effects of EGFR inhibition during CRC treatment, our lab has used mice homozygous for the *Egfr*^{wa2} hypomorphic allele. The *Egfr*^{wa2} produces a receptor with a reduced kinase activity due to a substitution of a glycine for a valine residue near the amino terminus of the tyrosine kinase domain. *Egfr*^{wa2}-encoded receptors have up to a 90% reduction in kinase activity depending on the assay used (Fowler et al., 1995; Luetteke et al., 1994). Historically the *Egfr*^{wa2} allele was maintained on a B6EiC3H mixed background (Luetteke et al., 1993). In our laboratory, it has been bred to congenicity on several genetic backgrounds including C57BL/6J (B6), 129S1/SvImJ (129), A/J (A),

and BTBR/J (BTBR). The F1 hybrids are better at supporting pup survival to adulthood, allowing for detailed phenotypic analysis in these adult mice. Recent studies have shown that genetic background affects the development of aortic stenosis and cardiac function in *Egfr*$^{wa2/wa2}$ adult mice (Barrick et al., 2007). The 129 background dominantly protects against calcific aortic stenosis in *Egfr*^{wa2} mice, while the B6-*Egfr*$^{wa2/wa2}$ mice are more susceptible and *Egfr*^{wa2} homozygotes on the B6.129 F1 background have a mild phenotype.

Since strain-based variation in phenotypes associated with reduced or absent EGFR activity are well established, we hypothesized that EGFR-dependent *Apc*$^{Min/+}$ tumor growth would also vary by genetic background. Therefore, to identify potential strain-specific differences in EGFR-dependent *Apc*$^{Min/+}$ tumor growth, we took advantage of the availability of four congenic *Egfr*^{wa2} strains and crossed them with B6-*Apc*$^{Min/+}$, *Egfr*^{wa2} mice. In this way, we generated *Apc*$^{Min/+}$, *Egfr*$^{wa2/wa2}$ F1 hybrids to compare tumor response from EGFR inhibition on multiple genetic backgrounds including B6, B6.BTBR F1, B6.A F1, and B6.129 F1. We found that genetic background modifies tumor multiplicity and size, confirming that genetic background modifiers influence tumor phenotypes. More importantly, strain-specific variability in tumor multiplicity associated with reduced EGFR activity was observed, demonstrating that genetic background affects response to EGFR inhibition in *Apc*$^{Min/+}$ mice.

4.2 Materials and methods

Mice. C57BL/6J (B6)-*Apc*^{Min} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice of the stock B6EiC3h-a/A-*Egfr*^{wa2}*Wnt3a*^{vt} were obtained from The Jackson Laboratory (Bar Harbor, ME). The *Egfr*^{wa2} allele was backcrossed to the inbred

strains 129S1/SvImJ, BTBR/J, A/J, and C57BL/6J as previous described (Roberts, 2003). Mice were fed Purina Mills Lab Diet 5058 under specific-pathogen-free conditions in an American Association for the Accreditation of Lab Animal Care–approved facility. Mice were euthanized by CO_2 asphyxiation for tissue collection.

Genotyping. Mice were genotyped for the *Apc*^{Min} and *Egfr*^{wa2} as previously described (Roberts et al., 2002; Roberts, 2003).

Tissue collection. The small intestine and colon were removed from each mouse. The small intestine was cut into thirds. Each segment were gently flushed with PBS to remove fecal material, cut longitudinally, and splayed flat. Representative tumors were scored before cutting in half under the dissecting microscope. One half were used for RNA extraction and array experiment; another half were fixed in 10% neutral buffered formalin at 4 °C overnight for histological analysis, or snap-frozen for use in cryo-sectioning.

Macroadenoma counts. The tumor number and diameter were obtained for the entire length of the small intestine and colon, with a dissecting microscope and in-scope micrometer at 5x magnification. The smallest tumors that can be counted are approximately 0.3 mm in diameter. Tumor scoring was performed without knowledge of genotype by the investigator. Changes in tumor growth rate were recorded grossly as tumor size. In addition to tumor size, tumors were carefully scored based on number and location along the gastro-intestinal (GI) tract.

Histology and Immunohistochemistry: Intestinal tissues or colon samples were rolled into a jelly-roll before fixing in 10% neutral buffered formalin. The processed tissues were embedded in paraffin and sectioned (7 μm). Every 50 μm, sections were taken and stained with H&E.

Statistics. The nonparametric Mann–Whitney U test was used to analyze all comparisons. One-sided P values are given.

4.3 Results

4.3.1 Tumor multiplicity and size in *Apc*$^{Min/+}$ mice on different backgrounds

To examine the effects of genetic background on EGFR-dependent tumor growth, intercrosses between B6-*Apc*$^{Min/+}$, *Egfr*$^{wa2/+}$ and three *Egfr*$^{wa2/+}$ congenic strains was established (Figure 4-1). Female *Egfr*$^{wa2/+}$ mice from B6, BTBR, A, and 129 strains were crossed with male B6-*Apc*$^{Min/+}$, *Egfr*$^{wa2/+}$ mice. Litters were primarily produced from crosses in which *Apc*$^{Min/+}$ was carried by males since *Apc*$^{Min/+}$ females had fewer and smaller litters than the *Apc*$^{+/+}$ females (unpublished observations, data not shown).

The resulting F1-*Apc*$^{Min/+}$ progeny homozygous for *Egfr*^{wa2} exhibited wavy coats and, along with *Apc*$^{Min/+}$, *Egfr*$^{wa2/+}$ or *Egfr*$^{+/+}$ littermate controls, were scored for tumors along the entire intestinal tract at three-months of age. The distribution of tumor number in the small intestines among F1 hybrids with at least one wildtype *Egfr* allele was significantly shifted to few tumors compared to the parental B6 background (Figure 4-2). A comparison of polyp numbers from all B6 mice and F1 hybrids revealed the influence of BTBR, A and 129 backgrounds on polyp number (Table 2; Figure 4-3A). The average total tumor number for the F1 hybrids was 125.8/mouse for B6.BTBR F1- *Apc*$^{Min/+}$ mice, which is significantly less that that for B6-*Apc*$^{Min/+}$ mice (mean = 208.0 polyps/mouse, $p < 0.005$). B6.A F1 mice had a further reduction in tumor number, developing on average 62.3 polyps/mouse (Figure 4-3A, $p < 0.0001$). Of the four genetic backgrounds examined, the B6.129 F1 background was the most resistant to polyp formation. These mice developed

on average 33.4 polyps ($p < 0.0001$). In contrast to the B6-*Apc*$^{Min/+}$ mice that developed an average 1.6 colonic polyps, none of the F1-*Apc*$^{Min/+}$ mice developed any colonic polyps (Table 2). Consistent with previous reports (Roberts et al., 2002), there was no difference in tumor number between mice carrying one or two wildtype *Egfr* alleles.

The sizes of polyps were estimated by measuring their maximum diameter. The average diameter decreased from 1.19 mm in B6-*Apc*$^{Min/+}$ mice to 1.03 mm in B6.A F1-*Apc*$^{Min/+}$ mice, with a further decrease to 0.87 mm in B6.BTBR F1-*Apc*$^{Min/+}$ mice. Consistent with resistance in tumor number, B6.129 F1 mice displayed the smallest average polyp diameter among all four groups, averaging 0.74 mm in diameter (Table 3, Figure 4-3B).

Taken together, these results suggest the existence of tumor resistance alleles carried by BTBR, A and 129 that can modify the B6-*Apc*$^{Min/+}$ phenotype, resulting in a significant decrease in both the number and size of tumors. Although fewer in number and smaller in size, the polyps present in the F1 mice were histologically similar to polyps in B6-*Apc*$^{Min/+}$ mice (data not shown).

4.3.2 Tumor multiplicity in *Apc*$^{Min/+}$, *Egfr*$^{wa2/wa2}$ mice is modified by genetic background.

Previous studies have shown that homozygosity for the *Egfr*^{wa2} allele has a profound effect on tumor multiplicity in adult animals on a mixed genetic background (Roberts et al., 2002). Consistent across all backgrounds, *Apc*$^{Min/+}$ mice homozygous for the *Egfr*^{wa2} allele developed significantly fewer polyps than their respective *Apc*$^{Min/+}$ littermate controls (Table 4, Figure 4-4A). Previous studies suggested that the growth of the residual polyps present on the *Egfr*$^{wa2/wa2}$ background is not dependent on EGFR

activity (Roberts et al., 2002). On the B6 background, the polyp number decreased from 208.0 polyps/mouse in wildtype *Egfr* controls to 93.4 polyp/mouse in $Egfr^{wa2}$ homozygous mice, representing a 48% reduction in polyp number (Table 4, Figure 4-4B). A similar reduction was observed on the B6.BTBR F1 background, where $Egfr^{wa2}$ homozygous $Apc^{Min/+}$ animals developed an average of 75.8 polyps, compared to 125.8 polyps in $Egfr^{wa2/+}$ or $Egfr^{+/+}$ controls. On the B6.A F1 background, EGFR-independent tumor growth was significantly less pronounced, with $Egfr^{wa2}$ homozygous mice developing an average of 21.4 polyps, or a 67.7% reduction compared to the 62.3 polyps in $Egfr^{wa2/+}$ or $Egfr^{+/+}$ littermate controls. On the B6.129 F1 background, $Egfr^{wa2/wa2}$ mice developed a small number of polyps (5.9 ± 4.85) when compared to the 33.4 polyps that developed in wildtype *Egfr* controls. This resulted in a significantly higher 75.2% reduction in tumor number. Overall, EGFR-independent polyp growth is highly influenced by strain background. The B6 and B6.BTBR F1 backgrounds are more resistant to reduced EGFR activity, supporting about 50% of tumors being EGFR-independent,. Conversely, the B6.A F1 and B6.129 F1 backgrounds are more sensitive to EGFR inhibition, exhibiting an approximate 70% reduction in EGFR-dependent tumors, which represents about 30% EGFR-independent tumors.

4.4 Discussion

Mouse models of human cancer are powerful tools for studying cancer pathogenesis and susceptibility, as exemplified in the Apc^{Min} mouse model. This model has an advantage over other experimental models of CRC, such as cancer cells grown as xenografts in nude mice, in that the phenotypes of Apc^{Min} tumors closely resemble many important aspects of the initiation and early progression of human colonic tumors. First,

the initiation of *Apc*^{Min} tumors is caused by the loss of APC expression, which molecularly recapitulates the most common initiation event seen in human CRC. Second, *Apc*^{Min} mice develop numerous countable intestinal neoplasms, similar to the numerous polyps that develop in FAP patients. Third, the colorectal tumor phenotype in FAP patients is modified by known and unknown genetic factors, a trait that is mirrored in *Apc*^{Min} mice. Among the genetic modifiers that have been localized, at least four specific '*modifiers of min*' (*Mom*) have been characterized using inbred mouse strains that modulate the *Apc*^{Min} phenotype (reviewed in Table 1). Inbred mouse stains can carry multiple *Mom* loci, with the *Apc*^{Min} tumor phenotype being a net result of modification by all loci, in addition to non-genetic, environment factors. For example, the B6 strain is known to harbor both the *Mom1*^{s} enhancer and the *Mom7* suppressor alleles (Dietrich et al., 1993; Kwong et al., 2007); the dominant polyp-suppressing *Mom7* allele is counteracted at least in part by the polyp-enhancing *Mom1*^{s} allele. Thus 100% of *Apc*$^{Min/+}$ mice on a B6 background develop numerous intestinal polyps by two-to-three months of age. This phenotype contrasts sharply with the AKR background, where 75% of *Apc*$^{Min/+}$ mice are tumor free at six months of age (Dietrich et al., 1993). The effect of the recessive, tumor enhancing *Mom7* allele in AKR is offset by the effect of the potent tumor-resistant *Mom1*^{r} allele in the same mouse (Dietrich et al., 1993; Kwong et al., 2007). Thus, a full characterization of the *Apc*^{Min} phenotype in different inbred mouse strains requires further analysis to identify novel *Mom* loci.

In our study, we provide evidence for the modification of intestinal tumor phenotypes in B6-*Apc*$^{Min/+}$ mice by alleles from BTBR, A and 129. In F1 hybrids carrying *Apc*$^{Min/+}$, alleles coming from BTBR, A or 129 strains confer a suppression of polyp

multiplicity and size when compared to B6-*Apc*$^{Min/+}$. The effect of polyp resistance varied among the backgrounds, indicating an array of alleles within the BTBR, A and 129 genomes affect polyp development. Previous studies have shown that all four strains harbor the tumor-enhancing *Mom1*^{s} allele. Therefore, our observed decrease in tumor number on the three F1 backgrounds, when compared to B6, is not likely due to the *Mom1* genotype. Additionally, the observed decrease in the F1 hybrids is probably not a result of the *Mom3* or *Mom7* alleles, since neither exerts tumor-enhancing effects on BTBR, A or 129 backgrounds. To determine whether the decrease in polyp number is the result of the tumor-resistant effects of *Mom2* requires further analysis. Alternatively, since previous studies have shown that mutations in *Dnmt1* (DNA methyltransferase) and *Mmp7* (matrix metalloproteinase matrilysin) can influence polyp number and size in *Apc*^{Min} mice (Cormier and Dove, 2000; Wilson et al., 1997), differences in the *Dnmt1* and *Mmp7* genes may contribute to tumor phenotype. Undoubtedly other unidentified modifier genes also play a significant role in *Apc*^{Min}-mediated tumor development.

The F1 hybrids used in our studies arose from a cross where parental strains were congenic for the *Egfr*^{wa2} allele on chromosome 11. Although we have not observed any differences in phenotypes between *Egfr*$^{wa2/+}$ and *Egfr*$^{+/+}$mice, it is possible that the size of the *Egfr*^{wa2} congenic region derived from the B6EiC3H mixed background could confound our results. Though unlikely, there may be *Apc*^{Min} modifiers flanking the *Egfr*^{wa2} locus that are ultimately responsible for the effects we observed. Irrespectively, differences in the sizes of the congenic intervals would not alter the conclusions since strain-dependent modifiers would still be the cause of the background-dependent tumor effects of EGFR inhibition.

B6-*Apc*^{Min} mice maintained in our colony develop a relatively high number of intestinal polyps compared with published studies. The tumor number in B6-*Apc*^{Min} mice is commonly reported as 60-100 polyps/mouse at three-months of age, while we observe an average of 208.0 polyps/mouse. However, such discrepancies in tumor number could not have affected the validity of our results since we performed all analyses with littermate controls. A high tumor number in our *Apc*^{Min} mice may reflect differences in environmental factors. For example, differences in the pathogen-free status and the gut microbiota may contribute to variation in polyp multiplicity. Variation in tumor number in B6-*Apc*^{Min} mice has been reported in previous studies (Haines et al., 2005; Silverman et al., 2002; Song et al., 2000). For example, Song et al. (2000) reported that B6-*Apc*^{Min} mice developed an average of 24.4 polyps at three-months of age, while Silverman et al. (2002) reported an average number of 89.9 polyps in three-month old B6-*Apc*^{Min} mice. Interestingly, *Mom3* was identified as a novel modifier closely linked to *Apc*^{Min} that results in a dramatic increase in polyp number (range 289-494 polyps/mouse) and a shorter life span (less than three-months of old) (Haines et al., 2005). Thus, another possible explanation for the increase in poly number within our colony is the presence of polyp-promoting mutations in our B6-*Apc*^{Min} colony. This can be tested by crossing our B6-*Apc*^{Min} mice with B6 mice from other sources.

A significant interest exists in defining factors that could be used to identify patients more likely to respond to EGFR-targeted therapeutics, so that treatments can be delivered to the appropriate subjects. Our studies were designed to provide a preliminary glimpse into the genetic complexity of this trait. Phenotypes of *Egfr*^{wa2} homozygotes represent the result of reduced EGFR activity *in vivo*, and when crossed to the *Apc*^{Min}

mouse model of CRC, the resulting mice are an excellent model to experimentally examine potential causes underlying the variable response of human CRC patients to EGFR-targeted therapies. Previous data showing modification of Apc^{Min} tumorigenesis by the $Egfr^{wa2}$ allele was reported for a B6EiC3H mixed background (Roberts et al., 2002). Although a mixed genetic background creates genetic heterogeneity that is relevant to an outbred human population, the random segregation of the mixed backgrounds produce excessive experimental variation, which can confound phenotypic analysis. To reveal the existence of potential modifiers that can modulate interactions between Apc^{Min} and $Egfr^{wa2}$, we took advantage of the availability of four congenic $Egfr^{wa2}$ strains by crossing with Apc^{Min} mice. In this way, we generated Apc^{Min}, $Egfr^{wa2/wa2}$ F1 hybrid mice that developed significantly fewer polyps when compared to their Apc^{Min} littermates with normal EGFR activity, which was consistent with previously published results (Roberts et al., 2002). Indeed, by using congenic $Egfr^{wa2}$ strains, our studies partitioned potential genetic background effects influencing tumor response to reduced EGFR activity; on the B6 and B6.BTBR F1 backgrounds a higher percentage of tumors survive with reduced EGFR activity than on the B6.A F1 and B6.129 F1 backgrounds. Our results strongly indicate that genetic differences influence the relative frequency of EGFR-independent versus dependent tumors. The background-associated variation in Apc^{Min}, $Egfr^{wa2}$ tumor phenotype closely resembles the variation in response to EGFR-targeted therapies observed in human patients. Being genotypically identical, each F1 hybrid population produces a more homogenous phenotype, which can be compared with responses to EGFR inhibition in a subset of patients. By changing the F1 hybrid background it is possible to improve our view and to dissect the variable responses seen in CRC patients.

Our studies represent an initial attempt to dissect the genetic complexity underlying variable response to EGFR-targeted therapies in heterogeneous human patients. This information will contribute to better patient selection, leading to improvements in the efficacy of cancer therapeutics.

Table 4-1 Genetic modifiers of *Apc*$^{Min/+}$ mice

Name (location)	Modifier effect	Mode of action	Candidate genes	References
Mom1	Reduces small intestinal adenoma size and number	Control the net growth rate of tumors	The secretory type II nonpancreatic phospholipase A2 (Pla2g2a) gene	Dietrich et al., 1993; Gould et al., 1996; MacPhee et al. 1995; Cormier et al. 1997, 2000
Dnmt1	Suppress tumor multiplicity and growth rate	Genomic hypomethylation associated with genomic instability	DNA methyltransfer-ase gene	Laird et al., 1995; Cormier and Dove, 2000
Mmp7$^{-/-}$	Suppress tumor multiplicity and size	Not determined	The matrilysin locus	Wilson et al., 1997
Blm$^{-/-}$	Increase tumor number	Stimulate inter-homolog recombination	Bloom syndrome helicase (*BLM*)	Luo et al., 2000; Goss et al., 2002; Suzuki et al., 2006
Mlh1$^{-/-}$, *Msh2*$^{-/-}$ or *Pms2*$^{-/-}$	Increase tumor multiplicity	Control tumor initiation	DNA mismatch repair gene	Shoemaker et al., 2000; Reitmair et al., 1996; Baker et al., 1998
*Mom*2	*Mom2*^{R} is polyp resistant in both small and large intestine by 90%	NA	The *Atp5a1* gene	Silverman et al., 2002; Baran et al., 2007
Egfr^{wa2}	Reduce tumor multiplicity	Affect tumor establishment	The epidermal growth factor receptor	Roberts et al., 2002
Rb(7/18)9Lub (Rb9)	Reduce tumor multiplicity	Suppress homologous somatic recombination on chromosome 18	Robertsonian translocation	Haigis and Dove, 2003
Mom3	Increase polyp numbers	Possibly modify the frequency of wild-type allele loss at *Apc*: mice with severe disease showed elevated rates of loss	NA	Haines et al., 2005
Mom7	>5 alleles in inbred strains	Possibly affect tumor initiation by affecting somatic recombination	NA	Kwong et al., 2007

Table 4-2 Comparisons of average number of polyps in *Apc*$^{min/+,}$ *Egfr*$^{wa2/+}$ or *Egfr*$^{+/+}$ mice on four different backgrounds

		Average number of polyps ± SD			
Background	No. of mice	Small intestine	p-value	Colon	Total
B6	9	206.6 ± 64.6	NA	1.6 ± 1.0	198.3 ± 66.9
B6.BTBR F1	12	125.8 ± 34.6	< 0.005	0	125.8 ± 34.6
B6.A F1	7	62.29 ± 27.1	< 0.0001	0	62.29 ± 27.1
B6.129 F1	14	33.4 ± 26.4	< 0.0001	0	33.4 ± 26.4

B6 served as controls. P-values are shown from comparisons between B6 and F1 hybrids, using the non-parametric Mann-Whitney rank sum test.

Table 4-3 Comparisons of average size of polyps in *Apc*$^{min/+}$, *Egfr*$^{wa2/+}$ or *Egfr*$^{+/+}$ mice on four different backgrounds

Background	No. of polyps	Avg polyp size ± SD	p-value
B6	1365	1.185 ± 0.53	NA
B6.BTBR F1	1021	0.8728 ± 0.42	< 0.0001
B6.A F1	374	1.025 ± 0.42	< 0.0001
B6.129 F1	406	0.7367 ± 0.38	< 0.0001

B6 served as controls. P-values are shown from comparisons between B6 and F1 hybrids, using the non-parametric Mann-Whitney rank sum test.

Table 4-4 Comparisons of average number of polyps between *Apc*$^{min/+,}$ *Egfr*$^{wa2/wa2}$ and *Apc*$^{min/+}$, *Egfr*$^{+/+}$ mice on four different backgrounds

	Average number of polyps ± SD		
Background	*Egfr*$^{wa2/wa2}$	*Egfr*$^{wa2/+}$, or *Egfr*$^{+/+}$	p value
B6	93.38 ± 45.39	208 ± 64.5	< 0.05
No of mice	8	9	
B6.BTBR F1	75.8 ± 50.77	125.8 ± 34.6	< 0.05
No. of mice	15	12	
B6.A F1	21.43 ± 20.19	62.29 ± 27.1	< 0.05
No of mice	7	7	
B6.129 F1	5.93 ± 4.85	33.4 ± 26.4	< 0.005
No of mice	15	14	

P-values are shown from comparisons between *Apc*$^{min/+,}$ *Egfr*$^{wa2/wa2}$ and *Apc*$^{min/+}$, *Egfr*$^{+/+}$ mice on each different background, using the non-parametric Mann-Whitney rank sum test.

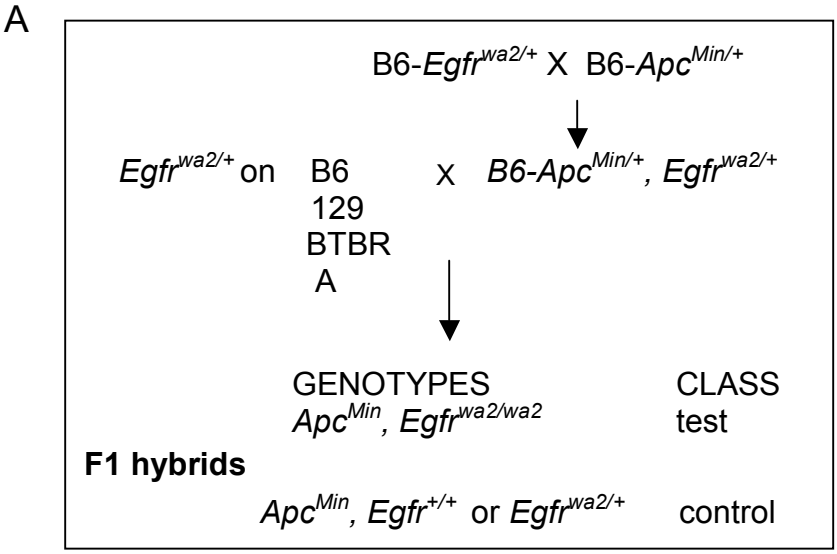

Figure 4-1Breeding scheme to generate *Apc*$^{Min/+,}$ *Egfr*$^{wa2/wa2}$ and *Apc*$^{Min/+}$, *Egfr*$^{wa2/+}$ or *Egfr*$^{+/+}$ mice on four different genetic backgrounds.

Figure 4-1 Breeding scheme to generate $Apc^{Min/+,}$ $Egfr^{wa2/wa2}$ and $Apc^{Min/+,}$ $Egfr^{wa2/+}$ or $Egfr^{+/+}$ mice on four different genetic backgrounds.

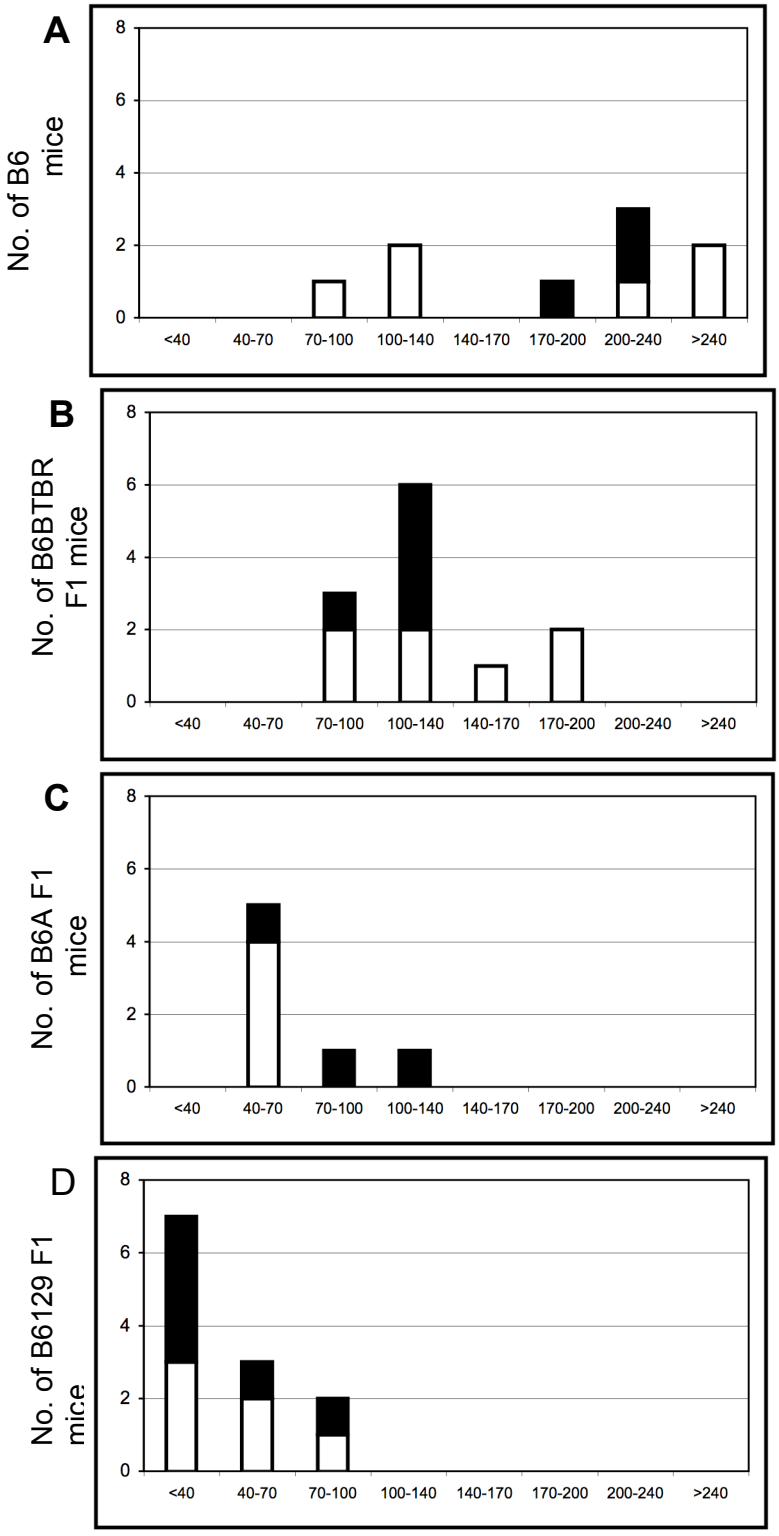

Figure 4-2 Poly number distribution in *Apc*$^{Min/+}$ mice on different genetic backgrounds

Figure 4-2 Poly number distribution in *Apc*$^{Min/+}$ mice on different genetic backgrounds. The polyp number in the entire small intestine and colon was determined in three-month old *Apc*$^{Min/+}$ mice on (A) B6, (B) B6.BTBR F1, (C) B6.A F1 and (D) B6.129 F1 backgrounds. Black column area represents male progeny and white column area represents female progeny. The Y-axis shows the number of mice within each strain background and the X-axis shows the number of polyps within each mouse.

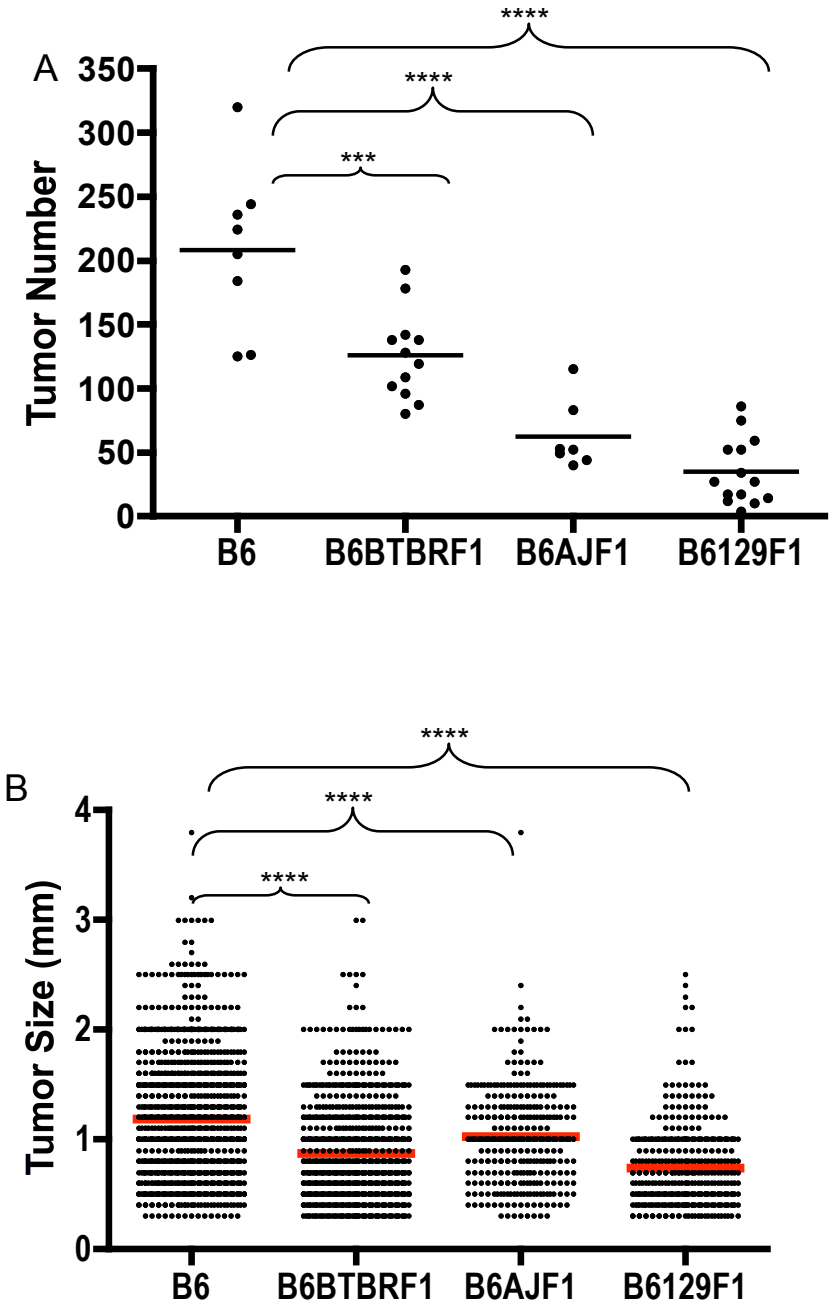

Figure 4-3 Effect of strain background on polyp number and size in *Apc*$^{Min/+}$ mice

Figure 4-3 Effect of strain background on polyp number and size in *Apc*$^{Min/+}$ mice. The horizontal bar indicates the position of the mean for each group. The scatter plot in (A) shows the distribution of the polyp number in *Apc*$^{Min/+}$ mice. Each dot represents the polyp number from a single three-month old mouse. The scatter plot in (B) shows the sizes of the polyps from *Apc*$^{Min/+}$ mice.

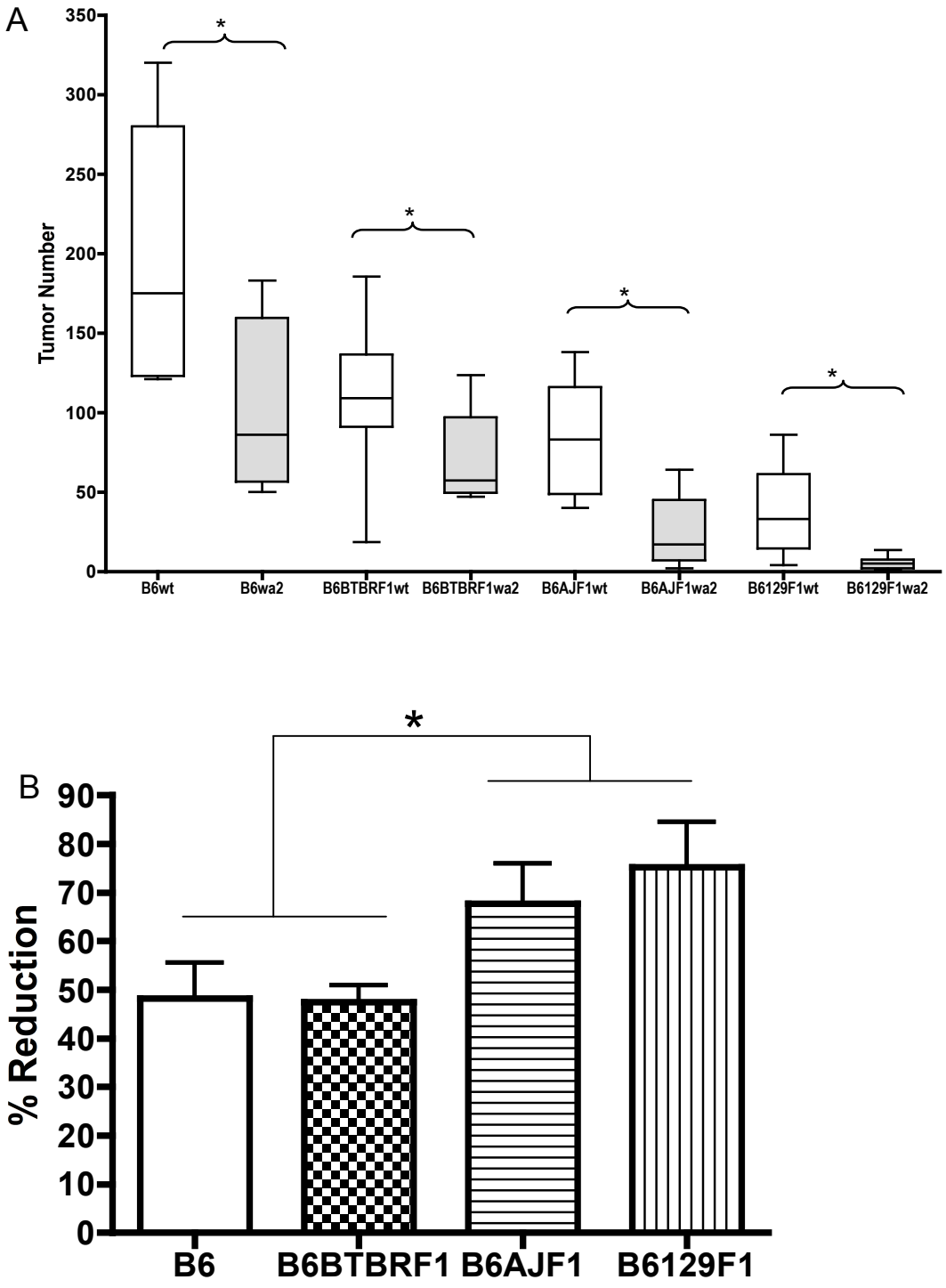

Figure 4-4 Effect of strain background on EGFR-independent tumor growth in *Apc*$^{Min/+}$ mice

Figure 4-4 Effect of strain background on EGFR-independent tumor growth in $Apc^{Min/+}$ mice. (A) Comparison of the mean tumor number in three-month old $Apc^{Min/+}$, $Egfr^{wa2/wa2}$ mice versus littermate controls. Grey columns represent $Egfr^{wa2/wa2}$ mice and white columns represent wildtype *Egfr* littermate controls. (B) Percentage of the reduction in tumor number when comparing $Apc^{Min/+}$, $Egfr^{wa2/wa2}$ mice versus littermate controls. White columns, B6; checkered columns, B6.BTBR F1; horizontal stripe columns, B6.A F1; vertical stripe columns, B6.129 F1. (* $p < 0.05$, ** $p < 0.005$, Mann-Whitney rank sum test)

Chapter 5 Conclusions and future directions

The ERBB family of receptor tyrosine kinases consist of four closely related members: epidermal growth factor receptor (EGFR or ERBB1), ERBB2, ERBB3 and ERBB4. Signaling through ERBB family members modulates many aspects of cellular activity, including proliferation, differentiation, migration, apoptosis and survival (Yarden and Sliwkowski, 2001). As such, it is required for proper development, maintenance and repair of most organ systems, including skin, brain, lungs and the GI tract. Conversely, dysfunction in ERBB signaling is associated with a number of disease states. For examples, defective EGFR activity causes intrauterine growth retardation or delayed embryonic development while hyperactivity of EGFR and other ERBB family members are implicated in a variety of epithelial malignancies derived from epithelial tissues, including CRC. Given the proposed roles of EGFR in tumor development, a number of small molecule and antibody-based therapies targeting EGFR have been developed as therapeutic agents. Although preclinical and early clinical studies with the EGFR-targeted therapies were encouraging, large-scale clinical trials clearly demonstrate that the majority of patients do not respond. This discrepancy demonstrates that little is known about the mechanisms underlying tumor response to EGFR-targeted therapies. In the work presented in this dissertation, we used a multi-faceted approach towards identifying an anti-EGFR therapy response signature in CRC to indicate directions for combinatorial and targeted therapies for tumors resistant to EGFR inhibitors. We also established the importance of ERBB3 in intestinal tumorigenesis and suggest that a major role of ERBB3 is to mediate EGFR-independent tumor growth. Lastly, since one of the greatest challenges in optimizing the use of EGFR-targeted therapies is posed by patient genetic heterogeneity in

patients, we began an investigation into how genetic background affects tumor response to EGFR inhibition.

The epithelial lining of the small and large intestine constantly undergo self-renewal. Signaling pathways including hedgehog (HH), TGFB, fibroblast growth factor (FGF) and WNT are required spatiotemporally during intestinal development (Fukuda and Yasugi, 2002; Sancho et al., 2003; Sancho et al., 2004; Wells, 1999). The role of ERBB family members in intestinal development and homeostasis is intriguing based on the analysis of mutant mice. On genetic backgrounds that support survival of *Egfr* nullizygous mice to three weeks of age, the intestinal epithelium of surviving neonates remains intact with a proliferative compartments in the crypts, despite severe abnormalities in other epithelial tissues such as skin (Sibilia and Wagner, 1995; Threadgill et al., 1995). Similarly, mice homozygous for the hypomorphic $Egfr^{wa2}$ allele manifest gastrointestinal phenotypes only upon exogenous stimulus (Egger et al., 2000; Helmrath et al., 1997). In mice with humanized EGFR, $hEGFR^{KI}$, no phenotype in GI tract was reported, although they display several abnormalities in other epithelial tissues like skin and hair (Sibilia et al., 2003). In our study, inactivation of EGFR specifically in intestinal epithelial cells did not affect crypt architecture. Indeed, the proliferation rate was even enhanced in mutant mice. These results indicate the tissue-specific requirement for EGFR and suggest that EGFR is dispensable during intestinal development. Similarly, ERBB3 inactivation in intestinal epithelium did not lead to dramatic perturbation of the epithelia of the small intestine: no proliferation or apoptosis defect was observed in *Erbb3* mutant mice. However, the minor architectural abnormalities such as the bifurcated villi phenotype in

ERBB3 deficient mice indicate that ERBB3-dependent signaling is involved in intestinal epithelial proliferation and differentiation.

Our efforts towards identifying an anti-EGFR therapy response signature in CRC are particularly timely and clinically relevant, in that recent reports of completed large-scale clinical trials clearly demonstrate a less than ideal response to EGFR blockade with antibodies or small molecule inhibitors. Identifying biomarkers for tumors that are sensitive or resistant to anti-EGFR therapies is critical to select for patients who would benefit most from this targeted therapy. Using mouse models as genetic tools and pharmacological agents, previous studies showed that a subset of intestinal tumors arose despite impaired EGFR activity suggesting an EGFR-independent mechanism (Roberts et al., 2002; Torrance et al., 2000). However, the EGFR-independent status of some tumors could not be shown definitely in those studies due to the residual kinase activity. In this study, we employed a conditional *Egfr* allele (*Egfr*^{tm1Dwt}) enabling the generation of *Apc*^{Min} mice with intestinal-epithelia specific *Egfr* deletion. We clearly demonstrate that a subset of *Apc*^{Min} polyps grow in an EGFR-independent manner. The absence of EGFR in these polyps exerts little or no suppression on their growth. Indeed, the growth of these EGFR deficient tumors may even be enhanced. A molecular biomarker for this subset of EGFR-independent tumors was revealed by global gene expression profiling analysis, followed by IHC. The signature is marked by up-regulation of components in cell cycle regulation and RAS-MAPK pathway genes, correlating well with a dramatic increase in p42/44 MAPK activity and proliferation index in EGFR-independent tumors (Figure 5-1). Ultimately, our goal is to probe a human CRC database with these EGFR-independent signatures to determine whether these mouse model-derived signatures are present in human samples.

Our results have important implications for EGFR-targeted therapies. Our findings suggest that EGFR inhibitors, either reversible or irreversible, may not be effective in a subset of EGFR-independent tumors that show hyperactivation of RAS/MAPK. Determining whether tumors are EGFR-dependent or independent will have an effect on our ability to make wiser clinical decisions regarding who should be given EGFR TKIs. Furthermore, combination therapies with MAPK inhibitors should be considered for EGFR-independent tumors. Additionally, it is important to determine upstream-receptor(s) that activate the RAS-MAPK pathway in the absence of EGFR to identify additional targets for combinatorial therapy. Of particular interest is the heterodimer formed by ERBB2/ERBB3, considering the tight co-regulation and inter-dependence within the ERBB family. Indeed, this idea is supported by the fact that ERBB2 and ERBB3 protein levels are increased in EGFR-deficient tumors. Alternatively, EGFR compensatory pathways could be mediated by other signals such as the insulin growth factor (IGF) related receptors. The IGF network is important for carcinogenesis (Samani et al., 2007). In particular, the IGF1 and IGF2 system is implicated in CRC (Chang et al., 2002; Slattery et al., 2004). In addition, the IGF1 system and ERBB pathways share downstream signaling mediators including RAS-MAPK and PI3K-AKT that result in cell proliferation and survival. Perhaps more importantly, the intimate cross-talk between IGF1 network and ERBB family members has been well documented (Morgillo et al., 2006; Ornskov et al., 2006). To test these hypotheses, cell culture experiments could be set up using the pre-neoplastic murine cell line IMCE ($Apc^{Min/+}$) or human colon cancer cell lines (Caco-2, HT-116, or HT-29). Identification of the compensatory pathways activated in EGFR inhibitor resistant clones generated by exposing cells to increasing concentrations of drug or by

transfecting cells with a dominant-negative *Egfr* construct (Murillas et al., 1995), could be used to validate our results. For human colon cancer cell lines, the recently available human phospho-receptor tyrosine kinase (RTK) array (R&D Systems) would allow comparison of how inactivation of EGFR would affect phosphorylation of other RTKs.

Elucidation of the role for EGFR during intestinal tumorigenesis should be continued. One hypothesis is that the mode of EGFR action in non-transformed epithelial cells contributes to tumor growth. This hypothesis can be tested by histological analysis of the non-transformed epithelial layer enveloping the tumor in the Apc^{Min} mice with an intestinal-epithelial specific *Egfr* deletion. Similarly, the temporal requirements for EGFR activity during CRC development could be addressed by using inducible CRE recombinase under control of the Villin promoter. Also, we have developed a B6-$Egfr^{flox}$ congenic line (> N10), which allows future studies to be carried on the B6 congenic background.

The role of ERBB3 in cancer biology has been under-appreciated, partly due to its defective intrinsic kinase activity. In this study, we established the importance of ERBB3-depdendent signaling in intestinal tumorigenesis by using a conditional knock out allele of *Erbb3*. Unexpectedly, deletion of *Erbb3* caused a more dramatic suppression effect on both tumor multiplicity and growth in Apc^{Min} mouse model. These results highlight the critical role of ERBB3, potentially by mediating the PI3K-AKT-MTOR pathway. Interestingly, although the majority of residual polyps forming on the ERBB3-deficent background have a reduced size in comparison to those on a wildtype *Erbb3* background, a few large intestinal polyps, greater than 2 mm in diameter, were present. The proliferation rates, the level of p42/44 MAPK activation and the level of S6K activity are comparable in those with wildtype *Erbb3.* Determination of their sensitivity to EGFR inhibitors would be

revealing, since ERBB3 has been proposed as a biomarker to predict sensitivity to EGFR inhibitors in NSCLC, pancreatic, and colon cancer cell lines (Buck et al., 2006; Engelman et al., 2005). Microarray-based gene expression profiling of ERBB3-independent tumors would also reveal their molecular characteristics in comparison to EGFR-independent tumors. Consequently, elucidation of additional aspects of ERBB3 biology during intestinal tumorigenesis should be continued. For example, microadenoma analysis would reveal if ERBB3-dependent signaling affects tumor initiation. Also, the spatio-temporal requirements for ERBB3 during tumor development and progression could be analyzed using appropriate CRE lines. ERBB3 is also required in a subset of colonic tumors induced by the carcinogen AOM. Since AOM tumors exhibit molecular alterations and morphological variation, detailed analysis of AOM colonic tumors from an ERBB3-deficient environment could reveal the nature of histopathology and pathway disruption in ERBB3-independent colonic tumors. Finally, all of the ERBB3-based methodologies used to study intestinal tumors could be applied to models of tumors in other tissues, such as lung and brain, where the ERBB3 activity is strongly implicated.

Finally, the studies presented here with different $Egfr^{wa2}$ congenic lines demonstrate the strong background modulation of tumor response to EGFR inhibition. To initiate mapping experiments to identify novel modifiers of this trait, F2 offspring of Apc^{Min}, $Egfr^{wa2/wa2}$ mice involving B6, BTBR, A and 129 backgrounds should be produced. The examination of genetic background effect in tumor phenotype presented in this thesis serves as an initial attempt to understand the genetic components of variability in tumor response to ERBB targeted therapies (Jimeno and Hidalgo, 2006; Li et al., 2006). Advances in understanding the genetic complexity of tumor response to EGFR targeted

therapies should allow application of individual tailored therapy to improve the efficacy of targeted therapies.

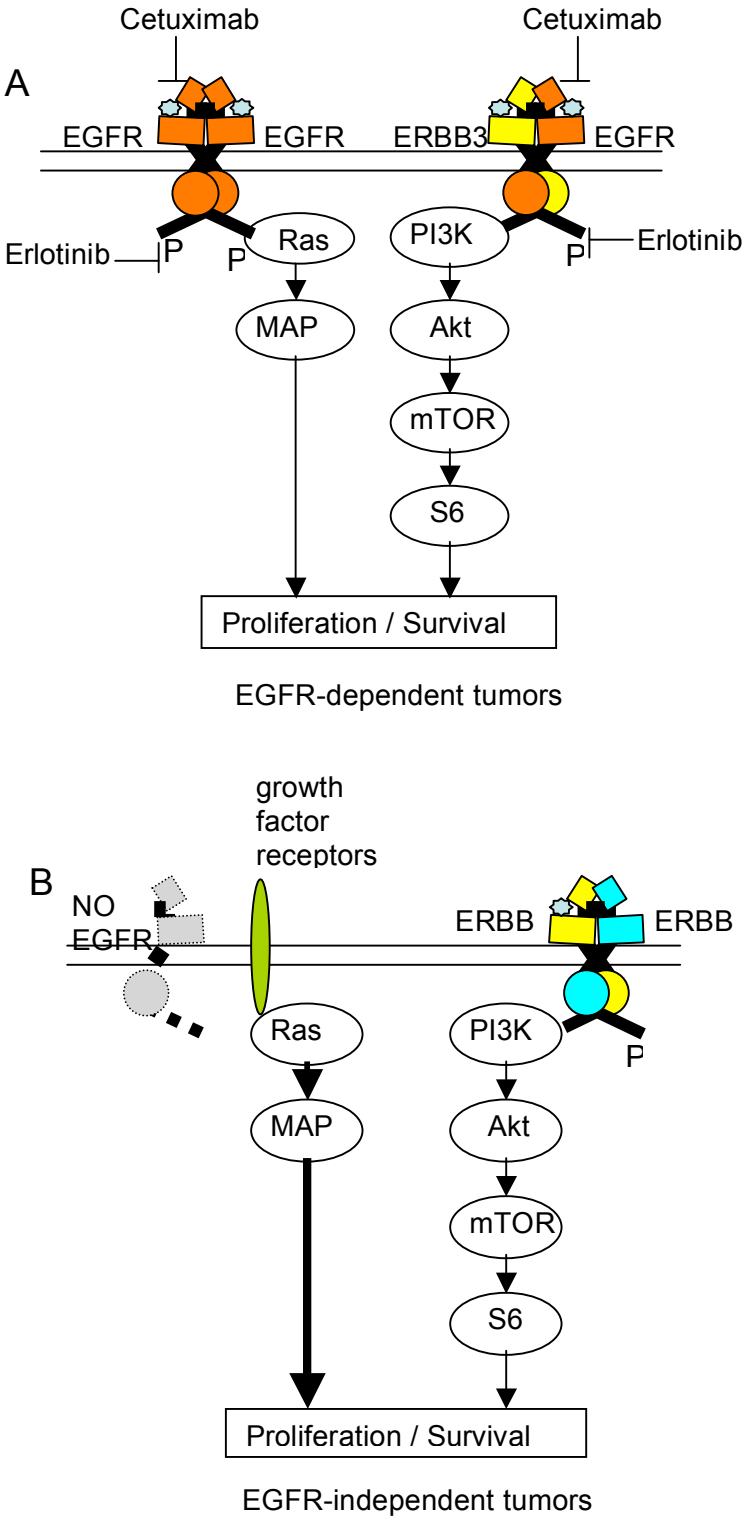

Figure 5-1 EGFR in intestinal tumor cells–to be there or not to be there?

Figure 5-1. EGFR in intestinal tumor cells–to be there or not to be there? Proposed mechanism for proliferation and survival in EGFR-dependent /-independent intestinal tumors. (A) In EGFR-dependent tumors, EGFR appears to have two major functions during tumor development, provides a mitogenic signal by activating the RAS-MAPK pathway and a survival signal by activating the PI3K/AKT/mTOR through heterodimerization with ERBB3. Therefore, these tumors are more likely to respond to EGFR-inhibition by small molecule inhibitors (such as Erlotinib) or monoclonal antibodies (such as Cetuximab). (B) In a subset of tumors lacking EGFR, the RAS-MAPK components are hyper-activated, probably as a compensatory pathway for loss of EGFR. The activation of RAS-MAPK is likely due to an alternative signal possibly involving other growth factors like insulin-like growth factor-I receptor or ERBB2/3 heterodimers. The ERBB2/3 heterodimer is likely responsible for activating the PI3K/AKT/mTOR pathway, highlighting the importance of ERBB3 in promoting development of both types of tumors. These EGFR-independent mechanisms render tumors resistant to EGFR-targeted therapeutics. In patients that harbor both types of tumors, combining Erlotinib or Cetuximab with another drug that targets IGF1R, MAPK, AKT or mTOR may lead to improvements in the efficacy of targeted therapies.

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