TNF-ALPHA CONVERTING ENZYME-DEPENDENT ERBB4 TRANSACTIVATION BY TNF PROMOTES COLONIC EPITHELIAL CELL SURVIVAL

By

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LIST OF ABBREVIATIONS

ADAM .....A disintegrin and metalloprotease

ADAM-TS .....ADAMs with thrombospondin domains

AOM .....Azoxymethane

AR .....Amphiregulin

BTC ..... Betacellulin

CAC ..... Colitis associated carcinogenesis

CRC ..... Colorectal cancer

DSS ..... Dextran sulfate sodium

EGF .....Epidermal growth factor

EGFR .....Epidermal growth factor receptor

FAP..... Familial polyposis colitis

FGF .....Fibroblast growth factor

FGFR .....Fibroblast growth factor receptor

GPCR ..... G-protein coupled receptor

HB-EGF .....Heparin-binding epidermal growth factor

HRG .....Heregulin

HNPCC .....Hereditary non-polyposis colorectal cancer

IBD .....Inflammatory bowel disease

INSR .....Insulin receptor

LRR .....Leucine-rich repeat

MAPK .....Mitogen-activated protein kinase

MMP ..... Matrix metalloproteinases

NOD .....Nucleotide-binding oligomerization domain

NRG ..... Neuregulin

NSCLC ..... Non-small cell lung cancer

PDGR .....Platelet derived growth factor

PI3K ..... Phosphatidylinositol 3-kinases

PKA ..... Protein kinase A

PMA .....Phorbol ester

PTB .....Phosphotyrosine binding

RTK .....Receptor tyrosine kinase

SH2 ..... Src homology 2

SVMP .....Snake venom metalloproteinases

TACE .....TNF-alpha converting enzyme

TGR-α .....Transforming growth factor- alpha

TIMP .....Tissue inhibitors of metalloproteinases

TLR .....Toll-like receptors

TNF .....Tumor necrosis factor

TNBS ..... Trinitrobenzene sulfonic acid

UC ..... Ulcerative colitis

CHAPTER I

INTRODUCTION

Protein kinases

Protein kinases are molecular switches that facilitate signal transduction by catalyzing the transfer of the γ-phosphate of ATP to the hydroxyl group of serine, threonine, or tyrosine. A catalog of the protein kinase complement of the human genome identified 518 putative protein kinase genes, which constitute about 1.7% of all human genes (Manning et al., 2002). Given that they constitute such a large component of the eukaryotic genome, it is no surprise that kinases contribute substantially to the signal transduction events that dictate cell physiology. Through tight regulation of their catalytic activity, kinases regulate cellular processes such as apoptosis, differentiation, metabolism, transcription, cell cycle progression, cytoskeleton rearrangement and cell motility. Mutations of protein kinases often leads to dysregulation of signaling pathways, which is known to cause a wide range of human diseases and cancers.

Since protein kinases have evolved unique mechanisms to impede catalytic activation, each class of kinase has a unique structure in its inactive state. When active, however, protein kinases share a striking similarity in their catalytic domain. In 1991, X-ray crystallographic studies solved the first protein kinase three-dimensional structure, that of cyclic AMP-dependent protein kinase

(also known as protein kinase A (PKA)), (Knighton et al., 1991). From this and other studies, it has been determined that the protein kinase domain is separated into two subdomains, termed lobes. The N lobe, which is the smaller N-terminal portion, consists of a five-stranded β sheet and one prominent α helix, while the larger C lobe is predominantly helical. ATP is bound in a deep cleft between the N and C lobes. A phosphate-binding loop, or P loop, that contains a conserved glycine-rich sequence motif (GXGXXG) is situated above the ATP. The glycine residues allow the loops to approach the phosphates of ATP close enough to facilitate coordination via backbone interactions. A centrally located loop known as the activation loop provides a platform for the peptide substrate, which binds across the front end of the nucleotide binding pocket, close to the γ-phosphate of ATP. When the kinase is active, this loop can be phosphorylated, which stabilizes it in an open conformation that is permissive for substrate binding. Completion of phosphotransfer requires precise spatial arrangement of several conserved catalytic residues that reside in the catalytic loops and that interact with the attacking hydroxyl side chain of the substrate. Proper coordination of ATP binding, association with substrate, and activation promote phosphotransfer, which often results in signal propagation in the cell (Hanks et al., 1988; Huse and Kuriyan, 2002).

Tyrosine kinases

In 1979, Eckhart et al. discovered protein kinase-like activity in immunoprecipitates from polyoma T antigens that had the unusual property of specifically phosphorylating tyrosine (Eckhart et al., 1979). A year later, Hunter and Sefton made the important finding that the transforming protein of Rous sarcoma tumor virus, v-SRC, is also able to phosphorylate tyrosine residues (Hunter and Sefton, 1980). This suggested that deregulated protein tyrosine phosphorylation could play a role in carcinogenesis. It is now known that src is one of 32 non-receptor tyrosine kinases (NRTK), which are also referred to as cytoplasmic tyrosine kinases. In addition to the src family kinases, there are eight other NRTK subfamilies: Csk, Ack, Fak, Tec, Fes, Syk, Abl and Jak classes. NRTKs have emerged as an important contributor to initiation and progression in a variety of human cancers. Src, the first identified oncogene, has particularly been implicated in tumorogenesis; upregulated or mutated src activity has been reported in breast, colon, pancreas, lung, ovarian, esophageal, gastric, and skin cancers (reviewed in (Irby and Yeatman, 2000)). Members of the other cytoplasmic tyrosine kinase families are also dysregulated in human cancers. For instance, activation of Ack (through overexpression or mutation) leads to enhanced cell motility in human breast cancer cell lines (van der Horst et al., 2005), which is crucial to metastasis and correlates with poor prognosis.

Of the 90 known tyrosine kinases, 58 are receptor tyrosine kinases (RTK), which are distributed among 20 subfamilies (Manning et al., 2002). More than

50% of these are mutated or overexpressed in human hyperproliferative diseases and are thus considered viable targets for cancer therapy. Mutations in receptor tyrosine kinases, as well as aberrant activation of their downstream signaling pathways, have been linked to inflammation, diabetes, arteriosclerosis, angiogenesis, and bone disorders, in addition to a multitude of different types of cancers (Lemmon and Schlessinger, 2010).

Signal transduction through receptor tyrosine kinases

One of the early challenges in the RTK field was determining the molecular mechanisms though which the receptors transduce signals across the plasma membrane. In the early 1980s, three reports showed that the RTKs epidermal growth factor receptor (Zhang et al.) (Ushiro and Cohen, 1980), the insulin receptor (INSR) (Kasuga et al., 1982), and the platelet derived growth factor (PDGF) receptor (Ek et al., 1982) are tyrosine kinases activated by their respective ligands. These data helped garner support for the concept of signaling mediated by tyrosine phosphorylation. Hunter and colleagues supplied additional experimental evidence for this concept by showing that stimulating NIH-3T3 cells with PDGF and A431 cells with EGF leads to rapid tyrosine phosphorylation of intracellular proteins downstream of the activated receptors (Cooper et al., 1982),(Hunter and Cooper, 1981). In a pivotal study published in 1986, Riedel et al. generated a chimeric receptor that fused the extracellular region of INSR with the transmembrane and intracellular domains of EGFR. They made the

unexpected discovery that insulin binding activated the EGFR kinase domain, suggesting that individual RTKs use similar mechanisms for signal transduction across the plasma membrane (Riedel et al., 1986).

Structure and activation mechanism of RTKs

In the last 25 years, the general structure and activation mechanism of receptor tyrosine kinases has been determined. All RTKs have a common molecular architecture, containing a ligand binding domain in the extracellular regions, a single transmembrane helix, a tyrosine kinase domain, and regulatory regions in the cytoplasmic domain (Lemmon and Schlessinger, 2010). Consistent with the key regulatory role of RTKs, the basic structure, mechanism, and cellular signaling pathways initiated by RTKs are highly conserved throughout evolution- from the nematode *Caenorhabditis elegans* to humans. In general, receptor tyrosine kinases are activated by ligand binding, which induces receptor oligimerization (Ullrich and Schlessinger, 1990). The bound ligand stabilizes the interaction between individual receptor molecules to form a receptor dimer. In some cases, the single membrane-spanning α helix also contributes to dimerization. Once ligand has bound to a receptor, self-association of the extracellular domains guides the intracellular domains into a dimeric conformation. Subsequently, one receptor in the dimer phosphorylates one or more tyrosines on its dimerization partner, which then serves as a site for

assembly and activation of intracellular signaling effectors (Lemmon and Schlessinger, 2010).

Although there is redundancy in the basic mechanisms leading to RTK activation, there are substantial differences in how each receptor binds to ligand, oligerimizes, transphosphorylates, and mediates downstream signaling. Early studies suggested a straightforward mechanism for ligand-induced dimerization whereby a bivalent ligand simultaneously binds two receptors and crosslinks them together into a dimer. This was supported by evidence from crystal structures of receptors such as the Eph receptor (Himanen and Nikolov, 2003), the nerve growth factor/ neurotrophin receptor TrkA (Wiesmann et al., 1999), the stem cell receptor KIT (Liu et al., 2007a) and the Flt1 vascular endothelial growth factor receptor (Wiesmann et al., 1997) bound to their respective ligands. However, recent structural studies have provided additional insight into other mechanisms that mediate dimerization. On the opposite end of the spectrum from bivalent ligand-induced dimerization is receptor-mediated dimerization. Ligand binding to ErbB receptors triggers conformational changes within the molecule that exposes a previously occluded dimerization arm (Garrett et al., 2002). In this type of dimerization, the ligand does not directly crosslink the dimer interface. Some studies suggest that EGF binds to and activates pre-existing EGFR oligomers, however the precise nature and size of the oligomers is not known (Clayton et al., 2005; Gadella and Jovin, 1995). Lastly, some RTKs utilize a combination of ligand-mediated and receptor-mediated dimerization. For

example, dimerization of the fibroblast growth factor receptor (FGFR) requires an FGF monomer to come into contact with two FGFRs simultaneously, and the resulting dimer is stabilized by heparin binding (Schlessinger et al., 2000).

Each RTK is uniquely autoinhibited by a specific set of intramolecular interactions. Upon ligand binding-induced dimerization, receptor tyrosine kinases release their autoinhibition to activate the intracellular tyrosine kinase domain. While crystal structures indicate that the activated forms of the tyrosine kinase domains are very similar, the inactive forms are substantially different from receptor to receptor and likely contribute mechanistically to receptor regulation (Huse and Kuriyan, 2002). Upon release from auto-inhibition, the first substrates that RTKs phosphorylate are tyrosines on the receptors themselves, and this autophosphorylation occurs in *trans* (Honegger et al., 1989; Till et al., 2002). The resulting phosphotyrosines serve as binding sites for the downstream signaling effecters and adaptors that are recruited to facilitate signal transduction, resulting in various biological outputs.

Discovery of EGF and EGFR

In 1962 Stanley Cohen isolated and characterized a salivary-gland protein that induced precocious tooth eruption and eyelid opening when injected into newborn mice (Cohen, 1962). This novel substance was termed epidermal growth factor, since it stimulated the proliferation of epithelial cells derived from chick embryos (Cohen, 1965). Subsequently, Carpenter et al. confirmed the

presence of specific sites that bound ^{125}I-labeled EGF on the surface of fibroblasts (Carpenter et al., 1975). These binding sites were later identified as the EGF receptor (Zhang et al.), a 170-kDa membrane component that showed increased ^{32}P incorporation in response to EGF treatment of epidermal carcinoma cells (Carpenter et al., 1978).

During the 1980's, cDNA cloning techniques facilitated the cloning of large gene transcripts. A team of scientists from Genentech, the Imperial Cancer Research Fund, and the Weizmann Institute of Science isolated and characterized the cDNA of human EGFR from A431 tumor cells and normal placental cells (Ullrich et al., 1984). This was the first complete amino-acid sequence of a cell-surface receptor with signaling ability, and it provided detailed insights into the molecular architecture of EGFR (Gschwind et al., 2004). Subsequently, a search amongst known protein sequences revealed a high level of similarity between the EGFR peptides and sequences of the avian oncogene v-erbB (Downward et al., 1984). This discovery was the first link between an animal oncogene and a human gene that encodes a cell growth-controlling protein.

ErbB family members

It is thought that humans and other jawed vertebrates have undergone two rounds of whole genome duplication, which duplicated every gene up to four fold (Dehal and Boore, 2005). While genome duplication can be a cataclysmic event for an organism, this double duplication enabled an enormous expansion in

functionality. While most duplicated genes, called ohnologs, were rapidly lost, 13% of human genes are still recognizable as duplicates (McLysaght et al., 2002). The ohnologs that remain are highly biased towards transcription factors and signaling genes (Manning and Scheeff, 2010). Currently, it is believed that the ErbB family originated from a single ancestral ErbB gene (Spring, 2002). All four ErbB ohnologs were retained after the genome quadruplication, with two remaining fully functional (EGFR and ErbB4), one losing its ligand-binding ability (ErbB2), and one lacking kinase activity (ErbB3) (Figure 1.1). The double duplication from a single ErbB gene gave rise to a complex array of homo- and hetero-dimeric receptors that mediate a diverse range of cellular signaling.

ErbB ligands

Ligand binding promotes dimerization and subsequent activation of the ErbB family members. EGFR, ErbB3, and ErbB4 have both overlapping and specific ligands; EGFR's cognate ligands are EGF, betacellulin (BTC), amphiregulin (AR), transforming growth factor-α (TGF-α), epiregulen (Peiretti et al.), heparin-binding EGF-like growth factor (HB-EGF), and epigen (EPG); while ErbB3's ligands are heregulin (HRG) 1, and HRG2. ErbB4 can bind the ligands HRG3 and HRG4, in addition to also binding BTC, HB-EGF, EPR, HRG1, and HRG2 (Harris et al., 2003) (Figure 1.2). ErbB2 has no known ligand; instead, it serves as the preferred dimerization partner of other ErbBs (Graus-Porta et al., 1997). All of the ligands are generated as type 1 transmembrane proteins that

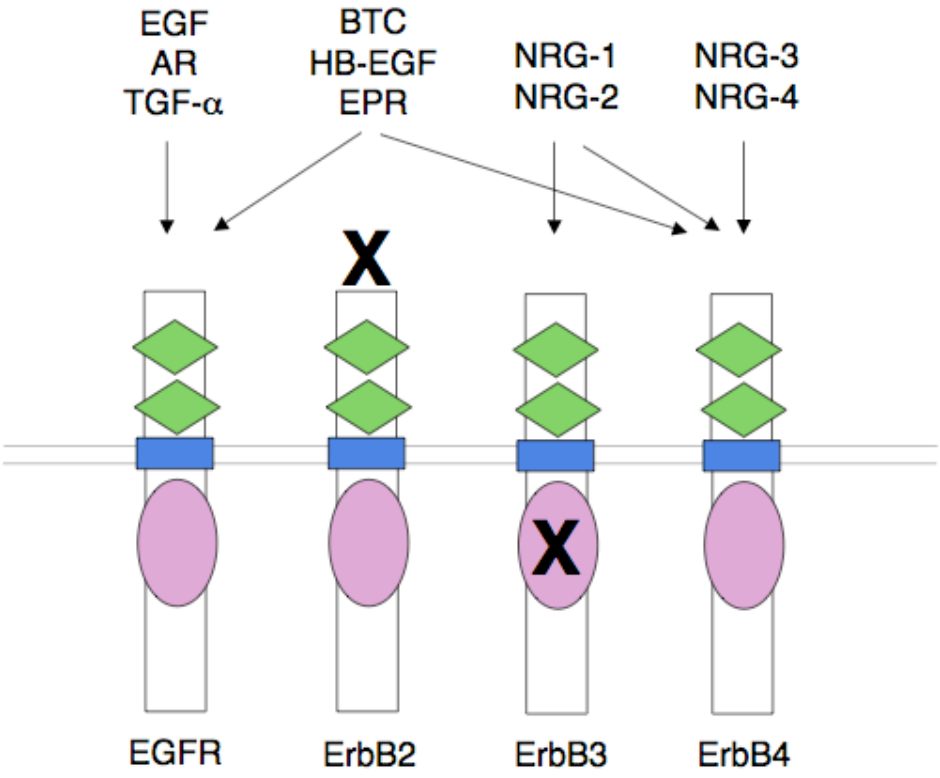

Figure 1.1 Schematic representation of the ErbB family with their respective ligands Each family member contains an extracellular region containing two cysteine-rich domains (green diamonds), a single transmembrane region (blue rectangle), and a cytoplasmic domain consisting of a tyrosine kinase domain (pink oval) and regulatory regions. ErbB2 has no known ligand, while ErbB3 has a deficient kinase domain.

are inserted into the plasma membrane. In response to cellular stimuli, proteases cleave the pro-form of the ligands to release mature growth factors. A defining feature of the ErbB ligands is the presence of an EGF motif, which is a consensus sequence consisting of six spatially conserved cysteine residues that form three intramolecular disulfade bonds. Additionally, AR and HB-EGF contain heparin-binding domains, while the HRGs contain an Ig-like domain (Falls, 2003; Harris et al., 2003) (Figure 1.2).

ErbB receptor transactivation

In addition to activation by direct ligand binding, receptor tyrosine kinases are transactivated by a wide variety of molecules, such as cytokines, bacterial products, G-protein coupled receptor (GPCR) ligands, and integrins. Previous reports demonstrate that transactivation occurs through two distinct mechanisms: the extracellular release of ligands or the intracellular activation of tyrosine kinases. A recent publication by our laboratory shows that EGFR and ErbB2 transactivation by TNF occurs in an intracellular, Src-dependant manner (Yamaoka et al., 2008). In contrast, EGFR transactivation by *Helicobacter pylori* is mediated through extracellular, metalloproteinase-dependent ligand cleavage (Yan et al., 2009). In 1996, Daub et al. discovered that the GPCR agonists endothelin-1, lysophosphatidic acid, and thrombin stimulate EGFR and ErbB2 phosphorylation in rat fibroblasts (Daub et al., 1997). Since then, EGFR transactivation by GPCR ligands has been observed in a myriad of cell types,

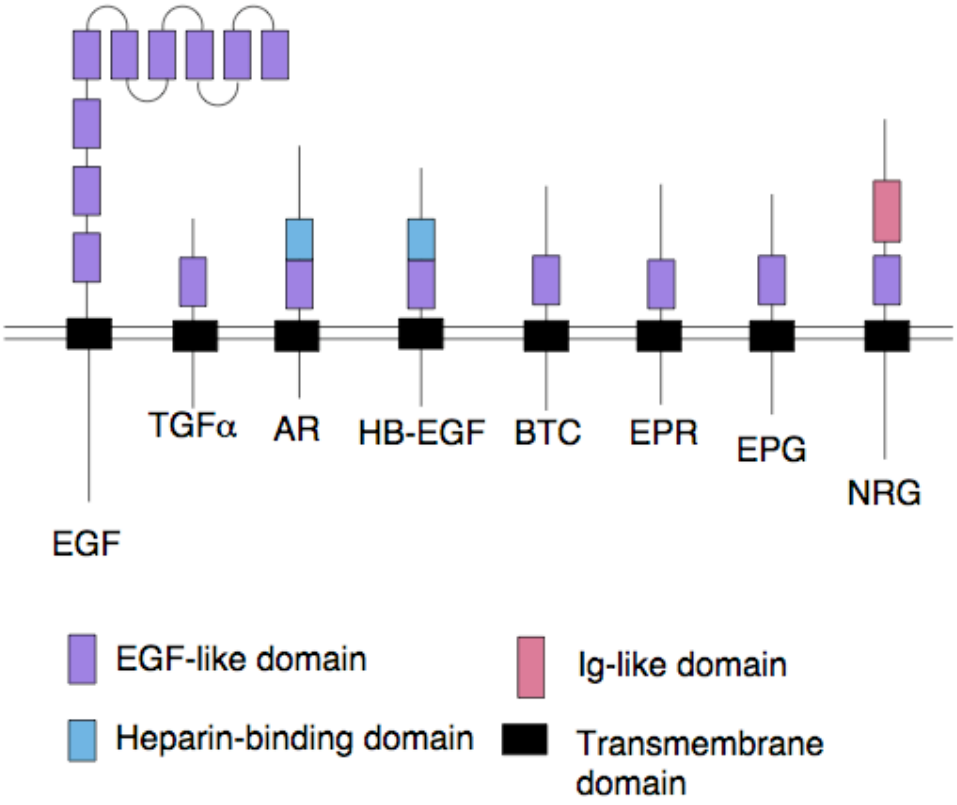

Figure 1.2 ErbB ligand schematic ErbBs are activated by a family of EGF-like ligands, as well as by the neuregulins. EGF: epidermal growth factor; TGFα: transforming growth factor alpha; AR: amphiregulin; HB-EGF: heparin binding epidermal growth factor; BTC: betacellulin; EPR: epiregulin; EPG, epigen; NRG: neuregulin

and has been shown to be an important mediator of GPCR-induced mitogen-activated protein kinase (MAPK) signal transduction. In the last two decades, EGFR has been recognized as a convergence point for diverse signal transduction pathways.

The ADAM family of metalloproteases

The a disintegrin and metalloprotease (ADAM) family are Zn^{2+}-dependent proteases that cleave membrane-bound proteins and/ or degrade the extracellular matrix. ADAMs are closely related to matrix metalloproteinases (MMPs), ADAM-TSs (ADAMs with thrombospondin domains), and snake venom metalloproteinases (SVMPs). Twenty-one ADAMs have been described in the human genome, but only 13 of these are proteolytically active (Edwards et al., 2008). ADAMs are involved in the proteolytic cleavage of a wide variety of membrane-bound proteins, and play a key role in growth factor receptor transactivation. All known ErbB ligands can be cleaved by members of the ADAM family. For instance, HB-EGF is cleaved by ADAMs 9, 10, 12, and 17; betacellulin is cleaved by ADAMs 10 and 17; epiregulin is cleaved by ADAM 17; and neuregulin is cleaved by ADAMs 17 and 19 (Higashiyama and Nanba, 2005; Montero et al., 2000; Shirakabe et al., 2001).

ADAMs often have tissue-specific functions and promote different physiological processes, which range from migration and cellular adhesion, to proliferation and fertilization. Given their diverse signaling targets, it is not

surprising that ADAMs have been implicated in numerous diseases, such as Alzheimer's, rheumatoid arthritis, diabetes, and heart disease (Gooz, 2010). Additionally, there is substantial evidence supporting a role for ADAMs in cancer progression and metastasis. ADAM12 overexpression promotes breast cancer progression (Kveiborg et al., 2005), while ADAM12 deficiency reduces prostrate tumor growth and progression (Peduto et al., 2006). ADAM 9 promotes colon cancer cell invasion *in vitro* (Mazzocca et al., 2005), and ADAM-15 loss decreases prostate cancer metastasis to bone (Najy et al., 2008).). It is not completely understood how metalloproteases are activated, but increased expression of metalloproteinases has been observed in almost every disease where inflammation is present (Manicone and McGuire, 2008). Interestingly, although metalloproteinases are often amplified in tumors, chemotherapies targeting metalloproteinases have not proven effective (Coussens et al., 2002). One plausible explanation for this is that metalloproteinases have both a protective function (repair after inflammation) as well as a harmful role (increased growth factor signaling).

TNF-alpha converting enzyme

TNF-alpha converting enzyme (TACE, or ADAM17) is a member of the ADAM family that is known to play a role in ligand cleavage, as well as in inflammation. TACE is a ubiquitously expressed protease involved in the ectodomain shedding of a wide variety of transmembrane proteins, such as

growth factor ligands, cytokines, and receptors. As its name implies, TACE was discovered in 1997 as the enzyme responsible for cleaving the membrane-bound TNF precursor into its soluble form (Black et al., 1997; Moss et al., 1997).

The structure of TACE is similar to that of other ADAMs: it contains a prodomain, a metalloenzyme or catalytic domain, a disintegrin domain, a cysteine-rich domain, followed by a transmembrane domain and a cytoplasmic C-terminus (Figure 1.3). The prodomain of TACE inhibits the protein's catalytic activity until it is removed by furin, a pro-protein convertase, in the trans Golgi network (Schlondorff et al., 2000). However, some studies show that removal of the prodomain in not necessary for TACE transport to the cell surface, since immature TACE is found in the plasma membrane even following furin inhibition (Peiretti et al., 2003). It is thought that the metalloprotease is packaged into lipid rafts during transport through the Golgi network, and that this aids in TACE activity regulation by keeping the enzyme separated from its substrates (Tellier et al., 2006). TACE activation is also regulated by tissue inhibitors of metalloproteinases-3 (TIMP-3), which inhibits TACE by binding to a hydrophobic pocket in its catalytic domain. While the interactions that inhibit TACE activation have been studied, much less is known about how the enzyme is actually activated and how it recognizes substrate.

Diaz-Rodriguez et al. have shown that phorbol ester (PMA) and EGF induce TACE phosphorylation on threonine 735 in the cytoplasmic tail of the metalloprotease. ERK mediates this activation, which results in cleavage of the

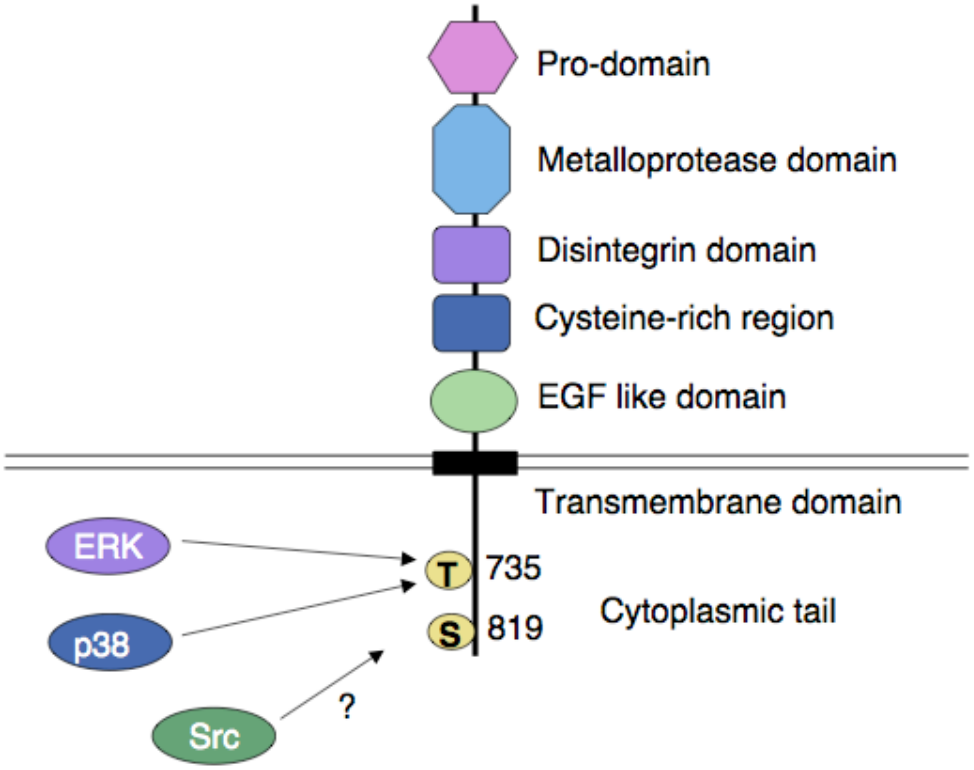

Figure 1.3 TACE structural schematic TNF-alpha converting enzyme (TACE), also known as a disintegrin and metalloprotease 17 (ADAM17), is a metalloprotease that is responsible for cleaving a variety of ligands, cytokines, and receptors. Threonine (735) and Serine (819) have been demonstrated as cytoplasmic phosphorylation sites. ERK and p38 activate TACE by phosphorylating T735; Src also activates TACE through an unknown mechanism.

transmembrane TrkA neurotrophin receptor in CHO cells and HEK293 cells (Diaz-Rodriguez et al., 2002). Other groups have also shown that PMA and EGF induce serine 819 phosphorylation on TACE in HeLa cells, also in an ERK-dependent manner (Fan et al., 2003). ERK is not the only MAP kinase capable of inducing TACE phosphorylation on T735; in C-α cells (derived from CHO cells) p38-dependent TACE activation leads to TGF-α cleavage and subsequent EGFR-dependent cellular proliferation (Xu and Derynck, 2010). Also, both constitutively active Src and endogenous Src induce TACE activation in COS7 and MCF7 cells, leading to TGF-α cleavage (Maretzky et al., 2011). Given these different activation mechanisms, it is likely that TACE catalytic activity is regulated in a tissue-specific and stimulus-specific manner.

Although the cleavage sites of several TACE substrates (such as TNF and growth hormone receptor) have been identified, there does not seem to be a classical TACE cleavage sequence. Instead, it is postulated that position of the cleavage site is more important that the actual sequence. There is some evidence that the “stalk region” between the transmembrane region and the first globular part of the substrate determines whether TACE can cleave a protein (Hinkle et al., 2004; Schlondorff and Blobel, 1999; Wang et al., 2002).

ErbB4

ErbB4 is the last identified member of the EGFR family of receptor tyrosine kinases. The 180 kD glycoprotein was discovered in 1993 by Gregory

Plowman. Plowman et al. designed degenerate oligonucleotide primers that matched a region of the mouse genome encoding conserved amino acids in the kinase domains of EGFR, ErbB2, and ErbB3. Following PCR to isolate genomic fragments of ErbBs1-3, they discovered a highly related, but distinct DNA fragment that they termed MER4. Subsequently, they used a similar strategy in the breast cancer line MDA-MB-453 to obtain a cDNA clone that corresponded to the human homology of MER4, which they termed HER4, or ErbB4 (Plowman et al., 1993). Like other members of the EGFR family, ErbB4 is characterized by two cysteine-rich domains in the ligand-binding extracellular region, a juxtamembrane domain, and a tyrosine kinase domain flanked by regulatory regions in the intracellular domain (Figure 1.4). ErbB4's extracellular domain shares the most similarity with ErbB3, while its kinase domain exhibits 79% and 77% identity with EGFR and ErbB2, respectively (Plowman et al., 1993).

ErbB4 expression is essential for normal mammalian development; ErbB4 null mice die at embryonic day 11 due to defects in myocardial trabeculae formation and nervous system development. Tidcombe et al. circumvented this dilemma by expressing ErbB4 under a cardiac-specific myosin promoter, which enabled studies of adult ErbB4 knockout mice. These mice exhibit aberrant cranial nerve architecture due to a defect in neural crest migration, as well as failure of the mammary lobuloalveoli to differentiate, which results in defective lactation (Tidcombe et al., 2003). In addition to neural, mammary, and cardiac tissue, ErbB4 is also expressed in the pancreas (Kritzik et al., 2000), esophagus,

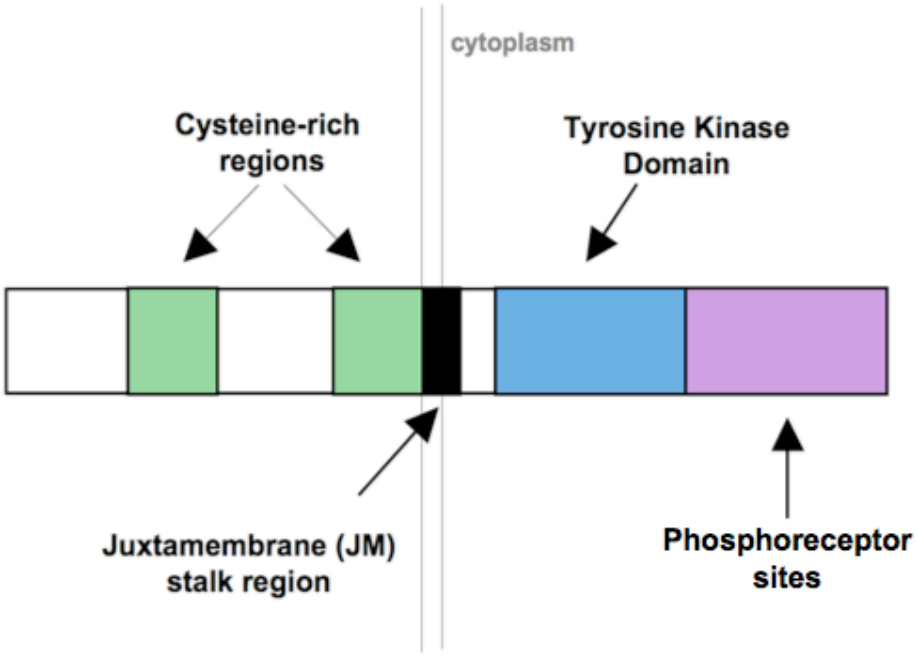

Figure 1.4 ErbB4 schematic ErbB4 is characterized by two cysteine rich domains in the extracellular ligand binding domain, a protease-sensitive stalk sequence, and a tyrosine kinase domain in the intracellular region. Multiple tyrosine phosphorylation sites in the C-terminal end serve as docking site for adaptor and signaling proteins.

stomach, duodenum (Kataoka et al., 1998), lung (Liu et al., 2007b), and utererus (Lim et al., 1998).

Mechanism of Activation

ErbB4 can be activated by any of several EGF-like ligands, such as HB-EGF, betacellulin, and epiregulin, as well as members of the heregulin/ neuregulin family, which includes heregulins 1-4. In the absence of ligand, ErbB4 is made up of four discrete subdomains that adopt an autoinhibited conformation (Bouyain et al., 2005). Upon ligand binding, a domain rearrangement occurs that exposes previously obscured surfaces and allows them to mediate dimerization (Burgess et al., 2003). Crystallographic studies show that active ErbB4 kinase adopts an asymmetric dimer conformation that is almost identical to that observed for EGFR (Qiu et al., 2008). The C lobe of a "donor" receptor contacts the N lobe of an adjacent "acceptor" receptor and induces conformational changes that activate the acceptor kinase (Zhang et al., 2006b). This particular asymmetric interaction is similar to the activation interface between cyclins and the N lobes of cyclin-dependent kinase (Jeffrey et al., 1995). Since the ErbBs control such a diverse range of cellular processes, it is vital that they have evolved an activation state that minimizes inappropriate kinase activity caused by intermolecular collisions between receptors.

Signaling downstream of ErbB4

Trans-phosphorylation of ErbB4's C-terminal tyrosine residues provides docking sites for signaling molecules with phosphotyrosine binding (PTB) or src homology 2 (SH2)-domains. A tandem mass spectroscopy approach identified 19 sites of tyrosine phosphorylation on ErbB4, which is more than have been identified for any other RTK (Kaushansky et al., 2008). Interestingly, all but one of the tyrosines that are located outside the kinase domain in the cytoplasmic region of the receptor are phosphorylated, and these sites seem to be more selective in their preference for binding partners than tyrosines in the other ErbBs (Kaushansky et al., 2008). Although it is possible that some of the sites of tyrosine phosphorylation are merely an artifact from the tandem mass spectroscopy approach used, it is encouraging to note that the authors did not find any evidence for phosphorylation of tyrosine residues in the extracellular portion of the receptor.

Although a wide range of binding partners has been identified for EGFR, there have been far fewer studies concerning ErbB4's signaling partners. Signaling targets of ErbB4 include PI 3-kinase (PI-3K), phospholipase C-γ (PLC-γ), Mdm-2, Yes-associated proteins (YAP), nucleolin, Stat-5, and Mtg16 (Kaushansky et al., 2008). Additionally, multiple Grb2 and Shc interaction sites and at least one binding site each for Nck and Crk have been identified through pull-down assays (Schulze et al., 2005). There are several regions in the C-terminal regulatory domain that are conserved between EGFR, ErbB2, and

ErbB4. In particular, Y-1284 in ErbB4, Y-1248 in ErbB2, and Y-1197 in EGFR are in regions of high sequence homology between the receptors, and are binding sites for Shc (Schulze et al., 2005), an important adaptor protein that can lead to Ras activation via Grb2 interactions (Luzi et al., 2000). Due to Shc's role in promoting signaling downstream of growth factor receptors, ErbB4 phosphorylation on Y-1284 is used as a marker for the receptor's activation. Interestingly, kinase-dead ErbB4 mutants isolated from cancerous cells maintain the ability to form heterodimers with ErbB2 and to activate MAPK and AKT pathways (Tvorogov et al., 2009).

Alternative splicing generates four ErbB4 isoforms

ErbB4 undergoes alternative splicing to generate four distinct isoforms that differ in their juxtamembrane and cytoplasmic domains. The two juxtamembrane domain variants are characterized by their susceptibility to cleavage by TACE and γ-secretase. The JM-a isoforms contain a 23 amino acid stretch in their juxtamembrane domain that confers cleavage in response to ligand binding or PMA stimulation, releasing a 120 kDa ectodomain and an 80 kDa intracellular domain. The alternate 13 amino acid sequence in the JM-b isoforms is resistant to cleavage (Elenius et al., 1997). The two CYT variants differ in their cytoplasmic tail. A 16 amino acid sequence in the C-terminal domain of the CYT-1 isoforms serve as a binding site for PI-3 kinase and Nedd4, while the CYT-2 isoforms lack

this sequence and cannot signal through PI-3 kinase (Elenius et al., 1999) (Figure 1.5).

Physiological functions of ErbB4 isoforms

Although ErbB4 is expressed in a variety of epithelial tissues including the lining of the gastrointestinal, urinary, reproductive, and respiratory tracts, its function in these tissues has not been fully investigated. ErbB4 promotes chemotaxis in fibroblasts (Kainulainen et al., 2000), proliferation in primary breast cancer cells (Maatta et al., 2006), survival in neuronal cells (Erlich et al., 2001), and suppresses anoikis in Ewing tumor cells (Kang et al., 2007). Paradoxically, ErbB4 also stimulates apoptosis in breast cancer cells and differentiation in the mammary gland (Long et al., 2003; Naresh et al., 2006). These contradictory roles can partially be explained by the differing functions of each ErbB4 isoform. Although the majority of the published papers on ErbB4 do not distinguish between the specific isoforms, recently several groups have investigated how the alternative splicing variants affect cell survival and tumorigenesis. While nuclear translocation of the JM-a, CYT-2 isoform promotes both survival and proliferation in breast cancer cells (Junttila et al., 2005), targeting of the JM-a, CYT-1 isoform to the mitochondria stimulates apoptosis (Naresh et al., 2006). Alternately, the Elenius laboratory has shown that the CYT-1 isoforms confer survival through PI 3-kinase signaling (Elenius et al., 1999). PI3K promotes survival by activating

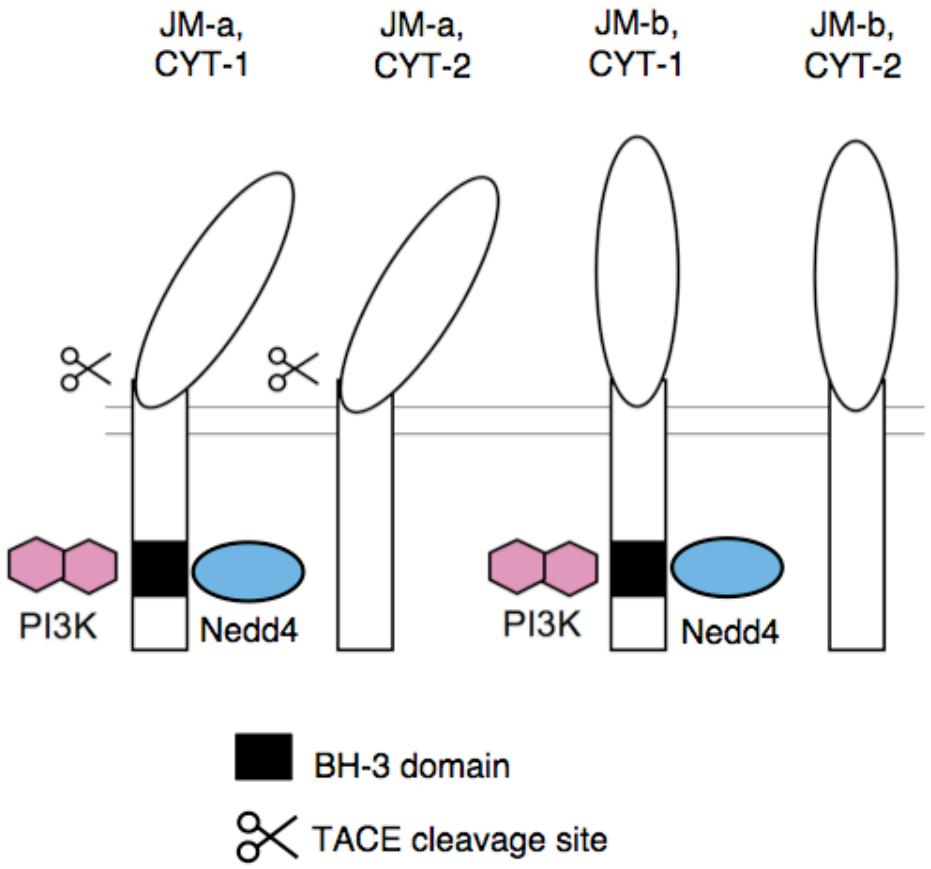

Figure 1.5 Schematic of the four ErbB4 isoforms Alternative splicing in the regions coding for the juxtamembrane and cytosolic domains generates four different isoforms of ErbB4. The JM-a isoforms are subject to a two-step proteolytic cleavage by TACE and γ-secretase, while the JM-b forms are resistant to metalloprotease-dependent cleavage. CYT-1 isoforms contain a PI3-kinase docking site as well as a Nedd4 binding site, which is absent in CYT-2 variants.

Akt, which then either activates pro-survival proteins or inactivates apoptotic proteins (Figure 1.6).

The seemingly disparate findings concerning ErbB4's role in biological processes may indicate a tissue and isoform-specific role for ErbB4 as either a tumor suppressor or tumor promoter. Both ErbB4 juxtamembrane splice variants and both cytoplasmic splice variants are expressed in mouse colon epithelial tissue and cultured cells (Frey et al., 2009), but very little is known about the function of each isoform in the GI tract.

Role of ErbB4 in cancer

There has been a great deal of interest in the last two decades concerning the role of ErbB family members in promoting oncogenic signaling. Given the proliferation and survival pathways downstream of ErbB activation, small molecule inhibitors and antibodies directed toward the receptors are attractive therapeutic agents. ErbB2 is overexpressed and/ or gene amplified in about 20% of all breast cancers; currently, the ErbB2 monoclonal antibody trastuzumab and the dual EGFR/ ErbB2 small molecule tyrosine kinase inhibitor lapatinib are approved for metastatic ErbB2-positive breast cancers (Gutierrez and Schiff, 2011). Additionally, there are large scale, population based studies treating non-small cell lung cancer (NSCLC) patients with the EGFR tyrosine kinase inhibitors erlotinin and gefitinib (Mok et al., 2009; Rosell et al., 2009). While the utility of targeting EGFR and ErbB2 in cancerous malignancies has been proven effective,

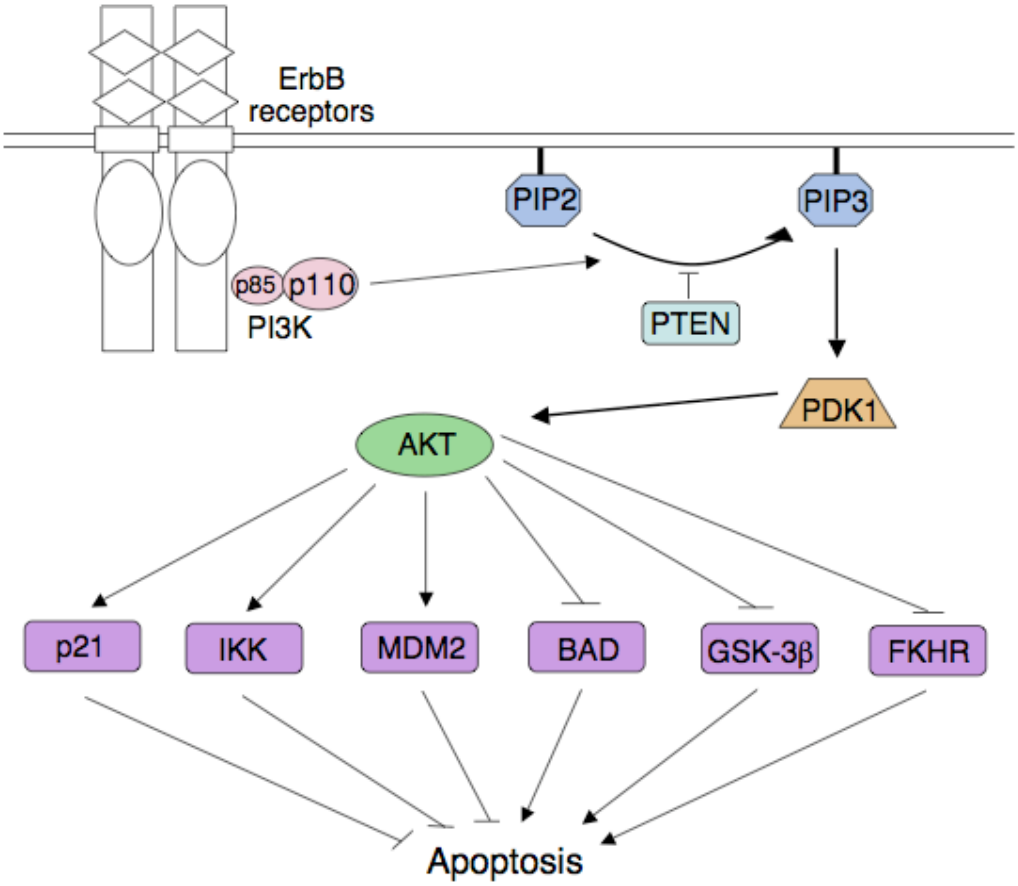

Figure 1.6 Schematic representation of the PI3K-AKT signaling pathway Following dimerization and activation of ErbB receptors, PI3K is translocated to the cell membrane and binds to phosphotyrosine consensus residues of the RTK through its p85 adaptor subunit. This results in activation of the p110 catalytic subunit, leading to production of PIP3. Through interactions with its pleckstrin homolgy (PH) domains, PIP3 recruits AKT. PTEN is a PIP3 phosphatase that negatively regulates the PI3K-AKT pathway. The interaction of PIP3 with the PH domain of AKT induces conformational changes in AKT, exposing the two main phosphorylation sites at T308 and S473, which are phosphorylated by PDK1 and PDK2. Activated AKT then interacts with a variety of signaling proteins to inhibit apoptosis.

it has not been definitively established if ErbB4 is an appropriate target for cancer treatment.

The role of ErbB4 as a tumor suppressor or an oncogene is currently quite controversial, with support for both claims evident in the literature. In support of a tumor-suppressing role, a constitutively active ErbB4 mutant inhibits the formation of drug-resistant colonies in the human prostate tumor cell lines DU-145 and PC-3 (Williams et al., 2003). Another constitutively active ErbB4 mutant harboring an activating transmembrane mutation potentiated apoptosis in ovarian, prostate, and breast cancer cells lines (Vidal et al., 2007). Additionally, cytosolic, but not membrane-bound, ErbB4 expression in primary human breast tumors was associated with tumor apoptosis. In the same cell type, the s80 cleavage product was shown to act as a BH3 only protein that interacts with BAK to promote apoptosis (Naresh et al., 2006).

As evidence that ErbB4 is a tumor promoter, colonic epithelial cells overexpressing ErbB4 acquire the ability to form colonies in soft agar (Frey et al., 2010). Likewise, ErbB4 mutations have been found in gastric carcinomas, colorectal carcinomas, non-small cell lung cancer, and breast tumors (Parsons et al., 2005) (Davies et al., 2005; Soung et al., 2006). Pricket et al. found that ErbB4 is the most highly mutated protein tyrosine kinase in a melanoma screen, with 19% of patients harboring a somatic mutation in the receptor (Prickett et al., 2009). A follow up to this study determined the melanoma samples exclusively expressed the cleavable ErbB4 isoform (JM-a, CYT-1 and JM-a, CYT-2), with no

detection of the non-cleavable JM-b isoforms (Rudloff and Samuels, 2010). These findings highlight the contribution individual isoforms make towards cancer progression, and underscore the importance of assessing which isoforms are expressed when studying the receptor.

Gastrointestinal tract homeostasis

The gastrointestinal tract consists of a single epithelial monolayer that extends from the mouth to the anus. Both the small intestine and the large intestine are comprised of a columnar epithelium that is invaginated into the crypts of Lieberkühn. Additionally, the epithelium of the small intestine contains finger-like projections termed villi. Cells from 6 to 10 different crypts migrate upwards in ordered columns to populate each villus (Schmidt et al., 1985). Intestinal stem cells reside in the base of crypts, where they produce progenitor cells that proliferate and migrate up the crypt axis. These cells differentiate into several principle types: mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, and absorptive enterocytes (Cheng, 1974a; Cheng and Leblond, 1974a, b). The small intestine also contains antimicrobial Paneth cells, which do not migrate up the axis, but instead migrate to the base of the crypt (Cheng, 1974b). Once at the tip of the villlus (in the small intestine) or the top of the crypt (in the colon), the intestinal epithelial cells undergo apoptosis and are shed into the lumen. Through a process of sequential proliferation, migration, differentiation, and apoptosis, the intestinal epithelium is completely renewed

every five days (Hall et al., 1994). Disruptions in the delicate balance of these physiological processes can cause chronic diseases such as inflammatory bowel disease (IBD) (Figure 1.7).

Inflammatory Bowel Disease

History/ Discovery

Inflammatory bowl diseases, which include Crohn's disease and ulcerative colitis (UC), are chronic diseases of the gastrointestinal tract that are characterized by cytokine-driven inflammation. Although the exact etiology of IBD is unknown, it is thought that a combination of genetic, environmental, and immunological factors play a role in the pathogenesis of these diseases. Crohn's disease was first seen in 1623 by the German surgeon Wilhelm Fabry (also known as Guilhelmus Fabricus Hildanus) following the autopsy of a young boy with a fibrous and ulcerated cecum (reviewed in (Fielding, 1988)). In 1939, the disease was described by and named after Burril B. Crohn, an American physician (Crohn et al., 1984). Ulcerative colitis was first described in 1859 by the British physician Sir Samuel Wilks (Wilks, 1859).

Symptoms and physiology

Although Crohn's disease and ulcerative colitis share many symptoms, such as abdominal pain and cramping, diarrhea, loss of appetite, and bloody

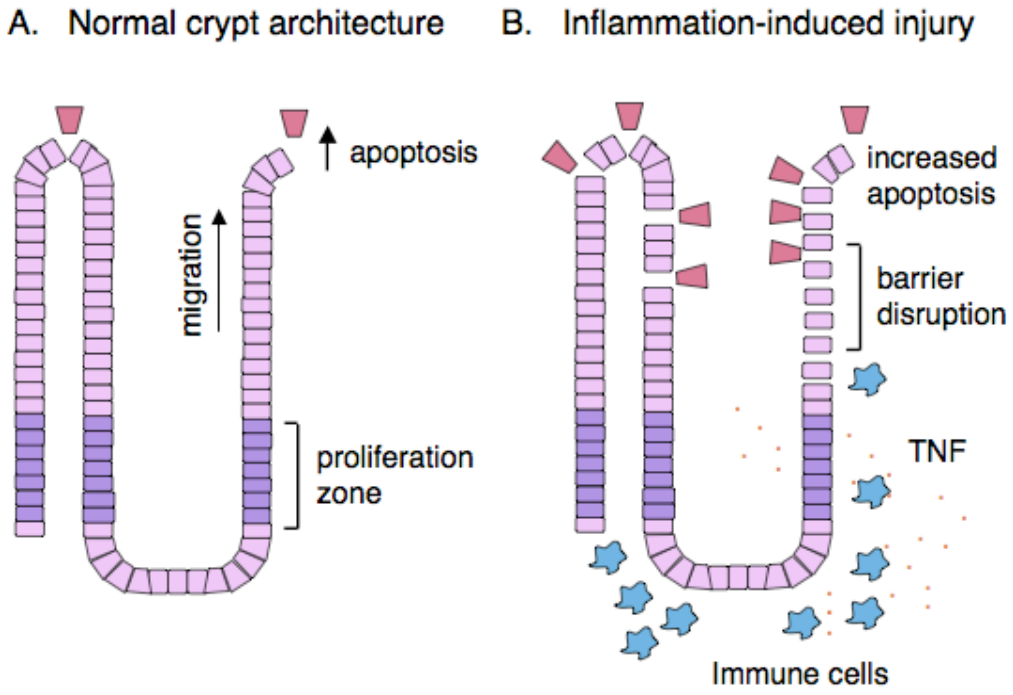

Figure 1.7 Schematic of colonic epithelial crypt architecture under normal and inflamed conditions A) Under normal conditions, colonic epithelial cells migrate from the base of the crypt upwards toward the tip of the crypt, where they undergo apoptosis and are shed into the lumen. B) During inflammatory conditions, colonic epithelial cells undergo increased apoptosis, which results in barrier defects. Bacterial pathogens in the lumen are then able to migrate into the lamina propria, which causes recruitment of immune cells such as neutrophils and macrophages.

stools, there are many physiological and molecular differences in the diseases. Crohn's disease can affect any portion of the digestive tract from the mouth to the anus, while ulcerative colitis only affects the large bowel. Additionally, Crohn's disease causes discontinuous ulceration and full thickness bowel wall inflammation in both the small intestine and the colon, while ulcerative colitis is characterized by continuous inflammation in only the mucosa of the colon. Patients with Crohn's often have excess IL-12/IL-23 and IFN-γ/IL-17 production, and those with UC typically have increased IL-13 production (Fuss et al., 2006; Fuss et al., 2004). Both diseases are chronic and relapsing, but ulcerative colitis can be cured by surgical removal of the colon.

Colitis Associated Carcinogenesis

One of the complications of recurrent bouts of intestinal inflammation in patients with IBD is an increased risk of colorectal cancer (CRC). IBD ranks as the third highest risk factor for CRC behind the genetic conditions familial polyposis coli (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). The risk of developing CRC rises with increased duration and extent of ulcerative colitis. Irrespective of disease extent, the cumulative probability of developing CRC is 2% after 10 years, 8% after 20 years, and 18% after 30 years of UC onset (Eaden et al., 2001). Drugs that reduce severity of inflammation tend to decrease the risk of developing CRC (Rutter et al., 2004). Typically, colitis-associated carcinogenesis (CAC) arises in grossly diseased mucosa and in the

microscopic colitis proximal to gross colitis. The progression of CAC is similar in many ways to that of CRC. First, mutations accumulate in oncogenes or tumor suppressor genes, which often result in the aberrant activation of β-catenin signaling. Subsequently, single preneoplastic cells transition into aberrant crypt foci, followed by adenoma and then colorectal carcinoma. While CRC is thought to originate from environmental mutagens and hereditary mutations, CAC is initiated by chronic amplified levels of cytokines such as TNF, IL-17, 1L-23, IFN-γ, and IL-6 and the tissue injury and repair these molecules cause. While both forms of colon cancer exhibit increased K-Ras signaling and decreased p53 levels, CAC also displays dysregulated NF-κB, Stat3, and GSK3β signaling (Terzic et al., 2010).

The role of the immune system in IBD

Although the cause of IBD is not completely understood, it is thought that a dysregulated, aberrant immune response to intestinal flora plays a significant role in pathogenesis. The intestinal inflammation associated with IBD is controlled by a dynamic interplay of the innate and adaptive immune systems. Following disruption of the intestinal epithelial barrier, antigen presenting cells (APC) underlying the intestinal epithelial monolayer are able to interact with the bacterial flora, which are normally compartmentalized in the lumen. Healthy individuals show immune tolerance to normal microbial antigens, but the mucosal immune system of patients with IBD severely overreacts to normal bacterial flora.

Subsequently, APC such as activated dendritic cells and macrophages secrete cytokines, which trigger and differentiate the T cells that activate the adaptive immune system. These cytokines also trigger increased apoptosis in the intestinal epithelium, which leads to further barrier disruption, and additional exposure to microbial antigens (Strober et al., 2007). This cyclical mechanism of barrier disruption leads to the chronic bouts of inflammation that are a hallmark of IBD.

The role of genetics in IBD

There has been a sharp rise in the rates of IBD since the 1950s, although it is not entirely understood why. The highest prevalence of ulcerative colitis and Crohn's disease is in North America, the United Kingdom, and in northern Europe. In these countries, the rates of IBD are beginning to stabilize. However, in low-incidence areas such as Asia, southern Europe, and most developing countries, IBD rates continue to rise (Loftus, 2004).

In addition to geographical location, there also seems to be a link between ethnicity and IBD incidence; ulcerative colitis is three to five times more prevalent in Jewish people, and children of North American Ashkenazi Jews with Crohn's disease typically experience an earlier onset of IBD (Heresbach et al., 1998; Niv et al., 2000). The largest independent risk factor for Crohn's disease or ulcerative colitis is a positive family history for IBD, which further supports the role of

genetics in these diseases. People with Crohn's disease have a first degree relative with the disease in 2-16% of cases, and those with ulcerative colitis have a first degree relative with UC in 6-16% of cases (Satsangi et al., 1996). Additionally, multiple studies have shown that monozygotic twins are significantly more likely to be concordant for IBD than dizygotic twins (Orholm et al., 2000; Thompson et al., 1996; Tysk et al., 1988).

Both ulcerative colitis and Crohn's disease are polygenic diseases; several genome-wide scans have identified IBD susceptibility regions on 12 different chromosomes (Hampe et al., 1999; Peltekova et al., 2004; Stoll et al., 2004). One gene in particular that has been linked to inflammatory bowel disease is the *caspase recruitment domain protein 15* (*CARD15)*, which encodes NOD2. Between 10%-20% of Caucasians with Crohn's disease have a mutation in NOD2, typically presenting with Arg702Trp, Gly908Arg, or a frameshift deletion at Leu1007 (Hugot et al., 2001; Ogura et al., 2001a). NOD2 belongs to a family of intracellular proteins containing a nucleotide-binding oligomerization domain (NOD) linked at its carboxy terminus to a leucine-rich repeat (LRR) domain. The NOD domain activates the molecule following ligand recognition, while the LRR domain is a microbial component recognition unit that is structurally similar to the LRR recognition unit found in Toll-like receptors (TLR) (Tanabe et al., 2004). NOD2 recognizes a bacterial peptidoglycan product termed muramyl dipeptide, and is expressed in antigen presenting cells and in Paneth cells. Dysfunction of NOD2 in either of these cell types could be involved in the pathogenesis of IBD,

since both APCs and Paneth cells help regulate the intestinal immune response to bacteria. In a healthy person, the resident microflora of the intestine does not provoke an inflammatory response. However, it is hypothesized that when the bacterial recognition protein NOD2 is dysregulated, commensal bacteria can induce pro-inflammatory signaling through NOD2-mediated NF-κB signaling in a similar manner as pathogenic bacteria (Ogura et al., 2001b).

Tumor Necrosis Factor signaling

History/ Discovery

In 1975 Carswell et al. identified a serum factor that induced hemorrhagic necrosis of sarcomas when passively transferred to tumor-bearing mice (Carswell et al., 1975). This protein, termed tumor necrosis factor (TNF), also killed tumor cells *in vitro* (Helson et al., 1975). Also referred to as cachectin because of its ability to induce cachexia, TNF is a proinflammatory cytokine that is primarily produced by macrophages and monocytes, although it can also be produced by T cells, fibroblasts, smooth muscle cells, granulocytes, keratinocytes, neurons, mast cells, and natural killer cells. TNF exists in the cell as a membrane-bound homotrimer of 26-kDa monomers (mTNF). Following cleavage by TACE, the cytokine is released from the cell as a soluble trimer of 17 kDa monomers (sTNF). TNF can signal either in the membrane-bound or soluble

form, and the relative levels of each are determined by the inducing stimuli, the cell type involved, and the amounts of biological TACE inhibitors (such as members of the tissue inhibitor of metalloproteinase (TIMP) family) (Smookler et al., 2006). Both mTNF and sTNF can bind either one of the two TNF receptors: TNFR1 (p55) or TNFR2 (p75), which are both also trimers. Although both TNFRs bind TNF, they differ in cellular expression profiles, affinity for ligand, cytoplasmic tail arrangement, and signaling mechanism. For instance, TNFR1 is constitutively expressed on virtually all cell types, whereas TNFR2 is generally inducible and is typically expressed on endothelial and hematopoietic cells. Additionally, soluble TNF preferentially binds to TNFR1 (with a dissociation constant Kd ~20pM) over TNFR2 (Kd ~400 pM), due to a 30-fold faster dissociation rate from TNFR2 than from TNFR1 (Grell et al., 1998). While physiological levels of TNF (1 ng/mL) enhance wound closure through TNFR2, pathological levels of TNF (100 ng/mL) inhibit migration of intestinal epithelial cells though TNFR1 (Corredor et al., 2003). Likewise, physiological concentrations of TNF stimulate proliferation in colonic epithelial cells through TNFR2, while signaling through TNFR1 inhibits proliferation in response to TNF at pathological levels (Kaiser and Polk, 1997).

TNF signaling in disease

Signaling through TNF can often be a double-edged sword; at low concentrations TNF can augment host defense mechanisms against infection, but at high concentrations the cytokine can lead to excessive inflammation and

tissue injury. TNF signaling is mediated by adaptor proteins that bind to the cytoplasmic domain of the TNFRs upon extracellular ligand binding. TNFR1 contains a death domain in its cytoplasmic region that binds TNFR-associated death domain (TRADD) and couples the receptor to either a nuclear factor kappa-B (NF-κB)-activating pathway or to an apoptosis-inducing pathway. NF-κB comprises a family of transcription factors that control a large number of inflammatory genes. Normally, NF-κB activation suppresses the apoptosis pathway. If, however, NF-κB activation is compromised, apoptotic signaling through caspase 8 and caspase 3 becomes the dominant TNFR1 mediated pathway (Van Antwerp et al., 1998) (Figure 1.8).

TNF plays an important role in regulating the inflammatory cascade that causes pathogenesis in chronic diseases such as psoriasis, rheumatoid arthritis, Crohn's disease, and asthma. Mice genetically engineered to overexpress TNF develop Crohn's-like inflammatory bowel disease, which seems to be mediated by mature T and B cells (Kontoyiannis et al., 1999). Additionally, TNF protein and mRNA are expressed in mast cells (Bischoff et al., 1999), macrophages, monocytes, and T cells in the intestinal mucosa of Crohn's disease and ulcerative colitis patients (Van Deventer, 1997). Patients with Crohn's disease and ulcerative colitis also have elevated concentrations of TNF in their intestinal mucosa (Van Deventer, 1997).

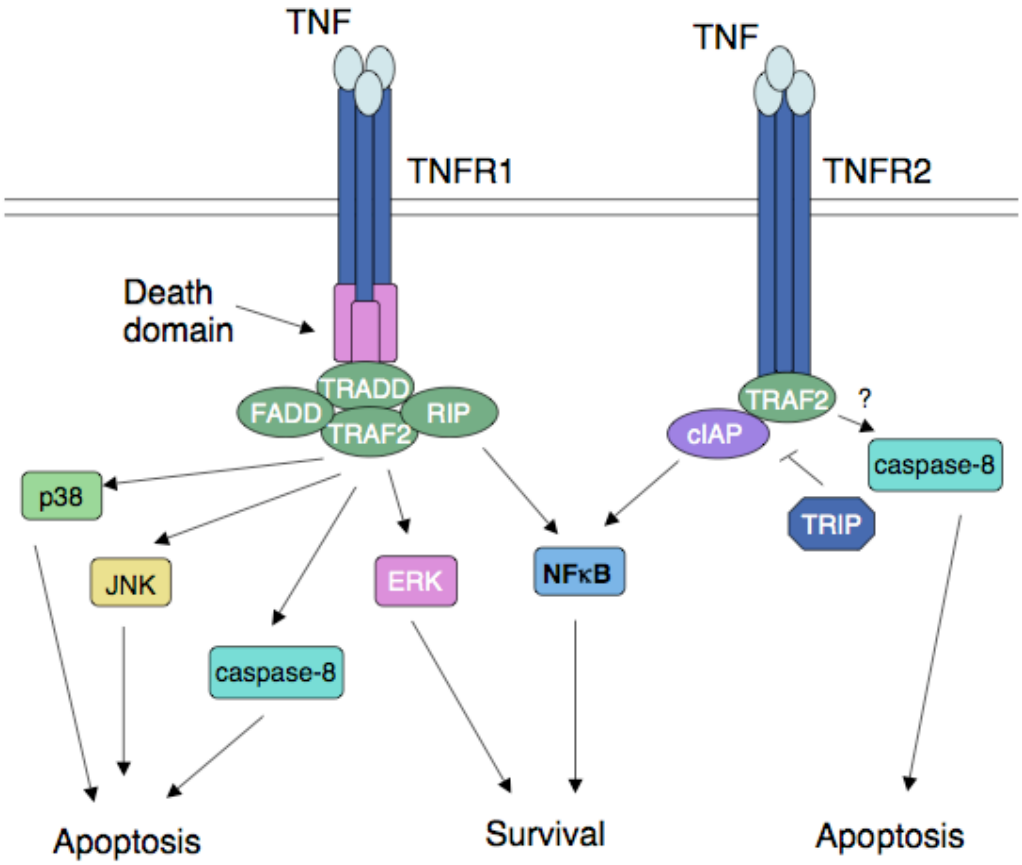

Figure 1.8 TNF receptors stimulate both apoptotic and survival signaling Through recruitment of adaptor proteins to its death domain, TNFR1 promotes apoptosis via downstream p38, JNK, and caspase-8 signaling. In certain cellular situations, TNFR1 promotes survival though NFκB and ERK signaling. It is uncertain how TNFR2 stimulates apoptosis, since it lacks a death domain. However, it is thought that TNFR2 interacts with TRAF2 through a domain in its cytoplasmic tail, and subsequently activates caspase cleavage.

Drugs targeting TNF

Infliximab (brand name Remicade) and adalimumab (brand name Humira) are monoclonal antibodies that specifically bind TNF, while etanercept (brand name Enbrel) is a TNF-receptor Fc-fusion protein that binds TNF family members (Tracey et al., 2008). Some clinical studies have found that TNF antagonists increase the overall risk of infection, particularly to tuberculosis (Harris and Keane, 2010; Listing et al., 2005). There have also been reports that patients treated with anti-TNF antibodies have an increased risk of lymphomas; however, the data are not yet conclusive, and the risk is currently considered limited enough not to be a deciding factor in whether or not to treat those with high disease activity (Bongartz et al., 2006).

Objectives

The mammalian gastrointestinal lining plays critical roles in nutrient absorption and in protecting the organism from the contents of the intestinal lumen. Disruption of the intestinal epithelial monolayer by alterations in apoptosis, proliferation, and wound healing is a hallmark of inflammatory bowel disease. While the exact etiology of IBD is unknown, it is believed that hyperpermeability of the intestinal barrier to bacterial products couples with an abnormal immune response to contribute to the pathogenesis of IBD. One of the mechanisms that increases hyperpermeability of the gut barrier is an increased level of pro-inflammatory cytokines, such as TNF and interferon-γ (Bruewer et al.,

2003). TNF also activates apoptotic pathways in epithelial cells, potentially leading to the ulcerations characteristic of IBD.

Members of the ErbB family of receptor tyrosine kinases are vital for monolayer restitution and injury repair. Our laboratory has shown that ErbB4 is upregulated in mouse models of colitis and is activated in response to TNF (Frey et al., 2009). Coupled with our observation that ErbB4 is necessary for cell survival in the presence of TNF (Frey et al., 2009), this suggests that ErbB4 is a key mediator of intestinal epithelial layer homeostasis during inflammatory insult. On the other hand upregulation and increased signaling through ErbB4 during colitis could have severe consequences for the GI tract, since several types of cancer display ErbB4 amplification. ErbB4 activation in response to inflammatory cytokines may contribute to the colorectal carcinogenesis that is a common complication of IBD. Although the roles of EGFR, ErbB2, and ErbB3 during intestinal pathologies have been explored, very little is known about how ErbB4 functions in the gastrointestinal tract. The experiments detailed in this thesis are designed to fill this gap in knowledge by exploring the role of ErbB4 transactivation in maintaining intestinal epithelial monolayer integrity during pathologies such as colitis.

Chapter II

MATERIALS AND METHODS

Cell Culture

Conditionally immortalized young adult mouse colon (YAMC) epithelial cells (Whitehead et al., 1993), Madin-Darby Canine Kidney (MDCK), and Immorto-Min Colon Epithelial (IMCE) cells were provided by Dr. Robert Whitehead (Vanderbilt University); TACE $^{-/-}$ lines stably expressing wildtype TACE and vector were obtained from Dr. Peter Dempsey (University of Michigan) (Garton et al., 2003). YAMC, IMCE, and TACE $^{-/-}$ lines were all maintained in RPMI 1640 medium containing 5% FBS, 5 U/mL of murine interferon (IFN)-γ (Intergen, Norcross, GA), 5 μg/mL insulin, 5 μg/mL transferron, 5 μg/mL selenous acid (Eto et al.) (BD Biosciences, San Jose, CA) and 100 U/mL penicillin and streptomycin and grown under permissive conditions at 33°C with 5% CO_2. Prior to all experiments, cells were transferred to 37°C (nonpermissive) conditions and cultured in medium with streptomyocin, penicillin, and with either 0.5% FBS or no FBS for 24 hours. MDCK cells were maintained in DMEM containing penicillin/ streptomycin and 10% FBS at 37°C. Prior to experiments, MDCK cells were starved of FBS overnight.

Transfections and constructs

Generation of ErbB4-expressing YAMC cells has previously been published (Frey et al., 2009). Briefly, inserts from pCDNA3.1-ErbB4 (JM-b/CYT-2 isoform) expression vectors (provided by Graham Carpenter, Vanderbilt University) were PCR amplified then cut and ligated into linearized bicistronic LZRS-GFP vector, which was obtained from Albert Reynolds (Vanderbilt University). LZRS-GFP or LZRS-ErbB4-GFP was transiently transfected into Phoenix packaging cells (Steve Hanks, Vanderbilt University) and YAMC or $TACE^{-/-}$ MCE cells were subjected to 5 rounds of infection with filtered supernatant supplemented with 4 µg/ml polybrene. Infected populations were expanded and GFP-positive cells were sorted at the Vanderbilt Medical Center Flow Cytometry Shared Resource using a Becton-Dickinson FACSAria. All ErbB-infected cell lines utilized in this study were infected with the JM-B, CYT-2 isoform, which is not cleavable by TACE in the juxtamembrane domain.

Nontargeting control and EGFR-specific siRNA pools were purchased from Dharmacon (Lafayette, CO, USA) and transfected into YAMC cells (100 nM siRNA) with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Table 2.1: ErbB ligand primer sequences

Primer	Sequence 5' to 3'
HB-EGF F	ATGAAGCTGCTGCCGTCG
HB-EGF R	TCAGTGGGAGCTAGCCAC
Epigen F	ATGGCTCTGGGGGTTCTGATAGC
Epigen R	TCACAATGGGCTCCCTCCAG
Epiregulin F	GTGATCCCATCATGCATCCC
Epiregulin R	ATTCTTTGCTCAAGGGTTGG
Amphiregulin F	CGCTGCTACCGCTGGCGCGC
Amphiregulin R	TATTCCCTGAAGTATCGTT
Betacellulin F	GGTAGCAGTGTCAGCTCCCT
Betacellulin R	GTGCAGACGCCGATGACTAA
EGF F	TACTCAGCGTCACAGCATGG
EGF R	AGCCACCCTCATAATCACAG
TGA-alpha F	CGACCGGACAGCTCGCTCTG
TGA-alpha R	TGGGTGTACTGAGCGAGCCC
Heregulin1 F	TGAATTTATGGAAGCGGAGG
Heregulin1 R	ATGTGCAGGGTTGTGATGAA
Heregulin2 F	TGTGCACTGACTGCGCCACC
Heregulin2 R	CGACCAGCAGGGCCACACAG
Heregulin3 F	AGGAGCCCCCAGTACGCAGG
Heregulin3 R	TGTCCTGGGCTGTGGTGCTTG
Heregulin4 F	TCCTGTGCAGGAAGGGCCAC
Heregulin4 R	TTGGCTGGAGGCCAGGTGGA

Antibodies, Cytokines, and Growth Factors

Polyclonal antibodies used for Western blot include phosphoERK1/2 (Promega) phospho-Y1068 EGFR, phospho-Y1248 ErbB2, active caspase-3, cleaved PARP, phospho-Y925 FAK, total FAK, phospho-T581 MSK1 (Cell Signaling), TACE, ErbB4 (Santa Cruz), and phospho-T735 TACE (Abcam). Monoclonal antibodies used include phospho-Y1284 ErbB4, phospho-Y1289 ErbB3, phospho-S473 Akt, total Akt (Cell Signaling), and actin (Sigma). Neutralizing antibodies used in cell culture include ErbB4 (Millipore), HRG, HB-EGF, and BTC (R&D Systems). Recombinant murine TNF was purchased from Peprotech (Rocky Hill, NJ). Recombinant HRG, BTC, and HB-EGF were purchased from R&D Systems (Minneapolis, MN). Recombinant EGF was a gift from Stanley Cohen (Vanderbilt University, Nashville, TN). TAPI-1 and GM6001 were purchased from EMD Chemicals (Darmstadt, Germany). PMA was purchased from Enzo Life Sciences. AG1478, U0126, CGP77675, and SB202190 were purchased from Calbiochem.

Cell lysates and Western blot analysis

Cell lysates were prepared by scraping cells in lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1 % SDS, 0.2% NaDeoxycholate, and 0.1% phosphatase and protease inhibitor cocktails (Sigma)), incubating on ice for 10 min, clearing by centrifugation, then boiling the

supernatants in Laemmli sample buffer. Cell lysates were run on SDS-PAGE gels (7.5-20% as needed) and transferred onto nitrocellulose membranes.

Apoptosis assays

Apoptosis was measured by cleaved PARP and active caspase-3 Western blot analysis following 3 hours of 100 ng/mL TNF and 1 μg/mL cycloheximide treatment. The ApopTag terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling kit (TUNEL; Millipore, Danvers, MA) was also used to label apoptotic cells. YAMC cells expressing either vector or ErbB4 were seeded in 4-well chamber slides (Lab-Tech) at $5x10^4$ cells and placed under non-permissive conditions overnight prior to apoptosis assays. Cells were treated with TNF/cycloheximide for 5 hours. The TUNEL assay was then performed following manufacturer's instructions, and cells were visualized by differential interference contrast microscopy on a Leica DM-IRB microscope.

Barrier Function Assay

Barrier function across YAMC monolayers was assessed by measuring transepithelial electrical resistance (TEER) using a World Precision Instrument, EVOM (Sarasota, Fl). YAMC cells were plated at $1x10^4$ in 24 well plates with transwells and grown under permissive conditions for five days. Following a 24 h shift to nonpermissive conditions, TEER readings were taken at 0 h and 24 h 100

ng/ml TNF stimulation. An average of five readings was calculated for each TEER reading.

Wound healing assay

Cells were seeded onto 35-mm plates coated with 2.5 μg of human fibronectin (BD Bioscience, Bedford, MA). Using a rotating silicon tip attached to a drill press, eight circular wounds were made in each confluent plate. Cells were washed twice with PBS and incubated under nonpermissive conditions with 1 ng/mL TNF, 100 ng/mL HRG, or 10 ng/mL EGF. Wounds were photographed at 0 and 24 h after wounding, and the size of each wound was determined using ImageJ software (National Institutes of Health, Bethesda, MD). The percent wound closure after 24 h was calculated. Values shown are the average wound closure rates for all eight wounds ± SD.

Proliferation Assay

Cells were plated in 96-well dishes ($5x10^3$ per well) at 37°C, in RPMI containing ITS, penicillin, and streptomycin. Cultures were exposed to 1ng/mL TNF, 100 ng/m HRG, 10 ng/mL EGF or vehicle for 24 h. Cells were counted at 0 and 24 h using an MTS-based colorimetric proliferation assay kit (Promega Corp.).

Statistics and replicates

All data with error bars are representative of at least three independent experiments. Statistical analyses were performed using Prism software (GraphPad Inc., La Jolla, CA). Statistical significance of differences was assessed by ANOVA analysis with Bonferroni post-test. Error bars indicate standard error of means.

Chapter III

TNF-ALPHA CONVERTING ENZYME-DEPENDENT ERBB4 TRANSACTIVATION BY TNF PROMOTES COLONIC EPITHELIAL CELL SURVIVAL

Introduction

Damage to the intestinal epithelium elicits multiple signal transduction pathways necessary for the cell survival, proliferation, and migration responses that repair injury. Coordinating these responses is critical for maintenance of a healthy colonic epithelial barrier, while disruption in these pathways can lead to conditions such as inflammatory bowel disease (IBD). We have shown that the tyrosine kinase ErbB4 is upregulated in the damaged epithelium of IBD patients and in mice with chemically induced colitis (Frey et al., 2009). ErbB4 is the most recently discovered member of the ErbB family of growth factor receptors, which includes epidermal growth factor receptor, ErbB2 (HER2), and ErbB3 (HER3). Binding of ErbB4 ligands, which include heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EPI), and heregulins (HRG) 1-4, induces receptor homodimerization or heterodimerization with ErbB1-3. Dimerization triggers activation of intrinsic tyrosine kinase activity and auto-phosphorylation of the cytoplasmic domain of the receptor, thus providing docking sites for a variety of adaptor proteins involved in the activation of

downstream cascades such as the phosphoinositide 3-kinase (PI3K)/Akt pathway (Kaushansky et al., 2008).

In addition to direct ligand stimulation, ErbB receptors can be indirectly activated by other stimuli such as G protein-coupled receptor agonists (Prenzel et al., 1999), cytokines (Yamaoka et al., 2008), and bacterial products (Yan et al., 2009). This cross communication enables the ErbB family to respond to diverse cellular signals and yield a myriad of biological responses, including survival, proliferation, and migration. Two distinct mechanisms of transactivation have been described: the activation of intracellular kinases and the extracellular release of ligands. Recently we have shown that EGFR and ErbB2 transactivation by the pro-inflammatory cytokine TNF occurs in an intracellular, Src-dependent manner (Yamaoka et al., 2008). However, other groups have shown that metalloprotease-driven ligand cleavage mediates EGFR transactivation following stimulation with GPCR-agonists (Schafer et al., 2004). Synthesized as membrane-anchored precursors, ErbB ligands are cleaved by members of the a disintegrin and metalloprotease family (Rose-John et al.), releasing soluble growth factors capable of paracrine and autocrine signaling (Sanderson et al., 2006). TNF-α converting enzyme (TACE/ADAM17) can cleave several members of the EGF-like ligand family, including HB-EGF, EPI (Hinkle et al., 2004), and HRG (Montero et al., 2000). The mechanism by which cellular stimuli activate TACE is not completely defined; however, data suggest a role for the mitogen-activated protein (MAP) kinases p38 or extracellular signal-regulated

(ERK1/2) kinase, which have both been shown to phosphorylate TACE on Thr735 (Diaz-Rodriguez et al., 2002; Xu and Derynck).

We have recently shown that ErbB4 expression is upregulated in the colonic epithelium of patients with inflammatory bowel disease and in mouse models of colitis (Frey et al., 2009). Additionally, 24 hour TNF exposure stimulates ErbB4 expression and activation in cultured young adult mouse colon (YAMC) epithelial cells, through an undetermined mechanism. One of the hallmarks of IBD is disruption of the intestinal epithelial monolayer due to increased apoptosis (Edelblum et al., 2006). ErbB4 induction by TNF presumably provides a check on colonocyte apoptosis by promoting cell survival in the presence of inflammatory cytokines (Frey et al., 2009).

The purpose of this study was to identify the mechanism by which TNF transactivates ErbB4, and to determine the role of this mechanism in anti-apoptotic signaling. Herein, we report that HB-EGF release by TACE is necessary for ErbB4 activation following TNF stimulation. Additionally, we show that TNF activates TACE in a MEK-dependent fashion, and that TACE activity and ErbB4 ligand binding are required for ErbB4-mediated cell survival in the presence of inflammatory cytokines, as well as activation of the pro-survival PI3K/Akt pathway. By providing the mechanism of the first example of ErbB4 transactivation, this study fills a gap in the knowledge of how ErbB4 functions to protect the gastrointestinal epithelium from inflammation-induced injury, which

may lead to novel treatment strategies for protecting the intestinal epithelium from cytokine-induced injury in patients with IBD.

Results

TNF Transactivates ErbB4 in Colon Epithelial Cells

Since ErbB4 expression is upregulated in the colonic epithelium of patients with IBD and in mice with DSS-induced colitis (Frey et al., 2009), we tested whether TNF could activate ErbB4 in young adult mouse colon (YAMC) epithelial cells stably over-expressing ErbB4 (termed YAMC-ErbB4 cells). TNF induced ErbB4 phosphorylation in a dose-dependent manner (Figure 3.1A), with the response peaking at 30 minutes and sustaining for at least 24 hours after treatment (Figure 3.1B). TNF also activated endogenous ErbB4 in an alternate colon epithelial cell line, Immorto-Min Colon Epithelial (IMCE) cells (Figure 3.2) as well as in Madin-Darby Canine Kidney (MDCK) cells (Figure 3.3), demonstrating that this phenomenon is not restricted to a single cell line or organ.

TNF-induced ErbB4 Activation in YAMC Cells is mediated by HB-EGF

Transactivation of ErbB receptors can occur through either ligand-dependent (Yan et al., 2009) or ligand independent (Yamaoka et al., 2008) mechanisms. To determine which route mediates TNF activation of ErbB4 in intestinal epithelial cells, we utilized a neutralizing antibody that blocks the

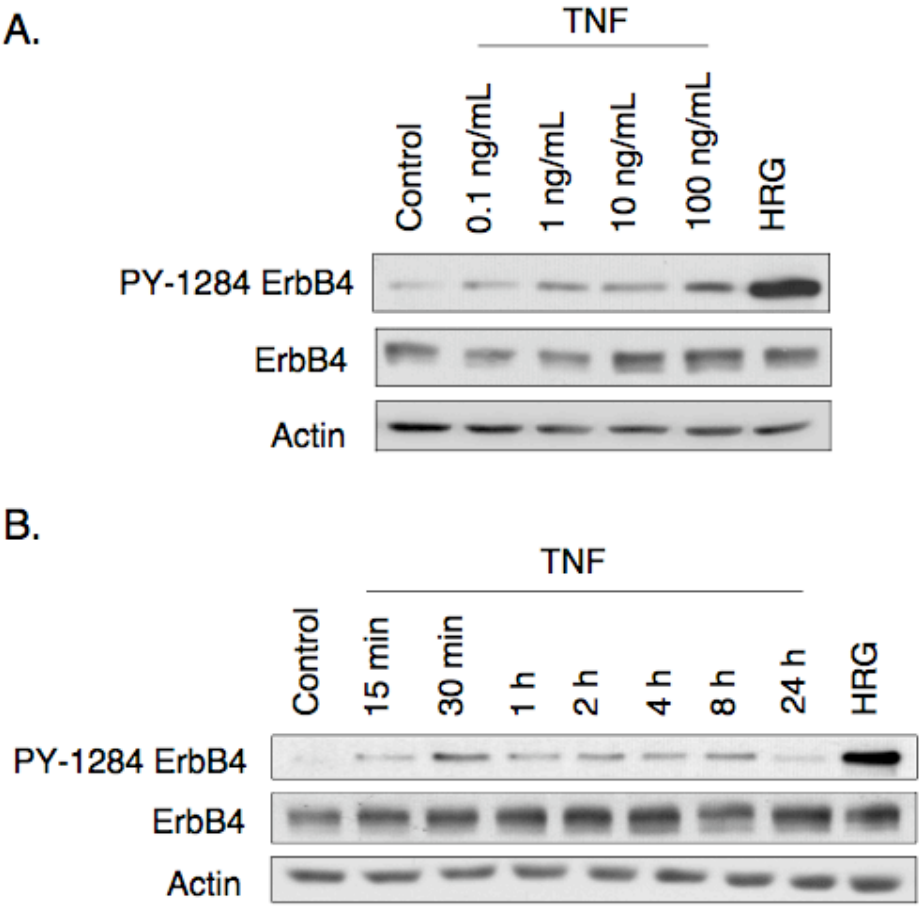

Figure 3.1: TNF transactivates ErbB4 in colonic epithelial cells A) YAMC cells expressing ErbB4 (YAMC-ErbB4 cells) were treated with TNF for 30 min at the indicated concentrations or with 10 ng/mL HRG for 10 min. Cell lysates were analyzed by Western blot with antibodies specific for phosphorylated (PY-1284) ErbB4, total ErbB4, or actin (loading control). B) YAMC-ErbB4 cells were treated with TNF (100 ng/mL) for the indicated time points or with HRG (10 ng/mL) for 10 min, as a positive control for activation.

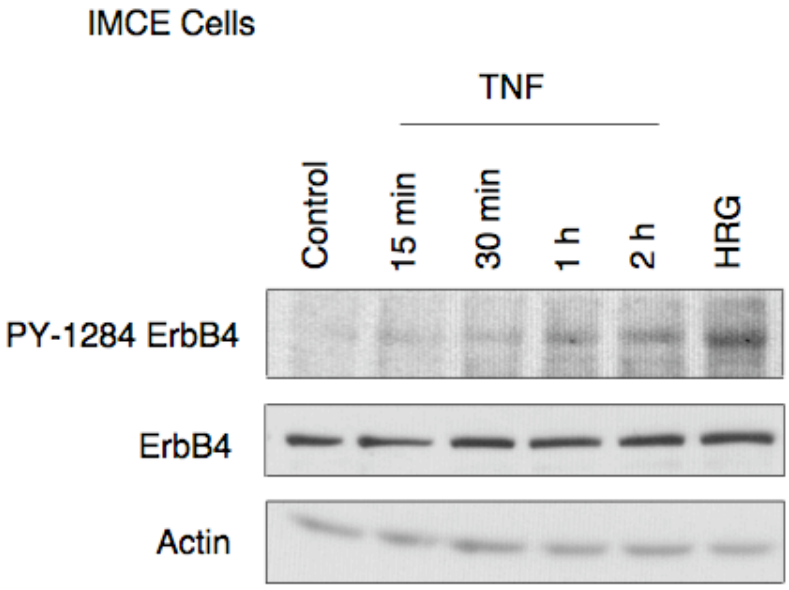

Figure 3.2 TNF transactivates ErbB4 in IMCE cells Immorto-Min Colon Epithelial (IMCE) cells were treated with 100 ng/mL TNF for the indicated times or with 10 ng/mL HRG for 10 min. Cell lysates were then analyzed by Western blot with antibodies specific for phosphorylated (PY-1284) ErbB4, total ErbB4, or actin.

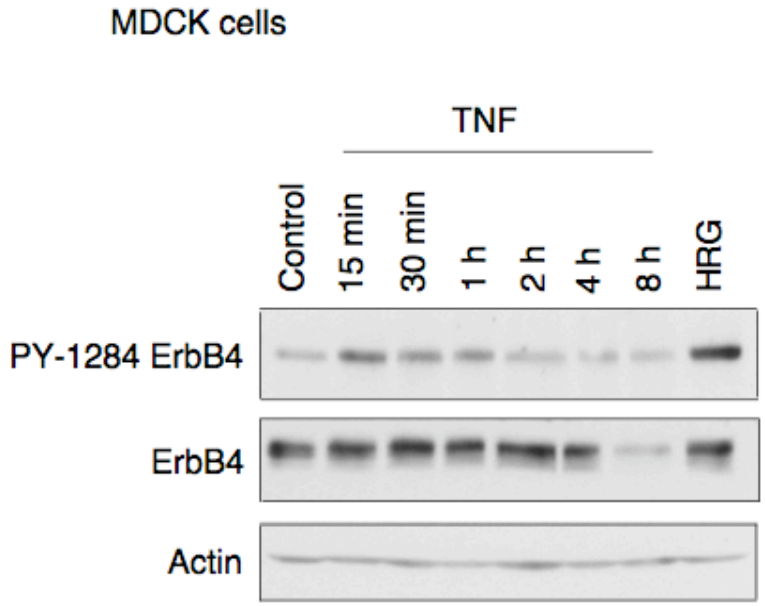

Figure 3.3 TNF transactivates ErbB4 in MDCK cells MDCK cells were treated with TNF (100 ng/mL) for the indicated time points or with HRG (10 ng/mL) for 10 min. Cell lysates were then analyzed by Western blot with antibodies specific for phosphorylated (PY-1284) ErbB4, total ErbB4, or actin.

ligand binding domain of ErbB4. YAMC-ErbB4 cells were incubated with the blocking antibody for 1 h before treatment with TNF for 30 min or HRG for 10 min. ErbB4 activation was then assessed by Western blot analysis. The ErbB4 blocking antibody inhibited both TNF-induced activation of ErbB4 and direct ligand stimulation (Figure 3.4), indicating that transactivation by TNF requires binding of an extracellular factor.

ErbB4 recognizes a subset of EGF-like ligands, such as HB-EGF and BTC, in addition to members of the heregulin family. To ensure that the ErbB4 blocking antibody did not cross-react with any of the closely related EGFR family members, YAMC-ErbB4 cells were treated with HRG (which binds ErbB3 and ErbB4), BTC (which binds EGFR and ErbB4), and EGF (which only binds EGFR) for 10 min. Figure 3.5 shows that ErbB4 blocking antibody inhibits ErbB4 phosphorylation by its own ligands, but not by EGF. Importantly, the ErbB4 blocking antibody did not inhibit EGFR activation by BTC and EGF or ErbB3 phosphorylation by HRG.

To narrow down the identity of the ligand responsible for ErbB4 transactivation by TNF, we utilized qualitative PCR to determine which ligands were expressed in YAMC cells. Table 3.1 shows the results of this PCR; EGF, HB-EGF, BTC, HRG-1, and HRG-2 were expressed in these cells. To determine which of the known ErbB4 ligands is responsible for ErbB4 transactivation by TNF, we treated YAMC-ErbB4 cells with TNF in the presence of HRG, HB-EGF, or BTC neutralizing antibodies. HB-EGF neutralizing antibody blocked both TNF

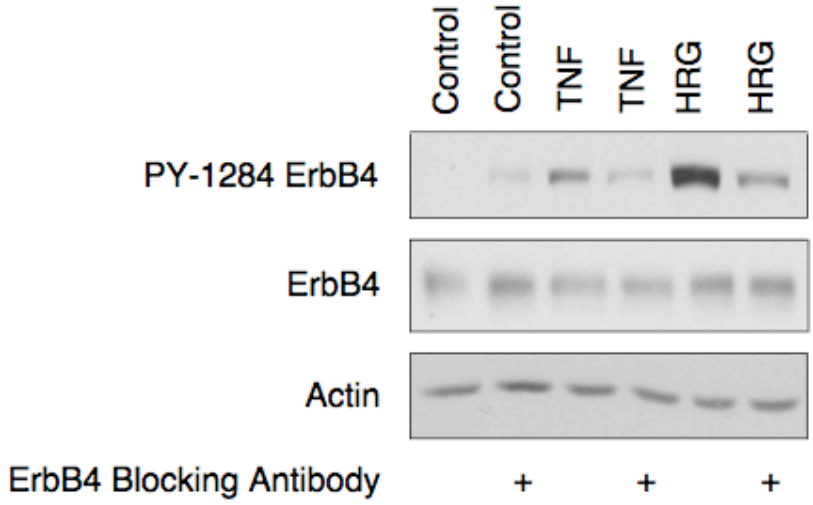

Figure 3.4 Ligand binding is required for ErbB4 transactivation by TNF YAMC-ErbB4 cells were incubated with either control mouse IgG or an ErbB4 blocking antibody for 1 h before the addition of TNF (100 ng/mL) for 30 min or HRG (10 ng/mL) for 10 min. Treatment with mouse IgG served as the negative control. Cell lysates were analyzed by Western blot with antibodies specific for phosphorylated ErbB4 (PY-1284), total ErbB4, or actin.

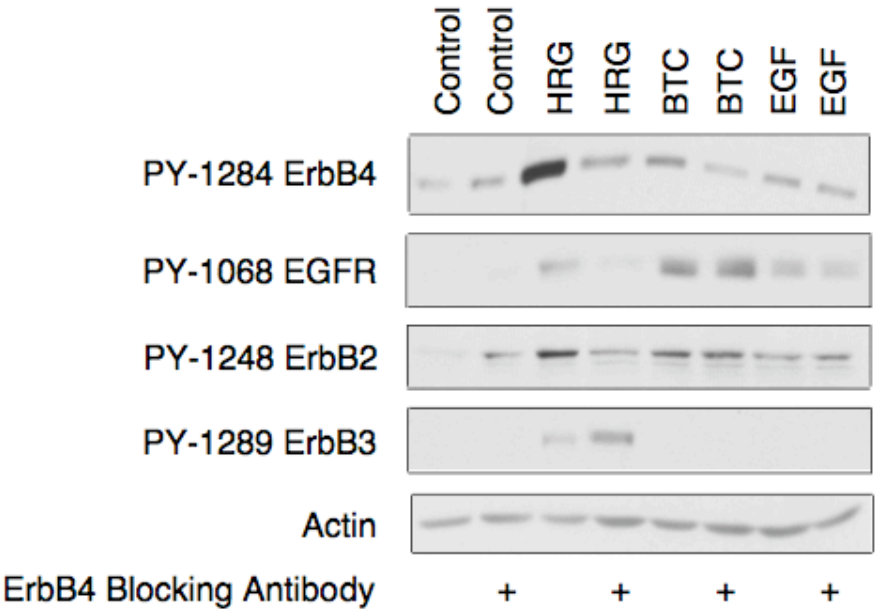

Figure 3.5 ErbB4 blocking antibody specifically blocks ErbB4 ligand binding YAMC-ErbB4 cells were incubated with an ErbB4 blocking antibody for 1 h before the addition of 10 ng/mL HRG, 1 ng/mL BTC, or 1 ng/mL EGF for 10 min. Phosphorylated ErbB4 (PY-1284), EGFR (PY-1068), ErbB2 (PY-1248), ErbB3 (1289), or total actin was assessed by Western blot.

Table 3.1: Expression of ErbB ligands is YAMC cells Qualitative PCR with primers specific for each ErbB ligand was used to determine which ligands are expressed in YAMC cells

Ligand	Expressed?
HB-EGF	Yes
Epigen	No
Epiregulin	No
Amphiregulin	No
Betacellulin	Yes
EGF	Yes
TGR-α	No
HRG-1	Yes
HRG-2	Yes
HRG-3	No
HRG-4	no

and HB-EGF-stimulated ErbB4 phosphorylation (Figure 3.6A). In contrast, neither HRG neutralizing antibody (Figure 3.6B) nor BTC neutralizing antibody (Figure 3.6C) attenuated TNF activation of ErbB4, although the antibodies were able to inhibit HRG and BTC-induced phosphorylation, respectively. We therefore conclude that HB-EGF is required for ErbB4 transactivation by TNF.

TACE-stimulated Release of HB-EGF Mediates TNF Transactivation of ErbB4

TNF signaling through TNFRs is known to activate metalloproteinases, which are proteases that can cleave membrane-anchored ligands (Huovila et al., 2005). To investigate whether ligand cleavage is necessary for ErbB4 transactivation, we treated YAMC-ErbB4 cells with the broad-spectrum metalloproteinase inhibitor GM6001 (50 μM) for 30 minutes, followed by TNF for 30 minutes or HRG for 10 minutes. GM6001 blocked ErbB4 activation in response to TNF, but not HRG (Figure 3.7A). Since the metalloproteinase TACE has been specifically implicated in the cleavage of ErbB4 ligands (Hinkle et al., 2004), we also treated YAMC-ErbB4 cells with the selective TACE inhibitor TAPI-1 (10 μM, 30 min) before TNF or HRG exposure. TNF-mediated ErbB4 phosphorylation was completely reversed by TACE inhibition, while HRG-induced activation was not altered (Figure 3.7B), suggesting that TACE-mediated cleavage of an ErbB4 ligand mediates ErbB4 transactivation.

To confirm that TACE is activated by TNF in YAMC-ErbB4 cells, cultures were treated with TNF for up to 30 min or with 20 ng/mL PMA for 1 minute, then

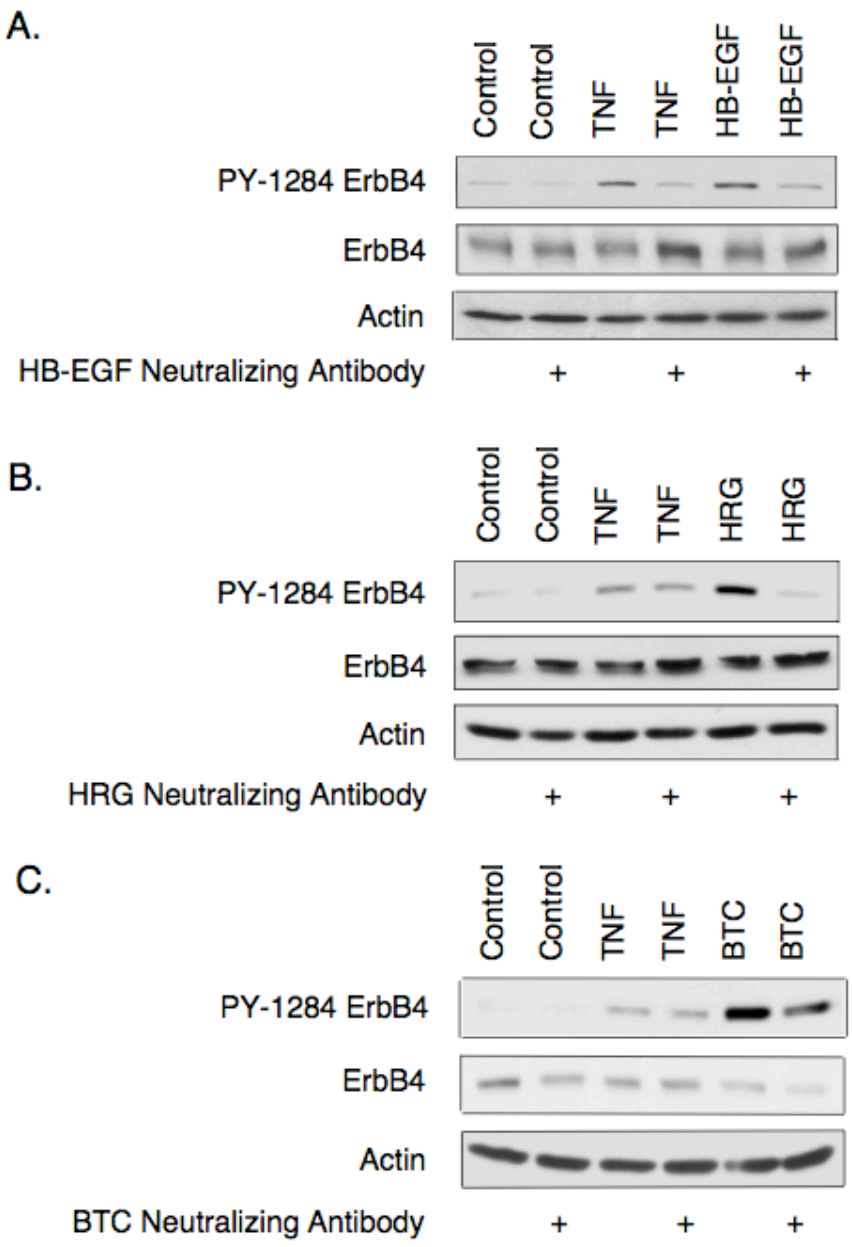

Figure 3.6 HB-EGF ligand binding mediates ErbB4 transactivation by TNF YAMC-ErbB4 cells were incubated for 1 h with A) HB-EGF neutralizing antibody B) HRG neutralizing antibody or C) BTC neutralizing antibody before stimulation with 100 ng/mL TNF for 30 min and with 50 ng/mL HB-EGF, 10 ng/mL HRG, or 10 ng/mL BTC for 10 min. Treatment with mouse IgG served as the negative control. Cell lysates were analyzed by Western blot with antibodies specific for phosphorylated ErbB4 (PY-1284), total ErbB4, or actin.

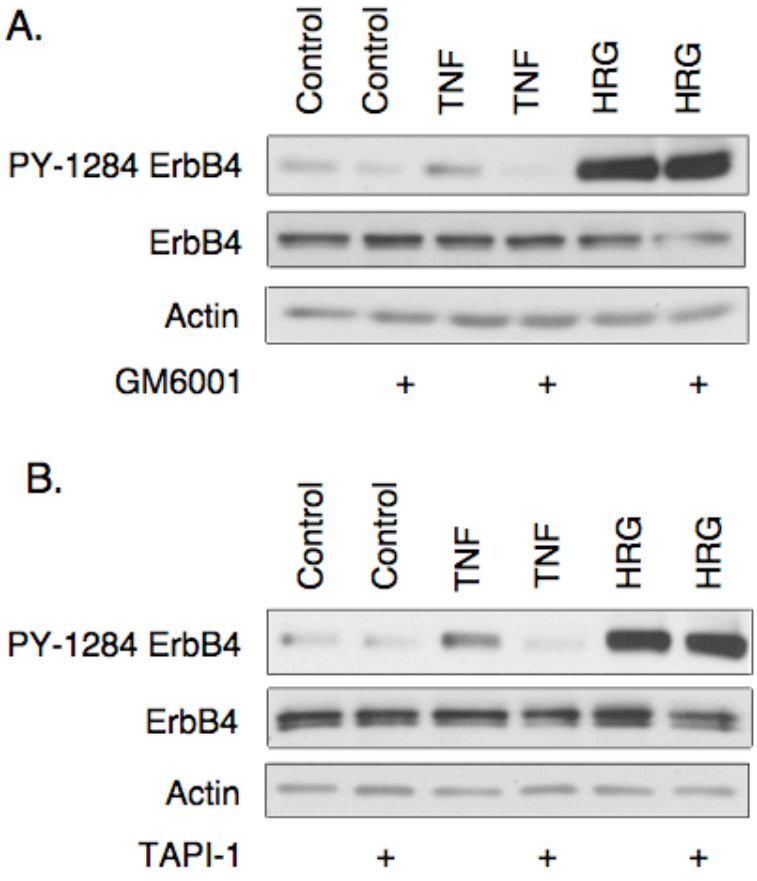

Figure 3.7 Metalloproteases mediate TNF transactivation of ErbB4 in colonic epithelial cells YAMC-ErbB4 cells were incubated for 30 min with A) 50 mM of the broad spectrum metalloproteinase inhibitor GM6001 or B) 10 mM of the TACE selective inhibitor TAPI-1, then stimulated with TNF (100 ng/mL, 30 min) or HRG (100 ng/mL, 10 min). Cell lysates were then analyzed by Western blot with antibodies specific for phosphorylated ErbB4 (PY-1284), total ErbB4, or actin.

lysed and analyzed for phosphorylation at a known TACE activation site (Diaz-Rodriguez et al., 2002). In response to TNF, TACE was phosphorylated at T735, with activation peaking between 2 and 5 minutes. PMA, a known TACE stimulus (Hahn et al., 2003), also induced TACE phosphorylation (Figure 3.8). To further verify that TACE is in fact the metalloproteinase responsible for TNF transactivation of ErbB4, we stably expressed ErbB4 in TACE $^{-/-}$ mouse colon epithelial (MCE) cells transfected with either wild type TACE or vector. ErbB4 was not phosphorylated in response to TNF in TACE $^{-/-}$ MCE cells expressing vector; however, re-expression of wild type TACE restored TNF-induced ErbB4 activation (Figure 3.9). We then took advantage of the observation that TNF cannot promote ErbB4 phosphorylation in the TACE $^{-/-}$ MCE line, by transferring conditioned media from YAMC-ErbB4 cells treated with TNF to the TACE null line and assessing ErbB4 phosphorylation by Western blot. In TACE $^{-/-}$ MCE cells, ErbB4 was phosphorylated by conditioned media transferred from YAMC cells treated with 100 ng/mL TNF for 15 min (Figure 3.10), further supporting the hypothesis that ErbB4 transactivation by TNF is mediated by TACE cleavage of an ErbB4 ligand.

ErbB4 Activation by TNF requires MEK kinase activity

There are multiple routes by which TNF could stimulate TACE. One common mechanism of TNF-induced TACE activation is through intracellular kinases. Specifically, ERK1/2 stimulates TACE phosphorylation at threonine 735

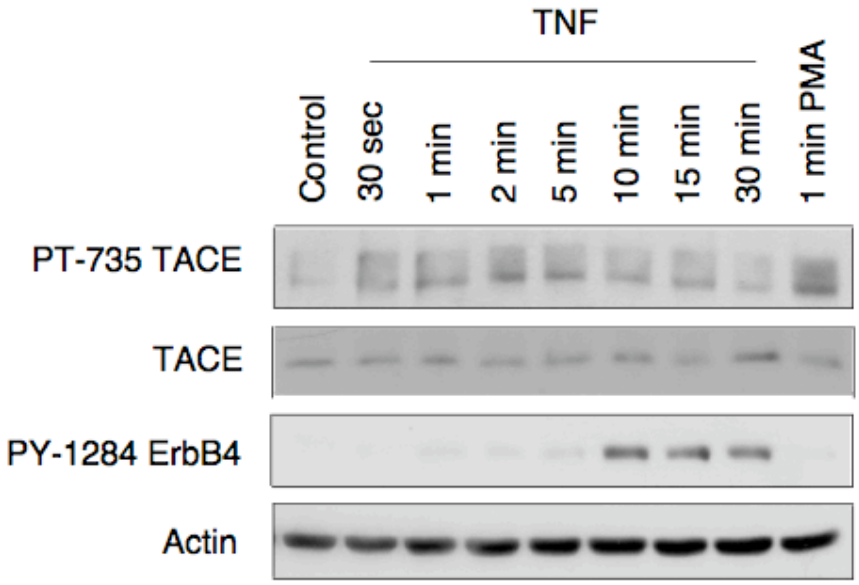

Figure 3.8 TACE is activated in YAMC cells treated with TNF YAMC-ErbB4 cells were treated with 100 ng/mL TNF for various time points or with 20 ng/mL PMA for 1 min. Cell lysates were analyzed by Western blot with antibodies specific for phosphorylated TACE (PT-735), ErB4 (PY-1284), ErbB4, or actin.

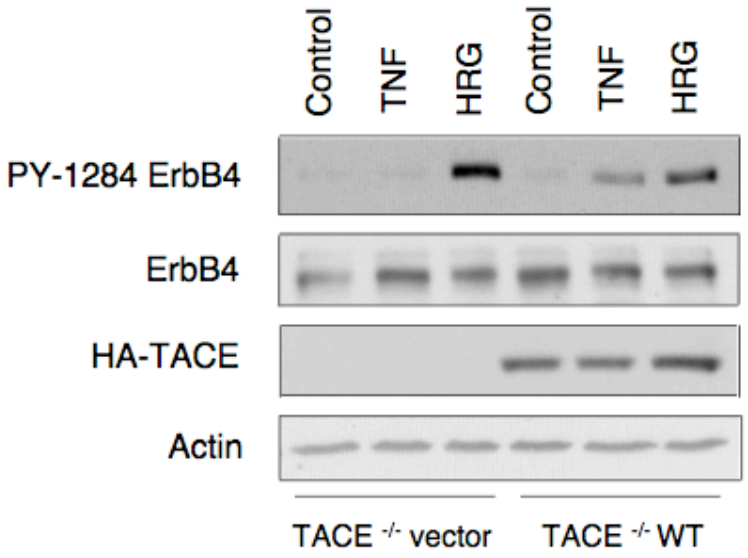

Figure 3.9 TACE mediates TNF transactivation of ErbB4 in colonic epithelial cells TACE $^{-/-}$ colonic epithelial cells infected with ErbB4 and with either wild type TACE or vector added back were treated with 100 ng/mL TNF for 30 min or 10 ng/mL HRG for 10 min. Cell lysates were then analyzed by Western blot with antibodies specific for phosphorylated ErbB4 (PY-1284), total ErbB4, TACE, or actin.

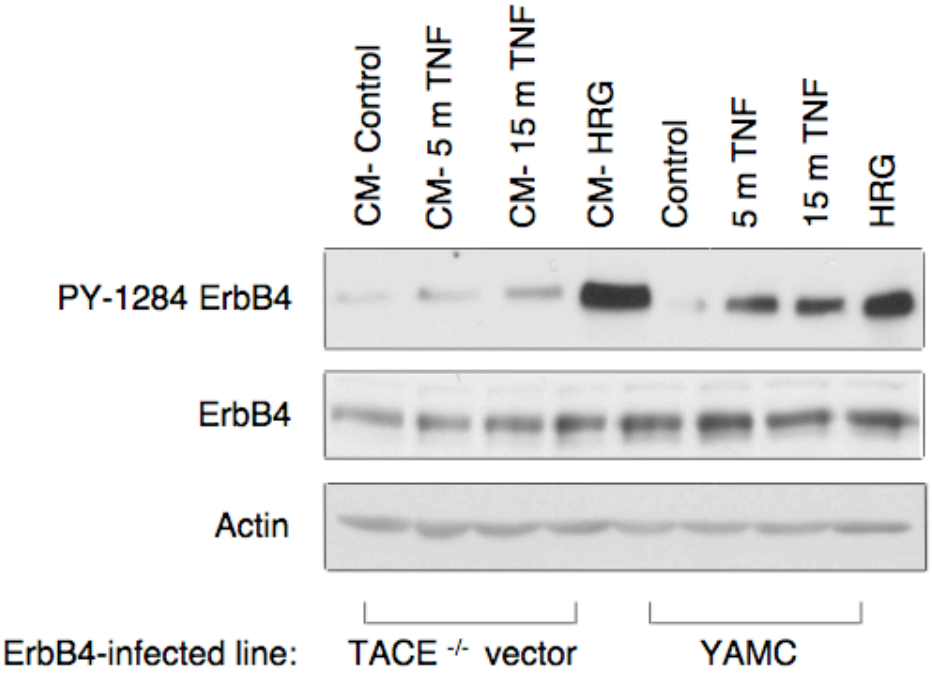

Figure 3.10 An extracellular factor mediates TNF transactivation of ErbB4 in colonic epithelial cells YAMC-ErbB4 cells were treated directly with 100 ng/mL TNF for 5 or 15 min or 10 ng/mL HRG for 10 min. TACE $^{-/-}$ colon epithelial cells expressing ErbB4 were treated with conditioned media from the corresponding YAMC cells for 10 min (indicated CM). Cell lysates were then analyzed by Western blot with antibodies specific for phosphorylated ErbB4 (PY-1284), total ErbB4, or actin.

in response to TNF treatment of HeLA cells (Diaz-Rodriguez et al., 2002), p38 mediates TACE activation in lymphocytes and monocytes treated with TNF (Killock and Ivetic, 2010), and Src is required for gastrin-releasing peptide-induced TACE activity in squamous cell carcinoma of the head and neck cell lines (Zhang et al., 2006a). Since we demonstrated TNF-induced TACE phosphorylation at the ERK site T735 (Figure 3.8), we tested the possibility that the MEK/ERK cascade is required for TNF transactivation of ErbB4. YAMC-ErbB4 cells were treated with the MEK inhibitor U0126 for 30 min before addition of TNF or HRG. MEK inhibition completely blocked ErbB4 phosphorylation in response to TNF, as assessed by Western blot analysis (Figure 3.11A). To further test the hypothesis that ERK-induced TACE activation promotes ErbB4 transactivation by TNF, we pretreated YAMC-ErbB4 cells with UO126 before TNF stimulation and assessed TACE activation. Blocking MEK activity reversed the ability of TNF to phosphorylate TACE on T-735, supporting the role of ERK being upstream of TACE in ErbB4 transactivation (Figure 3.11B). In contrast to MEK inhibition, Src inhibition by CGP77675 (Figure 3.12A) or p38 inhibition by SB202190 (Figure 3.12B) did not affect transactivation.

EGFR is required for TNF transactivation of ErbB4

Following ligand binding, ErbB4 can either homodimerize with another ErbB4 receptor or heterodimerize with another member of the ErbB family. To

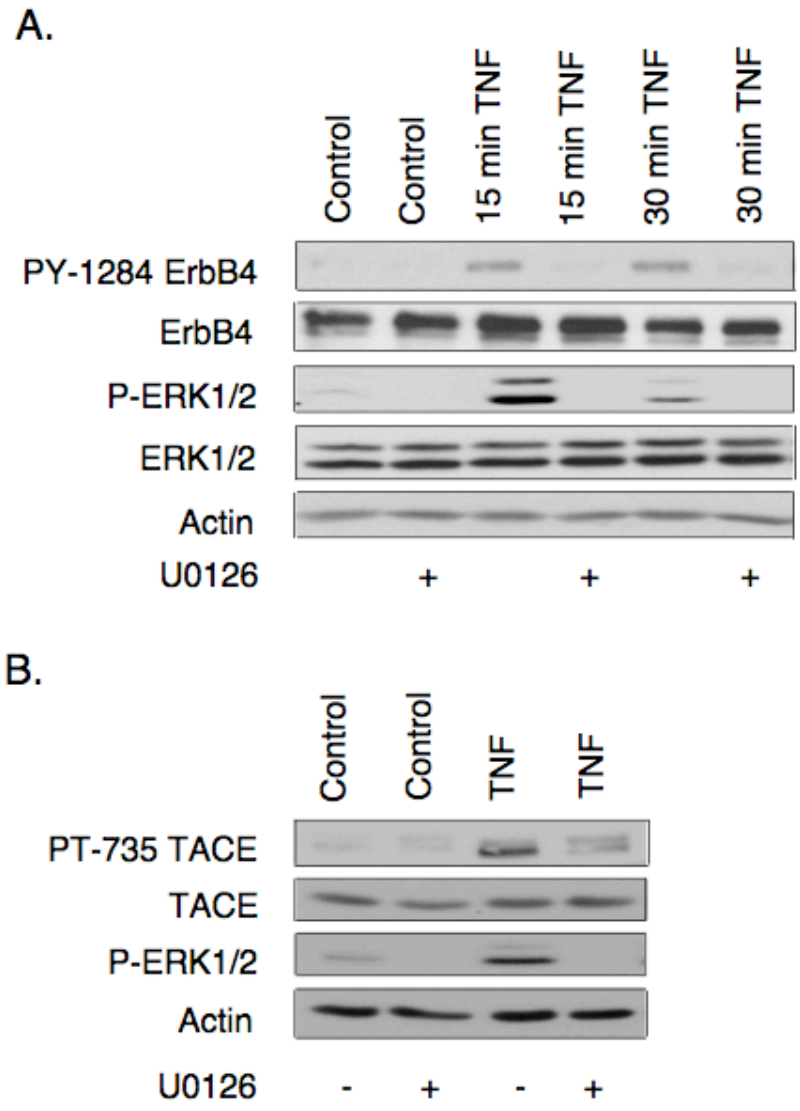

Figure 3.11 ErbB4 transactivation by TNF requires MEK A) YAMC-ErbB4 cells were incubated with 10 mM U0126 (MEK inhibitor) for 1 h before the addition of TNF (100 ng/mL) for either 15 or 30 min. B) YAMC-ErbB cells were pretreated with UO126 before 5 min TNF treatment. Phosphorylation of ErbB4 (PY-1284), TACE (PT-735), ERK1/2, or total ErbB4, TACE, ERK1/2, or actin was determined by Western blot analysis.

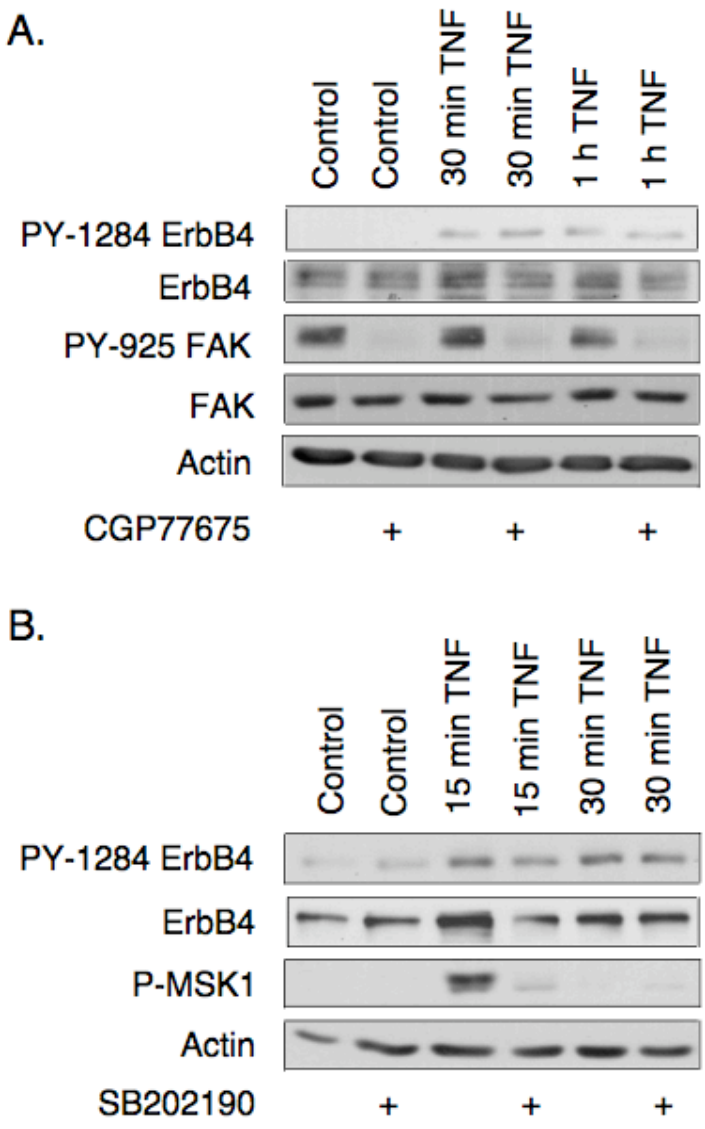

Figure 3.12 ErbB4 transactivation by TNF does not require p38 or Src kinases A) YAMC-ErbB4 cells were incubated with 2 mM of the Src inhibitor CGP77675 for 1 h before the addition of TNF for the indicated time points B) YAMC-ErbB4 cells were incubated with 10 mM SB20190 (p38 inhibitor) for 1 h before TNF treatment for 15 or 30 min. Phosphorylation of ErbB4 (PY-1284), MSK1 (positive control for p38 inhibition), or FAK (PY-925, positive control for Src inhibition) or total ErbB4, FAK, or actin was determined by Western blot analysis.

determine if EGFR activity was required for TNF transactivation of ErbB4, YAMC-ErbB4 cells were incubated with the EGFR tyrosine kinase inhibitor AG1478, and then treated with TNF, HRG, or EGF. Inhibiting EGFR kinase activity with AG1478 completely blocked TNF's ability to transactivate ErbB4 (Figure 3.13A). AG1478 has been shown to also inhibit ErbB4 kinase activity in some studies, so another possible interpretation of these data is that ErbB4 kinase activity, and not EGFR kinase activity, is required for TNF-induced ErbB4 activation. Likewise, transfection with EGFR-specific siRNA abrogated ErbB4 activation by TNF (Figure 3.13B). A limitation of these results is that the decrease in ErbB4 activation could be attributed to off-target affects from the EGFRs siRNA. To control for this, we used scrambled non-targeting siRNA; however, the non-targeting siRNA was not scrambled specifically from the EGFR siRNA sequences, so it is still possible that the results were influenced by non-specific affects from the siRNA. As a further control against off-target affects from the EGFR siRNA, we blotted for ErbB4 expression. Figure 3.13B shows that while the EGFR siRNA significantly knocks down EGFR expression, there is no change in ErbB4 levels. Both AG1478 and EGFR siRNA also attenuated HRG-induced activation, which is an observation we have recently published (Frey et al., 2010). These data suggest that heterodimerization with EGFR is necessary for optimal ErbB4 activation by TNF in colonic epithelial cells.

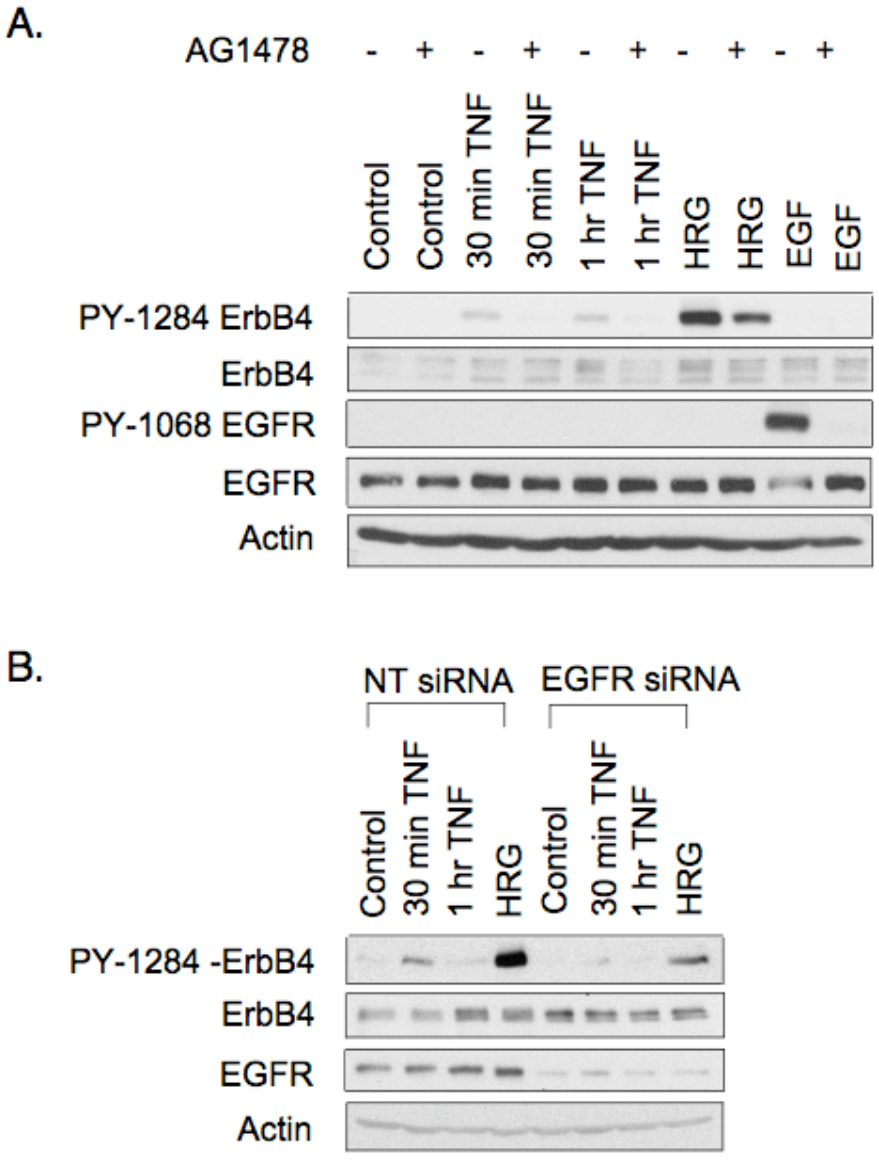

Figure 3.13 EGFR is required for ErbB4 transactivation by TNF A) YAMC-ErbB4 cells were treated with the EGFR tyrosine kinase inhibitor AG1478 (150 nM) for 30 min before treatment with 100 ng/mL TNF, 10 min 100 ng/mL HRG, or 10 min 10 ng/mL EGF. B) YAMC-ErbB4 cells were transfected with either EGFR or non-targeting siRNA and treated with TNF or HRG. Cell lysates were then analyzed by Western blot for phospho-ErbB4 (1284), phospho-EGFR (1068), or total ErbB4, EGFR, or actin.

ErbB4 Transactivation by TNF Promotes Colonic Epithelial Cell Survival

We have previously shown that ErbB4 overexpression protects colon epithelial cells from inflammatory cytokine-induced apoptosis (Frey et al., 2009). To test the hypothesis that TACE-mediated transactivation of the receptor is necessary for this survival response, we compared apoptosis levels in YAMC-ErbB4 cells and YAMC-vector cells following 3 hour treatment with 100 ng/mL TNF and 1 μg/mL cycloheximide (to stimulate apoptosis) with or without 30 min pretreatment with 10 μM TAPI-1 (to inhibit transactivation). After TNF/cycloheximide exposure, YAMC cells overexpressing ErbB4 exhibited lower levels of the apoptotic markers cleaved PARP and active caspase-3 than cells expressing vector alone. TAPI-1 treatment blocked the ability of ErbB4 to protect against apoptosis, indicating a requirement for TACE activity (Figure 3.14A). To corroborate these results, YAMC cells expressing either ErbB4 or vector were subjected to the same apoptosis assay after incubation with ErbB4 blocking antibody. Similar to TACE inhibition, the blocking antibody reversed the ability of ErbB4 to protect against cytokine-induced apoptosis (Figure 3.14B). Finally, TUNEL staining was used as an independent method to verify apoptosis. After 5 hours of TNF/cycloheximide exposure, substantially fewer YAMC-ErbB4 cells were TUNEL positive compared with vector controls. However, blocking TACE activity (by 30 min pretreatment with TAPI-1) (Figure 3.15) or ligand binding (by 1 hr pretreatment with ErbB4 blocking antibody) (Figure 3.16) abrogated the ability of ErbB4 to protect from apoptosis. Next, we used treated YAMC cells expressing

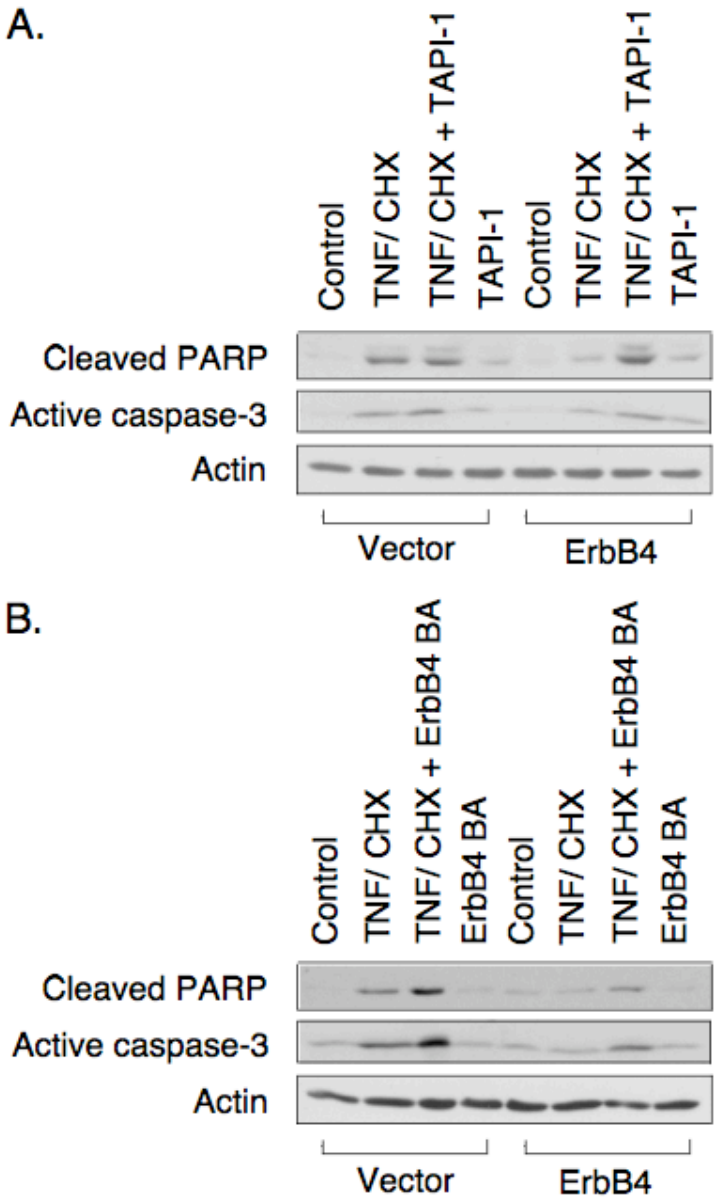

Figure 3.14 ErbB4 transactivation by TNF protects colonic epithelial cells from cytokine-induced apoptosis. YAMC cells expressing either vector (YAMC-vector) or ErbB4 (YAMC-ErbB4) were incubated with A, 10 mM TAPI-1 for 30 min or B, ErbB4 blocking antibody for 1 h before treatment with 1 mg/mL cycloheximide and 100 ng/mL TNF for 3 h. Caspase-3 activation and PARP cleavage were assessed by Western blot analysis of whole cell lysates. Actin was used as a loading control.

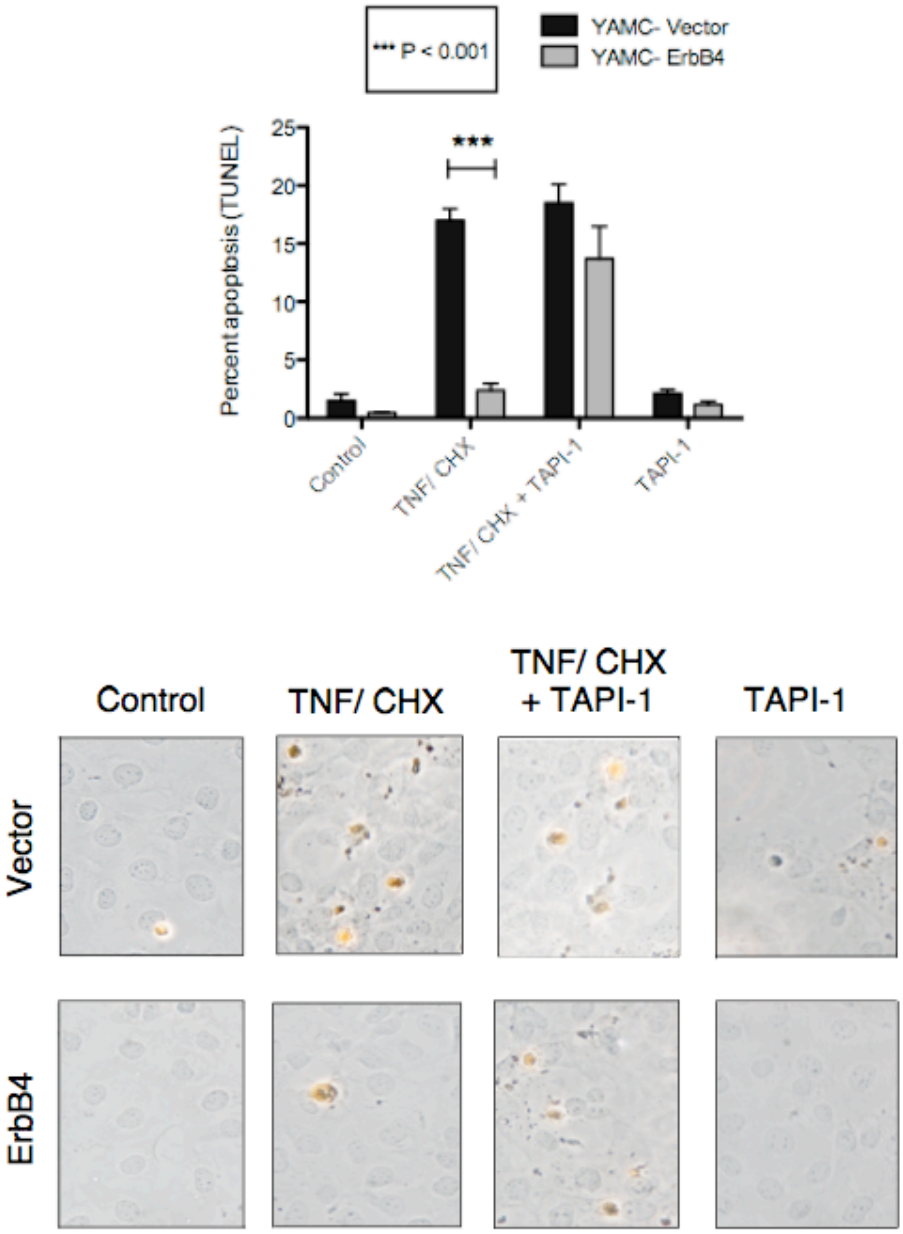

Figure 3.15 TACE is required for the cell survival advantage mediated by ErbB4 during exposure to TNF Vector and ErbB4-expressing YAMC cells were incubated with 10 mM TAPI-1 for 30 min before treatment with 1 mg/mL cycloheximide and 100 ng/mL TNF for 5 h. Subsequently, cells were subjected to TUNEL apoptosis assay. Labeled nuclei from at least 5 random fields per condition were counted in each experiment.

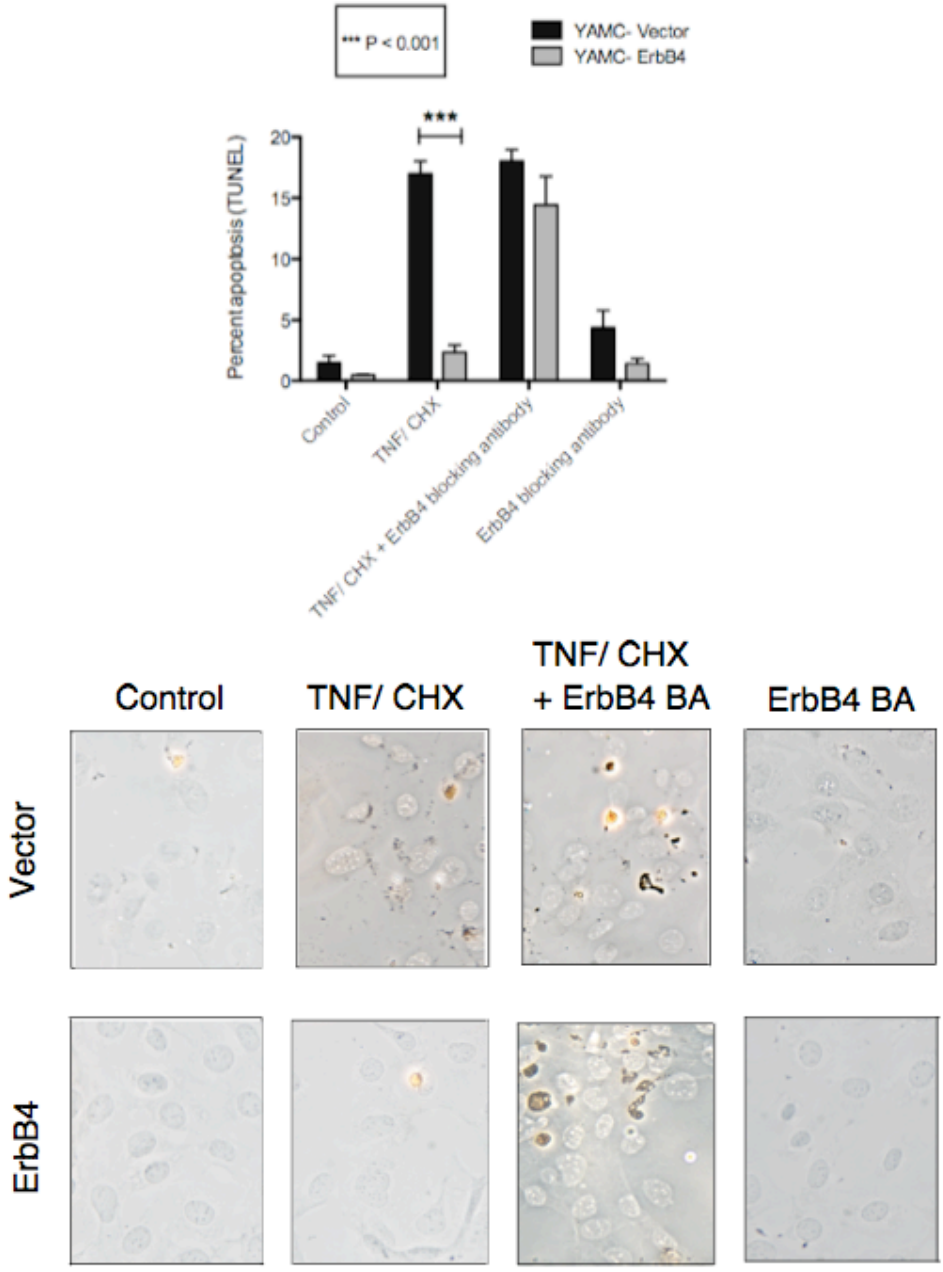

Figure 3.16 ErbB4 transactivation by TNF protects colonic epithelial cells from cytokine-induced apoptosis. Vector and ErbB4-expressing YAMC cells were incubated with an ErbB4 blocking antibody for 1 h before treatment with 1 mg/mL cycloheximide and 100 ng/mL TNF for 5 h. Subsequently, cells were subjected to TUNEL apoptosis assay. Labeled nuclei from at least 5 random fields per condition were counted in each experiment.

vector or ErbB4 with a cytokine cocktail (100 ng/mL TNF, 280 ng/mL IL-1b, 180 units/mL interferon-g) for 3 hours in the presence or absence of TAPI-1, then measured apoptosis by Western blot. When treated with physiological levels of pro-inflammatory cytokines, ErbB4 protects YAMC cells from apoptosis. However, inhibiting TACE with TAPI-1 reversed ErbB4's ability to promote survival in the presence of cytokines (Figure 3.17). Thus, ErbB4 transactivation via TACE protects colonic epithelial cells from cytokine-induced apoptosis.

Members of the ErbB family can promote survival through activation of the PI3K/Akt signaling pathway (Danielsen and Maihle, 2002; Frey et al., 2009). To determine if this is a result of TACE-mediated transactivation, YAMC-vector and YAMC-ErbB4 cells were incubated with TAPI-1 for 30 min before treatment with TNF for 15 minutes. Western blot analysis with an antibody specific for Akt phosphorylation (S473) showed that ErbB4 expression stimulates increased levels of TNF-induced Akt activation, and that inhibiting transactivation with TAPI-1 abrogates this response (Figure 3.18A). Pretreatment of YAMC-ErbB4 cells with an ErbB4 blocking antibody also inhibits ErbB4's ability to promote Akt activation following TNF stimulation (Figure 3.18B). In conjunction with our previous results showing that PI3K blockade reverses ErbB4-induced cell survival (Frey et al., 2009), these data indicate Akt signaling is involved in the pro-survival pathway elicited by TNF-induced ErbB4 transactivation in colon epithelial cells.

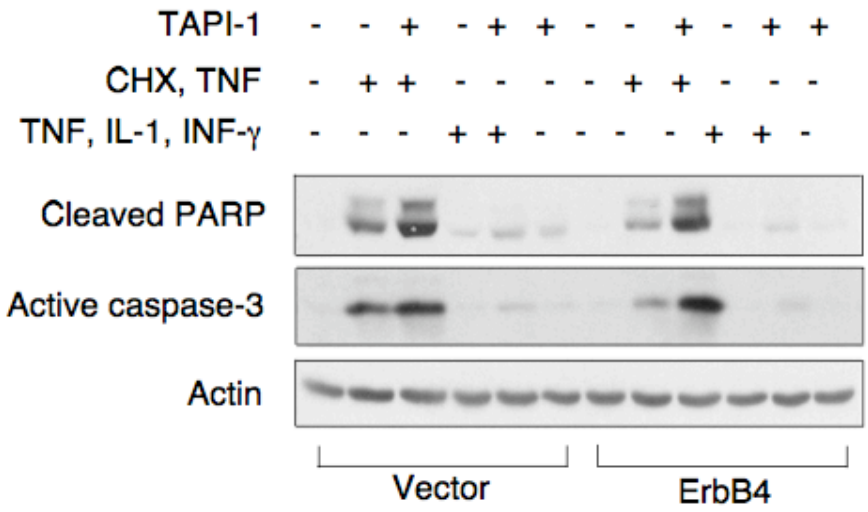

Figure 3.17 TACE is required for the cell survival advantage mediated by ErbB4 YAMC cells expressing vector or ErbB4 were incubated with 10 mM TAPI-1 for 30 min before treatment with 1 mg/mL cycloheximide and 100 ng/mL TNF or with a cytokine cocktail (100 ng/mL TNF, 280 ng/mL IL-1b, 180 units/mL interferon-g) for 3 hours. Densitometry from Western blots of cleaved PARP and active caspase 3 from three experiments was quantified

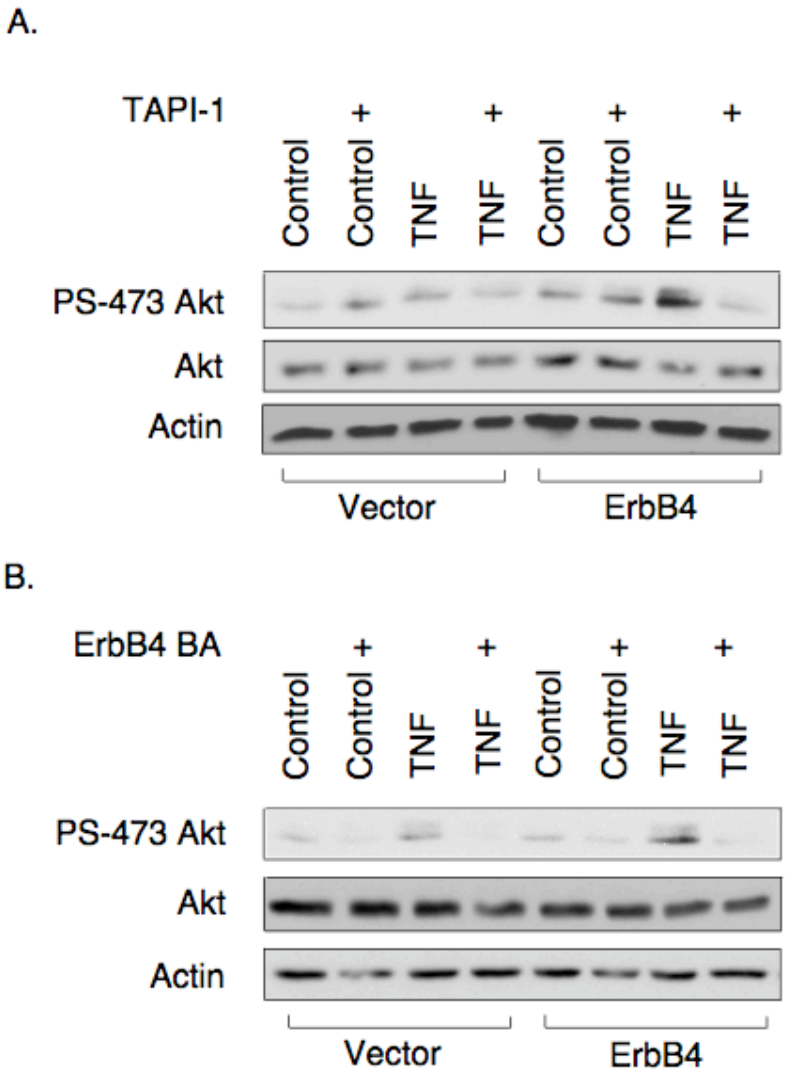

Figure 3.18 ErbB4 transactivation by TNF promotes AKT activation A) YAMC-vector and YAMC-ErbB4 cells were incubated with 10 mM TAPI-1 for 30 min or B) with ErbB4 blocking antibody for 1 hour before treatment with 100 ng/mL TNF for 15 min. Cell lysates were then analyzed by Western blot with antibodies against phosphorylated Akt (PS-473), total Akt, ErbB4, or actin.

The role of ErbB4 in maintaining barrier function in the colonic epithelium

Increased apoptosis due to TNF in the gastrointestinal epithelial monolayer can cause breeches in the normally tight barrier (Schulzke et al., 2006). This causes dual problems in patients with IBD: a loss of ions and water into the lumen causes diarrhea, while bacterial antigens crossing the epithelium perpetuate the inflammatory response. Since ErbB4 overexpression protects from cytokine-induced apoptosis, we hypothesized that ErbB4 could also protect from TNF-induced barrier disruption. To test this, YAMC cells were grown to confluence in transwells, and transepithelial electrical resistance (TEER) was measured before and after 24 h TNF treatment. Figure 3.19 shows that in this assay TNF stimulation increases TEER, instead of decreasing it as expected. This likely indicates that in this particular system, the YAMC cells are not forming tight junctions, and thus are not generating a barrier. As such, we could not determine whether ErbB4 protects from TNF-induced barrier disruption in this system. To further test whether ErbB4 plays a role in maintaining the integrity of the colonic epithelial monolayer, cells that form tight junctions (such as Caco-2 cells) could be infected with ErbB4 and TEER examined.

ErbB4 overexpression does not promote proliferation or migration in colon epithelial cells

Following inflammation-induced apoptosis, restitution of the colonic epithelium can occur through increases in proliferation and/or migration of

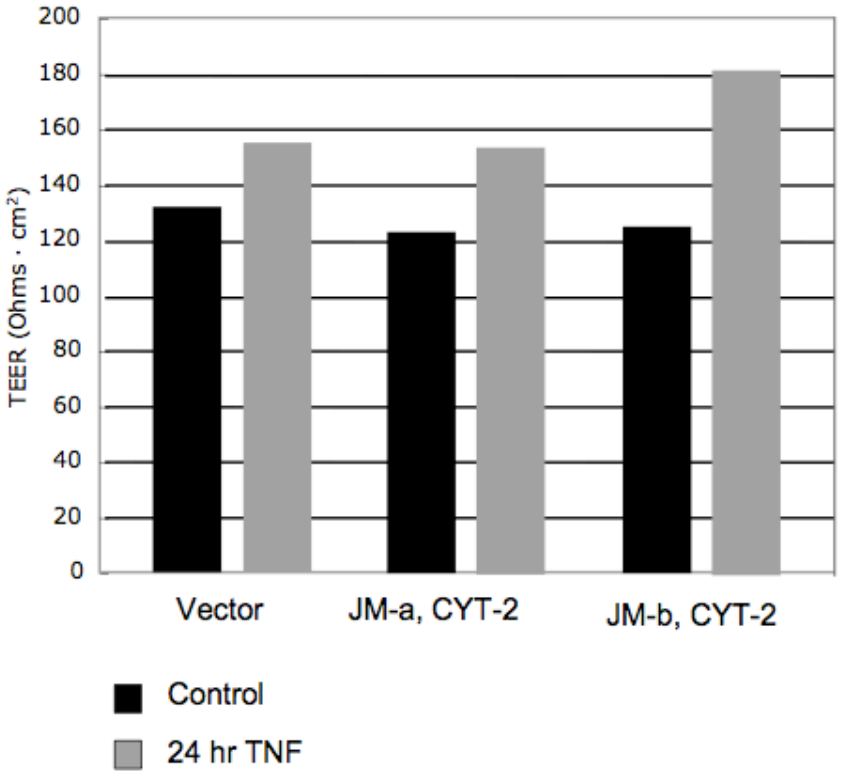

Figure 3.19 The role of ErbB4 isoforms in barrier function: YAMC cells expressing vector, the JM-a, CYT-2 isoform, or the JM-b, CYT-2 isoform were allowed to grow to confluence, and then TEER readings were taken both before and after 24 h 100 ng/mL TNF treatments

intestinal epithelial cells. We have previously shown that low doses of TNF stimulate YAMC cell proliferation and migration (Kaiser and Polk, 1997). Additionally, ErbB4 has been shown to promote proliferation in cardiomyocytes (Bersell et al., 2009), neuroblasts (Ghashghaei et al., 2006), and cervical cells (Eto et al., 2010). ErbB4 has also been shown to promote migration of neural progenitor cells (Gambarotta et al., 2004).

To determine if ErbB4 signaling is involved in TNF-induced proliferation, YAMC cells expressing vector, JM-a, CYT-2 isoform, or JM-b, C YT-2 isoform were treated with TNF, HRG, or EGF for 24 h and subjected to the MTS proliferation assay (Figure 3.20). As expected, EGF promoted proliferation in all cell lines, as did TNF (to a lesser degree). However, there was no real difference in the levels of TNF-induced proliferation in the lines expressing the ErbB4 isoforms. Similarly, the ErbB4 ligand HRG also did not promote increased proliferation, supporting the finding that ErbB4 signaling is not involved in TNF-induced increases in proliferation in YAMC cells.

To determine whether TNF-induced migration occurs through ErbB4 signaling, YAMC cells expressing vector, JM-a, CYT-2, or JM-b, CYT-2 isoforms were grown to confluence and wounded with a circular rotating silicon tip. Following wounding, cells were incubated for 24 h with TNF, HRG, or EGF. Figure 3.21 shows that while EGF promotes wound healing in all three cell lines, TNF only marginally increased migration. There did not appear to be a difference in migration between either ErbB4 isoform or vector. Additionally, HRG treatment

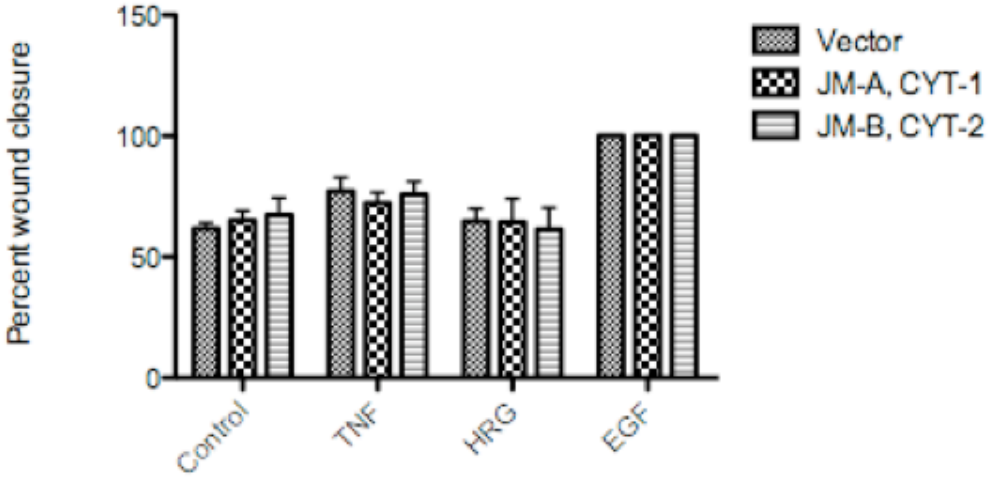

Figure 3.20 ErbB4 overexpression does not affect wound healing Circular wounds were created in monolayers of YAMC cells expressing vector, JM-a, CYT-1, or JM-b, CYT-2 isoforms of ErbB4. Cells were treated with 100 ng/mL TNF, 100 ng/mL HRG, or 10 ng/mL EGF for 24 h, then percent wound closure was measured.

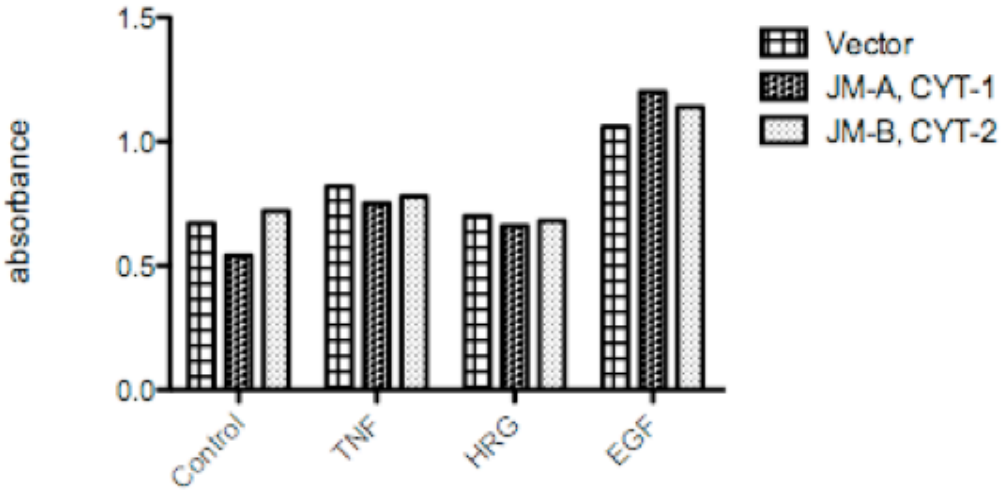

Figure 3.21 ErbB4 overexpression does not affect proliferation YAMC cells expressing vector, JM-a, CYT-1, or JM-b, CYT-2 isoforms of ErbB4 were treated with 100 ng/mL TNF, 100 ng/mL HRG, or 10 ng/mL EGF for 24 h. Proliferation was measured by MTS assay, with the absorbance readings from 0h subtracted from absorbance at 24h.

did not stimulate wound healing. It is possible that any ErbB4-induced migratory responses were hidden by the weak TNF response, since previous data from the lab showed a more robust TNF-induced migration.

Discussion

This study describes a novel signaling cascade that promotes epithelial cell survival following TNF exposure. We determined the pathway through which TNF induces ErbB4 activation, providing the first mechanism of ErbB4 transactivation by an inflammatory cytokine. The data show that TNF stimulates ErbB4 phosphorylation through TACE-dependent HB-EGF cleavage. Additionally, MEK, but not p38 or Src, kinase activity was required for TNF-induced ErbB4 activation. Furthermore, TACE-dependent ErbB4 transactivation is necessary for resistance from cytokine-induced apoptosis through increased Akt signaling. These results suggest that in the inflamed colon, TNF-induced ErbB4 activation may protect the epithelial monolayer from the high levels of epithelial apoptosis and ulcerations that are a hallmark of IBD (Hagiwara et al., 2002; Iwamoto et al., 1996).

Interestingly, although EGFR and ErbB4 are highly related members of the same receptor tyrosine kinase family, in colon epithelial cells ErbB4 transactivation by TNF occurs through a different mechanism than EGFR transactivation by TNF. TNF activates EGFR through an intracellular, Src-

dependent mechanism (Yamaoka et al., 2008), whereas TNF activates ErbB4 through extracellular, TACE-mediated ligand release. Given the wealth of data implicating EGFR in the pathogenesis of colon cancer (Ponz-Sarvise et al., 2007) and the emerging role of ErbB4 in protecting colon cells from apoptosis, delineating the differences in activation and signaling pathways between these two receptors is important for potential therapies. Understanding the mechanisms by which inflammatory cytokines cross-talk with ErbB family members may also provide more targets for treatment of colitis or for interfering with the pro-survival signaling that lead to carcinogenesis.

In IBD, high levels of inflammatory cytokines, such as TNF and interferon-γ, contribute to colon epithelial cell apoptosis, resulting in loss of mucosal integrity and barrier dysfunction (Edelblum et al., 2006). ErbB4 is likely important for protecting colon epithelial cells and limiting tissue damage during active inflammation. However, there is also a growing literature that suggests ErbB4 may be involved in colorectal cancer signaling. Inactivating somatic mutation of the ErbB4 kinase domain occurs in colorectal cancers (Soung et al., 2006), increased ErbB4 coexpression with ErbB2 has been found in late stage carcinomas (Lee et al., 2002), and high ErbB4 expression is seen in colorectal tumors (Leung et al., 2008). We have recently demonstrated that ErbB4-overexpressing colon epithelial cells acquire the ability to form colonies in soft agar in a COX-2 dependent manner (Frey et al., 2010). Furthermore, ErbB4 deletion in human colon cancer lines increases apoptosis (Lee et al., 2009).

It is interesting that we observed ErbB4 transactivation leading to increased Akt phosphorylation; aberrant PI3K/AKT activity is implicated in apoptosis resistance in a variety of cancers (Hennessy et al., 2005). An endocrine resistant human breast carcinoma cell line that displays both ErbB4 upregulation and increased levels of ErbB ligands (including HB-EGF, the ligand which activates ErbB4 in the current study) shows constitutive activation of the PI3K/Akt pathway (Ghayad et al., 2009). HB-EGF is expressed in a variety of cancers, including gastric and colon cancer (Wu et al., 2009). In breast cancer cells, inhibiting HB-EGF with a mutant form of the diptheria toxin, CRM197, attenuated Akt phosphorylation and led to significant apoptotic cell death (Yotsumoto et al. 2010). Our demonstration that HB-EGF-mediated ErbB4 transactivation by TNF leads to increased Akt activation may suggest a mechanism for chronic inflammation-induced carcinogenesis. While ErbB4 transactivation by TNF may provide the short-term benefit of protecting the colon epithelium from apoptosis-induced ulceration during inflammatory conditions, the long-term consequences could potentially lead to colitis-associated carcinogenesis.

Although TACE levels and activity are upregulated in patients with IBD and TACE-mediated TNF cleavage could potentially lead to the increased levels of soluble TNF seen in inflammatory diseases (Brynskov et al., 2002), it has not yet been resolved whether inhibiting TACE would be a viable therapy for IBD. For instance, in mice with TNBS-induced colitis, TACE activity was elevated,

resulting in concomitant increases in soluble TNF. Treatment with a pharmacological TACE inhibitor ameliorated TNBS-induced colonic damage and inflammation (Colon et al., 2001). On the other hand, when TACE expression and activity is reduced to <5% normal levels via genetic strategies, mice are far more susceptible to DSS-induced colitis (Chalaris et al., 2010; Terzic et al., 2010), supporting a role for TACE in protecting the GI tract from acute injury-induced inflammation. Likewise, when HT29 colon cells are grown to confluence, inhibiting TACE pharmacologically (with TAPI-2) or physiologically (with recombinant TIMP3) sensitizes the intestinal epithelial monolayer to TNF-induced barrier disruption, as measured by FITC-dextran flux (Freour et al., 2009). Our studies underscore the potentially complex outcomes of TACE blockade; while the metalloproteinase may enhance TNF release it is also required for the survival advantage conferred by TNF-induced ErbB4 activation. When TACE is inhibited, TNF is no longer able to activate ErbB4 in colonic epithelial cells and instead, the pro-apoptotic effects of TNF are increased.

While it is generally accepted that TACE plays a pivotal role in a range of inflammatory diseases such as IBD, rheumatoid arthritis (Newton et al., 2001), and heart disease (Wang et al., 2009), it is not fully understood how TACE activity is induced and regulated. Given the variety of stimuli that activate TACE and the wide range of substrates that TACE cleaves, there must be tight control over enzymatic activity. There have been multiple reports that phosphorylation at threonine 735 in the cytoplasmic tail of TACE by either p38 or ERK is important

for trafficking and regulation of the metalloproteinase (Diaz-Rodriguez et al., 2002; Soond et al., 2005; Xu and Derynck, 2010). In our study, we show that TACE is phosphorylated at T735 following TNF stimulation, and that inhibiting MEK attenuates both TNF-induced TACE phosphorylation as well as ErbB4 transactivation by TNF. These data indicate that TACE is activated by ERK at T735 following TNF stimulation in colonic epithelial cells, and that this activation is essential for ErbB4 transactivation by TNF. It is important to note that p38 activity is not required for transactivation, since in some cell lines p38 is responsible for TACE activation (Xu and Derynck, 2010).

In summary, our data demonstrate that ErbB4 transactivation by the proinflammatory cytokine TNF protects colon epithelial cells from apoptosis through a TACE–dependent mechanism leading to downstream Akt signaling. This study links two known mediators of inflammatory signaling, TNF and TACE, to activation of the ErbB4 growth factor receptor. We have determined the mechanism by which TNF transactivates ErbB4. First, TNF stimulates ERK phosphorylation. Activated ERK then phosphorylates TACE at Threonine 735. Once TACE is activated, it cleaves and releases membrane-bound HB-EGF. Soluble HB-EGF can then bind ErbB4, inducing its phosphorylation, which leads to Akt activation and subsequent downstream survival signaling (Figure 1.6). Since TNF upregulates and activates ErbB4, and ErbB4 has recently been implicated in colorectal carcinogenesis, delineating the steps of this mechanism

could provide additional therapeutic targets for treatment of IBD and prevention of colitis-associated carcinogenesis.

Chapter IV

SUMMARY AND FUTURE DIRECTIONS

Summary of Findings

The epithelium of the mammalian gastrointestinal lining must maintain a functional barrier to protect the organism from the contents of the lumen. Following injury to the gastrointestinal monolayer, a rapid epithelial migratory response coupled with tightly-regulated proliferation preserves the integrity of the barrier. If, however, bacteria manage to translocate across the epithelium, then the intestinal immune system responds by releasing antimicrobial compounds, initiating phagocytosis by macrophages and dendritic cells, and by producing inflammatory cytokines (reviewed in (Winter et al. 2010). One of the challenges of the intestinal immune system is to avoid an excessive immune response against the normal lumenal microbiota, while maintaining the ability to trigger an appropriate inflammatory response against pathogenic bacteria. In particular, excessive inflammation through cytokine production can lead to increased epithelial cell apoptosis, and which leads to breaks in the monolayer and barrier disruption.

We have shown that TACE-dependent ErbB4 transactivation by TNF is important for cell survival in the presence of inflammatory cytokines in conditionally immortalized colon epithelial cells. Additionally, we have defined the

mechanism by which TNF transactivates ErbB4 *in vitro*; TNF signaling activates the MAP kinase ERK, which stimulates TACE proteolytic activity. Membrane anchored HB-EGF is then released, and is able to bind ErbB4. Following ligand binding, ErbB4 phosphorylation stimulates downstream Akt activation. Through Akt-mediated signaling pathways, TNF transactivation of ErbB4 then protects the colon epithelial cells from apoptosis (Figure 4.1). This is the first example of transactivation of the ErbB4 receptor tyrosine kinase, and shows an important mechanism for how the colonic epithelium maintains an intact barrier following cytokine disruption.

While cytokine signaling is an important defense against pathogenic bacteria that manage to cross the epithelial barrier through breaks in the monolayer or by invading epithelial cells, excessive TNF production can cause damaging levels of apoptosis. This can be particularly dangerous when there are already breaks in the epithelial monolayer, as increased apoptosis can result in the ulcerations of the digestive tract that are a main symptom of IBD. By protecting the colonic epithelium from TNF-induced apoptosis, ErbB4 transactivation provides a check on inflammation and promotes homeostasis of the gastrointestinal lining. As further support for this hypothesis, we have shown that ErbB4 is upregulated and activated in the colonic epithelium of patient's with Crohn's disease (Frey et al., 2009). It is possible that short-term treatment with an ErbB4 ligand could prove therapeutic to IBD patients with uncontrolled

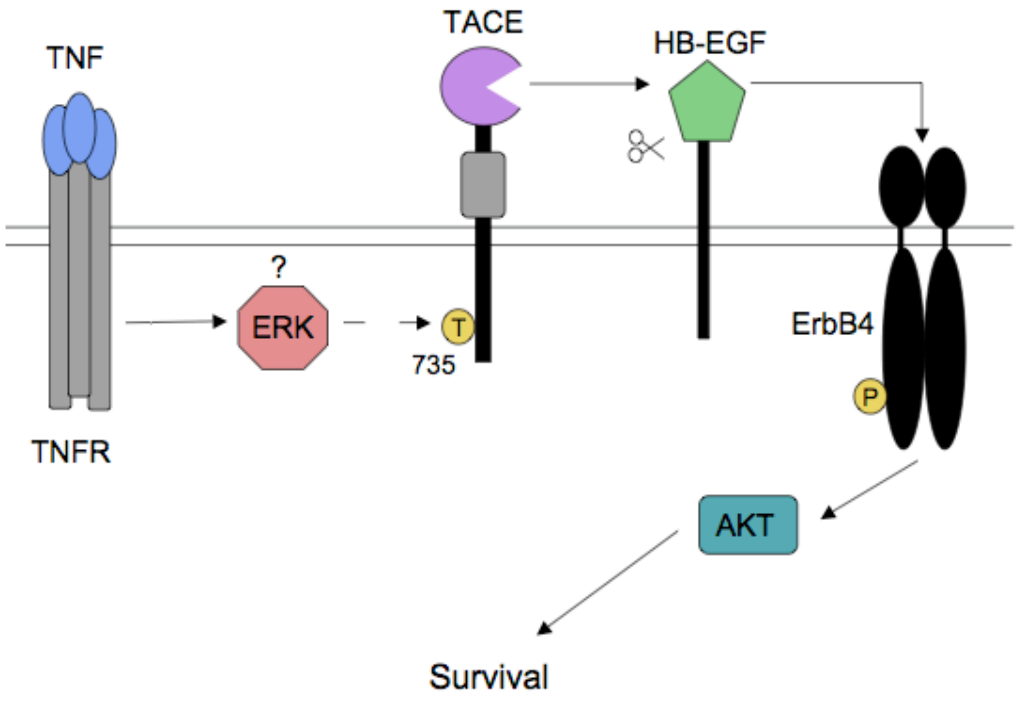

Figure 4.1 Model of ErbB4 transactivation by TNF in colonic epithelial cells TNF stimulation of colonic epithelial cells leads to activation of ERK1/2, which stimulates TACE activity. TACE cleaves membrane-bound HB-EGF, which then binds to ErbB4 and stimulates phosphorylation and activation. ErbB4 transactivation by TNF then mediates colonic epithelial cell survival through increased Akt signaling

inflammation-induced ulcerations. However, long-term activation of ErbB4 might be a contributor to colitis-associated carcinogenesis, since the growth factor receptor can stimulate survival signaling through pathways known to play a role in cancer. Further studies into the delicate balance between ErbB4-mediated protection against inflammation and activation of oncogenic signaling pathways will be necessary to determine the exact role the receptor plays in both IBD and CAC.

Future Directions

Future directions for this project will include confirming our results *in vivo*, further exploring the role that ErbB4 plays in colitis-associating carcinogenesis, and examining the role that ErbB4 plays during different inflammatory conditions in the GI tract.

Confirming findings in vivo

We have previously shown that mice injected with TNF for 24 h show increased ErbB4 expression (Frey et al., 2009). Additionally, we see that ErbB4 is upregulated and phosphorylated in response to TNF *in vitro*. An interesting experiment will be to determine if ErbB4 is activated *in vivo* in direct response to TNF, since it is possible that the conditionally immortalized YAMC cells have developed a different mechanism to respond to TNF stimulation than colonic epithelial cells in *vivo*. Wild type mice would be injected either with TNF or PBS,

and colon sections would be analyzed by immunohistochemistry for ErbB4 phosphorylation. In order to confirm the mechanism for ErbB4 transactivation by TNF *in vivo,* mice would be injected intraperitoneally with a pharmacological TACE inhibitor before TNF treatment, and then ErbB4 phosphorylation of colon sections would be analyzed. Another experiment that would confirm these results would be to use TACE knockout mice, and to compare the ability of TNF to activate ErbB4 in mice lacking the metalloprotease to wild type mice. We expect that mice treated with TACE inhibitors and mice that do not express TACE would no longer show increased ErbB4 activation following TNF treatment.

Determining the role of TACE in ErbB4 transactivation *in vivo* could have significant implications for IBD treatments. It has been suggested that TACE inhibition could be a valuable treatment for inflammatory diseases, since the metalloprotease is responsible for TNF shedding. However, mice that express less than 5% normal levels of TACE in the GI tract are more susceptible to DSS-induced colitis (Chalaris et al., 2010). Potentially, TACE might also be important for protecting the colonic epithelium from inflammatory insult by promoting transactivation of ErbB4.

On the other hand, it is possible that TACE-induced release of membrane-bound TNF and subsequent increases in TNF signaling might override the beneficial advantages of ErbB4 transactivation. To test this hypothesis, we could treat TIMP-3 knockout mice with either TNF injections or chemically induced colitis and then assess inflammation and injury scores of histological colon

sections. Since TIMP-3 is an endogenous inhibitor of TACE, genetic ablation of TIMP-3 should lead to increased TACE activity- effectively generating an activated TACE *in vivo*. If TACE activity is in fact deleterious and causes increased inflammation, we would see increased inflammatory levels in the TIMP-3 knockout mice compared to wildtype mice. As evidence to support this hypothesis, TIMP-3 knockout mice have elevated levels of TNF and severe inflammation in the liver (Black, 2004). One limitation of this approach is that TIMP-3 can also inhibit matrix metalloproteinases, so it might be hard to interpret whether TACE activity or MMP activity is responsible for the results. Gaining insight into how TACE both protects and damages the gastrointestinal lining during inflammation will aid development of treatments for IBD.

Determine the role of ErbB4 isoforms in promoting survival

There are conflicting reports about ErbB4's contribution to oncogenic signaling. Some of the confusion surrounding ErbB4's role in carcinogenesis may be alleviated by further determining which isoforms are expressed and activated in the colon, both basally and in response to stimuli. While we have seen by PCR that both juxtamembrane variants and both cytoplasmic variants are expressed in the colonic epithelium of mice, we do not know the exact composition of the isoforms that are expressed in the GI tract. (For instance, we cannot tell at this point if JM-a, CYT-1 and JM-b, CYt-2 isoforms are expressed, if JM-a, Cyt-2 and JM-b, CYT-1 are expressed, or if all four isoforms are expressed. The sequences

that encode for the juxtamembrane and cytoplasmic variants are too far apart to design primers that amplify a stretch of the gene that encompasses both regions.) We are further limited in that there are no isoform specific antibodies available at present. Currently, there are no colonic ErbB4 knockout cells, so while we are able to overexpress ErbB4 isoforms in YAMC cells, the endogenously expressed isoforms may still be contributing to survival signaling.

Colon epithelial cells derived from an ErbB4 knockout mouse could be stably infected with each individual isoform of ErbB4, and then survival assays could be performed with each line. ErbB4 deletion is lethal at embryonic day 11 due to defects in myocardial trabeculae formation. To circumvent this problem, Tidcombe et al. generated an ErbB4 knockout mouse that expresses ErbB4 under a cardiac-specific myosin promoter (Tidcombe et al., 2003). This mouse could be crossed to the Immorto-mouse to generate conditionally immortalized ErbB4 knockout colon epithelial cells. Another approach to generate ErbB4 knockout cells would be to cross mice harboring a loxP-flanked ErbB4 exon with mice expressing Cre recombinase driven by a villin promoter, which would cause intestinal-specific knockout of ErbB4. Then, using the LZRS retroviral infection system, these ErbB4$^{-/-}$ cells will be infected with JM-a, CYT-1; JM-a, CYT-2; JM-b, CYT-1; and JM-b, CYT-2. Subsequently, each individual line would be subjected to TNF/ CHX treatment, and apoptosis levels would be examined by TUNEL staining and by Westerns for cleaved PARP and active caspase-3. Given that the CYT-1 isoforms contain a PI3K binding site, we hypothesize that the

CYT-1 isoforms will be anti-apoptotic, since PI3K initiates several AKT pro-survival pathways (Figure 1.6). However, Naresh et al. have shown that the JM-a, CYT-1 s80 intracellular fragment contains a BH-3 domain that signals though BAK to promote apoptosis (Naresh et al., 2006). It seems ErbB4 must undergo proteolytic cleavage in order to stimulate apoptosis, since a JM-a, CYT-1 ErbB4 mutant that cannot be processed by γ-secretase fails to accumulate in the mitochondria, and the pro-apoptotic activity of ErbB4 was abolished in cells expressing the mutant. Examining the role of each isoform in a completely ErbB4 null background could shed light onto the paradoxical roles of the receptor.

We hypothesize that in cells devoid of other isoforms, the JM-a, CYT-1 isoform will actually promote apoptosis, while the JM-b isoforms would likely still promote survival signaling (since they cannot be cleaved). It is likely that the non-cleavable isoforms (JM-b, CYT-1 and JM-b, CYT-2) are able to promote PI3K/Akt activation and subsequent anti-apoptotic signaling through heterodimerization with ErbB3, which contains several PI3K binding sites. Once processed, the cleavable isoforms (JM-a, CYT-1 and JM-a, CYT-2) are no longer anchored at the membrane and thus cannot dimerize with ErbB3. Likewise, once the JM-a isoforms are cleaved, they are able to enter the nucleus and interact with a subset of proteins that they JM-b isoforms are sequestered from. Some of these proteins could facilitate the pro-apoptotic signaling of the JM-a isoforms.

Determine the role of ErbB4 in CAC

An important future direction for this project will be to determine ErbB4's contribution to cell survival during inflammatory conditions *in vivo*. One approach would be to chemically induced colitis with dextran sulfate sodium (DSS) or trinitrobenzene sulfonic acid (TNBS) treatment in cardiac-rescued ErbB4 null mice, or in mice with an intestinal specific ErbB4 deletion (mediated through loxP/villin-Cre). Once these mice have been treated with DSS or TNBS and sacrificed, colon sections stained for H&E could be scored for inflammation. Based on our data that ErbB4 expression protects colon epithelial cells from TNF-induced apoptosis, we would hypothesize that histological sections from ErbB4 knockout mice with chemically-induced colitis would exhibit increased inflammation, marked by neutrophil infiltration, ulcerations of the epithelium, and crypt distortion and hyperplasia.

It will also be interesting to further determine the role that ErbB4 plays in colitis-associated carcinogenesis. Cardiac-rescued ErbB4 null mice could be treated with azoxymethane (AOM, a potent carcinogen) followed by 4-7 days of DSS exposure. The mice would then be sacrificed and intestinal tumor number/severity would be scored from histological sections. Given that ErbB4 promotes survival in inflammatory conditions, we would expect that ErbB4 knockout mice treated with AOM/DSS would have fewer tumors/ less severe carcinogenesis than similarly treated wild-type mice. This would also fit with the

observation that ErbB4-overexpressing cells acquire that ability to form colonies in soft agar, which is indicative of cellular transformation (Frey et al., 2010).

Identify the role of ErbB4 in H. pylori-mediated apoptosis

It will also be interesting to determine the role of ErbB4 during other types of inflammation in the gastrointestinal tract. In addition to cytokine-induced inflammation that results from barrier defects, the GI tract is also exposed to the bacteria *Helicobacter pylori*, which can cause constant gastritis, tissue damage, and even carcinogenesis. We have recently seen that gastric epithelial cells co-cultured with *Helicobacter pylori* show a marked increase in expression of the s80 intracellular fragment. It will be interesting to determine if this fragment originates from JM-a, CYT-1 or JM-a, CYT-2 isoforms, since in some tissues the isoforms differ in their ability to promote either survival or apoptosis. We have developed CYT-specific antibodies, which we will utilize to determine the ErbB4 isoform that is upregulated following *H. pylori* incubation in gastric cells. Since JM-a isoforms are cleaved by γ-secretase and TACE, we will treat *H. pylori* infected gastric epithelial cells with inhibitors to these proteins and compare apoptosis levels between the cell lines. If JM-a, CYT-1 is the isoform upregulated, we hypothesize that inhibiting ErbB4 cleavage will lead to decreased levels of *H. pylori*-induced apoptosis, since the intracellular fragment of this isoform interacts with BAK to facilitate apoptosis. However, since the JM-a, CYT-2 isoform lacks a Nedd4 ubiquitination site (Figure 1.5) and thus more

efficiently translocates to the nucleus and interacts with STAT family members (Zeng et al., 2009), it might promote survival signaling following *H. pylori* infection. Since *H. pylori* is the leading cause of gastric cancer and ErbB4 mutations have been identified in gastric carcinomas (Soung et al., 2006), it will be important to determine the role that ErbB4 plays in *H. pylori*-mediated signaling.

Concluding Remarks

ErbB4 is unique among the ErbB family members in that it is alternatively spliced into four isoforms with different signaling capabilities. Currently there is not a consensus as to whether ErbB4 is an oncogene or a tumor suppressor. While in some tissues ErbB4 promotes apoptosis, in others (including the colonic epithelium in this study) ErbB4 promotes survival. There have been several cancer treatments targeting ErbBs that have proven effective; for instance, the ErbB2 inhibitor herceptin has shown efficacy in treating breast cancer patients. Determining the role that each ErbB4 isoform plays in oncogenesis will be important for future therapeutics, not only for ErbB4 targeting drugs, but for drugs that target other ErbBs as well. Since members of this RTK family can heterodimerize to promote different biological outcomes, the signaling patterns of each ErbB4 isoform could affect how drugs targeting EGFR or ErbB2 function as well. Analyzing the apoptotic potential of individual ErbB4 isoform on a background devoid of other isoforms could shed light onto the specific signaling

cascades that each isoform initiates. Once ErbB4's position as an oncogene or tumor suppressor has been fully characterized in different tissues, it will be possible to determine whether ErbB4 activation could possibly aid in the restitution of cytokine-damaged intestinal epithelium in patients with IBD.

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