# TALES OF THREE SIGNALING PATHWAYS: EGFR, TGFB, AND WNT SIGNALING IN THE GI TRACT

By

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# CHAPTER I

## THE EGFR, TGFB, AND WNT SIGNALING PATHWAYS

My research journey through the gastrointestinal tract has had its share of twists and turns. Ultimately, it has been educational. During my time in the Coffey lab, I have studied three signaling pathways in the context of three gastrointestinal (GI) disorders: the epidermal growth factor receptor (EGFR), transforming growth factor β (TGFB) family, and canonical WNT signaling pathways in the context of colorectal cancer (CRC) and two premalignant disorders of the GI tract - Ménétrier's disease and Juvenile Polyposis syndrome (JPS). My successes and failures in attempting to gain a better understanding of how these pathways function in human health and disease will serve as the body of my thesis.

Because each of the three pathways is integral to development, normal physiology, and many disease states, a review encompassing all that is known about them is beyond the scope of the present discussion. In this chapter, I will introduce the three diseases I have studied, providing further detail when necessary in the subsequent chapters. I will then provide an overview of the three pathways, focusing on how they are normally activated, the mechanisms by which the signals are transduced inside the cell, and modes of negative regulation. Lastly, given my focus on a family of E3 ubiquitin ligases, I will conclude this chapter by discussing the known roles of ubiquitylation in each of

the pathways.

The chapters following this introduction will focus on my studies of particular pathways. In the second chapter, I will discuss efforts by the Coffey lab to better understand and treat the hyperproliferative gastropathy, Ménétrier's disease, which has been found to be amenable to EGFR signaling blockade by the anti-EGFR monoclonal antibody, cetuximab. I will also discuss my observation that JPS, a gastrointestinal hamartoma syndrome caused by germline inactivating mutations of members of the TGFB family pathway, shares clinical and histological features with Ménétrier's disease, suggesting an antagonistic relationship between the EGFR and TGFB family pathways in the stomach. In the third chapter, I will discuss my findings that suggest NEDD4L, an E3 ubiquitin ligase, may be a tumor suppressor in the colon, which works by inhibiting canonical WNT signaling. In the final chapter, I will propose future directions for both the Ménétrier's disease/JPS and NEDD4L projects.

**Colorectal Cancer**

CRC cancer remains the second leading cause of cancer-related deaths in the United States (Siegel, Naishadham et al. 2013). The majority of CRC is sporadic, resulting from the accumulation of genetic and epigenetic aberrations over time; the lifetime risk of developing CRC in the U.S. is 5% (Hammoud, Cairns et al. 2013). From a genomic standpoint, there are three subtypes of CRC, though there is some crossover between them: chromosomal instability

(CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) (Al-Sohaily, Biankin et al. 2012; Markowitz and Bertagnolli 2009). Approximately 70% of all CRCs are of the CIN subtype. This subtype is characterized by karyotypic abnormalities; these cancers will commonly be aneuplodic, with frequent loss of heterozygosity (LOH) and gene amplification. The MSI subtype occurs when a cell loses the ability to repair mismatches in microsatellites following the loss of mismatch repair enzymes. Consequently, tumor suppressor genes with microsatellites will commonly have frameshift mutations in MSI cancers. Finally, the CIMP subtype is characterized by the hypermethylation of many gene promoters, most importantly those of tumor suppressors, which leads to their transcriptional repression.

A significant proportion (15%) of the total number of CRC cases is due to a hereditary cancer syndrome (Kinzler and Vogelstein 1996). Most commonly, the hereditary syndromes are caused by mutations in either the tumor suppressor adenomatous polyposis coli (APC), which causes familial adenomatous polyposis (FAP), or by mutations in one of a number of mismatch repair enzymes (MSH2, MLH2, MSH6, and PMS2), which underlie hereditary nonpolyposis colon cancer (HNPCC). Each of these syndromes is linked to a germline mutation in a tumor suppressor gene, and is therefore subject to the requirement of a second hit, or somatic inactivation of the other copy of the gene.

FAP is defined phenotypically by hundreds of adenomatous polyps in early adulthood, some of which will progress to CRC (Arvanitis, Jagelman et al. 1990). Patients with FAP typically receive a prophylactic colectomy, as virtually all

patients will develop colon cancer by age 40. HNPCC patients, on the other hand, are generally free of polyps, and have a later onset of cancer with a lower penetrance. Patients with either of these two syndromes are also at increased risk for other cancers (Vasen, Blanco et al. 2013). Patients with FAP are at increased risk for carcinomas of the duodenum and stomach, as well as medulloblastoma. Women with HNPCC have a significant risk (80%) for developing endometrial cancer, while all HNPCC patients are at increased risk for gastric cancer.

As I have performed my graduate work in a lab with a major focus on CRC, it is unsurprising that I would have significant exposure to the three signaling pathways mentioned above when considering the model of colorectal neoplastic progression now referred to as the Vogelgram (Kinzler and Vogelstein 1996; Vogelstein, Papadopolous et al. 2013) (Figure 1). The Vogelgram is a product of the pioneering research in cancer genetics performed in the lab of Bert Vogelstein. His lab monitored the progression from colonic polyp/early adenoma to poorly differentiated, invasive CRC from the standpoint of genetic alterations, charting the most commonly mutated genes and pathways, and the temporal point in progression at which they become altered. They found that aberrant activation of WNT signaling is the predominant first step in colon cancer initiation, which most commonly, as in FAP, is achieved through the loss of functional APC. Mutational inactivation of APC occurs in approximately 80% of sporadic CRCs. In many of the remaining CRC cases, WNT signaling is affected by activating

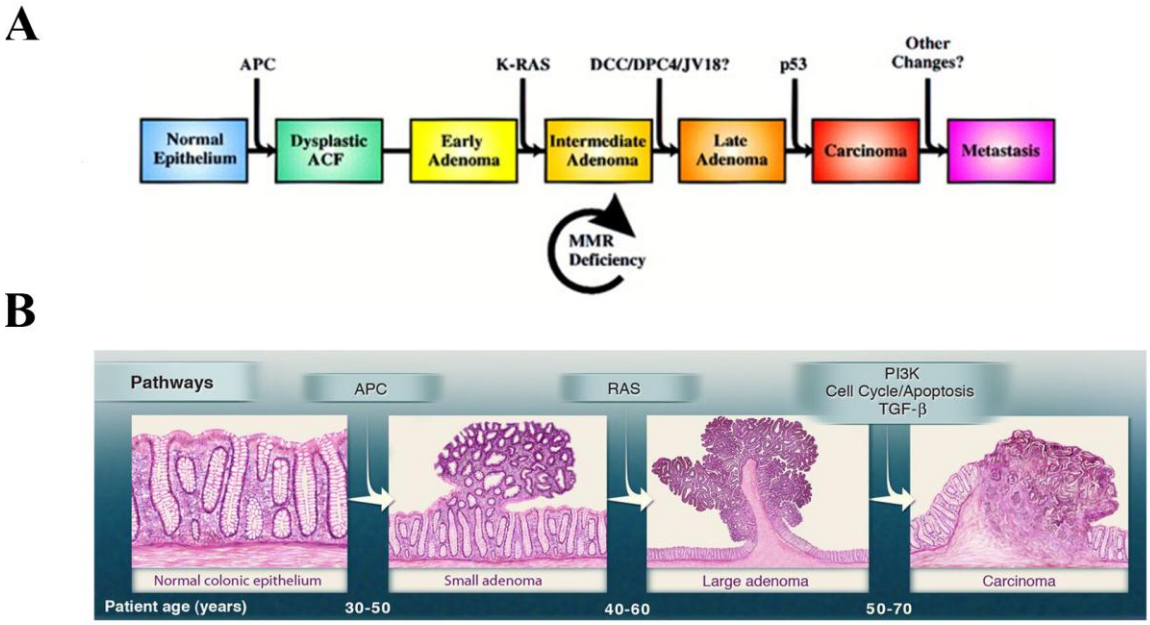



**Figure 1. Sporadic CRC develops in a step-wise manner.** (A) The original model of CRC tumorigenesis (the Vogelgram) was proposed by the Vogelstein lab. It is thought that a single colonic epithelial cell will develop aberrant activity in the WNT signaling pathway, most commonly through the acquisition of a loss-of-function mutation in *APC*. This cell will then begin to outgrow neighboring cells, resulting in a visible adenoma. Once established, the epithelial cells comprising the adenoma will then acquire activating mutations in oncogenic pathways (*KRAS* for example), and inactivating mutations in tumor suppressor pathways (TGFB and p53), ultimately resulting in an invasive carcinoma. (B) Here, the morphological changes that are thought to follow the common tumorigenic mutations of CRC are shown. (Adapted from Kinzler and Vogelstein, 1996; Vogelstein, Papadopoulos et al., 2013).

mutations of β-catenin.

The expression levels or mutational status of central members of the EGFR and TGFB family signaling pathways are also often affected in CRC (Bos, Fearon et al. 1987; Davies, Bignell et al 2002). KRAS and BRAF, downstream mediators of EGFR signaling, are commonly mutationally activated in CRC. The phosphatidylinositol-3-kinase/AKT (PI3K/AKT) arm of EGFR signaling is frequently activated in CRC through activating mutations or overexpression of the p110 catalytic subunit of PI3K (Figure 2) (Zhao and Vogt 2008). This pathway can also become activated through reduced levels or mutations of the PI3K antagonist, phosphatase and tensin homolog (PTEN). The EGFR itself is rarely mutationally activated in CRC, though there are studies suggesting it is overexpressed in 30-90% of CRCs, but this is based only on immunohistochemical staining (IHC) (Saif 2010).

TGFB signaling can be affected in CRC when the intracellular mediators, SMAD 2 and 4, are lost, either through the loss of the chromosomal locus of both (18q.21), or through mutational inactivation (Vogelstein, Papadopolous et al. 2013). The transformimg growth factor-β receptor II (*TGFBRII*) gene, which contains a 10 bp polyadenine tract in the 5' coding region of exon 3, is mutated in 90% of MSI cancers, typically with the addition or subtraction of one adenine, causing an inactivating, frameshift mutation (Grady and Markowitz 2002).

Virtually all CRCs have a mutation in an integral member of one of the three cell signaling pathways, and mutations commonly occur in all three pathways. The general process in the transformative steps from normal colonic

epithelium to adenoma and early carcinoma involves the activation of WNT and EGFR signaling, and the abrogation of TGFB signaling. The caveat is that later in tumor progression, particularly with respect to metastasis, the TGFB pathway can switch from a tumor suppressor to a tumor promoter (Ikushima and Miyazono 2010).

**Ménétrier's disease**

The hyperproliferative gastropathy, Ménétrier's disease, is a rare, acquired, precancerous disorder of the stomach (Coffey and Tanksley 2012). Patients with Ménétrier's disease will commonly present with nausea and vomiting, and edema thought to be due to hypoalbuminemia. On endoscopy, Ménétrier's disease is characterized macroscopically by diffusely enlarged gastric folds. Microscopically, an increase in the number of mucus-producing pit cells, and a loss of acid-producing parietal cells and zymogen-producing chief cells, is seen. This is termed foveolar hyperplasia with glandular atrophy, and explains the tendency for Ménétrier's disease patients to have a basic gastric pH.

A spontaneously remitting form of Ménétrier's disease, which most often occurs in children, has been linked to acute cytomegalovirus infection. However, the pathogenesis of chronic Ménétrier's disease, which I studied, is not well understood, though a significant proportion of patients have coexisting immune-mediated diseases (most commonly ulcerative colitis (UC)) (Fiske, Tanksley et al. 2009). EGFR signaling is thought to play a role in the pathogenesis of

Ménétrier's disease based on the finding that a transgenic mouse engineered to overexpress the EGFR ligand, transforming growth factor-α (TGFA), in the stomach phenocopies the human condition (Dempsey, Goldenring et al. 1992). This has proven to be the case in humans as well, given our success in treating Ménétrier's disease with EGFR blockade. Despite this, no genetic explanation for activated EGFR signaling has been made. And though there are claims of hereditary Ménétrier's disease, these have thus far, upon further investigation, been classified as other disorders, in many cases JPS (Rich, Toro et al. 2010).

**Juvenile Polyposis Syndrome**

JPS is one of the heritable, hamartomatous polyposis syndromes, which is a group of disorders that also includes Peutz-Jeghers syndrome (PJS) and the PTEN hamartoma tumor syndromes (Cowden and Bannayan-Riley-Ruvalcaba syndromes) (Gammon, Jasperson et al. 2009). Common features of these syndromes are GI polyps, a predisposition to cancer, and a mutational linkage to a gene encoding a tumor suppressor. A study of a JPS kindred suggests a lifetime cancer risk of 55%, with a 38% and 21% risk for colorectal and gastric cancers, respectively (Brosens, Langeveld et al. 2011). Individuals with JPS typically present in their late teens or early twenties with rectal bleeding, iron deficiency anemia, and/or abdominal pain, and the mean age at diagnosis of CRC is 43 years. Histologically, juvenile polyps are characterized by a dense, immature stroma, from which the name is derived, with overlying normal-

appearing epithelial cells.

JPS has been linked to germ-line mutations in *SMAD4* and bone morphogenetic protein receptor IA (*BMPRIA*), both members of the TGFB family signaling pathway (Howe, Roth et al. 1998; Howe, Bair et al. 2001). There are generally no extraintestinal manifestations of JPS, though some *SMAD4* mutations are also linked to a syndrome called JP-HHT, in which patients show signs of both JPS and Hereditary Hemorrhagic Telangiectasia (HHT), a vascular disorder most commonly linked to mutations in activin receptor-like kinase 1 (*ALK1*) and endoglin (*ENG*), both components of TGFB signal transduction in endothelial cells (Gallione, Richards et al. 2006).

**The EGFR signaling pathway**

A major goal of the Coffey lab has been to gain a better understanding of the role of the EGFR signaling pathway in the gastrointestinal tract, particularly in the context of CRC. There has been an emphasis on understanding how the seven ligands of the EGFR are transcriptionally and post-translationally regulated, how they are normally trafficked, and what happens physiologically when they are aberrantly expressed or mistrafficked (Fiske, Threadgill et al. 2009). Much of the trafficking work has been done in a battery of human colon cancer cell lines that are capable of forming a uniform polarized monolayer on Transwell filters, thus separating the plasma membrane into an apical and basolateral surface. A more recent focus has been to understand the roles of

negative regulators of EGFR signaling in the context of normal and abnormal gastrointestinal physiology, with particular emphasis on colonic stem cells.

The EGFR pathway is central to human cellular biology, affecting cell proliferation and survival, differentiation, and adhesion and migration, doing so by activating a number of downstream signaling cascades (Yarden and Sliwkowski 2001) (Figure 2). EGFR is an 1186-residue type I transmembrane receptor. It is the first member of the ERBB family of receptor tyrosine kinases, which are homologs of the erythroblastic leukemia oncogene. The family is also known as the human epidermal growth factor receptor (HER) family. The ERBB family contains four members: ERBB-1 (EGFR or HER1), ERBB-2 (HER2), ERBB-3 (HER3), and ERBB-4 (HER4). As EGFR is capable of functionally oligomerizing with any of the other three family members in a ligand-dependent manner, it is perhaps more accurate to call the EGFR signaling pathway the ERBB signaling pathway, though I will refer to it as EGFR signaling throughout (Lemmon and Schlessinger 2010).

There are seven EGFR ligands: epidermal growth factor (EGF), TGFA, amphiregulin (AREG), epiregulin (EREG), betacellulin (BTC), heparin-binding EGF-like growth factor (HBEGF), and epigen (EPGN). Of these, EGF, TGFA, AREG, and EPGN bind strictly to EGFR, while HBEGF, BTC, and EREG can also bind to ERBB-4. Each is synthesized as a type I transmembrane protein, and typically requires cleavage by a matrix metalloprotease to become an active soluble ligand. Cleavage is not always requisite, as membrane-anchored ligand can also be active. In turn, soluble and cell membrane-anchored ligand can

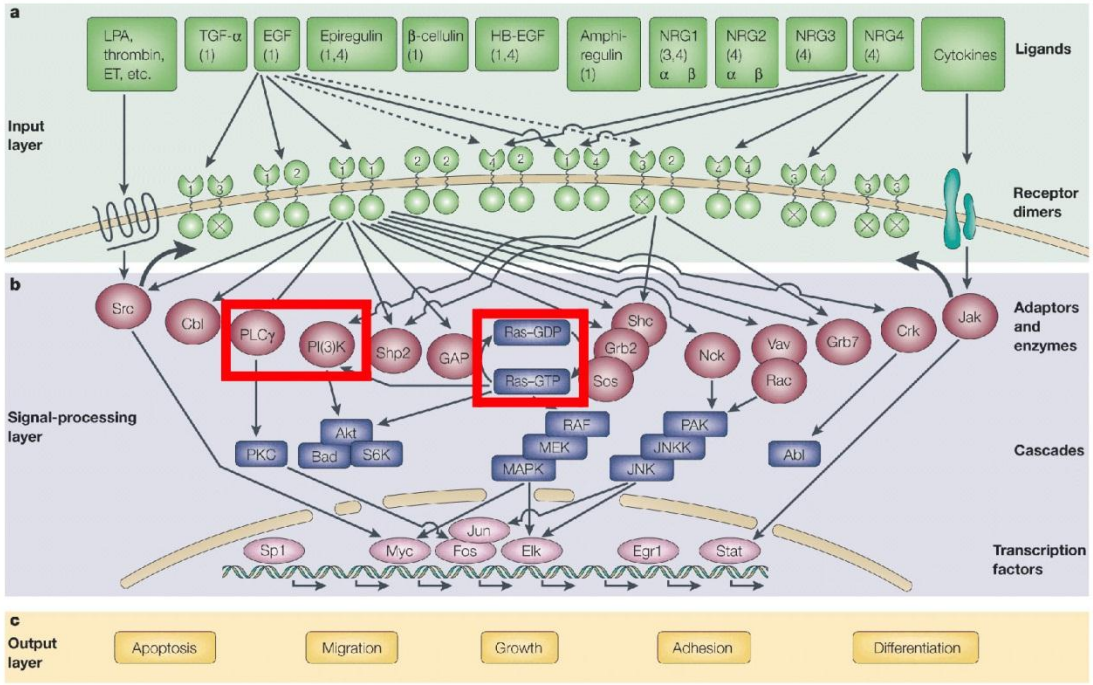



**Figure 2. The EGFR signaling pathway.** Seven mammalian ligands (including Epigen) bind EGFR, represented at the cell surface as (1). Upon ligand binding, EGFR oligomerizes with itself or other ERBBs. Subsequently, there is activation of intrinsic tyrosine kinase activity, transphosphorylation of other ERBB tyrosine residues, binding of SH2-domain-containing proteins, and activation of downstream signaling cascades. Central to CRC are the RAS and phospholipid metabolism pathways, which activate AKT and PKC (red boxes). The transcriptional programs affected by EGFR activation can lead to a number of different physiological outcomes depending upon context (c). However, in CRC, aberrant EGFR activation, or that of downstream targets, results primarily in growth promotion, invasion, or metastasis. (Adapted from Yarden and Sliwkowski, 2001).

induce autocrine, juxtacrine, paracrine, and even endocrine signaling (Singh and Harris 2005). Recently, the discovery of EGFR ligands packaged into exosomes has led to a new form of EGFR signaling termed exosome targeted receptor activation (ExTRAcrine) signaling, in which exosomes expressing ligands on their outer surface activate the EGFR on recipient cells (Higginbotham, Demory Beckler et al 2011).

In addition to the different ways in which the EGFR ligands are trafficked and secreted, there are also subtle differences in the ways in which the EGFR is trafficked following activation by different ligands, and in turn the duration of activity and rate of degradation/recycling of the receptor. A paper comparing the effect of six of the ligands on internalization and degradation of the EGFR found that HBEGF and BTC always cause receptor degradation, and EGF generally causes degradation (Roepstorff, Grandal et al. 2009). Conversely, TGFA, EREG, and AREG lead to complete receptor recycling. These ligand-dependent trafficking consequences, in turn, may help to explain different physiological outcomes following the administration of different ligands. For example, when AREG is administered to the Madin-Darby canine kidney (MDCK) epithelial cell line in culture, it causes a redistribution of E-cadherin and more spindle-like morphology, while TGFA administration does not (Chung, Graves-Deal et al. 2005).

Following ligand binding, EGFR will oligomerize with EGFRs or other ERBBs in a 2:2 ligand-to-receptor ratio (Lemmon and Schlessinger 2010). The exception to this is HER2, which, while being the preferred dimerization partner

of EGFR, has no known ligand (Lonardo, Di Marco, et al. 1990). Essentially, ligand-binding stabilizes the extracellular domain of EGFR in a conformation that allows a functional oligomerization with other ERBBs, though receptors can dimerize in the absence of ligand, presumably by transiently attaining the proper physical configuration to allow for dimerization (Low-Niam, Lidke et al. 2011; Dawson, Berger et al. 2005). The advantage of overcoming this inactive state in cancer is exemplified by the EGFRvIII mutant, commonly found in the human brain cancer, glioblastoma multiforme, which lacks much of the extracellular domain, and is constitutively active (Gan, Kaye et al. 2009).

The cytoplasmic portion of EGFR contains a tyrosine kinase domain that, following ligand-induced oligomerization, transphosphorylates tyrosine residues in the C-terminal domain of its ERBB partner, or a number of cytoplasmic proteins. Of note, ERBB-3 has a less active kinase domain, and has been found to be capable of autophosphorylation, but incapable of transphosphorylation (Shi, Telesco et al. 2010).

The phosphotyrosine residues in the C-termini of the ERBBs serve as docking sites for a number of SH2-domain-containing cytoplasmic proteins, many of which serve as the initial step in the activation of cellular signaling cascades. Additionally, certain phosphotyrosines serve as docking sites for negative regulators. For example, the E3 ubiquitin ligase, CBL, which polyubiquitylates and causes the lysosomal degradation of EGFR, binds to EGFR in a phosphotyrosine-1045-dependent manner (Grøvdal, Stang et al. 2004).

Given the four dimerization partners of EGFR, and the numerous binding

motifs created by phosphorylation of the tyrosine residues in the C-terminal regions of the ERBBs, myriad cellular proteins can bind to the activated ERBBs upon EGFR stimulation. Consequently, many, if not all, cellular signaling pathways can be affected at some level by an activated EGFR. In this review, I will limit the mechanistic discussion to the two best characterized: the RAS/ERK pathway and those pathways related to phospholipid metabolism.

Perhaps the intracellular signaling cascade most commonly tied to the activation of EGFR is the RAS/ERK pathway (Yarden and Sliwkowski 2001). Phosphorylated EGFR initiates activation of the RAS/ERK pathway by serving as a binding site for GRB2. GRB2 is a scaffold protein, which is constitutively bound in the cytoplasm to SOS, a RAS exchange factor. Upon activation of EGFR, the GRB2-SOS complex interacts with EGFR, either directly through an interaction between the SH2 domain of GRB2 and phosphotyrosines 1068 and 1086 of EGFR, or indirectly through a mutual interaction with SHC, which interacts with EGFR through its PTB domain. The recruitment of SOS to the membrane brings it into close proximity with membrane-associated RAS, which allows SOS to activate RAS by exchanging RAS-bound GDP for GTP.

Activation of RAS begins signal propagation to the nucleus via several intermediate kinases. RAS activates the serine/threonine kinase RAF-1. RAF-1 then phosphorylates and activates MEK1/2, also known as mitogen-activated protein kinase (MAPK) kinase, which in turn results in the phosphorylation of ERK1/2, also known as MAPK. Phosphorylated ERK then translocates to the nucleus where it phosphorylates a number of transcription factors, including SP1,

E2F, ELK1, and AP1. Additionally, ERK can activate the p70-S6 kinase in the cytoplasm, stimulating protein synthesis.

The effect of EGFR activation on phospholipid metabolism hinges on the direct activation of two enzymes: Phospholipase C-γ (PLCγ) and PI3K. Like GRB2, PLCγ contains an SH2 domain, and has been found to interact with phosphotyrosines 992 and 1173 of EGFR (McNamara, Dobrusin et al. 1993). Though EGFR phosphorylates PLCγ, it is unclear exactly how it becomes activated, as phosphorylation appears to be dispensible. Once activated, PLCγ catalyzes the conversion of PtdIns(4,5)-$P_2$ to the second messengers 1,2-diacylglycerol (DAG) and inositol 1,3,5-triphosphate ($IP_3$). DAG then serves as an activator of protein kinase C, while $IP_3$ causes the release of intracellular stores of $Ca^{2+}$, which can also activate PKC, as well as the NFκB pathway.

PI3K contains two subunits: the regulatory p85 subunit and the catalytic p110 subunit. The interaction of PI3K with an ERBB is dependent upon an SH2 domain in the p85 subunit. However, the major binding partner of PI3K is ERBB3, which is activated by EGFR upon oligomerization. The p110 subunit of active PI3K then catalyzes the phosphorylation of phosphatidylinositols, most importantly generating PtdIns(3,4,5)$P_3$ (PIP3) from PtdIns(4,5)$P_2$. PIP3 then serves as a binding site for a number of cytoplasmic proteins with lipid-binding domains, thus recruiting them to the membrane. Chief amongst these with respect to the cell biological outcomes of EGFR activation is AKT (PKB) (Burgess 2008). Once at the membrane, AKT is phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK-1). AKT performs its functions in

both the cytoplasm and nucleus, playing a major role in cell survival through inhibition of apoptosis via phosphorylation and inactivation of Bad, promotion of protein translation through mTOR and p70-S6, and, in the nucleus, promotion of cell cycle progression via the eventual suppression of cyclin-dependent kinase inhibitors.

There are a number of negative regulators that oppose EGFR signaling at various levels, some of which are induced following receptor activation. Interestingly, TGFA administration leads to the induction of TGFA transcription, thus setting up a positive feedback loop (Coffey, Derynck et al. 1987). Many of these ligands have also been shown to cross-induce the expression of other EGFR ligands (Barnard, Graves-Deal et al. 1994). At the same time, ligand administration leads to the internalization of degradation of the EGFR, excepting cases of receptor recycling. The degradation of EGFR is thought to be primarily mitigated by the aforementioned E3 ubiquitin ligase, CBL. A secreted protein, Argos, has been found to inhibit EGFR signaling in *Drosophila* by binding and sequestering ligand, though a similar protein in humans remains to be discovered (Klein, Nappi et al. 2004). The tumor suppressor and colonic stem cell marker, leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1), is thought to negatively regulate EGFR signaling by enhancing the recruitment of CBL to the activated receptor (Gur, Rubin et al. 2004). Mitogen-inducible gene 6 (MIG6) and receptor protein tyrosine phosphatase-κ (RPTPκ) affect the ability of EGFR to become active and to stay active, respectively (Zhang, Pickin et al. 2007; Xu, Tan et al. 2005). MIG6 negatively impacts the activity of the kinase

domain through steric hindrance, while RPTPκ dephosphorylates and deactivates the receptor. There are a number of important phosphatases that target the downstream mediators of EGFR signaling. Chief amongst these are the dual specificity phosphatases -5 and -6 (DUSP -5 and -6), PTEN. DUSP-5 and -6 target phosphorylated ERKs (Mandl, Slack et al 2005). PTEN is an important tumor suppressor in CRC, which essentially reverses the action of PI3K, thus decreasing PIP3 levels and inhibiting AKT activity (Shi, Paluch et al. 2012).

**The TGFB/BMP signaling pathway**

Like the EGFR pathway, the TGFB family signaling pathway is a ligand-activated pathway that affects cell proliferation and survival, differentiation, and adhesion and migration. Unlike the EGFR pathway, the intracellular mediators of the TGFB family signaling pathway are not shared with other signaling cascades. It is worth noting that many of the physiological outcomes of TGFB family signaling are opposed to those of EGFR signaling, most notably growth inhibition and cell differentiation versus cell proliferation.

Canonical TGFB family signaling, or SMAD4-dependent signaling, begins when a dimeric ligand (of which there are nearly 40 family members) binds the extracellular domain of a type II receptor dimer, a constitutively active serine/threonine kinase (STK) (Shi and Massagué 2003) (Figure 3). The ligand itself is secreted in a latent, inactive form; extracellular proteases, low pH, or

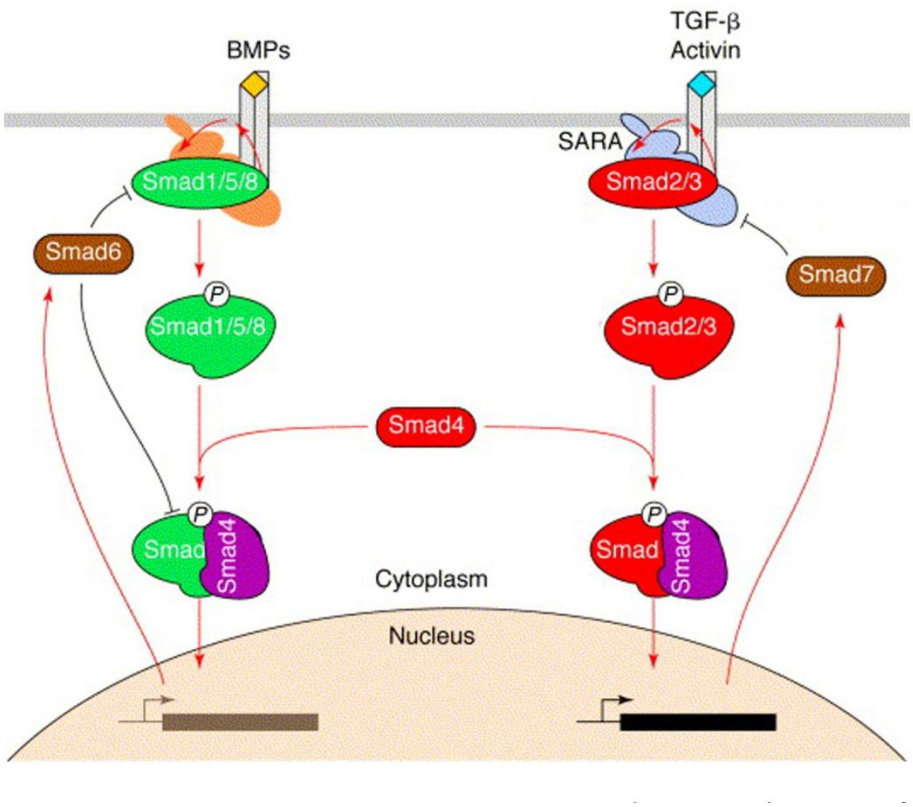



**Figure 3. Canonical TGFB/BMP signaling.** The two major arms of TGFB family signaling are represented here (the BMP and TGFB arms). An active ligand will bind a type II receptor, which is a constitutively active serine/threonine kinase. The type II receptor will then phosphorylate and activate a type I receptor. From there, the signal is passed to the R-SMADs (see text) through phosphorylation. The R-SMADs, which are considered to be BMP arm-specific, are SMAD 1, 5, and 8, while SMAD 2 and 3 are considered specific to the TGFB arm. Activated R-SMADs will then interact with SMAD4, enter the nucleus and initiate a transcriptional program. Two transcriptional targets are shown here: the I-SMADs (SMAD 6 and 7), which inhibit the BMP or TGFB arms, respectively. As stated in the text, there are cases of SMAD4- and SMAD-independent TGFB family signaling, thus making the physiological consequences of TGFB family receptor activation context-dependent. (Adapted from Zhang and Derynck 1999).

interactions with other proteins like thrombospondin or integrins are required to release the active ligand.

The ligand-bound type II receptor complex can then interact with a type I receptor dimer, also an STK, thus activating the type I receptor kinase domain. Five type II and seven type I receptors comprise the TGFB family of receptors. It is not the case that each ligand will interact with each type II receptor. Nevertheless, a given ligand may interact with multiple type II receptors, thus allowing TGFB family signaling to produce many context-dependent, and sometimes unexpected, consequences, which will be discussed further in the context of colon cancer.

From the tetrameric receptor complex, the signal is then passed to receptor-regulated SMAD proteins (R-SMADs: SMAD 1, 2, 3, 5, and 8) via phosphorylation at specific serine residues. This phosphorylation changes the conformation of the R-SMAD, which can then complex with the common-mediator SMAD (SMAD4) in the cytoplasm, translocate to the nucleus, and initiate a cell-type and context-dependent transcriptional program (Siegel and Massagué 2003). It is important to note that SMAD4 is not required for the entry and activity of R-SMADs in the nucleus, which allows for a SMAD4-independent mode of TGFB family signaling (Nakao, Röijer et al. 1997).

Broadly, canonical TGFB family signaling can be divided into two arms: the TGFB and BMP arms. This distinction is based upon differences in the amino acid sequences of the ligands, the different receptors with which they interact, and, in turn, the intracellular pathways activated by one versus the other ligand.

Generally, the TGFB and BMP arms signal through different receptors and cause the phosphorylation and activation of different R-SMADs, with TGFBs activating SMAD 2 and 3 and BMPs activating SMAD 1, 5, and 8. However, there are contexts in which TGFB can lead to the activation of SMAD 1, 5, and 8. In endothelial cells, for example, due to the expression of a different set of type I and II receptors and the coreceptor, ENG, TGFB can elicit activation of the BMP SMADs (Lebrin, Goumans et al. 2004).

Additionally, non-canonical (SMAD-independent) TGFB family signaling has been described (Holm, Habashi et al. 2011). Though both the type I and type II TGFB family receptors are categorized as serine/threonine kinases, they have also been found to auto- and transphosphorylate tyrosine residues. Importantly, the activated type I receptor has been found to directly activate SHCA, a scaffolding protein involved in the RAS/ERK pathway (Lee, Pardoux et al. 2007). More generally, in different contexts and cell-types, TGFB directly activates or inhibits a number of other well-characterized signaling networks, for example the PI3K/AKT pathways (Zhang 2009). A twist on canonical TGFB signaling comes from the observation that in erythrocytes, transcription intermediary factor 1-γ (TIF1γ) can substitute for SMAD4 in an active SMAD complex and lead to differentiation as opposed to growth inhibition (He, Dorn et al. 2006).

The best-characterized negative regulators of TGFB family signaling are the inhibitory SMADS (I-SMADs): SMAD 6 and 7 (Miyazono, Maeda et al. 2005). Each I-Smad is structurally similar to the other R-SMADs and SMAD4 with the

exception of the N-terminus, which is involved in DNA binding. In addition, the I-SMADs lack a DNA binding domain. SMAD6 is thought to compete with SMAD4 for SMAD1 binding, suggesting one role for the I-SMADs is to make the active R-SMADs transcriptionally inactive or less active (Hata, Lagna et al. 1998). Additionally, the I-SMADs have been shown to recruit E3 ubiquitin ligases to activated TGFB family receptors, as well as activated R-SMADs and SMAD4 (Kimaya, Miyazono et al. 2010; Inoue and Imamura 2008).

Other antagonists of TGFB family signaling include noggin, chordin, cerberus, and other ligand-binding agonists, which work by binding up ligand and making it unavailable for receptor interaction (Yanagita 2005). In general, ligand-binding antagonists seem to be most involved in the inhibition of the BMP arm of TGFB family signaling, possibly due to the need to properly control the large-scale gradient of BMP activity during morphogenesis. Inhibition of signaling at the level of the receptor can occur through BMP and activin membrane-bound inhibitor homolog (BAMBI), which is structurally similar to type I receptors, but lacks the intracellular domain (Onichtchouk, Chen et al. 1999). Thus, BAMBI will mimic and block the ability of the type I receptor to interact with a ligand-bound type II receptor, creating a functionally inactive complex that is incapable of phosphorylating the R-SMADs.

In the cytoplasm, the major mediators of TGFB family inhibition are the I-SMADs (SMADs 6 and 7), which can affect both the receptors and the R-SMADs, in some cases through the recruitment of E3 ligases. Another mediator of E3 ligase recruitment to R-SMADs is ERK, which phosphorylates many of the

SMADs in the linker region, creating a point of interaction with members of the NEDD4 family of E3 ligases (Gao, Alarcón et al. 2009; Aragón, Goerner et al. 2011). This family of nine E3 ligases, all of which contain at least two WW domains, interact with the SMADs and type II receptors through their PY motifs or PXpS/pTP motifs. The NEDD4 family will be discussed more thoroughly in Chapter III, as one of the specific foci of my thesis work.

In the nucleus, a number of transcriptional corepressors interact with the SMADs. Chief amongst these are TG-interacting factor (TGIF) and c-Ski, and the c-Ski-related protein, SnoN. These repressors work in a number of ways, but appear to mostly, if not strictly, inhibit the transcriptional activity of the TGFB-related SMADs (2 and 3), and not the BMP-related SMADs (1, 5, and 8). For example, both TGIF and c-Ski have been shown to compete with the transcriptional coactivator p300/CBP for binding to the active R-SMADs. Once bound, they then recruit histone deacetylases (HDACs) to the transcriptional complex, inhibiting transcription (Wotton, Lo et al. 1999).

**The WNT signaling pathway**

WNT signaling is also a well-defined, ligand-activated signaling pathway, with both canonical and non-canonical modes. Similar to the TGFB family signaling pathway, many of the mediators of WNT signaling are specific to the WNT pathway.

There are currently 19 known mammalian WNT genes (Willert and Nusse

2012; Clevers and Nusse 2012). Typical of secreted ligands, they are cysteine-rich, as disulfide links are essential to proper ligand activity. Importantly, they are secreted as hydrophobic proteins due to a palmitoylation on one of these cysteine residues, which is required for both proper trafficking and activity. The process of palmitoylation is performed by the enzyme Porcupine (Porc), which has recently been proposed as a pharmacologic target for WNT-signaling inhibition (Takada, Satomi et al. 2006; Covey, Kaur et al. 2012).

Canonical WNT signaling begins when a WNT ligand interacts with the receptors Frizzled (Fz) and the low density lipoprotein complex (LRP5/6) (Figure 4). Certain WNT ligands (1, 3A, and 8) are thought to preferentially activate the canonical WNT pathway, while others (5A and 11) are thought to preferentially activate non-canonical signaling. Fzs, of which there are ten known genes in humans, are G protein-coupled receptors (GPCRs). All are thought to play a role in WNT signaling, though some are better characterized than others. There are also a number of human LRP genes, though not all appear to be involved in WNT signaling. Further influencing the activation state of the canonical WNT pathway are the proteins leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) and R-Spondin (Barker, van Es et al. 2007; Kim, Zhao et al. 2006). LGR5 is a GPCR receptor, and R-spondin is its secreted ligand. LGR5, as well the closely-related, LGR4, mediate R-spondin signaling via an interaction with the LRP6/Fz5 complex at the surface of the cell. Though not required for canonical WNT signaling, LGR5 and R-spondin enhance WNT3A-induced activation substantially (de Lau, Barker et al. 2011; Glinka, Dolde et al. 2011;

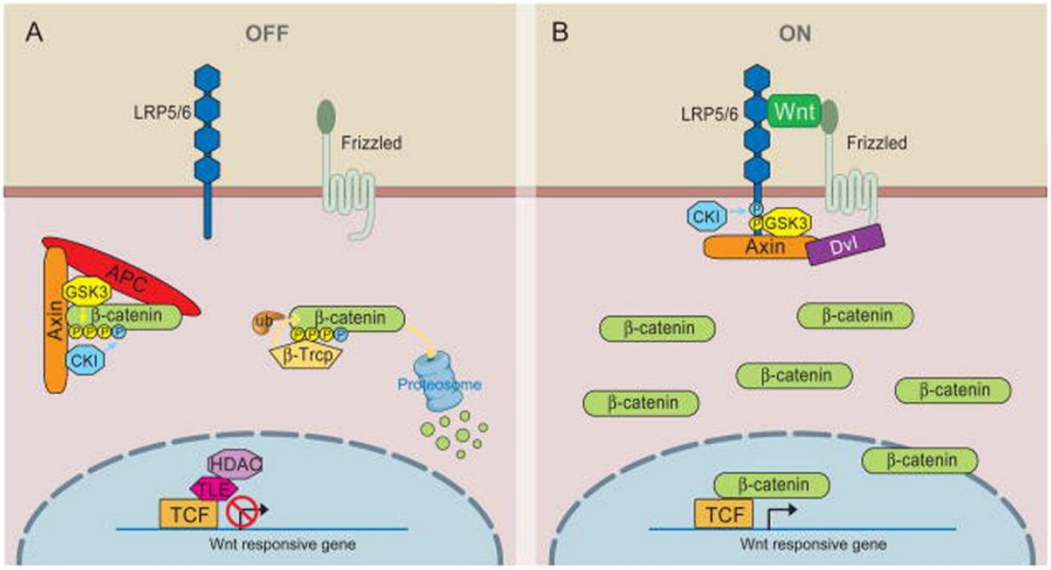



**Figure 4. Canonical WNT signaling.** In the absence of ligand (A), the β-catenin destruction complex, which consists of the scaffolds, APC and AXIN, and the kinases, CK1α and GSK3β, recruits and phosphorylates the cytoplasmic pool of β-catenin. Phosphorylated β-catenin is recognized by the multi-subunit E3 ligase, β-TRCP, which ubiquitylates β-catenin and targets it to the proteasome for degradation. When ligand is present (B), it interacts with and activates the Frizzled/LRP5/6 receptor complex. This process is enhanced by the receptor, LGR5, and its ligand, R-Spondin, which interact with the LRP/Fz complex (see text). The activated receptor complex then recruits AXIN to the membrane. This causes dissolution of the β-catenin destruction complex, which allows β-catenin to enter the nucleus, replace the transcriptional repressor Groucho (TLE), which is bound to TCF, and activate a transcriptional program. (Adapted from Macdonald, Tamai et al. 2009).

Carmon, Lin et al. 2012).

A process of derepression underlies canonical WNT signaling, culminating with the entry of β-catenin into the nucleus (Clevers and Nusse 2012). In the absence of ligand, β-catenin is subject to ubiquitin-mediated degradation. Following ligand stimulation, the cytoplasmic tail of LRP5/6 is phosphorylated, resulting in the recruitment of AXIN1 to the membrane. This is thought to involve the protein dishevelled (DVL), which interacts with both Fz (through its PDZ domain) and AXIN (through its DIX domain), thereby serving the roles of both recruiter and scaffold, as DVL-AXIN polymers are formed at the membrane.

The recruitment of AXIN1 to the membrane causes the dissolution of the β-catenin destruction complex. In addition to AXIN1, the β-catenin destruction complex is composed of the scaffold, adenomatous polyposis coli (APC), and the kinases, casein kinase I (CK1α) and glycogen synthase kinase β (GSK3B). In the absence of ligand, this complex binds and sequentially phosphorylates the cytoplasmic pool of β-catenin on three N-terminal Ser/Thr residues (Ser33/37 and Thr41). In turn, the N-terminal phosphorylated β-catenin is bound by the Skp, Cullin, F-box (SCF) complex, which contains the E3 ubiquitin ligase, β-transducin repeat containing protein (β-TRCP). β-TRCP polyubiquitylates β-catenin, which causes β-catenin to be recognized by the 26S proteasome and degraded.

Disassembly of the β-catenin destruction complex results in a stabilized pool of cytoplasmic β-catenin, which can then enter the nucleus. In the nucleus, β-catenin forms an active transcriptional complex with members of the

transcription factor family (TCF), doing so by displacing the transcriptional repressor Groucho, which binds TCF family members and recruits HDACs in the absence of ligand. Additionally, the proteins B-cell CLL/lymphoma 9 protein (BCL9), BCL9-like (BCL9L), and Pygopus, which interact with β-catenin in the nucleus, are thought to also be nuclear mediators of canonical WNT signaling (Hoffmans, Städeli et al. 2005; Brembeck, Schwarz-Romond et al. 2004; Deka, Wiedemann et al. 2010).

The two best-studied, and perhaps most important, target genes of canonical WNT signaling are the proto-oncoproteins Cyclin D1 and C-MYC. Cyclin D1 is commonly overexpressed in CRC, and is involved in driving the cell cycle through the $G_1$/S checkpoint in conjunction with cyclin-dependent kinase 4 (CDK4). C-MYC, the transcription factor that underlies Burkitt's lymphoma, drives cell cycle progression and DNA replication, and is also commonly dysregulated in CRC.

Two β-catenin-independent modes of WNT signaling exist: the non-canonical or planar cell polarity pathway (PCP) and the WNT/calcium pathway. Like canonical WNT signaling, each of these pathways begins when a WNT ligand binds a Fz receptor that then recruits DVL to the membrane. They diverge from there, most notably in the fact that canonical WNT signaling results in transcriptional changes, while non-canonical WNT signaling and the WNT/calcium pathway leads to more immediate cytoskeletal alterations.

In PCP Signaling, a number of coreceptors can serve in place of LRP5/6, including those related to receptor tyrosine kinase (RYK) and receptor tyrosine

kinase-like orphan receptor (ROR). Following stimulation, DVL interacts with dishevelled associated activator of morphogenesis (DAAM), which then activates Rho, and in turn, Rho-associated protein kinase (ROCK). In the PCP pathway, DVL also activates Jun Kinase 1 (JNK1) through Rac1. The WNT/calcium pathway causes the activation of PLC, which cleaves PIP2 into the second messengers IP3 and DAG, increasing intracellular calcium. There is controversy about the role of non-canonical WNT signaling in CRC, with evidence supporting its role as both a promoter of invasion and a suppressor of canonical WNT signaling (Jessen 2009).

The components of the β-catenin destruction complex (APC, AXIN1, CK1α, and GSK3B) and SCF complex are the major negative regulators of the canonical WNT signaling pathway. In CRC, as will be discussed, affecting the ability of the destruction complex to degrade β-catenin is integral to tumor initiation. It is notable that GSK3B, while a negative regulator in the context of the destruction complex, has also been found to be involved in the activation of canonical WNT signaling through DVL and Fz phosphorylation (Liu, Rubin et al. 2005). In addition, other members of the casein kinase family are distinctively involved in pathway activation. For example, CK1ε phosphorylates and activates DVL2, while CK1γ phosphorylates and activates LRP5/6 (Cong, Schweizer et al. 2004; Davidson, Wu et al. 2005).

Other negative regulators of WNT signaling include the secreted proteins WNT inhibitory factor 1 (WIF1), soluble Frizzled related protein (sFRP), and members of the Dickopf family (DKKs). WIF1 and sFRP, which inhibit both

canonical and non-canonical WNT signaling, function by binding and sequestering extracellular ligand (Hsieh, Kodjabachian et al. 1999; Rattner, Hsieh et al. 1997). DKK1 inhibits signaling by binding to and causing the internalization of LRP5/6 (Bafico, Liu et al. 2001). In the cytoplasm, members of the Naked family (NKD), NKD1 and NKD2, antagonize WNT signaling by causing the degradation of DVL1 (Schneider, Schneider et al. 2010; Hu, Li et al. 2010). As DVL1 is involved in both canonical and non-canonical WNT signaling, the NKDs can inhibit each arm.

Inside the nucleus are a number of proteins that affect the ability of β-catenin to interact with TCF, generally working through competitive inhibition or by affecting nuclear localization. As mentioned, the transcriptional repressor Groucho is constitutively bound to TCF in the nucleus. Similarly, myeloid translocation gene related-1 (MTGR-1) binds TCF and creates a transcriptionally inactive complex (Moore, Amann et al. 2008). Chibby and Inhibitor of catenin and T cell factor (ICAT) have been shown to bind and sequester β-catenin in the nucleus, while the kinase Nemo-like kinase (NLK) has been shown to phosphorylate and inhibit the activity of TCF, in some cases causing its nuclear export in a process that involves Par5 (Takemaru, Yamaguchi et al. 2003; Tago, Nakamura et al. 2000; Meneghini, Ishitani et al. 1999).

**Ubiquitylation in the EGFR, TGFB/BMP, and WNT signaling pathways**

The process of ubiquitylation is generally associated with protein turnover,

with a chain of ubiquitins serving to target a protein to the proteasome for degradation. Ubiquitylation also targets proteins to the lysosome for degradation, is required for the proper trafficking of some proteins, and, less commonly, stabilizes or activates a protein.

Regulation via ubiquitylation is central to WNT signaling, as the ubiqutin-mediated destruction of cytoplasmic β-catenin by β-TRCP is the key point of regulation of canonical WNT signaling, particularly in the context of CRC. In addition to the negative regulation by β-TRCP, a recent publication found that the stem cell-enriched E3, RNF43, inhibits WNT signaling by mediating the endocytosis and degradation of Fzs (Koo, Spit et al. 2012). Downstream of Fz, a number of members of the NEDD4 family of E3 ligases have been shown to inhibit canonical and non-canonical WNT signaling by causing the degradation of DVL (Wei, Li et al. 2012; Ding, Zhang et al. 2013). Conversely, DVL can be activated through ubiquitylation, though the E3 responsible for this remains to be identified (Tauriello, Haegerbarth et al. 2010). It is known, however, that the deubiquitylating enzyme (DUB), CYLD, removes these activating ubiquityl moieties from DVL, and blocks WNT hyperactivation (Tauriello, Haegebarth et al. 2010).

The EGFR is primarily ubiquitylated by CBL, which leads to its degradation. Counteracting this are NEDD4 and ITCH, which can ubiquitylate and cause the degradation of CBL (Magnifico, Ettenberg et al. 2003). NEDD4 and NEDD4L have been shown to negatively regulate EGFR levels by causing the degradation of ACK1, a non-receptor tyrosine kinase that stabilizes EGFR

(Chan, Tian et al. 2009). Additionally, WWP1 and ITCH have been shown to downregulate the EGFR dimerization partner, ERBB4 (Feng, Muraoka-Cook et al. 2009; Omerovic, Santangelo et al. 2007).

The NEDD4 family plays the most pronounced role in the regulation of TGFB family signaling. SMURFs 1 and 2, WWP1, NEDD4L, and ITCH have all been shown to downregulate TGFBRI and/or one or more of the SMADs (Dupont, Inui et al. 2012; Xu, Liu et al. 2012). In all cases, these E3s serve to abrogate TGFB family signaling. Additionally, the E3 ligases Skp2, CHIP, and Ectodermin/TIF1γ have been shown to cause the degradation of SMAD4. One ubiquitin ligase, Arkadia, has been shown to enhance TGFB signaling by causing the degradation of the pathway inhibitors SMAD7 and SnoN. Interestingly, Arkadia achieves this with the cooperation of the β-catenin destruction complex scaffolding protein, AXIN (Liu, Rui et al. 2006).

**Summary**

In this chapter, I have introduced the three human diseases and three cell signaling pathways on which I have worked while in the Coffey lab. For each pathway, I have discussed the ways in which the signals are propagated from the cell-surface receptor to effector proteins in the cytoplasm or nucleus. Each pathway is commonly dysregulated in CRC, with the EGFR and WNT signaling pathways being activated, and the TGFB family pathway being inhibited early and activated late. Additionally, I have studied the EGFR and TGFB family

pathways in the contexts of two premalignant diseases: Ménétrier's disease and JPS. In each disease, something has caused the given pathway to become dysregulated in a manner that leads to hyperproliferation. In Ménétrier's disease, the genetic or acquired cause of EGFR overactivation remains to be discovered, while in JPS, there is clear linkage to dysregulation of the TGFB family pathway.

As will be discussed more in the following chapters, during my time in the Coffey lab, I have been a part of the clinical trial involving the first effective medical therapy against Ménétrier's disease, and in determing a new algorithm to properly diagnose Ménétrier's disease (Fiske, Tanksley et al. 2009; Rich, Toro et al. 2010). Additionally, I was involved in showing a new modality for imaging the stomach of Ménétrier's disease patients (McKinley, Smith et al. 2012). My work concerning JPS will be written up in the following year, some of which depends upon the findings in a patient, the nature of which will be discussed in Chapter IV. Lastly, work concerning the expression of the NEDD4 family of E3 ubiquitin ligases is in submission.

# CHAPTER II

## MÉNÉTRIER'S DISEASE AND JUVENILE POLYPOSIS SYNDROME

I joined the Coffey lab as a decade-long clinical trial was concluding; I was able to participate in its completion, as well as a subsequent analysis of the patients referred to Vanderbilt for diagnosis of Ménétrier's disease. The purpose of the study was to determine whether cetuximab, an anti-epidermal growth factor receptor (EGFR) monoclonal antibody, is efficacious in the treatment of the premalignant hypertrophic gastropathy, Ménétrier's disease. The results of the trial were exciting, from both clinical and scientific standpoints. Clinically, we confirmed that EGFR blockade ameliorated most, and in some cases, all of the signs and symptoms of the disease. Scientifically, we learned much about the role of EGFR signaling in the maintenance of lineage allocation in the human stomach.

In this chapter, I will discuss features of that trial, what we learned about Ménétrier's disease during and following the trial, and what remains to be discovered about the actual cause(s) of Ménétrier's disease. I will conclude this chapter with a discussion of my observation that the gastrointestinal hamartoma syndrome, Juvenile Polyposis syndrome (JPS), shares some clinical and histological features with Ménétrier's disease, and, in turn, may share some pathophysiological mechanisms.

## EGFR and TGFB/BMP signaling in the stomach

The signs, symptoms, and gross and histological appearance of Ménétrier's disease are ameliorated by EGFR blockade (Burdick, Chung et al. 2000; Settle, Washington et al. 2005; Fiske, Tanksley et al. 2009). JPS is linked to inactivating mutations in the TGFB family members, SMAD4 and BMPRIA (Howe, Roth et al. 1998; Howe, Bair et al. 2001). Thus, these two pathways play a role in the genesis of these diseases in the stomach. Given this, a brief discussion of the cellular composition of the stomach, and what is known about the roles of the EGFR and TGFB family pathways in the development and maintenance of the human stomach is warranted.

The stomach is the initial site of food digestion. It can grossly be divided into the following three segments: the cardia, the fundus/body, and the antrum (Figure 5). The gastric cardia is generally the shortest segment (if present), and the mucus-secreting cells of the cardiac glands serve to protect the esophagus from damage by the highly acidic gastric environment. The fundus and body are the sites of the gastric, or oxyntic, glands, which contain the cell types responsible for maintaining the harsh environment in the stomach (the parietal cells and chief cells). The glands of the antrum are where the majority of the gastric enteroendocrine cells (G-cells and D-cells) reside. These cells modulate the function of the cells of the fundus/body through the regulated secretion of the peptide hormones gastrin and somatostatin, respectively, into the bloodstream (Karam and Leblond 1993 (1-4); Karam 1993). The effects of cetuximab are

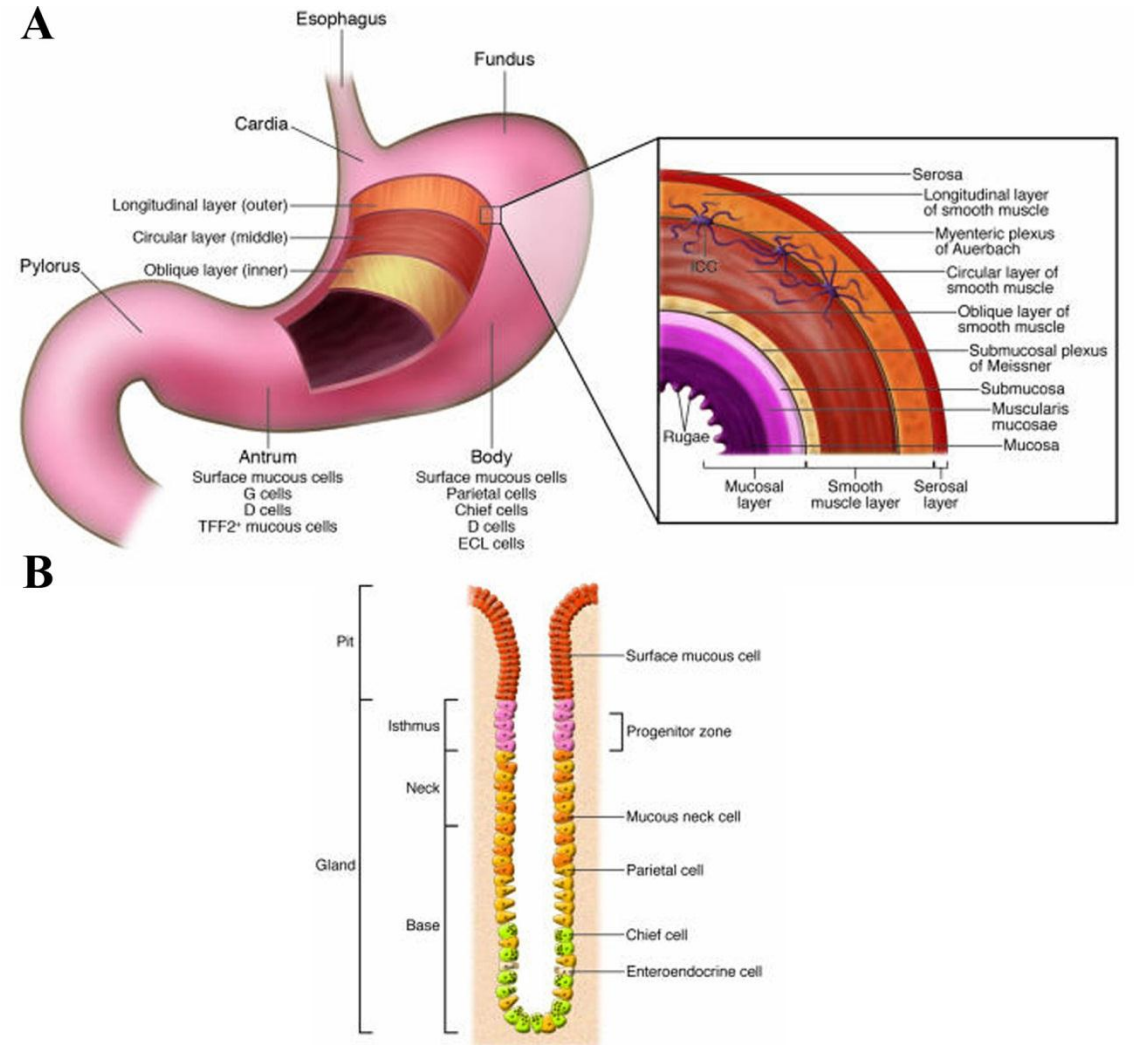



**Figure 5. Anatomy of the human stomach.** (A) The stomach consists of three major parts: the cardia, fundus/body, and antrum. The proximal (cardia) and distal (antrum) ends of the stomach lack the acid-secreting parietal cells and pepsin-secreting chief cells that are present in the fundus/body. This buffers the acidic environment of the stomach and the neutral enviroments of the esophagus and duodenum. Additionally, the antrum contains enteroendocrine cells, most importantly the G- and D-cells, which are responsible for secreting gastrin and somatostatin, respectively, into the bloodstream. The upper portion of gastric glands is lined by surface mucous cells, which help to protect the gastric lining from the acidic environment. It is only the surface mucous cells atop the glands lining the fundus/body (oxyntic glands) that hyperproliferate in Ménétrier's disease. At the base of the oxyntic glands are the parietal and chief cells, which are reduced in number or lost in Ménétrier's disease. (B) The oxyntic glands lining the fundus/body have a stem cell compartment (isthmus) that generally resides about one-fifth of the distance from the gastric lumen to the base of the gland (see text). Differentiating cells then migrate upwards to become surface mucous cell, or downward to become parietal, chief, or enteroendocrine cells. (Adapted from Coffey, Washington et al. 2007).

most marked in the fundus/body, though changes in the cell composition of the antrum following treatment may prove to be important, and have not yet been investigated.

The initiation of digestion is the product of two properties of the gastric luminal environment. Firstly, the environment in the stomach is highly acidic, with a normal pH of 1 to 3. This is accomplished by the parietal cells, which are triangular, eosinophilic cells containing an intricate tubulovesicular system, the membranes of which are studded with H+/K+ ATPases, or proton pumps. Following stimulation, this vesicular system fuses with the luminal membrane and the proton pumps actively secrete hydrochloric acid (HCl) into the lumen. This process can be inhibited by somatostatin, EGF, and TGFA, and promoted by gastrin, carbachol, and histamine. Secondly, there is a high concentration of the non-specific protease, pepsin, in the lumen, which begins the process of breaking proteins down into peptides and amino acids. Pepsin is secreted by the basophilic chief cells in its inactive form, termed pepsinogen, which becomes active following self-cleavage, a process induced by a low pH. The parietal and chief cells reside at the base of the gastric gland, below the isthmus, which is the oxyntic gland stem cell compartment. The area below the isthmus normally comprises 75-80% of the length of the gastric glands. A number of other cells reside at the base of the gastric gland, some in the process of differentiating (mucous neck cells that will become chief cells, for instance), and some that are fully differentiated (caveolated cells and enteroendocrine cells).

Above the isthmus is the area referred to as the pit. Here, the surface

mucous cell, or pit cell, resides. The surface mucous cell is responsible for secreting mucus into the lumen in order to prevent damage to the gastric mucosa by the acidic environment. Immediately beneath the epithelial layer and basement membrane is an interconnected layer of myfibroblasts, which are thought to serve in supporting the overlying epithelia. While there are also smooth muscle cells and extracellular matrix, in the normal stomach, there is very little visible stroma between adjacent glands.

Following production in the isthmus, cells can then migrate upward or downward, differentiating as they do so. Those cells that migrate upward become the short-lived surface mucous cells, with half-lives on the order of days, which are eventually sloughed into the lumen. Those cells that migrate downward become the long-lived chief and parietal cells, with half-lives on the order of weeks to months, which die by apoptosis. EGFR and TGFB family signaling have been shown to play a role in this lineage allocation process (Li, Karam et al. 1998).

The first identified EGFR ligand, EGF, is not made in the stomach (Murphy 1998). It is, however, synthesized and secreted by the salivary glands, and thought to serve the role of a luminal surveillance peptide, only affecting the primarily basolaterally located EGFR following damage, resulting in a proliferative and reparative response (Playford and Wright 1996). TGFA, on the other hand, is prevalent in the gastric glands, and findings in a mouse engineered to overexpress Tgfa in the stomach spurred the initial interest of our lab in Ménétrier's disease (Beauchamp, Barnard et al 1989).

It was known that treatment with TGFA caused a reduction in histamine-stimulated acid secretion from isolated rabbit parietal cells (Lewis, Goldenring et al. 1990). Furthermore, TGFA had been shown to stimulate the growth of guinea pig and canine mucous cells in culture, while systemic administration in rats led to a large increase in the amount of gastric mucin (Rutten, Dempsey et al. 1993; Guglietta, Lesch et al. 1994). These initial findings initiated the development of a transgenic mouse model engineered to overexpress rat Tgfa in the stomach (Dempsey, Goldenring et al. 1992). In this mouse, Tgfa expression was driven by the metallothionein promoter (MT-Tgfa), which was induced by including zinc in the diet. Histologically, these mice had fewer parietal and chief cells, and more surface mucous cells. The length of the pit increased, while the length of the base of the gland decreased (foveolar hyperplasia and glandular atrophy, respectively). Confirming this result were mice overexpressing human TGFA, and recently, mice overexpressing BTC in the stomach (Dahlhoff, Gerhard et al. 2012). Additionally, the waved2 mouse, which expresses a kinase-deficient Egfr, was found to secrete more acid into the gastric lumen, though there did not appear to be an effect on lineage allocation (Joshi, Ray et al. 1997).

The TGFB family pathway has been studied more in the context of tumorigenesis in the gastrointestinal tract, and less in the context of lineage allocation. However, it is known that isolated canine parietal cells upregulate expression of the proton pump when incubated with BMP-4, which also attenuates the downregulation of proton pump expression by EGF (Nitsche, Ramamoorthy et al. 2007). In this same paper, it was shown that TGFB1

administration led to apoptosis.

Gastric epithelial phenotypes in mouse models of altered TGFB family signaling are harder to interpret due to the contribution of stromal alterations, which are not present in the MT-Tgfa mouse. Mice engineered to express only one copy of Smad4 develop gastric polyps with an intense stromal component with overlying, relatively normal epithelium, which is in line with what is found in JPS (Xu, Brodie et al. 2000). Further supporting the contribution of the stroma to these phenotypes are the findings that a T-cell-specific knockout of Smad4, and a fibroblast-specific knockout of the type II TGFB receptor, cause gastric epithelial cancer (Kim, Li et al. 2006; Bhowmick, Chytil et al. 2004). Lastly, expression of the secreted BMP inhibitor, noggin, by parietal cells, results in a reduced number of parietal cells and an increased number of surface mucous cells (Shinohara, Mao et al. 2010).

In summary, evidence supports the idea, mentioned in the first chapter, that the EGFR and TGFB family pathways work in opposition to each other. Data from cell culture and mouse models suggest that activating the EGFR inhibits acid secretion, promotes mucus secretion, and decreases the number of cells below the stem cell compartment (chief and parietal cells), while increasing the number of cells above (surface mucous cells). Conversely, TGFB family signaling, more specifically the BMP arm, upregulates expression of the proton pump and increases acid secretion, while abrogation of the BMP signaling pathway in the stomach causes a phenotype with some similarities to the MT-Tgfa mouse, including fewer parietal cells, more mucous cells, and

hypochlorhydria.

Exploring how these pathways oppose each other in human disease was the basis of my initial thesis proposal. Before discussing the arc of that project, I will discuss my work on Ménétrier's disease, which has served as a considerable "side project" during my time in the Coffey lab. In the discussion of Ménétrier's disease that follows, I will borrow from three of the papers we published during this time.

**The history of Ménétrier's disease** (from Coffey and Tanksley 2012)

The French pathologist Pierre Ménétrier (1859–1935) first described the disease that bears his name in Archives Physiologie Normale et Pathologique in 1888. The editors of this prestigious French journal were Charles-Edouard Brown-Sequard (1817–1894) and Jean-Martin Charcot (1825–1893). These were eponymous times; if one described a disease or identified a syndrome, it was commonplace to append one's name to that entity.

In two separate autopsy reports that year in this journal (Ménétrier 1888), Ménétrier described seven individuals exhibiting two macroscopically distinct patterns of gastric hypertrophy: polypoid adenomas and sheet-like polyadenomas. He aptly likened the thickened gastric mucosa to cerebral convolutions. Four of the seven had the latter condition, which came to be referred to as Ménétrier's disease. Two of these four individuals had gastric cancer. He reported that the disorder affected the proximal portion of the

stomach (body and fundus) and spared the distal stomach (antrum); a link to gastric cancer and antral sparing continue to be recognized features of the disease. Ménétrier's original drawing captured many of the features of the disease (Figure 6). There is marked expansion of the surface mucous cells (histologically referred to as foveolar hyperplasia) and reduced numbers of acid-producing parietal cells and pepsinogen-producing chief cells (referred to as glandular or oxyntic atrophy). The foveolae (small pits) are often tortuous and undergo cystic dilatation. Normally, the pit to gland ratio is 1:4, but in Ménétrier's disease this ratio is often reversed, as depicted by Dr. Ménétrier. If Ménétrier had been able to perform pre-mortem gastroscopy on these patients, he would also have noted thick tenacious gastric fluid with reduced gastric acidity (gastric juice pH is often 4–7 rather than 1–3), reflecting the reduced parietal cell mass.

Ménétrier's disease is also known by several other names, including giant hypertrophic gastritis and hypoproteinemic hypertrophic gastropathy. There are no pathognomonic features to diagnose Ménétrier's disease, and it continues to be a clinicopathological diagnosis. Patients, more often males than females, usually between the ages of 30 and 60 years old, typically present with abdominal pain, nausea, vomiting, and edema of peripheral tissues (due to leakage of protein selectively across the gastric lining). The disease tends to be progressive in adults; there are no studies of spontaneous regression of the disease in patients with symptoms longer than 6 months duration (Barbosa, Nogueira et al. 1987; Charton-Bain, Paraf et al. 2000; Choi, Park et al. 2007; Hsu, Ito et al. 1991; Johnson, Spark et al. 1995; Wood, Bates et al. 1983). It is

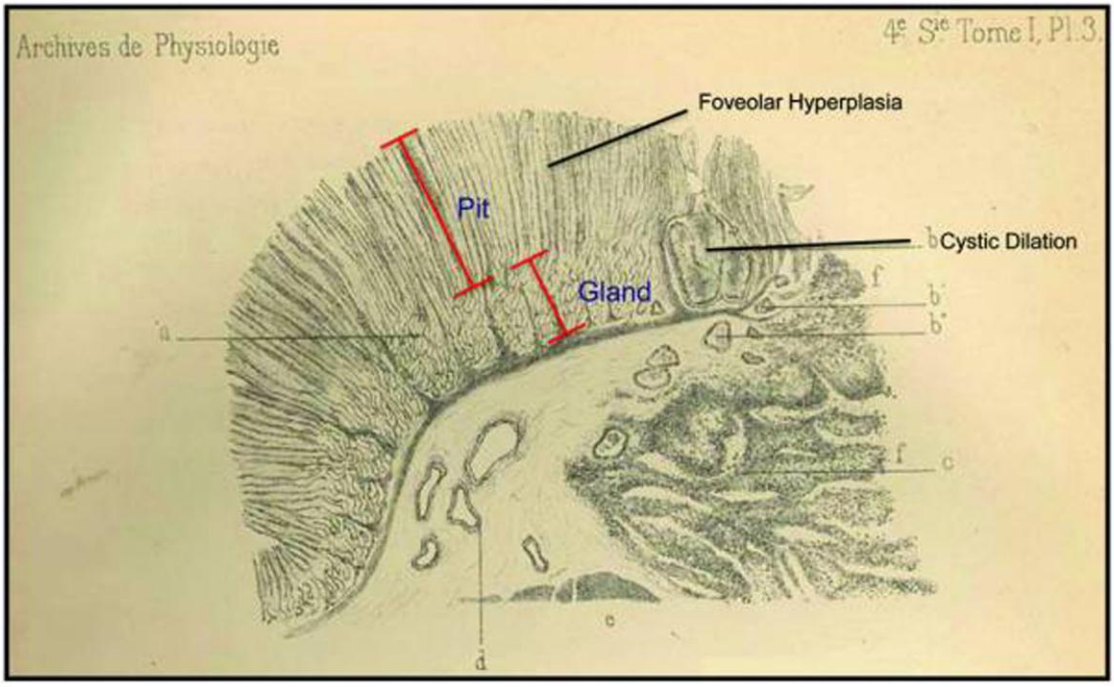



**Figure 6. Pierre Ménétrier's original drawing**. The original drawing of Ménétrier's disease highlights the most characteristic feature, foveolar hyperplasia. Foveolar hyperplasia is a product of the hyperproliferation of the surface mucous cells, which line the foveolus, and the loss of glandular lineages, which is most likely the product of the stem cells in the isthmus choosing the differentiate into mucous cells due to some extracellular cue. (Adapted from Ménétrier, 1888).

essential to obtain a full-thickness biopsy of the involved gastric mucosa when entertaining the diagnosis of Ménétrier's disease (Rich, Toro et al. 2010). Foveolar hyperplasia, often massive, is a histological sine qua non; a histological variant, hypertrophic lymphocytic gastritis, has been described, but is much less common in our experience, and may not truly be Ménétrier's disease (Wolfsen, Carpenter et al. 1993). As mentioned above, there are usually reduced numbers of parietal cells and chief cells. Additional histological features include retention of overall mucosal architecture with prominent eosinophils and/or plasma cells in the lamina propria, along with smooth muscle hyperplasia and edema in the lamina propria. Serum gastrin tends to be normal despite the reduced gastric acidity. Until recently, there has been no effective medical therapy and patients often undergo partial or total gastrectomy.

In addition to being a pathologist, Pierre Ménétrier was a medical historian who specialized in Byzantine and Greco-Roman medicine. In considering the underlying pathogenesis of this disorder, Ménétrier, ever the historian, lamented, "we regret very much not having been able to investigate the mode of multiplication of these epithelial elements due to the conditions in which we find ourselves." However, he presciently noted that "the glandular epithelial coat loses its highly differentiated functional character to acquire instead a new proliferative power, rather similar to that with which embryonal elements are endowed."

**The Clinical Trial** (from Fiske, Tanksley et al. 2010)

Evidence from both mice and humans has implicated increased signaling through the EGFR in the pathogenesis of Ménétrier's disease (Dempsey, Goldenring et al. 1992; Coffey, Romano et al. 1995). TGFA, one of seven mammalian EGF receptor ligands, increases gastric epithelial cell proliferation, stimulates gastric mucin production, and suppresses gastric acidity (Coffey, Romano et al. 1992; Bockman, Sharp et al. 1995; Goldenring, Ray et al. 1995). Transgenic mice that overexpress Tgfa in the stomach exhibit all of the histological features of the disorder. Patients with Ménétrier's disease exhibit increased TGFA immunoreactivity in the areas of abnormal gastric mucosa (Bluth, Carpenter et al. 1995).

On the basis of this evidence and the lack of any effective medical therapy, the U.S. Food and Drug Administration gave compassionate-use approval to treat a patient with cetuximab, a recombinant, chimeric, immunoglobulin G1 (IgG1) monoclonal antibody that binds specifically to the extracellular portion of the EGFR and inhibits binding of ligands such as TGFA. Treatment of this individual resulted in marked clinical and biochemical improvement (Burdick, Chung et al. 2000). This outcome led us to conduct a single-arm clinical trial to evaluate the effectiveness of cetuximab in the treatment of Ménétrier's disease. All seven patients who completed the 1-month course of cetuximab showed improvement in both quality-of-life index (QLI) and biochemical index of the disease and elected to continue treatment. Four of the

seven patients had near-complete histological resolution of the findings of Ménétrier's disease.

Of the nine patients enrolled, five were men and four were women. The ages of the patients at the time of initial presentation with Ménétrier's disease ranged from 29 to 79 years. Of note, four patients (44.4%) also had ulcerative colitis. Of the four patients with ulcerative colitis, two (patients 3 and 7) had been treated previously with both immunomodulator therapy [6-mercaptopurine (6-MP)] and a chimeric monoclonal antibody to tumor necrosis factor–α (TNFA) (infliximab). Patient 8 received 6-MP alone and patient 5 received no immunomodulator therapy. Only one patient was maintained on immunomodulator therapy while enrolled in the trial (patient 3).

All seven patients who completed the course of treatment reported improvement in their individual predominant symptom(s) within 1 week of starting treatment, usually within 1 to 2 days. There was a statistically significant increase in the primary outcome, both the overall QLI (Ferrans and Powers QLI; P = 0.02) and the QLI Health and Functioning subscale (P = 0.01). Parietal cell mass, as measured by quantitative immunohistochemistry of the parietal cell marker H+/K+-ATPase, increased by a factor of 3 after 1 month of therapy (P = 0.01) and was accompanied by a decrease in mean gastric pH from 6.0 to 4.0 (P = 0.05). Mean stomach wall thickness decreased from 13.7 to 9.6 mm, although this was not statistically significantly different (P = 0.06). No significant change was detected in other secondary outcomes. Shown as an example in Figure 7, patient 4 exhibited a large reduction in gastric wall thickness [as assessed by

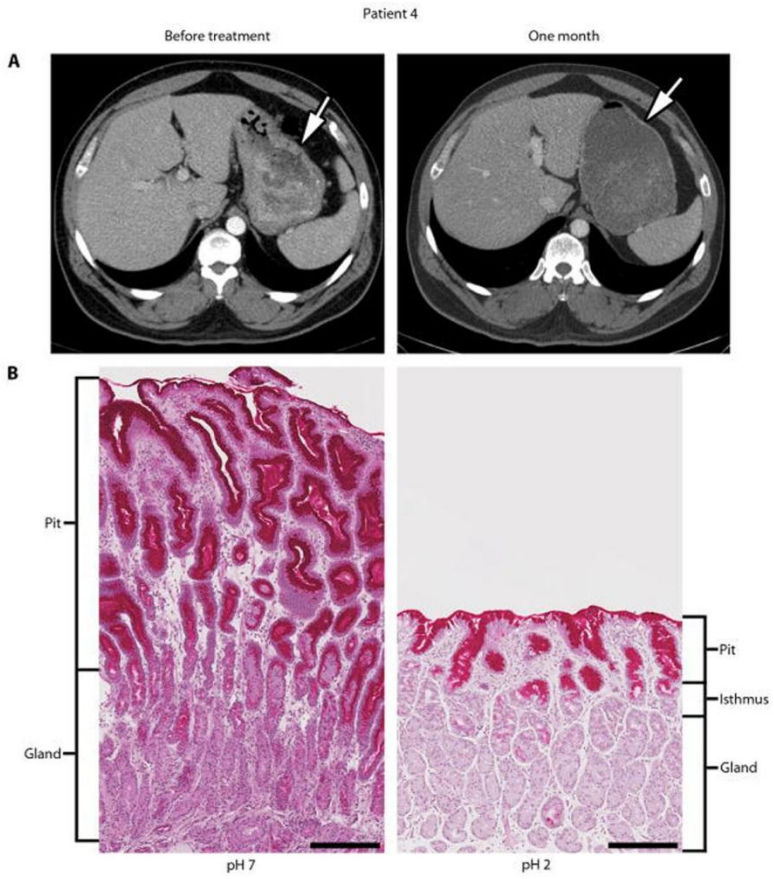



**Figure 7. Response to one-month course of cetuximab in patient 4.** (A) Patient 4 showed a marked reduction in stomach wall thickness by CT scan. An equivalent amount of VoLumen (an oral contrast agent) was administered prior to the scans. Arrows, thickness of gastric wall. (B) Biopsies before and 1 month after treatment show regression of foveolar hyperplasia and restoration of glandular mucosa with return to normal pit to gland ratio of 1:4. Surface mucous cells are strongly positive and mucous neck cells are weakly positive by diastase-resistant periodic acid-Schiff staining. Gastric pH decreased from 7 to 2 after 4 weekly infusions of cetuximab. Scale bar is 250 microns.

computed tomography (CT) scan], regression of foveolar hyperplasia, and reappearance of parietal cells with restoration of gastric acidity after four weekly doses of cetuximab.

Although the primary end point (overall QLI) was evaluated after four weekly infusions of cetuximab, we performed gastroscopy 24 hours after the first infusion and obtained grossly involved gastric tissue for analysis. After this short time interval, the parietal cell mass had increased from 20.9 to 41.1 ($P = 0.02$). Figure 8 shows H+/K+-ATPase immunostaining (a selective marker of parietal cells) at baseline, after 1 day, and after 1 month of cetuximab in patients 1 and 3. To confirm this finding, we pooled pretreatment samples and 1-day posttreatment samples from abnormal gastric tissue from eight patients and performed in-depth shotgun proteomics. In shotgun proteomic data sets, proteins are identified by mass spectrometry spectra, where higher numbers of observed spectra are interpreted as a measure of protein abundance in the specimen. There was a statistically significant factor of 3 increase in normalized spectral counts for the α subunit of H+/K+-ATPase after 1 day of cetuximab treatment (from 7 to 36 spectral counts, quasi-$P = 0.049$); the β subunit showed a smaller increase after treatment that did not reach statistical significance (from 15 to 24 spectral counts, quasi-$P = 0.98$). We also identified significant increases in proteins previously identified as markers of parietal cells, mucous neck cells, and chief cells. For example, carbonic anhydrase II and calmodulin 2 are expressed by parietal cells (Mills, Syder et al. 2001), mucin 6 is expressed by mucous neck cells (Ho, Roberton et al. 1995), and gastric lipase and leucine aminopeptidase 3 are

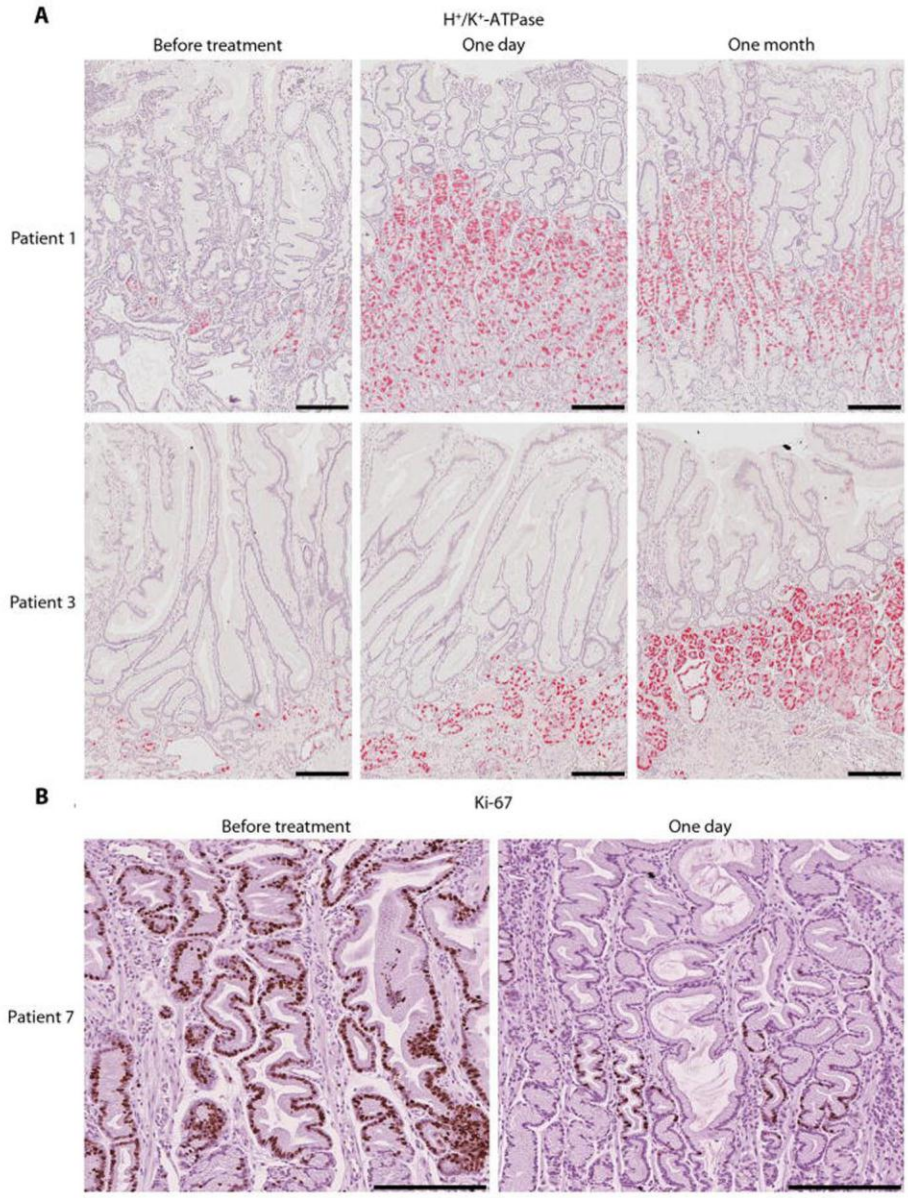



**Figure 8. Recovery of parietal cells and decrease in proliferation one day after initiation of cetuximab treatment.** (A) Patients 1 and 3 demonstrated rapid (one day) and sustained (one month) increases in H+/K+−ATPase α-subunit immunoreactivity. Scale bar is 250 microns. (B) Patient 7 had a dramatic decrease in Ki-67 staining one day after first dose of cetuximab. Scale bar is 250 microns.

Table 1. Proteins identified in involved gastric mucosa of Ménétrier's disease patients by shotgun proteomic analysis.

| Protein | Gene Name | Full Gene Name | Pre-Treatment counts | Post-Treatment counts | Fold-change | p-value |
|---|---|---|---|---|---|---|
| **Proteins with higher spectral counts post-treatment** | | | | | | |
| IPI00027350.3 | PRDX2 | peroxiredoxin 2 | 0 | 16 | ∞ | 0.002309 |
| IPI00032851.1 | COPZ1 | coatomer protein complex, subunit zeta 1 | 0 | 14 | ∞ | 0.002309 |
| IPI00025100.1 | BCKDHA | branched chain keto acid dehydrogenase E1, alpha polypeptide | 0 | 10 | ∞ | 0.002309 |
| IPI00293867.7 | DDT | D-dopachrome tautomerase | 0 | 10 | ∞ | 0.002309 |
| IPI00418262.4 | ALDOC | aldolase C, fructose-bisphosphate | 0 | 10 | ∞ | 0.002309 |
| IPI00550021.4 | RPL3 | ribosomal protein L3 | 0 | 10 | ∞ | 0.002309 |
| IPI00291328.3 | NDUFV2 | NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa | 0 | 9 | ∞ | 0.002309 |
| IPI00012119.1 | DCN | decorin | 0 | 10 | ∞ | 0.013969 |
| IPI00024915.2 | PRDX5 | peroxiredoxin 5 | 0 | 10 | ∞ | 0.013969 |
| IPI00006114.4 | SERPINF1 | serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 | 0 | 9 | ∞ | 0.013969 |
| IPI00017526.1 | S100P | S100 calcium binding protein P | 0 | 9 | ∞ | 0.013969 |
| IPI00018246.5 | HK1 | hexokinase 1 | 0 | 9 | ∞ | 0.019652 |
| IPI00015141.4 | CKMT2 | creatine kinase, mitochondrial 2 (sarcomeric) | 0 | 8 | ∞ | 0.019652 |
| IPI00003734.1 | LOC347701 | calgizzarin-like | 0 | 8 | ∞ | 0.019652 |
| IPI00006721.3 | OPA1 | optic atrophy 1 (autosomal dominant) | 0 | 8 | ∞ | 0.013969 |
| IPI00026665.2 | QARS | glutaminyl-tRNA synthetase | 0 | 8 | ∞ | 0.015987 |
| IPI00604664.4 | NDUFS1 | NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase) | 0 | 14 | ∞ | 0.024941 |
| IPI00401776.9 | MUC6 | mucin 6, oligomeric mucus/gel-forming | 0 | 11 | ∞ | 0.045686 |
| IPI00026087.1 | BANF1 | barrier to autointegration factor 1 | 0 | 10 | ∞ | 0.029086 |
| IPI00216694.3 | PLS3 | plastin 3 (T isoform) | 2 | 26 | 7.978694 | 0.020992 |
| IPI00009893.1 | LIPF | lipase, gastric | 5 | 47 | 5.769210 | 0.043437 |
| IPI00075248.11 | CALM2 | calmodulin 2 (phosphorylase kinase, delta) | 5 | 34 | 4.173471 | 0.044332 |
| IPI00010896.3 | CLIC1 | chloride intracellular channel 1 | 3 | 19 | 3.887056 | 0.044332 |
| IPI00645078.1 | UBE1 | ubiquitin-activating enzyme E1 | 9 | 52 | 3.546086 | 0.013969 |
| IPI00456969.1 | DYNC1H1 | dynein, cytoplasmic 1, heavy chain 1 | 6 | 32 | 3.273310 | 0.040341 |
| IPI00218919.7 | ATP4A | ATPase, H+/K+ exchanging, alpha polypeptide | 7 | 36 | 3.156406 | 0.048786 |
| IPI00009342.1 | IQGAP1 | IQ motif containing GTPase activating protein 1 | 10 | 48 | 2.945979 | 0.025964 |
| IPI00419237.3 | LAP3 | leucine aminopeptidase 3 | 5 | 23 | 2.823230 | 0.029700 |
| IPI00022200.2 | COL6A3 | collagen, type VI, alpha 3 | 23 | 105 | 2.801883 | 0.012067 |
| IPI00306959.10 | KRT7 | keratin 7 | 9 | 40 | 2.727759 | 0.036465 |
| IPI00218414.5 | CA2 | carbonic anhydrase II | 98 | 384 | 2.404881 | 0.007074 |
| IPI00017855.1 | ACO2 | aconitase 2, mitochondrial | 22 | 83 | 2.315495 | 0.016882 |
| Protein | Gene Name | Full Gene Name | Pre-Treatment counts | Post-Treatment counts | Fold-change | p-value |
| **Proteins with lower spectral counts post-treatment** | | | | | | |
| IPI00644766.3 | TOR1AIP1 | torsin A interacting protein 1 | 8 | 4 | 3.258679 | 0.013969 |
| IPI00021891.5 | FGG | fibrinogen gamma chain | 20 | 10 | 3.258679 | 0.018157 |
| IPI00045511.1 | CLCC1 | chloride channel CLIC-like 1 | 10 | 5 | 3.258679 | 0.024913 |
| IPI00410714.5 | HBA1 | hemoglobin, alpha 1 | 314 | 166 | 3.082003 | 0.013969 |
| IPI00032293.1 | CST3 | cystatin C | 15 | 8 | 3.055011 | 0.013969 |
| IPI00157820.3 | TXNRD2 | thioredoxin reductase 2 | 7 | 4 | 2.851344 | 0.027473 |
| IPI00217468.3 | HIST1H1B | histone cluster 1, H1b | 20 | 12 | 2.715566 | 0.027473 |
| IPI00025333.4 | CIAPIN1 | cytokine induced apoptosis inhibitor 1 | 11 | 7 | 2.560390 | 0.036465 |
| IPI00640417.1 | HP1BP3 | heterochromatin protein 1, binding protein 3 | 18 | 12 | 2.444009 | 0.024941 |
| IPI00007334.1 | ACIN1 | apoptotic chromatin condensation inducer 1 | 16 | 11 | 2.369948 | 0.027473 |
| IPI00021885.1 | FGA | fibrinogen alpha chain | 70 | 50 | 2.281075 | 0.008418 |
| IPI00010675.1 | TFF2 | trefoil factor 2 | 104 | 75 | 2.259351 | 0.004005 |
| IPI00011695.8 | PRSS2 | protease, serine, 2 (trypsin 2) | 12 | 9 | 2.172452 | 0.020737 |
| IPI00180240.2 | TMSL3 | thymosin-like 3 | 19 | 15 | 2.063830 | 0.013969 |
| IPI00008418.6 | DIABLO | diablo homolog (Drosophila) | 10 | 8 | 2.036674 | 0.009594 |

preferentially expressed in chief cells (Moreau, Bernadac et al. 1989; Mills, Andersson et al. 2003) (Table 1).

In addition, we assessed two markers of cellular proliferation, Ki-67 and phosphorylated mitogen-activated protein kinase (MAPK), after the first dose of cetuximab in all seven patients who completed the trial. The number of Ki-67–positive cells per gland decreased from 43.8 before treatment to 27.2 after 24 hours (P = 0.05). Results from patient 7 are shown in Figure 8. The ratio of phosphorylated (activated) MAPK to total MAPK by Western blotting decreased from 1.35 (±2.62) before treatment to 0.56 (±1.78) after 24 hours, although this did not reach statistical significance (P = 0.12). Together, these results show that monoclonal antibody blockade of the EGF receptor in this hyperproliferative disorder of the stomach caused a rapid response. In addition, these findings support the hypothesis that enhanced EGF receptor signaling directs gastric progenitor cells down a surface mucous cell lineage rather than a parietal cell lineage (Coffey, Washington et al. 2003) and underscore the remarkable plasticity of the adult stomach.

All seven participants who completed the trial reported that their predominant symptom(s) had improved to the extent that they elected to continue treatment (mean duration of follow-up, 18 months; range, 8 to 40 months). Table 2 presents the long-term clinical outcomes for each patient, along with the histological findings and gastric pH at last follow-up or at the time of gastrectomy.

After 18 months of cetuximab treatment, patient 1 had complete resolution of symptoms and a histologically normal stomach, leading to discontinuation of

Table 2. Long term clinical outcomes for those who continued treatment

| Patient | Duration of therapy | Long-term outcome | Gastric histology at last follow-up | Gastric pH at last follow-up |
|---|---|---|---|---|
| 1 | 18 months | Off Treatment | Minimal FH* | 1 |
| 2 | 16 months | Off Treatment | Minimal FH* | 2 |
| 3 | 40 months | On Treatment | Minimal FH* | 3 |
| 4 | 9 months | Gastrectomy | Normal | 2 |
| 5 | 24 months | Gastrectomy | Dysplastic lesion one year after treatment stopped | 7 |
| 6 | 9 months | Gastrectomy | FH* | 2 |
| 7 | 8 months | Gastrectomy | FH* | 3 |

*FH, foveolar hyperplasia

cetuximab. Nine months later, the stomach was grossly normal with minimal foveolar hyperplasia and a gastric pH of 1 (Figure 9). She remains asymptomatic 19 months after discontinuing cetuximab. Patient 2 was treated for 16 months, at.which time her gastric mucosa appeared grossly and histologically normal with a gastric pH of 2 (Figure 9). Cetuximab was stopped and she notes only occasional nausea 4 months later. Patient 3 has been treated with cetuximab for 40 months. Gastric histology at 31 months showed mild to moderate foveolar hyperplasia; however, 7 months later, gastric histology showed marked improvement with reduced mucosal thickness and minimal foveolar hyperplasiasignificant improvement in QLIs as well as significantly increased parietal cell mass and gastric acidity. As noted above, Ménétrier's disease is a rare disorder; it required 8 years to accrue nine patients, making it unfeasible to include a placebo control arm.

We were able to perform gastroscopy to obtain gastric tissue 24 hours after the initial infusion of cetuximab. Even at this early time, there was already a significant decrease in the number of Ki-67–positive cells in the involved gastric mucosa and a doubling of the parietal cell mass. The finding of increased parietal cell mass within 24 hours of cetuximab treatment is consistent with previous investigations in rodents with acute ablation of parietal cells with DMP-777, a cell-permeant neutrophil elastase inhibitor and parietal cell–specific protonophore (Goldenring, Ray et al. 2000). After cessation of treatment with DMP-777, parietal cells expressing H+/K+-ATPase rapidly return within 24 to 48 hours (Goldenring, Ray et al. 2000). Additionally, there was a factor of 7

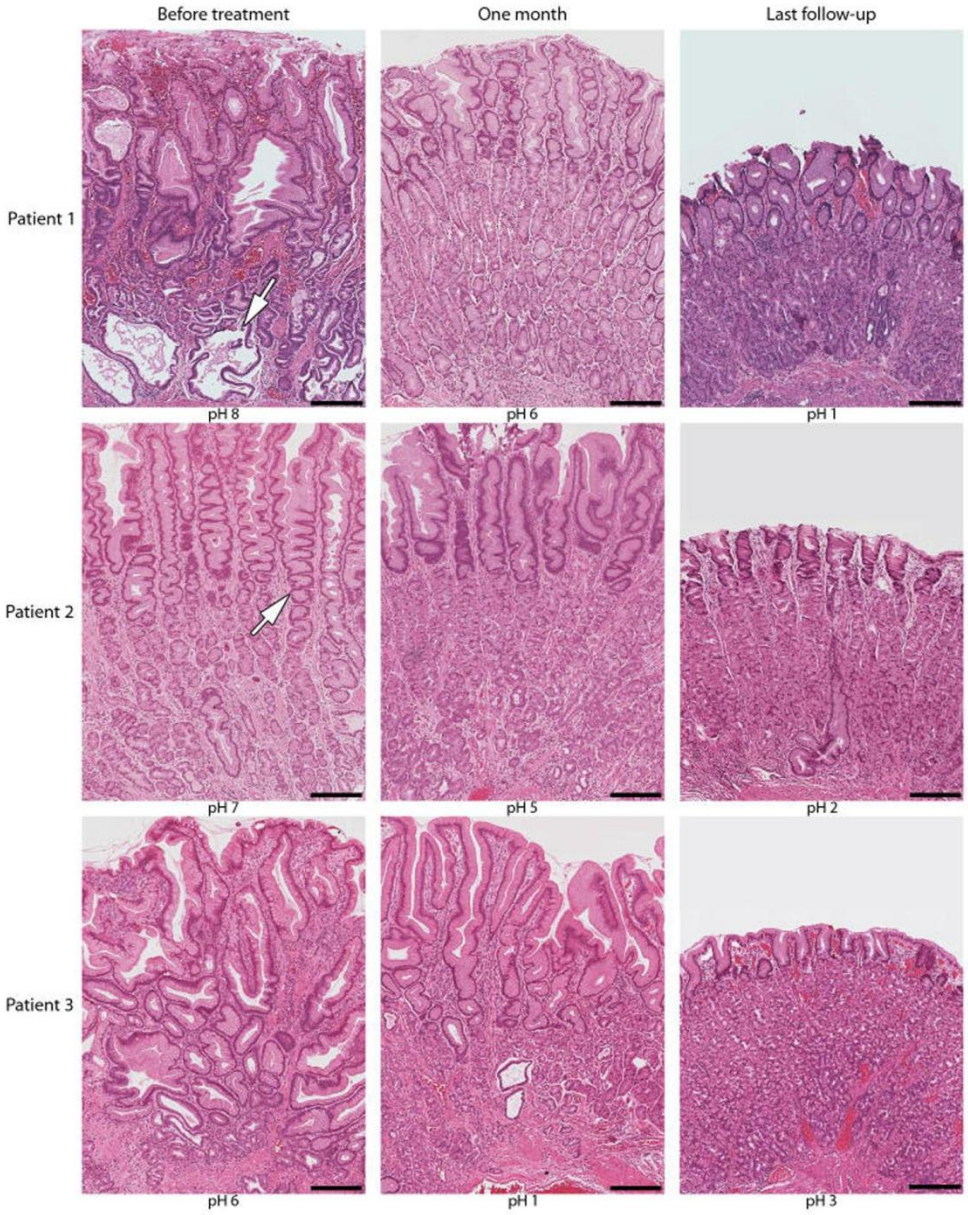



**Figure 9. Progressive improvement in gastric histology during cetuximab treatment.** Foveolar hyperplasia [with characteristic cysts (arrow in top left panel) and tortuous glands (arrow in middle left panel)] and glandular atrophy are evident the day before treatment by hematoxylin-eosin staining. At last follow-up, patients 1, 2 and 3 showed reduced thickness of gastric mucosa, regression of foveolar hyperplasia and restoration of glandular mucosa and gastric acidity. Cetuximab has been discontinued in patients 1 and 2. Patient 3 had the most distorted architecture at presentation. Progressive histological improvement was noted beginning at 31 months (Fig. S2) and last follow-up at 38 months. Scale bar is 250 microns.

increase in an ultrastructurally defined, immunohistochemically H+/K+-ATPase–negative, preparietal cell population in a transgenic mouse model in which parietal cells were ablated by H+/K+-ATPase promoter-driven expression of an attenuated diphtheria toxin A subunit (Karam and Leblond 1992; Li, Karam et al. 1996). We have hypothesized that excess EGFR stimulation in Ménétrier's disease results in overproduction of surface mucous cells at the expense of glandular lineages (Coffey, Washington et al. 2007); the results in animals cited above suggest the possibility that in patients with Ménétrier's disease, there is a relative abundance of H+/K+-ATPase–negative preparietal cells, which are held in a state of suspended maturation but poised to differentiate rapidly on EGFR blockade.

All seven participants who completed the trial elected to continue treatment because of improvements in their predominant symptom(s). There was no evidence that any of these patients treated with prolonged therapy developed resistance to cetuximab. The interval between infusions was increased to every 2 to 3 weeks and most showed continued clinical and histological improvement over time. Patients 1 and 2 were able to stop treatment altogether after remission of both symptoms and histological features of the disease. Patient 3 has been on treatment for more than 3 years and continues to show progressive histological improvement after 38 months compared to biopsies after 31 months (Figure 9). This suggests that prolonged therapy may provide continued benefit for some patients.

There have been anecdotal case studies of Ménétrier's disease patients

responding to various pharmacological interventions, including corticosteroids (Davis, O'Rourke et al. 1991); however, no consistent benefit has been observed. On the basis of the reported high incidence of infusion reactions to cetuximab in patients in the southeastern United States (O'Neill, Allen et al. 2007), dexamethasone (20 mg orally) was administered to five of the patients 20 min before cetuximab infusion. Although possible, we consider it highly unlikely that this contributed to the beneficial responses because the patients that have been treated successfully with corticosteroids for Ménétrier's disease received much higher continuous dosing. Moreover, five of our patients (patients 1, 3, 5, 7, and 8) had already been treated with continuous, high-dose corticosteroids for Ménétrier's disease–associated symptoms without any appreciable benefit before enrolling in this trial.

A notable feature of our study population was that four of nine (44%) participants also had ulcerative colitis. Seven patients with concurrent Ménétrier's disease and ulcerative colitis have been reported previously (Hatemi, Cagler et al. 2008; Hemmings 2007). EGF is involved in healing of the gastrointestinal mucosa, and EGF enemas are effective at reducing disease activity in ulcerative colitis (Sinha, Nightingale et al. 2003). As a result, there was concern that pharmacological blockade of EGFR could exacerbate colitis in these patients. However, none of the four patients with ulcerative colitis had worsening of their colitis symptoms, even with prolonged treatment, and periodic colonic biopsies from these patients did not show evidence of increased histological severity. The mechanism underlying the association of Ménétrier's disease and

ulcerative colitis is not known (Xavier and Podolsky 2007). All four patients with ulcerative colitis had pancolitis, although the duration and severity of the disease varied. In all cases, the diagnosis of ulcerative colitis preceded that of Ménétrier's disease. There was no common therapeutic regimen; two of the patients had received infliximab and 6-MP, one patient received 6-MP alone, and one patient received no immunomodulator therapy. Possible underlying factors that may link these two disorders include upregulation of EGFR ligands and a "leaky" mucosa that may provide a portal of entry for luminal contents (including commensal bacteria or their products). Whether these or other factors are operative or provide a link between the two disorders remains to be determined. Patient 2 had ankylosing spondylitis, suggesting a possible immunological underpinning to Ménétrier's disease.

From this trial, we cannot draw any firm conclusions regarding the ability of cetuximab to prevent malignant progression. One of our patients had coexisting gastric cancer that was only recognized after the second dose of cetuximab. The only patient in our study who developed a premalignant lesion during prolonged follow-up had stopped cetuximab therapy ~12 months before discovery of the lesion.

Although the precise etiology of Ménétrier's disease remains uncertain, our results suggest that a common feature of all cases of Ménétrier's disease is enhanced EGF receptor signaling. A form of the disorder, which is seen predominantly in children, has been associated with cytomegalovirus (CMV) infection (Eisenstat, Griffiths et al. 1995). These patients usually have an acute

presentation and spontaneous remission of symptoms and histological findings. When examined, increased TGFA immunoreactivity has been observed in the involved gastric mucosa of these cases (Xiao and Hart 2001). The principal envelope glycoprotein of CMV, glycoprotein B, binds EGFR, induces EGFR–HER3 heterodimers, and activates phosphoinositide 3-kinase–Akt signaling (Wang, Huong et al. 2003).

This trial suggests that blocking ligand binding to the EGFR with cetuximab is an effective treatment for patients with Ménétrier's disease. The patients in this study had severe disease, had failed all medical therapy, and were considering gastrectomy as the remaining treatment option. Before treatment, some were considered poor candidates for surgery because of their hypoalbuminemia and poor nutritional status. The seven patients who completed the 1-month trial experienced rapid clinical and biochemical improvement and elected to continue treatment. Four of the patients subsequently had near-complete histological remission, and two have remained asymptomatic off treatment for 19 and 4 months, respectively. Five of the patients ultimately required gastrectomy. In the subset of patients with Ménétrier's disease who are too ill to undergo gastrectomy, cetuximab may be a reasonable bridge to improve their operative risk. Because no other medical therapies have shown to be consistently beneficial, cetuximab should be considered as first-line therapy for Ménétrier's disease. It would be of interest to determine whether an oral EGFR tyrosine kinase inhibitor and/or a TNF-α–converting enzyme (TACE) inhibitor to block cell surface cleavage of TGFA would be as effective in this disorder as

intravenous delivery of the EGF receptor monoclonal antibody (Merchant, Voskresensky et al. 2008).

**Mimics of Ménétrier's disease**

During the cetuximab trial, we were referred a number of patients for treatment who turned out to have some other disorder. In fact, of the 48 patients referred to us, only 25 were confirmed to have Ménétrier's disease (Rich, Toro et al. 2010). This allowed us to investigate which signs and symptoms are specific to Ménétrier's disease, which are unlikely to be present in Ménétrier's disease, and which are shared with other gastric pathologies. In the end, we were able to formulate a decision-making tree for the proper diagnosis of Ménétrier's disease, which we hope will be used by clinicians confronted with the often confusing presentation of disorders caused by overgrowth of the gastric epithelium.

As mentioned, 48% of the patients referred to us for inclusion in the trial did not have Ménétrier's disease. The correct diagnosis in this group of patients was most commonly (13/23, 57%) gastric polyps or a polyposis syndrome. All the conditions referred to us are shown in Table 3.

We separated all patients referred to us into Ménétrier's disease, for those correctly diagnosed, and non-Ménétrier's disease, for those misdiagnosed. The non-Ménétrier's disease patients were then divided into two groups: those with polyps (non-Ménétrier's disease with polyps) and those without (non-Ménétrier's disease). The differences across groups are shown in Table 4 and Figure 10.

Table 3. Diagnoses of study patients.

| Disease state | Number |
|---|---|
| Ménétrier's disease | 25 |
| Juvenile Polyposis syndrome with gastric involvement | 3 |
| Gastric hyperplastic polyps | 4 |
| Hamartomatous polyp or unclassified polyposis syndrome | 4 |
| Cronkhite-Canada syndrome | 1 |
| Fundic gland polyps in setting of familial adenomatous polyposis | 1 |
| Proton pump inhibitor effect | 2 |
| Parietal cell hyperplasia | 2 |
| Chronic gastritis | 2 |
| Acute gastritis with G-cell hyperplasia | 1 |
| Gastric antral vascular ectasia | 1 |
| Linitis plastica with carcinomatosis | 1 |
| Diffuse signet cell gastric cancer | 1 |

Table 4. Patient characteristics and clinical complaints. Summary of the clinical comparisons made among MD patients, total non-MD patients, and non-MD patients with polyps

| Variable | MD (n=25) | Total non-MD (n=23) | Non-MD with polyps (n=13) |
|---|---|---|---|
| Age at diagnosis[1,2] | 40 (16 to 79) | 57 (39 to 73) | 52 (39 to 73) |
| Male:Female[3,4] | 18:7 | 9:14 | 5:8 |
| Nausea | 10 (40%) | 9 (39%) | 4 (31%) |
| Vomiting[3] | 12 (48%) | 3 (13%) | 2 (15%) |
| Peripheral oedema[5] | 11 (44%) | 5 (22%) | 3 (23%) |
| Pain | 12 (48%) | 13 (56%) | 5 (38%) |
| Weight loss | 8 (32%) | 7 (30%) | 3 (23%) |
| Anaemia[4] | 7 (28%) | 9 (39%) | 9 (69%) |
| Anaemia requiring transfusion | 2 (8%) | 4 (17%) | 4 (31%) |
| Early satiety | 1 (4%) | 3 (13%) | 1 (8%) |
| Diarrhea | 3 (12%) | 3 (13%) | 2 (15%) |
| Supplemental nutrition required | 3 (12%) | 0 | 0 |
| GERD-like symptoms | 3 (12%) | 2 (15%) | 2 (15%) |

[1] Wilcoxon rank sum test $p<0.05$ comparing MD to total non-MD.

[2] Wilcoxon rank sum test $p<0.05$ comparing MD to non-MD with polyps.

[3] Chi-square $p<0.05$ comparing MD to total non-MD.

[4] Chi-square $p< 0.05$ comparing MD to non-MD with polyps.

[5] Chi-square $p=0.11$ comparing MD to total non-MD.

These data suggest that along with foveolar hyperplasia and a basic gastric pH, Ménétrier's disease patients will more commonly present with vomiting and edema (likely due to hypoalbuminemia) when compared to all non-Ménétrier's disease patients. It is noteworthy in Figure 10 that despite having a median gastric pH of 6.5, Ménétrier's disease patients tend to have a normal serum gastrin, which is more surprising given that gastrin is known to be upregulated by EGFR stimulation and hypochlorhydria (Howell, Ziober et al. 1995). It is also worth noting that patients in the "non-Ménétrier's disease with polyps" category were more likely to be anemic, and trended towards a highly elevated serum gastrin despite a normal gastric pH, though there were not enough samples present for statistical significance.

In the end, we were able to suggest the clinicopathological decision-making tree shown in Figure 11. The diagnosis of Ménétrier's disease will, and should, involve both the gastroenterologist and pathologist, and possibly a radiologist. We expect a Ménétrier's disease patient to present with nausea, vomiting and peripheral edema. On endoscopy, the gastroenterologist will see enlarged gastric folds with thick mucus and a high gastric pH. We expect serum gastrin to be normal, and serum albumim to be low. On viewing a full thickness biopsy, we expect the pathologist to see foveolar hyperplasia, with tortuous, dilated glands that will generally maintain an orderly, parallel architecture, and possibly contain an increased number of eosinophils. Additionally, there is most often no family history of Ménétrier's disease. The absence of these qualities does not eliminate Ménétrier's disease, but suggests it is worth considering

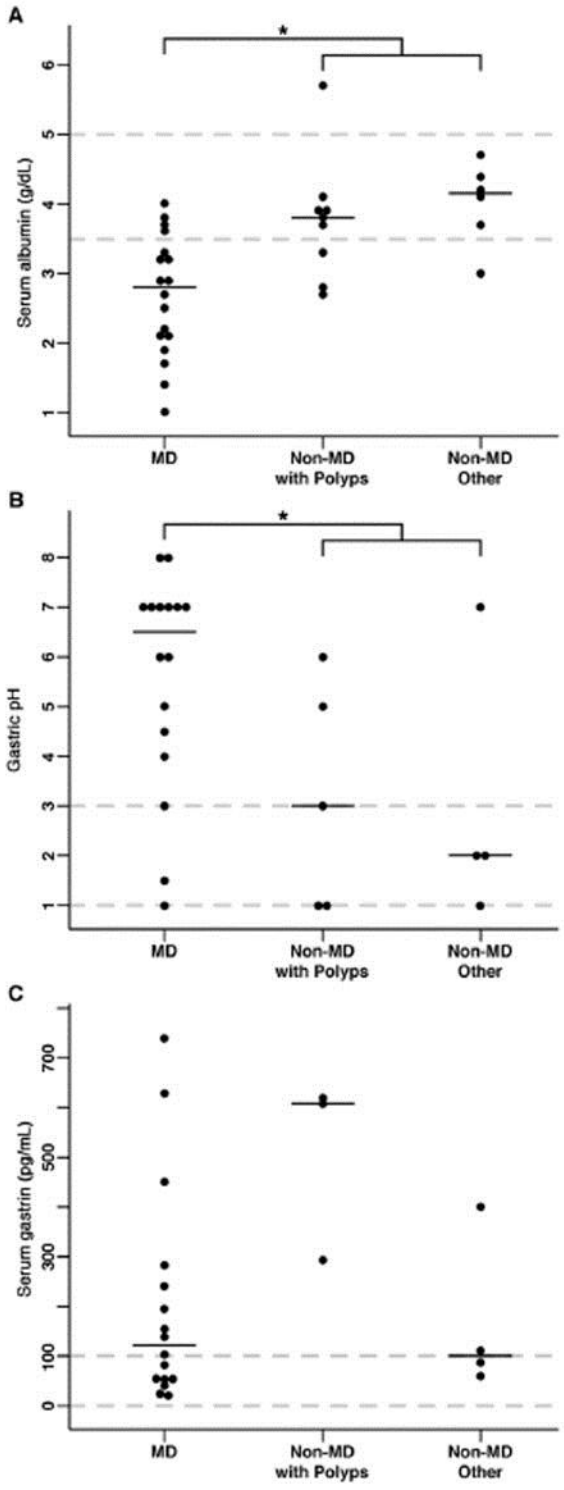



**Figure 10. Comparison of relevant lab values.** (A) Serum albumin. (B) Gastric pH. (C) Serum gastrin. Dotted lines signify normal range. Median values are designated with a bar for each group.

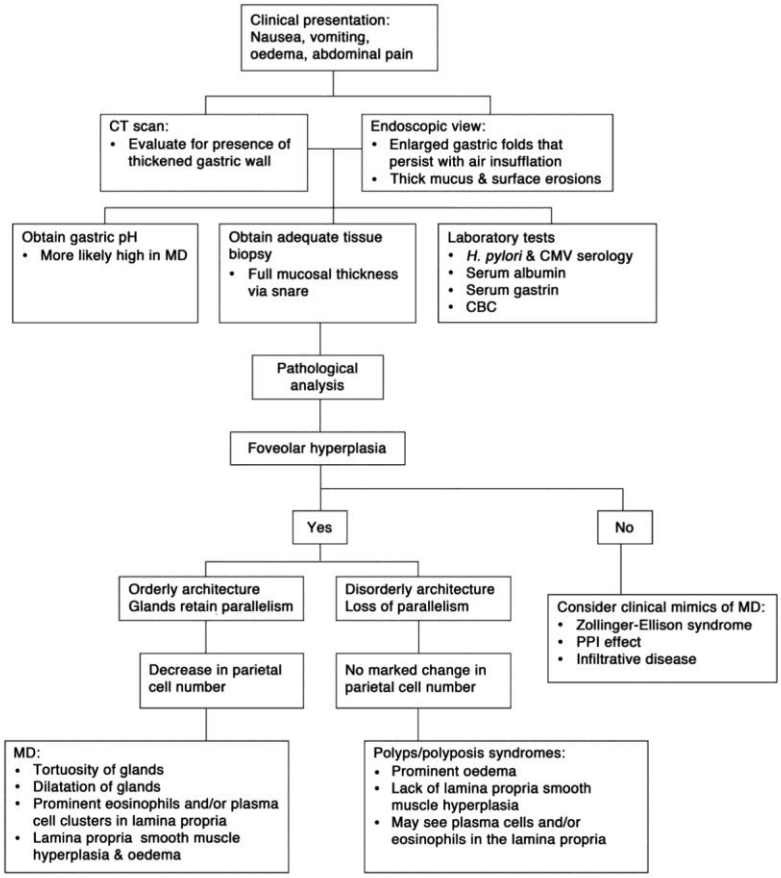



**Figure 11. Clinicopathological decision-making tree for the diagnosis of Ménétrier's disease.**

other diagnoses.

**Juvenile polyposis syndrome and Ménétrier's disease**

Unlike Ménétrier's disease, JPS can be confirmed genetically. Mutations in components of the TGFB pathway, *SMAD4* and *BMPRIA*, have been linked to JPS (Howe, Roth et al. 1998; Howe, Bair et al. 2001). It has also been reported that loss of *PTEN* might also cause JPS, but in hindsight, this turned out to be due to the loss of a portion of chromosome 10 (10q23), which is the locus of both *PTEN* and *BMPRIA*. Patients with JPS present with large, hamartomatous polyps in the colon and stomach, with gastric polyps being more common (and larger) in the presence of a SMAD4 mutation (Friedl, Uhlhaas et al. 2002). Histologically, these polyps are characterized by an immature stroma, from which the name is derived, with overlying normal-appearing epithelial cells (Figure 12). In addition to an increased risk for cancer, some JPS patients with *SMAD4* mutations also have the vascular disorder, Hereditary Hemorrhagic Telangiectasia (JP-HHT) (Gallione, Repetto et al. 2004).

I diagnosed the first patient I saw for inclusion in the Ménétrier's disease trial with JPS. This patient had a 4 bp deletion in one copy of *SMAD4*, which had been previously documented (Howe, Roth et al. 1998). It was noted using immunohistochemistry that she had sporadic SMAD4 immumoreactivity in her gastric epithelia, suggesting the somatic loss of the other SMAD4 allele. This finding was published by another group, showing the loss of wild-type (wt)

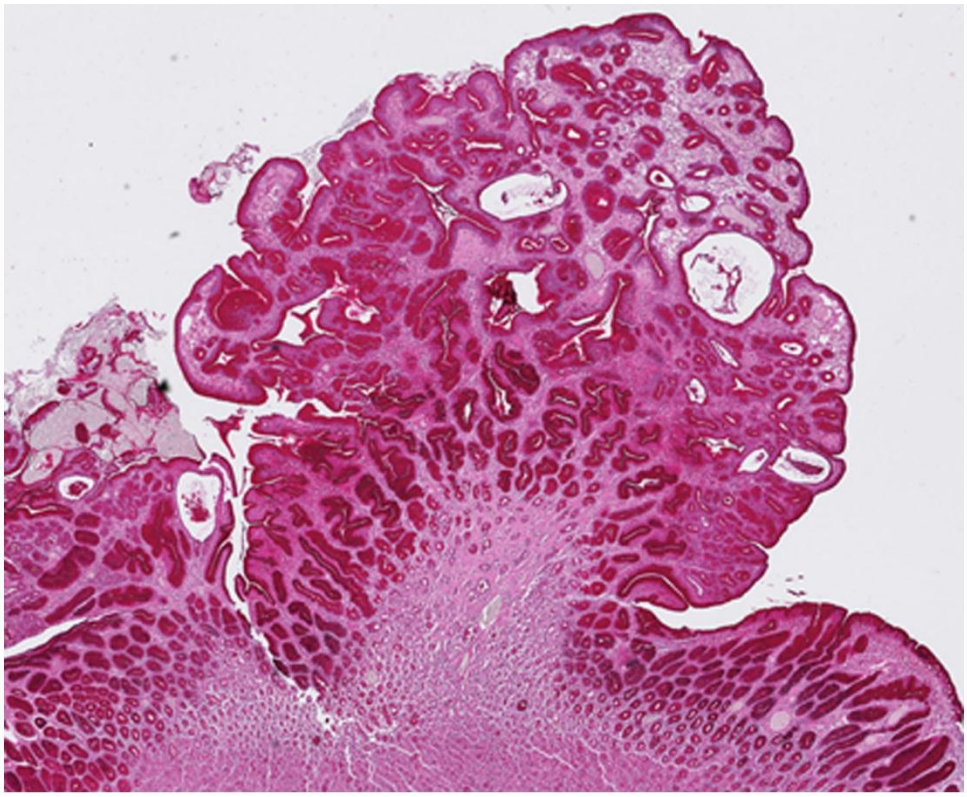


**Figure 12. A gastric juvenile polyp from a patient with a germ-line SMAD4 mutation.** Histologically, juvenile polyps are defined by an intense, immature stroma, with overlying normal-appearing epithelium. They are termed hamartomas, as opposed to adenomas, due to the normal appearance of the epithelium. However, there is an increased risk of carcinoma in these patients. In this specimen, which is stained with the Periodic-acid-Schiff stain to illustrate the surface mucous cells, it is apparent that the greatest architectural disorganization occurs above the isthmus. The base of the gastric glands remains relatively normal, and patients with JPS typically present with a normal gastric pH. Interestingly, on the lower right of this image, there is foveolar hyerplasia, but, unlike Ménétrier's disease, without glandular atrophy.

SMAD4 in the epithelium, stroma, or both by immunohistochemistry is common in SMAD4-linked JPS (Langeveld, van Hattem et al. 2010).

Patients with JPS are at increased risk for developing gastrointestinal carcinomas (Zbuk and Eng 2007; Calva and Howe 2008; Howe, Mitros et al. 1998). Though the mechanism of transformation remains unclear, it has been suggested that loss of wt SMAD4 in the epithelium is requisite (Alberici, Gaspar et al. 2008). However, as mentioned earlier, when Smad4 is specifically knocked out in T-cells, mice develop juvenile polyps and epithelial cancers throughout the GI tract, neither of which are seen upon targeted loss of Smad4 in the epithelium (Kim, Li et al. 2006). This argues against the necessity of wt SMAD4 loss in the epithelium of JPS patients, suggesting the increased risk of transformation in patients is a byproduct of a microenvironment that drives epithelial proliferation. Evidence suggesting that aberrant TGFB family signaling in stromal cells can lead to increased production of EGFR ligands comes from a mouse model in which specifically knocking out TGF-β type II receptor (TGFBR2) expression in fibroblasts results in gastric cancer; these fibroblasts increase the synthesis and secretion of TGFA and AREG (Cheng, Bhowmick et al. 2005; Bhowmick, Chytil et al. 2004).

I found an increase in phosphorylated EGFR (pEGFR) in the involved gastric mucosa of JPS patients, as well as the appearance of foveolar hyperplasia, which would explain the common misdiagnoses (Figure 13). These initial findings led to my original thesis proposal, which was based on the hypothesis that abrogation of TGFB family signaling in the stomach leads to

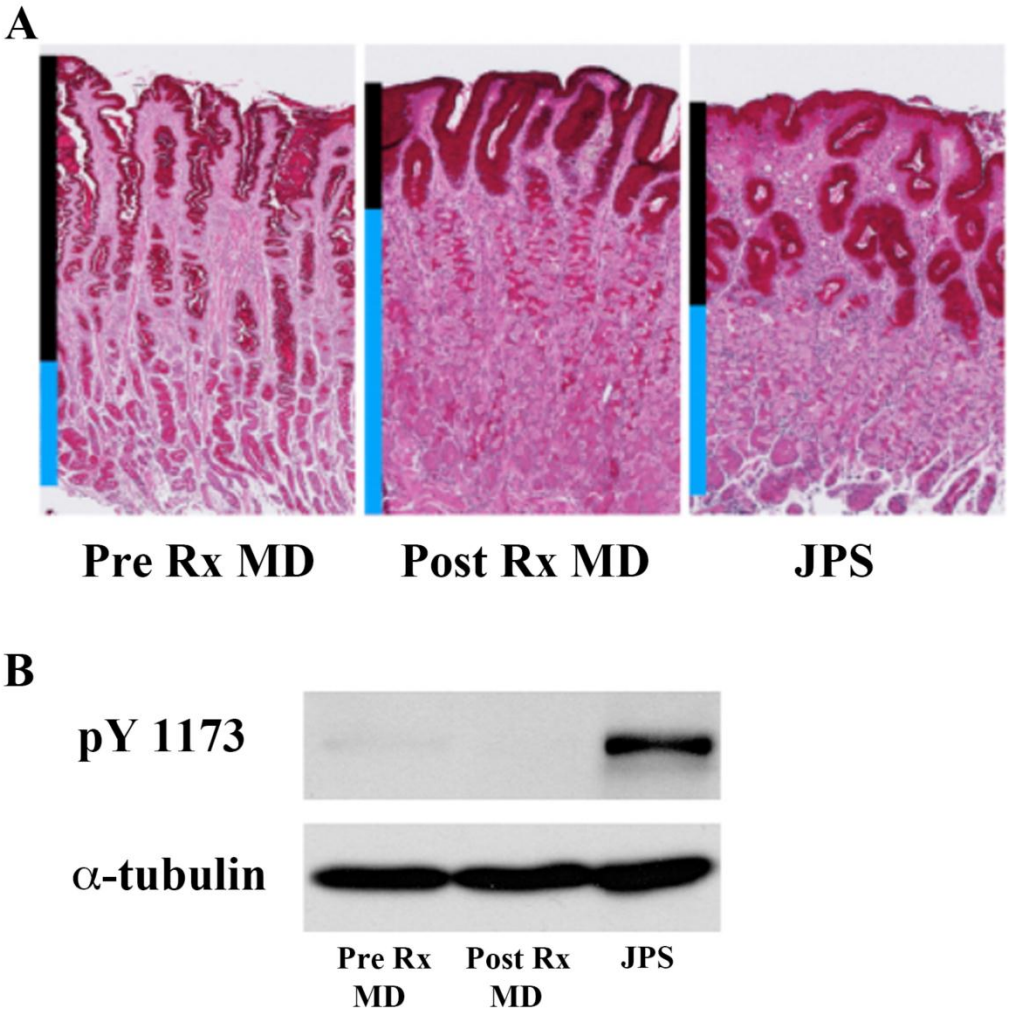



**Figure 13. Foveolar hyperplasia, and elevated phosphorylated EGFR are present in untreated Ménétrier's disease and JPS.** (A) Pre-treatment (Pre Rx) Ménétrier's disease patients show the histological sign of foveolar hyperplasia (FH), which is reversed upon treatment with cetuximab (Post 1 mo Rx). This is represented by the ratio of the black line (pit) to the blue line (gland). In the normal stomach, represented by Post Rx Ménétrier's disease, this ratio is 1:4. An unappreciated aspect of JPS, as seen on the right, is baseline FH, which we think precedes a polyp. Unlike Ménétrier's disease, there is no glandular atrophy. This may be a product of the significantly elevated gastrin levels common in JPS patients (see text), despite normal gastric pHs. Ménétrier's disease patients tend to have normal serum gastrin levels, despite hypochlorhydria. (B) From the same patients, tissue samples were analyzed for Western blot to determime the level of phosphorylated EGFR by probing for phosphotyrosine 1173.

increased EGFR signaling, specifically through the increased production of EGFR ligands by the epithelium and/or stroma.

To test this hypothesis, I began generating colon cancer cell lines in which I would alter the TGFB signaling pathway through the introduction of wt or mutant SMAD4 into human colon cancer cell lines via viral transduction, depending on their SMAD4 status. I would also do the same in stromal cell lines, and determine the status of EGFR ligand production in both.

As I embarked on these studies, a mouse model was published that essentially validated my hypothesis (Shinohara, Mao et al. 2010). In this mouse, expression of noggin, which is a secreted BMP inhibitor, was driven by the H+/K+ ATPase B subunit promoter. In turn, parietal cells were synthesizing and secreting Noggin. The histologic findings in this mouse had less in common with JPS and more in common with Ménétrier's disease. Like Ménétrier's disease patients, these mice had foveolar hyperplasia, fewer parietal cells, and hypochlorhydria. Importantly to my work, the authors also showed that *Tgfa* and *Areg* expression were increased three-fold in the gastric mucosa of these mice, and that levels of phosphorylated Erk2 were increased. I will further discuss these findings, particularly in the context of a future treatment for JPS, in the final chapter.

**Summary and rationale for studying rare diseases**

In this chapter, I have recapped my work on Ménétrier's disease and JPS. Since joining the lab, I have been involved in confirming the efficacy of the only known effective medical treatment for Ménétrier's disease, cetuximab. Further, based on the patients referred to us during the clinical trial, many incorrectly, I have assisted with the development of a clinco-pathological decison-making tree to aid in properly diagnosing Ménétrier's disease. Also during this trial, I observed many histopathological similarities between Ménétrier's disease and JPS, which might be due to some shared pathophysiological mechanisms. This led to my initial thesis project, which was to show a link between abrogation of TGFB family signaling and increased EGFR signaling in the stomach. I was to some extent "scooped' by the mouse model in which noggin was expressed in the stomach by parietal cells, but hope to publish a case report in the near future regarding similarities between these two diseases in humans.

Ménétrier's disease and JPS are both classified as rare diseases, which in the mind of some, might be reason enough to not waste time studying them, preferring instead to allocate resources towards things that are more common. This seems reasonable. However, rare diseases affect a lot of people. In the United States, a rare disease is defined as one that affects fewer than 200,000 Americans. By this criterion, there are 6,000 such disorders in this country affecting 25 million individuals.

Beyond the fact that rare diseases as a whole are common, studying a

rare disease may provide insight into disease pathogenesis, and even normal physiology, which extends far beyond the disease being studied. For example, Al Knudsen, a pediatrician in Philadelphia studying retinoblastoma, put forth the hypothesis that children affected with this rare disorder would have a germline mutation in one allele and tumors would result from a "hit" to the second. This proved to be correct, and fueled the still-dominant view in cancer biology that cancer is a disorder of mutated genes. Perhaps the 17th century medical pioneer William Harvery said it best: "Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path."

# CHAPTER III

# THE NEDD4 FAMILY OF E3 UBIQUITIN LIGASES

There are nine members of the NEDD4 family of E3 ubiquitin ligases: NEDD4, NEDD4L, WWP1, WWP2, ITCH, SMURF1, SMURF2, NEDL1, and NEDL2. Only in the last several years have they begun to be characterized, and their substrates identified. Each member is a modular protein, consisting of an N-terminal C2 domain, two to four centrally located WW domains, and a C-terminal, catalytic HECT domain. In this chapter, ubiquitylation, and the general mechanism by which the members of this family of E3 ligases recognize and ubiquitylate their substrates will be discussed. Further, the possible relevance of NEDD4 family members in cancer, with a particular focus on colorectal cancer (CRC), will be discussed in light of findings concerning their expression levels in different cancer types, and the currently known targets of NEDD4 family members. Finally, I will conclude this chapter by discussing my finding that one of the family members, NEDD4L, is downregulated in CRC, and offer evidence that it might act as a tumor suppressor via the inhibition of canonical WNT signaling.

## Ubiquitylation

Ubiquitylation is the three-step process that results in the covalent linkage

of ubiquitin(s), a 76 amino acid protein, to a Lys residue(s) in a substrate, or target, protein. It is a process central to cellular homeostasis, which is due primarily to its role in marking proteins for degradation by the 26S proteasome. More recently, ubiquitylation has also been shown to play important roles in protein trafficking, localization, and activity.

Activation of ubiquitin itself is the first step in the process of ubiquitylation. In this ATP-requiring step, one ubiquitin is linked via the carboxyl group of its C-terminal Gly residue to an internal Cys residue of an ubiquitin-activating enzyme, or E1, of which there are two in humans. This activated ubiquitin is then passed to a Cys residue of an ubiquitin-conjugating enzyme, or E2, of which more than 30 family members have been identified. Lastly, the E2-ubiquitin complex then interacts with one of more than 600 E3 ubiquitin ligases, allowing for highly specific substrate targeting. In this final step, the C-terminal carboxyl group of ubiquitin is ligated to the ε-amine group of a Lys residue in the target protein.

From the standpoint of the target protein, the process of ubiquitylation may not conclude with the addition of only one ubiquitin. If no more ubiquitins are added, it is termed monoubiquitylation. In general, monoubiquitylation causes the protein to be trafficked or localized differently, or activated or inactivated. Monoubiquitylation of membrane proteins, for instance, leads to their internalization by endocytosis, at which point they may be deubiquitylated and recycled, or further targeted to the lysosome for degradation. Additionally, a given protein can contain multiple monoubiquitylated Lys residues. This is termed multiubiquitylation, and the same general rules apply as to

monoubiquitylation.

Polyubiquitylation is a product of ubiquitin having 7 Lys residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63), each of which can serve as a sight for subsequent ubiquitin attachments. In turn, chains of ubiquitins can be formed. Most often, polyubiquitylation of a protein results in its degradation. The presence of four or more ubiquitins linked together at Lys48 residues leads to degradation by the proteasome, though it has recently been shown that chains formed using any Lys residue, with the exception of Lys63, can lead to proteasomal degradation. Chains linked by Lys63 residues may be marked for degradation by the lysosome, or, conversely, may result in the activation of a signaling pathway; for example, Lys63-linked polyubiquitylation of DVL can activate WNT signaling (Tauriello, Haegebarth et al. 2010). These generalities, however, will be subject to ongoing modifications given the only recently characterized differences in the structural conformations of the possible polyubiquitin chains, which will dictate the proteins with which these chains will interact (Ye, Blaser et al. 2012). In this chapter, the type of ubiquitin chain formed, if known, and the outcome of the modification of a substrate protein by a NEDD4 family member will be stated.

**E3 ubiqutin ligases, HECT-type E3s, and the NEDD4 family**

E3 ligases are divided into two major groups based upon the structure of their catalytic domain. The vast majority (~95%) of E3s are members of the

really interesting new gene (RING) or RING-related groups. A RING domain is a cysteine-rich, zinc-finger domain of about 40-60 amino acid residues. RING domains are not directly involved in the transfer of ubiquitin; rather, they serve as a site of interaction with E2s. In this scenario it is the E2 that is responsible for directly catalyzing the ubiquitylation of the substrate, with the RING-type E3, as a whole, serving as a scaffold by bringing into close proximity and proper orientation an E2, which binds with the RING domain, and a substrate, which binds with some other domain(s) in the RING-type E3. In some instances, the RING-type E3 may also serve as an allosteric activator of the E2 (Ozkan, Yu et al. 2005).

The HECT (Homologous to the E6-AP Carboxyl Terminus) family of E3s (~5%), on the other hand, directly catalyzes the transfer of ubiquitin from the E2 to the substrate. The HECT domain is an approximately 350 amino acid domain, consisting of an N-terminal region that is responsible for E2 binding, and a C-terminal region where an active-site Cys residue resides.

The NEDD4 family of E3 ligases is a subfamily of the HECT-type E3 family, and is also known as the C2-WW-HECT family, owing to its modular nature and the relative locations of the three requisite domains. At the N-terminus is one C2 domain, which binds $Ca^{2+}$ and phospholipids, and in turn, will target NEDD4 family members to intracellular membranes. Additionally, the C2 domain has been shown to be a source of auto-inhibition. This is thought to occur when the C2 domain interacts with the HECT domain, folding the E3 in a way that blocks protein-protein interaction (Wiesner, Ogunjimi et al. 2007).

C-terminal to the C2 domain are two to four WW domains, which are tryptophan-based motifs of approximately 23-30 amino acids. They are the primary means of substrate recognition by the NEDD4 family. WW domains target PY motifs in substrate proteins, which are simple, four amino acids motifs of the sequence PPXY or LPXY. More recently, it has been found that WW domains will also bind phospho-serine/threonine based motifs (PXpS/pTP), proline-rich sequences, and in the case of C-X-C chemokine receptor type 4 (CXCR4), an uncharacterized serine-based motif (Lu, Zhou,et al. 1999; Bhandari, Robia et al. 2009). In many instances, target proteins contain more than one of these motifs, thus allowing for multiple interactions between the substrate and NEDD4 family member (Plant, Lafont et al. 2000).

Finally, at the C-terminus of each of the NEDD4 family members is the HECT domain. As stated above, this domain is responsible for both the recruitment of the E2 and the direct catalysis of the ubiquitin ligation reaction. This reaction proceeds through a thioester complex formed between the C-terminal carboxyl group of ubiquitin and the active-site Cys residue in the HECT domain, concluding with the transfer of ubiquitin to the ε-amine group of a Lys residue in the target protein.

**The NEDD4 family members**

There are nine members of the NEDD4 family (NEDD4, NEDD4L, WWP1, WWP2, ITCH, SMURF1, SMURF2, NEDL1, and NEDL2) (Figure 14), which are

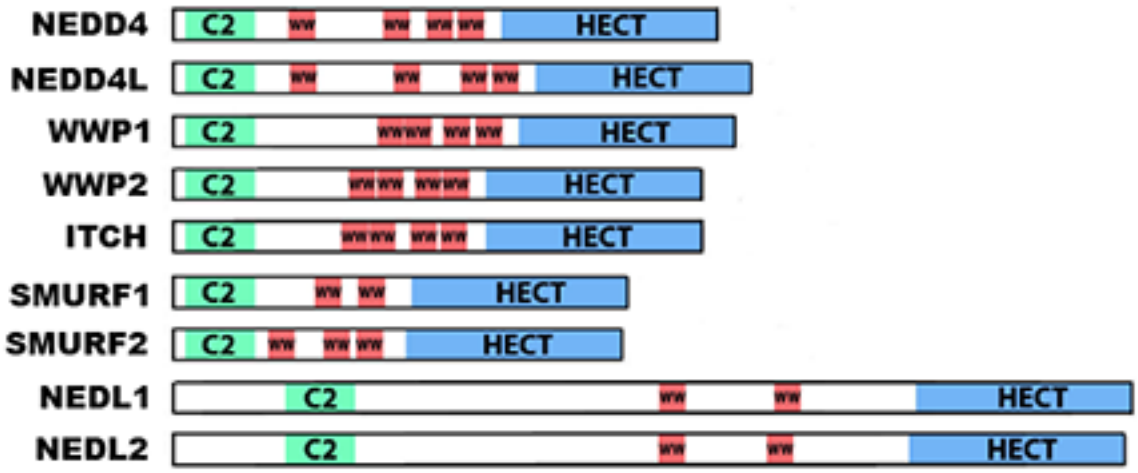



**Figure 14. The NEDD4 family of E3 ubiquitin ligases.** The nine members of the NEDD4 family are modular proteins. At their N-terminus, each has a C2 domain, which plays a role in proper cellular localization. The central portion of each protein contains two to four WW domains, which are responsible for target recognition. WW domains recognize PY motifs (PPXY, LPXY), as well as some phospho-Ser- or -Thr-based motifs. At the C-terminus is the HECT domain, which directly conjugates a ubiquityl moiety to the target protein. The family members are grouped together by name and homology. ITCH is more closely homologous to WWP 1 and 2 than to other family members.

further paired by name based upon greater sequence and structural similarity. The NEDD4 family itself derives its name from a screen meant to find genes expressed by neural-precursor cells (NPCs) that are downregulated with development (NPC-Expressed Developmentally Downregulated), which identified NEDD4 (Kumar, Tomooka et al. 1992). Shortly thereafter, NEDD4 was determined to be an E3 ligase (Kumar, Harvey et al. 1997). Later, NEDD4L was found, which shares 62% sequence homology with NEDD4 (Chen, Ross et al. 2001). For the first several years after its discovery, NEDD4, and eventually NEDD4L and WWP2, were studied predominantly in the context of their ability to negatively regulate levels of the epithelial sodium channel (ENaC) through ubiquitin-mediated degradation (Staub, Dho et al. 1996; Harvey, Dinudom et al. 2001; McDonald, Western et al. 2002). In fact, Liddle's syndrome, a disorder of improper sodium/potassium balance has been linked to mutations of the sodium channel that result in the loss of the PY motif, thus abrogating an interaction with members of the NEDD4 family, which can then target ENaC to the proteasome (Staub, Dho et al. 1996).

SMURF1 and SMURF2 (the SMAD Ubiquitination Regulatory Factors) were characterized around the same time, and share 74% sequence homology with each other. SMURF1 contains two WW domains while SMURF2 contains three. SMURF1 and SMURF2, were named when identified as E3 ligases for the receptor-regulated SMADS (R-SMADS), and consequently, as negative regulators of the BMP/TGFB signaling pathways (Podos, Hanson et al. 2001). The other two pairs, WWP1 and WWP2 (the WW domain-containing proteins;

68% homologous) and NEDL1 and NEDL2 (the NEDD4-Like Ubiquitin-Protein Ligases; 74% homologous), share the same modular layout. Lastly, ITCH, which has not been paired by name with any of the other family members, does share significant sequence homology (>65%) with the WWPs, and shares the same modular layout, with four WW domains located in the same approximate position. ITCH received its name based upon an immunological phenotype observed in a mouse model in which its function is abrogated (Itchy) (Perry, Hustad et al. 1998). WWP1, WWP2, and ITCH were orginally identified as interacting partners of atrophin-1, and grouped together by name as atrophin-1-interacting proteins (AIPs) (Wood, Yuan et al. 1998).

Despite this significant homology amongst the NEDD4 family members and their similar modular layout, it has been found that there is much diversity in target selection. Even amongst the highly homologous pairs, target selection differs, suggesting that factors beyond the C2 and WW domains are responsible for target selection. And while the HECT domains could also be responsible for differential substrate selection, it is worth noting that NEDD4 and NEDD4L share the same E2s, suggesting that the HECT domain plays an insubstantial role in target selection (Fotia, Cook et al. 2006).

**NEDD4 family targets**

Since the initial studies concerning the regulation of ENaC by NEDD4, the number of NEDD4-family targets has expanded rapidly. The majority of the

proteins targeted by NEDD4 family members contain a PY or PXpS/pTP motif, but not all targets do. Some targets are ubiquitylated by multiple family members, while others are targeted by only one, despite the presence of a PY motif(s). However, given the ability of a WW domain to bind a PY motif so readily, any proposed target of a NEDD4 family member should be viewed with skepticism if the only supporting data were found in the context of overexpression. This is illustrated by an experiment in which both NEDD4 and NEDD4L were knocked down and levels of ENaC were monitored. It was found that levels of ENaC were increased when NEDD4L was knocked down, but unchanged when NEDD4 was knocked down. This finding supports the idea that NEDD4L may be the true, physiologic E3 ligase of ENaC (Snyder, Steines et al. 2004).

Given that ENaC is a transmembrane protein, it would be expected that the phospholipid-binding C2 domain of NEDD4 is indispensable for the proper regulation of ENaC, by targeting NEDD4 family members to the cell membrane. However, this was shown to not be the case, as the C2 domain is not required for inhibition of ENaC, though multiple WW domains are required (Snyder, Olson et al. 2001). In yeast, the NEDD4 ortholog, Rsp5, was able to ubiquitylate a transmembrane substrate, the gap1 permease, following deletion of the C2 domain, but was unable to cause its endocytosis (Springael, De Craene et al. 1999). Further adding to the confusion regarding the predominant cellular localization of NEDD4, and more broadly, all NEDD4 family members, is the finding that NEDD4 can enter into and function in the nucleus (Hamilton,

Tcherepanova et al. 2001). Ultimately, as will be seen, the members of the NEDD4 family generally target cytoplasmic and membrane-associated or transmembrane proteins. Targeting can be dependent upon the C2 domain, the WW domains, and/or interacting partners (Sullivan, Lewis et al. 2007; Plant, Lafont et al. 2000).

The following discussion of NEDD4 family targets will focus on those targets that have a defined role as either a tumor suppressor or tumor/metastasis promoter, unless the target is otherwise noteworthy. Proteins targeted, and signaling pathways affected by the NEDD4 family can be found in Table 5.

NEDD4 targets a number of proteins involved in the pathways central to CRC development mentioned in Chapter I (Table 5). NEDD4 has been found to target both CBL, an EGFR E3 ligase, and PTEN, the phosphatase that antagonizes PI3K, thus allowing for prolonged or increased activation of both the RAS/ERK and PI3K/AKT pathways, respectively (Magnifico, Ettenberg et al. 2007; Drinjakovic, Jung et al. 2010). The BMP arm of TGFB family signaling is inhibited by NEDD4, which has been found to target activated SMAD1 to the proteasome; phosphorylated SMAD1 has also been shown to be a target of NEDD4L (Kim, Lee et al. 2011). NEDD4 has been shown to activate the Hedgehog pathway by causing the degradation of Patched (Luo, Chen et al. 2012). The Hedgehog signaling pathway has been shown to promote colon cancer cell growth (Mazumdar T, DeVecchio et al. 2011). NEDD4 has also been shown to ubiquitylate and degrade a number of growth-promoting receptor tyrosine kinases, including insulin-like growth factor 1 receptor (IGF1R), vascular

Table 5. The known targets of the NEDD4 family, the CRC pathways they affect, and the current knowledge of their expression levels in human cancers.

| E3 | Known targets | CRC pathways affected | Expression in cancer |
|---|---|---|---|
| NEDD4 | CBL, PTEN, SMAD1, PTC, IGF1R, VEGFR2, FGFR1, Notch, ENaC, RNAPII, CNrasGEF, AR, Sprouty2, Beclin1, Bcl10, Ptc, p63, p73 | EGFR, PI3K/AKT, BMP, Hh, Notch | Gastric, Colon, NSC Lung |
| NEDD4L | ACK1, SMAD 2 & 3, TGFBR1, DVL2, ENaC, Kv1.3, SGLT1, NaV1.5, ATA2, Fe65, DAT, Occludin, TrkA, hERG1, p130 | EGFR, TGFB, WNT | Prostate/Prostate, Gallbladder, Gastric, Glioma |
| WWP1 | CDKN1B, SMAD4, TGFBRI, HER4, p27, Ezrin, KLF5, JunB, RNF11 | TGFB, p27 | Prostate, Breast |
| WWP2 | Oct4, PTEN, SMAD 2, 3 & 7, ENaC, EGR2, GluR2, Gsc | PI3K/AKT, TGFB/TGFB | |
| ITCH | CBL, HER4, DVL2, p63, p73, CXCR4, Bcl10, HER4 | EGFR, HER4, WNT, p53 | |
| SMURF 1 | SMAD 1, 4, 5 & 8, TGFBR1, MDM2, STAT1, RhoA, ING2, JunB, Talin | BMP, TGFB/TGFB, BMP, p53 | Pancreas |
| SMURF 2 | CBL, GSK3B, SMAD 1, 2, 3 & 7, Prickle, RNF11, SMURF1, Id1, KLF5 | EGFR, TGFB, BMP | Renal Cell, Esophageal |
| NEDL1 | HER4, DVL2, p53 | HER4, WNT, p53 | Neuroblastoma |
| NEDL2 | p73 | p53 | |

In the “CRC pathways affected” and “Expression in cancer” columns, red indicates inhibition and downregulation, respectively, while green indicates activation and upregulation.

endothelial growth factor receptor 2 (VEGFR2), and fibroblast growth factor receptor 1 (FGFR1) (Vecchione, Marchese et al. 2003; Murdaca, Treins et al. 2004; Persaud, Alberts et al. 2011). Finally, in *Drosophila*, Nedd4 has been found to ubiquitylate Notch1, leading to its degradation (Koncarevic, Jackman et al. 2007). Notch signaling is thought to support tumorigenesis, though recent evidence suggests it may also suppress canonical Wnt signaling (Kim, Koo et al. 2012).

NEDD4 and NEDD4L share the same E2s, which correlates with the more highly homologous sequence of their respective HECT domains relative to that of their other domains (Fotia, Cook et al. 2006). While closely homologous to NEDD4, NEDD4L targets a number of different proteins for ubiquitylation. Interestingly, NEDD4L also abrogates TGFB family signaling, but does so by targeting different members than NEDD4. NEDD4L targets phosphorylated SMAD2 and 3, which is accomplished following an ERK-dependent phosphorylation of the SMADs (Gao, Alarcón et al. 2009; Aragón, Goerner et al. 2011). Further, NEDD4L has been found to down-regulate the type I TGFB receptor (TGFBRI) following recruitment by SMAD7 (Kuratomi, Komuro et al. 2005). In contrast to NEDD4, NEDD4L causes the degradation of the EGFR via an association with activated CDC42 kinase 1 (ACK1) (Chan, Tian et al. 2009). Recently, NEDD4L was shown to inhibit both canonical and non-canonical WNT signaling through the degradation of DVL1, 2, and 3 (Ding, Zhang et al. 2013).

WWP1, WWP2, and ITCH, are more highly homologous to each other, with respect to both sequence and domain layout, than to other members of the

NEDD4 family. Despite this, there is much divergence in their targets. Important targets of WWP1 include the tumor suppressors, cyclin-dependent kinase inhibitor 1B (CDKN1B), which controls cell cycle progression, and SMAD4, which is central to all canonical TGFB family signaling (Cao, Xue et al. 2011; Morén, Imamura et al. 2005). WWP1 also abrogates TGFB signaling through the destruction of TGFBRI in a SMAD7-dependent manner (Komuro, Imamura et al. 2004). WWP1 targets the ERBB family member, HER4, leading to its degradation (Feng, Muraoka-Cook et al. 2009).

Like WWP1, WWP2 has been shown to degrade SMADs. However, the resultant outcome, whether abrogation or promotion of TGFB signaling, is dependent upon which splice variant of WWP2 is present (Soond and Chantry 2011). The authors of this study found that there are three isoforms of WWP2: a full-length form (WWP2-FL), a C-terminal form that lacks the C2 domain and the three most N-terminal WW domains (WWP2-C), and an N-terminal form that lacks the three most C-terminal WW domains and the HECT domain (WWP2-N). They then showed that WWP2-N, in conjunction with WWP2-FL, targets inactive SMAD2 and 3 for degradation, while WWP2-C targets SMAD7 for degradation, which would enhance TGFB signaling. In contrast to the other NEDD4 family members that target SMAD7, WWP2 does not also ubiquitylate TGFBRI.

ITCH has been found to target a number of proteins important to cancer that are also targeted by other NEDD4 family members: CBL, HER4, and phosphorylated DVL2 (Magnifico, Ettenberg et al. 2003; Li, Zhou et al. 2009; Wei, Li et al. 2012). Importantly, ITCH ubiquitylates two members of the p53

family of tumor suppressors: p63 and p73. ITCH directly binds and ubiquitylates p63, while the mechanism by which p73 is ubiquitylated is less well-defined, but is dependent upon the E3 mouse double minute 2 homolog (MDM2) (Rossi, Aqeilan et al. 2006; Kubo, Okoski et al. 2010). Interestingly, ITCH enhances the ability of SMAD7 to interact with TGFBRI, but does not directly cause its degradation (Lallemand, Seo et al. 2005).

Smurf1 was initially identified in Xenopus as a regulator of the BMP-specific Smads (Smad1, 5, and 8) (Zhu, Kavsak et al. 1999). In this paper, it was also shown that by inhibiting the BMP arm of TGFB family signaling, Smurf1 promoted the TGFB arm by enhancing Smad2 signaling. However, it has since been show that SMURF1 can also inhibit the TGFB arm by degrading TGFBRI in conjunction with SMAD7 (Ebisawa, Fukuchi et al. 2001). SMURF1 also down-regulates SMAD4 when it is bound to SMAD1, 5, 7, or 8 (Morén, Imamura et al. 2004). The impact of SMURF2 on TGFB family signaling is more nuanced. In addition to ubiquitylating the BMP SMADs, SMURF2 ubiquitylates SMAD2 and 3 (Lin, Liang et al. 2000; Mizuide, Hara et al. 2003). However, in conjunction with SMAD2, SMURF2 has been shown to down-regulate the transcriptional corepressor SnoN, in turn, supporting SMAD-dependent TGFB signaling (Bonni, Wang et al. 2001).

In addition to TGFB family inhibition, SMURF1, as well as SMURF2, indirectly causes the degradation of p53 through the stabilization of MDM2, which then ubiquitylates p53, and targets it for degradation (Nie, Xie, et al 2010). Both Smurf1 and Smurf2 have also been shown to play a significant role in the non-

canonical Wnt, or planar cell polarity (PCP), pathway. They do this by degrading Prickle1, the precise localization of which is required for proper PCP signaling (Narimatsu, Bose et al. 2009).

The least-characterized members of the NEDD4 family are NEDL1 and NEDL2. They are the most structurally different when compared to the other NEDD4 family members. NEDL1 has been found to ubiquitylate HER4 and DVL1, like other family members (Li, Zhou et al. 2009; Miyazaki, Fujita et al. 2004). Additionally, NEDL1 has been found to enhance p53-mediated apoptosis (Li, Ozaki et al. 2008). The mechanism by which this works is not known, though the authors did show it requires both a direct interaction between the two proteins, and a functional catalytic unit in NEDL1. The only described function of NEDL2 is the stabilization of p73 (Miyazaki, Ozaki et al. 2003). This was shown to be ubiquitylation-dependent. However, it was found that NEDL2-mediated polyubiquitylation stabilized p73 and enhanced its transcriptional activity.

**The expression patterns of NEDD4 family members in human cancers**

As seen, there are a large number of proteins targeted by NEDD4 family members, and given the minimal nature and frequency of the PY binding motif, there are potentially thousands of other targets that have yet to be defined. With respect to CRC, the targets discussed thus far would seem to support the idea that the NEDD4 family would support tumor progression, with the exception of NEDL1 and NEDL2, which appear to oppose tumor progression. I say this

because the TGFB pathway is inhibited by each of the other seven family members. In addition, several family members appear to support RAS/ERK and PI3K/AKT signaling, while inhibiting the p53 pathway. An exception to this is the ability of two members, ITCH and NEDD4L, to inhibit canonical WNT signaling through the degradation of DVL. However, the level at which they inhibit WNT signaling is above the level at which the most common mutations affecting the pathway occur, possibly negating any substantial impact on WNT signaling these two family members may possess. In this section, I will discuss what is known about the expression of the NEDD4 family in cancer, and introduce my findings concerning the expression patterns in CRC.

Following the finding that PTEN is a target of NEDD4, it was discovered that NEDD4 is overexpressed in both CRC and gastric cancers by immunohistochemical staining (IHC) (Kim, Yoo et al. 2008). This was later confirmed in CRC by microarray analysis, though the authors went on to show that in culture that while NEDD4 overexpression increases cell proliferation in CRC cell lines, it does not impact PTEN levels (Eide, Cekaite et al. 2013). In contrast to this, a study in non-small cell lung cancer found that NEDD4 was overexpressed by IHC analysis, and in some instances amplified, and that NEDD4 levels correlated inversely with PTEN levels (Amodio, Scrima et al. 2010).

NEDD4L has been found to be both upregulated and downregulated in prostate cancer when compared to normal adjacent tissue or benign prostatic hyperplasia tissue (Hellwinkel, Asong et al. 2011; Hu, Xu et al. 2008). It has also

been shown to be increased in invasive gallbladder cancer cell lines, which the authors attributed to the increased expression of the matrix metalloproteinses (MMPs), MMP-1 and MMP-13 (Takeuchi, Adachi et al. 2011). In gastric cancer, higher NEDD4L expression by IHC analysis was associated with less aggressive cancer and a better prognosis (Gao, Pang et al. 2012). Lastly, it was found that NEDD4L is decreased by IHC in malignant glioma, and that patients with lower NEDD4L expression have a worse prognosis (He, Deng et al. 2012).

WWP1 has been shown to be increased, commonly through gene amplification, in both prostate and breast cancer tissues, which translates into an increase in both transcript and protein levels (Chen, Sun et al. 2007; Chen, Zhou et al. 2007). These authors went on to show that knock-down of WWP1 in human prostate or breast cancer cell lines resulted in growth suppression (Chen, Sun et al. 2007; Chen, Zhou et al. 2007).

SMURF1 has been found to be overexpressed in a subset of pancreatic cancers, commonly by gene amplification, and to promote invasiveness of pancreatic cell lines in culture (Birnbaum, Adélaïde et al. 2011; Kwei, Shain et al. 2011). SMURF2 has also been found to be overexpressed in renal cell carcinoma, with levels being inversely correlated to levels of TGFBRI (Fukasawa, Yamamoto et al. 2010); in both studies the analysis was done using semi-quantitative IHC.

With the exception of NEDL1, which was found to be downregulated in the most aggressive neuroblastomas, levels of the other NEDD4 family members have not been characterized or significantly altered in cancer (Li, Ozaki et al.

2008). More importantly, how the members of the NEDD4 family are regulated in CRC is unknown, excepting the discovery that NEDD4 is commonly overexpressed.

**The NEDD4 family in CRC**

The purpose of this study was to begin to understand the role of the NEDD4 family of E3 ubiquitin ligases in human CRC. Nine different E3s comprise the NEDD4 family. Each member is a modular protein, containing an N-terminal C2 domain, two to four central WW domains, and a C-terminal, catalytic HECT domain. Members of this family are known to affect pathways central to CRC development, including the WNT, TGFB, EGFR, and p53 pathways. Recently, *NEDD4* was found to be overexpressed in CRC (Eide, Cekaite et al. 2013). The expression patterns of the other family members in CRC have not been studied. Herein, we determined the expression patterns of all nine NEDD4 family members in CRCs from 250 patients. In agreement with the previous study, we noted an upregulation of *NEDD4*. Surprisingly, we found that *NEDD4L*, the closest homolog to *NEDD4*, was the most highly downregulated family member in CRC. We found that NEDD4L protein was significantly decreased by Western blotting in CRCs compared to adjacent normal mucosa. In addition, NEDD4L, but not catalytically inactive NEDD4L, inhibits canonical WNT signaling at or below the level of β-catenin *in vitro*. Our results suggest that *NEDD4L* may play a role as a tumor suppressor in CRC.

## Results

Expression levels of NEDD4 family members in CRC

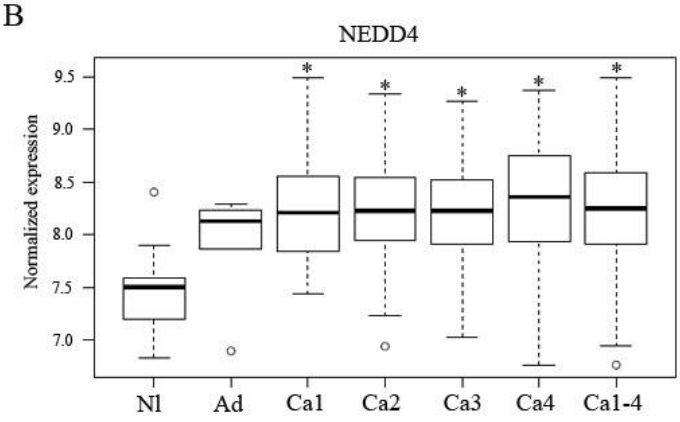
The NEDD4 family of E3 ubiquitin ligases has nine members, each of which is a modular protein (Figure 14). As a whole, the NEDD4 family is known to be involved in the regulation of a number of proteins and pathways that are central to the development of CRC (Table 5). There is high sequence homology amongst the family members, which explains the shared targets of many of the family members. However, there are also examples of different family members having opposing effects on a particular signaling pathway. Thus, there is the potential for a given member of the NEDD4 family to play the role of tumor suppressor or promoter in CRC.

In order to determine a potential role for the NEDD4 family in the initiation or progression of CRC, we subjected 250 CRC patient tumor samples of differing stages, 6 adenoma samples, and 10 adjacent normal samples to microarray analysis, noting how the expression of each family member changed with progression (Figure 15A). Interestingly, the closely homologous pair of *NEDD4* and *NEDD4L* are oppositely regulated (Figure 15B, C). *NEDD4* trends upward at the adenoma stage, and remains elevated during tumor progression. Conversely, *NEDD4L* expression decreases, trending downward in adenomas, and is significantly decreased in all stages of CRC (Figure 15C).

A

| | Nl | Ad | Ca1 | Ca2 | Ca3 | Ca4 |
|---|---|---|---|---|---|---|
| NEDD4 | 1 | 1.359428 | 1.682959 | 1.694665 | 1.69584 | 1.818816 |
| NEDD4L | 1 | 0.85825 | 0.614091 | 0.595596 | 0.593347 | 0.601971 |
| WWP1 | 1 | 1.685872 | 1.331455 | 1.226894 | 1.28493 | 1.47361 |
| WWP2 | 1 | 0.825141 | 0.839467 | 0.84211 | 0.824961 | 0.840126 |
| ITCH | 1 | 1.055959 | 1.433556 | 1.372546 | 1.411432 | 1.40497 |
| SMURF1 | 1 | 1.192641 | 1.192875 | 1.162142 | 1.197492 | 1.22434 |
| SMURF2 | 1 | 1.309341 | 1.222462 | 1.223586 | 1.367164 | 1.350394 |
| NEDL1 | 1 | 0.810875 | 0.853608 | 0.851682 | 0.804909 | 0.80196 |
| NEDL2 | 1 | 0.913225 | 1.160865 | 1.204133 | 1.281427 | 1.224281 |

B




C

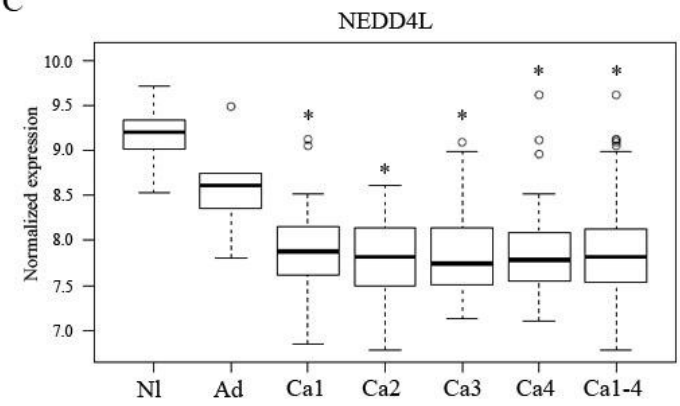



**Figure 15. Expression levels of the NEDD4 family in CRC.** (A) The expression of all nine NEDD4 family members was examined in CRC by microarray profiling. Shown here is the average fold-change of all probes in Nl (N=10), Ad (N=6), Ca1 (N=33), Ca2 (N=76), Ca3 (N=82), and Ca4 (N=59). (B) *NEDD4* is the most highly upregulated member of the NEDD4 family in CRC. (C) *NEDD4L* is the most highly downregulated member of the NEDD4 family in CRC. Nl = normal, Ad = adenoma, and Ca1 = stage 1 CRC, etc. *P<0.05.

NEDD4L protein is down in CRC

As transcript levels of a particular gene do not always correlate with protein levels, we next determined whether the decrease in *NEDD4L* mRNA resulted in a decrease in NEDD4L protein. To do this, we performed Western blot analysis on human CRC tumor and adjacent normal tissue samples from 20 patients using tissue collected at VUMC (Figure 16A, B). We observed a significant decrease in NEDD4L protein levels (~42%) in human CRC tumors compared to normal tissue, which was consistent with the microarray analysis.

*NEDD4L* expression levels and disease-specific survival in CRC patients

We next sought to determine whether NEDD4L expression levels correlate with disease-specific survival in our cohort of patients. As NEDD4L was found to be down in 75% (15/20) of the tumor samples analyzed by Western blot, and up or unchanged in 25%, we compared the survival of patients in the highest quartile of *NEDD4L* expression with that of those in the lowest (Figure 17). We found that those patients with highest *NEDD4L* expression trended towards a longer period of disease-specific survival than those patients with the lowest expression levels. This difference, however, did not reach statistical significance (P=0.079).

NEDD4L suppresses canonical WNT signaling

The significant decrease in NEDD4L expression in CRC suggests the possibility that NEDD4L plays a tumor-suppressive role in CRC. Given the

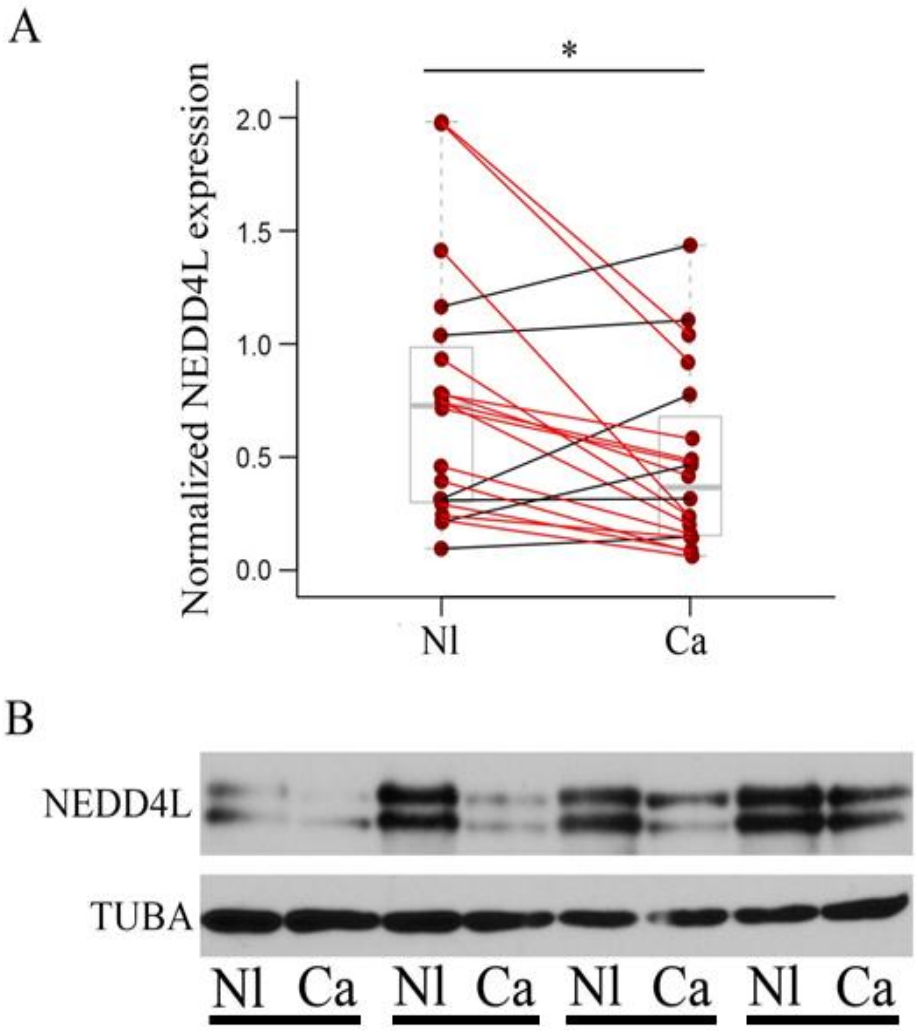



**Figure 16. NEDD4L protein levels are down in CRC.** (A) NEDD4L levels were determined by Western blotting of CRC (Ca) and adjacent normal (Nl) mucosa from twenty individuals. Levels were normalized to TUBA and Nl was compared to Ca. NEDD4L was significantly downregulated (~42%) in CRC (*P<0.05). Data is represented in box and whisker plot format. The lines connecting data points show the relative NEDD4L levels in a given Nl-Ca matched pair. Red denotes decreased NEDD4L levels in Ca, while black denotes an increase. (B) Lysates generated from Nl-Ca matched pairs were blotted for NEDD4L and TUBA. Shown here are four representative pairs.

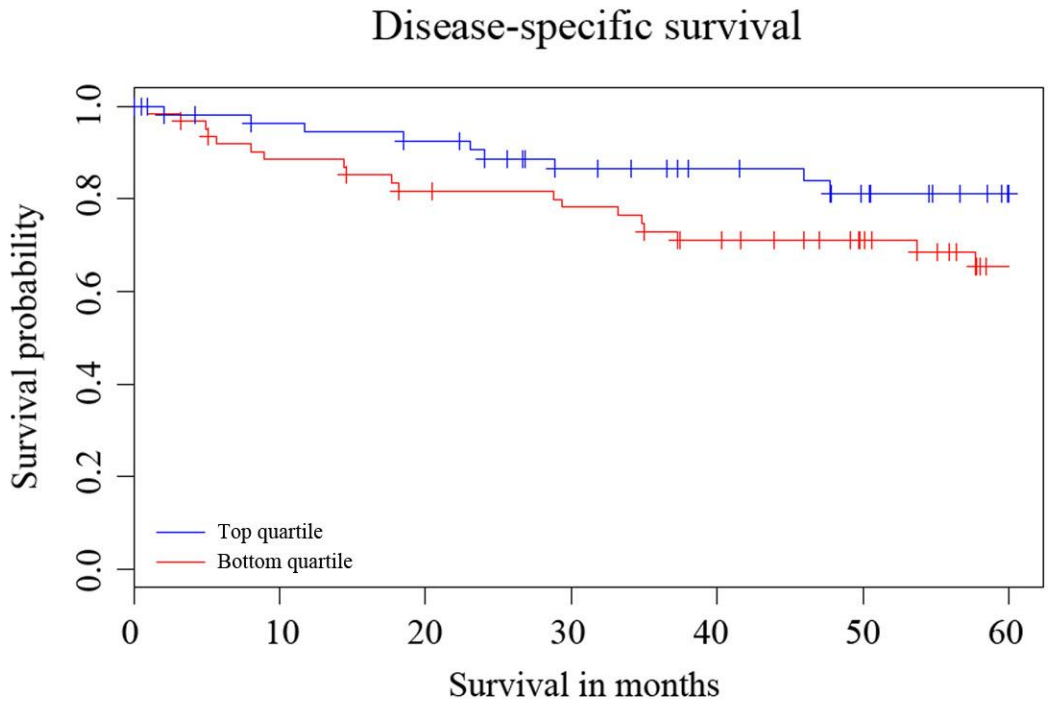



**Figure 17. Patients with highest *NEDD4L* expression show a trend towards longer disease-specific survival compared to those with the lowest.** Disease-specific survival in CRC patients with the highest and lowest *NEDD4L* expression was compared using Kaplan-Meier analysis. Those patients with tumor samples in the highest quartile of *NEDD4L* expression showed a trend towards longer disease-specific survival over a 60-month period. P=0.079.

centrality of canonical WNT signaling to the initiation and progression of CRC, we investigated whether NEDD4L can suppress canonical WNT signaling by performing the TOPFlash reporter assay. When canonical WNT signaling was activated by the addition of WNT3A and the coactivator, R-Spondin, and activity was measured using the TOPFlash assay, there was a significant reduction in canonical WNT signaling activity when a construct containing NEDD4L is cotransfected compared to empty vector or a construct containing a catalytically inactive NEDD4L mutant (NEDD4L C>A) (Figure 18A). This is in line with the recent finding that NEDD4L inhibits WNT signaling by targeting DVL for degradation. In this study, the investigators noted that DVL1, 2, and 3 contain a PY motif, which will interact with a WW domain in NEDD4L. However, we also found that NEDD4L inhibits canonical WNT signaling when wild-type (wt) or mutant (ΔN89) β–catenin is used as an activator (18B), suggesting NEDD4L is able to suppress WNT signaling downstream of DVL in the presence of mutant *APC* or *CTNNB1*.

Two mediators of canonical WNT signaling downstream of β–catenin activation that contain a PY motif are the transcriptional coactivators B-cell CLL/lymphoma 9 (BCL9) and BCL-9 like (BCL9L). These proteins interact with β–catenin in the nucleus when it is bound to TCF4 and transcriptionally active. To date, we have been unable to see an effect on or interaction with either BCL9 or BCL9L by NEDD4L.

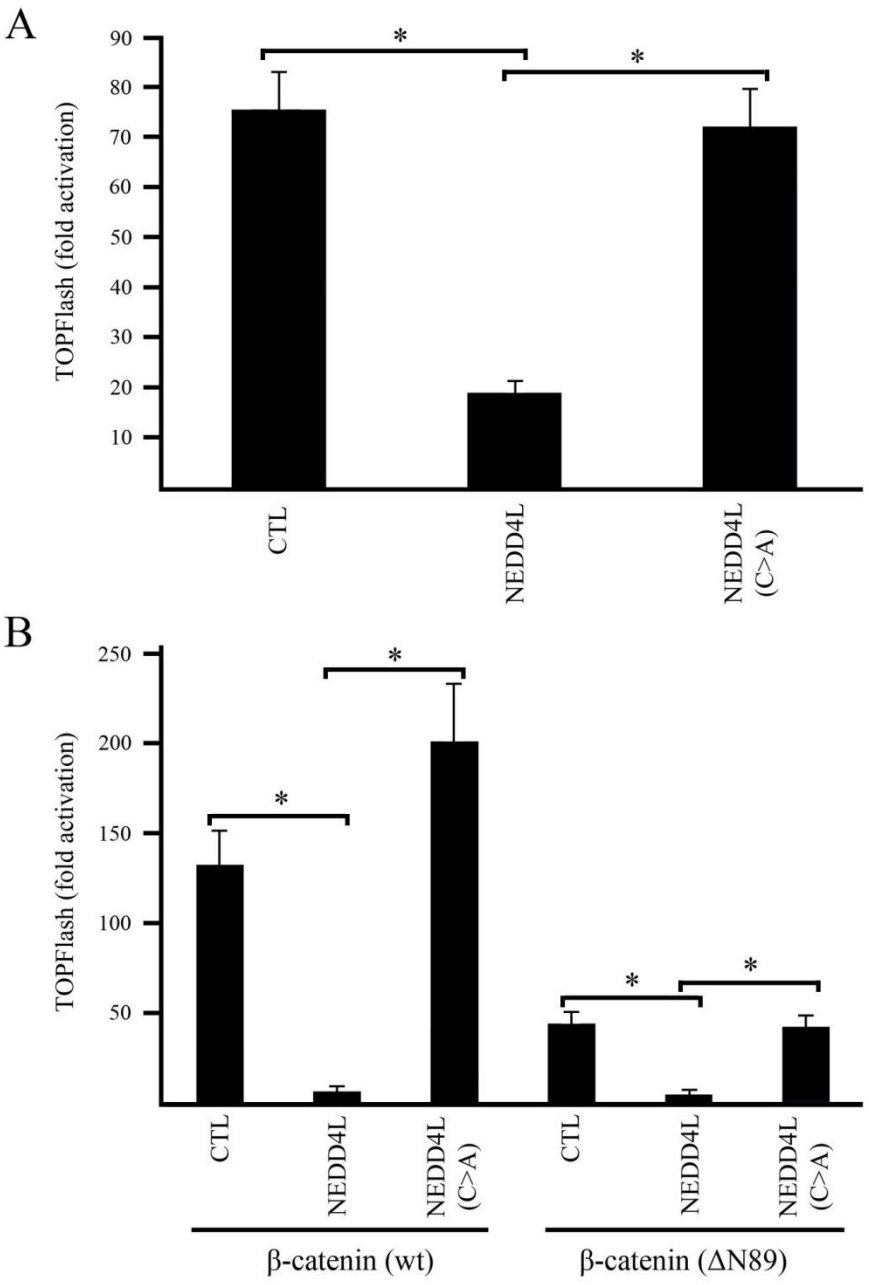



**Figure 18. NEDD4L inhibits canonical WNT signaling at or below the level of β-catenin activation.** (A) NEDD4L inhibits TOPFlash activity in HEK293 cells compared to empty vector (CTL) or catalytically inactive NEDD4L [NEDD4L (C>A)]. WNT3A (20 ng/ml) and R-Spondin (100 ng/ml) were used to activate canonical WNT signaling. (B) When two different β-catenin constructs, β-catenin (wt) and β-catenin (ΔN89), are used to activate canonical WNT signaling, a significant reduction in TOPFlash activity was observed in the presence of NEDD4L overexpression, as compared to empty vector or NEDD4L (C>A). All results are normalized to FOPFlash activity. *P<0.05.

Lack of functional consequences of NEDD4L knockdown in growth assays

Using two human CRC cell lines (DLD-1 and RKO), in which I have stably knocked down *NEDD4L*, I have attempted to determine how NEDD4L affects the growth of CRC cells. DLD-1 cells contain a truncating mutation in *APC*, while RKO cells have neither a mutation in *APC* nor *CTNNB1*, but do have an inactivating mutation in *NKD1*, an inducible negative regulator of WNT signaling. After verifying knockdown by Western blotting (~80%), I performed several different assays in order to determine whether NEDD4L knockdown increased the proliferation of either of these cells lines. NEDD4L knockdown had no effect on proliferation on plastic, growth in soft agar or collagen, or tumorigenesis in nude mice (data not shown).

**Discussion**

The present study was performed in order to begin to understand if there is a role for members of the NEDD4 family of E3 ubiquitin ligases in CRC. Nine members comprise this family, and each has been shown, in various contexts, to impact at least one of the cellular pathways central to the genesis and progression of CRC. Many of the members have been shown to be dysregulated in a number of different cancers. However, a systematic evaluation of the expression levels of all family members in human CRC has yet to be done. Notably, NEDD4 was recently shown to be upregulated in CRC (Eide, Cekaite et al. 2013). We felt that the first step in beginning to understand this family of E3s

in CRC was to examine the expression levels of each member in CRCs from a large cohort of patients. We chose to perform our analysis across all stages, including adenomas, as there is a well-defined, chronological order of major cell signaling pathways altered during CRC tumorigenesis (Kinzler and Vogelstein, 1996). Furthermore, some of these pathways can perform different functions at different stages. For instance, the TGFB pathway is a tumor suppressor early in CRC, and can promote progression and metastasis later (Padua and Massagué, 2009).

Herein, we evaluated how the expression of each NEDD4 family member changes with progression from adenoma to Stage IV CRC. The most highly upregulated family member was *NEDD4*, in accord with the aforementioned findings. The most highly downregulated family member in our data set was *NEDD4L*, suggesting that NEDD4L could be a tumor suppressor in CRC. We found this surprising given that NEDD4L shares the highest sequence homology with NEDD4, and has been shown to have a significant overlap with NEDD4 in target selection (Persaud, Alberts et al. 2009).

Recently, NEDD4L was shown to be downregulated in gastric cancer, and those patients with the lowest expression by immunohistochemical analysis had a poorer prognosis (Pang, Ren et al. 2012). Also, NEDD4L has been found to be both upregulated or downregulated in prostate cancer, increased in invasive gallbladder cancer cells, and downregulated in more aggressive malignant gliomas (Hellwinkel, Asong et al. 2011; Hu, Xu et al. 2009; Takeuchi, Adachi et al. 2011; He, Deng et al. 2012). These disparate findings are not unexpected given

the number of targets and cell signaling pathways upon which NEDD4L has been found to act.

Our data are consistent with NEDD4L serving as a tumor suppressor in CRC. A recent report showed that NEDD4L inhibits both the canonical and non-canonical WNT signaling pathways by ubiquitylating DVL2, and targeting it to the proteasome for destruction (Ding, Zhang et al. 2013). Here, we found that NEDD4L can also inhibit canonical WNT signaling at the level of, or downstream from, β-catenin. This is in contrast to the aforementioned paper, in which the investigators showed that NEDD4L could not inhibit TOPFlash when signaling was activated by a β-catenin mutant (S37A). In their study, isoform 2 of NEDD4L (NM_001144964.1) was used, whereas we used a different NEDD4L isoform (KIAA00439). These two constructs differ at their N-terminus; the construct used in this study has an additional 141 amino acid residues at the immediate N-terminus, which modifies the C2 domain, and 20 fewer residues in a segment preceding the second WW domain. This could explain differential localization or substrate targeting. Additionally, different β-catenin constructs were used; here, wt and mutant β-catenin (ΔN89) were used, and there, mutant β-catenin (S37A) was used.

In conclusion, we have begun the characterization of the role of the NEDD4 family in CRC. The most highly upregulated member is *NEDD4*, which has been previously shown, and the most highly downregulated is *NEDD4L*, which became statistically significant at Stage I. It trended downward at the adenoma stage, which could become statistically significant with a larger sample

size. In agreement with previous findings, we find that NEDD4L inhibits canonical WNT signaling, though we go on to show it inhibits WNT signaling when β-catenin is used as an activator. As the most common mutations in the WNT signaling pathway are at this level, it is possible that NEDD4L could be an important tumor suppressor even at an advanced tumor stage. Patients with the highest *NEDD4L* expression in their tumors showed a trend towards longer disease-specific survival. Therefore, our findings suggest that NEDD4L may act as a tumor suppressor in CRC by inhibiting canonical WNT signaling.

**Materials and Methods**

Human tissue sample microarray analysis

A total of 250 human CRC tissue samples were collected at Vanderbilt University Medical Center (VUMC) (N=55) and Moffitt Cancer Center (MCC) (N=195). Of these CRC samples, 33 were Stage I (13.2%), 76 Stage II (30.4%), 82 Stage II (32.8%), and 59 Stage IV (23.6%). From ten of these patients, adjacent normal tissue was collected at MCC. Of these, two were collected from Stage I, five from Stage II, and three from Stage II patients. An additional six adenomas were collected. RNA from these tissues was obtained and hybridized to the Affymetrix Human Genome U133 Plus 2.0 GeneChip Expression Array.

Patients in the VUMC group had a median follow-up of 50.2 months, with a minimum follow-up of 0.4 months and a maximum of 111.3. Those in the MCC group had a median follow-up of 44.7 months, with a minimum of 0.92 and a

maximum of 142.8 months. All of these samples were collected and analyzed in accord with a protocol approved by the Institutional Review Boards at both VUMC and MCC.

Western blot analysis

Tissue samples from twenty patients with CRC, who were seen at VUMC, were collected and snap frozen in liquid Nitrogen. Adjacent normal samples were also collected from each patient and snap frozen. Samples were then lysed in 8.0 M urea, sonicated, and centrifuged at 14,000 rpm. The supernatant was removed, and the protein quantified by measuring absorbance at 280 nm. The samples were then run on a 7.5% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blotted with anti-NEDD4L (1:2000) (Bethyl) and anti-α-tubulin (1:2000) (TUBA) (CalBiochem) antibodies, and detected using appropriate HRP-conjugated secondary antibodies and the chemiluminescence method. The intensities of the blots were then quantified using the ImageJ software, and analyzed.

TOPFlash Assay

Transfections were performed using Metafectene (Biontex) according to the manufacturer's instructions. HEK293 cells ($2 \times 10^5$) were plated in a 12-well dish, allowed to attached, and transfected the following day with 0.1 μg of each plasmid. The following plasmids were used: TOPFlash reporter plasmid, FOPFlash reporter plasmid, pcDNA3.1, NEDD4L (KIAA0439) (Addgene plasmid

27000), and NEDD4L(C>A) (Addgene plasmid 27001) (Gao, Alarcón et al. 2009). Constructs containing wt β-catenin and mutant β-catenin (ΔN89) were a gift from Ethan Lee. The day after transfection, the media was removed, and serum-free media containing 20 ng/ml WNT3A and 100 ng/ml R-Spondin (Vanderbilt Antibody and Protein Resource Core) was added. The following day, cells were lysed and processed in accord with the luciferase assay kit (Promega). TOPFlash luciferase activity was normalized to FOPFlash activity, and fold-activation was determined by normalizing to TOPFlash and FOPFlash activity in unstimulated cells. All experiments were performed in triplicate at least 3 times.

Statistical analysis

To compare the expression level of each probe between the normal, adenoma, and the cancer tissue samples, the Wilcoxan rank sum test was used. NEDD4L protein levels were normalized to TUBA levels, log-transformed, and compared using a paired t-test. Significance levels in the TOPFlash reporter assay experiment were determined using the Student's t-test.

# CHAPTER IV

# GENERAL DISCUSSION

In the preceding chapters, I have discussed three of the projects on which I worked during the last four years. During my time in the Coffey lab, I participated in a clinical trial testing the efficacy of cetuximab in the treatment of Ménétrier's disease, in addition to formulating a way to more accurately diagnose Ménétrier's disease. I have been involved in the study of Juvenile Polyposis syndrome (JPS), and in the conception of the idea that foveolar hyperplasia, a major histological finding in Ménétrier's disease, may precede the formation of a juvenile polyp. Lastly, I have led the study of the NEDD4 family of E3 ubiquitin ligases in colorectal cancer (CRC). This led to the finding that NEDD4L is significantly downregulated in CRC, and may be a tumor suppressor through the inhibition of the WNT signaling pathway. In this final chapter, I will sum up what I have learned during my graduate career, discuss the most important unanswered questions for each project, and propose experiments that could help us begin to answer these questions.

## Ménétrier's disease

During my time in the Coffey lab, we demonstrated the efficacy of epidermal growth factor receptor (EGFR) blockade by cetuximab in the treatment

of chronic Ménétrier's disease, a disorder that had been considered refractory to medical therapy. Ménétrier's disease is characterized histologically by an increased production of surface mucous cells and a decreased production of the glandular lineages, the parietal and chief cells. This is termed foveolar hyperplasia with glandular atrophy, and explains the common finding on endoscopy of a hypermucinous and hypochlorhydric gastric lumen. Surprisingly, these patients also tend to have normal serum gastrin, which we contend is abnormally normal. Hypochlorhydria should drive gastrin production, which will increase the activity and production of parietal cells. Commonly, these patients also present with edema due to hypoalbuminemia, which is thought to be due to leakage of protein into the gastric lumen as a consequence of compromised epithelial cell-cell junctions. Following cetuximab treatment, all of these clinical and histologic findings are ameliorated, and, in some cases, return to normal.

This trial began following the successful use of cetuximab in a single patient with chronic Ménétrier's disease, who received compassionate-use approval from the U.S. Food and Drug Administration. The evidence that supported treating that patient with EGFR blockade was from a mouse model the Coffey lab utilized, in which the EGFR ligand, transforming growth factor-α (TGFA), was overexpressed in the stomach. This mouse displayed a Ménétrier's disease-like phenotype. More recently, a mouse model of overexpression of heparin-binding EGF-like growth factor (HB-EGF), another EGFR ligand, in the stomach, also showed features of Ménétrier's disease. Also, there is a spontaneously remitting form of Ménétrier's disease in humans, which most

commonly affects children, and is associated with acute cytomegalovirus (CMV) infection. As discussed in Chapter II, it has been shown that the EGFR is a receptor for the outer envelope glycoprotein, glycoprotein B (gB), of CMV, and becomes activated upon gB binding.

Beyond hints from the mouse models, the effective use of cetuximab, and a link to acute CMV infection, nothing is known about the true pathophysiological mechanisms underlying the development of Ménétrier's disease in humans. It is true that there is elevated TGFA by immunohistochemical analysis in the affected mucosa of Ménétrier's disease patients, but it is known that TGFA induces its own expression. As well, EGFR ligands have been shown to cross-induce the production of other EGFR ligands. So while TGFA appears to be important in the pathogenesis of Ménétrier's disease, the initial insult remains to be characterized.

Ultimately, the pathogenesis of chronic Ménétrier's disease, like all diseases, will be explained by one of three possibilities: an environmental insult, a germ-line or somatic genetic alteration, or a genetic predisposition to respond improperly to an environmental insult. Arguing for the former is the fact that the spontaneously remitting form of Ménétrier's disease seen most commonly in children has a viral cause. A caveat to this is that many people are affected by an acute CMV infection, but very few develop Ménétrier's disease. There have been no studies done to address a germ-line or acquired genetic cause. Cases of familial Ménétrier's disease have been reported, but I believe these were, in every case, incorrectly diagnosed. As to an acquired mutation, we possess the

tissue to perform this analysis, and with the current ease of whole exome/genome sequencing, this should be considered. This experiment would be done by analyzing the genomic DNA of the affected tissue, and comparing it to genomic DNA from each patient's blood sample, with the hope that there is a shared mutation(s) in the gastric mucosa of our patient population, or that there are mutations affecting components of the EGFR signaling pathway.

It seems most likely that Ménétrier's disease is the result of a genetic predisposition to respond to an environmental cue, infectious or otherwise, in an abnormal way. As we pointed out in the paper, more than half of the patients had a coexisting immune-mediated disease. Four had ulcerative colitis (UC), and one had ankylosing spondylitis. UC exemplifies a disease that results from a complex relationship between genetics and the environment. Only recently have we begun to get a foothold in the search for genes that predispose one to UC. These include genes related to MHC class and epithelial barrier function. It is intriguing that the hypoproteinemic edema in Ménétrier's disease is related to aberrant epithelial barrier function, which is quickly mended on EGFR blockade. Unlike Ménétrier's disease, UC runs in families (though we have yet to see Ménétrier's disease run in the families of any of our patients with UC). However, there are many environmental factors that contribute to or cause UC, as well. Ultimately, UC is classified as an autoimmune disease, with T-cells infiltrating the affected portion of the colon. However, we rarely see significant inflammation in the mucosa of Ménétrier's disease.

The abnormally normal serum gastrin levels in our patients are intriguing.

As noted, their serum gastrin should be significantly elevated in the setting of hypochlorhydria. This suggests a possible role for the enteroendocrine cells that mostly reside in the antrum. In Ménétrier's disease, the antrum is grossly unaffected. However, a systematic analysis of possible changes in the cell population occupying the antral glands has yet to be performed. Somatostatin is known to suppress gastrin levels. It is possible that the initial insult in Ménétrier's disease causes an increased production of somatostatin, which curbs gastrin production, and exacerbates the problem of acid production. In the future, an analysis of serum somatostatin, and the presence of D-cells in the antrum, which produce and secrete somatostatin, should be performed.

Based on this trial, we have gained insight into the allocation of cell lineages in the human stomach. The inhibitory effect of TGFA administration on parietal cell acid secretion and proliferation *in vitro* and *in vivo* was well known prior to this trial. However, all those findings were from a number of different animal models. One would expect similar result in a human engineered to overexpress TGFA in the stomach, but that is not feasible, or ethical. In the setting of the clinical trial, we were essentially able to see what happens when one abrogates EGFR signaling in a human stomach: within 24 hours, there are more parietal cells (as determined by $H^+/K^+$ ATPase staining). Additionally, the number of Ki-67 positive cells per gland is significantly reduced, and shortly thereafter, the number of pit cells is reduced. The explanation for this rapid alteration remains to be determined. It is doubtless that a change in the proliferative index is important, but I believe an alteration in the differentiation

process in the presence of ceutximab plays the most significant role in the rapid return of parietal cells. Essentially, we feel that in the gastric epithelium of Ménétrier's disease patients, there is a large cell population waiting final cue(s) to differentiate into a particular cell-type (Nalle and Turner, 2009). In turn, I believe that the results from the trial support the idea that EGFR overactivation stimulates proliferation of fundic stem cells, and directs them down a surface mucous cell lineage. EGFR blockade, on the other hand, slows proliferation, and allows for differentiation into parietal cells and chief cells.

Should someone following me in the Coffey lab be interested in pursuing a more basic characterization of the cause of Ménétrier's disease, I have spent considerable time generating primary cell strains from one of the patients. These cells have been passaged and expanded multiple times, and frozen back. Initial characterization suggests that these cells are primarily stromal/fibroblasts, though it is possible epithelial cells remain in the earlier passages. As the symptoms of Ménétrier's disease are caused by an effect on the epithelial population of the stomach, it is likely more important that epithelial cells be studied in culture. However, it is also possible that Ménétrier's disease begins in the stroma. Ultimately, my expectation is that cell culture may be too simplistic to get at the underlying cause of Ménétrier's disease, unless a disease-linked mutation is discovered. Given the link to CMV, we did collaborate with an investigator (David Wang at Washington Unversity, St. Louis), whose lab can use chip-based technology to search a tissue sample for the presence of a large panel of viruses. Thus far, the results have been negative, but deeper

sequencing of the specimens is needed.

In summary, Ménétrier's disease is a hyperproliferative disease of the stomach. It is modified by EGFR blockade, and linked, in a more acute form, to CMV infection. Histologically, there are more surface mucous cells, and fewer parietal and chief cells in the gastric mucosa of patients with Ménétrier's disease. This is rapidly reversed following cetuximanb administration, suggesting the stem cell population of the fundus/body is being driven to proliferate and differentiate down a surface mucous cell path by some extracellular cue. The clinical trial showed that, regardless of the underlying cause of Ménétrier's disease, ligand-induced EGFR activation is vital to its chronicity. Additionally, we learned that the EGFR signaling pathway plays a significant role in the lineage allocation of the human stomach. We hope to one day have a true understanding of the cause, but it is likely a complex disease that is the product of both genetic and environmental factors. While Ménétrier's disease is a rare disease, and warrants study as a single entity for the reasons I noted earlier, the fact that our patient population had coexisting UC cannot be ignored. In turn, a more focused study of our patients, in particular their genomes, as well as the presence of any auto-antibodies in their serum, may lend insight into the pathogenesis of both Ménétrier's disease and UC.

**Juvenile polyposis and mimics of Ménétrier's disease**

Ménétrier's disease is ultimately a clinical diagnosis, as there are no

pathognomonic features. Foveolar hyperplasia must be present, but as we have come to learn, foveolar hyperplasia can be present in many gastric disorders. During the trial, we were referred a number of patients for inclusion who had some other gastrointestinal disease. In turn, this gave us a good overview of what signs and symptoms make clinicians and pathologists think of Ménétrier's disease when evaluating a patient. There were several cases referred to us by clinicians with an incomplete understanding of what Ménétrier's disease is. We were, for example, referred a patient with parietal cell hyperplasia, which is the antithesis of Ménétrier's disease. On the other hand, a number of diseases can reasonably be misdiagnosed as Ménétrier's disease (in fact, I will suggest shortly that we made a mistake in the diagnosis one of the patients in the trial). Once we conclusively diagnosed each of these other diseases, we found ourselves able to create a clinicopathologic decision-making tree in order to more accurately diagnose Ménétrier's disease. This chart was presented in Chapter II (Figure 11).

The most common single disease mistaken for Ménétrier's disease was JPS. As I mentioned earlier, JPS is caused by an inactivating mutation in a member of TGFB/BMP signaling. The patients have large hamartomas, filled with an immature stroma, and covered by a relatively normal epithelium. These patients are not hypochlorhydric, unless they are being treated with proton pump inhibitors, and commonly have an elevated serum gastrin. The cells covering the polyp are surface mucous cells, but they appear smaller than in Ménétrier's disease.

In contrast to the differences between these two diseases, there are two important similarities. Firstly, JPS patients seem to have foveolar hyperplasia throughout their stomachs, which is more pronounced in the tissue immediately adjacent to the polyp. It looks like a juvenile polyp "caps" a hyperplastic foveolus in JPS. Secondly, we see increased phosphorylated EGFR in JPS patients, suggesting that hyperactivation of the EGFR signaling pathway may cause foveolar hyperplasia in JPS.

These two findings spurred my interest in understanding whether and how the TGFB/BMP and EGFR signaling pathways might oppose each other in the stomach. The clinical payoff, in my mind, could ultimately be the use of cetuximab, or some other EGFR inhibitor, in the treatment of JPS patients. This could be important when considering another human disease caused by mutations of the TGFB/BMP signaling pathway: pulmonary arterial hypertension (PAH). Hereditary PAH has been linked to mutations of the BMP type II receptor, which causes a progressive occlusion of the pulmonary arteriolar vasculature. Though there is currently no treatment that effectively counteracts the abrogation of BMP signaling in the patients, the use of pulmonary vasodilators has significantly improved survival. In JPS patients, should the severity of their disease make them gastrectomy candidates, my feeling is that cetuximab would be a viable choice to address the symptoms, and not the cause, in the same manner the current PAH treatments do the same.

During my pursuit of this, a mouse model of the overexpression of the secreted BMP inhibitor, noggin, by parietal cells, showed that the abrogation of

TGFB/BMP *in vivo* can cause increased EGFR activation. In this mouse model, there was both foveolar hyperplasia and increased expression of the EGFR ligands, TGFA and amphiregulin (AREG). Unfortunately, the authors of this study did not try treating these mice with an EGFR inhibitor to determine if they could reverse the phenotype. Cetuximab does not work in mice, as it was generated in a mouse against the human EGFR, but there are alternatives that could have been used. I think EGFR blockade should be tried in a mouse model of JPS.

I believe we have already tried this experiment in a human patient. One of the earliest entrants into the trial may not have had Ménétrier's disease. To support this, I refer to the clinicopathologic decision-making tree we created. In our paper concerning mimics of Ménétrier's disease, we split the patients into three groups: those with Ménétrier's disease, those with polyps and without Ménétrier's disease, and those with neither. The patients that presented with Ménétrier's disease typically had low serum albumin, with hypochlorhydria and normal serum gastrin, and were rarely anemic (Figure 10, Table 4). On the other hand, patients with polyps, or a polyposis syndrome, had normal serum albumin, normal gastric pH, high serum gastrin, and were commonly anemic (Figure 10, Table 4). In every case, this patient fit more closely into the "Non-Ménétrier's disease with Polyps" group (Figure 19). Additionally, as a part of our clinicopathologic decision-making tree (Figure 11), we suggested that the biopsy specimens taken from the affected areas of Ménétrier's disease patients would show a maintenance of parallelism in the gastric unit. The gastric units in this patient clearly lost their parallelism (Figure 20). At the time this patient entered

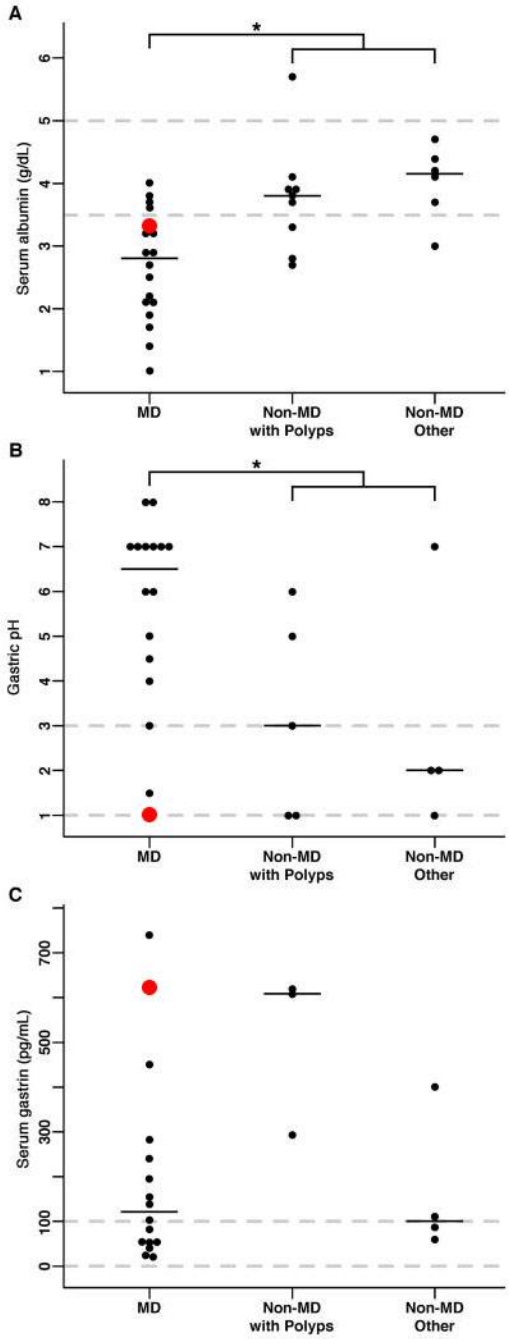



**Figure 19. A patient we diagnosed with Ménétrier's disease and treated with cetuximab fits better into the "Non-MD with Polyps" group of patients.** Here, I place a larger, red dot to represent the patient discussed in the text. My contention is that he fits better into the group with polyps based upon this modified version of Figure 10. Further, this patient was anemic, and required transfusions, which is also more common amongst the polyposis patients. However, this patient responded quickly to cetuximab.

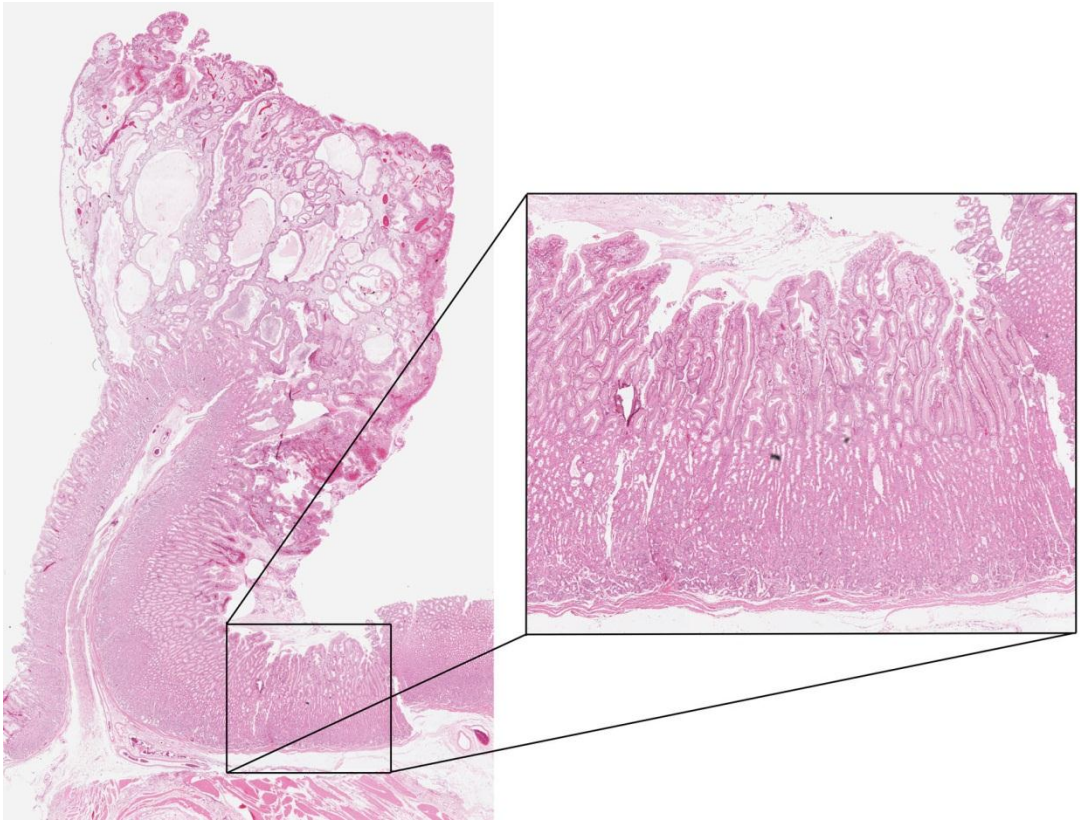


**Figure 20: A large polyp, with adjacent foveolar hyperplasia, was present in the gastric mucosa of a Ménétrier's disease patient at gastrectomy.** A number of polyps were present in the gastric mucosa of this patient preceding, throughout, and following his inclusion in the cetuximab trial. Shown here is a large polyp that was present in his gastrectomy specimen. The higher power view shows the foveolar hyperplasia that is immediately adjacent to the base of the polyp. As can be seen, the foveolae have a corkscrew appearance, which is common in the mucosa of Ménétrier's disease. However, there is no obvious glandular atrophy, which is common in Ménétrier's disease.

the trial (10 years prior to this improved method of properly diagnosing Ménétrier's disease), a thick mucosa and foveolar hyperplasia, in the absence of another diagnosis, justified calling this Ménétrier's disease.

This patient ultimately had his stomach removed due to concerns regarding long-term intravenous infusions of cetuximab and the associated rash and diarrhea. However, his "Ménétrier's disease" responded. In fact, during the trial, a case report was written regarding the amelioration of his anemia following cetuximab infusion, which had required bimonthly transfusions prior to treatment (Settle, Washington et al., 2005).

In my mind, this patient had JPS. Unfortunately, I have been unable to prove this. I initially sequenced each of his *SMAD4* exons in DNA derived from his affected tissue, finding all exons to be normal. I then performed whole exome sequencing on DNA from the affected tissue to search for uncharacterized mutations. This also yielded no obvious explanation. Larger chromosomal deletions involving *SMAD4* and *BMPRIA* commonly underlie JPS, and neither of those would have been seen by exon or whole exome sequencing. My sequencing analysis would have missed larger deletions, which can be common in JPS. In order to determine if there is a larger deletion present in either the *SMAD4* or *BMPRIA* locus, we will perform multiplex ligation-dependent probe amplification (MLPA) analysis, which is able to detect this type of anomaly. Supporting the need to continue searching for a *SMAD4* mutation or loss, is Figure 21, which shows the SMAD4 staining in the involved gastric mucosa of this patient. As mentioned earlier, JPS patients commonly lose the wt *SMAD4*

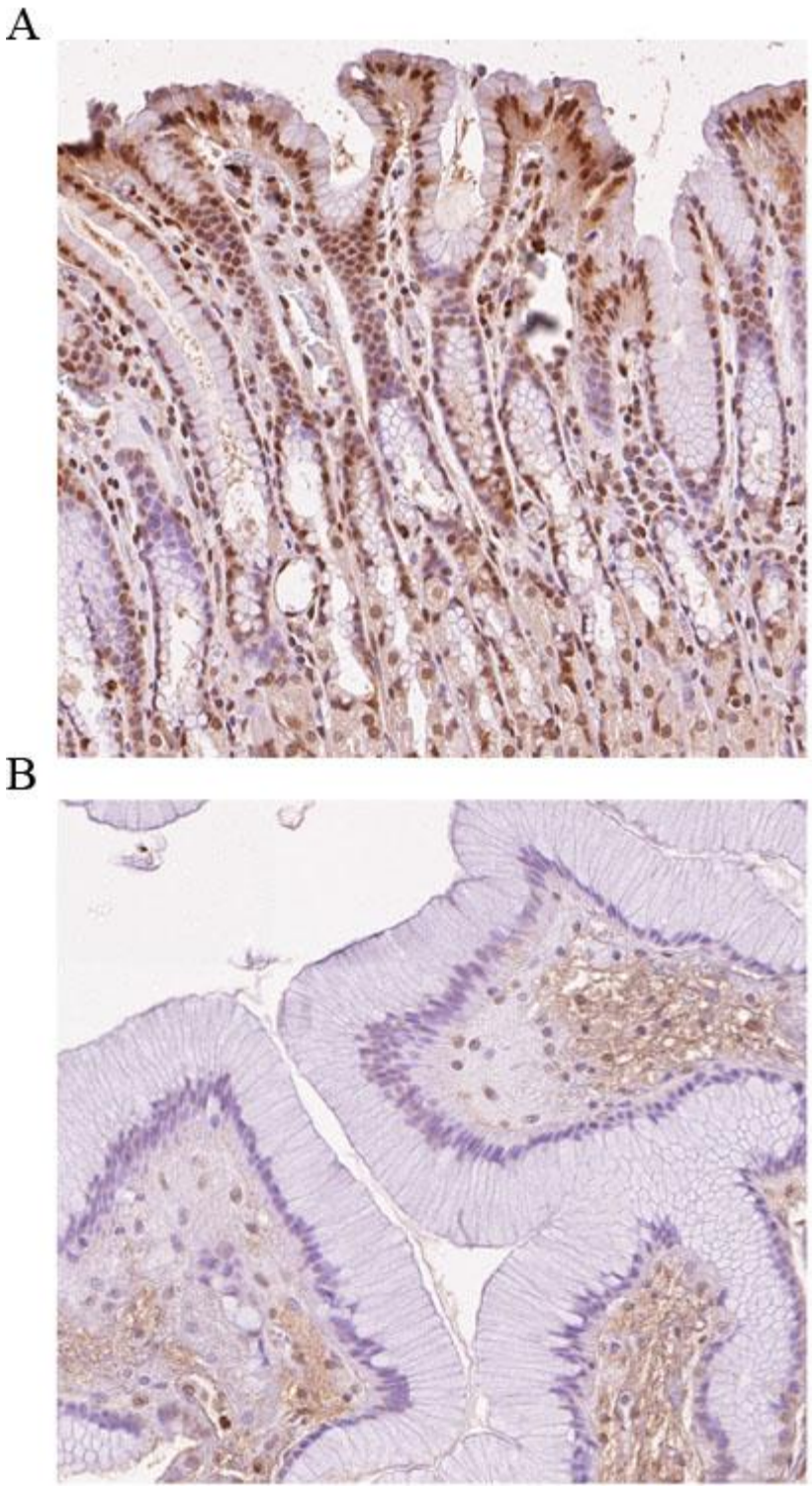



**Figure 21. SMAD4 staining is reduced or absent in a possible JPS case.** (A) SMAD4 staining in normal human gastric mucosa shows that many of epithelial cells have both positive nucleic and cytoplasmic staining. (B) Surface mucous cells from the involved mucosa of this patient show absent SMAD4 staining.

allele in the involved mucosa.

For future Coffey lab members interested in JPS, or in attempting to better understand the role of the TGFB/BMP signaling pathway in the human stomach, colon, or in CRC, I have generated two reagents. Firstly, I have engineered the human CRC cell line, Caco-2, to inducibly overexpress wt SMAD4 in a Tet-dependent manner; expression is turned on in the absence of Tet. This was accomplished using the Retro-X™ Tet-Off® Advanced Inducible Expression System from Clontech. Caco-2 cells have lost one copy of *SMAD4*, and contain a point mutation causing a D351H substitution, which has been found to abrogate proper function. In turn, they do not respond to TGFB administration in a growth inhibitory manner. The addition of wt SMAD4 should offer an investigator the chance to determine how the resumption of proper TGFB/BMP pathway function affects the biology of a polarizing human CRC cell *in vitro*.

I also generated primary cell strains from two JPS patients. The first was generated from the patient I diagnosed with JPS (Chapter II), and very little characterization has been done. The second strain(s) were generated from the colon, duodenum, and stomach of a patient with JP-HHT. This patient had a 1082G>A mutation, which causes an R361H substitution. These cells stain positive for α-smooth muscle actin by immunofluorescence, suggesting they are a myofibroblast-like cell. It is unknown whether these cells retain expression of wt SMAD4. Following further characterization, either of the cells strains could serve as tools to model JPS in culture.

In summary, I contributed to work describing the first effective medical

treatment for Ménétrier's disease and outlining a diagnostic approach to patients supposed to have this disorder. JPS, a disease linked to TGFB/BMP signaling, was the most commonly confused with Ménétrier's disease. In turn, we noted that patients with JPS have foveolar hyperplasia and increased phosphorylated EGFR in the involved gastric mucosa. This led us to hypothesize that abrogating TGFB/BMP signaling can activate EGFR signaling. This was shown to be the case in a mouse model of noggin overexpression. It remains to be seen if the EGFR signaling pathway is important to polyp formation in JPS. If this can be shown convincingly, it may serve as an impetus to use cetuximab in the treatment of JPS. As in Ménétrier's disease, cetuximab therapy may serve as a bridge to surgery, as many JPS patients require gastrectomy. At the same time, as in Ménétrier's disease, it may be curative.

**The role of NEDD4L in CRC**

What originally stimulated my interest in the NEDD4 family in CRC has not yet been discussed in this document. The Coffey lab has a long-term interest in the production, trafficking, and cell biological effects of the EGFR ligands. During these studies, the protein Naked2 (NKD2) was found to be integral to the proper, basolateral trafficking of TGFA. NKD2 coats TGFA-containing vesicles via a direct interaction with the cytoplasmic tail of TGFA. It then escorts these vesicles to the basolateral surface in a NKD2-myristoylation-dependent manner, where they dock and fuse, exposing TGFA to the extracellular environment (Li, Franklin

et al. 2004).

After dissociating from TGFA, NKD2 is then thought to perform its second function: the inhibition of WNT signaling by the degradation of Dishevelled-1 (DVL1). In fact, NKD2 and DVL1 accelerate each other's degradation (Hu, Li et al. 2010). The Coffey lab has shown that NKD2 directly interacts with a membrane-associated form of DVL1, which results in the ubiquitin-mediated degradation of both proteins. The E3 ligase responsible for this process remains to be discovered. It is known that the E3, ring finger protein-25 (RNF25), ubiquitylates NKD2 in the cytoplasm. However, it is not involved in the degradation of membrane-localized NKD2. Therefore, a search for other E3s was carried out. Near its C-terminus, NKD2 contains a PY motif, a short amino acid motif that can interact with a WW domain. As mentioned, all nine NEDD4 family members contain two to four WW domains. Subsequently, I was able to co-immunoprecipitate NKD2 with the family members, WWP2 and NEDD4L, but not WWP1, SMURF1, or NEDD4. I saw reduction in the activity of the TOPFlash reporter assay when NKD2 and NEDD4L constructs were co-transfected into HEK293 cells. However, in one of my controls, NEDD4L alone, I also saw a reduction in TOPFlash activity, suggesting NEDD4L can inhibit WNT signaling in the absence of NKD2 (HEK293s lack detectable NKD2). This does not mean that NEDD4L-mediated inhibition of WNT signaling is not enhanced by NKD2, only that I was unable to detect it using the currently available tools. The E3 that is responsible for ubiquitylating DVL1 in a NKD2-dependent manner is an important discovery that remains to be made, and the NEDD4 family may yet be

found to play a role in this phenomenon.

DVL1 also contains a PY motif, as do DVL2 and 3. Based on this, I hypothesized that NEDD4L would inhibit WNT signaling by ubiquitylating at least one, if not all three, of the DVLs. Another lab published this exact finding. In their paper, they showed that NEDD4L directly interacts with all three DVLs. They characterized the effect on DVL2, showing that it is ubiquitylated by NEDD4L and targeted to the proteasome for degradation. In turn, NEDD4L inhibits both canonical and non-canonical WNT signaling. They also showed that NEDD4L impacts WNT signaling only at this level, as there was no inhibition when a construct containing β-catenin, which is downstream of DVL2, was used to activate the TOPFlash reporter.

I have findings support the idea that DVL1 is ubiquitylated by NEDD4L (data not shown). However, I have also found that NEDD4L significantly inhibits the TOPFlash reporter when both wild-type and a mutant β-catenin (with the N-terminal 89 amino acids deleted) are used to activate WNT signaling. This finding, in conjunction with my finding that *NEDD4L* is significantly downregulated in CRC, suggests that NEDD4L might be a tumor suppressor in CRC via its ability to inhibit canonical WNT signaling.

I am unable at this time to offer a definitive mechanism regarding the ability of NEDD4L to inhibit canonical WNT signaling downstream of β-catenin. Two important members of canonical WNT signaling that are downstream of β-catenin, B-cell CLL/lymphoma 9 (BCL9) and BCL9L, contain PY motifs. I have been unable to co-immunoprecipitate overexpressed or endogenous NEDD4L

with either of these proteins, or see an effect on BCL9 or BCL9L stability.

Though BCL9 and BCL9L both contain PY motifs, and therefore seem like the likeliest targets, it is possible that some other downstream effector of canonical WNT signaling is the target of NEDD4L. This protein could interact directly with NEDD4L even in the absence of a PY motif, as has been shown before. Moreover, some other protein could serve as a scaffold, which functions to recruit and link NEDD4L with its target.

Though I have findings that suggest NEDD4L inhibits WNT signaling at or below the level of β-catenin activation, I have yet to see evidence of this in CRC cells. In hindsight, it may have been wise to also create DLD-1 and RKO cells (and other human CRC cell lines) that either inducibly or constitutively overexpress NEDD4L. As my data supports the idea that NEDD4L is a tumor suppressor, it may be the case that these cell lines have already downregulated NEDD4L to a point that further downregulation is inconsequential with respect to proliferation. I found that NEDD4L is down approximately 42% in human CRC, suggesting I should overexpress it at least two-fold *in vitro*. It may also be the case that the loss of NEDD4L allows for the increased activity of non-canonical WNT signaling, which could be important in the promotion of metastasis. I saw no metastasis in my mouse experiments, but a more informative experiment to determine if this is the case would be an invasion or wound-healing assay.

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