IMPLICATIONS OF ERBB RECEPTOR TYROSINE KINASE SIGNALING IN RESISTANCE TO EGFR-TARGETED THERAPIES IN COLORECTAL CANCER

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2019

Major Subject: Genetics

ABSTRACT

Colorectal cancer (CRC) ranks among the third most common human malignant diseases and is one of the leading causes of cancer-related deaths globally. The epidermal growth factor receptor (EGFR) is recognized as an important player in CRC initiation and progression. EGFR is a transmembrane receptor tyrosine kinase that is commonly upregulated in many human epithelial cancers including CRC (Roskoski 2014). The activation of these receptor is tightly regulated via ligand binding leading to downstream signaling that influence biological process like cell proliferation, apoptosis, angiogenesis, cell adhesion, mobility, invasion and metastasis. EGFR-targeted therapies have been approved for CRC treatment. However, increasing evidence suggests that only 15% of CRC patients initially respond to these therapies due to high levels of primary resistance, and those who show initial response eventually become refractory to treatment and relapse under the treatment (secondary resistance). Efficacy of anti-EGFR therapy in humans is proven to be influenced by the mutational context of the cancer. In this dissertation we review the mechanisms of resistance to anti-EGFR therapies and the critical roles of ERBB members in CRC, with an emphasis on different approaches to overcome this resistance and potential future directions for more tailored cancer therapies. In chapter 2, we demonstrated that colorectal tumors can initiate through an EGFR-independent mechanism, characterized by activation of IL10 and ERBB4 signaling, with an accelerated growth rate mediated by a state of anergy. The existence of this EGFR-independent mechanism of CRC progression could explain the lack of response in a subset of CRC,

and suggests that targeting EGFR would be most effective for those cancers that are dependent upon EGFR signaling. Additionally, in chapter 3, we also demonstrate the importance of ERBB3-mediated intestinal tumorigenesis through activation of PI3K/AKT signaling pathway activation, providing a valuable target for therapeutic intervention. The differential expression of ERBB family and the high resistance to anti-EGFR treatment in CRC patients suggest that therapies targeting more than signaling pathway might be the most effective treatment in the future. The studies done in this dissertation advances our understanding of ERBB family biology during colonic tumorigenesis, ultimately contributing to better therapies for CRC.

DEDICATION

A todos sus memorables discursos – A mi mamá

To Marcus and Asher because I am a better person with you in my life

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. David Threadgill. In his laboratory and under his leadership, I was able to become an independent researcher. And to my committee members, Dr. Robert Chapkin, Dr. Robert Coffey, Dr. Weston Porter, and Dr. Sally Ward, for their guidance and support throughout the course of this research.

Thanks also go to all past and current members of the Threadgill lab. During the last five years, I was lucky to meet great scientists and friends like Dr. Danila Cuomo, Dr. Melanie Warren, Dr. Diana Medina and the future Dr. Anna Salvador who listened, offered me advice and supported me through this process. Thank you to the Genetics faculty, graduate students and staff for making my time at Texas A&M University a great experience.

This journey wouldn't have been possible without the continuous support of my mom and my brother. Because of them I became the person I am today. Finally, thanks to Marcus, for his love, encouragement and patience in this journey. Thanks for all the time, and for all.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by dissertation committee consisting of Dr. David Threadgill, Dr. Robert Chapkin, Dr. Robert Coffey and Dr. Weston Porter.

Transcriptomic data was analyzed by Dr. Andrew Hillhouse and Kranti Konganti in TIGSS.

All other work conducted for the thesis (or) dissertation was completed by the student independently.

Funding Sources

Graduate study was supported by a graduate research assistantship at Texas A&M University.

This work was also made possible in part by Texas A&M University Genetics Fellowship and Genetics Graduate Student Association Travel Award.

NOMENCLATURE

AdCre	Cre recombinase-expressing adenovirus
AKT	Protein Kinase B
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
AREG	Amphiregulin
BMP	Bone Morphogenetic Protein
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BTC	β-cellulin
CAC	Colitis-Associated Colorectal
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability Pathway
CKO	Knockout CMS Consensus Molecular Subtypes
CRC	Colorectal Cancer
DCR	Disease Control Rate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
EREG	Epiregulin
ERK	Extracellular signal Regulated Kinase
FAP	Familial Adenomatous Polyposis

GI	Gastro-intestinal
GTP	Guanosine Triphosphate
HB-EGFR	Heparin Binding EGF (HB-EGF)
HER	Human Epidermal Growth Factor Receptor
HGF	Hepatocyte Growth Factor
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
HRP	Horseradish Peroxidase
H&E	Hematoxylin and Eosin
IGF-1R	Type 1 Insulin-like Growth Factor Receptor
IL	Interleukin
IPA	Ingenuity pathway analyis (IPA)
JAK	Janus Kinase
KRAS	Kirsten Rat Sarcoma viral oncogene
LEF	Lymphoid Enhancer Family
LOH	Loss of Heterozygosity
mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
mCRC	Metastatic CRC
MEK	Mitogen-activated protein kinase kinase
MET	Mesenchymal-Epithelial Transition factor receptor
MSI	Microsatellite Instability Pathway
mTOR	Mammalian Target of Rapamycin

MYC	MYC Proto-Oncogene
NRAS	Neuroblastoma RAS viral oncogene homolog
NRG	Neuregulins
NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival
PFS	Progression-Free Survival
PI3K	Phosphatidylinositol 3-Kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase Catalytic subunit Alpha
PTEN	Phosphatase and Tensin homolog
qPCR	Quantitative-Polimerase Chain Reaction
RAS	Retrovirus-Associated DNA Sequences
RAF	Rapidly Accelerated Fibrosarcoma
RR	Response Rate
RTK	Receptor Tyrosine Kinase
SRC	Rous Sarcoma oncogene
STAT	Signal Transducer and Activator of Transcription
TCF	T Cell Factor
TGF-A	Transforming Growth Factor-Alpha
TGF-B	Transforming Growth Factor-Beta
TGFBR	TGFB receptor
TKI	Tyrosine Kinase Inhibitor
TP53	Tumor Protein p53

Treg	Regulatory T
TSG	Tumor-Suppressor Genes
VEGF	Vascular Endothelial Growth Factor
WT	Wild-type

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1. INTRODUCTION

1.1. Synopsis

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase (RTK) that is commonly upregulated in many human epithelial cancers including colorectal cancer (CRC). EGFR has been characterized as the prototypical member of the ERBB family that also includes HER2/neu (ERBB2), HER3 (ERBB3) and HER4 (ERBB4) (Roskoski 2014). The activation of these receptors is tightly regulated via ligand binding leading to downstream signaling that influence biological process like cell proliferation, apoptosis, angiogenesis, cell adhesion, mobility, invasion and metastasis (Shih, Telesco et al. 2011, Seshacharyulu, Ponnusamy et al. 2012, Yarden and Pines 2012). Several drugs have been developed to inhibit the activity of the EGFR by antibodies against the ligand binding domains (cetuximab, panitumumab and trastuzumab) or small molecules against the tyrosine kinase domains (erlotinib, gefitinib, and lapatinib) (Sartore-Bianchi, Siena et al. 2016, Giordano, Remo et al. 2019). Both approaches have shown considerable clinical promise. However, increasing evidence suggests that only 15% of CRC patients initially respond to these therapies due to high levels of primary resistance, and those who show initial response eventually become refractory to treatment and relapse under the treatment (secondary resistance) (Cremolini, Benelli et al. 2019, Rachiglio, Lambiase et al. 2019, Sandhu, Lavingia et al. 2019). Several mechanisms of resistance to EGFR inhibitors have been identified. Kirsten rat sarcoma viral oncogene (*KRAS*) codon12 activating mutation is a predominate mechanism of

resistance to EGFR inhibitors in around 40% of patients with advanced CRC (Montagut, Tsui et al. 2018, Mauri, Pizzutilo et al. 2019). Other potential mechanisms of resistance include increased EGFR copy number, alterations in ligand expression, mutations of v-Raf murine sarcoma viral oncogene homolog B (*BRAF*), phosphatase and tensin homolog (*PTEN*), excess activation of phosphatidylinositol 3-kinase (PI3K) and janus kinase /signal transducer and activator of transcription (JAK/STAT) signaling pathways, enhanced epithelial-mesenchymal transition (EMT) and activation of alternate signaling pathways (Gao, Maria et al. 2019, Garcia-Albeniz, Alonso et al. 2019, Rachiglio, Lambiase et al. 2019, Sandhu, Lavingia et al. 2019). More recently, evidence suggests that immune infiltrates in the tumor microenvironment appear to differentially modulate CRC development, depending on their pro-tumor or anti-tumor nature (Mager, Wasmer et al. 2016, Giordano, Remo et al. 2019). The purpose of this review is to discuss mechanisms of resistance to EGFR therapies in CRC with an emphasis on different approaches to overcome the resistance to anti-EGFR therapies and potential future directions for more tailored cancer therapies.

1.2. Colorectal cancer

Colorectal cancer (CRC) ranks among the third most common human malignant diseases and is one of the leading causes of cancer-related deaths globally (Siegel, Miller et al. 2019). According to the American Cancer Society, colorectal cancer is the third most common cancer in both females and males in USA. Early detection and more effective therapies have reduced mortality, yet approximately 51020 deaths are expected in 2019 (Miller, Nogueira et al. 2019).

The risk factors associated with CRC include dietary and lifestyle factors and inherited and somatic mutations. In fact, a Western style diet (high unsaturated fat/low fiber), excessive alcohol consumption, a sedentary life style, obesity and incidences of inflammatory bowel disease increase risk to develop CRC (Barrington, Wulfridge et al. 2018). A recent review also highlighted the probably complex interactions among energy balance, hormones, and gut flora and inflammation in the development of CRC (Huxley, Ansary-Moghaddam et al. 2009, Taylor, Burt et al. 2010). Molecular genetic studies in inherited and sporadic CRC have revealed critical mutations in adenomatous polyposis coli (*APC*), *KRAS*, *MYC,* mitogen-activated protein kinase (*MAPK*), transforming growth factor-beta/bone morphogenetic protein (*TGF-B/BMP*) and tumor protein p53 (*TP53*) underlying the pathogenesis of CRC (Sartore-Bianchi, Siena et al. 2016). However, the etiological factors and pathogenetic mechanisms underlying CRC development are complex and heterogenous.

CRC is a heterogeneous disease in terms of its clinical manifestations, molecular characteristics, sensitivity to treatments and prognosis. Over the past four decades, the development of CRC is generally thought of as a predictable progression of genetic and epigenetic alterations, resulting in distinct pathological changes. This cumulative multi-stage process usually occurs over many years (Nguyen and Duong 2018). In individuals with inherited mutations, the time course of initiation and/or progression to malignancy can be significantly shorter. For example, familial adenomatous polyposis (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 lynch syndrome or

hereditary nonpolyposis colorectal cancer (HNPCC) patients, the initiation step appears unchanged compared to sporadic cancers, but once initiated the progression from polyps into malignancy is much faster (Snyder and Hampel 2019).

Inherited and sporadic forms of CRCs share major genetic abnormalities. The study of inherited familial CRC syndromes such as FAP and HNPCC has provided a better understanding of the molecular pathogenesis underlying sporadic CRC (Lynch, Lynch et al. 2008). The chromosomal instability pathway (CIN), microsatellite instability pathway (MSI) and CpG island methylator phenotype (CIMP) are the three recognized mechanisms of carcinogenesis in CRC (Pino and Chung 2010). These alterations may occur, either individually or in combination, resulting in the growth of tumors with different clinical and pathological features (Bardi, Fenger et al. 2004).

The CIN pathway is also known as the adenoma-carcinoma sequence, and it follows a well-defined sequence of genetic events and corresponding histological changes. These genomic variations include activation of proto-oncogenes, like *KRAS* and inactivation of tumor-suppressor genes (TSG), including, *APC*, *TP53*, and loss of heterozygosity (LOH) (Di Fiore, Blanchard et al. 2007). Recently mutations involving other genes have been described, such as the TGFB receptor (*TGFBR*) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), that are required for the adenoma-carcinoma sequence model.

The *APC* gene is located on chromosome 5q21-q22, and encodes roughly 300-kDa protein that regulate cell-cell adhesion, cell migration, chromosomal segregation, and apoptosis in the colonic crypt (Hankey, Frankel et al. 2018). *APC* is the most common

initial gene mutated in familial/inherited and sporadic CRC; the exon 15 appears to be the most vulnerable region for both germline and somatic mutations of *APC* (Nemecek, Berkovcova et al. 2016). APC is a negative regulator of the WNT signaling pathway through its role in the destruction of B-catenin. In *APC* mutant colonocytes, excess cytosolic β-catenin forms complexes with E-cadherin, leading to enhanced cell-cell adhesion and changing the spatial organization and migratory pattern of the cells in the continuous renewal of crypts (Fearon 2011). β-catenin accumulation in the cytoplasm, also promotes the formation of complexes with DNA-binding proteins of the T cell factor/lymphoid enhancer family (TCF/LEF) family, and translocate to the nucleus. The collection of genes controlled by β-catenin/TCF may include proto-oncogenes, such as cMYC and cyclin D1 (Bogaert and Prenen 2014). Whether genomic instability initiates the adenoma-carcinoma sequence or whether it arises during the process and facilitates evolution to CRC is still controversial (Huxley, Ansary-Moghaddam et al. 2009).

TP53 gene is significantly involved in the control of the cell cycle and apoptosis and is commonly mutated in CRC. The p53 protein induces G1 cell-cycle arrest and facilitates DNA repair prior to DNA replication (Zwang, Oren et al. 2012). If p53 is mutated, a cancer cell will not induce apoptosis if DNA repair is unsuccessful. *TP53* mutations occur during the transition from adenoma to cancer. Mutations in p53 found in CRC patients may facilitate the uncontrolled growth and acquisition of invasive properties in the face of varied stresses that otherwise severely limit cell survival at the adenoma-carcinoma transition (Danielsen, Lind et al. 2008).

LOH is considered to be an important mechanism for inactivating one allele of specific TSGs in cancer. LOH in the 17p chromosome region occurs in approximately 70% of CRC patients, and the p53 gene is thought to be the main target of 17p LOH (Melcher, Hartmann et al. 2011). Additionally, 85% of the p53 mutations found in CRC patients are missense defects (Zauber, Wang et al. 2004, Wood, Parsons et al. 2007). Several studies suggest that mutations and LOH of p53 are closely associated with the adenoma-carcinoma transition. The basis for selection for p53 mutations at this point in colorectal tumorigenesis is uncertain.

Numerous studies have attempted to elaborate on the prognostic significance of *APC* and *TP53* mutation in CRC, with conflicting results. A recent study suggests that the combination of *APC* and *TP53* mutations may predict cetuximab sensitivity and that it may overcome *KRAS*/neuroblastoma RAS viral oncogene homolog (*NRAS*) mutation status (Sanchez de Abajo, de la Hoya et al. 2006, Pilozzi, Ferri et al. 2011).

MSI is the hallmark of HNPCC and is seen in more than 95% of these patients. In contrast, for most sporadic CRC, the mechanisms responsible for CIN remain elusive, and MSI is responsible for only 15%–20% of the cases. CRC patients with MSI frequently acquires 5-Fluorouracil (5-FU) resistance, and the exact mechanism underlying how CRC cells acquire chemo resistance to 5-FU remains unclear (Chen, Swanson et al. 2017).

Epigenetic mechanisms may be as significant as gene mutations in cancer but are less well understood. Various covalent histone modifications and methylation of cytosine residues in DNA represent prominent modes of gene regulation (Plass, Pfister et al. 2013). CRC shows 8%–15% lower total DNA methylation than normal tissue (Puccini, Loupakis

et al. 2019), even in precursor adenomas (Feinberg, Chesney et al. 2017). The most studied epigenetic events in the CRC are CpG island methylation and histone modifications, although there are other mechanisms of epigenetic modification, such as nucleosomal occupancy and remodeling, chromatin looping, and noncoding RNAs expression. A distinct subset of CRCs shows coordinate hypermethylation of many CpG-rich promoters, conferring the CpG island methylator phenotype (CIMP), with transcriptional attenuation of tumor suppressor genes (Cruz-Correa, Cui et al. 2004).

In the last decade, numerous factors, other than the tumor cells themselves, have been found to contribute to cancer progression. Non-cancerous cells in the vicinity of the tumor, commonly referred as tumor microenvironment or stroma, can promote CRC (Lytras, Nikolopoulos et al. 2014). Non-malignant cells, like immune infiltrates, appear to differently modulate CRC development, depending on their nature (Slattery and Fitzpatrick 2009, Mager, Wasmer et al. 2016). The inhibition or promotion of CRC depends on the expression of particular cytokine networks (Table 1-1). The identification of differential cytokine networks is important to improve therapeutic efficacy, as neutralization of individual cytokines may not be enough to benefit CRC patients (De Simone, Franze et al. 2015). Indeed, single cytokine inhibition does not significantly improve outcomes, as cytokine signals are often overlapping. For example, Interleukin (IL)-6 has a pro tumorigenic function through signal transducer and activator of transcription 3 (STAT3) activation, a signaling pathway also triggered by multiple other cytokines, such as IL-11, IL-21, IL-10 or IL-22 (Rokavec, Oner et al. 2014). Targeting a

combination of multiple cytokines has been shown to increase anti-tumor activity in a murine model of metastatic CRC (Yu, Steel et al. 2010).

Table 1-1 Cytokine networks in the pathogenesis of colorectal cancer

Cytokine effect	Cytokine
Anti-tumorigenic	IFN-y, IL12, IL15, IL17F, IL18
Double edged/unclear	IL1, IL9, IL10, IL21, GM-CSF
Pro-tumorigenic	IL4, IL6, IL8, IL11, IL17A, IL22, IL23, IL33, TNF, TGF-B, VEGF

The epidermal growth factor receptor (EGFR) is recognized as an important player in CRC initiation and progression. This membrane-bound receptor tyrosine kinase (RTK) is present on all epithelial and stromal cells. It is a multifunctional receptor that plays a key role in cell division, apoptosis, cell differentiation and dedifferentiation, migration, and organogenesis (Wells 1999). EGFR was one of the first targets to be exploited in cancer treatment and it has therefore become a key target of therapeutic strategies designed to treat metastatic CRC, in particular with monoclonal antibodies (mAbs) against the extracellular domain of the receptor (Markman, Javier Ramos et al. 2010).

1.3. Epidermal growth factor

EGFR is a glycoprotein of 170 kDa, encoded by a gene located on chromosome 7p12. EGFR is a member of the human tyrosine kinase receptor (HER/ERBB) family, which consists of EGFR (ERBB1/HER1), HER2/Neu (ERBB2), HER3 (ERBB3) and HER4 (ERBB4) (Roskoski 2014). These receptors are formed of single amino acid chain protein structure with an extracellular ligand binding domain (domains I, II, III, IV), a transmembrane domain, a juxta membrane nuclear localization signal and a tyrosine kinase intracellular portion (Troiani, Napolitano et al. 2016). Members of the ERBB family are ubiquitously expressed in many cell types of epithelial, mesenchymal and neuronal origin (Prenzel, Fischer et al. 2001). Under homeostatic conditions, the activation of these receptors is regulated by ligand availability. Major ligands are divided in three groups. The first includes epidermal growth factor (EGF), transforming growth factor α (TGF-α), and amphiregulin (AREG), which all bind specifically to the EGFR. The second group includes heparin binding EGF (HB-EGF), β-cellulin (BTC), and epiregulin (EREG), which bind to both EGFR and HER4. The third group is composed of the neuregulins (NRG1-4) and are further subdivided based on their ability to bind HER3 (NRG1 and NRG2) and HER4 (NRG3 and NRG4) (Hynes and MacDonald 2009, Tebbutt, Pedersen et al. 2013) (Figure 1-1). The receptor-ligand interaction induces a conformational change of the receptor leading to homodimerization or heterodimerization, thereby triggering the activation of several pathways involved in biological roles including development, proliferation and differentiation (Barnea, Haif et al. 2013, Tebbutt, Pedersen et al. 2013). The activation of complex intracellular signaling pathways are tightly

regulated by the presence and identity of the ligand, heterodimer composition, the availability of phosphotyrosine-binding proteins, and the duration of the signal. The two primary signaling pathways activated by EGFR are the retrovirus-associated DNA sequences/rapidly accelerated fibrosarcoma/mitogen-activated protein kinase (RAS/RAF/MAPK) and phosphatidylinositol 3-kinase/phosphatase and tensin homolog/protein kinase B (PI3K/PTEN/AKT) pathways (Figure 1-2). When activated, the PI3K/AKT pathway leads to protein synthesis, cell growth, survival, and mobility. The RAS/RAF/MAPK pathway leads to cell cycle progression and proliferation (14,15).

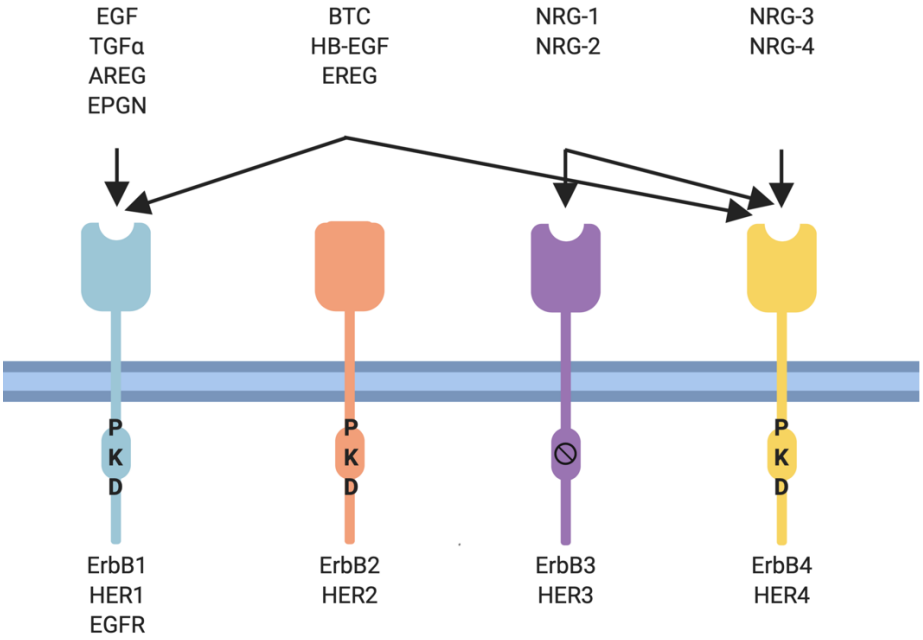


Figure 1-1 ERBB tyrosine kinase receptors and their cognate ligands

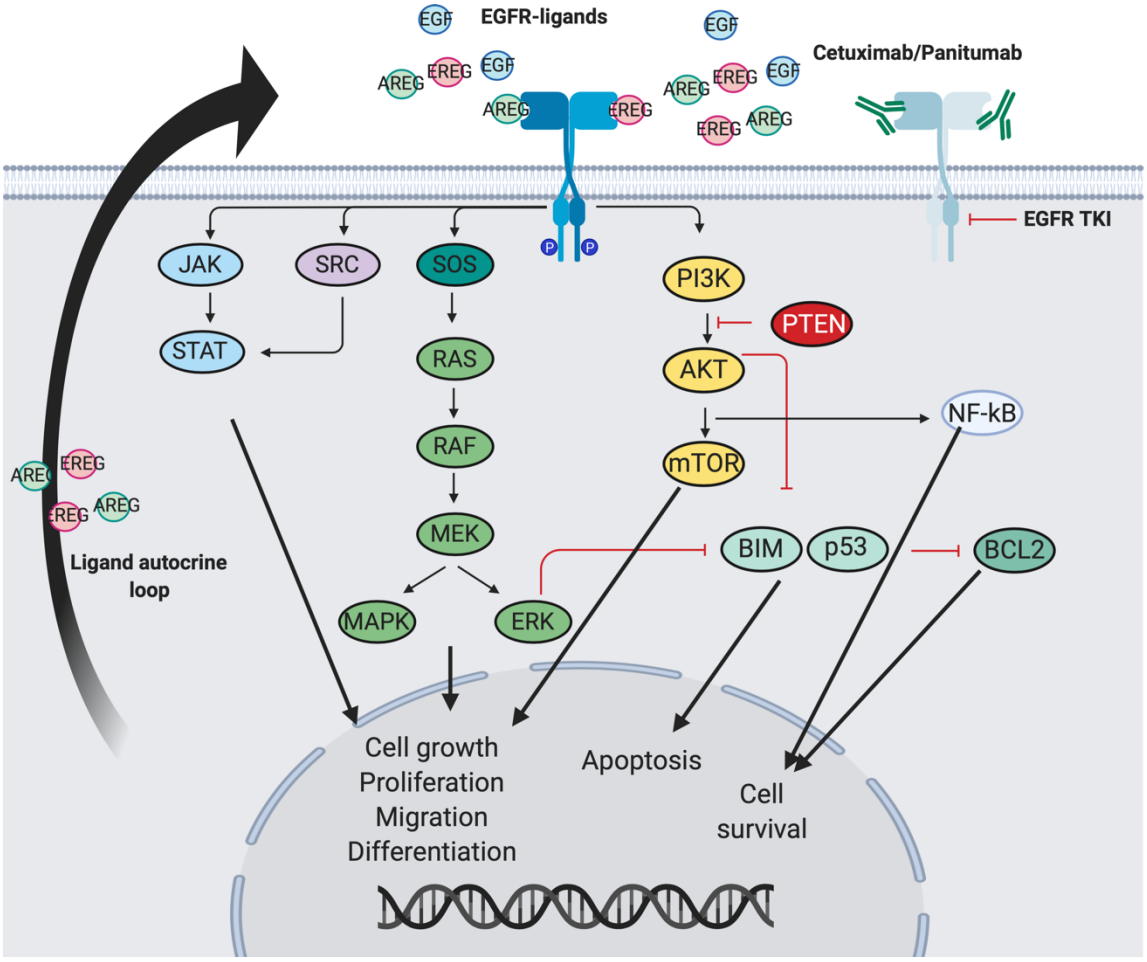


Figure 1-2 EGFR-mediated signaling pathways

In many epithelial cancers, EGFR expression is associated with decreased overall survival (OS) rates. Therefore, targeting the EGFR has been intensely pursued in the last three decades as a cancer treatment strategy. Based on the hypothesis that mAb against EGFR could prevent ligand binding and inhibit activation of the receptor's tyrosine kinase (Mendelsohn, Prewett et al. 2015), interest in anti-EGFR treatments for specific tumors such as CRC and NSCLC led to the development of mAbs and tyrosine kinase inhibitors (TKIs). In 2004 and 2006, the EGFR-directed mAbs cetuximab and panitumumab were approved for the treatment of chemo refractory metastatic CRC (mCRC) patients (Seshacharyulu, Ponnusamy et al. 2012). Cetuximab is a human/murine chimeric mAb that binds to the extracellular domain of EGFR. The interaction of cetuximab with the receptor partially blocks the ligand-binding domain preventing the correct conformation of the dimerization arm. Consequently, cetuximab stops both ligand binding and EGFR dimerization, avoiding the formation of heterodimers with other HER family members. In addition, cetuximab effectively blocks EGFR phosphorylation, promotes EGFR internalization, and reduces cellular proliferation (Pozzi, Cuomo et al. 2016). Panitumumab, is a fully humanized antibody directed against EGFR, having a similar mode of action as cetuximab (Van Cutsem, Peeters et al. 2007). In 2008, these treatments were restricted to wild-type *KRAS* (*KRAS-WT*) CRC patients (Karapetis, Khambata-Ford et al. 2008). Clinical trials showed large differences in treatment response among mCRC patients (Yang, Lin et al. 2014). Retrospective subgroup analyses have shown that the benefit of anti-EGFR treatment is limited to a molecularly distinct population (Martins, Mansinho et al. 2018).

When used as a single agent in unselected mCRC patients, cetuximab and panitumumab achieved only a 10-20% of response rate (RR) (Cunningham, Humblet et al. 2004, Van Cutsem, Peeters et al. 2007). High frequency of genetic or epigenetic alterations in proteins involved in EGFR regulation itself and downstream pathways (such as RAS, v-Raf murine sarcoma viral oncogene homolog B (BRAF), PI3K and PTEN) explain some of non-responding cases and the low RR. Actually, the subgroup of patients with WT markers for RAS, BRAF, PIK3CA and expressing normal levels of PTEN have the best response to mAbs (De Roock, Claes et al. 2010, De Roock, De Vriendt et al. 2011, Karapetis, Jonker et al. 2014). Nevertheless, still about 10% of these individuals remain resistant to anti- EGFR therapies, suggesting the existence of still unknown alternative mechanisms capable of influencing treatment effectiveness. The lack of dependency of colorectal tumors to EGFR signaling could explain the lack of response to anti-EGFR treatment and it is further discussed in the Chapter 2 of this dissertation. The importance of other ERBB members like ERBB3 and ERBB4 signaling as a compensatory mechanism activated in intestinal tumorigenesis in the lack of EGFR is also described in the following chapters.

1.4. Primary resistance to anti-EGFR treatment

1.4.1. *KRAS* activating mutations

Nearly 90% of CRC patients have heterogeneous genetic alterations in genes involved in EGFR signaling, which negatively affects response to mAbs cetuximab and panitumumab. A major signaling cascade initiated after EGFR activation compromises the KRAS/RAF/MAPK signaling pathway (Normanno, Tejpar et al. 2009, Arrington,

Heinrich et al. 2012). KRAS activation results in downstream signaling through the PI3K and extracellular signal regulated kinase (ERK) pathways (Vitiello, Cardone et al. 2019) (Figure 1-2). Therefore, KRAS is a critical component of EGFR-induced signaling cascades. The *KRAS* oncogene is among the most studied and best characterized of the known cancer-related genes. The detection of activating *KRAS* mutations has been associated with decreased RR to select chemotherapeutic agents.

Over 30% of CRC patients harbor *KRAS* mutations in exon 2 (codon 12 and 13) (De Roock, De Vriendt et al. 2011, Allegra, Rumble et al. 2016). The *KRAS* mutational status in CRC is one of the predictive biomarkers to anti-EGFR resistance to emerge in the clinic. In mCRC, increased response rates to therapy are correlated with improvements in progression-free survival (PFS) and OS. Thirty to fifty percent of CRC patients harbor activating *KRAS* mutations in codon 12 affecting many cellular functions including cell proliferation, apoptosis, migration, fate specification and differentiation (Zhao, Wang et al. 2017). It was first reported by Lievre *et al.*, followed by Di Fiore *et al*., that patients with *KRAS* mutations have a decreased response to anti-EGFR agents (Lievre, Bachet et al. 2006, Di Fiore, Blanchard et al. 2007). Additionally, it was noted that patients with *KRAS*-*WT* had better OS compared to patients with mutant *KRAS* (Lievre, Bachet et al. 2006). The data from several clinical trials showed that the addition of cetuximab to first-line chemotherapy (fluorouracil, leucovorin, and oxaliplatin (Bokemeyer, Bondarenko et al. 2009) or fluorouracil, leucovorin, and irinotecan (Van Cutsem, Labianca et al. 2009) is not beneficial for *KRAS*-mutated tumors, although these benefit from chemotherapy alone. Importantly, previous studies also show that addition of EGFR-targeted treatment to

chemotherapy may even be detrimental in such patients (Bokemeyer, Bondarenko et al. 2009, Bardelli and Siena 2010).

Based on these results, since 2008, the regulatory system is focused on the observation that mCRC patients carrying mutated *KRAS* should not receive anti-EGFR mAbs. However, not all specific point mutations in *KRAS* result in the resistance to anti-EGFR monoclonal antibodies. A pooled analysis from seven clinical trials examining mCRC patients demonstrated that patients' as a whole with *KRAS* mutations had decreased survival (De Roock, Piessevaux et al. 2008); nevertheless, the addition of cetuximab to first-line chemotherapy benefit patients with *KRAS* G13D by increasing OS and PFS (De Roock, Claes et al. 2010, McFall, Diedrich et al. 2019). Conversely, *KRAS* G13D mutation has also been associated with worse OS compared to patients with other *KRAS* mutations or *KRAS-WT* (Arrington, Heinrich et al. 2012). Therefore, there are still substantial and observable discrepancies between response to EGFR inhibitors and *KRAS* mutation status, which indicates that the KRAS biomarker is not fully predictive of which CRC cases will likely be responsive to anti-EGFR therapies.

1.4.2. Elevated EGFR expression

EGFR has been found to be abnormally activated in many human malignancies, through several mechanisms, including receptor overexpression, gene amplification, activating mutation, overexpression of receptor ligands, and/or loss of negative regulatory mechanisms (Fakih and Wong 2010). More than half of CRC patients show heterogeneous genetic EGFR alterations such as somatic mutations and gene copy number variations, both of which have been shown to negatively affect response to the mAbs

cetuximab and panitumumab (Martins, Mansinho et al. 2018). However, mutations in the EGFR kinase domain are an extremely rare event in patients with CRC, and when they do occur, they are not associated with patient response (Parseghian, Napolitano et al. 2019).

Approximately 80% of CRC patients show aberrant EGFR expression; elevated EGFR expression correlates with disease progression, metastatic spread and poorer prognosis (Fakih and Wong 2010). In the initial development of EGFR inhibitors, patients were selected to enroll on trials only if the tumors were positive for EGFR expression using immuno-histochemistry. This was based on the concept that lack of EGFR expression, results in resistance to EFGR inhibitors. However, several studies demonstrated that patients with no EGFR expression or low *EGFR* gene copy responsed to anti-EGFR treatment (Chung, Shia et al. 2005, Sartore-Bianchi, Moroni et al. 2007, Scartozzi, Bearzi et al. 2009, Shen, Chen et al. 2014). Therefore, the degree of EGFR expression, using immuno-histochemistry or molecular based assays, does not seem to fully correlate with effectiveness of EGFR inhibitors. Moreover, due to technical obstacles and considerable discrepancies between scoring systems, evaluation of sensitivity to anti-EGFR drugs through estimation of EGFR gene copy number is still ineffective in clinical practice (Personeni, Fieuws et al. 2008, Sartore-Bianchi, Fieuws et al. 2012, Zhao, Wang et al. 2017).

1.4.3. Low levels of expression of EGFR ligands, AREG and EREG

High levels of the EGFR ligands AREG and EREG have been shown to be strongly correlated to response to anti-EGFR therapy. These receptors have a key effect on intracellular signaling suggesting that the autocrine or paracrine loop generated by the

increased expression is responsible for driving the growth of these tumors (Bardelli and Siena 2010, Pietrantonio, Vernieri et al. 2017, Zhao, Wang et al. 2017).

Overall, high levels of *AREG* and *EREG* transcripts is linked to sensitivity to cetuximab, as shown by improvement in disease control rate (DCR) and longer PFS. In CRC patients with high transcript levels of *AREG* and *EREG*, cetuximab treatment tends to have a more potent antitumor activity. However, in patients with *KRAS* mutant tumors, there is no correlation between *AREG* and *EREG* gene expression and PFS and OS (Baker, Dutta et al. 2011). Additionally, *KRAS-WT* patients with low ligand expression behaved like *KRAS* mutant CRC patients. Therefore, the low expression of the ligands may be a mechanism of resistance to EGFR inhibitors as it indicates that the EGFR system may not be the main contributor of tumor growth or progression (Ruiz-Saenz, Dreyer et al. 2018). Recently, a methylation profile of *AREG* and *EREG* genes has been shown to inversely correlate with their expression in both colorectal cancer cells and human colorectal cancer samples (Bormann, Stinzing et al. 2019).

However, similar to the technical difficulties quantifying EGFR copy number, the levels of expression *AREG* and *EREG* have been challenging to evaluate systematically, therefore, these markers cannot be used to select patients eligible for cetuximab or panitumumab therapy (Baker, Dutta et al. 2011, Shaib, Mahajan et al. 2013).

1.4.4. Constitutively active *BRAF* mutations

The RAS status of CRC patients is a good predictor to help identify a patient population that is resistant to anti-EGFR therapy. However, only half of patients with *KRAS-WT* tumors respond to anti-EGFR treatment. Therefore, additional molecular

alterations in the downstream components of the EGFR signaling network are also likely to associate with resistance to anti-EGFR mAbs. Among these, the serine-threonine kinase BRAF is the principal downstream effector of KRAS in the EGFR pathway. *BRAF* mutations are found in less than 10% of CRC patients.

BRAF V600E mutation is the most common, counting for 95% of the mutations occurring in *BRAF* gene. *BRAF* V600E mutation promotes tumor cell proliferation and survival by constitutive activation of the MAPK signaling pathway (Figure 1-2). Moreover, preclinical and clinical studies have suggested that, regardless of EGFR blockade, mutations in *BRAF* lead to persistent activation of downstream signaling resulting in cell proliferation and survival (Wan, Garnett et al. 2004, Zhao, Wang et al. 2017). *BRAF* V600E mutation is an indicator of poor prognosis in patients with *KRAS-WT*, and a marker of resistance to anti-EGFR therapy in the chemotherapy-refractory setting (Siroy, Boland et al. 2015). Furthermore, *BRAF* mutations are mutually exclusive with *KRAS* mutation indicating common downstream effects (Rajagopalan, Bardelli et al. 2002). Numerous meta-analyses show that the addition of cetuximab or panitumumab treatment did not significantly improve PFS and OS among *KRAS-WT*/*BRAF*-mutated patients (Cremolini, Benelli et al. 2019).

A combination therapy of BRAF and EGFR inhibitors has been used in clinical trials with *BRAF*-mutant CRC patients, and in some of the cases it resulted in improved RR (Sartore-Bianchi, Siena et al. 2016, Cremolini, Marmorino et al. 2019). These findings demonstrate a strong association between the presence of the *BRAF* V600E mutation and resistance to anti-EGFR therapy in mCRC (Capalbo, Marchetti et al. 2014). Therefore,

consideration of both *BRAF* and *KRAS* mutations in tumors before administering anti-EGFR therapy can help identify more than half of the primary non-responders.

Despite *KRAS* and *BRAF WT* status, there have still been a significant number of non-responders (41%) to anti-EGFR therapy questioning further pathways that are important in defining resistance to these treatments (Douillard, Oliner et al. 2013).

1.4.5. MAPK/MEK alterations

The MAPK signaling pathway is critically involved in many important cellular processes, including cellular proliferation, survival, differentiation and motility. As a consequence, dysregulation of the MAPK pathway is found in a majority of cancers including CRC (Wagle, Kirouac et al. 2018, Vitiello, Cardone et al. 2019).

The mitogen-activated protein kinase kinase (MEK) protein is another downstream target of KRAS. MEK activates ERK-1 and ERK-2 which are responsible for cell cycle activation through the phosphorylation of several factors involved in the G to S transition of the cell cycle progression. ERK phosphorylation is a biomarker of MAPK signaling pathway activation which has direct implications for tumorigenesis (Burotto, Chiou et al. 2014). Targeting MEK protein or EGFR only does not fully block the ERK signal (Yaeger and Corcoran 2019). It has been described that resistance to EGFR-targeted therapy could also be mediated through alternate ERK1/2 activation that bypasses EGFR either via alternative receptors at the plasma membrane or constitutively active downstream components (Hopper-Borge, Nasto et al. 2009).

MEK inhibitors in combination with anti-EGFR have been shown to inhibit RAS-mutant mCRC cell viability *in vitro* (Ducreux, Chamseddine et al. 2019). Preliminary

data from *in vitro* and *ex vivo* experiments also shown that the use of anti-MEK such as trametinib or cobimetinib in combination with cetuximab overcomes resistance to anti-EGFR in KRAS-mutated CRC (Cheon, Kim et al. 2018, Ledys, Derangere et al. 2019). However, further clinical trials are required to confirm these results.

1.4.6. Activation of *PIK3CA/PTEN/AKT* signaling pathway

In addition to the RAS/RAF/MAPK pathway, EGFR also activates the PIK3CA/PTEN/AKT signaling pathway (Figure 1-2.). Molecular alterations in this pathway, including active mutations of PIK3CA or the loss of PTEN expression, can also trigger the activation of downstream signaling pathways through EGFR-independent mechanisms (Parseghian, Napolitano et al. 2019). Consequently, the function of activated PIK3CA/PTEN signaling in the resistance to anti-EGFR treatment has been explored.

1.4.6.1. Active mutations in *PIK3CA*

PIK3CA, is a downstream effector of EGFR signaling. About 10% to 18% of mCRC patients harbor mutations in *PIK3CA* gene; these mutations can be found in patients with both *KRAS* and *BRAF* mutations (Barault, Veyrie et al. 2008, Nosho, Kawasaki et al. 2008). Mutations in exon 9 (E542K, E545K) or exon 20 (H1047R) lead to constitutive activation of the protein kinase and its downstream signaling pathway, resulting in tumor cell proliferation and survival (De Roock, Claes et al. 2010).

The role of *PIK3CA* mutations as a predictor of resistance to anti-EGFR therapy have been evaluated in several studies, with highly inconsistent results (Vitiello, Cardone et al. 2019). A retrospective study that investigated the effect of *PIK3CA* mutations on the response to cetuximab therapy found that patients with *KRAS-WT/PIK3CA* exon 20

mutated showed significantly lower RR compared to *PIK3CA-WT*, and no significant effect in patients with *PIK3CA* exon 9 mutated (De Roock, Claes et al. 2010). The differential response to treatment with EGFR mAbs could be explained by the differential effects of the mutations. Exon 20 mutations trigger gain of function independent of interaction with RAS-Guanosine triphosphate (GTP), whereas exon 9 do so through RAS-GTP binding (Zhao and Vogt 2008). Therefore, *PIK3CA* exon 20 mutations are associated with a lack of response and resistance. A strategy to improve the treatment of CRC patients could be established on the combination of an anti-EGFR therapy and MEK or PI3K/AKT inhibitors. However, considering the low frequency of incidence of these mutations, large randomized clinical trials should be conducted to draw a definitive conclusion.

1.4.6.2. PTEN activity loss

The *PTEN* gene, located on chromosome 10, is a tumor suppressor gene that negatively regulates the PI3K/AKT signaling pathway through its lipid phosphatase activity (Zhao and Vogt 2008). PTEN inactivation can occur through either *PTEN* gene silencing or mutation (Goel, Arnold et al. 2004). In mCRC loss of PTEN expression is estimated to occur in about 20-40% of patients through mixed genetic/epigenetic mechanisms (Siena, Sartore-Bianchi et al. 2009). PTEN loss results in constitutive activation of the PI3K/AKT signaling pathway, leading to tumor cell proliferation and survival (Sansal and Sellers 2004). The prognostic and predictive role of PTEN in CRC response to cetuximab and panitumumab treatment has been analyzed by several studies,

which reported conflicting and inconclusive results (Laurent-Puig, Cayre et al. 2009, Loupakis, Pollina et al. 2009, Parseghian, Napolitano et al. 2019).

In 2009, Sartore-Bianchi *et al.,* showed that PTEN loss is associated with decreased RR, PFS, and OS in mCRC patients treated with anti-EGFR mAbs (Sartore-Bianchi, Martini et al. 2009, Zhao, Wang et al. 2017). A retrospective study also reported that loss of PTEN expression, was associated with non-responsiveness to cetuximab. *In vitro* studies in colon cancer cells have also confirmed these results by showing that PTEN loss may predict the efficacy of cetuximab treatment (Rachiglio, Lambiase et al. 2019). However, Laurent-Puig *et al.,* reported that PTEN loss is no associated with RR, PFS, or OS (Laurent-Puig, Cayre et al. 2009); Loupakis *et al.,* confirmed that PTEN expression and the response to cetuximab treatment was different between primary tumors and metastases (Loupakis, Pollina et al. 2009). Supporting these data, Razis *et al.,* did not find any association between PTEN protein expression and response to anti-EGFR treatment (Chen, Shi et al. 2015).

In conclusion, the differences in the analysis of PTEN levels and the variable expression in primary and metastatic tumor samples of CRC, makes loss of PTEN an unreliable biomarker of the efficacy of anti-EGFR mAbs. Further investigation and large randomized clinical trials are still required to confirm the role of PTEN in anti-EGFR therapy resistance.

The intersection of KRAS/MAPK/PIK3CA pathway has direct effects for tumorigenesis and the resistance to anti-EGFR therapy. Suggesting that synergistic drugs can be used to treat resistant tumors with lower concentrations and decreased side effects.

For example, in a *KRAS* mutant lung cancer model, it was shown that the combination of PI3K and MAPK pathway inhibition by treatment with a dual PI3K/mammalian target of rapamycin (mTOR) inhibitor (NVP-BEZ235) and a MEK inhibitor (ARRY-142886) led to significant tumor regression (Sartore-Bianchi, Martini et al. 2009).

1.4.7. Excess activation of JAK/STAT signaling pathway

The Janus family of tyrosine kinases (JAK) and the STAT family are key factors of cytokine receptor signaling which are involved in cellular survival, proliferation, differentiation, and apoptosis (Ma, Wang et al. 2004). STAT3 is the most studied member of the STAT family of transcription factors mediating cellular responses to cytokines and growth factors and acting as an oncogene in many cancers, including CRC (Rokavec, Oner et al. 2014).

Autocrine and paracrine production of cytokines lead to persistent activation of STAT3 through the JAK family, as well as activation of tyrosine kinases, such as EGFR and rous sarcoma oncogene (SRC), playing a critical role in oncogenesis, angiogenesis, invasion, metastasis and immune system suppression (Kisseleva, Bhattacharya et al. 2002, Corvinus, Orth et al. 2005). Several studies support the role of STAT proteins in resistance to EGFR inhibitors in several preclinical models, including glioma, HNSCC, and non-small cell lung cancer (NSCLC) (Kijima, Niwa et al. 2002, Haura, Sommers et al. 2010). In 2015, Qiong Li *et al.,* demonstrated that STAT3 phosphorylation (pSTAT3) correlated with gefitinib resistance in CRC cells. Furthermore, the addition of Stattic, a STAT3-specifc inhibitor, or STAT3-specifc siRNA significantly increased the efficacy of gefitinib against CRC cells, both *in vitro* and *in vivo* (Li, Zhang et al. 2015). Yar Saglam *et al.,* in

2016, also demonstrate that combined treatment with cucurbitacin B, a JAK/STAT3 pathway inhibitor, and gefitinib could lead to enhanced anti-tumor activity in human CRC cells (Yar Saglam, Alp et al. 2016). These findings suggest that activation of the JAK/STAT3 pathway contribute to EGFR inhibition resistance in CRC. Therefore, combining EGFR blockade with suppression of JAK/STAT3 signaling may enhance the anti-tumor effects of EGFR inhibitors alone and overcome anti-EGFR therapy resistance.

1.4.8. Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is a complex biological process during which epithelial cells fail to keep their original morphology and properties like cell polarity and cell-cell contact, and simultaneously gain mesenchymal characteristics, such as increased motility (Prieto-Garcia, Diaz-Garcia et al. 2017). In CRC, EMT is associated with an increased invasive or metastatic phenotype, and treatment resistance (Tan, Miow et al. 2014).

Previous research proposes that the EMT-like alterations that develop in carcinoma cells mitigate the role of EGFR signaling in regulating cell proliferation and survival *in vitro* (Thomson, Petti et al. 2008). It has been suggested that EMT acts as a kinase switching mechanism; during EGFR kinase blockade, alternative tyrosine kinases activate compensatory signaling pathways. Consequently, EMT-like transition has been implicated as a potential mechanism of anti-EGFR therapy resistance. Preclinical data suggests that mesenchymal-like CRC cells have a reduced sensitivity to EGFR-inhibitors compared to epithelial CRC cell lines (Buck, Eyzaguirre et al. 2007). Furthermore, the combination of inhibitors for EGFR and CRIPTO, an important signaling node that

induces EMT, overcome the resistance of mesenchymal-like CRC cells to anti-EGFR drugs (De Luca, Arra et al. 2000, Lazarova and Bordonaro 2017). In 2013, Byers *et al.,* proposed that EMT gene signatures predicted resistance to EGFR and PI3K inhibitors (Byers, Diao et al. 2013). Overall, the data support a possible role of EMT as a mediator of resistance to anti-EGFR drugs in CRC cells. Nonetheless, it is unclear if the cellular mesenchymal-like alterations are generated during the course of anti-EGFR treatment, contributing to acquired resistance as well. However, there is no clinical evidence for EMT-induced resistance to EGFR inhibitors in mCRC. Although limited data are available for mCRC, further evaluation of EMT related resistance to EGFR targeting could provide novel therapeutic opportunities for CRC treatment.

1.5. Acquired resistance to anti-EGFR therapy in CRC

Most CRC patients that initially respond to EGFR monoclonal antibodies ultimately show disease progression. This progression after anti-EGFR therapy is identified as acquired or secondary resistance. Clinical data suggest that response to anti-EGFR therapies is transitory and most tumors become refractory within 3-18 months (Van Emburgh, Sartore-Bianchi et al. 2014). Numerous mechanisms that might contribute to acquired resistance to anti-EGFR antibodies have been studied and classified in two categories. The first category includes mutations in EGFR or activation of parallel RTKs (Haura, Sommers et al. 2010, Li, Zhang et al. 2015), such as HER2 and MET (Roskoski 2014, Adams, Brown et al. 2018, Bregni, Sciallero et al. 2019); and the second category involves mutation in the bypass points of the pathway, such as RAS and BRAF (Barault, Veyrie et al. 2008, Algars, Sundstrom et al. 2017, Cremolini, Benelli et al. 2019).

1.5.1. EGFR mutations

Genetic alterations affecting drug binding and preventing kinase activation play a critical role in acquired resistance. In CRC, acquired mutations in the extracellular domain of EGFR, such as S492R, G465E and G465R mutations (Van Emburgh, Arena et al. 2016), have been associated with secondary resistance to EGFR mAbs.

Several structural analyses determine that mutations in S492 region affect the cetuximab binding site, reducing the affinity of the receptor to the ligand and interfering with binding to cetuximab (Martinelli, De Palma et al. 2009, Bertotti, Papp et al. 2015). Additionally, the S492R mutation does not inhibit binding of panitumumab to EGFR. Therefore, following disease progression upon cetuximab treatment, treatment with panitumumab appears to be a reasonable strategy for patients harboring the S492R mutation (Montagut, Tsui et al. 2018). On the other hand, G465 is located in the center of the region in which the epitopes of both cetuximab and panitumumab overlap, preventing the binding of both antibodies (Braig, Marz et al. 2015). Patient-derived tumor-grafts (Bertotti, Papp et al. 2015), as well as cell culture studies (Arena, Siravegna et al. 2016), showed that new-generation anti-EGFR antibodies, that bind EGFR epitopes different from those recognized by cetuximab and panitumumab, are very effective in opposing the growth of tumor samples harboring mutations in the G465 residue.

Mutations in the EGFR kinase domain may also be involved in resistance to EGFR mAbs as identified by cell-free DNA analysis (Bettegowda, Sausen et al. 2014), and studies in cetuximab-resistant patient-derived tumor-grafts (Bertotti, Papp et al. 2015). Small-molecule inhibitor or cetuximab alone have not been effective in the treatment of

tumor samples harboring mutations in the EGFR kinase domain, but the combination resulted in substantial and durable inhibition of tumor growth (Bertotti, Papp et al. 2015).

1.5.2. Activation of alternative growth factor receptor pathways

Acquired resistance to anti-EGFR has been associated with the activation and upregulation of alternative and compensatory signaling through growth-factor receptors other than EGFR. Numerous growth factor receptors, such as type 1 insulin-like growth factor receptor (IGF-1R), mesenchymal-epithelial transition factor receptor (MET receptor), and the human epidermal growth factor receptor-2 (HER2) (Weber, Fottner et al. 2002, Wheeler, Huang et al. 2008, Bardelli, Corso et al. 2013), can trigger EGFR downstream effectors and activate intracellular signaling pathways by bypassing EGFR, thus inducing tumor cell proliferation and resistance to apoptosis (Figure 1-3).

1.5.2.1. Activation of the IGF1 signaling pathway

The IGF-1R is a member of a family of transmembrane tyrosine kinases that includes the insulin receptor and the insulin receptor-related receptor. The IGF-1R signaling pathway includes transduction of the IGF signal by the MAPK and PI3K/AKT pathway and its activation has been associated with anti-EGFR resistance in CRC (Weber, Fottner et al. 2002).

Evidence suggests a cross-talk between IGF-1R and EGFR, which might be crucial for the mitogenic and transforming activity of EGFR. Simultaneous blockade of IGF-1R and MEK has been shown to effectively prevent the occurrence of the EGFR-IGF1R cross-talk in BRAF mutated CRC preclinical models (Buck, Eyzaguirre et al. 2008). Preclinical data shows that combination of IGF-1R and EGFR kinase inhibitors results in

growth inhibition in CRC cell lines (Reinmuth, Liu et al. 2002). It has been proposed that IGF-1 signaling induce EGFR independent PIK3CA and AKT activity, which might be another explanation for the lack of response to anti-EGFR mAbs in *KRAS-WT* CRC patient (Hu, Patil et al. 2008). Bohula *et al.,* showed that IGF-1R activates both RAS/ERK and PI3K/AKT signaling pathways, which regulate growth, differentiation and survival (Bohula, Playford et al. 2003). However, a phase II clinical trial showed that just one *KRAS-WT* patient out of 21 patients treated with a combination therapy of anti-EGFR and anti-IGF-1R achieved a partial response (Reidy, Vakiani et al. 2010). According to the reported results, the role played by IGF-1R as a marker of resistance is still unclear and further clinical trials have to be done before concluding the role of IGF-1R on resistance to anti-EGFR therapy in CRC.

1.5.2.2. MET overexpression and amplification

The MET tyrosine kinase receptor for hepatocyte growth factor (HGF) is a proto-oncogene that activates cell proliferation and survival through the RAS/ERK cascade, the PI3K/AKT axis, and stimulation of SRC and STAT (Benvenuti and Comoglio 2007, Comoglio, Giordano et al. 2008, Gherardi, Birchmeier et al. 2012). Alterations in MET signaling may occur by numerous mechanisms, including genetic abnormalities such as MET amplification, mutations in the MET kinase domain and as a consequence of increased HGF expression/activity (Trusolino and Bertotti 2012).

MET amplification has been documented in CRC patients as a mechanism of acquired resistance to cetuximab and panitumumab (Bardelli, Corso et al. 2013). MET amplification was observed in tumor tissues from patients who developed resistance to

cetuximab or panitumumab (Bardelli, Corso et al. 2013). Further analysis showed that pharmacological inhibition and silencing of MET could restore the effects of cetuximab in CRC samples (Krumbach, Schuler et al. 2011). A complex cross-signaling network between EGFR and MET has been described to be involved in the resistance to anti-EGFR treatment (Gherardi, Birchmeier et al. 2012, Troiani, Martinelli et al. 2013). Overexpression of TGF-a has been proposed to activate the MET pathway through EGFR/MET interactions, (Troiani, Martinelli et al. 2013). In these studies, only concomitant inhibition of both MET and EGFR substantially reduced tumor growth *in vivo* (Luraghi, Reato et al. 2014). Additionally, a case report suggests that MET amplification in CRC patients also prevents sensitivity to drugs targeting the downstream RAS pathway and dual blockade of both BRAF and MET proved to be clinically effective (Pietrantonio, Oddo et al. 2016).

Several studies have identified that amplification of MET associates with a negative response to cetuximab (Krumbach, Schuler et al. 2011, Bardelli, Corso et al. 2013). However, only 1% of untreated mCRC patients show MET amplification (Bardelli, Corso et al. 2013), and for this reason, MET amplification is not an effective biomarker of primary resistance to anti-EGFR therapy in mCRC.

1.5.2.3. HER2 amplification and other ERBB receptors

HER2 is the only member of the HER family of receptor tyrosine kinases that is not activated by growth factor ligands. HER2 has the ability to trigger the MAPK and PI3K/AKT pathways by forming heterodimers with other ligand-stimulated receptors like EGFR, HER3 and HER4 (Wheeler, Huang et al. 2008, Yonesaka, Zejnullahu et al. 2011).

Overexpression of HER2 has also been associated with aberrant constitutive activation of the signaling pathway in CRC (Martinelli, Troiani et al. 2018). HER2/HER3 heterodimers generate the most powerful growth-promoting signals (Ciardiello and Normanno 2011). HER2 has been characterized as a potential biomarker of resistance to anti-EGFR therapy because it leads to the activation of a signaling pathway shared with EGFR.

Accumulating evidence has shown that HER2 amplification is a predictor of poor response to anti-EGFR antibodies (Bertotti, Migliardi et al. 2011, Ciardiello and Normanno 2011). Analysis of genotype-response correlations in a platform of patient-derived mCRC xenografts demonstrated that HER2 gene amplification was significantly associated with resistance to cetuximab (Bertotti, Migliardi et al. 2011). Particularly, in CRC patients, HER2 amplification was found in just 14% of pretreatment tumor cells and in 71% of after anti-EGFR therapy cells (Bregni, Sciallero et al. 2019). Therefore, considering its low frequency, HER2 is not likely to be a significant player in primary resistance to anti-EGFR therapy, but it plays an important role in acquired resistance.

Additionally, the administration of a HER2 inhibitor overcomes the resistance to anti-EGFR *in vitro*. Bertotti et al., and Yonesaka et al., suggested, in two independent studies, that combinations of selective inhibitors targeting HER2 and EGFR were able to significantly inhibit the growth of cetuximab-resistant CRC cells, and stimulate long-lasting tumor regression in experimental models (Bertotti, Migliardi et al. 2011, Yonesaka, Zejnullahu et al. 2011).

The signaling activated by HER2/HER3 has attracted extensive attention considering the effect of HER3 in cancer progression (Hofmann, Seeboeck et al. 2016).

HER2 and HER3 increased co-expression previously found in breast cancer might explain the lack of responsiveness to TKIs (Hofmann, Seeboeck et al. 2016). Ten percent of colon and gastric cancers present somatic mutations in HER3 (Lee, Ma et al. 2014, Zhang, Wong et al. 2016). Previous preclinical studies indicate that the ERBB3 pseudo-kinase has essential roles in supporting intestinal tumorigenesis, suggesting that ERBB3 may be a promising target for the treatment of CRC (Lee, Yu et al. 2009). In Chapter 3 of this dissertation, we provide additional evidence of the importance of regulators of intestinal tumor progression that are dependent on the ERBB3 signaling pathway.

HER4 involvement in cancer progression has been less well characterized (Rudloff and Samuels 2010, Lau, Killian et al. 2014). Recently, it has been shown that HER4 is overexpressed in CRC, indicating an important role in promotion of colorectal carcinogenesis (Williams, Bernard et al. 2015). Additionally, activating mutations in *ERBB4* have been described in the kinase (D931Y and K935I) as well as extracellular (Y285C and D595V) domain in non-small cell lung cancer (Kurppa, Denessiouk et al. 2016). In Chapter 2, we also showed that ERBB4 signaling is required to increase cell proliferation in a subset of colorectal tumors in mice.

The diverse array of intracellular signaling networks initiated by ERBB proteins drives tumor progression in almost all solid cancers in humans. Hence, a new generation of drugs that selectively target the ERBB oncoproteins has demonstrated impressive therapeutic efficacy in the clinic (Perez, Crombet et al. 2013, Arteaga and Engelman 2014, Roskoski 2014).

1.5.2.4. Alterations of VEGF signaling

Vascular endothelial growth factor (VEGF) plays a fundamental role in angiogenesis and EGFR signaling has been associated with important functions on tumor cell survival, migration, and invasion (Figure 1-3) (Kaplan, Riba et al. 2005). Elevated expression levels of VEGF have been shown to be correlated with acquired EGFR inhibitor resistance (Ciardiello, Bianco et al. 2004, Bianco, Rosa et al. 2008). Furthermore, silencing of VEGFR1 or the treatment with vandetanib, an orally available TKI that inhibits EGFR, VEGFR1, and VEGFR-2 tyrosine kinases, decrease the growth and proliferation of EGFR inhibitor-resistant cells. These findings suggest that the combined inhibition of VEGFR and EGFR results in increased sensitivity to anti-EGFR drugs, and further supports an association between increased expression of VEGF/VEGFR1 and anti-EGFR treatment resistance. However, this preclinical strategy was not successful in human clinical trials (Hecht, Mitchell et al. 2009, Tol, Koopman et al. 2009, Derangere, Fumet et al. 2016). The combination of the anti-VEGF mAb bevacizumab, and the anti-EGFR mAb cetuximab or panitumumab did not result in improved PFS or OS (Hecht, Mitchell et al. 2009, Tol, Koopman et al. 2009). An increase in drug associated toxicity might cause reduction in survival, since it contributed to increases in dose delays, decreases in dose intensity, and increases in mortality in the dual EGFR/VEGF inhibition arm. In addition, several studies indicate a potential negative pharmacodynamic interaction between anti-EGFR mAbs and anti-VEGF mAbs (Hecht, Mitchell et al. 2009). Therefore, further research is still required in order to develop a

more comprehensive understanding of the role of VEGF signaling in the resistance of anti-EGFR therapy.

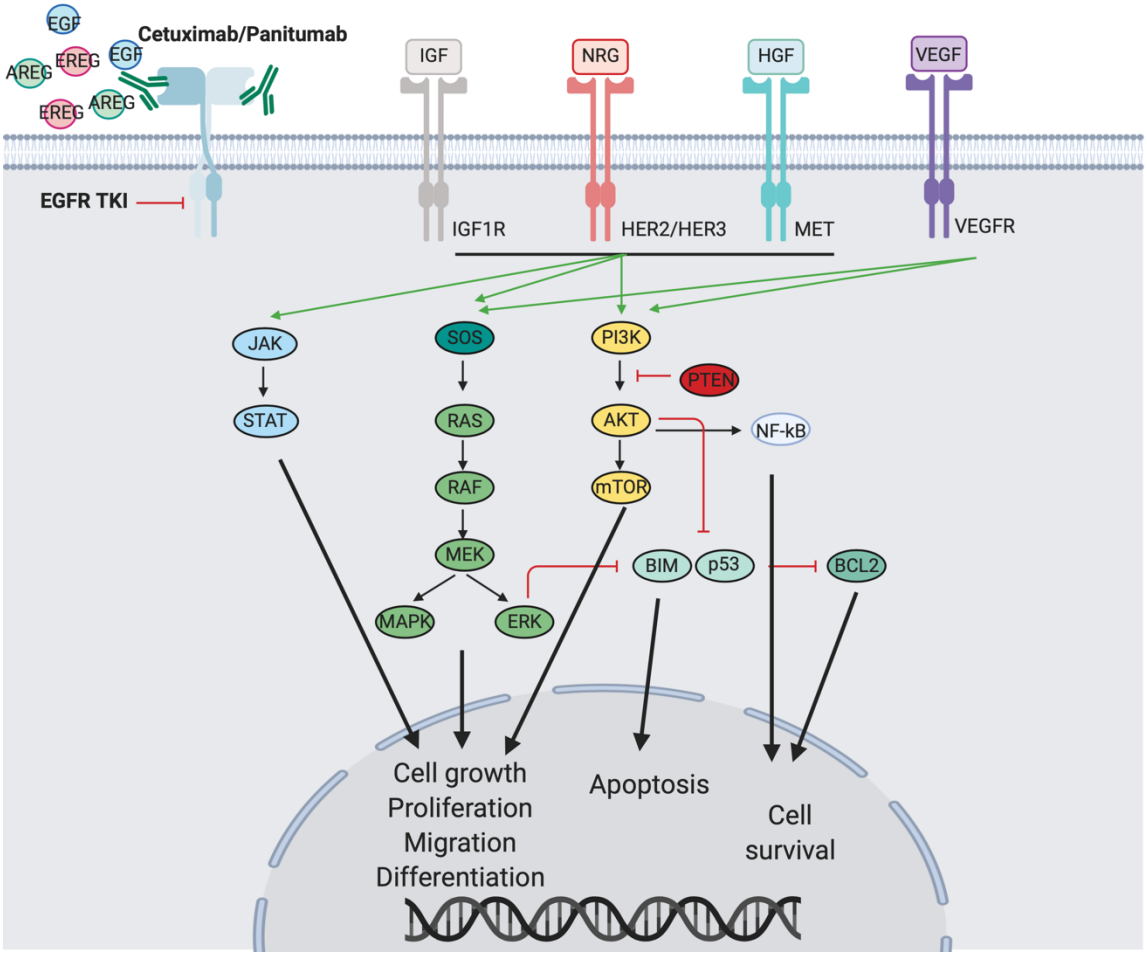


Figure 1-3 Alternative growth factor receptors activated as compensatory pathways of anti-EGFR resistance

1.5.3. Secondary alterations in the RAS/RAF signaling pathway

RAS activating mutations are the main mechanism for primary resistance to anti-EGFR therapy in CRC. In addition, genetic alterations in RAS/RAF are also found in 40–60% of CRC patients who progress on cetuximab or panitumumab (Van Emburgh, Arena et al. 2016, Yaeger and Corcoran 2019). Preclinical and clinical studies have detected the development of *KRAS* mutations after cetuximab treatment (Bouchahda, Karaboue et al. 2010). Several patients that were *KRAS-WT* prior to treatment showed *KRAS* mutations after receiving cetuximab. In contrast, *KRAS* mutations were not detected in patients that received chemotherapy alone. Similarly, secondary mutations in *NRAS* and *BRAF* are also associated with secondary resistance (Misale, Arena et al. 2014). However, it is unclear if these "acquired genetic alterations" in RAS/RAF signaling are novel spontaneous mutations or whether they are selected from pre-existing resistant sub-clones by the anti-EGFR therapy.

Despite the rapid advancement in EGFR targeted therapy, the mechanisms of resistance in CRC has yet to be uncovered. Clearly, *KRAS* codon 12 mutation is a leading cause of resistance to EGFR inhibitors. Nevertheless, even a group of *KRAS-WT* CRC patients do not respond to anti-EGFR treatment. And among the various KRAS mutations, that which encodes the G13D mutant protein ($KRAS^{G13D}$) behaves differently. Furthermore, for unknown reasons, $KRAS^{G13D}$ CRC patients benefit from the EGFR-blocking antibody cetuximab. Controversy surrounds this observation, because it contradicts the well-established mechanisms of EGFR signaling with regard to RAS mutations. Previous studies suggest that KRAS mutants, that strongly interacted with and

competitively inhibited NF1, drove WT RAS activation in an EGFR-independent manner, whereas $KRAS^{G13D}$ weakly interacted with and could not competitively inhibit NF1 and, thus, $KRAS^{G13D}$ cells remained dependent on EGFR for WT RAS activity (McFall, Diedrich et al. 2019).

Several contributing factors seem to influence this resistance, however none of them are conclusive. It has been established that increased Wnt signaling confers cetuximab resistance, by activating a double-negative feedback loop between MIR100HG and the transcription factor GATA6, however the mechanism of EGFR and Wnt signaling crosstalk is still uncertain (Lu, Zhao et al. 2017). Understanding the underlying biology of colon tumors is necessary to develop effective personalized therapies.

1.6. Conclusions and outlook

CRC is a frequently lethal disease with heterogenous outcomes and drug response. Molecular biological factors related to the primary site of colon tumor development and therapeutic effects of anti-EGFR antibodies have been characterized. However, it is thought that there are genetic and epigenetic factors associated with resistance to anti-EGFR treatment that have not yet been identified.

Given that there are molecular differences in the development and progression of colon tumors, a classification system for CRC was proposed in 2015 (Guinney, Dienstmann et al. 2015). The consensus molecular subtypes (CMS) classification is one of the most robust CRC classifications based on comprehensive gene expression profiles. However, biomarkers that can predict the therapeutic effect of anti-EGFR have not yet been established. It is expected that after verification via prospective or retrospective

analysis, this classification system could be used as a predictive biomarker. Furthermore, from a clinical perspective, it is still unknow whether combinations of these features are needed for accurate prediction of prognosis and drug response.

Additionally, the ERBB family of tyrosine receptors share a complex cross talk between all the members and other tyrosine kinase receptors, that have not been associated with the current CMS classification, and that can increase resistance to selective EGFR treatment. In this chapter, several mechanisms of resistance to anti-EGFR therapies and the critical roles of ERBB members in colon cancer progression were described (Table 1-2).

The importance of EGFR and ERBB2 in CRC development have been extensively examined. However, the importance of ERBB3 and ERBB4 in intestinal tumorigenesis is still unclear.

Table 1-2 Biomarkers of primary and acquired resistance to anti-EGFR mAbs in CRC patients

Gene/Pathway	Genetic evidence	Study
***Ras/Raf/MEK* pathway**		
KRAS and *NRAS*	Missense mutations	Preclinical and clinical
BRAF	Missense mutations	Clinical and meta-analysis
MEK1	Missense mutations	Preclinical and clinical
Receptors and ligands from EGFR family		
EGFR	Missense mutations	Preclinical and clinical
HER2	Amplification	Preclinical and clinical
HER3/4 ligand	Overexpression	Preclinical and clinical
Heregulin	Overexpression	Clinical
TGF-a	Overexpression	Preclinical
Other tyrosine kinase receptors		
MET	Amplification	Preclinical and clinical
IGF1R	Overexpression	Preclinical
VEGF/VEGFR	Overexpression	Preclinical
EGFR downstream signaling		
PI3K/Akt pathway	Hyperactivation	Preclinical and clinical
MEK/ERKs pathway	Hyperactivation	Preclinical and clinical
Foxo3	Upregulation	Preclinical and clinical

Aberrant activation of EGFR characterizes the transition from early adenoma to intermediate adenoma, in the classical adenoma-colorectal carcinoma sequence. However, the role of EGFR has not been fully explored and the lack of dependency of EGFR signaling in colon tumorigenesis could explain the low response to anti-EGFR treatment. Activation of HER2 pathway as a bypass signaling pathway has been identified as a mechanism of resistance for anti-EGFR in CRC treatment. Overexpression of ERBB3 often accompanies EGFR or ERBB2 overexpression and has been frequently detected in a variety of cancers, including CRC (Maurer, Friess et al. 1998, Jaiswal, Kljavin et al. 2013). Recently, it has been shown that HER4 is overexpressed in CRC, indicating an important role in promotion of colorectal carcinogenesis (Williams, Bernard et al. 2015).

A diverse set of second and third generation ERBB targeting drugs (TKIs and antibodies) has been developed in the last decade. These drugs counteract *de novo* or acquired resistance to anti-EGFR and will further prolong the overall survival time of cancer patients experiencing an advanced disease. Currently, the major challenge with current cancer treatment is determining predictive biomarkers to optimize patient selection. The value of clinically relevant mutations can be improved by evaluating circulating plasma DNA and non-tumor cell autonomous signaling, which might help to explain acquired resistance mechanisms. Available knowledge on the molecular and immunological landscape of colorectal cancer can help to improve the therapeutic management of patients with CRC.

2. ACTIVATION OF IL10 SIGNALING IN EGFR-INDEPENDENT COLORECTAL CANCER

2.1. Synopsis

Epidermal growth factor receptor (EGFR)-targeted therapies have been approved for colorectal cancer (CRC) treatment. However, previous studies observed that efficacy of anti-EGFR therapy in humans is influenced by the mutational context of the cancer. Mutations in kirsten rat sarcoma viral oncogene (*KRAS*) explain some non-responding CRCs, but even in cancers lacking *KRAS* mutations, little is known about which cancers are likely to respond to EGFR targeted treatment. In this study, we used a mouse model with a conditional *Egfr* allele, (*Egfr*^{tm1dwt} also called *Egfr*^{f}) to demonstrate that colorectal tumors can initiate through an EGFR-independent mechanism. EGFR-independent tumors also had an accelerated growth rate in two different models, *Apc*$^{Min/+}$ and in tumors focally induced by adenomatous polyposis coli (APC) deficiency and activated KRAS. Transcriptomic analysis revealed a set of diagnostic genes for EGFR-independent colon tumors in mice. In both models, one of the top upstream molecules associated with the differentially expressed genes in EGFR-independent colon tumors was interleukin 10 receptor alpha (IL10RA). Quantitative gene expression analysis for several genes involved in the IL10RA pathway, including *Sult1a1*, *Il10, Il10ra, Maob, Aadac,* and *Socs3,* confirmed differential pathway activation. In addition, we found anergy-inducing genes to be overexpressed in EGFR-independent colon tumors, suggesting that tumor cells without EGFR might escape cell-mediated immune defense by increasing production of

IL10, which was detectable in serum from animals with EGFR-independent colon tumors. These findings demonstrate the existence of an EGFR-independent mechanism by which CRC can progress, and that EGFR-independent tumor progression is supported by IL10RA mediated anergy.

2.2. Introduction

Colorectal cancer (CRC) is the third most common, cancer-related cause of death in the United States and is responsible for nearly 50000 deaths each year (Siegel, Miller et al. 2019). Preventative screening and more effective therapies have contributed to a moderate decline in CRC incidence. Although promising new molecular targeted therapies have been approved, or are in clinical trials, primary or acquired resistance to therapy remains a major limitation at the patient level indicating a need for additional and novel therapies that have greater efficacy against defined subclasses of CRC.

CRC is primarily caused by alterations in wingless-type MMTV integration site family (WNT) signaling, with a substantial number of cases also having activated kirsten rat sarcoma viral oncogene (KRAS) (Van Emburgh, Arena et al. 2016). Consistent with a role for epidermal growth factor receptor (EGFR) in CRC development, genetic or pharmacologic inhibition of EGFR results in a significant decrease in tumor incidence in the adenomatous polyposis coli (*APC*) model for multiple intestinal neoplasia (Min) ($Apc^{Min/+}$) mouse model of CRC (Torrance, Jackson et al. 2000, Roberts, Min et al. 2002). Numerous agents targeting EGFR, including small molecule kinase inhibitors (Martins, Mansinho et al. 2018, Rachiglio, Fenizia et al. 2019) and ligand-blocking antibodies (Mauri, Pizzutilo et al. 2019), are currently being used in the clinic for the treatment of

CRC. However, increasing evidence suggests that only a small percentage of CRC patients respond to these therapies, and those who show initial response eventually become refractory to treatment. Mutations in *KRAS* explain some resistant CRCs (Uhlyarik, Piurko et al. 2019), but even in cancers lacking *KRAS* mutations, little is known about which patients are likely to respond to EGFR targeted treatment.

Although many factors contribute to high levels of resistance to EGFR inhibitors, the lack of dependency on the EGFR signaling pathway in a subset of CRCs could explain some of the lack of clinical response. In support of this hypothesis, previous studies using the $Apc^{Min/+}$ mouse model of CRC and the hypomorphic $Egfr^{wa2}$ allele showed that although polyp development is greatly reduced with decreased EGFR activity, a subset of intestinal polyps still arises on a background with lower EGFR activity, and these polyps are larger than those in $Apc^{Min/+}$ mice with normal EGFR levels (Torrance, Jackson et al. 2000, Roberts, Min et al. 2002). These results suggested that a subset of CRCs may arise independently of EGFR activity. However, these results were inconclusive as to whether colon tumors can arise and grow independently of EGFR activity. Limitations in the experimental design of previous studies could not distinguish EGFR independence from variable residual EGFR activity. In this study we used a conditional knockout allele of *Egfr* ($Egfr^{tm1dwt}$ also called $Egfr^{f}$) to conclusively demonstrate that colorectal tumors can initiate through an EGFR-independent mechanism in two different mouse models.

Accumulating evidence also suggests that the formation of a microenvironment enriched in cancer-associated fibroblasts, macrophages, and regulatory T (Treg) cells is a key step in tumor development mediated by secretions of chemokines and cytokines

(Parseghian, Napolitano et al. 2019). Several studies utilizing mouse models support the requirement of epithelium-derived cytokines, like Interleukin 11 (IL11), for the development of CRC (Putoczki, Thiem et al. 2013). In this study, we definitively proved the existence of an EGFR independent route of CRC development and provided evidence that not only IL11 but Interleukin 10 (IL10), a TH2 cytokine secreted by almost all leukocytes and intestinal epithelium cells (Fiorentino, Bond et al. 1989, Moore, de Waal Malefyt et al. 2001, Wolk, Kunz et al. 2002), regulates immune activity by suppressing inflammatory responses in EGFR independent tumors. Moreover, in this study we showed that IL10 favored the differentiation of monocytes toward M2- like macrophages in colon tumors (Jackute, Zemaitis et al. 2018), which accumulate in the tumor microenvironment (Solinas, Germano et al. 2009). Furthermore, high levels of IL10 correlated with high levels of ERBB4 in EGFR-independent colon tumors suggesting the existence of a compensatory mechanism activated by the absence of EGFR. However, the activating mechanisms of IL10 signaling are not well understood, and it remains unclear whether cancer cells alone or in combination with other immune cells secrete IL10 and whether IL10 has an impact on the aggressiveness and malignancy of cancer cells.

2.3. Materials and methods

Animal experiments

All animal studies were maintained and protocols followed in accordance with Texas A&M University Institution Animal Care and Use Committee guidelines. 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. *Apc*^{tm1Tno}, *Kras*^{tm4Tyj}, *Egfr*^{tm1dwt}, and *Erbb4*^{tm1Fej} mice were maintained on C57BL/6J background. Mice were housed five per cage, fed Purina Mills Lab Diet 2919 and maintained at 22° under a 12-hr light cycle. Mice were euthanized by CO2 asphyxiation for tissue collection.

Genotyping

Mice were genotyped for the *Apc*^{Min} allele as previously described (Roberts, Min et al. 2002). Mice were genotyped for the *Egfr*^{tm1Dmt} allele as previously described (Lee and Threadgill 2009). Cre transgenic mice were determined using PCR with Cre-S1, 5'-GTGATGAGGTTCGCAAGAAC; and Cre-AS1, 5'-AGCATTGCTGTCACTTGGTC primers. Mice were genotyped for the *Erbb4*^{tm1Fej} allele using B4-1, 5'-TATTGTGTTCATCTATCATTGCAACCCAG; B4-2, 5'-CAAATGCTCTCTCTGTTCTTTGTGTCTG; and B4-3, 5'-TTTTGCCAAGTTCTAATTCCATCAGAAGC. *Kras*^{tm4Tyj} allele was genotyped using K004, 5'-GTCGACAAGCTCATGCGGGTG; K005, 5'-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA; K006, 5'-CCTTTACAAGCGCACGCAGACTGTAGA. *Apc*^{tm1Tno} was genotyped using APC-Int12-F2, 5'- GAGAAACCCTGTCTCGAAAAAA; and APC-Int12-R2, 5'-AGTGCTGTTTCTATGAGTCAAC.

Nonsurgical exposure to Adeno Cre virus

Polyethylene tubing (I.D. 1.4 mm, O.D. 1.90 mm; Becton Dickinson, Sparks, MD) was cut to sizes appropriate for mice (10 cm). A 1 cm window was notched into the tubing and the end of the tubing was closed with edges being rounded to avoid perforation of the

bowel. Marks corresponding to 1 cm intervals were made on the tubing. A longitudinal stripe was also applied corresponding to the orientation of the window. Mice were anesthetized using 2% isoflurane. The colon was irrigated with phosphate buffered saline (PBS). A narrow ribbon of GelFoam (Pharmacia and Upjohn, NY, NY) was inserted into the window cut into the polyethylene tube. 200 μL of 0.05% trypsin (Hyclone, Logan, VT) was injected into the tubing and inserted into the mouse colon at the desired depth and radial orientation. After 10 minutes, the slotted tube was removed and the soft brush was introduced at the same intraluminal location. The brush was then used to abrade the epithelium for up to 3 minutes. After PBS irrigation, a slotted tubing containing GelFoam was then filled with 200μL PBS containing 109 PFU of Cre recombinase-expressing adenovirus (AdCre) (Ad5CMVCre and Ad5CMVEmpty, University of Iowa Gene Transfer Vector Core, IA). After 30 minutes of incubation, the tubing was removed. Owing to the anatomical limitations of the mouse, only the most distal half of the colon (~4 cm) could be inoculated in this way. Mice recovered quickly after the procedure and did not exhibit overt signs of pain or distress following the procedure, as they quickly became active. Note that nonsteroidal anti-inflammatory drugs were not used for post-procedure analgesia as these agents have been shown to suppress intestinal tumorigenesis in both humans and laboratory mice.

Murine colonoscopy

Mice were anesthetized using 2% isoflurane and the colons were flushed with PBS. The Coloview System was used to monitor tumor formation and growth in the distal half of the colon every two weeks as previously described (Karl Storz, Goleta, CA) (Becker,

Fantini et al. 2005). ImageJ analysis was utilized to measure the percent lumen occlusion as previously described (Hung, Maricevich et al. 2010).

Mouse IL10 neutralizing antibody treatment

For inhibiting IL10 *in vivo*, mice with AdCre induced colonic tumors, were intraperitoneally (IP) injected with the anti-IL10 (200μg/mouse was injected at the beginning of the treatment; 100μg/mouse and 20μg/mouse injections were IP administrated once/week with two days between injections for 4 weeks to neutralize IL10.

Macroadenoma count

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 hematoxylin and eosin staining

Intestinal tissues or colon samples were collected and fixed 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.

Colon tumors were deparaffinized in xylene followed by rehydration in 100%, 90%, and 70% ethanol and distilled water. The slides were then incubated in fresh hematoxylin (Merck, Darmstadt, Germany) for 5 min and washed in distilled water,

followed by incubation in acidified eosin solution (Sigma, Deisenhofen, Germany) for 1 min and washing. Finally, the slides were dehydrated in 90% and 100% ethanol, air dried, and mounted. The H&E stained colon sections were assessed by pathologist for differentiating the tumor part from the non-tumor part.

Immunohistochemistry

Immunohistochemical procedures were performed as described (Paul Olson, Hadac et al. 2014). 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% specific serum and 0.1% Triton X- 100, and then incubated with primary antibodies and HRP-conjugated specific anti-rabbit secondary antibody (Vector Laboratories, Inc). Antigen-antibody complexes were detected with DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Suppliers for primary antibodies were Abcam, (CD163-ab182422, iNOS-ab15323, Ki67-ab15580, Tunel Assay Kit-ab206386); Santa Cruz Biotechnology, (CD3-sc-1127); Biolegend, Inc (F4/80-BM8), eBioscience, (CD45-14-0451). The total number of cells and all the positively stained cells in tumor area of the core were counted using Fiji and expressed as a percentage of the total number of cells in the tumor core.

Intestinal organoids

Epithelial-only intestinal minigut cultures from freshly isolated *Egfr*WT and *Egfr*CKO mouse colonic tumors were established essentially as described previously (Sato, Stange et al. 2011), from $Egfr^{+/+}$, $Apc^{f/f}$, $Kras^{LSL/+}$ and $Egfr^{f/f}$, $Apc^{f/f}$, $Kras^{LSL/+}$ mice

on a C57Bl/6 background. Crypts were isolated by calcium chelation and mechanical agitation, then embedded in Matrigel (Corning™ Matrigel™ GFR Membrane Matrix, ThermoFisher – CB40230C) droplets with N2 and B27 supplements (ThermoFisher – 17502001 and 17504001). Once established, cultures were broken up by adding 0.25% of trypsin and incubating for 10 minutes. Single cell suspensions were allowed to proliferate for 24 hours before adding respective treatment. Treatment with mouse IL10 recombinant protein, eBioscience™ (ThermoFisher – 14-8101-62), IL10 neutralizing antibody (Clone JES052A5 from R&D – MAB417), or EGFR inhibitor AG1478 was left for 72 hours and cell proliferation was measured using CyQUANT™ Cell Proliferation Assay Kit, for cells in culture, (ThermoFisher – C7026). RNA from tumor organoids was isolated using PicoPure™ RNA Isolation Kit (ThermoFisher – KIT0214).

Enzyme-linked immunosorbent assay (ELISA)

IL10 concentration in mouse serum was measured using IL10 Mouse Instant ELISA™, (ThermoFisher – BMS614INDT). The measurement was performed according to the manufacturer's instructions.

Transcriptomic analysis

A total of 3 sequencing runs were performed to sequence 56 samples on NextSeq 500 sequencing instrument at Texas A&M Institute for Genome Sciences and Society using high output kit v2. A total of 1.5 billion 75 bp single-end reads were checked for adapter sequences and low-quality bases using Trimmomatic (Bolger, Lohse et al. 2014), resulting in approximately 1.4 billion filtered reads (96%). RNA-Seq reads were aligned to mouse assembly mm10 using HISAT2 version 2.0.5 (Kim, Landmead et al. 2015) with

an overall mapping rate of approximately 97%. Raw gene counts were generated with feature Counts package (Liao, Smyth et al. 2014) while discarding ambiguous read mappings. Normalized read counts and gene expression tests were performed using DESeq2 (Love, Huber et al. 2014) following recommended guidelines by the authors. Ingenuity Pathway Analysis (IPA) was used to analyzed differentially expressed genes between the different groups.

Quantitative real time PCR

Genes with significant changes in expression between *Egfr*WT tumors and *Egfr*CKO tumors, based on ANOVA analysis, were confirmed by qRT-PCR. cDNA was synthesized from total RNA from each tumor using the QuantiTect Reverse Transcription Kit (Qiagen 205314). PCR reactions were set up in 96-well plates, all samples were run in triplicate. Analysis was performed on a LightCycler 96 Thermocycler (Roche) using LightCycler 480 Sybr Green I Master reaction mix. Specific primers were designed to amplify a fragment from the genes in Supplementary Table 2-1.

Statistics

The nonparametric Mann–Whitney U test was used to analyze tumor data. To compare the statistical difference between 2 groups, student's *t* test was used. The *p-value* less than 0.05 was considered as the significant difference.

2.4. Results

2.4.1. Intestinal polyps in the *Apc*$^{Min/+}$ mouse model can arise independently of EGFR activity

Using the *Apc*$^{Min/+}$ mouse model of human CRC, it was previously shown that a subset of intestinal polyps arises in the context of reduced or pharmacologically inhibited EGFR activity (Torrance, Jackson et al. 2000, Roberts, Min et al. 2002) suggesting the existence of an EGFR-independent subset of CRC. A conditional knockout allele of *Egfr* (*Egfr*^{tm1Dwt} also known and *Egfr*^{f}) was used to definitely demonstrate the existence of an EGFR-independent intestinal tumors in mice. At 100 days of age, tissue-specific deletion of *Egfr* (referred as *Egfr*CKO - *Apc*$^{Min/+}$, *Egfr*$^{f/f}$, *Tg(Vil1-Cre)*) displayed a 80% reduction in total polyp number compared to *Egfr* wild-type (referred as *Egfr*WT - *Apc*$^{Min/+}$, *Egfr*$^{f/f}$) littermates (*p-value<0.0001*) (Figure 2-1A). There was no significant difference in the number of colon polyps that arise under knockout levels of *Egfr* (Figure 2-1B). Similar to the original study using a hypomorphic allele of *Egfr* (*Egfr*^{wa2}) (Roberts, Min et al. 2002), the polyps that developed in the absence of *Egfr* were significantly larger (*p-value<0.05*) than those developing under normal levels of *Egfr* (Figure 2-1C).

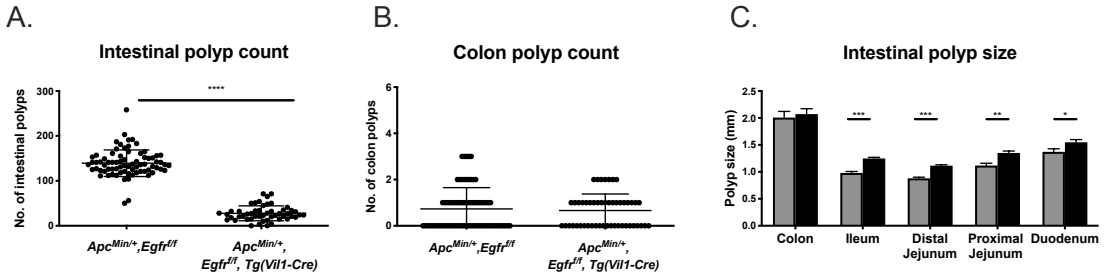


Figure 2-1 EGFR-independent intestinal adenoma growth in *Apc$^{Min/+}$* mice
(A). Intestinal polyps' multiplicity in *Apc$^{Min/+}$* mice of different *Egfr* genotype. (B). Colon polyps' multiplicity in *Apc$^{Min/+}$* mice of different *Egfr* genotype. Each dot represents the polyp number in each 100-day-old mice. (C). Macroadenoma size in *Apc$^{Min/+}$* mice of different *Egfr* genotype. Grey bars represent the size of *Egfr*WT (*Apc$^{Min/+}$, Egfr$^{f/f}$*) and black bars represent *Egfr*CKO (*Apc$^{Min/+}$, Egfr$^{f/f}$, Tg(Vil1-Cre*) intestinal tumors. * *p-value<0.05*, ** *p-value<0.01*, *** *p-value<0.001*, **** *p-value*<0.0001

2.4.2. EGFR-independent colon tumors have accelerated growth

Considering that EGFR-independent intestinal polyps are larger than those arising with normal EGFR levels, we tested whether colon tumors could arise independently of EGFR. We used a previously described mouse model of sporadic CRC (Hung, Maricevich et al. 2010) that contains conditionally inactivated *Apc* alleles (*Apc^{tm1Tno}* or *Apc$^{f/f}$)* in combination with a conditionally activatable allele of the oncogenic *Kras* (*Kras^{tm4Tyj} or Kras$^{LSL-G12D}$*). Colon tumors developing under normal levels of EGFR (*Egfr$^{+/+}$*, *Apc$^{f/f}$, Kras $^{LSL-G12D/+}$- Egfr*WT+*Apc*CKO+*Kras*OE) were considered EGFR-dependent tumors, while tumors arising in the absence of EGFR were considered EGFR-independent tumors (*Egfr$^{f/f}$*, *Apc$^{f/f}$, Kras$^{LSL-G12D/+}$ - Egfr*CKO+*Apc*CKO+*Kras*OE)*. We also included mice containing just the *Apc$^{f/f}$* allele (*Egfr$^{+/+}$*, *Apc$^{f/f}$, Kras$^{+/+}$ - Egfr*WT+*Apc*CKO+*Kras*WT) and animals with the *Apc$^{f/f}$* allele in combination with *Egfr$^{f/f}$* allele (*Egfr$^{f/f}$, Apc$^{f/f}$, Kras $^{+/+}$- Egfr*CKO+*Apc*CKO+*Kras*WT).

We demonstrated that following delivery of Cre recombinase-expressing adenovirus (AdCre) to the distal colon there is a set of colon tumors that grow independent of EGFR (*Egfr$^{f/f}$, Apc$^{f/f}$, Kras$^{+/+}$ and Egfr$^{f/f}$, Apc$^{f/f}$, Kras $^{LSL-G12D/+}$*). No tumors developed by inactivating *Apc* alone. Interestingly, mice with *Egfr$^{f/f}$* allele showed increased tumor penetrance compared to mice with wild-type levels of EGFR (*Egfr$^{+/+}$*) (Figure 2-2A). Additionally, we compared the tumor multiplicity of EGFR-dependent versus EGFR-independent tumors. When the *Egfr$^{f/f}$* allele was present the overall tumor multiplicity increased in a statistically significant manner (*p-value<0.001*) (Figure 2-2B).

We next assessed the rate of tumor growth by colonoscopy (Figure 2-2C). Endoscopic images were obtained every two weeks and analyzed using ImageJ software as previously described by Hung *et al*., (Hung, Maricevich et al. 2010). Briefly, we determined the ratio of the tumor and lumen cross-sectional areas. We generated growth curves for the three different groups of colon tumors at each endoscopic time of assessment. The percentage of lumen occlusion showed a significant increase until week 15 (*p-value<0.05*) in the EGFR-independent group compared to EGFR-dependent tumors (Figure 2-2D). Taken together, these results suggest that there is a group of colon tumors that arise independent of EGFR and that in absence of EGFR, tumors are more likely to arise and they showed an accelerated growth rate.

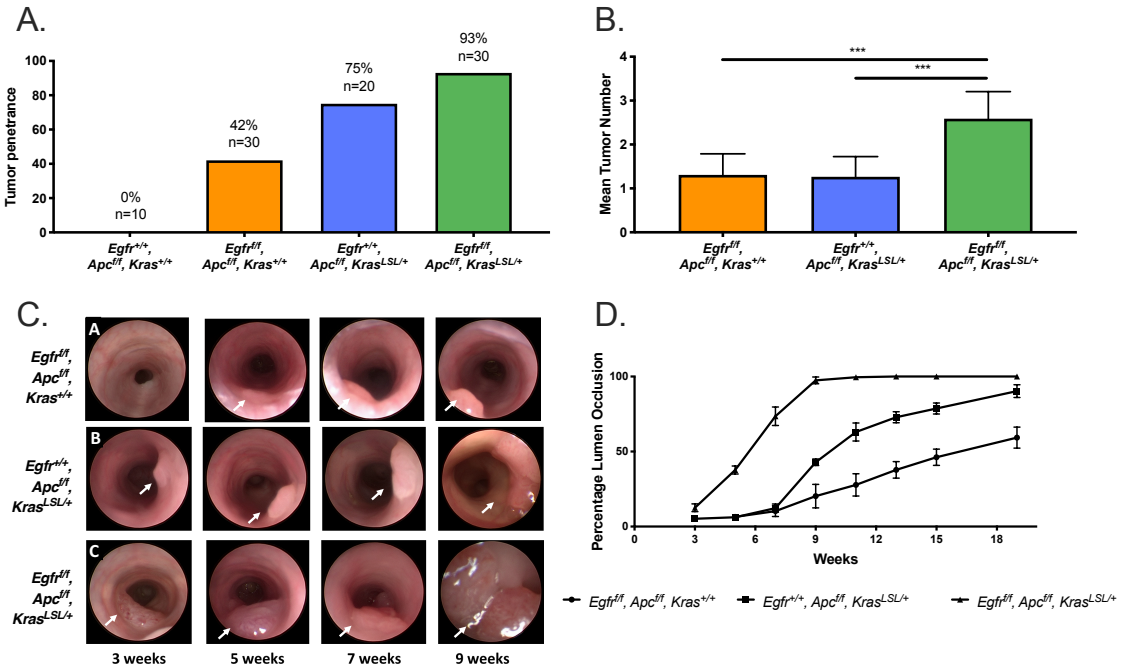


Figure 2-2 Genetic ablation of EGFR resulted in an increase of tumor penetrance. (A). Lack of EGFR increase tumor penetrance in mice. (B). Early endoscopic determination in mutant animals after AdCre induction showed an increase in tumor multiplicity in *Egfr*$^{f/f}$, *Apc*$^{f/f}$, *Kras* $^{LSL\text{-}G12D/+}$ mice (*p-value<0.001*). (C). Representative serial endoscopic images after AdCre administration to the distal colon of mice. (D). Growth curves of *Egfr*CKO AdCre-induced tumors show a significant increase (*p-value<0.05*) until 15 weeks after induction compared to *Egfr*WT AdCre-induced tumors.

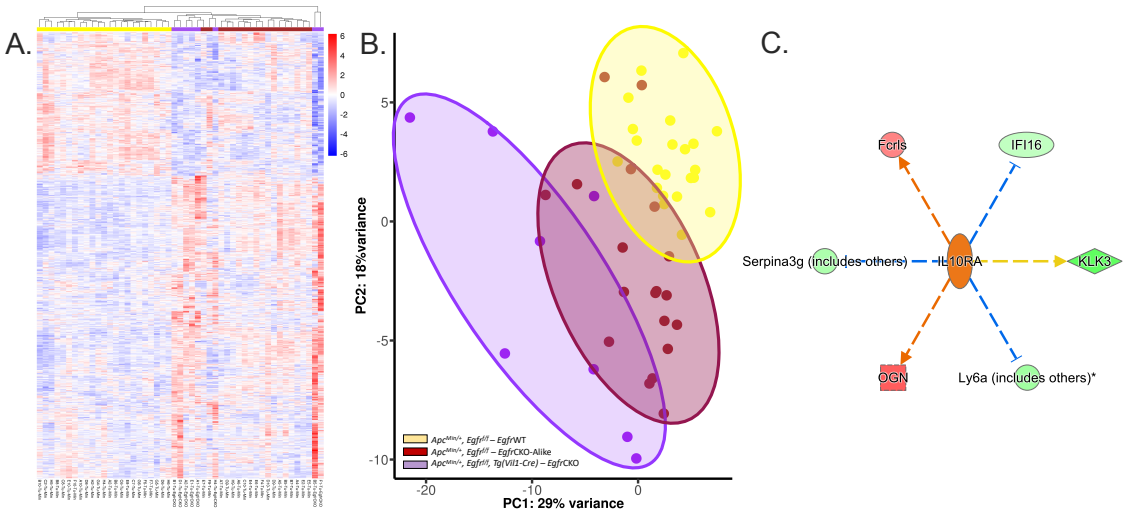
2.4.3. EGFR-independent intestinal polyps are not dependent upon absence of EGFR

To determine whether EGFR-independent tumors have a distinct gene expression profile, we analyzed RNA from intestinal polyps with *Egfr*WT versus *Egfr*CKO. Hierarchical clustering (Figure 2-3A) and PCA plot (Figure 2-3B) from the transcriptomic analysis demonstrated a clear distinction between *Egfr*WT and *Egfr*CKO intestinal tumors. We were also able to demonstrate that a subset of intestinal tumors that developed under *Egfr*WT conditions had expression patterns more similar to *Egfr*CKO tumors and

they clustered together forming a subgroup that we have called *Egfr*CKO-Alike (*Egfr*CKO-A), confirming the existence of an EGFR-independent mechanism of CRC progression in mice even when EGFR is present. These data also showed that there is a group of upstream regulators, including IL10RA (Figure 2-3C), that characterize the observed gene expression changes in *Egfr*CKO when compared to *Egfr*WT intestinal tumors (Figure 2-3D). Together, these results suggest that the EGFR-independent pathway is not dependent upon loss of EGFR, but occurs in a subset of tumors even in the presence of EGFR.

2.4.4. Differentially expressed genes derived from EGFR-independent colon tumors

Comparative transcriptomics also revealed differential gene expression related to the *Egfr* and *Kras* status in colon tumors (Figure 2-4A). When compared to their respective normal colon tissue the transcriptomic analysis demonstrated that there is a group a genes that significant characterize the three groups of tumors as shown in the venn diagram (Figure 2-4B). Ingenuity pathway analyis (IPA) showed that there are several upstream regulators that characterize the dependency of colon tumors to EGFR (Figure 2-4C).



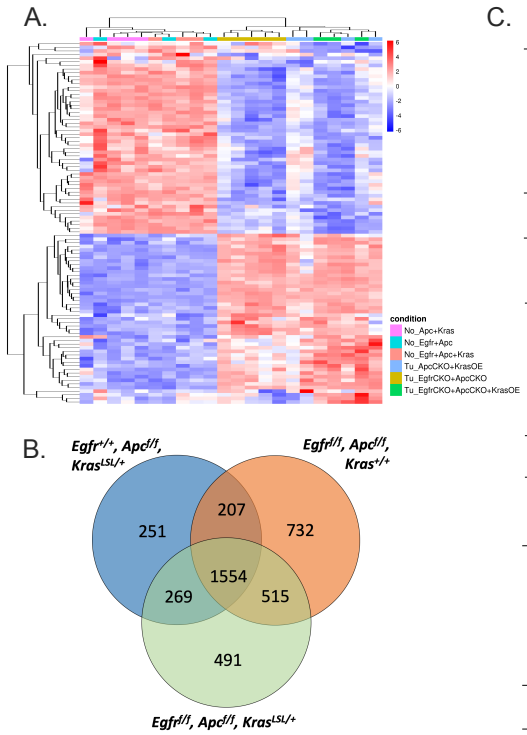
D.

Upstream regulator	Molecule type	Predicted activation state	Activation z-score	p-value of overlap
TRIM24	Transcription regulator	Activated	2.000	$2.40x10^{-04}$
Epigallocatechin-gallate	Chemical drug	Activated	1.987	$5.06x10^{-03}$
SOCS1	Other	Activated	1.982	$7.16x10^{-04}$
IL10RA	Transmembrane receptor	Activated	1.664	$3.73x10^{-04}$
IL10	Cytokine	-	1.412	$2.50x10^{-02}$
SIM1	Transcription regulator	Inhibited	-2.433	$4.63x10^{-05}$
ARNT2	Transcription regulator	Inhibited	-2.433	$4.05x10^{-05}$
MYC	Transcription regulator	Inhibited	-2.393	$5.08x10^{-02}$
TP53	Transcription regulator	Inhibited	-2.049	$1.11x10^{-01}$
Beta-estradiol	Chemical	Inhibited	-2.017	$1.30x10^{-01}$

TRIM24, Tripartite Motif Containing 24; SOCS1, Suppressor of cytokine signaling 1; IL10RA, Interleukin 10 Receptor Subunit Alpha; IL10, Interleukin 10; SIM1, Single Minded homolog 1; ARNT2, Aryl Hydrocarbon Receptor Nuclear Translocator 2; MYC, Myelocytoma Proto-Oncogene; TP53, Tumor Protein p53.

Figure 2-3 Transcriptomic analysis from *Egfr*CKO intestinal polyps in the *Apc*$^{Min/+}$ mouse model.

(A). Gene expression patterns analyzed by clustering differentially expressed genes between *Egfr*WT (yellow) and *Egfr*CKO (purple) intestinal polyps. (B). Principal Component Analysis plot classified intestinal polyps in three different subgroups *Egfr*WT (yellow), *Egfr*CKO (purple) and *Egfr*CKO-A (maroon). (C-D). Prediction of upstream regulators involved in progression of *Egfr*CKO intestinal polyps.



C. ***Tumor Egfr$^{f/f}$, Apc$^{f/f}$, Kras$^{+/+}$***

Upstream regulator	Molecule type	Predicted activation state	Activation z-score	p-value of overlap
IL10RA	Transmembrane receptor	Activated	6.466	$5.53x10^{-35}$
Dexamethasone	Chemical drug	Activated	6.568	$1.78x10^{-43}$
Alpha catenin	Group	Activated	4.579	$1.42x10^{-12}$
DKK1	Growth factor	Activated	4.579	$5.02x10^{-10}$
PPARGC1A	Transcription regulator	Activated	4.385	$1.46x10^{-13}$
TNF	Cytokine	Inhibited	-6.440	$3.01x10^{-56}$
ERK	Group	Inhibited	-5.980	$1.47x10^{-12}$
PDGF BB	Complex	Inhibited	-5.724	$6.59x10^{-21}$
EGF	Growth factor	Inhibited	-5.697	$1.41x10^{-22}$
MYD88	Other	Inhibited	-5.641	$1.34x10^{-14}$

IL10RA, Interleukin 10 Receptor Subunit Alpha; DKK1, Dickkopf WNT Signaling Pathway Inhibitor 1; PPARGC1A, Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha; TNF, Tumor Necrosis Factor; ERK, Extracellular-signal-regulated Kinase; PDGF BB, Platelet-derived growth factor BB; EGF, Epidermal Growth Factor; MYD88, Myeloid differentiation primary response 88.

Tumor Egfr$^{+/+}$, Apc$^{f/f}$, Kras$^{LSL/+}$

Upstream regulator	Molecule type	Predicted activation state	Activation z-score	p-value of overlap
Alpha catenin	Group	Activated	6.293	$1.87x10^{-25}$
DUSP1	Phosphatase	Activated	4.277	$2.27x10^{-09}$
DKK1	Growth factor	Activated	4.256	$1.46x10^{-09}$
NR3C1	Ligand-dependent nuclear receptor	Activated	4.029	$1.81x10^{-09}$
SFTPA1	Transporter	Activated	3.928	$2.75x10^{-08}$
TNF	Cytokine	Inhibited	-7.804	$3.24x10^{-40}$
Ige	Complex	Inhibited	-6.882	$9.11x10^{-14}$
PDGF BB	Complex	Inhibited	-6.508	$9.71x10^{-15}$
MYD88	Other	Inhibited	-6.410	$1.41x10^{-11}$
IL1B	Cytokine	Inhibited	-6.320	$3.75x10^{-27}$

DUSP1, Dual Specificity Phosphatase 1; DKK1, Dickkopf WNT Signaling Pathway Inhibitor 1; NR3C1, Nuclear Receptor Subfamily 3 Group C Member 1; SFTPA, Surfactant Protein A; TNF, Tumor Necrosis Factor; Ige, Immunoglobulin E; PDGF BB, Platelet-derived growth factor BB; MYD88, Myeloid differentiation primary response 88; IL1B, Interleukin 1B.

Tumor Egfr$^{f/f}$, Apc$^{f/f}$, Kras$^{LSL/+}$

Upstream regulator	Molecule type	Predicted activation state	Activation z-score	p-value of overlap
Alpha catenin	Group	Activated	6.763	$5.58x10^{-34}$
IL10RA	Transmembrane receptor	Activated	6.568	$1.78x10^{-43}$
SFTPA1	Transporter	Activated	4.579	$1.42x10^{-12}$
DKK1	Growth factor	Activated	4.579	$5.02x10^{-10}$
PPARGC1A	Transcription regulator	Activated	4.385	$1.46x10^{-13}$
TNF	Cytokine	Inhibited	-6.440	$3.01x10^{-56}$
ERK	Group	Inhibited	-5.980	$1.47x10^{-12}$
PDGF BB	Complex	Inhibited	-5.724	$6.59x10^{-21}$
EGF	Growth factor	Inhibited	-5.697	$1.41x10^{-22}$
MYD88	Other	Inhibited	-5.641	$1.34x10^{-14}$

IL10RA, Interleukin 10 Receptor Subunit Alpha; SFTPA, Surfactant Protein A; DKK1, Dickkopf WNT Signaling Pathway Inhibitor 1; PPARGC1A, Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha; TNF, Tumor Necrosis Factor; ERK, Extracellular-signal-regulated Kinase; PDGF BB, Platelet-derived growth factor BB; EGF, Epidermal Growth Factor; MYD88, Myeloid differentiation primary response 88.

Figure 2-4 Transcriptomic analysis from induced colon tumors with differential EGFR and KRAS levels

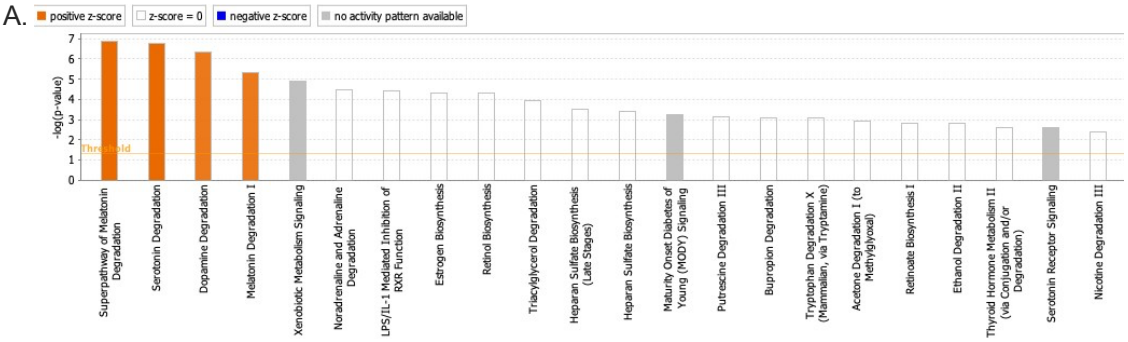
(A-B). Gene expression patterns analyzed by clustering differentially expressed genes between induced colon tumors with differential *Egfr* levels (*Egfr*CKO+*Apc*CKO+*Kras*WT (orange), *Egfr*WT+*Apc*CKO+*Kras*OE (blue) and *Egfr*CKO+*Apc*CKO+*Kras*OE (green)). (C). Tables describing the prediction of top upstream regulators involved in development of induced colon tumors with differential *Egfr and Kras* levels.

Forty four genes were found to be differentially expressed between EGFR-dependent (*Apc$^{f/f}$, Kras $^{LSL-G12D/+}$*) and EGFR-independent (*Egfr$^{f/f}$, Apc$^{f/f}$, Kras $^{LSL-G12D/+}$*) colonic tumors (Table 2-1). Upregulated genes include *Aadac, Sult1a1, Itpka Sult1c2, Bmp3 and Aldh1a*. Downregulated genes include *Ngf, Rnaset2b, Col5a3, and Erdr1*. IPA indicated that these genes are enriched in canonical pathways involving superpathway of melatonin degradation, serotonin degradation, dopamine degradation and melatonin degradation I (Figure 2-5A). We assessed upstream regulators using IPA (Figure 2-5B). The top upstream molecules associated with the differentially expressed genes in our data were IL10RA (z-score = 3.148, *p-value* = 1.10×10^{-10}), HNF1A (z-score = 2.392, *p-value* = 1.09×10^{-5}), glucocorticoid (z-score = 1.982, p-value = 1.19×10^{-3}), and beta-estradiol (z-score = 1.367, p-value = 1.69×10^{-2}) (Figure 2-5C). IPA also showed that the top three enriched signaling networks were dermatological diseases and conditions, organ morphology, small molecule biochemistry (Network 1) (Fifure 2-5D-left); lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism (Network 2) (Figure 2-5D-middle); cell to cell signaling interaction, drug metabolism, post-translational modification (Network 3) (Figure 2-5D-right). The core nodes in the signaling network 1 include ERK1/2, MAPK and PI3K complex. For network 2 and network 3, the core nodes include IL1B and beta-estradiol, respectively.

Table 2-1 Differentially expressed genes between EGFR-dependent and EGFR-independent induced colon tumors.

Gene Name	Gene Symbol	Expr Fold Change	Expr p-value	Location	Type(s)
Sucrase-isomaltase	Sis	22.642	1.07E-14	Cytoplasm	Enzyme
Mucosal pentraxin 1	Mptx1	7.296	4.76E-08	Other	Other
Transcription factor AP-2 gamma	Tfap2c	7.117	2.35E-05	Nucleus	Transcription regulator
Carboxypeptidase N subunit 2	Cpn2	6.738	1.07E-05	Extracellular Space	Peptidase
Carbonic anhydrase 4	Car4	6.032	1.03E-07	Plasma Membrane	Enzyme
Cytochrome P450 family 2 subfamily C member 18	Cyp2c55	5.902	1.91E-05	Cytoplasm	Enzyme
Prolactin receptor	Prlr	5.447	0.000151	Plasma Membrane	Transmembrane receptor
Solute carrier family 26 member 3	Slc26a3	5.433	9.05E-05	Plasma Membrane	Transporter
RIKEN cDNA 2010106E10 gene	2010106E10Rik	5.412	2.13E-05	Other	Other
Cytochrome P450 family 2 subfamily F member 1	Cyp2f2	5.05	0.000103	Cytoplasm	Enzyme
Aquaporin 8	Aqp8	5.015	0.000152	Plasma Membrane	Transporter
Calcium voltage-gated channel subunit alpha1 E	Cacna1e	4.884	4.04E-05	Plasma Membrane	Ion channel
Syncollin	Sycn	4.87	1.13E-05	Extracellular Space	Other
Carboxylesterase 1E	Ces1e	3.932	6.73E-07	Cytoplasm	Enzyme
Regulated endocrine specific protein 18	Resp18	3.916	4.47E-06	Extracellular Space	Other
Arylacetamide deacetylase	Aadac	3.865	3.62E-05	Cytoplasm	Enzyme
Carboxylesterase 1	Ces1d	3.8	9.69E-05	Cytoplasm	Enzyme
Sulfotransferase family 1A, phenol-preferring, member 1	Sult1a1	3.693	3.7E-08	Cytoplasm	Enzyme
Solute carrier family 6 member 4	Slc6a4	3.645	1.97E-05	Plasma Membrane	Transporter
Fatty acid binding protein 2	Fabp2	3.574	0.000108	Cytoplasm	Transporter
Inositol-trisphosphate 3-kinase A	Itpka	3.413	1.02E-05	Cytoplasm	Kinase
RIKEN cDNA 1810063I02 gene	1810063I02Rik	3.41	0.000145	Other	Other
Hydroxysteroid 17-beta dehydrogenase 13	Hsd17b13	3.374	2.27E-05	Extracellular Space	Enzyme
Interleukin 11	Il11	3.373	3.49E-05	Extracellular Space	Cytokine
Estrogen related receptor gamma	Esrrg	3.336	0.000136	Nucleus	Ligand-dependent nuclear receptor
Glutathione S-transferase alpha 5	Gsta1	3.315	7.16E-05	Cytoplasm	Enzyme
RIKEN cDNA 0610005C13 gene	0610005C13Rik	3.212	5.22E-06	Other	Other
Angiotensin I converting enzyme 2	Ace2	3.1	0.000141	Plasma Membrane	Peptidase
Monoamine oxidase B	Maob	3.056	2.26E-07	Cytoplasm	Enzyme
Selenium binding protein 1	Selenbp1	2.989	2.5E-08	Cytoplasm	Other
Caspase 14	Casp14	2.969	6.18E-06	Cytoplasm	Peptidase
Sulfotransferase family 1C member 2	Sult1c2	2.906	9.97E-05	Cytoplasm	Enzyme
Alcohol dehydrogenase 1C (class I), gamma polypeptide	Adh1	2.853	3.73E-05	Cytoplasm	Enzyme
Bone morphogenetic protein 3	Bmp3	2.817	6.46E-07	Extracellular Space	Growth factor
Predicted gene 5485	Gm5485	2.709	7.16E-05	Other	Other
Alkaline ceramidase 1	Acer1	2.65	8.92E-06	Cytoplasm	Enzyme
Aldehyde dehydrogenase 1 family member A1	Aldh1a1	2.244	1.05E-06	Cytoplasm	Enzyme
Aldehyde dehydrogenase family 1, subfamily A7	Aldh1a7	2.212	1.34E-05	Cytoplasm	Enzyme
Guanylate cyclase activator 2A	Guca2a	2.156	2.92E-05	Extracellular Space	Other
Nerve growth factor	Ngf	-2.089	1.18E-06	Extracellular Space	Growth factor
Ribonuclease T2	Rnaset2b	-2.277	2.19E-06	Cytoplasm	Enzyme
Collagen type V alpha 3 chain	Col5a3	-3.155	0.000041	Extracellular Space	Other
Erythroid differentiation regulator 1	Erdr1	-4.192	0.000037	Other	Other

A.

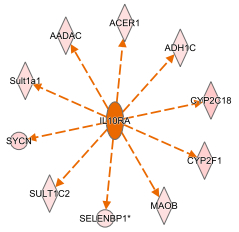


B.

Upstream regulator	Molecule type	Predicted activation state	Activation z-score	p-value of overlap
IL10RA	Transmembrane receptor	Activated	3.148	$1.04x10^{-10}$
HNF1A	Transcription regulator	Activated	2.392	$1.55x10^{-05}$
Beta-estradiol	Chemical drug	-	1.367	$1.74x10^{-02}$
Glucocorticoid	Chemical drug	-	0.762	$1.71x10^{-03}$
Gentamicin	Chemical drug	-	-1.342	$6.39x10^{-05}$
Methylprednisolone	Chemical drug	-	-1.192	$2.30x10^{-03}$
AGT	Growth factor	-	-1.103	$1.08x10^{-03}$
Dexamethasone	Growth factor	-	-0.781	$7.78x10^{-03}$

IL10RA, Interleukin 10 Receptor Subunit Alpha; HNF1A, Hepatocyte nuclear factor 1 homeobox A; AGT, Angiotensinogen.

C.



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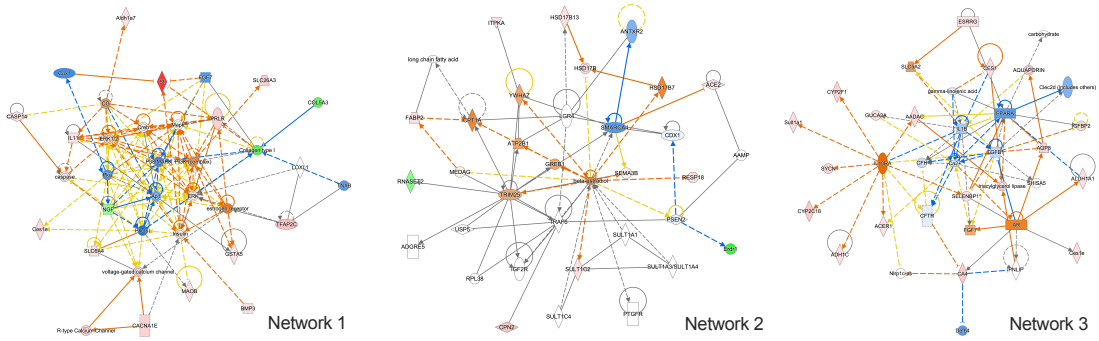


Figure 2-5 Transcriptomic analysis from EGFR-independent colon tumors
(A). Significant canonical pathways identified from IPA distinguishing *Egfr*CKO+*Apc*CKO+*Kras*OE induced colon tumors. (B). Top upstream regulators of genes significantly differentially expressed in EGFR-independent colon tumor. (C). IL10RA upstream regulator activated in EGFR-independent colon tumors. (D). Top three enriched signaling networks in EGFR-independent colon tumors.

We validated by quantitative-PCR (qPCR) five common genes (*Aadac*, *Sult1a1, Aldh1a1, Maob, and Sult1c2*) between the top five enriched canonical pathways and the upstream regulator IL10RA (Figure 2-6). We showed that the transcript levels of *Aadac*, *Sult1a1*, *Aldh1a1* are upregulated in tumors lacking EGFR, independent of KRAS status. *Maob* and *Sult1c2* is upregulated just in tumors lacking EGFR and overexpresed levels of KRAS.

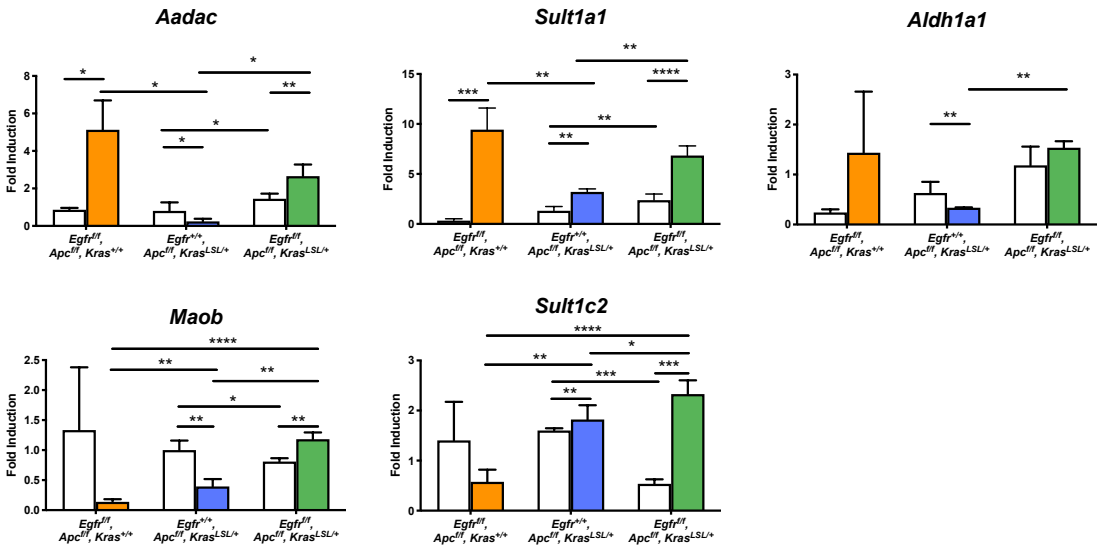


Figure 2-6 Validation of differentially expressed genes in EGFR-independent colon tumors in mouse models

White bars represent the mean transcript level of specific genes in the adjacent normal tissue associated to each tumor type, color bars represent the mean transcript level of specific genes in the colon tumor with different genotype. * *p-value<0.05*, ** *p-value<0.01*, *** *p-value<0.001*, **** *p-value*<0.0001

2.4.5. IL10RA signaling is enhanced in EGFR-independent colon tumors

To examine the role of IL10, we measured *Il10ra* and *Il10* expression in *Egfr*WT and *Egfr*CKO induced colon tumors in mice. We did not observe any differences in the transcript levels of *Il10ra*, however we did see an increase in the transcript levels of *Il10* and a well-known downstream target of IL10RA, *Socs3* suggesting that IL10RA is activated (Figure 2-7A). We also observed an increase in IL10 levels in serum of animals with *Egfr*CKO induced colon tumors compared with levels of IL10 in animals with *Egfr*WT induced colon tumors (Figure 2-7B). Increased levels of IL10 in serum have been associated with poor prognosis and lower survival in CRC patients.

2.4.6. Infiltration of M2 macrophages is increased in EGFR-independent colon tumors

Because IL10 is known to regulate the differentiation of M2 macrophages, we first evaluated whether accumulation of immune infiltrates, especially macrophages is increased in EGFR-independent tumors. As shown in Figure 2-8, *Egfr*CKO colon tumors exhibited thriving clusters of leukocytes, represented by CD45+ marker and total macrophages by marker F4/80+, specifically CD163+ representing M2 macrophages and no iNOS, biomarker of M1 macrophages. An accumulation of CD3+ (T-cells) cells was observed in both *Egfr*WT and *Egfr*CKO colon tumors with no significant difference. Based on these results, the combination of *Egfr*CKO and *Kras*OE appears to be more important in the formation of the tumor microenvironment than *Egfr*CKO or *Kras*OE alone.

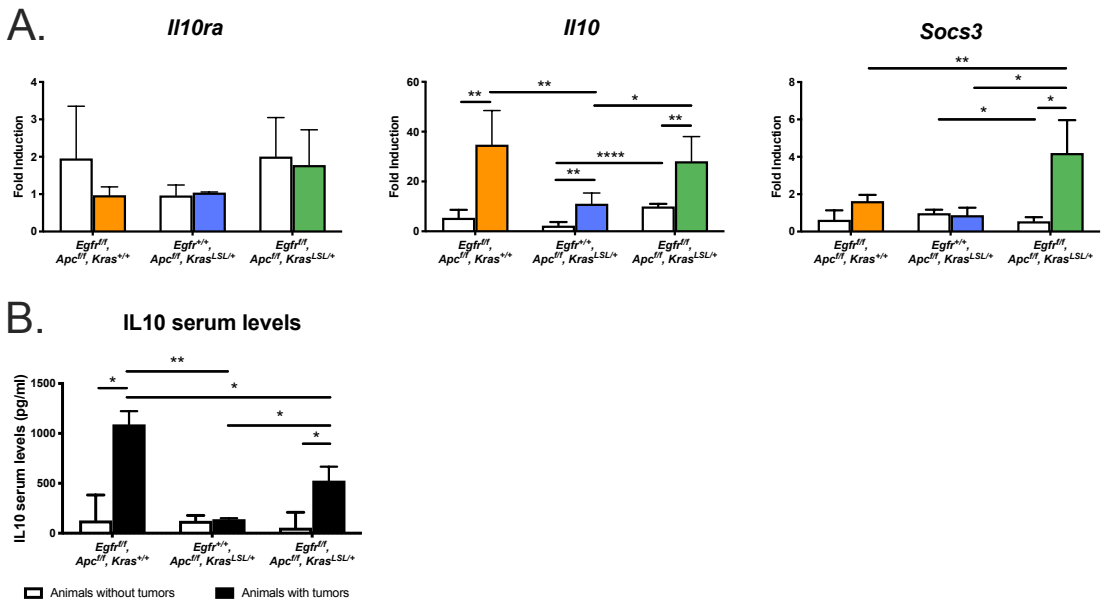


Figure 2-7 IL10 is overexpressed in EGFR-independent colon tumors
(A). Transcript levels of *Il10ra* (left), *Il10* (middle) and *Socs3* (right) were measured by qPCR. White bars represent the mean transcript level of specific genes in the adjacent normal tissue associated to each tumor type, color bars represent the mean transcript level of specific genes in the colon tumor tissue with different genotype. (B). IL10 levels in the serum of mice were increased in animals with *Egfr*CKO colon tumors. White bars represent the mean levels of IL10 in serum in animals without tumors, black bars represent the mean levels of IL10 in serum in animals with tumors. * *p-value<0.05*, ** *p-value<0.01*, *** *p-value<0.001*, **** *p-value*<0.0001

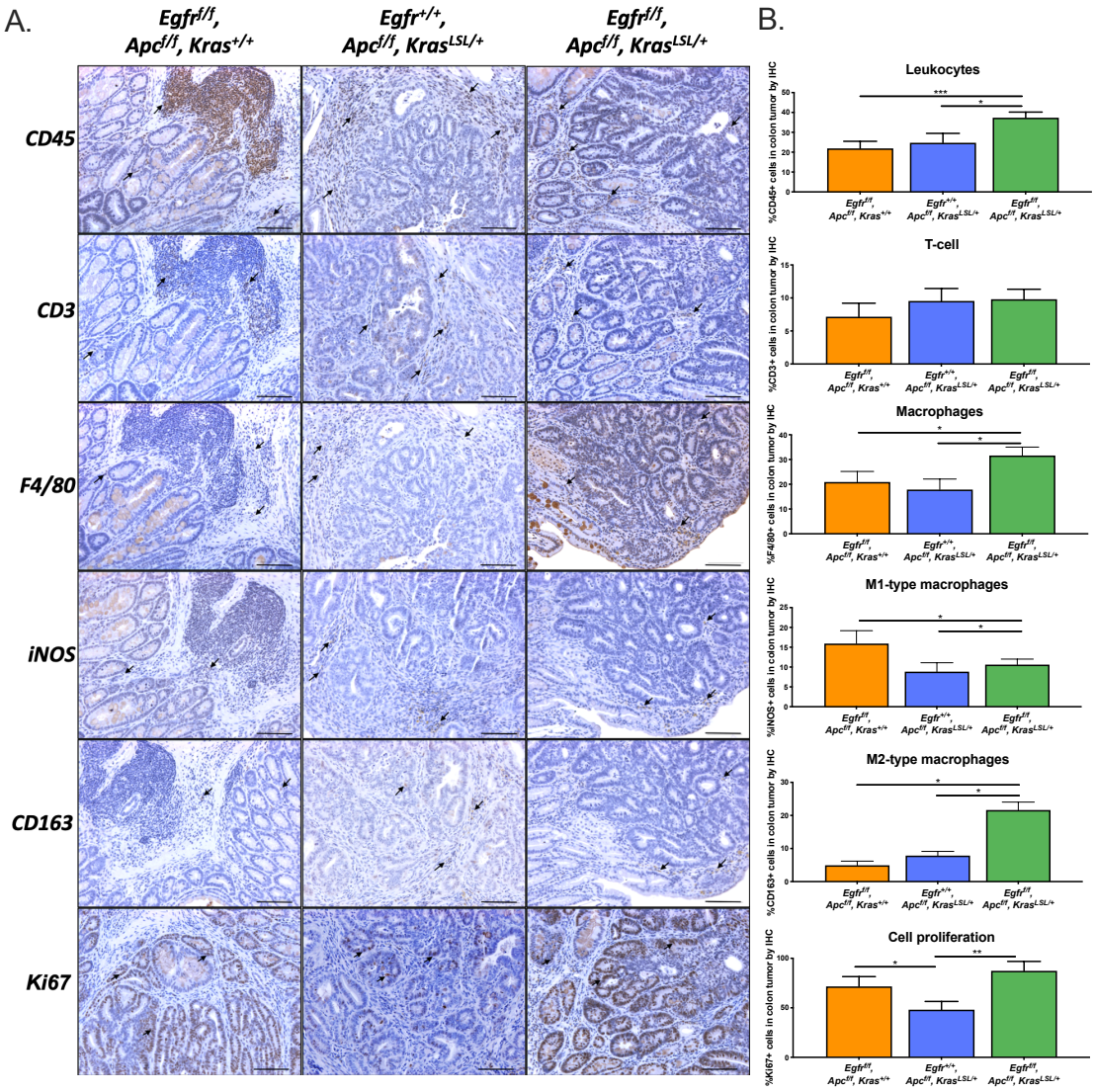


Figure 2-8 Increased number of immune infiltrates, especially M2 macrophages, in EGFR-independent colon tumors

(A). Histological evaluation of $CD45^+$, $CD3^+$, $F4/80^+$, iNOS, $CD163^+$ and Ki67 marker in induced colon tumors. (B). Immunohistochemistry quantification of specific markers in induced colon tumors. * *p-value*<*0.05*, ** *p-value*<*0.01*, *** *p-value*<*0.001*, **** *p-value*<0.0001

2.4.7. EGFR-independent tumors showed activation of anergy-associated genes

The immunosuppressive cytokine IL10 has been associated with poor prognosis in colon cancer (Herbeuval, Lelievre et al. 2004). Although macrophages are involved in anti-tumor defenses, production of IL10 by tumor cells may permit malignant cells escape to cell-mediated immune defense. In order to determine if IL10 induces an anergic state in EGFR-independent colon tumors, we examined the expression of several anergy-associated genes compared to control normal colon tissue and EGFR-dependent tumors (Figure 2-9). Anergy-inducing genes was activated in EGFR-independent colon tumors, suggesting that *Egfr*CKO tumors escape the immune system by increasing IL10 levels allowing these tumors to grow faster.

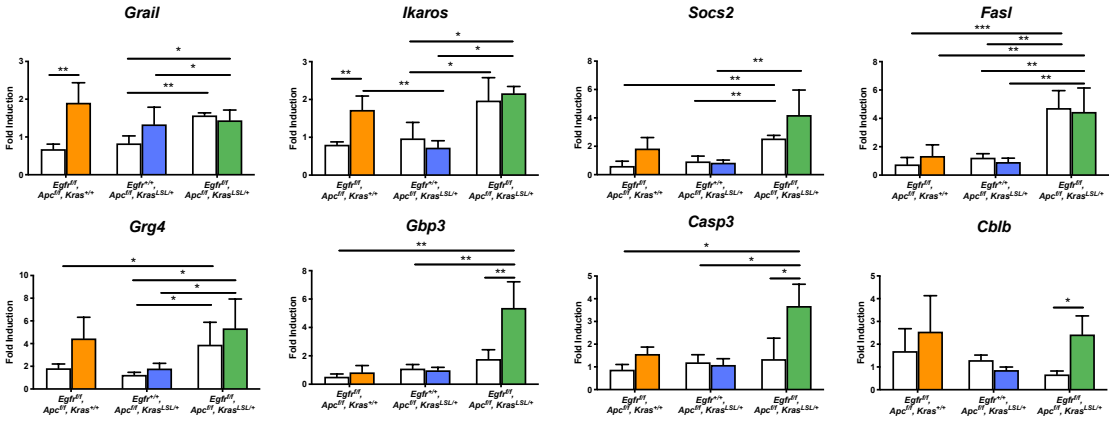


Figure 2-9 EGFR-independent colon tumors showed increased levels of anergy-associated genes when compared to colon tumors with normal levels of EGFR.
White bars represent the mean transcript level of specific anergy-associated genes in the adjacent normal tissue associated to each tumor type, color bars represent the mean transcript level of specific anergy-associated genes in the colon tumor with different genotype. * *p-value*<*0.05*, ** *p-value*<*0.01*, *** *p-value*<*0.001*, **** *p-value*<0.0001

2.4.8. IL10 signaling is required to increase cell proliferation in EGFR-independent colon tumors

We showed that IL10 levels increased in the serum of mice with *Egfr*CKO induced colon tumors as well as the transcript levels of IL10 in the colon tumor tissue. EGFR-independent colon tumors also showed an increase in cell proliferation when compared with *Egfr*WT tumors. Therefore, we performed *in vitro* experiments in which colon tumor organoids were treated with IL10 supplement or IL10 neutralizing antibody to evaluate the effect of IL10 signaling in the colon tumor growth.

In accordance to our *in vivo* results, *Egfr*CKO organoids grew faster than organoids with *Egfr*WT levels (Figure 2-10). We also found increased levels of IL10 in the conditioned media of *Egfr*WT and *Egfr*CKO organoids, suggesting that epithelial cells were the source of elevated IL10 (Figure 2-10). After serum starvation for 12 hours, the organoids were treated with conditioned media with or without treatment. As shown in Figure 2-11A, when the organoids were treated with mouse recombinant IL10 protein, *Egfr*WT organoids significantly increased cell proliferation in a dose-dependent manner. This effect was not observed in *Egfr*CKO organoids already producing high levels of IL10. Conversely, proliferation is attenuated in *Egfr*CKO organoids when IL10 neutralizing antibody was added to the media (Figure 2-11B). Additionally, *Egfr*WT organoids showed an increase in cell proliferation rate and the levels of IL10 in the media increased after treatment with EGFR inhibitor AG1478 (Figure 2-11C). Furthermore, a cooperative effect of EGFR inhibitor and IL10 neutralizing antibody was observed in organoids with

and without EGFR, by decreasing cell proliferation and tumor size (Figure 2-11D). Together these data confirm that IL10 is required for EGFR-independent cell proliferation.

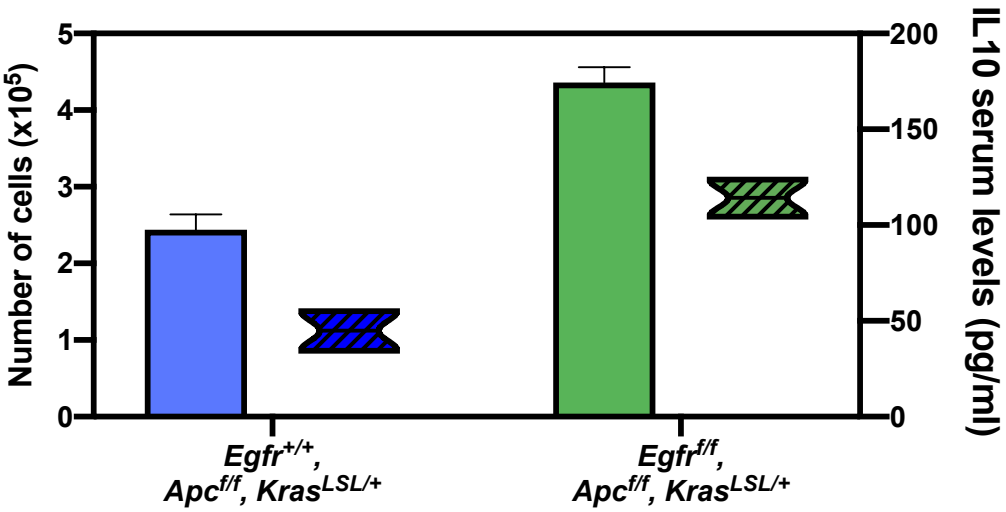


Figure 2-10 EGFR-independent organoids showed increased cell proliferation associated to increased levels of IL10 in serum.
Bars represent number of alive cells and violin plot represent IL10 serum levels of colon tumor organoids with *Egfr*WT (*Egfr*$^{+/+}$, *Apc*$^{f/f}$, *Kras*$^{LSL/+}$) levels (blue) and *Egfr*CKO (*Egfr*$^{f/f}$, *Apc*$^{f/f}$, *Kras*$^{LSL/+}$) levels (green). * *p-value<0.05*, ** *p-value<0.01*, *** *p-value<0.001*, **** *p-value*<0.0001

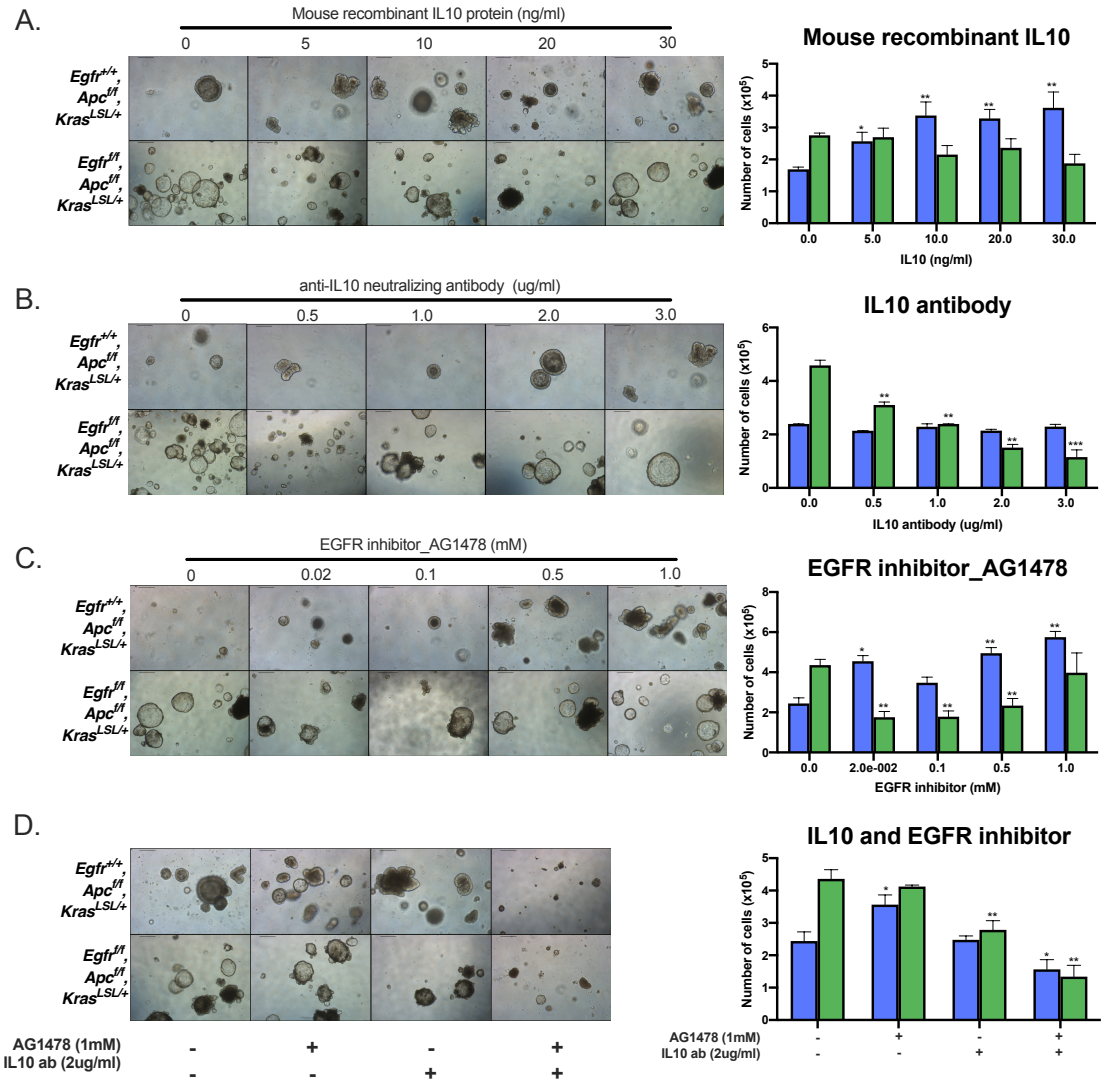


Figure 2-11 Effect of IL10 in cell proliferation in colon tumor organoids

(A). Colon tumor organoids were treated with conditional media in the absence or presence of recombinant IL10 for 72h, cell number was counted. (B). After treatment with anti-IL10 antibody for 72h, cell number was counted. (C). After treatment with EGFR inhibitor (AG1478) for 72h, cell number was counted. (D). After 1mM of AG1478 and 2ug/ml anti-IL10 antibody treatment, cell number was counted. Blue bars represent mean number of alive cells of colon tumor organoids with *Egfr*WT (*Egfr*$^{+/+}$, *Apc*$^{f/f}$, *Kras*$^{LSL/+}$) levels and green bars represent mean number of alive cells colon tumor organoids with *Egfr*CKO (*Egfr*$^{f/f}$, *Apc*$^{f/f}$, *Kras*$^{LSL/+}$). * *p-value*<0.05, ** *p-value*<0.01, *** *p-value*<0.001, **** *p-value*<0.0001

To evaluate the potential effect of an anti-IL10 treatment on colon cancer, mice that had developed *Egfr*WT and *Egfr*CKO colon tumors were administrated either anti-IL10 neutralizing antibody or vehicle (PBS) by intraperitoneal injection. Anti-IL10 antibody administration significantly reduced *Egfr*CKO colon tumor size, with one tumor disappearing completely upon anti-IL10 treatment, when compared to mice injected with PBS (Figure 2-12).

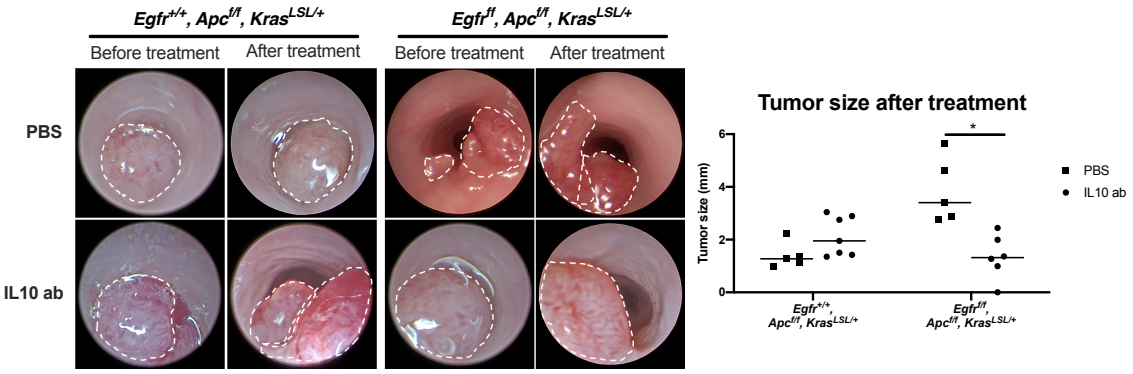


Figure 2-12 Treatment with anti-IL10 neutralizing antibody reduced *Egfr*CKO tumor size
Left panel shows a representation of colon tumor size of *Egfr*WT and *Egfr*CKO before and after the treatment by colonoscopy. Right panel shows the tumor size after the treatment during necropsy. * *p-value*<*0.05*, ** *p-value*<*0.01*, *** *p-value*<*0.001*, **** *p-value*<0.0001

2.4.9. Relationship between IL10RA and ERBB family members and their cognate ligands

The relationship between activation of IL10RA and ERBB family members has been studied in different types of cancers, including CRC. We measured expression levels of *Egfr, Erbb2, Erbb3 and Erbb4* in EGFR-dependent and EGFR-independent intestinal polyps in the $Apc^{Min/+}$ model (Figure 2-13) as well as induced-colon tumors (Figure 2-14). EGFR-independent tumors showed an increase in *Erbb2* transcript levels in both models, but the levels of *Erbb4* transcript were only increased in *Egfr*CKO induced colon tumors (Figure 2-14). Levels of ERBB ligands were also measured in both models (Figure 2-13 and Figure 2-14). EGFR-independent tumors showed an increased in both *Btc*, *Nrg1*, while *Nrg4* levels was only increased in *Egfr*CKO intestinal polyps. The transcript levels for *Egf*, *Areg* and *Ereg* were decreased in both models of EGFR-independent CRC in mice. In *Egfr*CKO intestinal polyps we also observed decreased levels of *Tgfa* and *Epgn*. There was no difference in *Tgfa, Epgn* levels. These results are consistent with previous human data that suggest the low expression of EREG and AREG as predictive biomarkers for lack of efficacy to anti-EGFR treatment (Pietrantonio, Vernieri et al. 2017, Kanat, Ertas et al. 2018, Ruiz-Saenz, Dreyer et al. 2018, Takegawa, Tsurutani et al. 2019). Higher levels of HER2, and activation of HER2/HER3 are also predictive of resistance to current treatment in CRC (Meric-Bernstam, Johnson et al. 2019).

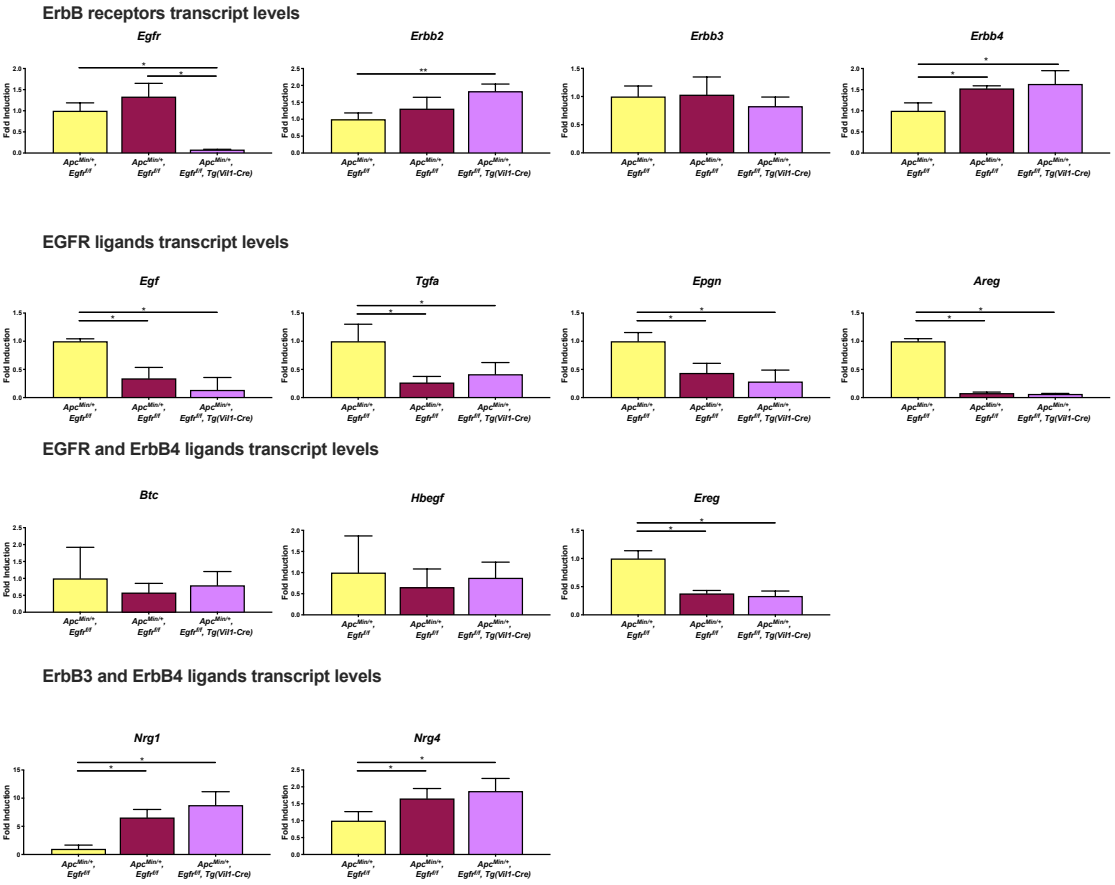


Figure 2-13 Changes in expression of ERBB family members in EGFR-independent tumors in the *Apc$^{Min/+}$* mouse model

*Egfr*CKO (purple) and *Egfr*CKO-A (maroon) intestinal polyps show similar transcript levels of ERBB receptors and ligands. * *p-value<0.05*, ** *p-value<0.01*, *** *p-value<0.001*, **** *p-value*<0.0001

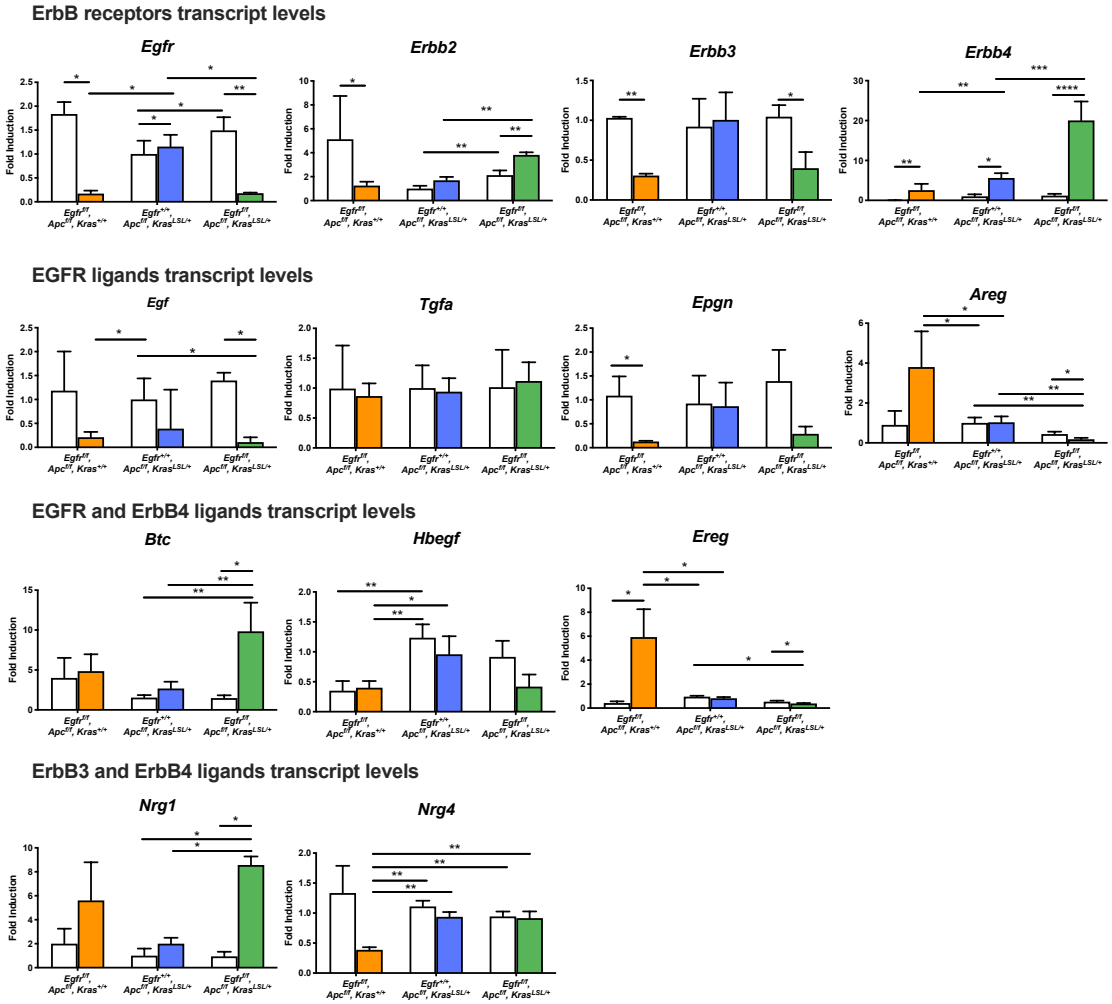


Figure 2-14. Changes in expression of ERBB family members in EGFR-independent tumors in induced colon tumors

Induced colon tumors show different expression levels dependent on EGFR, APC and KRAS status. White bars represent the mean transcript level of specific genes in the adjacent normal tissue associated to each tumor type, color bars represent the mean transcript level of specific genes in the colon tumor with different genotype. * *p-value<0.05*, ** *p-value<0.01*, *** *p-value<0.001*, **** *p-value*<0.0001

Levels of ERBB members and cognate ligands were measured after the different treatments used *in vitro* in *Egfr*WT and *Egfr*CKO colon tumor organoids as well as *in vivo* in colon tumors after the treatment with anti-IL10 neutralizing antibody (Figure 2-15). These results suggest that the absence of EGFR can trigger *Erbb4* upregulation as a result of negative feedback signaling disruption. Concomitant with this, we also observed an increase in *Btc* and *Nrg1* transcript levels, specific ERBB4 ligands, after EGFR disruption. Levels of ERBB family member significant changed after treatment with anti-IL10 neutralizing antibody *in vitro* and *in vivo*. Levels of *Erbb4* were significantly decreased after anti-IL10 treatment *in vitro* and *in vivo*.

2.4.10. EGFR-independent colon tumors use IL10 and ERBB4 to increase cell proliferation

Based on the involvement of IL10 in the *Egfr*CKO tumor development and the increased levels of *Erbb4* transcript, we evaluated the effect of IL10 in *Erbb4*CKO colon tumors. First, we investigated if loss of ERBB4 affects colon cancer development *in vivo*. ERBB4-deficient $Apc^{Min/+}$ mice (*Erbb4*CKO – $Apc^{Min/+}$, $Erbb4^{f/f}$, *Tg(Vil1-Cre)*) were established and used to show that *Erbb4*CKO mice had a significant decrease in the number of intestinal and colon polyps (Figure 2-16A). We measured the transcript levels of ERBB family members on intestinal tumors with ERBB4-deficient levels and compared to *Erbb4*WT polyps (Supplementary Figure 2-1). We observed a decreased in the transcript levels of *Erbb2* and *Erbb3*, suggesting a possible effect of ERBB4 heterodimers in the development of intestinal tumors.

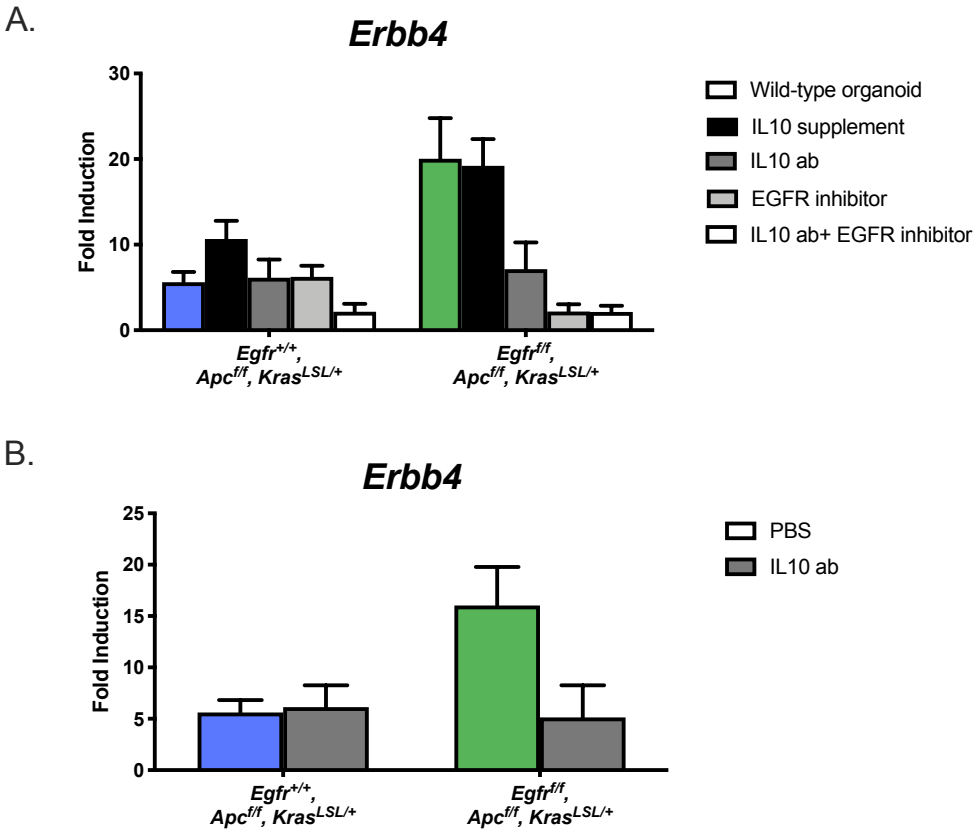


Figure 2-15 Transcript levels of ERBB4 were decreased after anti-IL10 treatment. (A). Erbb4 transcript level decreased after anti-IL10 ab treatment *in vitro* and (B) *in vivo*. Blue bars represent mean transcript levels of *Erbb4* of *Egfr*WT (*Egfr*$^{+/+}$, *Apc*$^{f/f}$, *Kras*$^{LSL/+}$) tumors and green bars represent mean transcript levels of *Erbb4* of *Egfr*CKO (*Egfr*$^{f/f}$, *Apc*$^{f/f}$, *Kras*$^{LSL/+}$) tumors. * *p-value*<*0.05*, ** *p-value*<*0.01*, *** *p-value*<*0.001*, **** *p-value*<0.0001

Consistent with the previous results, transcriptomic analysis of ERBB4-deficient intestinal tumors predicted downregulation of IL10RA signaling (Supplementary Figure 2-2) and quantitative PCR validates that transcript levels of *Il10* were decreased in *Erbb4*CKO tumors (Figure 2-16B), and correlates with a decrease of IL10 in the serum of *Erbb4*CKO animals (Figure 2-16C). In addition, we showed that the treatment with NRG1

(Figure 2-16D), an ERBB4 ligand, promoted cell proliferation and increased levels of IL10 in serum in organoids lacking EGFR. Together, these data suggested that ERBB4 is required for colon tumor formation by decreasing IL10 levels with or without EGFR.

2.4.11. Effect of EGFR and SRC inhibitor combinatorial therapy *in vivo*

Rous sarcoma oncogene (*Src*) is an important upstream activator of ERK and AKT, and it increases cell survival related signaling. Previous studies in lung cancer showed that IL10 increased the levels of phospho-SRC in a dose dependent manner, promoting increase in tumor cell proliferation. In this study, we showed that genetically and chemically inhibition of SRC reduce the number and size of intestinal polyps at three months of age of $Apc^{Min/+}$ mice (Figure 2-17).

To evaluate the effect of SRC in EGFR-independent colon tumors, $Apc^{Min/+}$, $Src^{+/+}$ and $Apc^{Min/+}$, $Src^{tm1Sor/+}$ mice were treated with the small molecule EGFR inhibitor, AG1478. At three-months of age AG1478 caused a significant reduction in small intestinal tumor number in both groups by 65.4% and 70.4% (p-value < 0.0001), respectively, although no cooperative effect was observed between EGFR and SRC reduction (p = 0.1418; Figure 2-17-left). Despite the lack of a cooperative effect in small intestinal tumor number, AG1478 reduced colonic tumor number in $Apc^{Min/+}$, $Src^{tm1Sor/+}$ mice by 70.3%, with a cooperative effect when comparing $Apc^{Min/+}$, $Src^{+/+}$ with $Apc^{Min/+}$, $Src^{tm1Sor/+}$ mice on AG1478 (p = 0.0444) (Figure 2-17-middle).

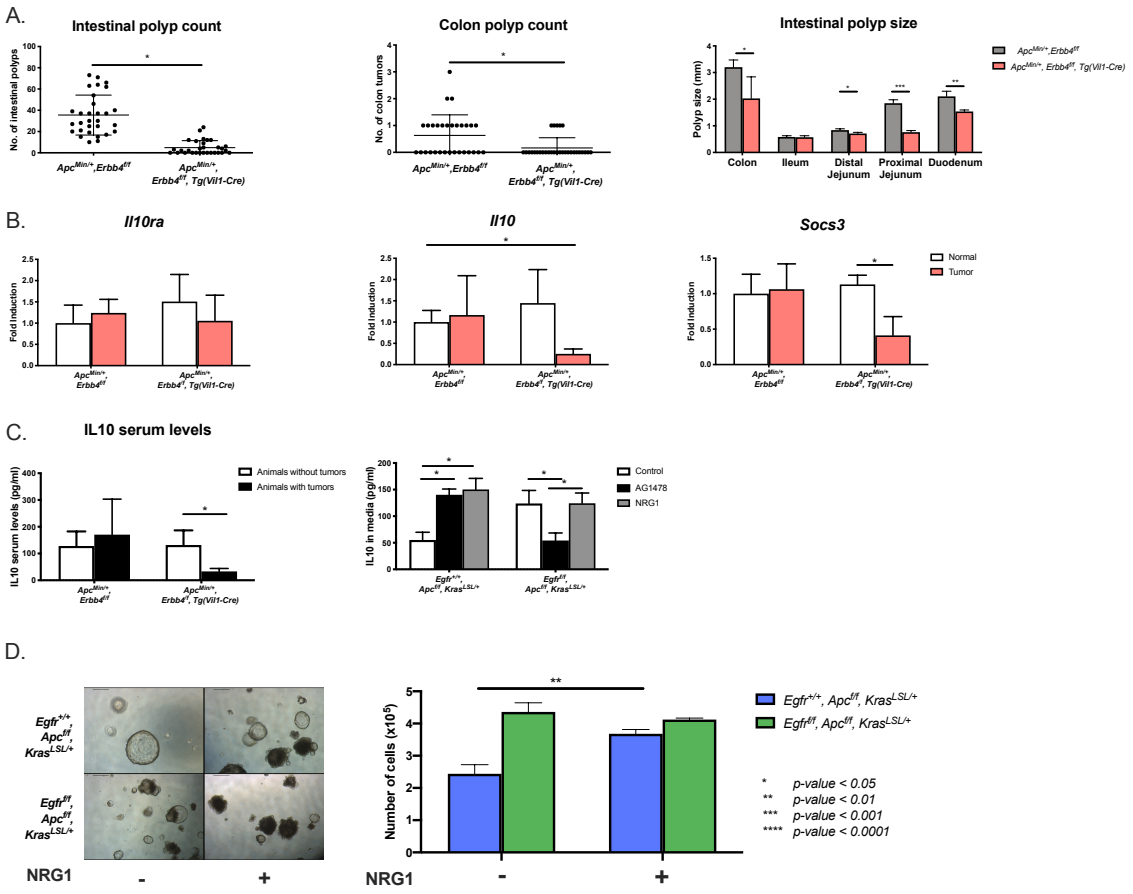


Figure 2-16 Effect of ERBB4 signaling in CRC mouse model

(A). Intestinal polyps' multiplicity and size in $Apc^{Min/+}$ mice of different *Erbb4* genotype. Each dot represents the polyp number in each 100-day-old mice. Grey bars represent mean tumor size of *Erbb4*WT intestinal polyps, salmon color bars represent mean tumor size of *Erbb4*CKO intestinal polyps (B). Transcript levels of *Il10ra* (left), *Il10* (middle) and *Socs3* (right). White bars represent the mean transcript level of specific genes in the adjacent normal tissue associated to each tumor type, salmon color bars represent the mean transcript level of specific genes in the colon tumor with different genotype (C). IL10 levels in the serum from mice with *Erbb4*CKO colon tumors (left) and levels of IL10 in media of colon tumor organoids after treatment with AG1478 or NRG1 (right). (D). Cell proliferation after NRG1 treatment in colon tumor organoids. * *p-value<0.05*, ** *p-value<0.01*, *** *p-value<0.001*, **** *p-value*<0.0001

Although small intestinal tumor number was not affected, AG1478 treatment did reduce small colon tumor size in both $Apc^{Min/+}$, $Src^{+/+}$ and $Apc^{Min/+}$, $Src^{tm1Sor/+}$ mice by almost 20% ($p < 0.05$; Figure 2-17-right), with a cooperative effect between AG1478 and $Src^{tm1Sor/+}$. Response of AG1478 treated $Apc^{Min/+}$, $Src^{+/+}$ mice were compared to $Apc^{Min/+}$, $Src^{tm1Sor/+}$ mice (0.876 mm versus 0.819 mm; $p = 0.0344$).

In summary, our data indicate that there is a subset of colorectal tumors that grow independent of EGFR activity. These tumors showed increased levels of IL10, which increases *Erbb4* transcript levels and promote increase of cell proliferation.

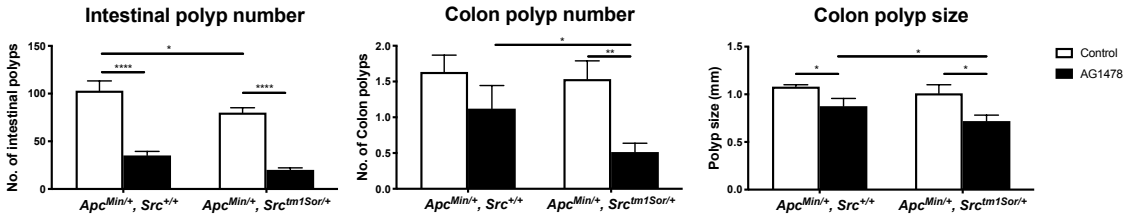


Figure 2-17 Effect of *Srctm1Sor* targeted mutation and small molecule EGFR inhibitor AG1478 on small intestinal and colonic tumor number in $Apc^{Min/+}$ mice at 3 months of age and intestinal polyp size.
White bars represent the mean number of intestinal polyps (left), colon polyps (middle) and polyp size (right) with different Src genotype. Black bars represent the mean number of intestinal polyps (left), colon polyps (middle) and polyp size (right) after EGFR inhibitor (AG1478) treatment. * *p-value<0.05*, ** *p-value<0.01*, *** *p-value<0.001*, **** *p-value*<0.0001

2.5. Discussion

In this study, we definitively established the existence of a subset of colon tumors that arise independently of EGFR and demonstrated its correlation with a more aggressive phenotype of colorectal tumors in mice. We also showed that increased IL10 contributes to elevated growth of EGFR-independent tumors. Additionally, levels of *Erbb4* in EGFR-independent colon tumors seemed to be increased in response to the lack of EGFR. IL10 and ERBB4 upregulation seemed to promote colon cancer development in the absence of EGFR.

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. Previous studies could not distinguish EGFR independence from variable residual EGFR activity. In the current study, we took advantage of a conditional knockout allele of *Egfr* to prove that a subset of colon tumors does arise independently of EGFR signaling (Figure 2-1 and Figure 2-2). Our genetic approach demonstrates that despite having far fewer polyps than $Apc^{Min/+}$ mice with a wild-type *Egfr*, $Apc^{Min/+}$ mice with intestinal-epithelia specific *Egfr* deletion do develop polyps and these polyps grow in an EGFR-independent manner (Figure 2-1). We also prove that under normal levels of EGFR, a subset of colon tumors arises with a similar gene expression profile to those without EGFR, suggesting that these colon tumors might have a constitutive mechanism to development independent of EGFR activity (Figure 2-3).

Surprisingly, the absence of EGFR in these polyps enhances their growth (Figure 2-1C and Figure 2-2C-D). 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, differential response to EGFR inhibition is well documented in non-small-cell lung carcinoma (NSCLC), where patients harboring EGFR activating mutations exhibit dramatic clinical responses to gefitinib (Lynch, Bell et al. 2004, Paez, Janne et al. 2004, Pao, Miller et al. 2004). Additionally, an induced model of colon cancer in mice showed that even in the presence of *Kras* mutation, EGFR status influences the development of colon tumors by activating compensatory pathways (Figure 2-2) (Troiani, Martinelli et al. 2013).

Transcriptomic analysis predicted that IL10RA signaling was upregulated in *Egfr*CKO intestinal and colon tumors (Figure 2-3C and Figure 2-5C), increased transcript levels of *Il10* and *Socs3* confirmed that IL10RA signaling was activated in EGFR-independent colon tumors (Figure 2-7). These data correlate with previous studies suggesting that IL10 promotes cancer development by inhibiting anti-tumor immune responses. Specifically, IL10 can impair the activation of cytotoxic T lymphocytes (CTLs) and Th1 CD4+ cells (Pizarro, Arseneau et al. 2000), and can inhibit the cytolytic activity of natural killer cells and CTLs (Kamizato, Nishida et al. 2009), which are responsible for the immune surveillance of cancer. We found that IL10 was increased in different mouse models of colon cancer lacking *Egfr* (*Apc*$^{Min/+}$, *Egfr*$^{f/f}$, *Tg(Vil1-Cre)*; *Egfr*$^{f/f}$, *Apc*$^{f/f}$, *Kras*$^{LSL/+}$; and *Egfr*$^{f/f}$, *Apc*$^{f/f}$, *Kras*$^{+/+}$). Treatment with IL10 neutralizing

antibody reduced cell proliferation and tumor size *in vitro* and *in vivo* (Figure 2-11 and Figure 2-12, respectively). We confirmed that *Egfr*CKO tumors showed an increased infiltration of M2-type macrophages (Figure 2-8). In addition, activation of anergy-associated genes in *Egfr*CKO colon tumors confirmed that IL10 may act as a suppressor of the immune system (Figure 2-9). Furthermore, several studies have shown that late-stage CRC patients had higher IL10 expression and patients with higher IL10 levels presented lower survival rates (Zhao, Wu et al. 2015, Mager, Wasmer et al. 2016). Although the *in vivo* role of IL10 in colon cancer has not been previously elucidated, IL10 expression by tumor-associated macrophages has been correlated with a poor prognosis (Herbeuval, Lelievre et al. 2004). In this study we showed that IL10 was highly increased in mice with *Egfr*CKO colon tumors (Figure 2-7B). However, the influence of IL10 on tumor development is still controversial because communication between the tumor microenvironment and tumor cells is critical for cancer development. An experimental model capable of mimicking the tumor environment had to be established to elucidate the role of IL10 in cancer progression. The mechanism of IL10 upregulation is not well understood, and it remains unclear whether cancer cells secrete IL10 and whether IL10 plays a role in the aggressiveness and malignancy of cancer cells.

We also observed that serum levels of IL10 were decreased in $Apc^{Min/+}$, $Erbb4^{f/f}$, *Tg(Vil1-Cre)* mice that develop colon tumors (Figure 16C – left), which indicates that lack of EGFR and ERBB4 activation may be involved in IL10 expression and might be positively correlated with colon cancer formation. Differences in the expression of ERBB family members have been reported in multiple solid tumor malignancies. Kaplan-Meier

survival curve using cases from The Cancer Genome Atlas indicates a survival disadvantage in colorectal carcinoma with ERBB4 alteration (Cerami, Gao et al. 2012, Gao, Aksoy et al. 2013). In addition, a combined Moffitt Cancer Center and Vanderbilt Medical Center CRC expression data set showed that ERBB4 transcript levels were increased at all tumor stages (Williams, Bernard et al. 2015). Overexpression of ERBB4 enhances the survival and growth of cells driven by Ras and/or WNT signaling (Williams, Bernard et al. 2015). Some studies suggest that chronic ERBB4 overexpression in the context of inflammation may contribute to colorectal carcinogenesis (Frey, Hilliard et al. 2010). Also, elevated co-expression of KITENIN and ERBB4-CYT-2 promoted the transition of colon adenoma to adenocarcinoma within an APC loss-associated tumor microenvironment (Bae, Kho et al. 2016). We confirmed by *in vivo* and *in vitro* experiments that ERBB4 transcript was upregulated in the absence of EGFR (Figure 2-13, Figure 2-14 and Figure 2-15). We also showed that the activation of ERBB4 by NRG1 treatment increased proliferation in colon tumor organoids (Figure 2-16D). By knocking out ERBB4 in the intestinal epithelium we observed a decrease in the number of tumors corelated to a decreased in IL10 levels in serum (Figure 2-16A). These results suggest that ERBB4 and IL10 in combination have an effect on the development of EGFR-independent colon tumors. The mechanism leading to tumor over-expression of ERBB4 at the RNA level is a key follow-up question for these studies. It is unclear if increased IL10RA signaling is responsible of the ERBB4 increased levels or if ERBB4 increases as a compensatory mechanism of the lack of EGFR in the intestinal epithelium.

Previous studies in lung cancer reported that IL10 increased the levels of phospho-SRC in a dose dependent manner, promoting increase in tumor cell proliferation (Herbeuval, Lelievre et al. 2004, Hsu, Wang et al. 2016). Accumulating evidence suggests that SRC also contributes to cancer development and may be a target in the treatment of CRC (Warmuth, Damoiseaux et al. 2003). SRC is a non-receptor tyrosine kinase that is activated in human colon, breast, liver, lung, and pancreatic cancers, and is increased in tumors arising in $Apc^{Min/+}$ mice (Moran, Hunt et al. 2004). Transgenic mice ectopically expressing *Src* have significantly more tumors in the liver and intestinal tract, which is further enhanced by loss of the *Cdkn1a* (p21) tumor suppressor gene (Kline, Jackson et al. 2008). Supporting an important role for SRC, inhibition of SRC activity reduces growth of human CRC cells as well as tumor growth in xenograft models (Golas, Lucas et al. 2005). Additionally, 64 CRC cell lines were shown to be depend on SRC family kinase (SFK) activity (Emaduddin, Bicknell et al. 2008).

In the current study, we demonstrated that the inhibition of SRC activity reduces tumorigenesis both *in vitro* and *in vivo*, and that combining SRC and EGFR inhibition is more efficacious than inhibiting either kinase alone. Furthermore, colonic tumor number was significantly reduced when combining the Src^{tm1Sor} mutation with the AG1478 inhibitor whereas neither AG1478 nor Src^{tm1Sor} alone was sufficient for reducing colonic tumor number in these mice (Figure 2-17). These data support the previous results that an EGFR independent mechanism could use IL10 and its SRC signaling to promote a more aggressive development of colon tumor.

Based on our data and evidence in the literature, we suggest that EGFR-independent colonic tumors show increase levels of IL10 activating IL10RA signaling. The activation of IL10RA might increase SRC and ERBB4 levels as a compensatory mechanism for the absence of EGFR (Figure 2-18). Nevertheless, direct evidence to clarify whether IL10RA or SRC regulates Erbb4 expression is lacking. The combinatorial effect of EGFR and IL10 inhibition indicate that the compensatory pathways might be inhibited by these two treatments. Finally, because IL10 inhibition decreases cell proliferation and decrease tumor size *in vivo*, the future development of anti-IL10 treatment in combination with anti-EGFR will be of benefit to colon cancer therapy.

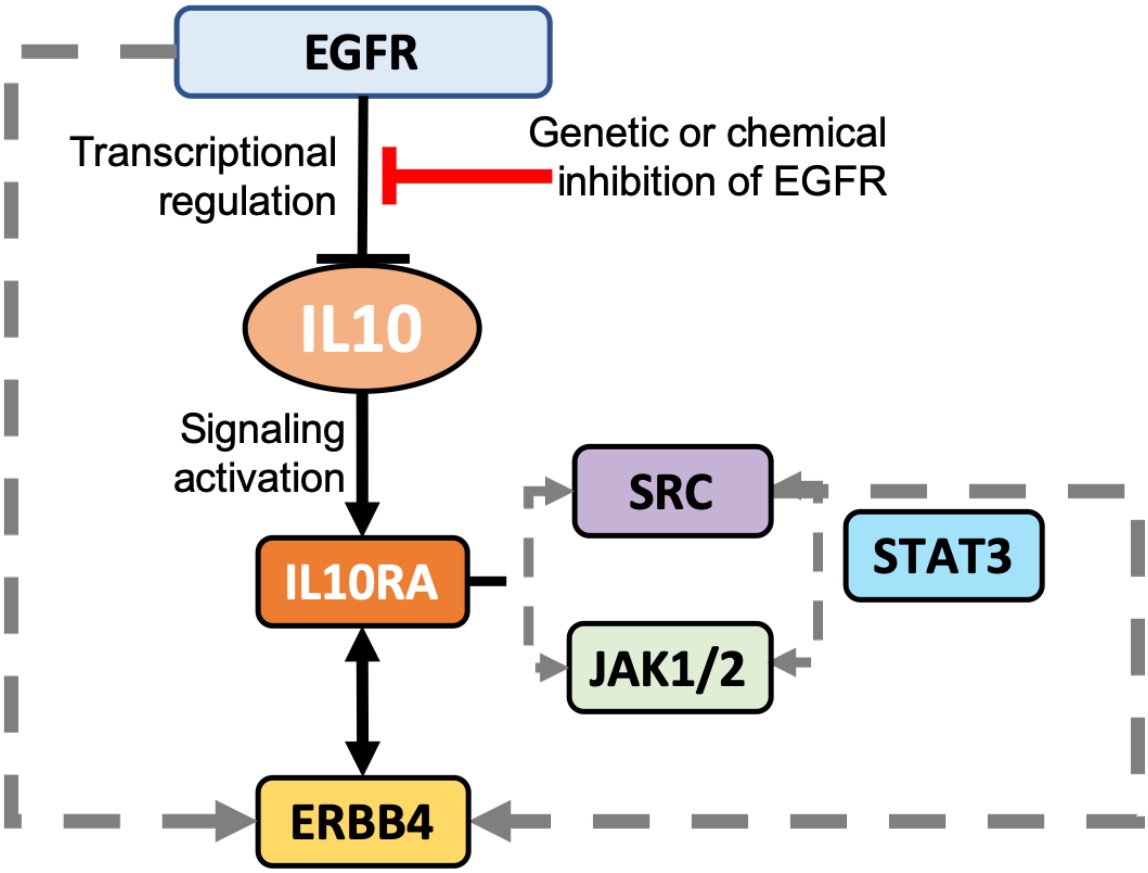


Figure 2-18 EGFR-independent mechanism of colorectal cancer progression
Black arrows represent proven mechanisms, grey arrows show proposed interactions from literature.

3. ERBB3 SIGNALING IN INTESTINAL TUMORIGENESIS

3.1. Synopsis

The ERBB family plays a crucial role in intestinal development and tumorigenesis. ERBB3, one of the members of the ERBB family, has been undervalued in the development of colorectal cancer (CRC). To examine the impact of ERBB3 deficiency on the $Apc^{Min/+}$ mouse model of familial adenomatous polyposis (FAP), we used a mouse model with Cre-mediated intestinal epithelia-specific ERBB3 deletion on C57BL/6 background mice. We observed a significant increase in total polyp number in 3-month-old $Apc^{Min/+}$, $Erbb3^{f/f}$, *Tg(Vil1-Cre)* – *Erbb3*CKO mice compared to wild-type ERBB3 littermates ($Apc^{Min/+}$, $Erbb3^{f/f}$ – *Erbb3*WT) (*p-value <0.0001*). The polyps forming in *Erbb3*CKO mice were significantly smaller than those forming in the controls (0.62 ± 0.48 mm versus 0.94 ± 0.45 mm; (*p-value<0.0001*), suggesting that normal levels of ERBB3 signaling are essential for tumor growth in $Apc^{Min/+}$ mice. Proliferation rate, measured by Ki67 staining, in ERBB3-deficient polyps was comparable to polyps with wild-type ERBB3, while an increase in TUNEL-positive cells were observed in polyps from *Erbb3*CKO mice. This data indicates that ERBB3-dependent signaling prevents apoptosis in $Apc^{Min/+}$ polyps. Transcriptomic analysis suggested a decrease of the mitogen-activated protein kinase (MAPK) signaling pathway compared to ERBB3 wild-type intestinal polyps. Using an established mouse model of colitis-associated colorectal (CAC) tumorigenesis induced by azoxymethane (AOM), we also observed a significant increase in tumor penetrance in ERBB3-deficient mice (*p-value<0.05*), compared to the wild-type

ERBB3 controls. However, no differences were detected in the size of AOM-induced colonic tumors between the 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. Taken together, this study reveals the importance of ERBB3-mediated phosphatidylinositol 3-kinase/protein kinase B/MAPK (PI3K/AKT/MAPK) in intestinal tumorigenesis, thus provides a valuable target for therapeutic intervention.

3.2. Introduction

Members of the ERBB/HER receptor family (EGFR or ERBB1, ERBB2, ERBB3, ERBB4) have been studied intensively and it is known that they play an important role in tumorigenesis and hold significant promise for development of cancer therapeutics (Jaiswal, Kljavin et al. 2013). 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 (Arteaga and Engelman 2014). Unlike other ERBB receptors, ERBB3 lacks intrinsic kinase activity and cannot auto-phosphorylate (Yarden and Pines 2012), as a consequence, activation of ERBB3 signaling is only possible by heterodimerization with other ERBB receptors. Upon binding of an ERBB3 ligand, neuregulin, the dimerization arm in the extracellular domain is exposed promoting receptor-receptor interaction, triggering a complex signaling downstream (Lee-Hoeflich, Crocker et al. 2008). ERBB3 can be trans-activated on cytoplasmic tyrosine residues by forming heterodimers with other ERBB family members, of which ERBB2 is the preferred partner. In contrast, the ERBB2 receptor has tyrosine kinase activity, but has no known

ligand (Yarden and Sliwkowski 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 phosphatidylinositol 3-kinase (PI3K) among ERBB receptors due to the nine binding docking sites for the p85 subunit of PI3K (Ruiz-Saenz, Dreyer et al. 2018). As a consequence, activation of ERBB3 frequently results in strong activation of the protein kinase B (AKT) signaling, a critical oncogenic stimulus whose aberrant activity is implicated in a wide range of cancers and it is a major cause of treatment failure in cancer therapy because of its role in therapeutic resistance (Yonesaka, Takegawa et al. 2019).

Of the four members of the ERBB family, EGFR and ERBB2 are well-documented protooncogenes and they have been actively pursued as anti-cancer target due to their aberrant activation in many human malignancies (Hynes and MacDonald 2009). In contrast, the function of ERBB3 has been less appreciated due to its defective kinase activity (Ma, Lyu et al. 2014). 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 (Lee-Hoeflich, Crocker et al. 2008, Watanabe, Yonesaka et al. 2019), colorectal cancer (CRC) (Maurer, Friess et al. 1998, Jaiswal, Kljavin et al. 2013), gastric cancer (Cao, Chen et al. 2016, Ahmed 2019), ovarian cancer (Chung, Kim et al. 2019) and pancreatic cancer (Wang-Gillam, Li et al. 2016). In ERBB2-driven tumors, ERBB3 functions as an intimate signaling partner that promotes the cell transformation

potency of ERBB2, usually by activating the PI3K/AKT pathway (Holbro, Beerli et al. 2003, DeSantis, Miller et al. 2019). For these cancers in particular, ERBB3 inhibition may be required to effectively eradicate cancerous cells (Adams, Brown et al. 2018). Recently it was reported that ERBB3 couples EGFR to the PI3K/AKT pathway in non-small cell lung cancer (NSCLC) cells that are sensitive to the EGFR inhibitors like gefitinib (Engelman, Zejnullahu et al. 2007). Consistent with a potential 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, Zejnullahu et al. 2007). Blocking of ERBB3 with monoclonal antibody (mAb) is one of the current strategies currently being investigated in preclinical (Schoeberl, Pace et al. 2009, Schoeberl, Faber et al. 2010) and clinical studies (http://www.clinicaltrials.gov). mAb blocking of the ligand-binding domain is the major approach to target this receptor, because ERBB3 lacks appreciable kinase activity (Citri, Skaria et al. 2003, Shi, Telesco et al. 2010). Several anti-ERBB3 mAbs that prevent ligand-induced activation of ERBB3, such as MM-121 and MM-111 (Merrimack Pharmaceuticals, Cambridge, MA) and U3-1287/AMG 888 (Amgen Inc., Thousand Oaks, CA) have shown significant antitumor activity *in vitro* and *in vivo* (Schoeberl, Pace et al. 2009, Schoeberl, Faber et al. 2010, McDonagh, Huhalov et al. 2012, Li, Brand et al. 2013). Additionally, Bautz *et al.,* suggested that prophylactic vaccination could represent a novel approach to prevent and reduce the occurrence of CRC in at-risk patient populations (Bautz, Sherpa et al. 2017).

Furthermore, ERBB3-dependent signaling, through ERBB2-ERBB3 heterodimers, has been shown to contribute to the enhanced invasiveness of mammary tumor cells (Xue, Liang et al. 2006). Altogether, it has become increasingly 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. To investigate the role of ERBB3-dependent signaling during intestinal tumorigenesis, we used a previously generated conditional *Erbb3* allele (*Erbb3*$^{tm1.1Dwt}$ referred as *Erbb3*^{f} (Lee, Yu et al. 2009)) and ablated ERBB3 specifically in the intestinal epithelium (*Apc*$^{Min/+}$, *Erbb3*$^{+/+}$, *Tg(Vil1-Cre)*). We showed 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 a mouse model of colitis-associated colorectal tumorigenesis (CAC) induced by the azoxymethane (AOM). In the *Apc*$^{Min/+}$ mouse model, we observed a strain-dependent effect in tumor development. In both mouse models, on a wild-type C57BL/6J (B6) background, ERBB3 deficiency dramatically increased colon tumor multiplicity. These colon tumors also showed increased transcript levels of *Erbb4* and *Egf*. In contrast, on a 129S1/SvlmJ and C57BL/6J (129/B6) F1 generation, the lack of ERBB3 does not seem to have an effect on intestinal tumorigenesis. Analysis of littermates in additional generations of backcrossing to 129S1/SvImJ (129) showed that ERBB3-deficiency promotes a significant decrease in tumor multiplicity in the *Apc*$^{Min/+}$ mouse model. Together these results suggest an important strain effect in the development

of colon tumors in mice. Overall, these results establish the importance of ERBB3-dependent signaling pathway in intestinal tumorigenesis.

3.3. Materials and methods

Animals experiments

All animal studies were maintained and protocols followed in accordance with Texas A&M University Institution Animal Care and Use Committee guidelines. 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. *Erbb3*$^{tm1.1Dwt}$ (*Erbb3*^{f}) and Gt(ROSA)26Sor ^{tm1Sor}/J (*R26R*^{f})mice were maintained on C57BL/6J background. Mice were housed five per cage, fed Purina Mills Lab Diet 2919 and maintained at 22° under a 12-hr light cycle. Mice were euthanized by CO2 asphyxiation for tissue collection.

Genotyping

Mice were genotyped for the *Apc*^{Min} allele as previously described (Roberts, Min et al. 2002). Cre transgenic mice were determined using PCR with cre-S1, 5-gtgatgaggttcgcaagaac and cre-AS1, 5-agcattgctgtcacttggtc primers which brings a 278-bp PCR product. Mice were genotyped for the *Egfr*^{tm1Dmt} allele as previously described (Lee and Threadgill 2009). Mice were genotyped for the *Erbb3*$^{tm1.1Dwt}$ allele using B3-F, 5'-TCCAGCGTGGAAAAGTTCAC; and B3-R, 5'- AAGCCTTCTCTATGGAAAGTG. Gt(ROSA)26Sor ^{tm1Sor} allele was genotyped using the following primers, rosaENDO, 5'-

GGAGCGGGAGAAATGGATATG; rosaCOM, 5'-AAAGTCGCTCTGAGTTGTTAT; and rosaNEO, 5'-GCGAAGAGTTTGTCCTCAACC.

Azozymethane (AOM) treatment

A single lot of AOM was obtained from Sigma- Aldrich (St. Louis, MO) in 100 mg isovials and stored at -80°C. Each 100 mg vial was resuspended in 2 ml phosphate-buffered saline (PBS) and individual 250 ul aliquots stored at -80°C until use. A working stock of 1.25 mg/ml AOM was made by diluting individual 250 ul aliquots into 10 ml of saline (0.9% NaCl). Three months of age mice were injected intraperitoneal (IP) 10 mg AOM per kg body weight once a week for 4 weeks. Age-matched controls were injected with saline.

Tissue collection

The small intestine and colon were removed from each mouse. The small intestine was cut into fourths. Each segment was gently flushed with PBS to remove fecal material, cut longitudinally, and splayed flat. Representative tumors were scored before sectioning in half under the dissecting microscope. One half was used for molecular analysis; the other half was 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 collected and fixed 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 (Paul Olson, Hadac et al. 2014). Colon tumors were 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% specific serum and 0.1% Triton X- 100, and then incubated with primary antibodies and HRP-conjugated specific anti-rabbit secondary antibody (Vector Laboratories, Inc). Antigen-antibody complexes were detected with DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Suppliers for primary antibodies were Abcam, (Ki67-ab15580, Tunel Assay Kit-ab206386).

Transcriptomic analysis

A total of 3 sequencing runs were performed to sequence 56 samples on NextSeq 500 sequencing instrument at Texas A&M Institute for Genome Sciences and Society using high output kit v2. A total of 1.5 billion 75 bp single-end reads were checked for adapter sequences and low-quality bases using Trimmomatic (Bolger, Lohse et al. 2014),

resulting in approximately 1.4 billion filtered reads (96%). RNA-Seq reads were aligned to mouse assembly mm10 using HISAT2 version 2.0.5 (Kim, Landmead et al. 2015) with an overall mapping rate of approximately 97%. Raw gene counts were generated with feature Counts package (Liao, Smyth et al. 2014) while discarding ambiguous read mappings. Normalized read counts and gene expression tests were performed using DESeq2 (Love, Huber et al. 2014) following recommended guidelines by the authors. Ingenuity Pathway Analysis (IPA) was used to analyzed differentially expressed genes between the different groups.

Quantitative real time PCR (qRT-PCR)

Genes with significant changes in expression between *Egfr*WT tumors and *Egfr*CKO tumors, based on ANOVA analysis, were confirmed by qRT-PCR. cDNA was synthesized from total RNA from each tumor using the QuantiTect Reverse Transcription Kit (Qiagen 205314). PCR reactions were set up in 96-well plates, all samples were run in triplicate. Analysis was performed on a LightCycler 96 Thermocycler (Roche) using LightCycler 480 Sybr Green I Master reaction mix. Specific primers were designed to amplify a fragment from the genes in Supplementary Table 3-1.

Statistics.

The nonparametric Mann–Whitney U test was used to analyze tumor data. To compare the statistical difference between 2 groups, student's *t* test was used. The *p-value* smaller than 0.05 was considered as the significant difference.

3.4. Results

3.4.1. Effect of ERBB3 on tumor development in *Apc*$^{Min/+}$ mice is dependent genetic background

ERBB3 signaling has been associated with CRC development. To evaluate the role of ERBB3 signaling during intestinal tumorigenesis, in this study, we used the *Apc*$^{Min/+}$ mouse model with wild-type (*Apc*$^{Min/+}$, *Erbb3*$^{f/f}$) or intestinal epithelium specific deletion of ERBB3, using the conditional knockout allele of *Erbb3*^{tm1Dwt2} (also called *Erbb3*^{f}) (*Apc*$^{Min/+}$, *Erbb3*$^{f/f}$, *Tg(Vil1-Cre)*) . On the C57BL/6J (B6) background, *Apc*$^{Min/+}$ mice lacking ERBB3 in the intestinal epithelial showed increased number of polyps, surprisingly the polyps developed without ERBB3 were significantly smaller than polyps with normal levels of ERBB3. At three months of age, all *Apc*$^{Min/+}$ mice examined (n=30 ERBB3 wild-type, n= 15 ERBB3 deficient) developed visible polyps (>0.3mm in diameter) in the small intestine regardless of ERBB3 genotype. Interestingly, the number of intestinal polyps in *Apc*$^{Min/+}$ mice lacking ERBB3 were significantly increased compared with that in *Apc*$^{Min/+}$ controls (Figure 3-1A left). This ERBB3-dependent increase in the number of small intestine polyps was observed in all regions of the small intestine. Whereas 66% of the *Apc*$^{Min/+}$ control mice developed at least one colon polyp, all *Apc*$^{Min/+}$ lacking ERBB3 developed at least two colon polyps (Figure 3-1A middle). Altogether, these results demonstrated that epithelial-specific ERBB3 signaling is important during intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. This observation is contradictory to a previous study showing that in the absence of ERBB3, *Apc*$^{Min/+}$ had a reduced number of intestinal polyps (Lee, Yu et al. 2009). The fact that these two studies

were done using two different mouse strains suggested a strain-dependent effect of ERBB3 in the development of intestinal tumors in mice.

To assess the strain effect of ERBB3 in the development of intestinal tumors, a 129/B6 F1 population of *Apc$^{Min/+}$, Erbb3$^{f/f}$, Tg(Vil1-Cre)* was compared to littermates with wild-type ERBB3 (*Apc$^{Min/+}$, Erbb3$^{f/f}$)*. Surprisingly, there was no significant difference in the number of intestinal and colon polyps that developed in the absence of ERBB3 in the F1 population (Figure 3-1B left, middle). When crossed back to the 129 background mice for greater than three generations, we observed a decreased in the number of intestinal polyps compared to wild-type littermates, a consistent result with the previous study that used a 129/B6 mix background (Figure3-1C). Despite differences in tumor number, the size of ERBB3-deficient intestinal polyps was reduced in all three models (Figure 3-1 right panels). Histological analysis of size-matched polyps did not reveal morphological differences related to ERBB3 genotype (data not shown).

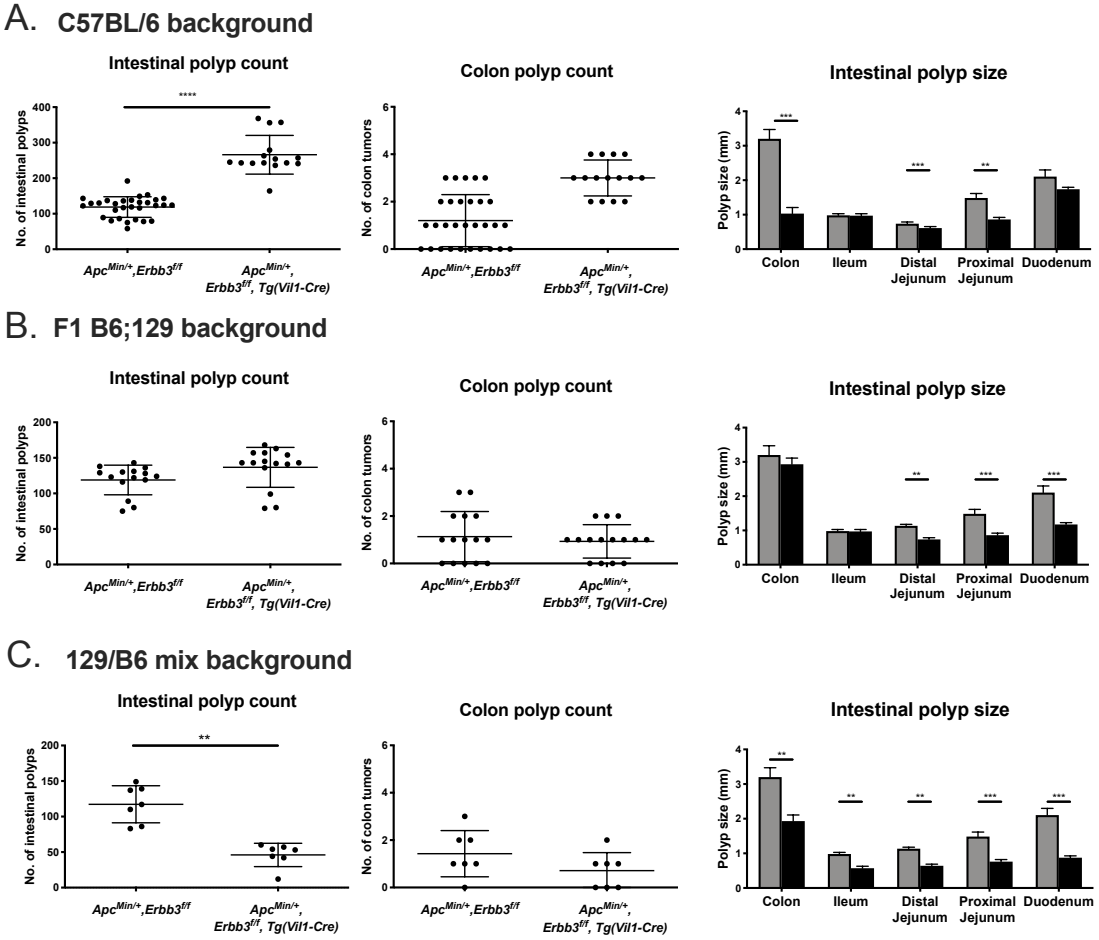
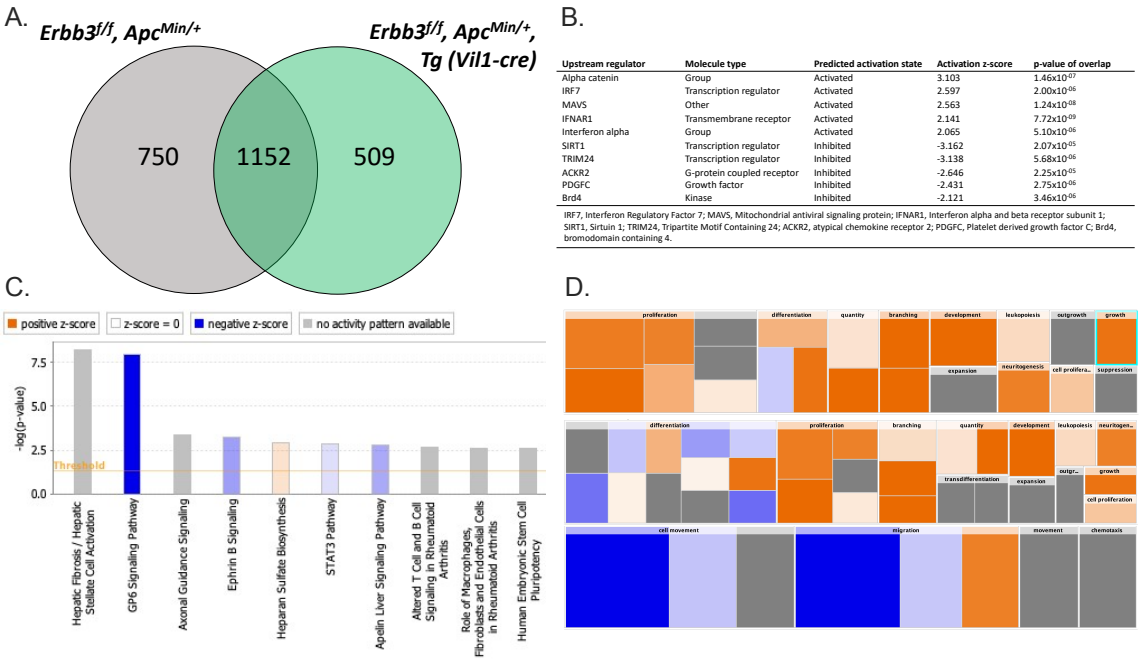


Figure 3-1 The effect of ERBB3 on intestinal polyp development

(A). The effect of ERBB3 on intestinal (left) and colon (middle) polyps' multiplicity and intestinal tumor size (right) in *Apc*$^{Min/+}$ mice on C57BL/6J background. (B). The effect of ERBB3 on intestinal (left) and colon (middle) polyps' multiplicity and intestinal tumor size (right) in *Apc*$^{Min/+}$ mice on B6/129 F1 background. (C). The effect of ERBB3 on intestinal (left) and colon (middle) polyps' multiplicity and intestinal tumor size (right) in *Apc*$^{Min/+}$ mice on 129/B6 mix background. Each dot represents the polyp number in each 100-day-old mice. Grey bars represent the mean polyp size of *Erbb3*WT (*Apc*$^{Min/+}$, *Erbb3*$^{f/f}$) and black bars represent the mean polyp size of *Erbb3*CKO (*Apc*$^{Min/+}$, *Erbb3*$^{f/f}$, *Tg(Vil1-Cre)*). * *p-value*<0.05, ** *p-value*<0.01, *** *p-value*<0.001, **** *p-value*<0.0001

3.4.2. ERBB3 signaling prevents apoptosis in *Apc*$^{Min/+}$ polyps

To determine the mechanisms underlying the increase of intestinal polyp number in the absence of ERBB3 in the intestinal epithelium on B6 mice, we analyzed transcriptomic data from colon polyps lacking ERBB3 compared to polyps from littermates with wild-type levels of ERBB3. We found 509 genes characteristic of ERBB3-deficient colon polyps (Figure 3-2A). Ingenuity pathway analysis (IPA) predicted several upstream regulators (Figure 3-2B) and canonical pathways (Figure 3-2C) that characterize ERBB3-deficient intestinal polyps including downregulation of MAPK1 (Supplementary Figure 3-1). Impairment of specific molecular and cellular functions such as cellular movement/migration (downregulated), cellular development and cellular growth (upregulated, and proliferation (upregulated) (Figure 3-2D) was also predicted using IPA. Differentially expressed genes involved in these cellular functions (*Socs3, Fas, Tcf3, IL15, Apoe*) were validated by quantitative PCR (qPCR) (Figure 3-3).



Upstream regulator	Molecule type	Predicted activation state	Activation z-score	p-value of overlap
Alpha catenin	Group	Activated	3.103	$1.46x10^{-07}$
IRF7	Transcription regulator	Activated	2.597	$2.00x10^{-06}$
MAVS	Other	Activated	2.563	$1.24x10^{-08}$
IFNAR1	Transmembrane receptor	Activated	2.141	$7.72x10^{-09}$
Interferon alpha	Group	Activated	2.065	$5.10x10^{-06}$
SIRT1	Transcription regulator	Inhibited	-3.162	$2.07x10^{-05}$
TRIM24	Transcription regulator	Inhibited	-3.138	$5.68x10^{-06}$
ACKR2	G-protein coupled receptor	Inhibited	-2.646	$2.25x10^{-05}$
PDGFC	Growth factor	Inhibited	-2.431	$2.75x10^{-06}$
Brd4	Kinase	Inhibited	-2.121	$3.46x10^{-06}$

IRF7, Interferon Regulatory Factor 7; MAVS, Mitochondrial antiviral signaling protein; IFNAR1, Interferon alpha and beta receptor subunit 1; SIRT1, Sirtuin 1; TRIM24, Tripartite Motif Containing 24; ACKR2, atypical chemokine receptor 2; PDGFC, Platelet derived growth factor C; Brd4, bromodomain containing 4.

Figure 3-2 Transcriptomic analysis of ERBB3-deficient intestinal polyps
(A). Venn diagram of differentially expressed genes between Erbb3-wild-type (*Apc*$^{Min/+}$, *Erbb3*$^{f/f}$) and ERBB3-deficient (*Apc*$^{Min/+}$, *Erbb3*$^{f/f}$, *Tg(Vil1-Cre)*) intestinal tumors. (B) Top upstream regulators characterizing ERBB3-deficient intestinal polyps. (C). Significant canonical pathways identified from IPA to be deregulated in ERBB3-deficient intestinal polyps. (D). Top molecular and cellular functions altered in ERBB3-deficient intestinal polyps.

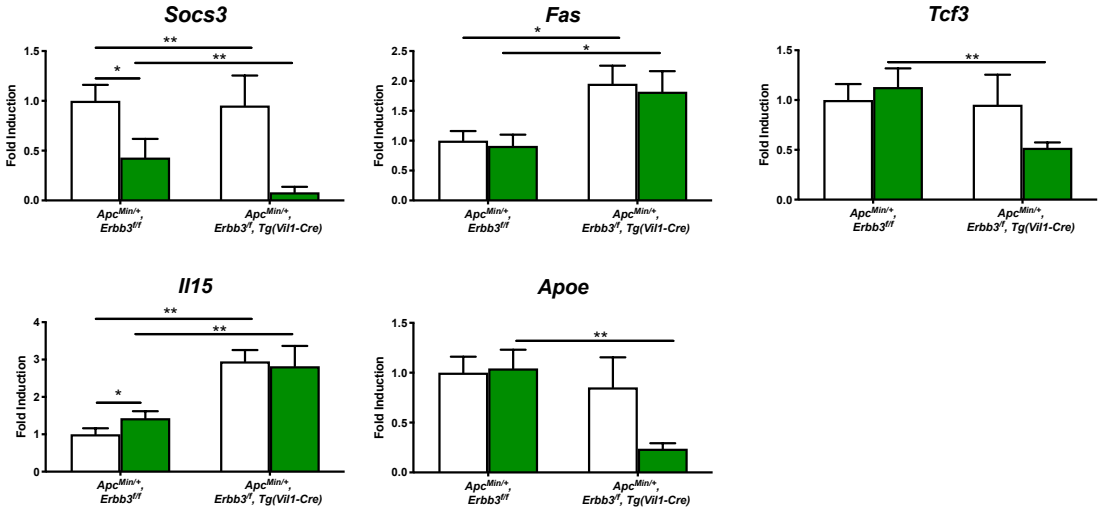


Figure 3-3 Validation of differentially expressed genes in ERBB3-deficient intestinal polyps

White bars represent the mean transcript level of specific genes in the adjacent normal tissue adjacent to tumor, green bars represent the mean transcript level of specific genes in the colon tumor with different *Erbb3* genotype. * *p-value*<*0.05*, ** *p-value*<*0.01*, *** *p-value*<*0.001*, **** *p-value*<0.0001

The proliferative and apoptotic rates within $Apc^{Min/+}$ polyps were measured to validate the IPA prediction. By staining with the proliferation marker Ki67, a slight increase in the proliferation of tumor cells as well as normal epithelium of ERBB3-deficient tissue was observed when compared to tumor and normal cells with normal levels of ERBB3 (Figure 3-4). The apoptotic rate of polyps was measured through TUNEL assay and a significant increase in the number of TUNEL-positive cells was observed in polyps from $Apc^{Min/+}$, $Erbb3^{f/f}$, *Tg(Vil1-Cre)* mice compared with polyps from $Apc^{Min/+}$, $Erbb3^{f/f}$ control mice (Figure 3-4). These results suggest that the increase in the number of polyps is due to accelerated proliferation of the normal epithelium.

Additionally, the reduced tumor size caused by epithelial deletion of ERBB3 is due to an elevated level of apoptosis, indicating that ERBB3 provides a survival signal for intestinal tumor cells.

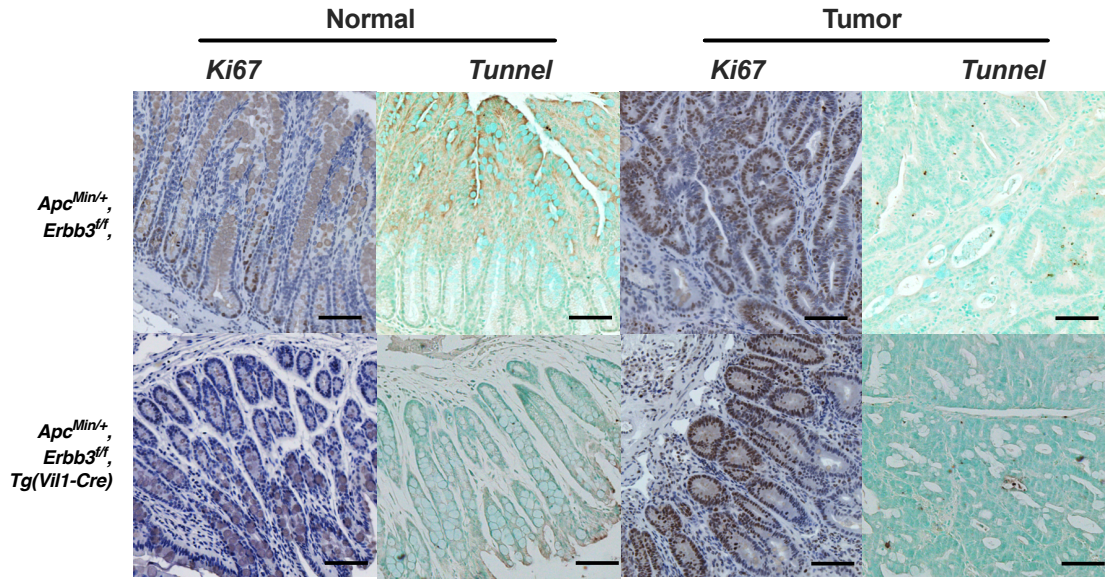


Figure 3-4 Effect of ERBB3 deficiency in proliferation and apoptosis.
Ki67 marker was used to detect number and location of proliferative cells. TUNNEL assay showed the number of apoptotic cells in colon normal and tumor tissue with different *Erbb3* genotype.

Previous study of ERBB3-deficient polyps with a mix 129/B6 background indicated that the number of proliferative cells in the normal epithelium is decreased and they are restricted to the bottom of the crypts (Lee, Yu et al. 2009). In this study, we showed that irrespective of ERBB3 genotype, Ki67 positive cells were increased in $Apc^{Min/+}$ polyps with no significant difference between groups. An increase in the number

of TUNEL-positive cells was observed in polyps from *Apc*$^{Min/+}$, *Erbb3*$^{f/f}$, *Tg(Vil1-Cre)* mice compared with polyps from *Apc*$^{Min/+}$, *Erbb3*$^{f/f}$ control mice.

3.4.3. ERBB3 is required in a subset of colon tumors

To confirm the dependency on ERBB3 signaling in the development of intestinal polyps observed in the *Apc*$^{Min/+}$ model, we also investigated the effect of ERBB3-deficiency on a mouse model of colitis-associated colorectal tumorigenesis (CAC) induced by the azoxymethane (AOM). In this study, we used the conditional knockout allele of *Erbb3*^{tm1Dwt2} (also called *Erbb3*^{f}) in combination with Cre-recombinase under the expression of the Villin promoter, *Tg(Vil1-Cre)*. The Rosa26 conditional reporter allele R26R (*R26R*^{f}) was used to verify that the expression of Cre-recombinase is restricted to epithelial cells. Similar to the *Apc*$^{Min/+}$ model, a significant increase in tumor multiplicity was observed in the absence of ERBB3 in the intestinal epithelium on a C57BL/6J background (Figure 3-5). More than half (53%) of ERBB3 wild-type mice (*Erbb3*$^{+/+}$, *R26R*$^{f/f}$, *Tg(Vil1-Cre)*) treated with AOM developed one or more colonic tumors, with an average of 1.7 tumors per mouse. ERBB3-deficiency (*Erbb3*$^{f/f}$, *R26R*$^{f/f}$, *Tg(Vil1-Cre)*) increased the susceptibility to AOM. About 90% of *Erbb3*CKO mice treated with AOM developed colonic tumors, with an average of 4.2 tumors per mouse. In contrast to the size-effect on residual polyps observed in the *Apc*$^{Min/+}$ mouse model, there was no significant difference in the size of AOM-induced colonic tumors between the two groups (3.65 ± 1.6 vs. 3.58 ± 1.1; p =0.92). These findings indicate that ERBB3 signaling contributes to intestinal and colonic tumors irrespective of the model.

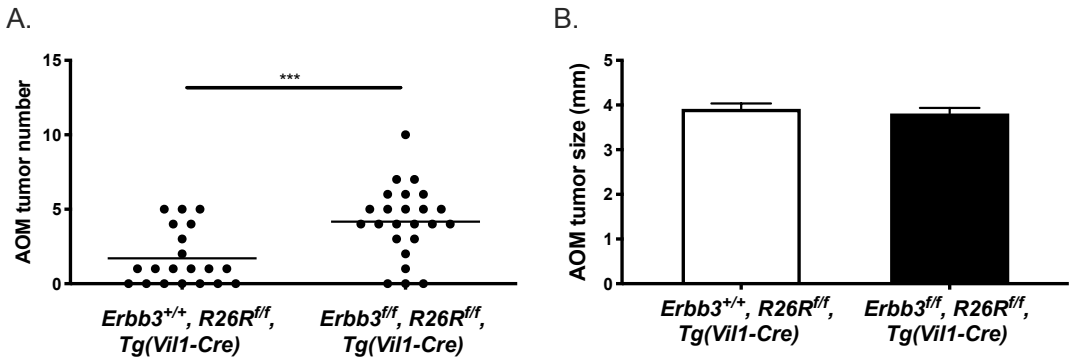


Figure 3-5 Effect of ERBB3-deficiency on AOM-induced colonic tumor development (A) Colonic tumor multiplicity and (B) colonic tumor size in AOM-treated ERBB3 wild-type and ERBB3-deficient mice. Each dot represents the number of colon tumors after induction by AOM. White bars represent the mean tumor size of *Erbb3*WT colon tumors and black bars represent the mean tumor size of Erbb3CKO colon tumors induced by AOM. * *p-value*<*0.05*, ** *p-value*<*0.01*, *** *p-value*<*0.001*, **** *p-value*<0.0001

3.4.4. The effect of EGFR-ERBB3 signaling in the development of intestinal polyps

The strain-dependent effect of ERBB3-deficiency in $Apc^{Min/+}$ mouse model suggests a critical role of EGFR-ERBB3 signaling in the development of intestinal polyps. To investigate the effect of EGFR-ERBB3 heterodimers, we generated a double intestinal-specific deletion of EGFR and ERBB3 in the $Apc^{Min/+}$ model ($Apc^{Min/+}$, *Erbb3*$^{f/f}$, *Egfr*$^{f/f}$, *Tg(Vil1-Cre)*). Surprisingly, fewer than expected double knockout animals were obtained and survived 100 days. In fact, only 5 out of 100 animals had the desired genotype, and 3 of them survived the time of study. Double knockout animals appeared to be anemic, denoted by clear white paws and tail, and future studies are needed to identify the mechanisms underlying this phenotype. When compared to wild-type littermates, fewer and smaller intestinal polyps were found in *Egfr*CKO+*Erbb3*CKO animals.

Taken together, these results showed that ERBB3 plays an important role in intestinal tumorigenesis and that this role is dependent on heterodimerization with EGFR.

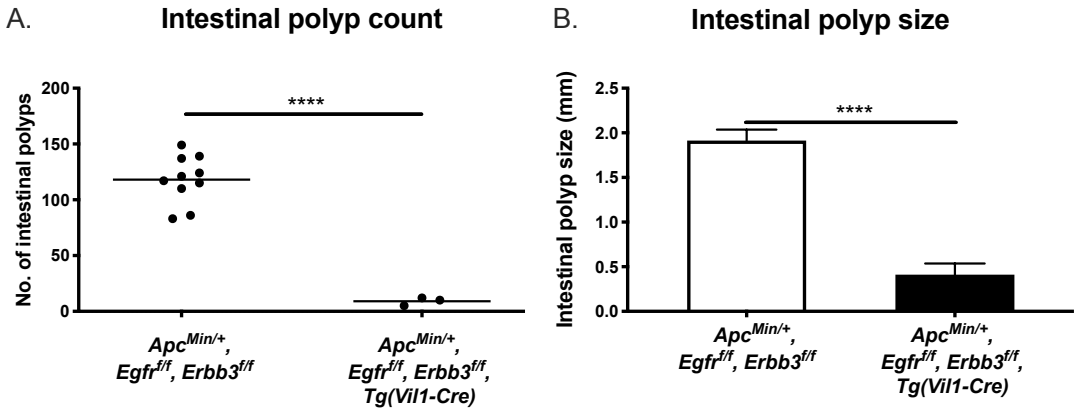


Figure 3-6 The effect of EGFR-ERBB3 signaling on intestinal polyp development
(A) EGFR-ERBB3 double knockout reduced the number of intestinal polyps developed on $Apc^{Min/+}$ mice. Each dot represents the number of colon tumors after induction by AOM. (B). EGFR-ERBB3 double knockout reduced intestinal polyps' size on $Apc^{Min/+}$ mice. White bars represent the mean tumor size of *Erbb3*WT colon tumors and black bars represent the mean tumor size of Erbb3CKO colon tumors induced by AOM. * *p-value*<0.05, ** *p-value*<0.01, *** *p-value*<0.001, **** *p-value*<0.0001

3.4.5. Discussion

ERBB3 is the only member of the ERBB family that lacks intrinsic kinase activity. Accumulating evidence suggests that activation of ERBB3-dependent pathways can modulate tumor phenotypes (Xue, Liang et al. 2006, Lee, Ma et al. 2014). By using an intestinal-specific deletion of ERBB3, we were able to examine intestinal polyp development in a genetic environment deficient in ERBB3 activity. In the $Apc^{Min/+}$ mouse model of FAP, ERBB3 deficiency had a profound effect on polyp number, increasing the mean tumor number on a B6 background (Figure 3-1A), while we did not observe an effect of ERBB3 deficiency in a F1 B6/129 background (Figure 3-1B), ERBB3-deficiency significantly decreased the number of intestinal tumors on a mix B6/129 background (Figure 3-1C). We demonstrated a strain-dependent effect of ERBB3 in the development of intestinal tumors (Figure 3-1), and a possible effect of ERBB3-EGFR heterodimers in the strain-dependent effect of ERBB3, consistent with previous research showing a strain-dependent effect of EGFR in intestinal tumorigenesis (Rinella and Threadgill 2012).

The role of ERBB3 in tumor development was confirmed by utilizing the AOM mouse model of colon cancer (Figure 3-5). Additionally, ERBB3-deficiency significantly decreased the average size of $Apc^{Min/+}$ polyps independent of the genetic background (Figure 3-1). The effect of ERBB3 in the reduction of intestinal polyps was opposite to the effect of EGFR in the size of intestinal and colon tumors. Previously, Roberts et al., demonstrated that reduced EGFR promoted the development of larger intestinal tumors (Roberts, Min et al. 2002). By using a conditional knockout allele of EGFR, we also confirmed that the EGFR-independent intestinal and colonic tumors are bigger,

highlighting the unique role of ERBB3-dependent signaling in regulating tumor growth. To our knowledge, we provide the first direct evidence that the effect of ERBB3 in the development of CRC is genetic background-dependent, which could have major implications for ERBB3 inhibitor therapy in the clinic.

A higher number of apoptotic cells were detected in the ERBB3-deficient polyps from $Apc^{Min/+}$ mice on B6 background (Figure 3-4), demonstrating an important role for ERBB3 in tumor cell survival. Transcriptomic analysis predicted that ERBB3-deficient polyps have decreased levels of p42/44 MAPK activation, which is the predominant mitogenic signal. Our results indicate that ERBB3 signaling contributes to tumor growth by increasing cell proliferation (Figure 3-4). In addition, ERBB3 can couple EGFR to the PI3K/AKT upon growth factor stimulation activating unique signaling (Schoeberl, Pace et al. 2009, Huang, Li et al. 2013).

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 MAPK pathway to promote cell proliferation (Supplementary Figure 3-1). EGFR also activate PI3K/AKT through association with the adaptor protein GAB1 in $Apc^{Min/+}$ polyps (Moran, Hunt et al. 2004). It is possible that PI3K/AKT is activated by EGFR via two mechanisms, association with GAB1 and coupling with ERBB3. Based on previous data and our results, we propose that ERBB3 activation of PI3K/AKT is one of the mechanisms in $Apc^{Min/+}$ polyps. The decrease of polyp number and polyp size in the double knockout of ERBB3 and EGFR suggest these two receptors are essential in the development of intestinal polyps. However, more data should be collected to determine

if this is a direct effect of deficiency of the receptors or an indirect effect of changes in intestinal morphology. 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 number of polyps were increased. 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, Park 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 intestinal polyps can grow independently of ERBB3, similar to previous results observed for EGFR (Roberts, Min et al. 2002).

In this study, we observed a profound strain effect in the tumorigenesis effect of ERBB3. This robust tumor activity of targeting ERBB3 may result from its unique link to PI3K/AKT and its downstream effector MAPK. Furthermore, as ERBB3 partners with EGFR/ERBB2/ERBB4 and triggers essential signaling downstream, a lack of ERBB3 would abolish EGFR/ERBB3, ERBB2/3, and ERBB3/4 heterodimers simultaneously, which may contribute to the antitumor effects. The decreased transcript levels of ERBB4 in ERBB3-deficient intestinal tumors suggests that elevated apoptosis may be due to loss of ERBB3-ERBB4 heterodimers. Previous studies suggested that ERBB3-ERBB4 heterodimer–dependent AKT pathway activation may be required to prevent colon cancer cell apoptosis (Lee, Yu et al. 2009). 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 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.

4. CONCLUSIONS AND FUTURE DIRECTIONS

ERBB family members (epidermal growth factor receptor (EGFR) or ERBB1, ERBB2, ERBB3, and ERBB4) have been found to be essential for cancer cell proliferation and survival in several tumor types (Roskoski 2014, Jacobi, Seeboeck et al. 2017). Tumor cells showed unique mutation and expression profiles of ERBB genes (Faber, Wong et al. 2010, Settleman 2012). Epigenetic alterations in ERBB signaling have a specific impact on cancer cell differentiation, proliferation, migration, and survival (Ljuslinder, Malmer et al. 2009). In the last few year, ERBB family members have been described as important biomarkers and drug targets for precision therapy (Fiske, Threadgill et al. 2009, Hynes and MacDonald 2009, Tebbutt, Pedersen et al. 2013). The inhibition of specific ERBB proteins using tyrosine kinase inhibitors (TKIs) or monoclonal antibody (mAb) has been proven to be sufficient to cause cancer cell death in specific types of cancer (Albanell and Gascon 2005, Roy and Perez 2009). However, high levels of resistance to anti-EGFR is observed in colorectal cancer (CRC) patients. Furthermore, resistance to treatment has been associated with mutations of ERBB family members (Presutti, Santini et al. 2015, Nagel, Semenova et al. 2016, Rossi, D'Argento et al. 2016, Shi, Zhang et al. 2016, Vavala, Follador et al. 2016, Wang, Cang et al. 2016, Kobayashi, Azuma et al. 2017, Normanno, Maiello et al. 2017), suggesting that in some cancers the survival of the tumor cells is strictly dependent on the mutant or overexpressed ERBB family receptor.

EGFR is the prototypical member of the ERBB family and has been related with a role in tumor development. Elevated levels of EGFR correlates with disease progression,

metastatic spread and poorer prognosis (Fakih and Wong 2010). However, EGFR targeted therapies in the treatment of CRC have achieved only 10% of the objective response rate (ORR) (Saltz, Meropol et al. 2004, Van Cutsem, Köhne et al. 2011, Troiani, Napolitano et al. 2016). More than half of CRC patients show heterogeneity in genetic EGFR alterations such as somatic mutations and gene copy number variations, both of which have been shown to negatively affect response to the mAbs cetuximab and panitumumab (Martins, Mansinho et al. 2018). 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 much remains to be discovered about the mechanisms underlying tumor response to EGFR-targeted therapies. In this dissertation, we have shown that one area not previously investigated is the existence of CRCs that arise independent of EGFR, which could be a major cause of primary resistance irrespective of other cooperating mutations.

The association of the other ERBB receptors in the progression of CRC and the resistance to anti-EGFR treatment has been described in the literature. ERBB2 amplifications are present only in 4% of mCRC patients. However, despite the low percentage of ERBB2 alterations in CRC, ERBB2 amplifications result in resistance to anti-EGFR antibodies. Patients with RAS/BRAF wild-type tumors show an enrichment of ERBB2 mutations (Cancer Genome Atlas 2012, Richman, Southward et al. 2016, Loree, Kopetz et al. 2017), and they are correlated with resistance to anti-EGFR therapy in *KRAS-WT* patients (Bertotti, Migliardi et al. 2011). Among the solid malignancies, CRC presents the highest rate (6.3%) of ERBB3 mutation (Lee, Yu et al. 2009, Lee, Ma

et al. 2014, Sartore-Bianchi, Trusolino et al. 2016). ERBB3 overexpression has been associated with concomitant increase in ERBB2 levels (Maurer, Friess et al. 1998, Lee, Yu et al. 2009, Watanabe, Yonesaka et al. 2019) and more severe clinical outcome (Zhao, Pan et al. 2016). Additionally, inhibition of ERBB3 with TKIs is non-effective, as ERBB3 is missing the kinase domain (Zhang, Chang et al. 2016). The involvement of ERBB4 in carcinogenesis has been less characterized so far (Rudloff and Samuels 2010, Lau, Killian et al. 2014). In 2015, Williams et al., reported that ERBB4 is over-expressed in human CRC, and in experimental systems ERBB4 enhanced the survival and growth of cells driven by Ras and/or WNT signaling (Williams, Bernard et al. 2015). However, intense research efforts are still necessary to understand the complex interaction of ERBB receptor and their role with CRC and resistance to anti-EGFR.

In CRC patients treated with anti-EGFR antibodies, resistance has been linked to three main mechanisms: mutations in the antibody-binding site (Montagut, Dalmases et al. 2012, Arena, Bellosillo et al. 2015), activation of alternative pathways (Yonesaka, Zejnullahu et al. 2011, Bardelli, Corso et al. 2013) or reactivation of downstream signaling (Misale, Arena et al. 2014). In the work presented in this dissertation, we used a multi-faceted approach towards identifying a novel EGFR-independent mechanism responsible for resistance to current treatments. We established the importance of ERBB3 and ERBB4 in intestinal tumorigenesis and suggest that a major role of ERBB4 is to mediate EGFR-independent tumor growth through activation of IL10RA and activation of an anergic state. We also proposed combinatorial and targeted therapies that could improve the response to treatment and to prevent resistance to EGFR inhibitors.

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-dependent signaling in intestinal tumorigenesis using a conditional knockout allele of ERBB3. Unexpectedly, deletion of ERBB3 had a strain-dependent effect on tumor multiplicity and growth in $Apc^{Min/+}$ mouse model. On a C57BL/6J genetic background, we found that ERBB3 increased tumor multiplicity while on a 129S1/SvImJ background, a decrease in the number of intestinal tumors was observed in the $Apc^{Min/+}$. Regardless of the strain, ERBB3-deficient polyps have a reduced size in comparison to those on a wild-type ERBB3 background. These results highlight the critical role of ERBB3, potentially by mediating the PI3K/AKT/MTOR or p42/44 MAPK pathway depending on the genetic background that these tumors develop and changing the proliferation rates. The strain-dependent effect of ERBB3-deficiency in $Apc^{Min/+}$ mouse model suggests a critical role of EGFR-ERBB3 signaling in the development of intestinal tumors. Determination of ERBB3 sensitivity to EGFR inhibitors should be further investigated, since ERBB3 has been proposed as a biomarker to predict sensitivity to EGFR inhibitors in NSCLC, pancreatic, and colon cancer cell lines (Engelman, Zejnullahu et al. 2007, Buck, Eyzaguirre et al. 2008).

Reports on the role of ERBB4 in cancer are contradicting, suggesting pro- as well as anti-tumor effects of ERBB4 depending on cancer subtypes and the ERBB4 isoform expressed (Sundvall, Iljin et al. 2008, Roskoski 2014, Canfield, Li et al. 2015). Previous studies suggest that chronic ERBB4 overexpression in the context of inflammation may contribute to colorectal carcinogenesis (Frey, Hilliard et al. 2010). We confirmed by *in*

vivo and *in vitro* experiments that ERBB4 transcript levels are upregulated in the absence of EGFR. We also showed that the activation of ERBB4 by NRG1 treatment increased proliferation in colon tumor organoids. By ablating ERBB4 in the intestinal epithelium we observed a decreased number of tumors suggesting that ERBB4 may be a valid therapeutic target in colorectal and perhaps other epithelial-based malignancies. However, because of the controversial role of ERBB4 in cancer development, this ERBB-family member is so far not considered a validated therapeutic target, thus no specific ERBB4-targeting antibodies have been tested in clinical trials yet.

Many cases of addiction of the ERBB family have been identified in the last decades (Yan, Parker et al. 2014). Investigating additional aspects of ERBB biology during intestinal tumorigenesis should be continued. The importance of ERBB-dependent signaling in tumor initiation, as well as the spatial-temporal requirements of ERBB during tumor development and progression should be further analyzed. Furthermore, the fact that the knockout of ERBB members have a different effect on tumor progression and development in the $Apc^{Min/+}$ model elucidates the complex and multi-faceted properties of CRC. The differential expression of ERBB family and the high resistance to anti-EGFR treatment in CRC patients suggest that combination therapies might be the most effective treatment in the future (Huang, Wang et al. 2013, Ioannou, Seddon et al. 2013, Noto, De Vitis et al. 2013, Torka, Penzes et al. 2014, Ichihara, Hotta et al. 2015, Ribeiro Gomes and Cruz 2015, De Pauw, Wouters et al. 2016, La Monica, Madeddu et al. 2016, Liu, Kambrick et al. 2016, Zhao, Pan et al. 2016).

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. We clearly demonstrate that colorectal tumors can initiate through an EGFR-independent mechanism, and that EGFR-independent tumors have an accelerated growth rate in two different models, $Apc^{Min/+}$ model and in tumors focally induced by APC deficiency and activated KRAS. The absence of EGFR in these polyps exerts little to no suppression on their growth. Indeed, the growth of these EGFR deficient tumors may even be enhanced. 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. A molecular signature for this subset of EGFR-independent colon tumors suggest that these tumors escape the immune system by enhancing the activation of IL10RA.

The immunosuppressive cytokine IL10 has been associated with poor prognosis in colon cancer (Herbeuval, Lelievre et al. 2004). Although macrophages are involved in antitumor defenses, production of IL10 by tumor cells may permit malignant cells to escape cell-mediated immune defense. Because cross-talk between the tumor microenvironment and tumor cells is critical for cancer development, an experimental model capable of mimicking the tumor environment had to be established to elucidate the role of IL10 in cancer progression. The mechanism of IL10 activation is not well understood, and it remains unclear whether cancer cells secrete IL10 and whether IL10 has an impact on the aggressiveness and malignancy of cancer cells. Cytokines in the tumor stroma critically influence CRC development and progression either by directly

stimulating neoplastic epithelial cells or by altering the function or activity of non-tumor cells in the CRC microenvironment.

Our efforts towards identifying an EGFR-independent signature in CRC are particularly timely and clinically relevant. Our data suggests that tumor cells without EGFR might escape cell-mediated immune defense by increasing production of IL10. Immune response has long been a question of great interest in a wide range of fields such as cancer therapies and anti-tumor immunity through checkpoint inhibitors. Recently, in CRC, immune checkpoint molecules such as programmed cell death-1 (PD-1), a member of the CD28 superfamily of T-cell regulators, PD ligand 1 (PD-L1) have been identified as a possible target for immunotherapy (Xiao and Freeman 2015, Yaghoubi, Soltani et al. 2019). Recent studies in ovarian cancer, showed that IL10 mediates PD-1, and that a combination therapy augmented the anti-tumor response in ID8 ovarian tumor bearing mice; leading to a decrease in tumor mass and a significant increase in survival. In our model, *Egfr*CKO tumors showed upregulation of *Pdcd1* transcript levels compared to normal tissue. Further investigation is needed to validate the relationship between IL10 and PD-1 in CRC.

In this study, we also showed a significant effect of SRC, a member of a superfamily of membrane-associated nonreceptor protein tyrosine kinases, and the development of EGFR-independent colon tumors. It is well known that SRC is stimulated by receptors of growth hormone, cytokines, and adipokines, and it regulates multiple signaling pathways, including PI3K/AKT, MAPK, STAT3, IL8, and VEGF pathways, and cytoskeletal pathways to cause a cascade of cellular responses (Yeatman 2004). Eighty

percent of patients with colon cancer overexpress Src in tumor tissue (Dehm and Bonham 2004). Evidence has shown that the overexpression of Src in colon cancer accelerates metastasis and causes chemotherapeutic drug resistance via multiple downstream signaling pathways (Haegebarth, Bie et al. 2006, Brauer, Zheng et al. 2011). Therefore, the inhibition of Src may be useful for the treatment of colon cancer. However, the inhibition of Src may also weaken immune responses that are essential for the eradication of cancer cells. Overcoming the problem of inhibiting Src in cancer cells while retaining immune system efficacy is the key to the successful application of Src-inhibition therapy. Different Src family members are used by the immune system and colon cancer. This differential use may provide a good opportunity to develop Src family member specific inhibitors to avoid immune inhibition. Targeting a downstream effector of SRC, like STAT3, a transcriptional mediator of oncogenic signaling, could solve some of the problems that arise while inhibiting SRC. However, the development of STAT3 inhibitors remains an active area of research as no inhibitors have yet to be approved for the treatment of CRC or any other cancers.

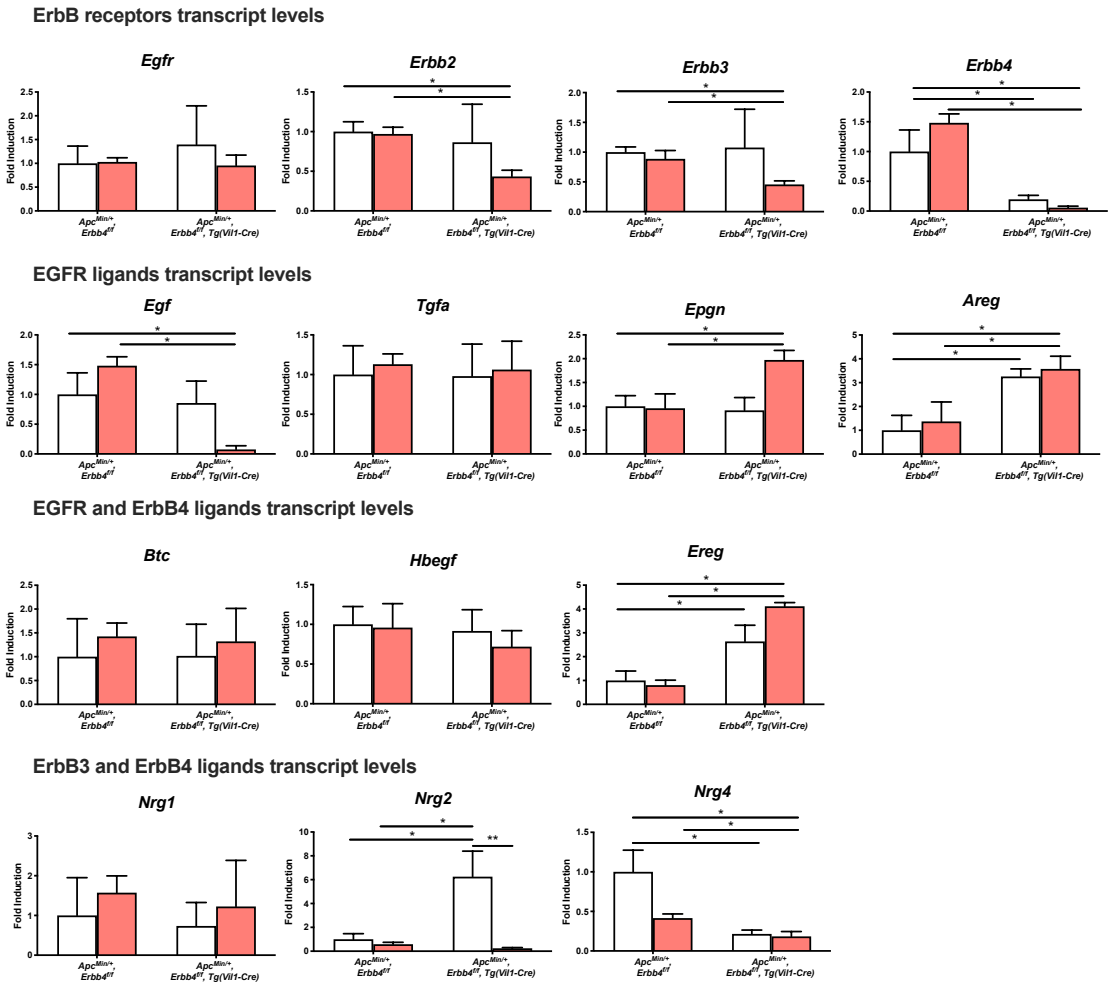
Our results have important implications for EGFR-targeted therapies. Our findings suggest that EGFR inhibitors, either reversible or irreversible, may not be effective in a subset of EGFR-independent tumors that show hyperactivation of IL10RA signaling. Determining whether tumors are EGFR-dependent or independent will improve our ability to make wiser clinical decisions regarding who should receive treatment against EGFR. Combination therapies with IL10 antibodies should be considered for EGFR-independent tumors or in combination with EGFR inhibitors. Additionally, it is important

to determine upstream-receptor(s) that activate the IL10RA pathway in the absence of EGFR to identify additional targets for combinatorial therapy. Of particular interest is the ERBB4 receptor, considering the effect of this receptor in the activation of IL10RA and the elevated levels of ERBB4 transcript in all stages of CRC, including the premalignant adenoma indicating that ERBB4 over-expression is an early event in tumorigenesis.

Finding and targeting the critical driver molecules of CRC is a primary goal of precision medicine (Luo, Solimini et al. 2009, Pagliarini, Shao et al. 2015, Nagel, Semenova et al. 2016). Successful targeting and inactivation of specific driver proteins would cause a systemic failure in tumor cell physiology. A new generation of drugs that selectively target the ERBB oncoproteins might have increased therapeutic efficacy in the clinic (Perez, Crombet et al. 2013, Arteaga and Engelman 2014, Roskoski 2014). 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.

APPENDIX A

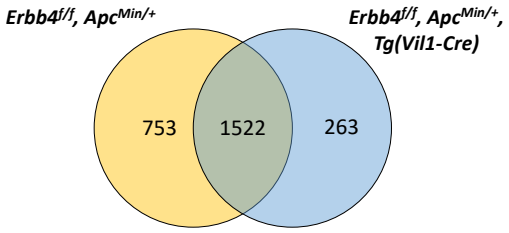
SUPPLEMENTARY FIGURES



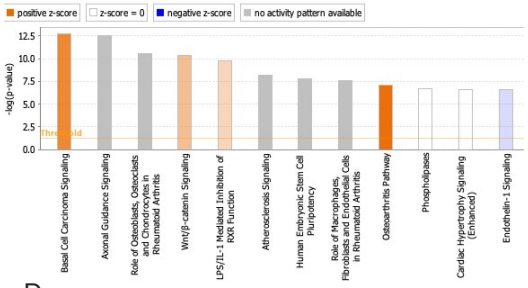
Supplementary Figure 2- 1 Changes in expression of ERBB family members in ERBB4-deficient tumors on *Apc*$^{Min/+}$ mice.

ERBB4-deficient intestinal tumors showed different expression levels on *Apc*$^{Min/+}$ mice compare to *Erbb4*WT tumors. White bars represent the mean transcript level of specific genes in the adjacent normal tissue associated to each tumor type, salmon color bars represent the mean transcript level of specific genes in the intestinal tumors with different genotype. * *p-value*<*0.05*, ** *p-value*<*0.01*, *** *p-value*<*0.001*, **** *p-value*<0.0001

A.



B.

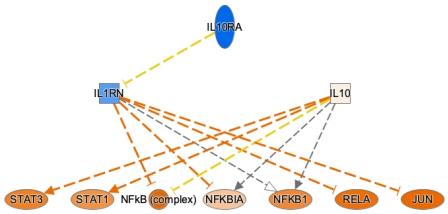


C.

Upstream regulator	Molecule type	Predicted activation state	Activation z-score	p-value of overlap
MYD88	Complex	Activated	4.429	$1.01x10^{-08}$
ERK1/2	Group	Activated	4.028	$7.75x10^{-05}$
CTNNB1	Transcription regulator	Activated	3.845	$1.23x10^{-21}$
ERG	Transcription regulator	Activated	3.845	$1.76x10^{-04}$
CSF2	Cytokine	Activated	3.562	$9.29x10^{-03}$
IL10RA	Transmembrane receptor	Inhibited	-6.870	$2.34x10^{-24}$
HNF4A	Transcription regulator	Inhibited	-4.345	$3.75x10^{-01}$
PPARGC1A	Transcription regulator	Inhibited	-4.271	$9.48x10^{-07}$
DKK1	Growth factor	Inhibited	-4.060	$4.56x10^{-07}$
Alpha catenin	Group	Inhibited	-3.654	$2.20x10^{-09}$

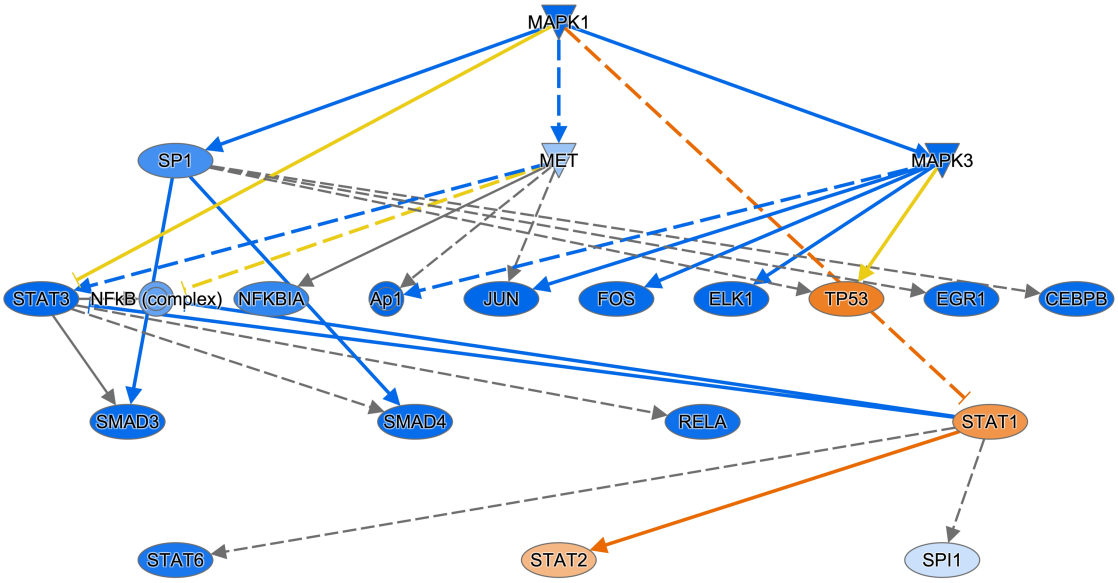
MYD88, Myeloid differentiation primary response 88; ERK, Extracellular-signal-regulated Kinase 1/2; CTNNB1, Catenin Beta 1; ERG, Erythroblast transformation-specific related gene; CSF2, Colony Stimulating Factor 2; IL10RA, Interleukin 10 Receptor Subunit Alpha; HNF4A, Hepatocyte Nuclear Factor 4 Alpha; PPARGC1A, Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha; DKK1, Dickkopf WNT Signaling Pathway Inhibitor 1.

D.



Supplementary Figure 2- 2 Transcriptomic analysis of ERBB4-deficient intestinal polyps.

(A). Venn diagram of differentially expressed genes between *Erbb4*WT (*Apc*$^{Min/+}$, *Erbb4*$^{f/f}$) and *ErbB4*CKO (*Apc*$^{Min/+}$, *Erbb4*$^{f/f}$, *Tg(Vil1-Cre)*) intestinal tumors. (B) Significant canonical pathways identified from IPA to distinguish ERBB4-deficient intestinal tumors. (C). Top upstream regulators characterizing ERBB4-deficient intestinal polyps. (D). Prediction of inhibition of IL10RA in ERBB4-deficient intestinal polyps.



Supplementary Figure 3- 1 Transcriptomic analysis of ERBB3-deficient tumors predicted downregulation of MAPK1 signaling pathway.

APPENDIX B

SUPPLEMENTARY TABLES

Supplementary Table 2- 1 Description of primers of selected genes tested by quantitative real time-PCR to validate EGFR-independent tumor transcriptomic

Gene symbol	Gene name	Gene function	Forward (sequence 5′ → 3′)	Reverse (sequence 5′ → 3′)
Anergy associated genes				
Gbp3	Guanylate Binding Protein 3	Cell signaling	AGGAAACCCTCACTGTTTGG	AGTGAGCCGAGGAATTTCAG
Ikzf1	Ikaros family zinc finger protein	Zinc protein -Transcription factor	CGGGATCCCTTTGAGTGTAA	AGCTCAGGTGGTAACGATGC
Casp3	Caspase 3	Apoptosis-related cysteine peptidase	ACGCGCACAAGCTAGAATTT	CTTTGCGTGGAAAGTGGAGT
Dgka	Diacylglycerol	Diacylglycerol kinase	CTGCCAATCTCAATTGCAC	AGTGCGGCCAAAATAATCAC
Socs2	Suppressor of Cytokine Signaling 2	Negative regulator in the growth hormone/IGF1 signaling pathway.	GTGCAAGGATAAACGGACAG	TCGACAGAAATGCTGCAGAG
FasL	Fas ligand gene	Triggers apoptosis through Fas	GCAAATAGCCAACCCCAGTA	ATTCCAGAGGGATGGACCTT
Grg4	Groucho related gene 4	Groucho transcription factor	TCACTCAAGTTTGCCCACTG	CACAGCTAAGCACCGATGAG
Grail	Gene Related to Anergy in Lymphocytes	Inhibitor of cytokine gene transcription	ATGCAAGAGCTCAAAGCAGGAAGC	GTGCGCAGCTGAAGCTTTCCAATA
Cbl-b	Casitas B-lineage Lymphoma - b	E3 ubiquitin-protein ligase	GCAGCATCATTGACCCTTTCAGCA	ATGTGACTGGTGAGTTCTGCCTGT
ErbB family members				
Egfr	Epidermal Growth Factor Receptor	ErbB tyrosine kinase receptor	GCATCATGGGAGAGAACAACA	CTGCCATTGAACGTACCCAGA
Erbb2	Epidermal growth factor receptor 2	ErbB tyrosine kinase receptor	GAGACAGAGCTAAGGAAGCTGA	ACGGGGATTTTCACGTTCTCC
Erbb3	Epidermal growth factor receptor 3	ErbB tyrosine kinase receptor	TCTGCATTAAAGTCATCGAGGAC	CAGCCGTACAATGTGGGCAT
Erbb4	Epidermal growth factor receptor 4	ErbB tyrosine kinase receptor	TCCCCCAGGCTTTCAACATAC	GCACCCTGAGCTACTGGAG
Egf	Epidermal Growth Factor	Growth Factor ligand	TTCTCACAAGGAAAGAGCATCTC	GTCCTGTCCCGTTAAGGAAAAC
Tnfa	Transforming growth factor –a	Growth Factor ligand	CACTCTGGGTACGTGGGTG	CACAGGTGATAATGAGGACAGC
Areg	Amphiregulin	Growth Factor ligand	GCCTCCGAAGTGTGGTATCC	CCTGGTACTGTCCAAACGCA
Epgn	Epigen	Growth Factor ligand	GGGGGTTCTGATAGCAGTCTG	TCGGTGTTGTTAAATGTCCAGTT
Btc	Betacellulin	Growth Factor ligand	AATTCTCCACTGTGTGGTAGCA	GGTTTTCACTTTCTGTCTAGGGG
Hbegf	Heparin-binding Egf	Growth Factor ligand	CGGGGAGTGCAGATACCTG	TTCTCCACTGGTAGAGTCAGC
Ereg	Epiregulin	Growth Factor ligand	CTGCCTCTTGGGTCTTGACG	GCGGTACAGTTATCCTCGGATTC
Nrg1	Neuregulin1	Growth Factor ligand	TCAGCAAGTTAGGAAACGACAG	ACATAGGGTCTTTCAGTTGAGGC
Nrg2	Neuregulin2	Growth Factor ligand	GGATGGCAAGGAACTCAACC	TCGGCCTCACAGACGTACT
Nrg3	Neuregulin3	Growth Factor ligand	TTACGCTGTAGCGACTGCATC	GCCTACCACGATCCATTTAAGC
Nrg4	Neuregulin4	Growth Factor ligand	CACGCTGCGAAGAGGTTTTTC	CGCGATGGTAAGAGTGAGGA
IL10 signaling				
Il10Ra	Interleukin 10 receptor alpha	Cell surface receptor	GCCCTTCCTATGTGTGGTTTG	TTGAGTTTCCGTACTGTTTGAGG
Il10	Interleukin 10	Anti-inflammatory cytokine	AGTGGAGCAGGTGAAGAGTG	TTCGGAGAGAGGTACAAACG
Socs3	Suppressor of cytokine signaling 3	Negative regulation of cytokines that signal through the JAK/STAT pathway	ATGGTCACCCACAGCAAGTTT	CTGGAGGCGGCATGTAGTG
RNA-sequencing validation				
Aadac	**Arylacetamide deacetylase**	Hydrolase activity and triglyceride lipase activity	TACCGCTTCCAGATGCTATTGA	ACTGATTCCCAAAAGTTCACCAA
Aldh1a1	Aldehyde Dehydrogenase 1 Family Member A1	Oxidoreductase activity and acyl-CoA dehydrogenase activity	ATACTTGTCGGATTTAGGAGGCT	R GGGCCTATCTTCCAAATGAACA
Maob	Monoamine Oxidase B	Protein homodimerization activity and electron transfer activity	ATGAGCAACAAAAGCGATGTGA	TCCTAATTGTGTAAGTCCTGCCT
Sult1c2	Sulfotransferase Family 1C Member 2	Sulfotransferase activity	ATGGCCTTGACCCCAGAAC	TCGAAGGTCTGAATCTGCCTC
Sult1a1	Sulfotransferase Family 1A Member 1	Sulfotransferase activity and flavonol 3-sulfotransferase activity	CAACATGGAGCCCTTGCGTAA	ATGAGCACATCATCAGGCCAG
IL17Ra	Interleukin 17 Receptor A	Proinflammatory cytokine signaling	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
Ndrg4	NDRG Family Member 4	Enhance growth factor signaling	TCCGGGGCTCTCCCAAAGGG	GGCATCCACGTGGCACACCA
Reference genes				
Actb	Beta Actin	Cytoskeletal structural protein	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Tbp	TATA-box-binding protein	General transcription factor	ACCGTGAATCTTGGCTGTAAAC	GCAGCAAATCGCTTGGGATTA
Gusb	Glucuronidase, Beta	Lysosomal exoglycosidase	GGCTGGTGACCTACTGGATTT	GGCACTGGGAACCTGAAGT
Eef2	Eukaryotic Translation Elongation Factor 2	Protein Synthesis	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA
Gapdh	Glyceraldehyde3-phosphate dehydrogenase	Glycolysis pathway enzyme	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

Supplementary Table 3- 2 Description of primers of selected genes tested by quantitative real time-PCR to validate ERBB3-deficient tumor transcriptomic analysis

Gene symbol	Gene name	Gene function	Forward (sequence 5′ → 3′)	Reverse (sequence 5′ → 3′)
ErbB family members				
Egfr	Epidermal Growth Factor Receptor	ErbB tyrosine kinase receptor	GCATCATGGGAGAGAACAACA	CTGCCATTGAACGTACCCAGA
Erbb2	Epidermal growth factor receptor 2	ErbB tyrosine kinase receptor	GAGACAGAGCTAAGGAAGCTGA	ACGGGGATTTTCACGTTCTCC
Erbb3	Epidermal growth factor receptor 3	ErbB tyrosine kinase receptor	TCTGCATTAAAGTCATCGAGGAC	CAGCCGTACAATGTGGGCAT
Erbb4	Epidermal growth factor receptor 4	ErbB tyrosine kinase receptor	TCCCCCAGGCTTTCAACATAC	GCACCCTGAGCTACTGGAG
Egf	Epidermal Growth Factor	Growth Factor ligand	TTCTCACAAGGAAAGAGCATCTC	GTCCTGTCCCGTTAAGGAAAAC
Tnfa	Transforming growth factor –a	Growth Factor ligand	CACTCTGGGTACGTGGGTG	CACAGGTGATAATGAGGACAGC
Areg	Amphiregulin	Growth Factor ligand	GCCTCCGAAGTGTGGTATCC	CCTGGTACTGTCCAAACGCA
Epgn	Epigen	Growth Factor ligand	GGGGGTTCTGATAGCAGTCTG	TCGGTGTTGTTAAATGTCCAGTT
Btc	Betacellulin	Growth Factor ligand	AATTCTCCACTGTGTGGTAGCA	GGTTTTCACTTTCTGTCTAGGGG
Hbegf	Heparin-binding Egf	Growth Factor ligand	CGGGGAGTGCAGATACCTG	TTCTCCACTGGTAGAGTCAGC
Ereg	Epiregulin	Growth Factor ligand	CTGCCTCTTGGGTCTTGACG	GCGGTACAGTTATCCTCGGATTC
Nrg1	Neuregulin1	Growth Factor ligand	TCAGCAAGTTAGGAAACGACAG	ACATAGGGTCTTTCAGTTGAGGC
Nrg2	Neuregulin2	Growth Factor ligand	GGATGGCAAGGAACTCAACC	TCGGCCTCACAGACGTACT
Nrg3	Neuregulin3	Growth Factor ligand	TTACGCTGTAGCGACTGCATC	GCCTACCACGATCCATTTAAGC
Nrg4	Neuregulin4	Growth Factor ligand	CACGCTGCGAAGAGGTTTTTC	CGCGATGGTAAGAGTGAGGA
RNA-sequencing validation				
Fas	TNF receptor superfamily member 6	Identical protein binding	TATCAAGGAGGCCCATTTTGC	TGTTTCCACTTCTAAACCATGCT
Tcf3	Transcription factor 3	DNA-binding transcription factor activity	GGGTGCCAGCGAGATCAAG	ATGAGCAGTTTGGTCTGCGG
IL15	Interleukin 15	Cytokine activity	ACATCCATCTCGTGCTACTTGT	GCCTCTGTTTTAGGGAGACCT
Apoe	Apolipoprotein E	Protein homodimerization activity	CTGACAGGATGCCTAGCCG	CGCAGGTAATCCCAGAAGC
Socs3	Suppressor of cytokine signaling 3	Negative regulation of cytokines that signal through the JAK/STAT pathway	ATGGTCACCCACAGCAAGTTT	CTGGAGGCGGCATGTAGTG
Reference genes				
Actb	Beta Actin	Cytoskeletal structural protein	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Tbp	TATA-box-binding protein	General transcription factor	ACCGTGAATCTTGGCTGTAAAC	GCAGCAAATCGCTTGGGATTA
Gusb	Glucuronidase, Beta	Lysosomal exoglycosidase	GGCTGGTGACCTACTGGATTT	GGCACTGGGAACCTGAAGT
Eef2	Eukaryotic Translation Elongation Factor 2	Protein Synthesis	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA
Gapdh	Glyceraldehyde3-phosphate dehydrogenase	Glycolysis pathway enzyme	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

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