Exploring a Novel Mechanism of Regulation of the TNFR Family Member FN14

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Abstract

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Fibroblast growth factor-inducible 14 (FN14) is a highly inducible cytokine receptor linked to a number of intracellular signaling pathways, including the nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, as well as to physiological processes, such as angiogenesis, tissue repair, and regeneration. Although FN14 is expressed at low levels in normal tissue, it is highly expressed after tissue injury and appears to mediate activities crucial to wound repair and regeneration. *In vitro*, FN14 expression is induced by numerous growth factors, as well as by its own ligand TNF-like weak inducer of apoptosis (TWEAK). Given these results, it is thought that FN14 plays an important physiological role in the response to acute injury, and that FN14 activity is primarily regulated at the level of ligand and receptor expression.

Various pathological states, however, also exhibit increased expression and activity of FN14. Tumor progression, chronic autoimmune disease, and neuroinflammation are just a few of the conditions in which increased expression or function of FN14 have been implicated. In addition, it has been shown, *in vitro*, that overexpression of FN14 can result in ligand-independent signaling by the receptor. This signaling in the absence of TWEAK has been associated with increased proliferation and invasiveness of several types of cancer cells. Thus, although FN14 plays an important physiological role after acute injury, it appears that dysregulation of FN14 expression may contribute to various chronic pathological states. Since FN14 protein expression is so highly inducible, and TWEAK-FN14 signaling can further amplify receptor levels, this begs the question whether unregulated receptor levels can influence the pathological switch through altered signaling and resultant cell proliferation, migration, or chemokine expression. It seems likely that there are cellular mechanisms in place to prevent excess accumulation of FN14. To date, however, the stability of FN14 or mechanisms of FN14 downregulation, factors which would presumably have dramatic effects on the sustained activity of the receptor, have not been explored.

The main focus of this project was to explore the cellular mechanisms involved in downregulation of FN14 levels or signaling capacity which are likely important for preventing inappropriate and uncontrolled signaling. One mechanism through which receptor signaling can be attenuated is by ligand-induced downregulation. In some cases, as with the receptor Notch, ligand-independent turnover can occur as well. During the course of this project, both of these turnover mechanisms were evaluated for FN14. It was determined that the receptor undergoes both rapid ligand-dependent and ligand-independent turnover, and that these two processes are distinct and synergistic. A described endocytic motif in the FN14 cytoplasmic tail does not seem to be required for either method of turnover, which means that the endocytic mechanism remains to be elucidated. We focused on dissecting the ligand-independent turnover of FN14 due to the novel nature of this regulatory mechanism. It appears that FN14 is constitutively expressed, trafficked to the cell surface, endocytosed, and degraded in lysosomes. This constitutive trafficking to sites of degradation seems to require only the extracellular domain of the receptor, suggesting that FN14 may interact with a trafficking partner using this domain. Although FN14 seems to be constitutively degraded in the steady-state, growth factor treatment stabilizes protein levels, in a manner independent of transcriptional upregulation. This finding supports the notion that FN14 levels are regulated not only at the level of expression, but also at the level of protein stability. Ultimately, this work describes previously unrecognized aspects of FN14 biology, which may enhance our understanding of how FN14 activity is dysregulated in chronic pathological states. Using this information, it might be possible to design peptides to alter FN14 trafficking patterns in a therapeutically applicable manner.

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General Introduction

TNFR superfamily members

Research over the past several decades has led to the discovery and description of the multi-functional and pluripotent TNF superfamily (TNFSF) of cytokines as well as the TNF receptor superfamily (TNFRSF) through which the TNF ligands act. There are 19 related ligands of the TNFSF, which are recognized by 29 receptors in the TNFRSF (1). The TNFRSF members are type I transmembrane proteins, which have an extracellular N-terminus and an intracellular C-terminus (1). They are characterized by homologous cysteine-rich domains (CRD) in their extracellular regions, which are stabilized by disulfide bonds between the conserved cysteine residues (1). The receptors can be categorized into three main groups, activating receptors, death receptors, and decoy receptors (1). The activating receptors can induce the NF-κB and MAPK signaling pathways. The death receptors contain an 80 amino acid intracellular death domain, which can directly trigger cell death pathways. Finally, the decoy receptors can sequester ligand, and thereby inhibit signaling. Despite the classifications, the different types of receptors do, in fact, have overlapping functions. For example, the death receptors TNFR1 and Fas can activate the NF-κB and MAPK signaling pathways in addition to cell death pathways. Conversely, the activating receptors TNFR2, CD40, and FN14 have been implicated in cell death under particular circumstances, despite the lack of a death domain.

The TNF ligands are involved in the regulation of a variety of normal physiological functions, including immune responses, hematopoiesis, and morphogenesis, but have also been implicated in tumorigenesis, transplant rejection, septic shock, viral replication, bone reabsorption, rheumatoid arthritis, and diabetes (1). At the cellular level, they can induce proliferation, survival, differentiation, and death (1). The TNF ligands are expressed primarily by the cells of the immune system, such as B cells, T cells, NK cells, monocytes, and dendritic cells (1). The TNFRs, however, are expressed by a variety of cell types. The receptors that we will focus on in this work are TNFR1, FN14, and CD40. TNFR1 has been found on all nucleated cells of the body so far examined, FN14 is found primarily on epithelial and

endothelial cells, and CD40 is expressed primarily on antigen-presenting cells such as dendritic cells, B cells, and monocytes (1,2).

TNFRSF signaling pathways

After binding of ligand, the members of the TNFRSF mediate diverse biological outcomes, such as apoptosis, survival, differentiation, or proliferation. This occurs through various signaling pathways, including NF-κB, JNK, MAPK and p38 MAPK (1). Almost all of the TNFRSF members utilize TNFR-associated factors (TRAFs) to initiate their signal cascades, and specifically, TRAF2, TRAF5, and TRAF6 have been shown to mediate activation of NF-κB and JNK (1,3). TNFR1 interacts with TRAFs through the TRADD adaptor, while some other TNFRSF members can interact directly with TRAFs through a specific motif in their cytoplasmic domains (1,3). The TRAF utilization of TNFR1, CD40, and FN14 is depicted in Figure 1.

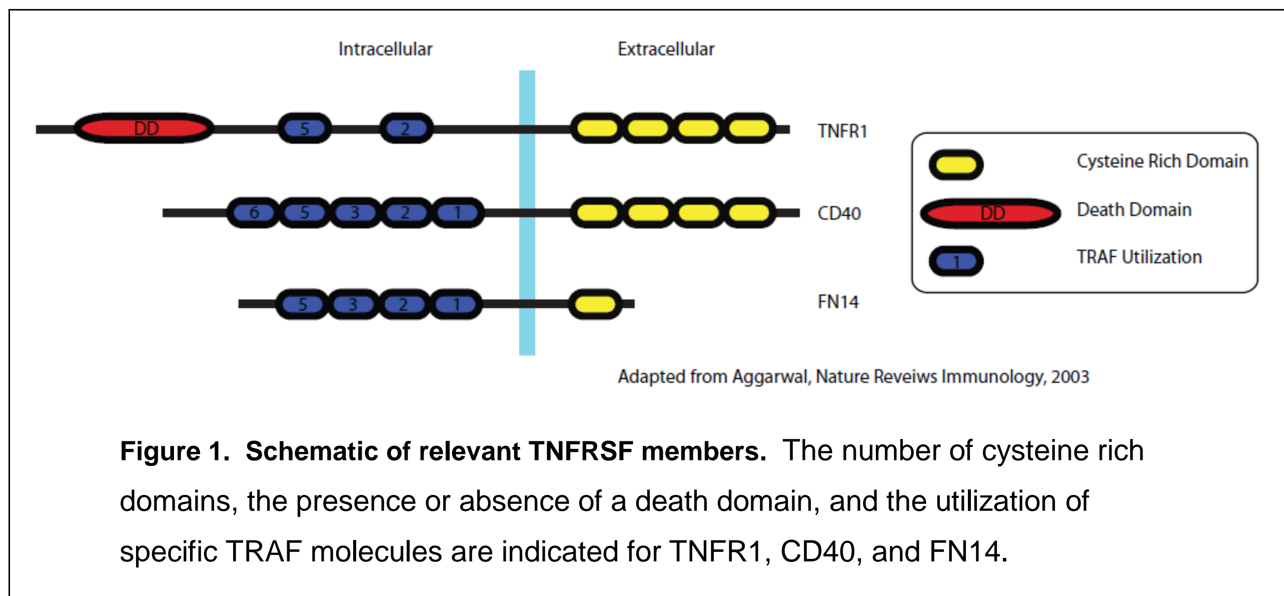

Figure 1. Schematic of relevant TNFRSF members. The number of cysteine rich domains, the presence or absence of a death domain, and the utilization of specific TRAF molecules are indicated for TNFR1, CD40, and FN14.

Many TNFRSF members can activate the canonical NF-κB pathway, and TNFR1 signaling can be used as a model to understand this pathway. TNFR1 self-associates through the pre-ligand-binding assembly domain (PLAD) found at the N-terminal residues 1-54, which overlap with the first CRD of the receptor (CRD1) (4). Deletion of the PLAD abrogates TNF binding, suggesting that self-assembly is

required for ligand binding (4). Currently it is thought that receptor trimerization occurs through one of two mechanisms, either binding of trimeric TNF or pre-assembly of the monomeric receptors through the PLAD. Ligand binding occurs through the second and third CRDs (CRD2 and CRD3) of the receptor (5), and allows the cytoplasmic tail to adopt a conformation which enables recruitment of the adaptor protein TNFR-associated death domain (TRADD) and the Ser/Thr kinase receptor-interacting protein (RIP1) (1,6). TRADD recruits TRAF2 or TRAF5, which then recruit the E3 ubiquitin ligases cIAP1 and cIAP2 (6). The cIAPs form ubiquitin linkages on various components of the signaling complex, and these linkages are thought to recruit the linear ubiquitin chain assembly complex (LUBAC) (6,7). LUBAC has been shown to add linear ubiquitin chains to RIP1 and NEMO (7,8), which may facilitate the recruitment of the TAB/TAK complex and the NEMO/IKK complex or may stabilize the IKK complex (6). Modified RIP1 recruits NEMO, and is also necessary for the activation of the IKK complex (3,6). Once the IKK complex is activated, either through auto-phosphorylation or trans-phosphorylation by another kinase such as TAK1, the IKKβ catalytic subunit phosphorylates IκBα, leading to its degradation (3). This releases NF-κB dimers, which are then free to translocate to the nucleus and transactivate NF-κB responsive genes (3). There are five different NF-κB subunits, and the activation of particular dimers over others likely contributes to the specificity of gene induction (3).

Only a handful of the TNFRSF members are able to activate the non-canonical NF-κB signaling pathway, including CD40, BAFF-R, LTβR, RANK, FN14, and CD27 (9). CD40 signaling can be used as an example to generally understand this signaling pathway. Receptor ligation of CD40 by CD40L causes degradation of the TRAF-cIAP complex that negatively regulates NF-κB-inducing kinase (NIK) (9). NIK is thus stabilized, upon ligand binding, and phosphorylates IKKα, likely in homodimeric complexes (9). Activated IKKα, in turn, phosphorylates p100 (9). This results in the recruitment of the $SCF^{\beta TrCP}$ ubiquitin ligase complex, which targets p100 for partial proteasomal processing (9). p100 is processed to p52, which in complex with RelB, is translocated to the nucleus and initiates transcription of target genes (9).

Some TNFRSF members can also activate the mitogen-activated protein kinase (MAPK) pathway. CD40 signaling can be used as a model to understand this pathway. Upon ligand binding of CD40, there is formation of a receptor signaling complex that includes TRAFs 2, 3, and 6, cIAP1/2, and the E2 ligase Ubc13 (10). Downstream of CD40, the TRAF2 signaling complex also contains NEMO and

MEKK1 while the TRAF6 signaling complex contains NEMO and TAK1 (10). MEKK1 and TAK1 are members of the MAP3K Ser/Thr kinase family involved in the initiation of the MAPK signal cascade (10). CD40 activation leads to the cIAP1/2-mediated degradation of TRAF3, and this releases the TRAF2-MEKK1 and TRAF6-TAK1 complexes into the cytoplasm (10). This cytoplasmic translocation is important for the activation of the MAP3Ks and begins the 3-tiered downstream phosphorylation cascade that culminates in activation of MAPKs such as JNK and p38 kinase (10,11). Although there is overlap between the upstream signaling components of the NF-κB and MAPK pathways, spatial and temporal separation between the active IKK and MAPK complexes contribute to differential downstream signaling effects.

There are a number of death receptors in the TNFRSF that can induce cell death pathways directly through their 80 amino acid cytoplasmic death domains. TNFR1 is an example of a death receptor that can not only induce apoptosis, but in some cases, can also initiate programmed necrosis. Normally, TNF binding to TNFR1 leads to anti-apoptotic NF-κB signaling downstream of TNFR1. NF-κB activation induces expression of proteins such as cFLIP, which inhibit caspase-8 activation (12). Upon ligand-binding and subsequent internalization of the receptor, however, pro-apoptotic signaling can be initiated (13). The death domain containing adaptor molecule TRADD associates with the death domain of TNFR1 through a homotypic protein interaction and recruits other signaling molecules, leading to formation of the death-inducing signaling complex (DISC) (13,14). In the case of the TNFR1, the DISC contains RIP1, TRADD, FADD, and caspase-8, and is thought to form in the cytoplasm after disassociation of the adaptor molecules from the receptor (13). DISC formation results in cleavage and activation of caspase-8, relaying the death signal by triggering a protease cascade of the effector caspases-3, -6, or -7 (15). During the final stages of apoptosis, the effector caspases cleave cellular substrates, and the cellular content is packaged into apoptotic bodies, which are recognizable by phagocytic cells (15). In addition to apoptotic signaling, it has recently been recognized that death receptors can also activate another type of cell death known as programmed necrosis or necroptosis (16). Necrosis is a morphologically distinct cell death pathway from apoptosis, and is characterized by plasma membrane permeabilization, cell swelling, and release of cellular contents into the extracellular environment (16). Although previously thought of as an unregulated process, it is now appreciated that

death receptors may be able to initiate necrosis under particular conditions. When caspase activity is inhibited, TNF signaling triggers this alternate cell death pathway (16). Necroptosis requires complex formation and function of RIP1, RIP3, mixed lineage kinase domain-like protein (MLKL), and the mitochondrial phosphatase PGAM5, and may involve disruption of mitochondrial or lysosomal activity, but is otherwise mediated through a poorly defined mechanism (16,17). Finally, a number of the other TNFRSF members have also been implicated in cell death pathways, even in the absence of death domains. TNFR2 and CD40, for example, are known to induce cell death through cross-talk with TNFR1 (18). TNFR2 ligation not only induces TNF production, but it also leads to depletion of TRAF2, which is necessary for pro-survival signals downstream of TNFR1 (19). In addition to indirectly initiating cell death, CD40 is also reported to directly induce apoptosis in various cancer cell types, in a manner that does not require *de novo* protein synthesis (20-22). However, this pathway is not well understood and seems to be quite cell type specific. The major signaling pathways reported to be downstream of TNFRSF members are depicted in Figure 2.

In addition to ligand-mediated signaling, it is known that overexpression of TNFRSF members can lead to various signaling outcomes. First, it is well established that TNFR1 overexpression can induce downstream NF-κB and JNK activation and apoptotic signaling (23). In fact, overexpression of most of the death receptors can lead to self-aggregation of the death domains which results in spontaneous, ligand-independent cell death (23,24). Regulatory proteins have been identified that retain the death domains in a silent conformation, thereby preventing spontaneous signaling under normal conditions (25). In addition, previous work from our lab, which will be discussed in more detail later, explores another cellular regulatory mechanism that prevents the steady-state accumulation of TNFR1.

Overexpression of TNFRSF members can have another outcome as well. Ectopic expression of TNFR1 induces shedding of the receptor. TNF and its main receptor TNFR1 undergo shedding mediated by the metalloprotease known as ADAM17 or TNF-α converting enzyme (TACE) (26). Protealytic cleavage of the receptor leads to the release of the 28 KD ectodomain of the receptor. It has also been reported that full-length 55 KD receptor can be released in exosome-like vesicles, without the need for receptor sheddases (27). The release of soluble TNFR1 is thought to neutralize or buffer the pro-inflammatory activity of TNF. For example, transgenic mice expressing high levels of sTNFR1 are

protected from LPS-induced septic shock, which is mediated by TNF (28). However, these mice are more susceptible to normally sublethal infections with *Listeria monocytogenes* or *Leishmania major* (28). Low-level transgenic expression of sTNFR1, on the other hand, results in partial resistance to LPS-induced shock as well as resistance to bacterial infection (28). These results suggest that low-level release of sTNFR1 contributes to the control of local TNF-mediated inflammation while still allowing for the extended, systemic protective functions of TNF.

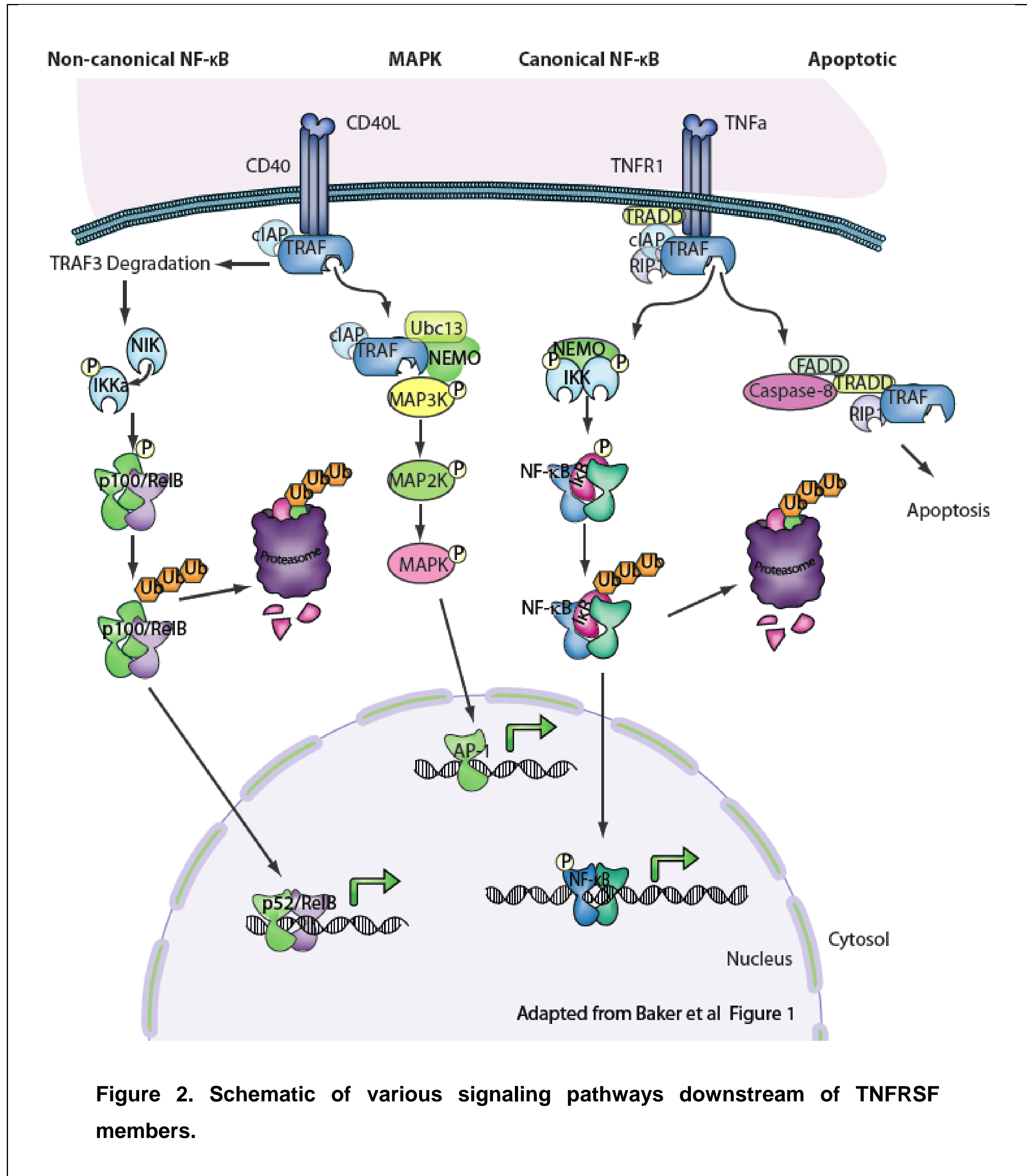

Figure 2. Schematic of various signaling pathways downstream of TNFRSF members.

Although the TNFRSF members utilize many of the same downstream signaling pathways, they have the ability to induce a plethora of cellular responses. There are many factors that can influence signaling outcomes (29). Cell-type specific expression of signaling proteins is one relevant factor. The

substrate specificity and different kinetics of action of key signaling enzymes is another important factor. The use of different scaffolding proteins and different combinations of downstream signal transducers can also add complexity to the signaling pathway. Finally, there are spatial and temporal control mechanisms that can regulate signaling capacity. This thesis will focus on the spatial and temporal regulatory factors that may affect the signaling outcomes of a particular TNFRSF member, FN14.

Effects of compartmentalization and localization on TNFRSF signaling

As mentioned above, spatial and temporal elements can influence receptor signaling specificity. Some examples of spatial regulatory factors are cellular localization, association with lipid rafts, and receptor endocytosis, while examples of temporal regulatory factors include ligand and receptor stability, turnover of downstream signaling mediators, and negative and positive feedback loops.

Receptor endocytosis has long been known as a mechanism involved in signal termination. TNFR1 undergoes ligand-induced internalization, with the maximum receptor downregulation occurring within 30 minutes of TNF treatment (30,31). This ligand-induced internalization leads to degradation of the ligand and receptor, leading to cessation of the signal (30,31). CD40 has also been shown to undergo ligand-induced internalization. CD40L expressed by T cells and by fibroblasts has been shown to induce internalization of CD40 in B cells, thereby downregulating the CD40L signal (32,33).

In addition to signal downregulation, it is now recognized that receptor internalization is necessary for the full activation of some signaling pathways. TNFR1 receptor is known to activate both anti-apoptotic and apoptotic pathways, and it has been proposed that TNFR1 signaling is bifurcated through the assembly of two molecularly and spatially distinct signaling complexes (13,34). Within minutes of TNF binding, a TNFR1 signaling complex, called complex I, assembles at the cell surface. This complex includes TNFR1, TRADD, RIP1, and TRAF2, and is thought to activate the NF-κB pathway through recruitment of the IKK complex (13). Then, at later times after TNFR1 internalization, apoptotic signaling is initiated. Two models have been proposed which describe the molecular mechanisms of TNFR1-mediated apoptosis. First, it has been suggested that RIP1, TRAF2, and TRADD dissociate from TNFR1 upon internalization and then recruit FADD and caspase-8 in the cytoplasm (13). This cytoplasmic

complex is known as complex II (13). A second model proposes that the TNFR1 signaling complex, containing TRADD, RIP1, and TRAF2, is internalized into endocytic vesicles using a defined internalization domain at amino acids 205 to 214, distal to the transmembrane region of TNFR1 (34). During endocytosis, FADD and caspase-8 are recruited to form the TNFR1 DISC (34). Despite the differences in these models, it is well accepted that TNFR1-mediate apoptotic signaling requires internalization of the signaling complex. In addition to TNFR1, CD95 (FAS) also seems to require compartmentalization to direct apoptotic signaling (35).

There is evidence to suggest that internalization of TNFR1 may also be necessary for TNF-mediated gene expression in endothelial cells (36). It has been shown using various methods to inhibit endocytosis, such as acidification of the media using acetic acid, hypertonicity using sucrose, metabolic inhibition using phenylarsine oxide, and treatment with primary amines, that TNF induction of the adhesion molecules ELAM-1, ICAM-1, and VCAM-1, requires endocytosis (36). Previous data from our lab has also indicated that internalization may be necessary for TNFR1-mediated NF-κB activity. The ligand-induced internalization of CD40 also seems to be important for its signaling outcomes. In vascular endothelial cells, soluble CD40L (sCD40L) induces internalization, while membrane-bound CD40L (mCD40L) seems to capture the receptor at the cell surface (37). sCD40L and mCD40L stimulation induce differential recruitment of TRAFs, an altered pattern of AKT phosphorylation, and altered kinetics of NF-κB signaling (37). In fact, only mCD40L appears capable of inducing expression of pro-inflammatory cytokines and cell adhesion molecules (37). On the other hand, forced internalization of CD40 is able to activate NF-κB signaling, suggesting that localization to the endosome can trigger formation of a signaling complex (37). Thus, spatial regulation may contribute to the bifurcation in signaling that occurs upon stimulation with the soluble and membrane forms of CD40L.

Localization to lipid rafts may also contribute to divergent signaling outcomes. It is hypothesized that lipid raft association facilitates clustering of receptors, exclusion of signaling inhibitors, and relocalization of cytosolic adaptors upon ligand binding (38). There are a number of reports of TNFR1 association with lipid rafts, either in the steady-state or upon ligand binding. TNFR1 has been shown to be constitutively associated with rafts in U937 and HeLa cells (39,40). The death domain of the receptor seems to be necessary for this raft association in HeLa cells (39). Another study suggests that, in human

fibrosarcoma HT-1080 cells, TNF binding results in the translocation of the ligand-receptor to lipid rafts, where formation of the TNF receptor signaling complex occurs (41). Disruption of the raft microdomains switches the outcome of TNF signaling from NF-κB activation to induction of apoptosis (41). Yet another study proposes that in mouse macrophages, TNFR1 association with lipid rafts is necessary for MAPK activation, but not for NF-κB activation (42). An interesting finding, which relates to ideas discussed later in this thesis, is that in airway smooth epithelial cells, TNF treatment leads to relocalization of both TNFR1 and the RhoA GTPase to lipid rafts, and that this localization is necessary for TNFR1-mediated activation of RhoA (43). It is clear from the varied findings of the aforementioned reports that the functional significance of TNFR1 association with lipid rafts is not well understood. The differing conclusions are thought to be a result of cell type differences in raft association or perhaps of differences in the raft isolation techniques utilized in each study. Finally, lipid raft association has also been shown for other TNFRSF members, including CD40 (44,45). A pertinent finding for this thesis, as will be discussed later, is that retention of CD40 in lipid rafts has been shown to actually augment receptor signaling. In B-cell lineage non-Hodgkin's lymphoma there is constitutive expression of NF-κB through the maintained assembly of a CD40 signaling complex or signalosome within lipid raft microdomains (46). Disruption of the signalosome by anti-CD40 or anti-CD40L antibodies downregulates constitutive expression of NF-κB in these cells, resulting in the inhibition of lymphoma cell growth and promotion of cell death (46).

Taken together, the above findings indicate that spatial and temporal regulation of the TNFRSF members influences both downstream signaling outcomes and duration of receptor signaling. To date, little is known about the spatiotemporal regulation of the smallest TNFRSF member, FN14. In this thesis, we will attempt to shed light on the regulatory mechanisms controlling activity of this receptor.

Chapter 1: FN14 Stability and Downregulation

Introduction

FN14 expression

FN14 is the smallest member of the TNFRSF described thus far, and is the only known signaling receptor for the ligand TWEAK (47). FN14 was first identified as a FGF-1-inducible gene in murine NIH 3T3 cells (48), and was later cloned from a HUVEC cDNA library and shown to be the TWEAK receptor (47). FN14 is expressed primarily on the surface of epithelial, endothelial, and non-hematopoetic cells (49). It is highly inducible by a variety of factors, including PMA, FBS, FGF-1, FGF-2, and PDGF in both murine and human fibroblasts (47,48,50). It has also been shown that TWEAK treatment can induce FN14 mRNA and protein expression in glioma cell lines (51). Specifically, TWEAK binding to FN14 stimulates expression of FN14 through a Rac1 and NF-κB dependent mechanism (51). FN14 has potential AP-1, cyclic AMP responding element (CREB) and NF-κB transcription factor binding sites in its promoter region, as well as highly conserved potential binding sites for muscle-specific transcription factors, such as MyoD, in the human and mouse FN14 promoters (51,52). Generally, FN14 is thought to be lowly expressed in normal tissues, and upregulated in solid tumors, in response to tissue injury, and during progression of chronic inflammatory disease (Figure 3).

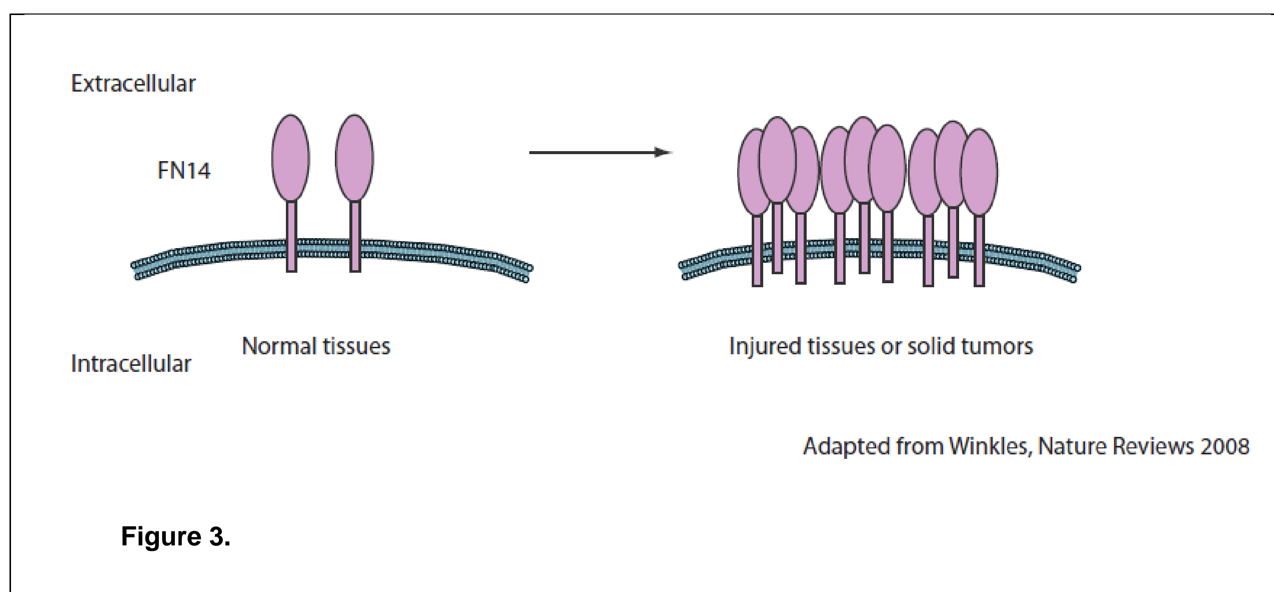

Figure 3.

Figure 3. FN14 expression is highly regulated. In normal tissues, FN14 expression is relatively low. FN14 levels are elevated after tissue injury, in solid tumors, and during progression of chronic inflammatory disease models.

TWEAK expression

TWEAK is the only known ligand for FN14. It was originally cloned from a mouse macrophage cDNA library, and was recognized as a member of the TNF superfamily based on the presence of TNF sequence motifs (53). TWEAK was first described as a weak inducer of apoptosis in HT-29 human adenocarcinoma cells that were pre-treated with IFN-γ (53). Like other members of the TNFSF, TWEAK is a type II-transmembrane protein that is thought to exist as a homotrimeric molecule (53). It can be cleaved by furin proteases to produce a soluble cytokine and both the membrane-bound and soluble versions of TWEAK are capable for signaling (53,54). The TWEAK-FN14 pathway is evolutionarily conserved, and appears in diverse species from zebrafish to humans (53).

TWEAK expression has been detected in many different cell types. TWEAK mRNA expression has been found in cells as diverse as macrophages, astrocytes, microglia, endothelial cells, and erythroid lineage precursors (49). TWEAK protein has been detected on the cell surface of several tumor cell lines (55,56). It has also been reported that resting NK cells, macrophages, and dendritic cells express TWEAK protein intracellularly, as assessed by permeabilization and FACS analysis, and that TWEAK protein levels are upregulated in these cells after stimulation with IFN-γ or phorbol myristate acetate (PMA) (57). Similarly, IFN-γ treatment has been shown to induce cell surface TWEAK expression in monocytes (58). Although TWEAK is primarily thought to be expressed in non-lymphoid cells, one study reported that activated T cells express increased levels of TWEAK in comparison to unstimulated cells (59).

Little is known about the transcriptional regulation of TWEAK. The 3’ UTR of TWEAK mRNA contains an AU rich element (ARE), which may be important in the regulation of the mRNA stability and translational efficiency of TWEAK (53). *In vitro*, IFN-γ, but not IFN-α or LPS, induces TWEAK protein

expression in monocytes (58). *In vivo*, TWEAK expression is increased in tissues in contexts of injury, inflammatory disease, and cancer (49).

Physiological roles of the TWEAK-FN14 pathway

FN14 is expressed at low levels in normal tissues, and is upregulated after tissue injury and during disease progression (50,60). It is thought that TWEAK produced by NK cells and macrophages acts on non-hematopoetic cells expressing FN14 to coordinate inflammation, tissue progenitor cell expansion, and fibrogenic responses after tissue injury. *TWEAK*-null and *FN14*-null mice, which develop normally and are viable and fertile (57,61), have been utilized in a variety of model systems to determine the contribution of the TWEAK/FN14 pathway in both physiological and pathological settings.

It was appreciated in the early studies of FN14, that the receptor plays an important role during wound healing. In a model of rat aortae injury, the endothelial cells at the wound edge and the proliferating smooth muscle cells lining the artery express much higher levels of FN14 in comparison to uninjured arteries (47). In another wound healing model using human renal microvascular endothelial cells (HRMEC), it was shown that the PMA- or EGF- stimulated migration of endothelial cells, which occurs during wound closure, is inhibited by FN14-Fc, a soluble decoy receptor that competes with endogenous FN14 for TWEAK binding (47).

The TWEAK/FN14 pathway has also been shown to be important in various models of tissue regeneration. To begin with, the pathway plays a major role during liver regeneration. TWEAK acts as a mitogen for oval cells, the liver progenitor cells that are critical for regeneration (61,62). Transgenic mice overexpressing TWEAK exhibit hyperplasia of oval cells, in an FN14-dependent manner, and endogenous TWEAK has been shown to promote oval cell proliferation in a diethoxycarbonyl-1-4-dihydrocollidine (DCC)-induced liver injury model (61). TWEAK has also been shown to have mitogenic effect on FN14-expressing liver progenitor cell lines *in vitro* (61,62). In addition to effects on progenitor cells, the TWEAK/FN14 pathway is also known to promote inflammatory and fibrogenic responses in response to liver injury. In another model of liver injury, choline-deficient ethionine (CDE)-supplemented diet, it was shown that after 14 days, FN14 levels were increased 17-fold in the CDE-fed mice in comparison to control-diet fed mice (62). By 21 days of the CDE diet, the FN14 levels had subsided to an

11-fold increase over controls (62). In line with these kinetics, most of the regenerative differences between wildtype and FN14 KO mice in this model were scored at the 14 day time point (62). In response to CDE-diet, TWEAK produced by NK cells and macrophages in the liver coordinated tissue regenerative responses, such as induction of the inflammatory cytokines TNF, IL-6, IFN-γ, and LT-β, and of the fibrosis markers, collagen-1 and timp-1/2 (62). In livers from FN14 KO mice subjected to the CDE-diet, however, there were reduced inflammatory infiltrates and decreased expression of inflammatory and fibrotic markers in comparison to the wildtype CDE-fed controls (62).

The TWEAK/FN14 pathway is also known to be important in skeletal muscle regenerative responses. Skeletal muscle progenitor cells express FN14 and it appears that TWEAK acts as a mitogen for these precursor cells of the mesenchymal lineage, namely skeletal muscle myoblasts, preadipocytes, and chondrocyte and osteoblast precursors (60). In addition, TWEAK treatment of the murine myoblast cell line C2C12 not only results in proliferation, but also inhibits differentiation into myotubes under differentiation conditions (60,63). It is thought that the TWEAK/FN14 inhibitory effects on myoblast differentiation occur through NF-κB dependent degradation of muscle-specific genes, including the MyoD family of transcription factors, as well as through induction of genes involved in cell cycle progression (63,64). In a model of skeletal muscle regeneration following cardiotoxin-induced injury, TWEAK and FN14 mRNA levels are upregulated by 3 days post-injury and there is MCP-3 chemokine expression and infiltration of inflammatory cells (60). In FN14 KO mice, however, there is reduced inflammation and delayed muscle regeneration in response to cardiotoxin induced injury (60). Also, while TWEAK levels subside by 5 days post-injury in muscle from the wild-type mice, increased TWEAK levels persist in the FN14 KO mice (60). This could be a result of delayed healing, or it could potentially indicate that FN14 is involved in the downregulation of TWEAK levels.

The TWEAK-FN14 pathway may also play a role in bone remodeling. Bone morphogenetic protein (BMP)-2 is a member of the TGF-β superfamily which can induce differentiation of the pre-osteoblastic cell line MC3T3-E1. It was shown that FN14 is expressed in MC3T3-E1 cells and that TWEAK treatment inhibits the BMP-2 induced expression of osteoblast differentiation markers such as alkaline phosphatase and osteocalcin (65). In addition, TWEAK by itself and in concert with TNF can

regulate osteoblast function through induction of sclerostin, an inhibitor of differentiation and an inducer of various signaling pathways in osteoblastic cell lines and primary cells (66).

Taken together, these results suggest that FN14 plays an important role in regulating tissue repair and remodeling (49,67). It is thought that after injury, upregulation of FN14, combined with basal levels of TWEAK expression by tissue resident cells, is responsible for the chemokine expression that recruits inflammatory cells and for the signaling that leads to expansion of tissue progenitor cells. The recruited inflammatory cells help with repair and remodeling by killing microbes, clearing matrix and cell debris, and releasing growth factors and cytokines that signal for tissue repair. The recruited inflammatory cells also produce more TWEAK. The positive feedback loop of increased TWEAK expression which leads to increased FN14 expression, will add to the recruitment of inflammatory infiltrates and progenitor cell expansion. Then, as the repair proceeds, expression of FN14 and TWEAK declines, which then allows the expanded progenitor cells to differentiate and regenerate the tissue. Little is known about the stability of FN14 and TWEAK or the methods of downregulation, which likely contribute to how long or robustly the inflammatory process will occur in these instances of tissue repair. Hypothetically, failure to downregulate the FN14/TWEAK pathway appropriately could result in the advent of inflammation-related disease.

Pathological roles of the TWEAK-FN14 pathway

In situations of chronic tissue injury and inflammation, persistent activation of the TWEAK/FN14 pathway produces harmful, pathological effects, and has been shown to contribute to the severity of various diseases. First of all, TWEAK activates a number of processes that could contribute to tumor progression, such as proliferation, angiogenesis, inflammation, and migration. Specifically, TWEAK has been shown to act on endothelial cells to stimulate angiogenesis and inflammation. TWEAK binding to FN14 on HUVEC can induce proliferation and migration of the cells, as well as upregulation of the adhesion molecules ICAM-1 and E-selectin and secretion of IL-8 and MCP-1 (68). Angiogenic factors such as FGF-2 and VEGFA are known to induce FN14 expression, and it has been suggested that these factors may act with TWEAK to induce synergistic growth responses in HUVEC *in vitro* (69,70). In an *in*

vivo model of corneal angiogenesis in mice, treatment with FN14-Fc reduces FGF-2 induced vessel growth, vascular area, and vessel density further suggesting that FN14 signaling does indeed play a role in angiogenesis (47). There is also a potential role for the TWEAK-FN14 pathway in tumor angiogenesis. It has been shown that subcutaneous injection in athymic mice of HEK293 cells-overexpressing TWEAK results in the formation of highly vascularized tumors (71).

FN14 is highly expressed in many solid tumors. Elevated FN14 protein expression has been detected in esophageal adenocarcinoma patient samples and (72) and elevated FN14 mRNA expression has been detected in HER2-positive/estrogen negative breast cancer patient samples (73). Enhanced FN14 mRNA levels have also been detected in patient-derived glioblastoma cells isolated from the migrating tumor rim as compared to the matched tumor core (51). In glioma cell lines, TWEAK treatment and FN14 overexpression enhance cell survival and resistance to pro-apoptotic stimuli (74). In glioma, breast cancer, and esophageal adenocarcinoma cell lines FN14 overexpression results in increased cell invasiveness, whereas FN14 knockdown decreases invasiveness (51,72,73). In gastric cell lines, FN14 overexpression results in increased proliferation (75). In all of the cancer types discussed, increased FN14 expression in patient samples seems to correlate with worsening disease and poor patient outcome (51,72,73,75). TWEAK signaling also has pro-tumorigenic activity. TWEAK has been shown to promote proliferation of a number of hepatocellular carcinoma cell lines (55), and as mentioned earlier, TWEAK has pro-angiogenic effects which can enhance tumor vasculature (71).

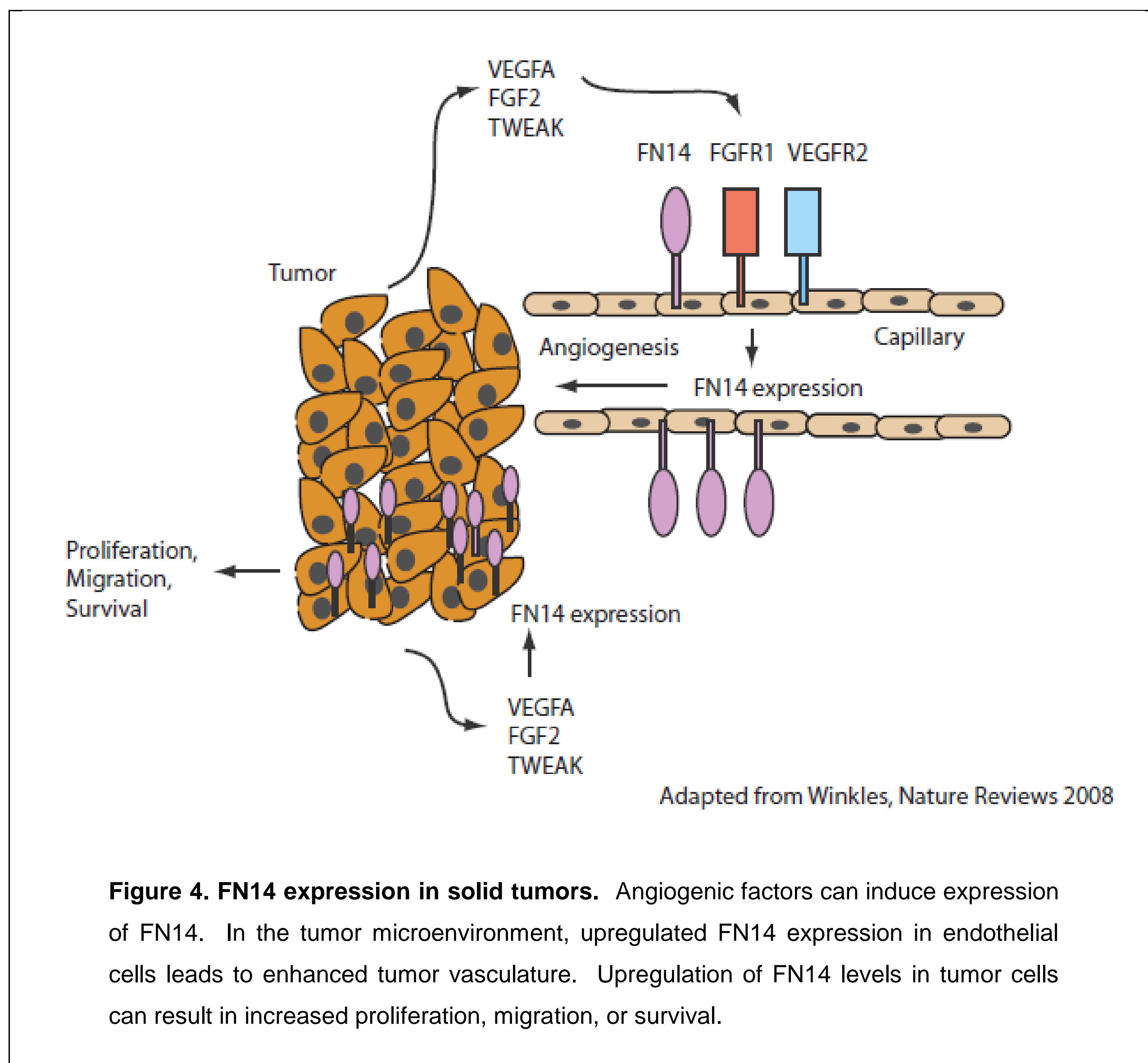

Figure 4. FN14 expression in solid tumors. Angiogenic factors can induce expression of FN14. In the tumor microenvironment, upregulated FN14 expression in endothelial cells leads to enhanced tumor vasculature. Upregulation of FN14 levels in tumor cells can result in increased proliferation, migration, or survival.

Using rodent models of human disease, it has been demonstrated that the TWEAK-FN14 pathway may contribute to the severity of various autoimmune diseases. For example, two groups have recently demonstrated that TWEAK is an arthritogenic cytokine, which contributes to pathogenesis in a mouse model of collagen-induced arthritis (CIA) (76,77). The CIA model is commonly used to study the human disease rheumatoid arthritis, a chronic inflammatory autoimmune disorder of the synovial joints, characterized by cartilage and bone destruction, elevation of cytokines in synovial tissues, and angiogenesis during disease progression. In both of the studies using the CIA model, intraperitoneal injection of an anti-TWEAK antibody reduced the severity of the disease induced by collagen injection

(76,77). Specifically, anti-TWEAK treatment resulted in the inhibition of cartilage and bone loss, reduced production of proinflammatory mediators, diminished proliferation of synovial cells, and decreased synovial angiogenesis (76,77). The anti-TWEAK treatment did not, however, affect B-cell or T-cell autoreactive responses to type II collagen, suggesting that TWEAK primarily mediates the innate immune response (76,77). These results suggest that the TWEAK-FN14 pathway could be a novel therapeutic target for human rheumatoid arthritis.

TWEAK may also be a renal biomarker and disease mediator in systemic lupus erythematosus (SLE). SLE is an autoimmune, inflammatory disease that is characterized by autoantibody production, with deposition of immune complexes in various organs. There is often associated kidney pathogenesis with SLE, known as lupus nephritis. In a chronic graft vs. host disease (cGVHD) induced mouse model of SLE, it was shown that FN14 KO mice had reduced renal IgG deposition and cytokine expression, as well as reduced proteinuria, in comparison to wildtype mice (78). Anti-TWEAK treatment had similar positive effects in the SLE model (78). Neither FN14 deficiency nor anti-TWEAK treatment affected autoantibody production in this model (78). It has also been seen that in patients with SLE, those with associated renal disease have higher urinary TWEAK levels than those without renal disease, suggesting that TWEAK could be used as a biomarker for renal involvement in SLE (79).

The TWEAK-FN14 pathway also contributes to pathogenesis in an experimental acute renal failure model induced by folic acid (80). After injection of folic acid in mice, TWEAK and FN14 expression was increased, and the resultant tubular injury was characterized by tubular cell apoptosis (80). As a result of these findings, the contribution of TWEAK to tubular cell death was examined. Cultured murine tubular epithelial MCT cells were shown to express TWEAK and FN14 (80). FN14 levels were inducible in these cells by TWEAK, TNF, and IFN-γ treatment (80). An interesting observation, which pertains to the theme of this thesis, is that the mRNA levels of FN14 were most upregulated 2 hours after stimulation, while the protein levels were also highest 2 hours after stimulation (80). This suggests that the increase in FN14 protein levels might be attributable to mechanisms other than expression, such as protein stabilization. Co-stimulation of the tubular cells with TNF/IFN-γ and TWEAK resulted in caspase-8, -9, and -3 activation, Bid cleavage, mitochondrial injury, and resultant apoptosis (80). Pre-treatment with a pan-caspase inhibitor sensitized the cells to necrosis through generation of reactive oxygen species (80).

Given these findings, it seems likely that the TWEAK, in conjunction with other inflammatory cytokines, may play an important role in renal tubular cell injury.

Although the TWEAK-FN14 pathway has important physiological functions in muscle regeneration after injury, the pathway is also implicated in skeletal muscle wasting after denervation. Transgenic expression of TWEAK in mice induced atrophy, fibrosis, switching to weaker muscle fiber types, and degradation of muscle-specific proteins (81). In a denervation mouse model in which the sciatic nerve was transected, there was an increase in FN14, but not TWEAK, expression (81). Denervation resulted in loss of muscle proteins, decreased muscle mass, and reduced strength, but these symptoms were rescued in TWEAK-deficient mice (81). It is thought that the upregulation of FN14 after denervation allowed for TWEAK activation of NF-κB (81). This resulted in the induction of the MuRF1 E3 ubiquitin ligase, thereby enhancing the activity of the ubiquitin-proteasome pathway, and leading to degradation of muscle-specific factors (81). A role for NF-κB mediated expression of MuRF1 and enhanced proteasome activity has been previously described in denervation-induced muscle wasting, and it has also been demonstrated in tumor-induced muscle wasting (82). Since FN14 levels are highly expressed in solid tumors, it is possible that the TWEAK-FN14 pathway could play a role in tumor-induced muscle wasting as well.

The TWEAK-FN14 pathway is also implicated in inflammation of the central nervous system. TWEAK promotes astrocyte proliferation, production of IL-6, IL-8, and expression of ICAM-1 (49). TWEAK may also increase blood-brain barrier permeability by inducing astrocyte expression of MMP-9, a known mediator of blood-brain barrier leakage and through induction of ICAM-1 and chemokine expression by astrocytes and endothelial cells, which recruits leukocytes to the CNS (83). Microglia also express FN14, and are subject to TWEAK mediated proliferation, which could contribute to CNS inflammation. Finally, it is reported that TWEAK directly induces neuronal cell death (84). Since the TWEAK-FN14 pathway can affect various CNS cell types and can contribute to CNS inflammation, it may be a novel target for therapeutic intervention of CNS diseases.

Specifically, the TWEAK-FN14 pathway may play a role in the pathogenesis of EAE, a model for the demyelinating disease multiple sclerosis. TWEAK mRNA expression is increased in the brain and spinal cord during development of myelin-oligodendrocyte glycoprotein (MOG)-induced EAE, and

transgenic overexpression of TWEAK in this model increases disease severity (85). Another group suggested that TWEAK contributes to inflammation and disease severity through induction of the T-cell attracting chemokine CCL2 (86). In this study, vaccination of rats with recombinant TWEAK or FN14 in the MOG-induced EAE model induced production of neutralizing antibodies, and in two different models of EAE in rats and in mice, vaccination ultimately provided protection from disease (86). Similarly, another group showed that intra-peritoneal injection with anti-TWEAK monoclonal antibodies can reduce inflammatory infiltrates into the spinal cord during MOG-induced EAE in mice, as well as severity of disease (87). In addition to the EAE-model, there is evidence that TWEAK and FN14 expression is upregulated in the postmortem brain tissue from MS patients and that serum TWEAK levels are elevated in MS patients in comparison to healthy controls (49). These results, taken together, suggest that targeting the TWEAK-FN14 pathway could be a novel way to treat multiple sclerosis.

In a mouse model of cerebral ischemia known as distal middle cerebral artery occlusion (MCAO) the TWEAK-FN14 pathway seems to play a role in ischemia-mediated cell death. It was shown that TWEAK and FN14 are expressed by astrocytes and neurons *in vitro* (88). Furthermore, after MCAO in mice, FN14 mRNA levels increased in the ischemic hemisphere of the brain, as did both TWEAK and FN14 protein in the area surrounding the necrotic core of the ischemic region (84,88). Administration of the soluble decoy receptor, FN14-Fc, after MCAO, resulted in decreased infarct volume, microglial activation, and ischemia-induced cell death (88). Intraperitoneal injection of a TWEAK neutralizing antibody in the MCAO model similarly decreased infarct volume, and it was suggested that this may have occurred through inhibition of TWEAK-mediated NF-κB activation which contributes to neuronal cell death (84). Intraventricular injection of the FN14-Fc decoy receptor in the MCAO model has also been shown to reduce ischemia-induced blood-brain barrier permeability and cerebral edema and to improve motor activity (83,89). The contribution of FN14 to the severity of stroke is further demonstrated by the fact that FN14 KO mice exhibit reduced ischemic lesions and blood brain barrier permeability and improved motor function after MCAO in comparison to wild-type mice (89).

Overall, it appears that TWEAK and FN14 expression are induced in many chronic disease states and that persistent activation of the pathway contributes to the pathology of these diseases (Figure 5). FN14 mediates pro-inflammatory, angiogenic, proliferative, and death inducing activities. FN14 pro-

inflammatory activities primarily occur through the mediation of the innate immune response through induction of cytokines, chemokines, and cell adhesion molecules. Anti-TWEAK and anti-FN14 treatment have therapeutic effects in many of the discussed model systems of disease, suggesting that manipulation of the TWEAK-FN14 pathway may have efficacy against human diseases as well. The question of how TWEAK and FN14 expression becomes dysreglated in the first place, however, remains elusive. A possible explanation could be that FN14 expression is upregulated by growth factor signaling, and then sustained by a positive feedback loop of expression. We hypothesize in this thesis, that there may be additional factors which could contribute to dysregulation of FN14 levels during chronic disease states. We propose that there are cellular mechanisms in place which constitutively destabilize the receptor in order to prevent inappropriate aggregation and signaling. Given this hypothesis, increased stabilization of the receptor could lead to altered signaling activity and ultimately pathological consequences during disease states.

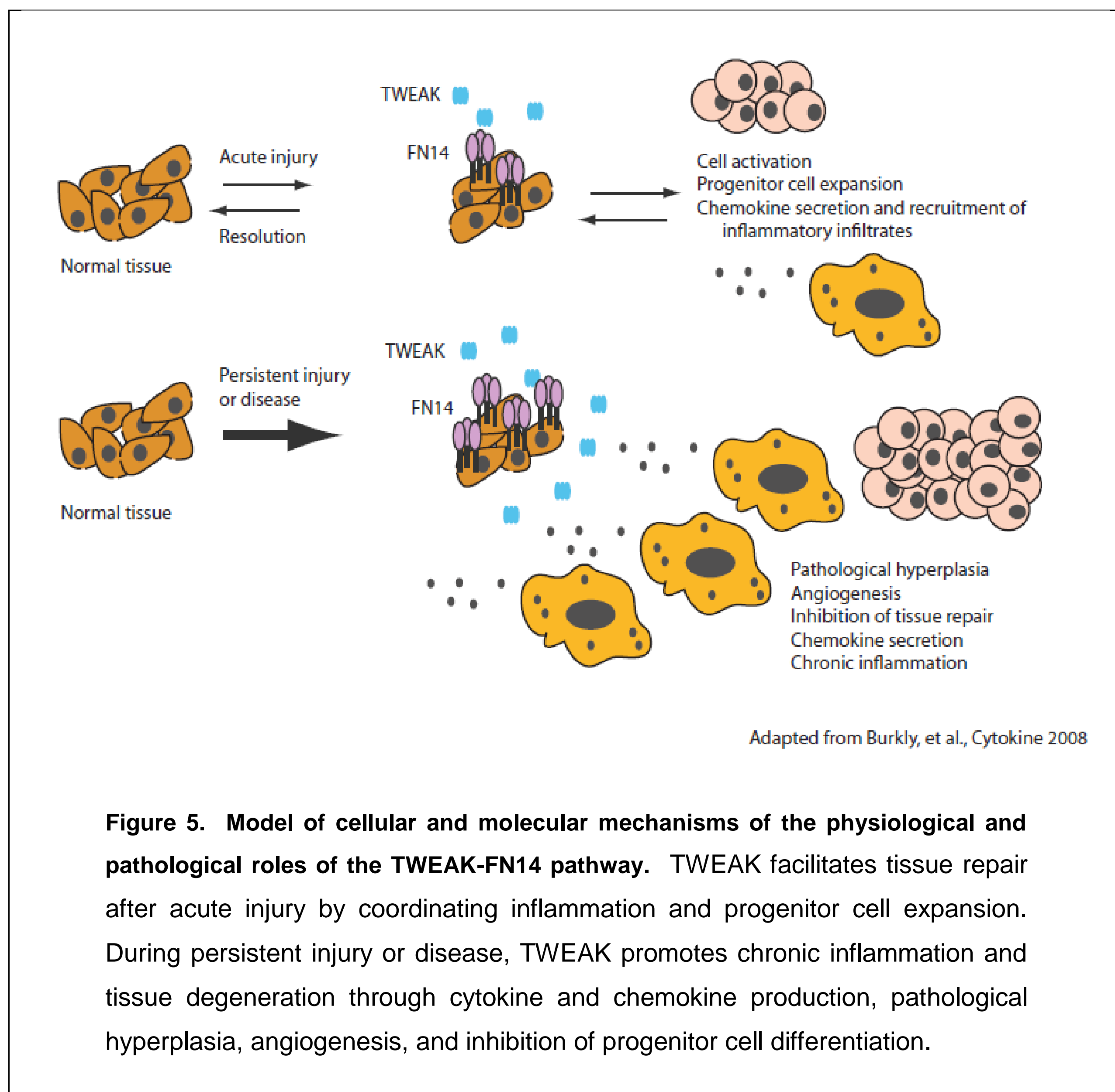

Figure 5. Model of cellular and molecular mechanisms of the physiological and pathological roles of the TWEAK-FN14 pathway. TWEAK facilitates tissue repair after acute injury by coordinating inflammation and progenitor cell expansion. During persistent injury or disease, TWEAK promotes chronic inflammation and tissue degeneration through cytokine and chemokine production, pathological hyperplasia, angiogenesis, and inhibition of progenitor cell differentiation.

Anti-FN14 therapy

As mentioned previously, anti-TWEAK and anti-FN14 therapy has been used successfully in a number of studies to modulate disease progression. In many of the disease models discussed above, the use of anti-TWEAK neutralizing reagents such as monoclonal antibodies or the FN14-Fc soluble decoy receptor has been efficacious in dampening inflammation and ameliorating disease. The FN14-Fc decoy receptor inhibits angiogenesis (47), and has also been shown to dampen ischemia-induced brain damage

in mice after MCAO, a model for stroke (83,84,88,89). Anti-TWEAK treatment decreases inflammatory disease symptoms in a CIA mouse model for human rheumatoid arthritis (76,77) and in a MOG-induced EAE model for multiple sclerosis (87). Anti-TWEAK treatment also ameliorates renal complications in a cGVHD mouse model of the human disease SLE (78), as well as muscle loss after denervation in a mouse model of skeletal muscle atrophy (81). Currently, there are a number of therapies on the market targeting TNF activity in inflammatory conditions. It has been suggested that blocking the TWEAK-FN14 pathway may be a safer means of achieving some of the same anti-inflammatory goals because, unlike TNF, TWEAK does not affect the homeostasis and activity of adaptive immune cells.

Anti-FN14 therapy has another potential application, which is just starting to be explored. Since FN14 is highly expressed in solid tumors, it is a potential tumor antigen that can be used as a therapeutic target. For example, an anti-FN14 monoclonal antibody, ITEM-4, has been fused to recombinant gelonin (rGel), which is a cytoxic ribosome-inactivating *N*-glycosidase (90). This reagent was internalized specifically by FN14-expressing tumor cell lines, and induced cell death (90). The conjugated antibody also inhibited tumor growth in mice bearing a bladder carcinoma line xenograft tumor (90). A point to take notice of, for the purposes of this thesis, is the fact that the anti-FN14 antibody was internalized quite rapidly, within 2 hours. The ITEM-4 antibody was first identified as a monoclonal antibody that can induce apoptosis in sensitive cell lines (91). To date, it has not been reported that FN14-undergoes ligand-dependent internalization, and the rapid internalization of ITEM-4 in this study was not addressed.

FN14 stability and downregulation

During this project, we attempted to discern novel mechanisms through which FN14 is regulated. It is known that FN14 signaling is tightly regulated at the level of expression. However, once expressed, there is a positive feedback loop which continues to induce expression of FN14. Since there is evidence of increased FN14 levels and activity in various autoimmune and chronic inflammatory conditions, it is important to study how the receptor might be regulated to keep inappropriate signaling from occurring. There are several lines of evidence from the literature which indicate that the stability of FN14 and mechanisms of FN14 downregulation require further investigation. For example, FN14 and TWEAK levels increase in response to liver and skeletal muscle injury, and then subside as healing progresses

(60,62). This process of downregulation of the pathway is not fully understood. Since FN14 signaling induces further expression of FN14, the stability of the receptor likely affects the duration of the TWEAK-FN14 response in response to injury. A related issue is that in FN14 KO mice, TWEAK levels persist after skeletal muscle injury (60). It is not clear in this case if induction of TWEAK is sustained by ongoing inflammation, or if TWEAK requires FN14 for downregulation, perhaps through receptor-mediated internalization. One piece of evidence that supports the possibility of receptor-mediated internalization is the fact that FN14 binding to the ITEM-4-rGel fusion leads to internalization of the antibody within 2 hours (90). Given the lack of clear information surrounding the stability and downregulation of FN14, these aspects of the receptor cell biology required further examination. Since the receptor is highly expressed in many cultured cell lines, an investigation of FN14 downregulation and stability in cultured cells seemed the natural place to begin our study.

Results

The regulation of other TNFRSF members serves as a model when exploring how FN14 levels might be regulated. TNFR1 (30,31) and CD40 (32,33) are known to undergo ligand-induced downregulation, and thus, it seems possible that FN14 might also undergo turnover upon binding of TWEAK. Since TWEAK is known to induce expression of FN14 (51), two different approaches were employed to address this question. We first looked at the levels of endogenous FN14 in HeLa cells upon treatment with increasing amounts of TWEAK. HeLa cells were switched to low-serum non-stimulating conditions 18 hours prior to treatment in order minimize induction of FN14 by serum growth factors. As will be discussed in more detail later, experiments performed under normal 10% serum conditions resulted in saturating levels of FN14, thereby masking perceptible changes in protein level. In parallel to the TWEAK treatment of HeLa cells, TWEAK-mediated turnover was also evaluated in FN14 KO cells that were stably reconstituted with V5 epitope tagged FN14. In the stably reconstituted cells, FN14 is not under the control of the endogenous promoter, and any FN14 loss would not be masked by TWEAK-mediated induction. The cells were treated with increasing concentrations of TWEAK for 30 minutes, a time point also chosen to preclude the contribution of FN14 transcriptional upregulation to receptor levels.

Finally, FN14 levels were assessed by Western blot. The results of both experimental approaches indicated that TWEAK treatment induces loss of FN14 (Figure 6). Furthermore, the ligand-induced turnover of FN14 occurs in the same time frame and in a dose-dependent fashion in both HeLa cells and in stably reconstituted FN14 KO MEFs.

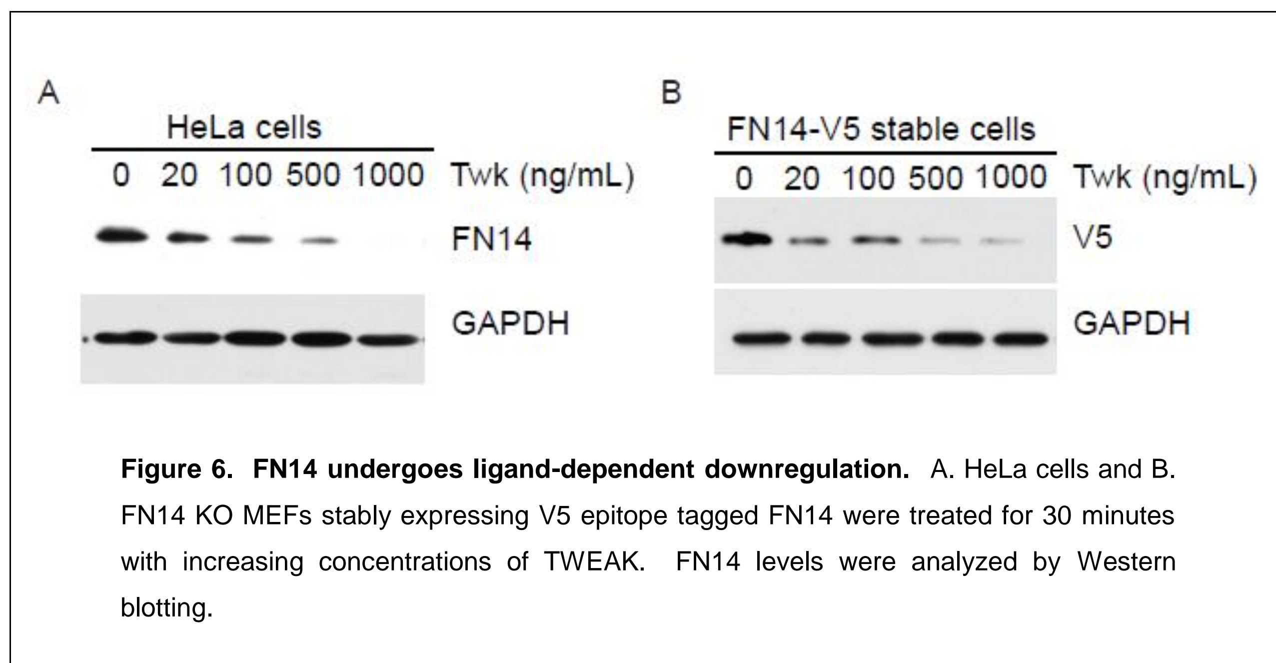

Figure 6. FN14 undergoes ligand-dependent downregulation. A. HeLa cells and B. FN14 KO MEFs stably expressing V5 epitope tagged FN14 were treated for 30 minutes with increasing concentrations of TWEAK. FN14 levels were analyzed by Western blotting.

Our results suggested that ligand destabilizes FN14 and induces turnover of the receptor, but the steady-state stability of the receptor in the absence of ligand has never been examined. Receptor stability can influence the availability of the receptor for signaling and consequently the signaling kinetics, making it an important temporal regulatory factor of receptor activity. In order to investigate FN14 stability, HeLa cells were treated with 20 µg/mL cycloheximide to inhibit protein synthesis for 0, 60, and 120 minutes. Then, FN14 levels were assessed by blotting for FN14 and for control proteins, specifically EGFR as a control receptor and GAPDH as a loading control. Surprisingly, the Western blots showed that FN14 undergoes rapid turnover, with a half-life of about 60 minutes (Figure 7). The levels of the EGFR control, on the other hand, were stable for the duration of the cycloheximide treatment, thereby confirming that rapid turnover is not a common property of cell surface receptors.

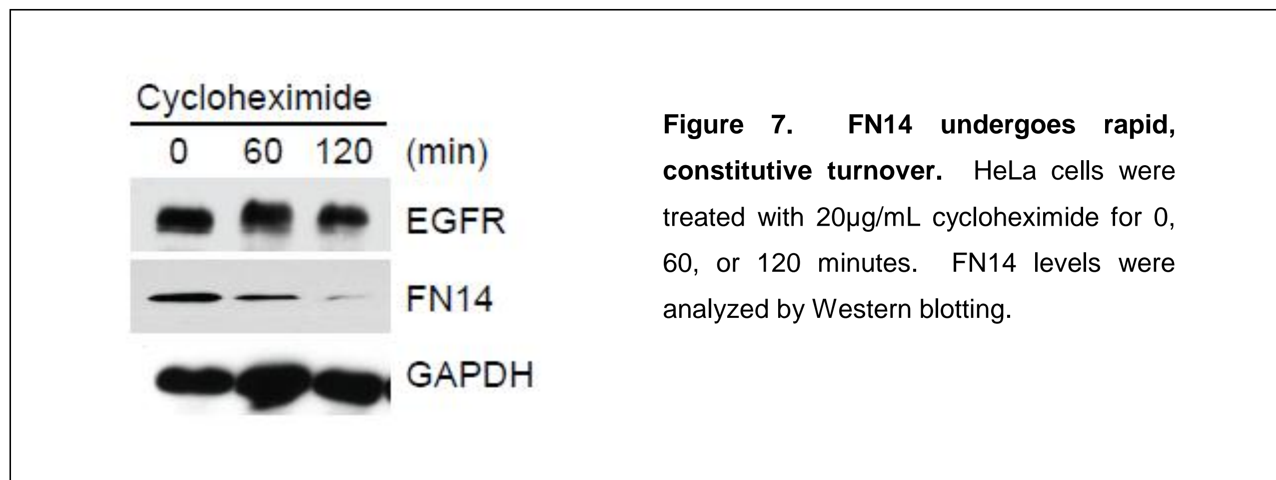

Figure 7. FN14 undergoes rapid, constitutive turnover. HeLa cells were treated with 20μg/mL cycloheximide for 0, 60, or 120 minutes. FN14 levels were analyzed by Western blotting.

In order to confirm that the rapid constitutive turnover of FN14 was truly independent of TWEAK and was not a result of basal levels of TWEAK in the media, it was necessary to neutralize TWEAK using a monoclonal antibody. Thus, HeLa cells were pre-treated with 10μg/mL of anti-TWEAK antibody, an established neutralizing concentration (91), or with an IgG isotype control. Then turnover was assessed in the presence of cycloheximide for 0, 60, or 120 minutes. To verify that the anti-TWEAK treatment was effective, HeLa cells were also treated with 100 ng/mL TWEAK pre-complexed with 10 μg/mL of an IgG isotype control antibody or with 10 μg/mL anti-TWEAK. The ability of the stimulus to induce IκBα phosphorylation was then assayed after 0, 5, and 30 minutes. The stimulation control experiment showed that the TWEAK complexed with anti-TWEAK did not stimulate the cells, confirming that the concentration of anti-TWEAK used was in fact neutralizing (Figure 8A). Even in the presence of the neutralizing antibody, however, there was rapid turnover of FN14 upon cycloheximide treatment (Figure 8B). This verifies that FN14 not only undergoes ligand-dependent turnover as shown previously, but that it also turns over constitutively even in the absence of ligand.

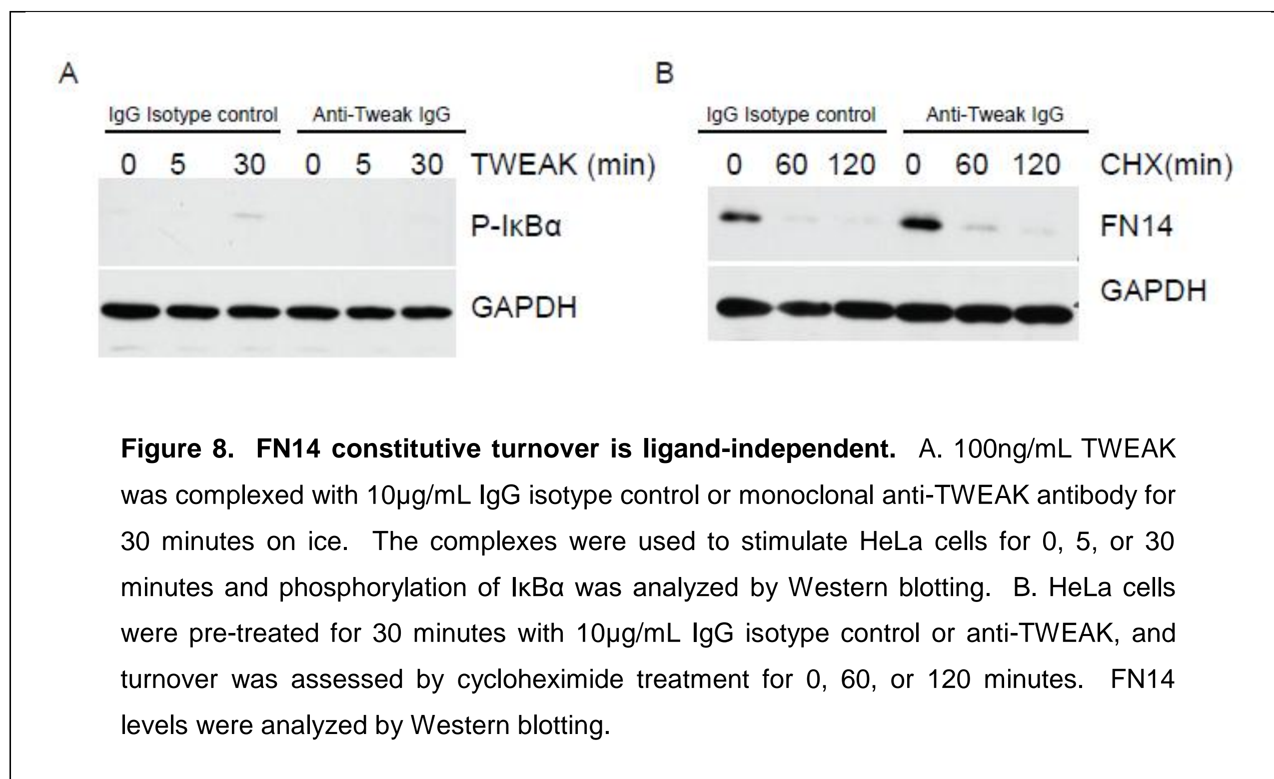

Figure 8. FN14 constitutive turnover is ligand-independent. A. 100ng/mL TWEAK was complexed with 10μg/mL IgG isotype control or monoclonal anti-TWEAK antibody for 30 minutes on ice. The complexes were used to stimulate HeLa cells for 0, 5, or 30 minutes and phosphorylation of IκBα was analyzed by Western blotting. B. HeLa cells were pre-treated for 30 minutes with 10μg/mL IgG isotype control or anti-TWEAK, and turnover was assessed by cycloheximide treatment for 0, 60, or 120 minutes. FN14 levels were analyzed by Western blotting.

One method to show that two pathways do in fact occur by distinct mechanisms is to show synergy between them. In order to examine the possibility of a synergistic effect of ligand-dependent and ligand-independent turnover, HeLa cells were treated with either cycloheximide alone, TWEAK alone, or the two together for 0, 30, or 60 minutes. Then once again, turnover was assessed by Western blot. The treatment with both TWEAK and cycloheximide resulted in more rapid and robust loss of FN14 than treatment with either compound alone (Figure 9). This result demonstrates that the two pathways are indeed synergistic, reinforcing that they represent distinct mechanisms of receptor turnover.

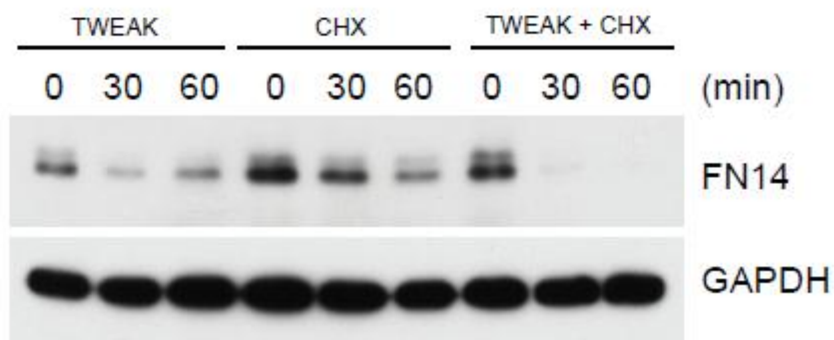

Figure 9. Ligand-dependent and constitutive turnover of FN14 are synergistic processes. HeLa cells were treated with 100ng/mL TWEAK, 20μg/mL cycloheximide, or the two compounds together for 0, 30, or 60 minutes. FN14 levels were analyzed by Western blotting.

In addition to HeLa cells, FN14 is reported to be expressed by many other epithelial and endothelial cell types. In order to verify that rapid constitutive turnover of the receptor is a universal occurrence, it was necessary to examine receptor turnover in various cells types. HEK 293 cells, primary murine keratinocytes, and primary human umbilical vein endothelial cells (HUVEC) were treated with cycloheximide for 0, 60, or 120 minutes and the lysates were blotted for FN14 and GAPDH. In all of the cells types tested, FN14 has a half-life of about 60 minutes or less, suggesting that rapid constitutive turnover is not cell-type specific and may be a general property of FN14 biology (Figure 10).

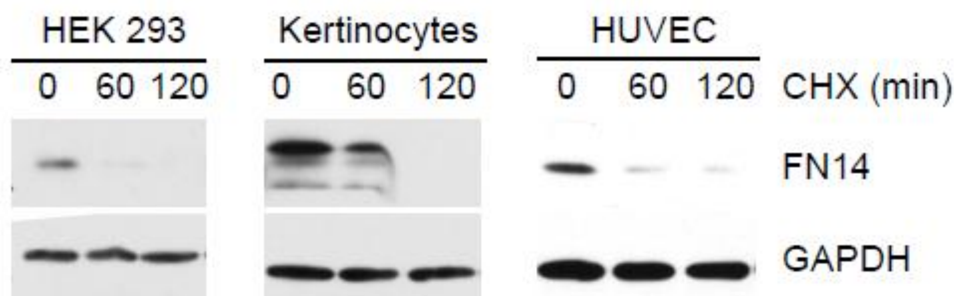

Figure 10. FN14 constitutive turnover occurs in various cell types. HEK 293 cells, primary murine keratinocytes, and human umbilical vein endothelial cells (HUVEC) were grown in low serum conditions and treated with 20µg/mL cycloheximide for 0, 60, or 120 minutes. FN14 levels were analyzed by Western blotting.

Discussion

We have determined that FN14 undergoes both ligand-dependent and constitutive turnover. These aspects of FN14 biology have not been previously described. Ligand-dependent downregulation of FN14 occurs within 30 minutes, while the half-life of unliganded FN14 is about 60 minutes. These two processes occur in an additive fashion, and thus seem to represent distinct processes. Furthermore, constitutive turnover of FN14 occurs in multiple tumor and primary cell types, suggesting that it is a general property of the receptor.

The finding that FN14 undergoes ligand-dependent turnover could help to explain how the TWEAK-FN14 pathway might be downregulated upon resolution of wound healing. TWEAK binding and subsequent receptor turnover might result in fewer cell surface receptors available for signaling. This would decrease the sensitivity to ligand, thereby limiting the positive feedback loop that leads to amplification of FN14 levels. This hypothesis is loosely supported by the skeletal muscle injury model in which TWEAK persists in FN14 KO mice (60). It is possible that TWEAK persists because it is normally downregulated through binding of FN14.

The finding that FN14 undergoes constitutive turnover, even in the absence of ligand, is novel. We will further dissect the mechanism of FN14 constitutive turnover in Chapter 2 of this thesis.

Chapter 2: Spatiotemporal Regulation of FN14

Introduction

Receptor endocytosis and vesicular trafficking

Endocytosis and endosomal sorting play an important role in regulating the surface expression of cellular receptors as well as the downstream signal cascades activated by receptor ligation. There are a number of mechanisms of receptor internalization. Receptors can be delivered to early endosomal vesicles through clathrin-dependent endocytosis, caveolin-dependent endocytosis, or through pathways that are independent of both clathrin and caveolin (92). Ubiquitination often serves as a sorting signal for receptors internalized through a clathrin-dependent mechanism (93). From the early endosome, the receptor can either enter a recycling pathway or a degradation pathway (94). The receptors that are ultimately degraded are transported through the late endosome to multivesicular bodies (MVB) (93). The receptors are sorted in the MVB by proteins of the ESCORT complex (Endosomal Sorting Complex Required for Transport), which can bind ubiquitinated cargo, deform the membrane, and pinch off vesicles (93). The receptors targeted for degradation are transported to the lysosome through temporary membrane fusion allowing for transfer of cargo (93,95). Alternatively, the receptors destined for the recycling pathway are sorted in the early/sorting endosome, and then transported back to the plasma membrane (93,94). In some cases, endocytosed receptors are also transported from the early endosome back to the endoplasmic reticulum or Golgi complex for reprocessing (93,95). Finally, endocytosis of receptors not only results in regulation of receptor levels, but can also influence downstream signaling pathways (93). For example, ubiquitination might label receptors for transport and retention in signaling endosomes, thereby allowing for activation of particular signal cascades (93). Prolonged retention in a signaling compartment may also promote recycling back to the plasma membrane (96). A schematic of receptor endocytosis and the subsequent fates of the receptor are depicted in Figure 11.

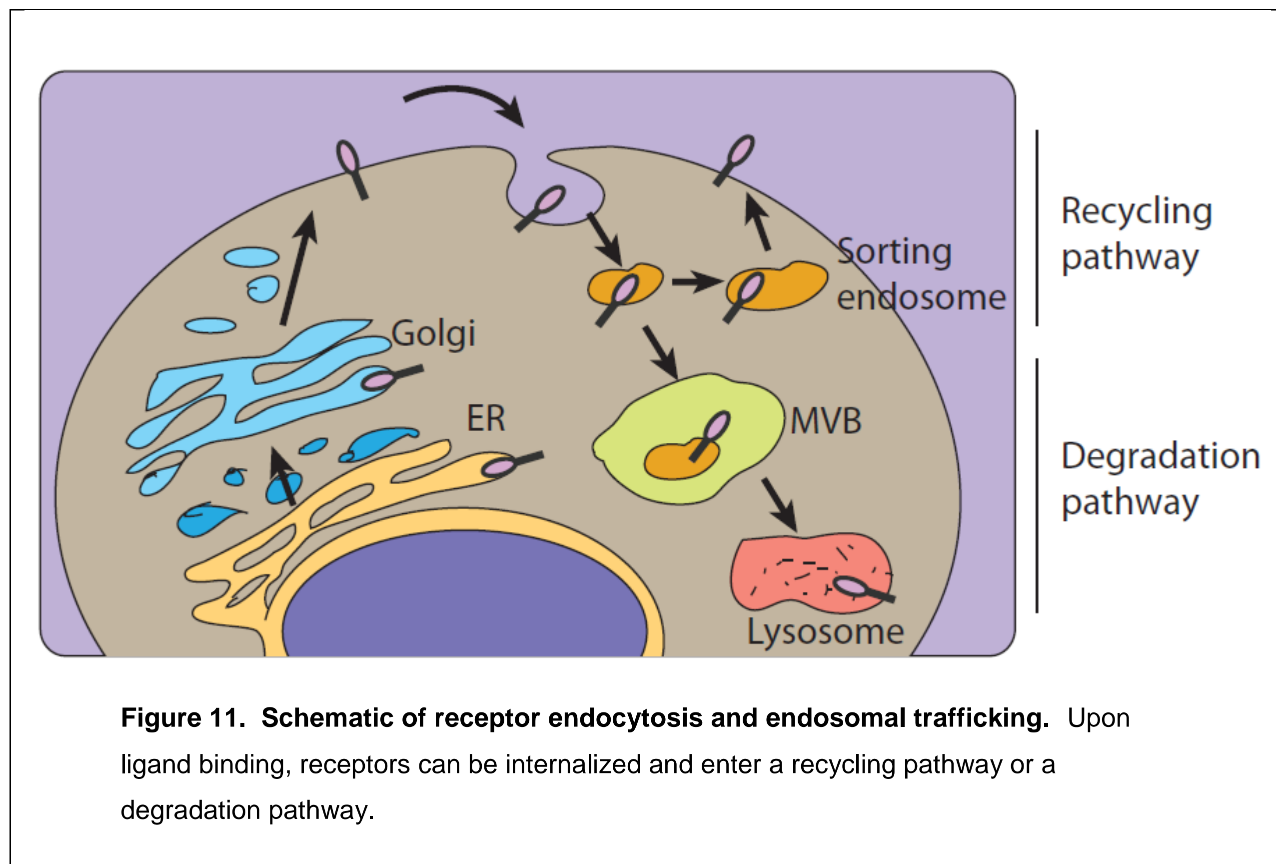

Figure 11. Schematic of receptor endocytosis and endosomal trafficking. Upon ligand binding, receptors can be internalized and enter a recycling pathway or a degradation pathway.

TNFRSF trafficking

The fate of TNFRSF members in the presence of ligand has been well studied. The cell biology of many of these receptors was originally characterized using iodinated ligand, often before the receptors were identified or cloned. As a result, little has been done to look at steady-state levels and localization of the receptors in the absence of ligand. These are important factors as well because they determine the availability of receptors for ligand-induced signaling, and also control the quiescence of the signaling pathway in the absence of stimulation.

It is predominantly thought that TNFR1 localizes to storage pools in the Golgi (97,98). However, previous work in the lab has suggested that this localization actually represents a receptor that is being constitutively synthesized, trafficked, and degraded. In the absence of ligand, TNFR1 seems to undergo rapid turnover, with a half-life of about 30 minutes. This facet of TNFR1 cell biology was proposed by another group many years ago (99). However, their results suggested that TNFR1 constitutive turnover

occurs only in a cell-type specific manner and at much slower kinetics, and their model was never incorporated into the main-stream view of how TNFR1 is regulated (99). Results from our lab suggest that the constitutive turnover of TNFR1 is rapid, occurs in all cells, and requires the extracellular domain of the receptor. Another receptor, Notch, is known to undergo this type of constitutive turnover. In the presence of ligand, Notch requires endocytosis for full activation (100). In the absence of ligand, Notch undergoes continuous endocytosis, thereby preventing spontaneous activation (100). It is thought that differential ubiquitination regulates the degradation/recycling routes of unliganded Notch and the activation of liganded Notch (100).

There is also preliminary evidence from our lab to suggest that, in the absence of ligand, TNFR1 may be internalized through association with a trafficking receptor of the Vps10-domain family. Another TNFRSF member, the neurotrophin receptor $p75^{NTR}$, has been shown to associate with sortilin, a member of this VPS10-domain family of trafficking receptors (101). Sortilin is internalized through clathrin-coated pits, delivers internalized ligands to lysosomes, and transports cargo from the Golgi network to endosomes (101). Sortilin association with $p75^{NTR}$ is crucial for the pro-apoptotic function of the receptor, suggesting that trafficking of the receptor may influence its functionality (101). Another recent study has suggested that low-density lipoprotein-related protein (LRP-1), which is known to function in receptor-mediated endocytosis and cell signaling, may regulate cell-surface levels of TNFR1 through an unknown mechanism (102). This LRP-1-mediated control of TNFR1 levels is thought to contribute to regulation of the IKK/NF-κB pathway (102). Given these findings, it seems likely that receptor trafficking, perhaps through association with a trafficking receptor, represents an important, but understudied, aspect of TNFRSF biology.

Receptor trafficking and endocytosis in TNFR1-related inflammatory disease

There is evidence to suggest that aberrant TNFR trafficking may actually contribute to disease. TNFR-associated periodic syndrome (TRAPS) is an autosomal dominant autoinflammatory disorder, which is characterized by prolonged fever attacks, abdominal and muscle pain, migratory rash, myalgia, and periorbital edema (103). As previously discussed, TNFR1 cell signaling pathways are regulated by intracellular trafficking and TNFR1 compartmentalization. TRAPS results from missense mutations in the

TNFR1 extracellular domain, which affect receptor folding and trafficking. However, different models of trafficking dysfunction have been proposed for different TRAPS mutations, making it difficult to understand the exact cause of the disease. For example, it has been suggested that the TRAPS mutations lead to cellular dysfunction and ultimately pathology through inhibition of shedding, inappropriate ligand-independent signaling, and intracellular receptor accumulation (103).

The shedding hypothesis resulted from the observation that patients with the C33Y, T50M, C52F, and C88R TNFR1 mutations demonstrate lower levels of soluble TNFR1 between attacks and during attacks in comparison to control patients (103). The ligand-independent signaling hypothesis was based on several observations. First, the T50K mutation results in increased cell surface expression of the receptor in comparison to wildtype TNFR1, and this stabilization is associated with increased NF-κB activation in a manner that is likely independent of TNF (103). Second, it has been shown that T50M and C33Y mutations in macrophages cause enhanced activation of JNK and p38 MAPK, and that LPS-induced MAPK activation in these cells is independent of TNF (103). It is thought that ligand-independent receptor activation of reactive oxygen species initiates MAPK signaling in these cells (103). Finally, the intracellular accumulation hypothesis resulted from several observations as well. It has been shown that some TRAPS mutations cause both intracellular accumulation and increased stability of the receptor (104,105). These mutants are often retained in the endoplasmic reticumulum (ER), likely due to abnormal oligomerization of the mutant receptors through non-physiological disulfide bonding (103). It is thought that the ER accumulation of these misfolded proteins might induce inflammatory signaling through ER stress and the unfolded protein response (103). In contrast, the C73R mutation has been shown to result in elevated levels of cell surface TNFR1 in PBMCs isolated from TRAPS patients, thereby leading to enhanced NF-κB activation (103).

Despite the various properties of the different TNFR1 mutations, the majority of the mutations seem to result in enhanced NF-κB activation (103). Furthermore, cells from TRAPS patients exhibit ongoing inflammatory activity even under resting conditions and TRAPS mutations in patients lead to elevated serum levels of pro-inflammatory cytokines (103). These results suggest a model in which misfolding of TNFR1 due to the TRAPS mutations causes aberrant trafficking and thereby influences receptor oligomerization and signaling capacity.

FN14 receptor trafficking

Given our finding that FN14 undergoes ligand-dependent and constitutive turnover, it seems that there are previously unrecognized aspects of FN14 spatiotemporal regulation that require further investigation. We expect that, as for other members of the TNFRSF such as TNFR1, this spatiotemporal regulation is very important for prevention of inappropriate spontaneous activation of FN14. In this chapter, we explore the mechanism of constitutive turnover of FN14 and follow the fate of receptor in the steady-state condition.

Results

The finding that FN14 undergoes rapid constitutive turnover is novel, and thus the mechanism of action has never been explored. Since it was reported that FN14 is primarily localized to the plasma membrane (48), it seems possible that the loss following cycloheximide treatment occurs at the level of cell surface expression. Thus, HeLa cells were treated with cycloheximide for the indicated times, and cell surface FN14 levels were assessed by staining and subsequent flow cytometry. Cycloheximide treatment over increasing amounts of time resulted in continued loss of FN14 cell surface expression (Figure 12A). This loss of cell surface expression of FN14 was also seen in HUVEC after cycloheximide treatment (Figure 12C). These results suggest that protein synthesis is necessary for the steady-state cell surface localization of FN14. Inhibition of protein synthesis likely prevents expression and trafficking of the receptor to the plasma membrane, thereby leading to lower surface expression. The FACS results do not, however, directly indicate that FN14 is actually lost from the cell surface in the absence of protein synthesis. To test whether FN14 loss occurs from the cell surface, a cell surface biotinylation assay was performed. The preliminary results of this experiment indicate that FN14 constitutive turnover occurs from the cell surface, presumably through receptor shedding or through internalization and degradation (Figure 13). However, these results need to be repeated and further validated.

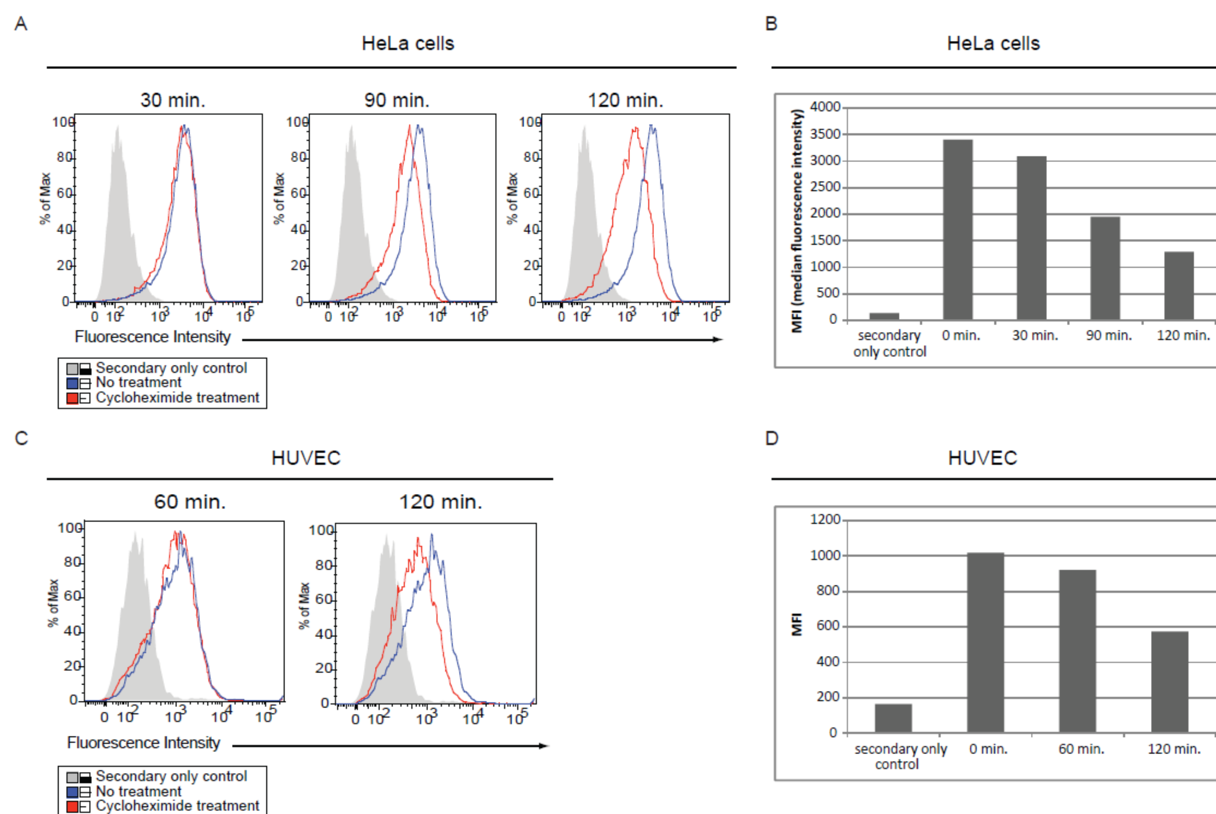

Figure 12. Inhibition of protein synthesis results in loss of FN14 cell surface expression. A. HeLa cells and C. HUVEC were treated with 20µg/mL cycloheximide for the indicated times. Harvested cells were stained for surface FN14 expression using the ITEM-4 primary mAb and an anti-mouse Alexa 546-conjugated secondary antibody. Stained cells were analyzed by flow cytometry. B. The bar graphs represent the median fluorescence intensities of the designated histograms, indicating the relative amount of cell surface FN14 after each time point of cycloheximide treatment in HeLa cells and D. HUVEC.

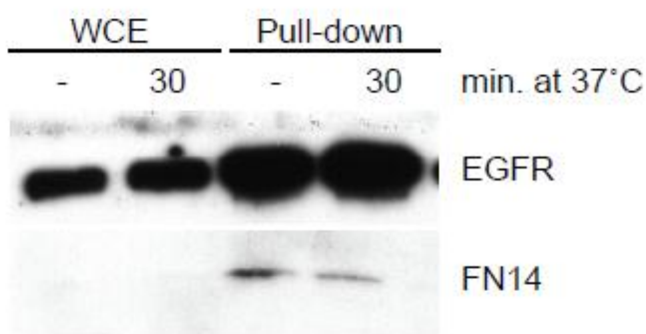

Figure 13. FN14 is lost from the cell surface. HeLa cells were cell-surface biotinylated on ice, and then either incubated on ice or at 37°C for 30 minutes. Lysates were harvested, and the biotinylated proteins were pulled-down using a streptavidin bead slurry. The pull-down samples were boiled in reducing SDS-sample buffer, and equal volumes were loaded on an SDS-PAGE gel. FN14 and EGFR levels were analyzed by Western blotting.

It is surprising that FN14 turns over so rapidly because, even in low-serum conditions, the receptor is easily detectable in cultured cells by Western blot and by FACS analysis. This suggests that there might be ongoing synthesis of the receptor to replace the rapidly lost receptors. In order to explore this possibility, HeLa cells grown in low-serum conditions were treated with an inhibitor of cellular trafficking, Brefeldin A, which collapses the Golgi complex into the ER (106). Treatment with Brefeldin A blocks receptor trafficking from the Golgi complex to the plasma membrane. If receptor loss occurs only at the cell surface, as indicated by the cell surface FACs analysis and the preliminary cell surface biotinylation results, then constitutive synthesis should lead to FN14 intracellular accumulation after Brefeldin A treatment. After 0, 60, and 120 minutes of inhibitor treatment of the cells, lysates were run on SDS-PAGE and blotted for FN14 and a control protein. There was noticeable accumulation of FN14 protein after the inhibition of trafficking to the cell surface (Figure 14A). In order to verify that inhibition of receptor trafficking to the cell surface is able to protect FN14 from constitutive turnover, HeLa cells were pre-treated for 30 minutes with Brefeldin A or monensin, another trafficking inhibitor which blocks acidification of the Golgi (107), before 120 minute treatment with cycloheximide. Brefeldin A and

monensin pre-treatment blocked the rapid turnover of FN14, indicating that constitutive receptor synthesis and trafficking to the cell surface is a property of the receptor that precedes constitutive turnover of the receptor (Figure 14B).

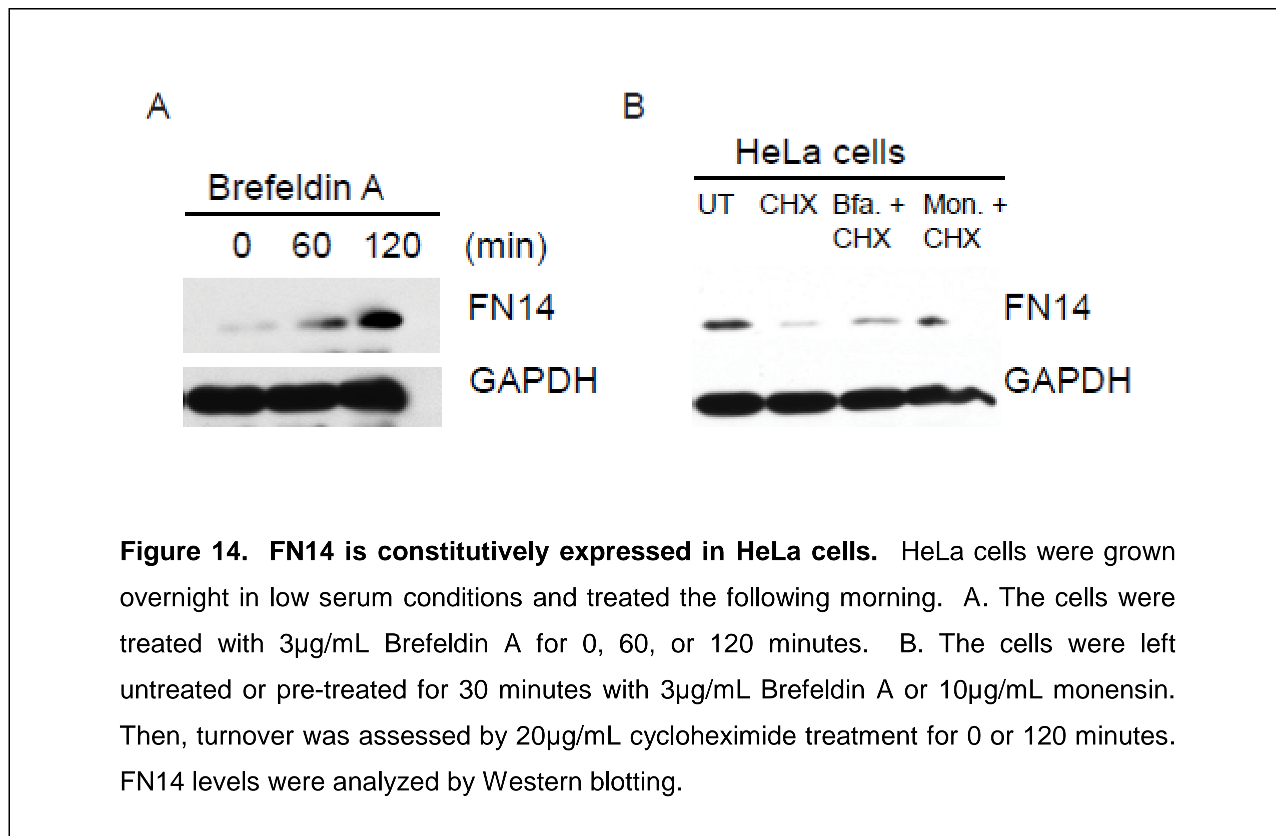

Figure 14. FN14 is constitutively expressed in HeLa cells. HeLa cells were grown overnight in low serum conditions and treated the following morning. A. The cells were treated with 3µg/mL Brefeldin A for 0, 60, or 120 minutes. B. The cells were left untreated or pre-treated for 30 minutes with 3µg/mL Brefeldin A or 10µg/mL monensin. Then, turnover was assessed by 20µg/mL cycloheximide treatment for 0 or 120 minutes. FN14 levels were analyzed by Western blotting.

The Western blotting analysis suggested that FN14 undergoes constitutive synthesis. In order to validate these results using another method, FACS analysis was employed. HeLa cells were treated with or without Brefeldin A for 0, 90, or 150 minutes, and then both the surface levels of FN14 and total levels of FN14 after fixation and permeabilization of the cells were assessed by flow cytometry. As expected, blocking trafficking from the Golgi complex to the plasma membrane resulted in increased levels of total FN14, validating that FN14 is being constitutively synthesized (Figure 15, top panels). The inhibition of trafficking, however, led to decreased levels of cell surface FN14 (Figure 15, bottom panels). This indicates that trafficking to the cell surface is also constitutive, and is needed to replenish surface levels of the receptor.

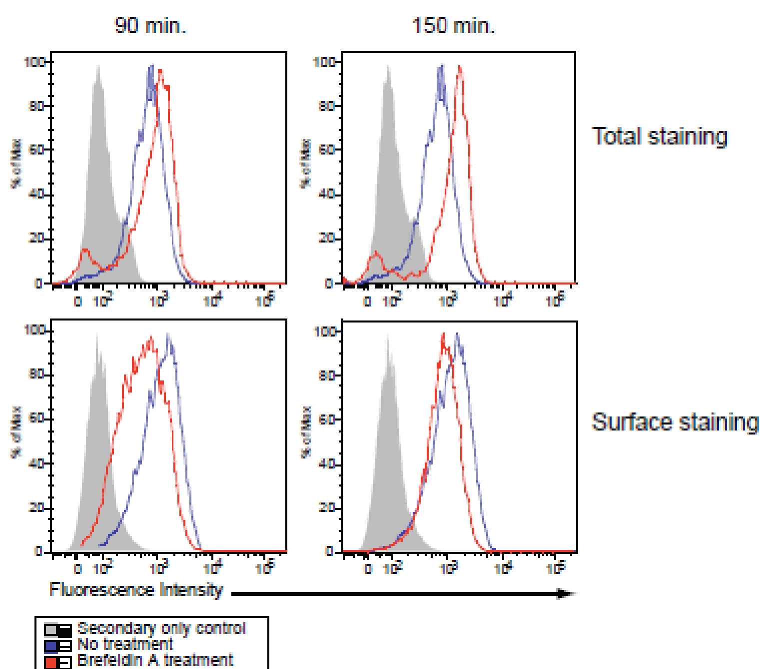

Figure 15. FN14 is constitutively synthesized and trafficked to the cell surface. HeLa cells were treated with Brefeldin A for 0, 90, or 150 minutes. Harvested cells were stained for FN14 surface levels or were permeabilized and stained for total FN14 levels. Stained cells were analyzed by flow cytometry.

Our results, thus far, indicate that FN14 is constitutively synthesized, trafficked, and then lost, which seems to be a very metabolically costly method of regulation of a receptor. In addition, some of the inhibitors used to define this mechanism have been proposed to have off target effects such as inhibition of endocytosis. In order to further validate our unexpected results, fluorescence microscopy was employed to visualize the trafficking and localization of the receptor. HeLa cells were grown on coverslips under low-serum conditions, and the cells were treated with various inhibitors to block protein synthesis or receptor trafficking. The cells were then fixed and permeabilized, and an anti-FN14 primary antibody and fluorophore-conjugated anti-mouse secondary antibody were used to stain the samples for FN14, along with DAPI to stain the nuclei. Both conventional fluorescent images and confocal z-stack images were taken to determine the localization of the receptor. The untreated control cells showed a punctate staining pattern for FN14 (Figure 16). After the cells were treated with cycloheximide, there were

noticeably less FN14 puncta, and the few remaining spots were smaller than those in the untreated sample (Figure 16). This correlates with the receptor loss that becomes apparent by Western blotting upon cycloheximide treatment. To test whether there is constitutive synthesis of the receptor, the cells were treated with Brefeldin A or monensin, before staining and visualization of FN14. Brefeldin A treatment for 3 hours resulted in a significant increase in FN14 levels, as assessed by the increased fluorescence (Figure 16). Since Brefeldin A causes collapse of the Golgi complex, there was a drastic alteration in localization of the accumulated receptor, as seen by the loss of the punctate staining. The 3 hour treatment with monensin also resulted in an accumulation of the receptor (Figure 16). Monensin should not destroy the Golgi structure, however, and accordingly, the staining after monensin treatment showed an increase in fluorescence at a peri-nuclear structure, most likely indicating accumulation at the Golgi complex. Taken together, these images confirm the model that FN14 is constitutively synthesized, trafficked, and then rapidly lost.

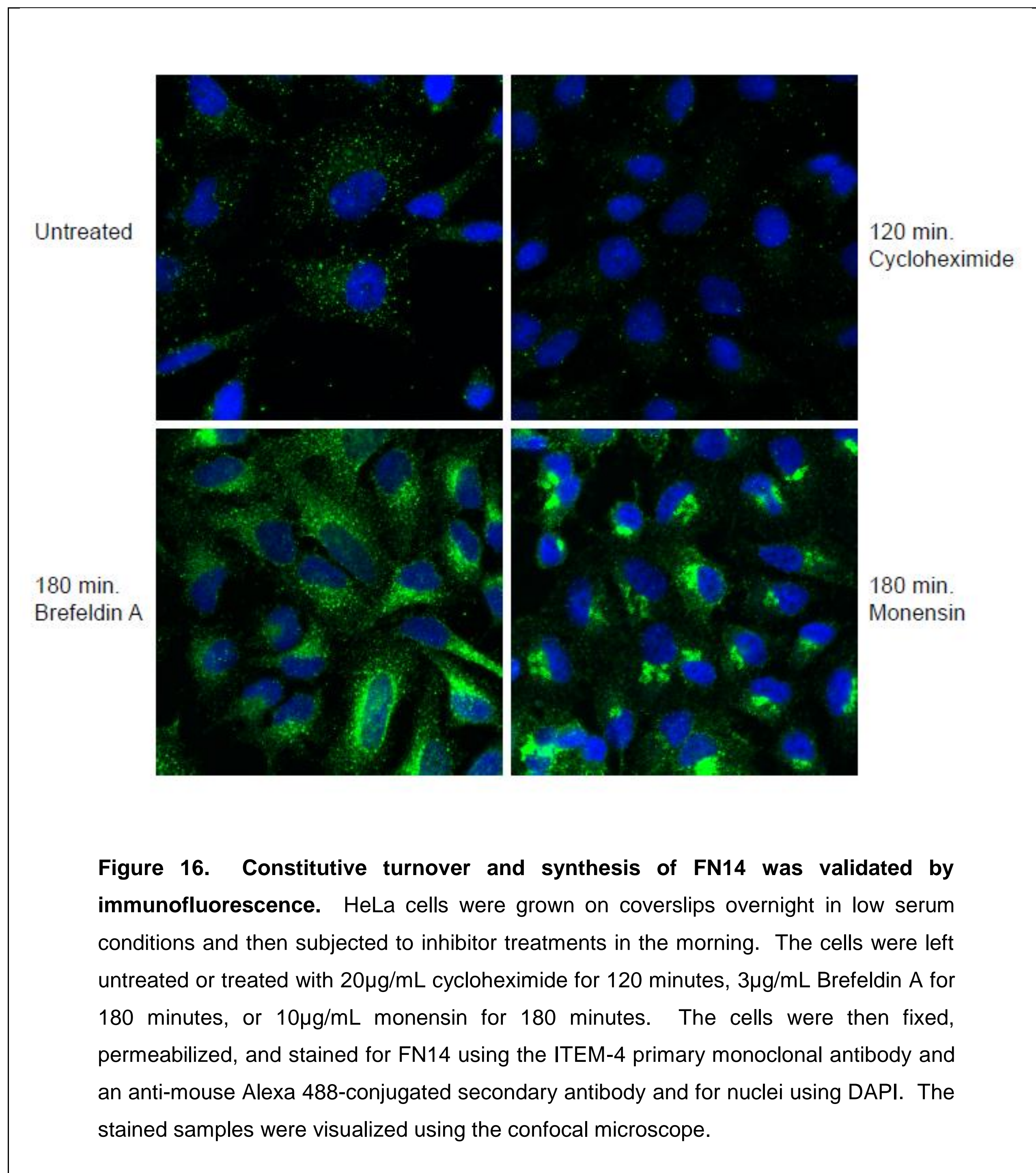

Figure 16. Constitutive turnover and synthesis of FN14 was validated by immunofluorescence. HeLa cells were grown on coverslips overnight in low serum conditions and then subjected to inhibitor treatments in the morning. The cells were left untreated or treated with 20µg/mL cycloheximide for 120 minutes, 3µg/mL Brefeldin A for 180 minutes, or 10µg/mL monensin for 180 minutes. The cells were then fixed, permeabilized, and stained for FN14 using the ITEM-4 primary monoclonal antibody and an anti-mouse Alexa 488-conjugated secondary antibody and for nuclei using DAPI. The stained samples were visualized using the confocal microscope.

Since constitutive turnover of FN14 seems to occur from the plasma membrane, there are two obvious potential mechanisms of receptor loss, shedding or internalization. FN14 does not have a predicted ectodomain cleavage site, so it is unlikely that the receptor is being shed. However, a number

of other TNFRSF members are subject to cleavage by TACE, with subsequent shedding. Thus, the TACE inhibitor TAPI-I was used to examine the role of TACE in FN14 constitutive turnover. HeLa cells were pre-treated with or without an established working concentration of TAPI-I for 30 minutes, and then treated with or without cycloheximide for 2 hours. Inhibition of TACE did not seem to affect FN14 turnover (Figure 17). It is notable that the Western blot from this experiment contained two bands for FN14, potentially suggesting glycosylation of the protein. Although FN14 glycosylation has not been previously reported, we noticed this FN14 doublet in occasional experiments. Thus, a more thorough examination of FN14 modification is required.

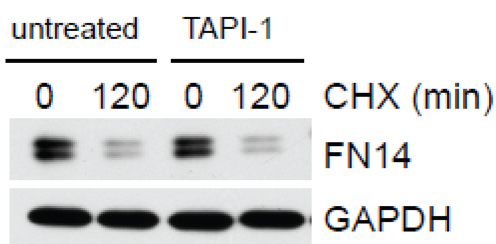

Figure 17. TACE is not involved in the constitutive turnover of FN14. HeLa cells were left untreated or pre-treated with 25uM TAPI-1 for 30 minutes, and then subjected to cycloheximide treatment for 0 or 120 minutes. FN14 levels were analyzed by Western blotting.

The result of the TACE inhibition experiment does not rule out a possible role of other sheddases in FN14 loss. Thus, an additional approach was taken to address the possibility of FN14 shedding. HeLa cells were left untreated, or treated with TWEAK or cycloheximide for 6 hours, and the cell culture supernatant was immunoprecipitated for shed FN14. The preliminary results indicate that TWEAK and cycloheximide treatment do not induce shedding of the receptor (data not shown). However, these results are in the process of being validated.

The role of endocytosis in FN14 receptor loss was next evaluated. To test this, HeLa cells were treated either with a DMSO control, with sucrose, an osmotic inhibitor of endocytosis (36), or with butanol, an inhibitor which disrupts formation of clathrin-coated pits (108). After a 30 minute pre-treatment with

these inhibitors, cycloheximide was added to the cells for 0, 60, or 120 minutes, as indicated. Treatment with the endocytic inhibitors stabilized receptor levels and blocked turnover of the receptor (Figure 18). Treatment with the endocytic inhibitors also generally decreased levels of FN14, potentially through an off target effect on cellular proteases. This possibility will be discussed in more detail later in this chapter.

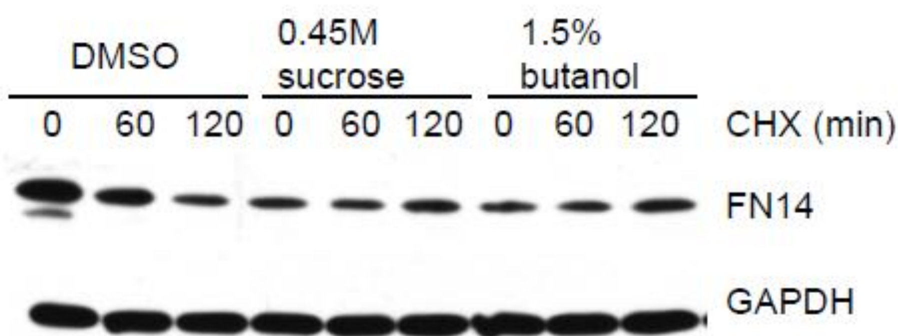

Figure 18. FN14 constitutive turnover is mediated by endocytosis. HeLa cells were pre-treated with DMSO, hyperosmotic sucrose, or butanol for 30 minute, and then subjected to cycloheximide treatment for 0, 60, or 120 minutes. FN14 levels were analyzed by Western blotting.

Endocytosis seems to be important for the turnover of FN14, but the mechanism of receptor loss after endocytosis is still elusive. In order to test whether proteasomal degradation could be involved in the rapid turnover of FN14, the proteasome inhibitors epoxomicin and MG-132 were employed. HeLa cells were pre-treated for 30 minutes with the proteasome inhibitors and then treated with cycloheximide for 0 or 120 minutes. Then FN14 levels were evaluated by Western blot. The results were quite surprising because epoxomicin had no effect on turnover, whereas MG-132 efficiently blocked turnover of FN14 (Figure 19A). To verify that the inhibitors were actually effective in blocking the proteasome, the ability of inhibitor pre-treatment to stabilize IκBα after stimulation was determined. The Western blot for this experiment contained a non-specific band above IκBα, which is an established artifact of the particular antibody used for blotting. However, the blot indicated that the inhibitors blocked degradation of IκBα, suggesting that the differential effects of the inhibitors on FN14 turnover were likely not a result of partial inhibition of the proteasome (Figure 19B). There are reports in the literature implicating

epoxomicin as a more specific inhibitor of the proteasome than MG-132. It has been suggested that MG-132 affects not only proteasomal degradation but also the activity of particular lysosomal hydrolases (109,110). Thus, the differential effects of epoxomicin and MG-132 in our assay could denote a role of lysosomal hydrolases in FN14 constitutive turnover.

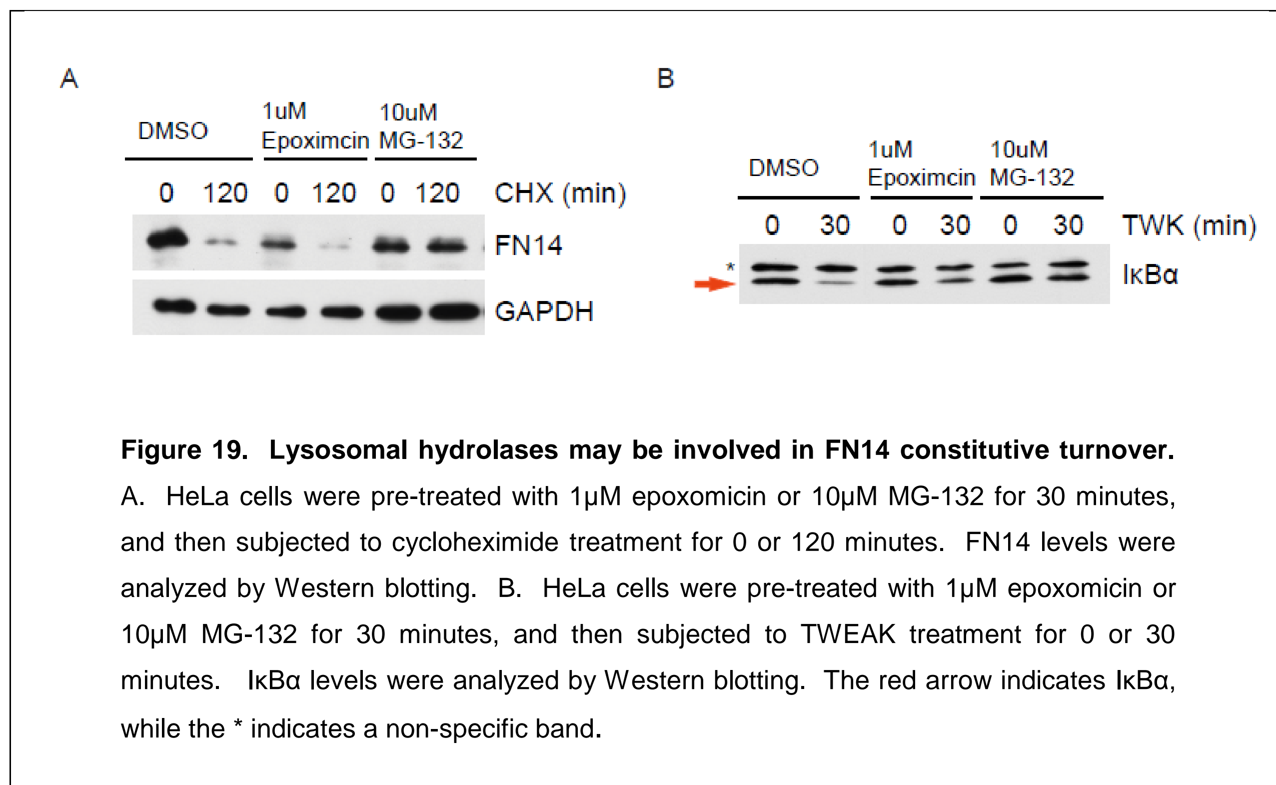

Figure 19. Lysosomal hydrolases may be involved in FN14 constitutive turnover. A. HeLa cells were pre-treated with 1μM epoxomicin or 10μM MG-132 for 30 minutes, and then subjected to cycloheximide treatment for 0 or 120 minutes. FN14 levels were analyzed by Western blotting. B. HeLa cells were pre-treated with 1μM epoxomicin or 10μM MG-132 for 30 minutes, and then subjected to TWEAK treatment for 0 or 30 minutes. IκBα levels were analyzed by Western blotting. The red arrow indicates IκBα, while the * indicates a non-specific band.

To further examine the role of lysosomal degradation in FN14 turnover, inhibitors of endosomal acidification were employed. HeLa cells were pre-treated with ammonium chloride or chloroquine for 4 hours, and then incubated with cycloheximide for 0 or 120 minutes. Unfortunately, treatment with these general inhibitors of endosomal acidification had somewhat unexpected results. Inhibitor treatment alone caused a decrease in receptor levels, even in the absence of cycloheximide (Figure 20A, left panel). We hypothesized that the inhibitors might be inducing loss through an off-target mechanism such as shedding. Thus, a more specific inhibitor of lysosomal hydrolases, leupeptin, was tested for effects on turnover. HeLa cells were pre-treated with leupeptin or ammonium chloride for 4 hours, and then incubated with cycloheximide for 0 or 120 minutes. Ammonium chloride treatment resulted in receptor loss, as seen previously, but leupeptin treatment resulted in slight receptor accumulation (Figure 20A,

right panel). The leupeptin treatment did not, however, completely block receptor turnover in HeLa cells. Since leupeptin mainly inhibits cysteine, serine, and threonine proteases, it is likely that other proteases are also involved in FN14 constitutive turnover. On a technical note, these experiments need to be repeated in HeLa cells for validation and for construction of figures containing proper loading controls.

Since the lysosomal inhibitors had varied effects on FN14 levels in HeLa cells, the inhibitors were also tested in FN14 KO MEF cells stably reconstituted with FN14. The reconstituted cells were pre-treated with leupeptin, ammonium chloride, and MG-132 for 4 hours, and then treated with cycloheximide for 0 or 120 minutes. All of the inhibitors blocked constitutive turnover of FN14 to some extent, although MG-132 had the strongest effect (Figure 20B). Thus, in these cells, lysosomal degradation seems clearly to be involved in FN14 constitutive turnover. The finding that the general inhibitors of endosomal acidification have varying effects in HeLa cells versus MEF cells could be due to cell type specific differences. We are exploring this possibility further, and will come back to this idea in the discussion section of this chapter.

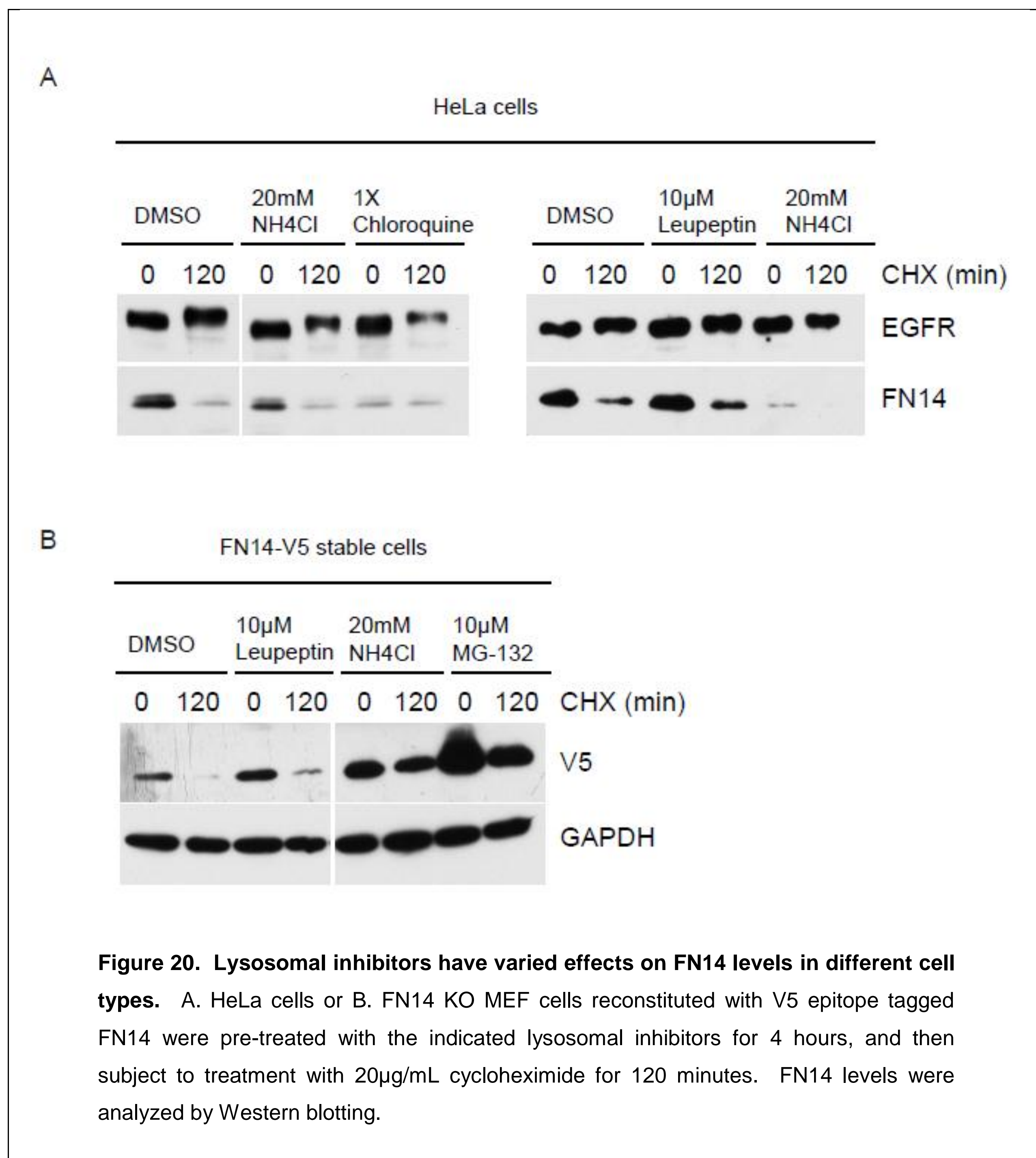

Figure 20. Lysosomal inhibitors have varied effects on FN14 levels in different cell types. A. HeLa cells or B. FN14 KO MEF cells reconstituted with V5 epitope tagged FN14 were pre-treated with the indicated lysosomal inhibitors for 4 hours, and then subject to treatment with 20µg/mL cycloheximide for 120 minutes. FN14 levels were analyzed by Western blotting.

The results of the lysosomal inhibition experiments were not entirely clear, and thus another approach was needed to address whether FN14 is truly degraded in lysosomes. In order to further examine this possibility, an FN14 C-terminal mCherry fusion was constructed and clonal cell lines stably expressing the fusion were isolated through transduction of FN14 KO MEFs. The FN14-mCherry

construct turned over upon cycloheximide treatment, although the kinetics were a bit slower than the kinetics that had previously been determined for the endogenous receptor (Figure 21A). In order to visualize localization of the receptor, cells stably expressing FN14-mCherry were plated in a glass-bottom dish to allow for live cell imaging. Untransduced FN14 KO MEFs were grown in parallel as a negative control for microscopy (Figure 21B, top panels). For the duration of the experiment, the FN14-mCherry stably expressing cells were grown in the presence of lysotracker, to stain the acidic compartments in the cell, namely the late endosomes and lysosomes. After lysotracker staining for 2 hours, confocal microscopy was utilized to assess co-localization of FN14-mCherry with the lysotracker-stained compartments. Multiple z-stack images were taken, and individual slices were compared to determine qualitative differences in localization due to the inhibitor treatment. In untreated cells there was a significant amount of co-localization of FN14-mCherry with the lysotracker stained compartments, denoted by white arrows in the figure (Figure 21B, middle panels). In order to verify that the apparent co-localization was real, Brefeldin A treatment was used to disrupt normal receptor trafficking. As expected, this treatment led to accumulation of FN14-mCherry, indicated by brighter and more prevalent mCherry fluorescence. In addition to the increase in amount of the fusion receptor, Brefeldin A treatment also disrupted the co-localization between FN14-mCherry and the acidic compartments, thereby leading to increased regions of FN14-mCherry that did not co-localize with the acidic compartments (Figure 21B, bottom panels). The steady-state co-localization of FN14 with acidic compartments suggests either that FN14 is degraded in lysosomes or that localization to endosomes represents a previously unknown aspect of FN14 biology.

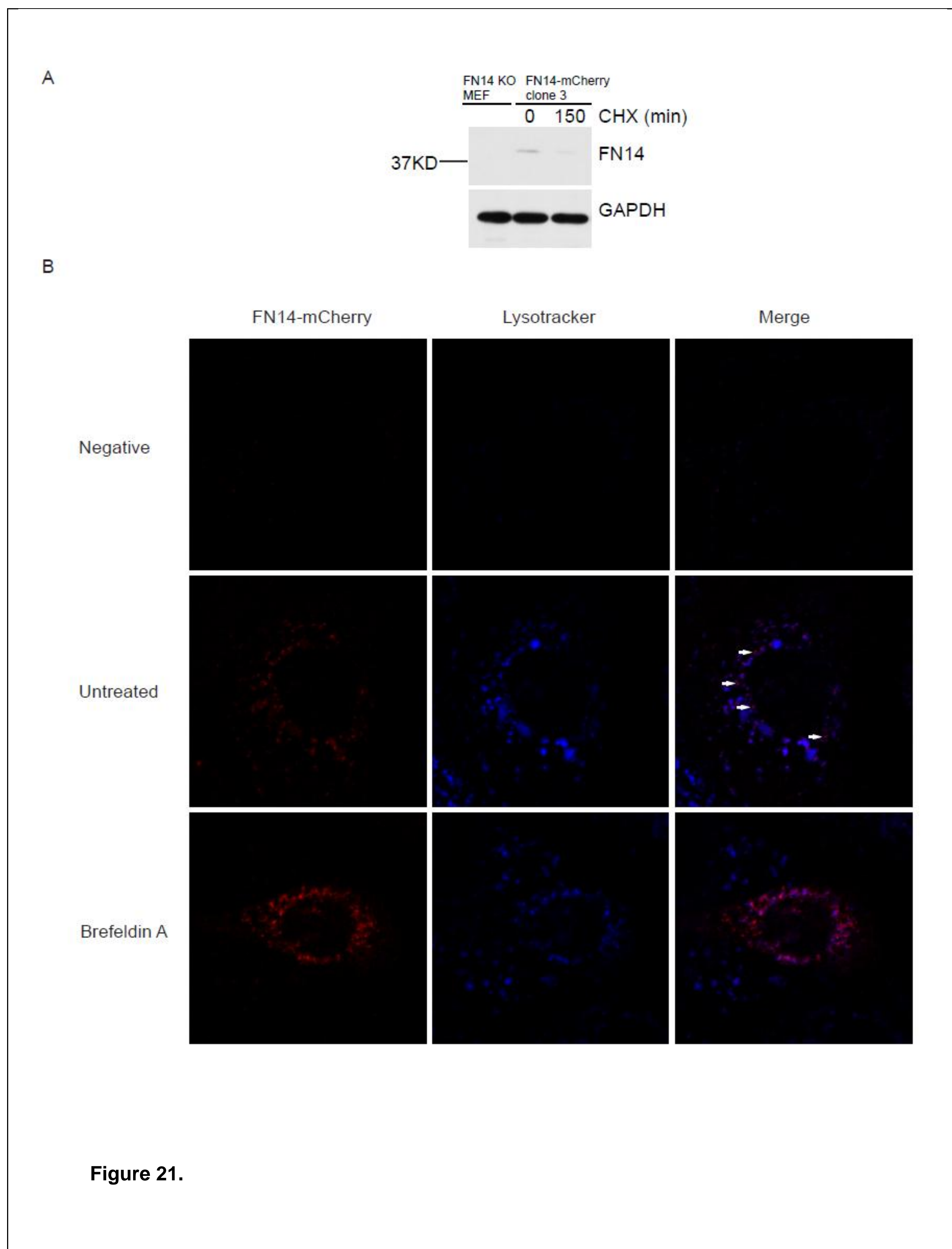

Figure 21.

Figure 21. FN14-mCherry co-localizes with late endosomes and lysosomes. A. A clonal FN14 KO MEF line stably expressing FN14-mCherry was treated with 20μg/mL cycloheximide for 0 or 150 minutes. FN14 levels were analyzed by Western blotting. B. FN14 KO MEFs (top panels) and the FN14-mCherry clonal cells (middle and bottom panels) were grown on glass bottom dishes overnight. Prior to visualization, the cells were incubated with 75nM lysotracker for 120 minutes, and were concurrently left untreated or treated with 3μg/mL Brefeldin A for 120 minutes. Co-localization of FN14-mCherry with lysotracker was assessed by confocal microscopy. Z-stacks were taken, and co-localization was evaluated for individual slices. White arrows indicate points of co-localization.

In order to address the discrepancies seen with the lysosomal inhibitors, the idea of off-target effects upon receptor shedding was revisited. It has been suggested that treatment with endocytic inhibitors can lead to increased cleavage of proteins retained at the plasma membrane by membrane localized γ-secretase (111). γ-secretase is a protease known to cleave in the intramembrane region of proteins containing short ectodomains, thereby releasing the cytoplasmic domains. Generally, γ-secretase substrates are pre-cleaved by a sheddase to produce an intermediate C-terminal fragment of the receptor, before further cleavage in the hydrophobic intramembrane region by γ-secretase. If FN14 is a γ-secretase substrate, this could explain why FN14 loss occurs upon treatment with many of the endocytic inhibitors tested. General inhibitors of endosomal acidification may also affect endosomal trafficking, which could explain why these inhibitors cause receptor loss as well. To test this possibility, HeLa cells were left untreated or treated with the γ-secretase inhibitor DAPT for 6 hours. During this incubation, various compounds and inhibitors, which were previously shown to induce receptor loss, were directly added to the media. TWEAK was added for 30 minutes, cycloheximide was added for 120 minutes, and Brefeldin A, hyperosmotic sucrose, and ammonium chloride were all added for the full 6 hours. Then FN14 levels were analyzed by Western blotting. Surprisingly, DAPT treatment resulted in an accumulation of FN14 in all cases except for in the Brefeldin A treated samples, which remained essentially the same (Figure 22). This fits our model since Brefeldin A treatment leads to intracellular accumulation of the receptor in the Golgi complex, while all of the other inhibitors block later stages of the

FN14 trafficking pathway. γ-secretase activity would likely occur at the plasma membrane or in endosomal compartments. In addition, a second, lower molecular weight band appeared in the DAPT treated samples, which was recognized by the anti-C-terminal-FN14 antibody. These results seem to indicate that FN14 is a substrate of γ-secretase, and that the intermediate C-terminal fragment of the receptor is stabilized upon inhibition of γ-secretase. However, this does not preclude the finding that FN14 is localized to acidic compartments and subject to degradation by lysosomal hydrolases. The DAPT treatment does not completely block ligand-dependent or constitutive turnover in this experiment. Thus, it seems likely that both lysosomal degradation and γ-secretase activity may regulate FN14 levels. This experiment is preliminary, however, and requires further validation.

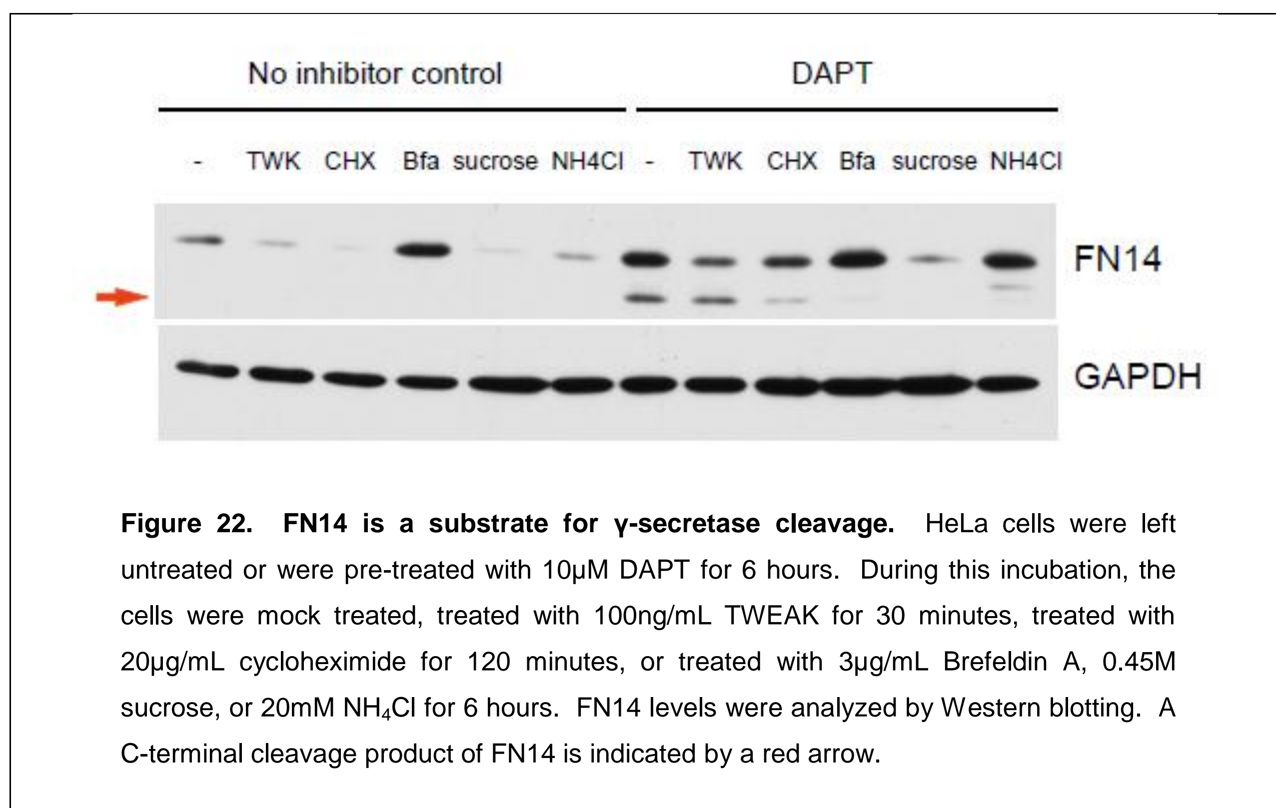

Figure 22. FN14 is a substrate for γ-secretase cleavage. HeLa cells were left untreated or were pre-treated with 10μM DAPT for 6 hours. During this incubation, the cells were mock treated, treated with 100ng/mL TWEAK for 30 minutes, treated with 20μg/mL cycloheximide for 120 minutes, or treated with 3μg/mL Brefeldin A, 0.45M sucrose, or 20mM NH_4Cl for 6 hours. FN14 levels were analyzed by Western blotting. A C-terminal cleavage product of FN14 is indicated by a red arrow.

Discussion

We have found that FN14 undergoes constitutive synthesis, trafficking to the cell surface, internalization, and then degradation in lysosomes. The receptor may also be a substrate for the protease γ-secretase, and may be released through shedding as well. Constitutive trafficking is a

relatively novel mechanism of receptor regulation, and a previously unrecognized property of FN14. We hypothesize that this mechanism prevents inappropriate aggregation of the receptor at the cell surface, while maintaining the proper sensitivity to ligand. In the addition, the possibility of shedding of the receptor suggests that soluble FN14 may also play a regulatory role in the buffering or neutralization of TWEAK.

To fully define the mechanisms of ligand-dependent and ligand-independent turnover, a number of questions will be addressed experimentally. To better understand the ability of FN14 to undergo shedding, further investigation of potential sheddases is required. While TACE activity does not seem to be involved, there is still the possibility of furin cleavage of the receptor. The involvement of furin in FN14 turnover can be assessed by utilizing proprotein convertase inhibitors. In addition, though the preliminary immunoprecipitation experiment did not indicate the presence of shed FN14 in the cell culture media, it is possible that this assay was not sensitive enough to detect shedding. There is a new, commercially available ELISA kit to detect FN14, which may be useful in pursuing this issue. It is possible that the receptor either undergoes ectodomain shedding mediated by a protease, or given the lack of a predicated ectodomain cleavage site, that it may be released in exosomes. These possibilities have not been ruled out, and require further examination.

Our data demonstrated that inhibitor treatments, such as ammonium chloride and chloroquine treatments, resulted in loss of FN14 in HeLa cells. This suggests that the inhibitor treatments might have induced shedding of endogenous FN14 as an off-target effect, perhaps through activation of γ-secretase cleavage. The differences seen in HeLa cells versus MEF cells upon treatment with the general inhibitors of endosomal acidification could be due to cell type specific expression or localization of γ-secretase. We do not yet know if FN14 is a normal substrate of γ-secretase or if the activity is occurring due to our manipulations of cellular trafficking. If FN14 is subject to γ-secretase cleavage under normal conditions, however, this further supports the possibility that the receptor also undergoes ectodomain shedding. The majority of γ-secretase substrates require ectodomain cleavage by a sheddase prior to γ-secretase intra-membrane cleavage (112). Given the many intriguing possibilities that have come out of the preliminary study using the γ-secretase inhibitor, we are in the process of further exploring the link between FN14 loss and γ-secretase activity.

The localization of FN14 to late endosomes and lysosomes is quite clear by confocal microscopy, but the exact lysosomal hydrolases involved in FN14 degradation remain to be elucidated. We have found that MG-132 and leupeptin both block constitutive turnover of FN14 to varying degrees. It has been reported that MG-132 inhibits certain calpains and cysteine proteases (110) and it is known that leupeptin blocks cysteine, serine, and threonine proteases. Interestingly, MG-132 has also been reported to inhibit γ-secretase activity (113). Since MG-132 blocks FN14 constitutive turnover more fully than the other inhibitors tested, it seems likely that both lysosomal degradation and γ-secretase activity are contributing to receptor loss. Alternatively, MG-132 may simply block additional proteases that are required for constitutive turnover.

The mechanism of ligand-induced turnover has not yet been examined, but the method of inhibitor pre-treatment and turnover evaluation can be utilized to address this issue. Based on the fate of other TNFRSF members upon ligand-binding, it seems likely that FN14 is internalized and degraded upon TWEAK binding. This possibility will be investigated in the near future.

Ultimately, our results indicate that FN14 is regulated at the protein level through the novel mechanism of constitutive trafficking and receptor turnover. This finding opens up a wealth of questions for further investigation. During this project, we were especially interested to explore the molecular details of the constitutive trafficking mechanism and the biological significance of this novel regulatory mechanism. The contribution of FN14 trafficking motifs and structural regions to the mechanism of constitutive turnover is further examined in Chapter 3.

Chapter 3: Mapping of FN14 Regions Involved in Receptor Turnover

Introduction

FN14 structure

FN14 was originally cloned from a HUVEC cDNA library and was predicted to be a type I transmembrane protein containing a 27-amino acid (aa) signal peptide, a 53-aa extracellular domain, a 21-aa transmembrane domain, and 28-aa cytoplasmic domain (47,48,50). After proteolytic processing by a signal peptidase, the mature FN14 protein is a 102-aa receptor that is found primarily at the plasma membrane and the trans-Golgi network near the nuclear membrane (48).

The extracellular domain of FN14 contains a single CRD, comprised of two structural modules known as A1 and C2 (114). The modules are categorized by sequence and structural similarity and by the number and pattern of disulfide bonds. The C2 module is quite unique, as CRD4 of TNFR1 contains the only other C2 module in the TNFRSF (114). TWEAK binding to the FN14 CRD depends on charged residues located in both the A1 and C2 modules (115). The intracellular domain of FN14 contains a TRAF consensus binding site (PIEET), which has been shown to interact with TRAFs 1, 2, 3, and 5 (47,116,117). FN14 also has a predicted endocytic leucine-isoleucine (LI) motif at the C-terminus (48). Di-leucine (LL) and LI motifs have been implicated in endocytosis and Golgi sorting to the endosomal/lysosomal pathway, but the importance of the FN14 LI-motif for receptor internalization or sorting has yet to be explored or validated (118,119).

FN14 trafficking motifs

In order to further examine the mechanism of constitutive turnover, we attempted to determine the region of FN14 required for this property. Important residues for ligand-binding have already been reported (115). However, functional internalization motifs and the self-association domain of the receptor have not yet been identified. In this chapter, we designed and characterized a number of receptor mutants and fusions in the hopes of better understanding the receptor requirements that allow specific

receptors to undergo constitutive turnover. Ultimately, this information can be used to design peptides for modulation of FN14 trafficking or protein levels.

Results

FN14 contains a putative endocytic motif (LI) in its cytoplasmic tail, which has not yet been evaluated for functional significance (48). To test whether this motif is important for either the ligand-induced or constitutive turnover of the receptor, a truncation of the receptor lacking the last 3 amino acids (LIQ) was constructed (FN14 ΔLIQ). FN14 KO cells were transduced with a lentivirus expressing this truncated mutant and stably expressing cells were isolated. The cells stably expressing FN14 ΔLIQ were treated with increasing amounts of TWEAK to examine ligand-induced turnover and with cycloheximide for increasing amounts of time to look at constitutive turnover. Surprisingly, the LI motif does not seem to be important for TWEAK-mediated turnover or for the constitutive turnover ability of FN14 (Figure 23B).

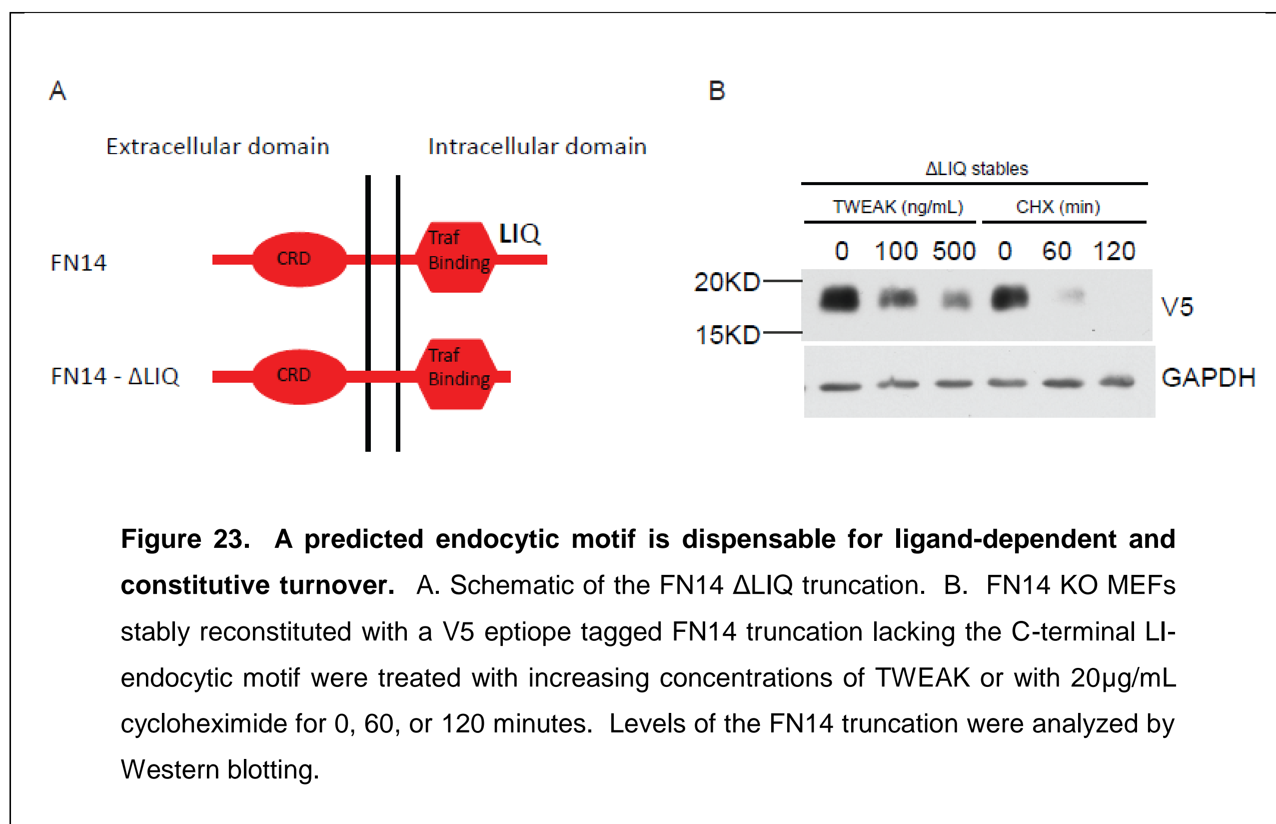

Figure 23. A predicted endocytic motif is dispensable for ligand-dependent and constitutive turnover. A. Schematic of the FN14 ΔLIQ truncation. B. FN14 KO MEFs stably reconstituted with a V5 eptiope tagged FN14 truncation lacking the C-terminal LI-endocytic motif were treated with increasing concentrations of TWEAK or with 20µg/mL cycloheximide for 0, 60, or 120 minutes. Levels of the FN14 truncation were analyzed by Western blotting.

In order to further examine regions of FN14 that might be involved in the constitutive turnover ability, several approaches were taken. To begin with, a truncation of the receptor lacking the cytoplasmic domain was constructed to look at the contribution of intracellular elements upon turnover. FN14 KO MEFs were transduced with a lentivirus expressing the truncated FN14, designated FN14 ECD-V5, and single-cell derived colonies were selected to create clonal, stably expressing cell lines. The ability of FN14 ECD-V5 to undergo rapid constitutive turnover was assessed by treating two different clonal lines with cycloheximide for 0 or 120 minutes and then blotting for the fused V5 tag. In all of the clonal lines tested, the FN14 truncation turned over within 120 minutes (Figure 24B). This indicates that the cytoplasmic domain is not important for turnover, and that the FN14 extracellular and transmembrane domains are sufficient to mediate constitutive turnover. This finding is quite surprising since it suggests that the FN14 internalization motif does not mediate endocytosis directly through intracellular trafficking adaptors. Rather, it indicates that the extracellular domain may be interacting with a trafficking receptor which could mediate its internalization.

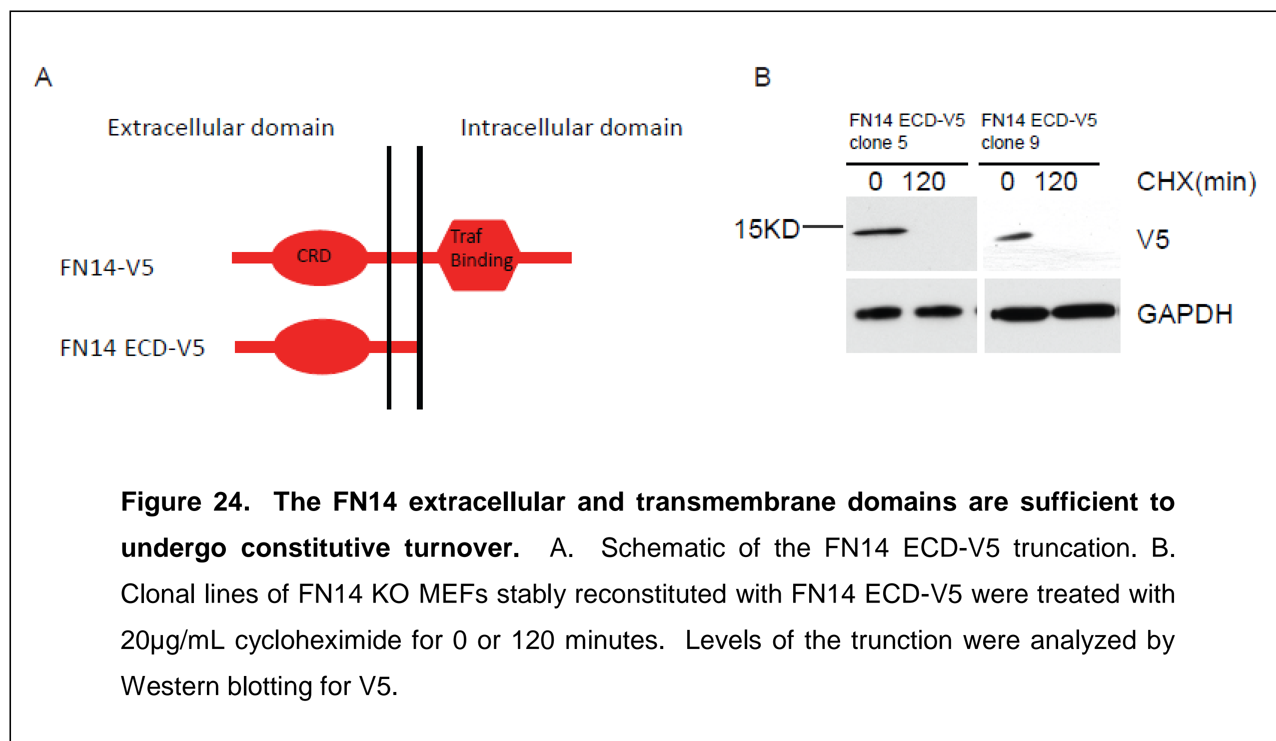

Figure 24. The FN14 extracellular and transmembrane domains are sufficient to undergo constitutive turnover. A. Schematic of the FN14 ECD-V5 truncation. B. Clonal lines of FN14 KO MEFs stably reconstituted with FN14 ECD-V5 were treated with 20μg/mL cycloheximide for 0 or 120 minutes. Levels of the trunction were analyzed by Western blotting for V5.

From previous results in the lab, we know that TNFR1 is another receptor that undergoes rapid constitutive turnover. When defining the mechanism of action of TNFR1 turnover, truncations of the

receptor were made and subjected to turnover analysis. It was determined that the CRD4 and transmembrane regions of TNFR1 were sufficient to undergo rapid turnover (Figure 25B). The CRD4 region of TNFR1 is interesting for the scope of our current project because it shares unique homology with FN14. Both the CRD4 of TNFR1 and the single CRD of FN14 contain a module known as C2, which is not found in any of the other TNFRSF members (114). As a result of this unique homology, it seems possible that the C2 module might be important for the rapid turnover ability common to both TNFR1 and FN14.

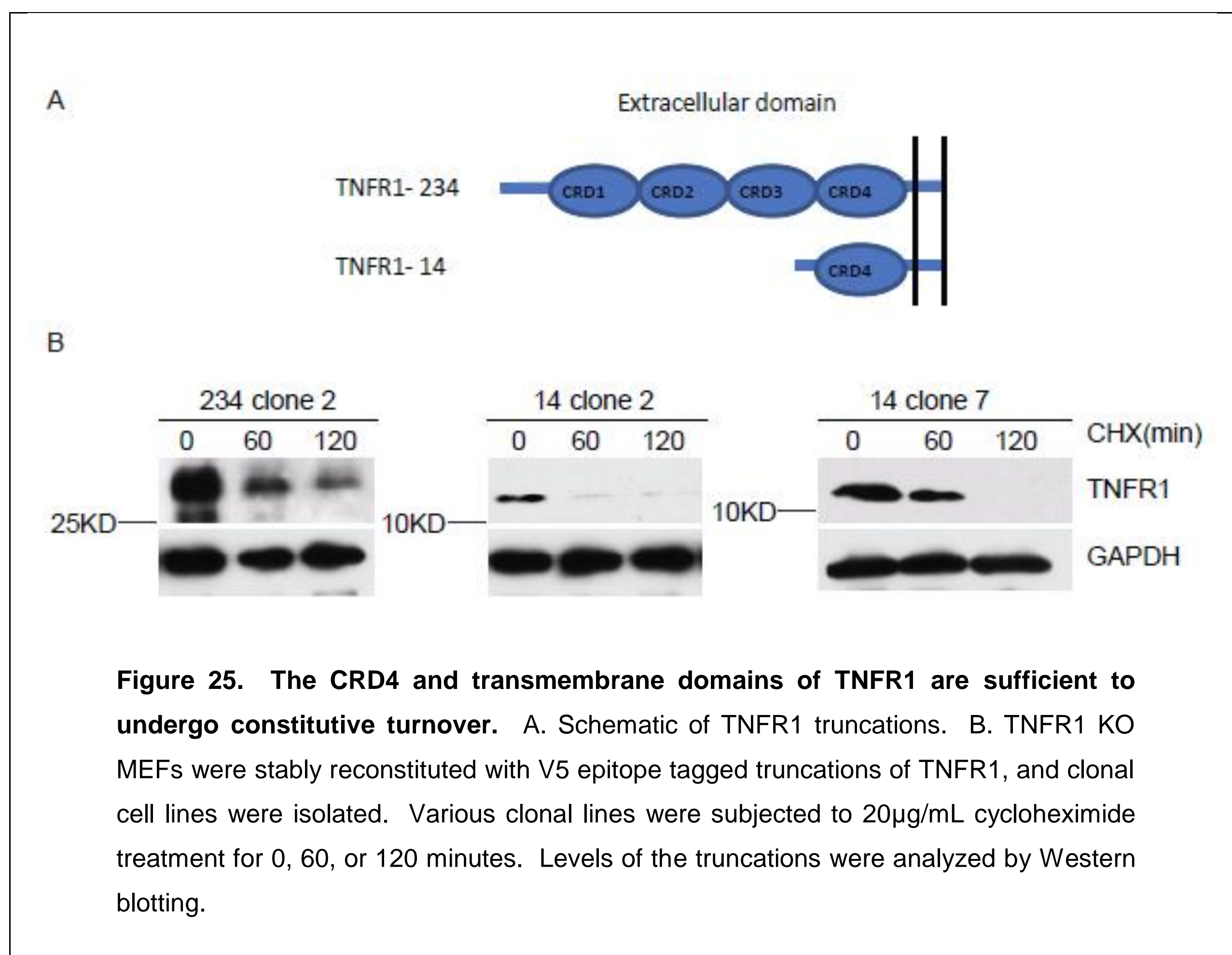

Figure 25. The CRD4 and transmembrane domains of TNFR1 are sufficient to undergo constitutive turnover. A. Schematic of TNFR1 truncations. B. TNFR1 KO MEFs were stably reconstituted with V5 epitope tagged truncations of TNFR1, and clonal cell lines were isolated. Various clonal lines were subjected to 20µg/mL cycloheximide treatment for 0, 60, or 120 minutes. Levels of the truncations were analyzed by Western blotting.

In order to explore the importance of the FN14 C2 region for turnover, several approaches were taken. First, fusions were made between TNFR1 and FN14, to determine whether swapping domains between the receptors would preserve the turnover ability. The FN14 extracellular domain (ECD) was fused to the TNFR1 transmembrane and cytoplasmic domains (TM/ICD), the TNFR1 ECD was fused to

the FN14 TM/ICD, and finally, the FN14 CRD domain was swapped into TNFR1 in place of the TNFR1 CRD4 (Figure 26A). The ability of these fusion receptors to undergo constitutive turnover was first assessed in an overexpression experiment. HEK 293 cells were transiently transfected with the three different constructs and cultivated for 24 hours to allow for expression of the constructs. Then, the cells were treated with cycloheximide for 0 or 120 minutes, and turnover was assessed by Western blotting analysis. All of the fusions between TNFR1 and FN14 turned over within 120 minutes (Figure 26B). This suggests that the regions that are necessary for the turnover ability are preserved in the fusions, and are supplied by one or the other receptor. In other words, the domain that is responsible for constitutive turnover is interchangeable between the two receptors. The disadvantage of performing this assay in an overexpression format is that the expression levels of the constructs differed after transfection, most likely because the cells that were overexpressing the constructs containing the TNFR1 intracellular death domain at high levels were subject to cell death. Thus, clonal cell lines stably expressing the different constructs were made in order to obtain cells with similar expression levels. The ability of the various constructs to undergo constitutive turnover was verified in these lines (data not shown). The results of the turnover analysis in the transfected and stably expressing cells support the hypothesis that the C2 region, which is unique to the two receptors, is interchangeable between TNFR1 and FN14, and could be important for the constitutive turnover ability.

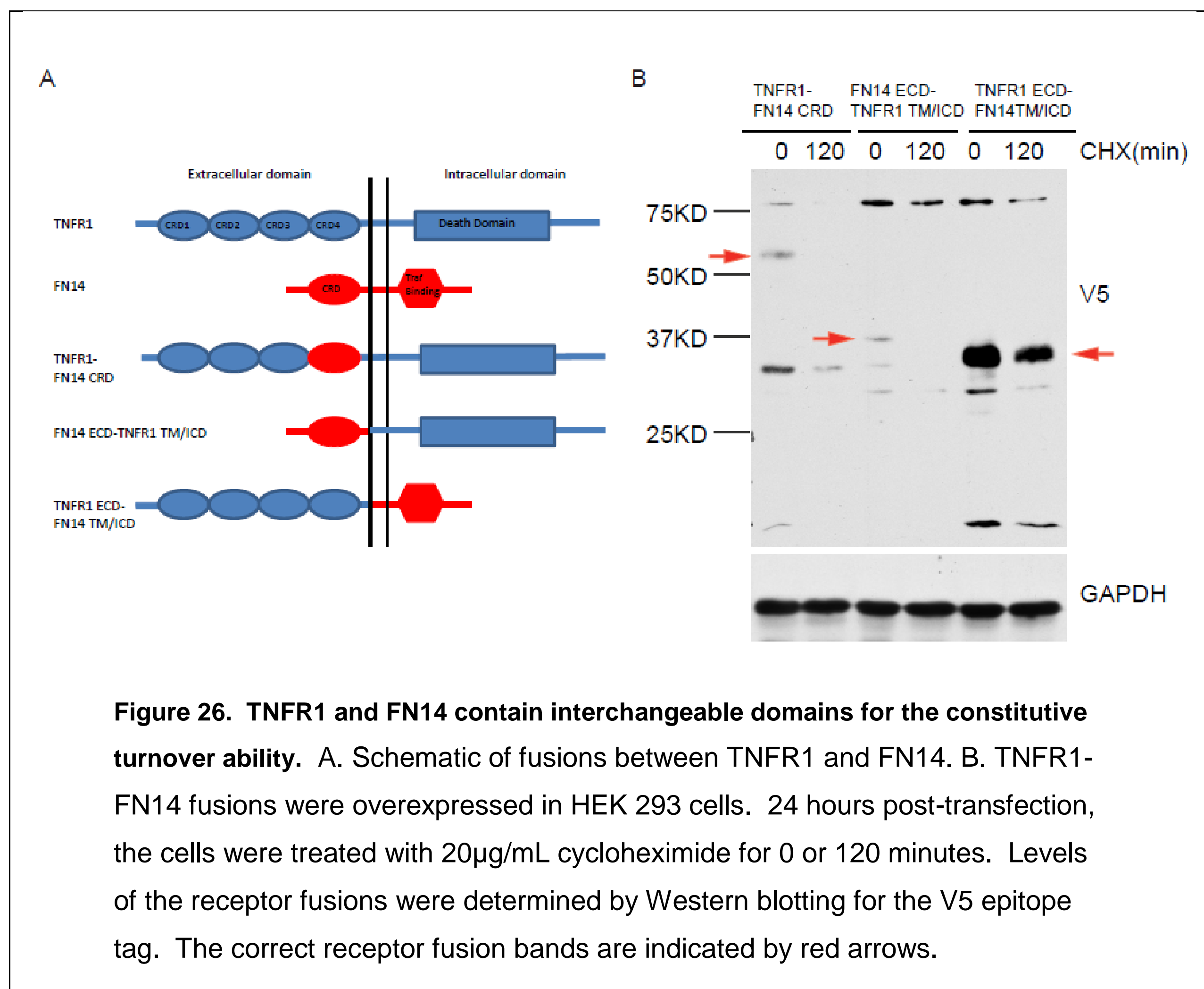

Figure 26. TNFR1 and FN14 contain interchangeable domains for the constitutive turnover ability. A. Schematic of fusions between TNFR1 and FN14. B. TNFR1-FN14 fusions were overexpressed in HEK 293 cells. 24 hours post-transfection, the cells were treated with 20µg/mL cycloheximide for 0 or 120 minutes. Levels of the receptor fusions were determined by Western blotting for the V5 epitope tag. The correct receptor fusion bands are indicated by red arrows.

Given the unique homology between the CRD4 of TNFR1 and the CRD of FN14 and the fact that the extracellular domains of both receptors can undergo rapid turnover, it seems possible that the homologous C2 module could be important for the turnover ability of the receptors. To examine this possibility further, mutations were made in the FN14 C2 module to disrupt the structure, and possibly the function, of this region. Three different point mutants, all tagged with V5, were generated. Cysteine residues were mutated to serine residues at positions 52 and 67 to disrupt the outer disulfide bond and at positions 55 and 64 to disrupt the inner disulfide bond. In addition, a conserved charged residue at position 62, which is known to be important for ligand-binding, was mutated from aspartic acid to alanine. The transfection of these constructs was optimized in HEK 293 cells to obtain moderate expression, thereby allowing for detection of protein levels changes upon inhibitor treatments. Similar to previous

experiments, turnover was assessed by cycloheximide treatment and subsequent Western blotting. In order to verify that the constructs were stable, and were not turning over due to misfolding and resultant proteasomal degradation, pre-treatment with epoxomicin was included as a control. The western blot indicates that the C52/67S mutant is unstable, and is subject to proteasomal degradation, which can be prevented by epoxomicin treatment (Figure 27). The C55/64S and D62A mutants, on the other hand, are stable, but undergo constitutive turnover similar to wildtype FN14 (Figure 23). It has been shown that the C55S and D62A mutations preclude receptor binding to TWEAK (115). Thus, the finding that our FN14 mutants undergo constitutive turnover confirms that turnover occurs independently of ligand. It also suggests that, contrary to our original hypothesis, the C2 region may not be important for FN14 constitutive turnover.

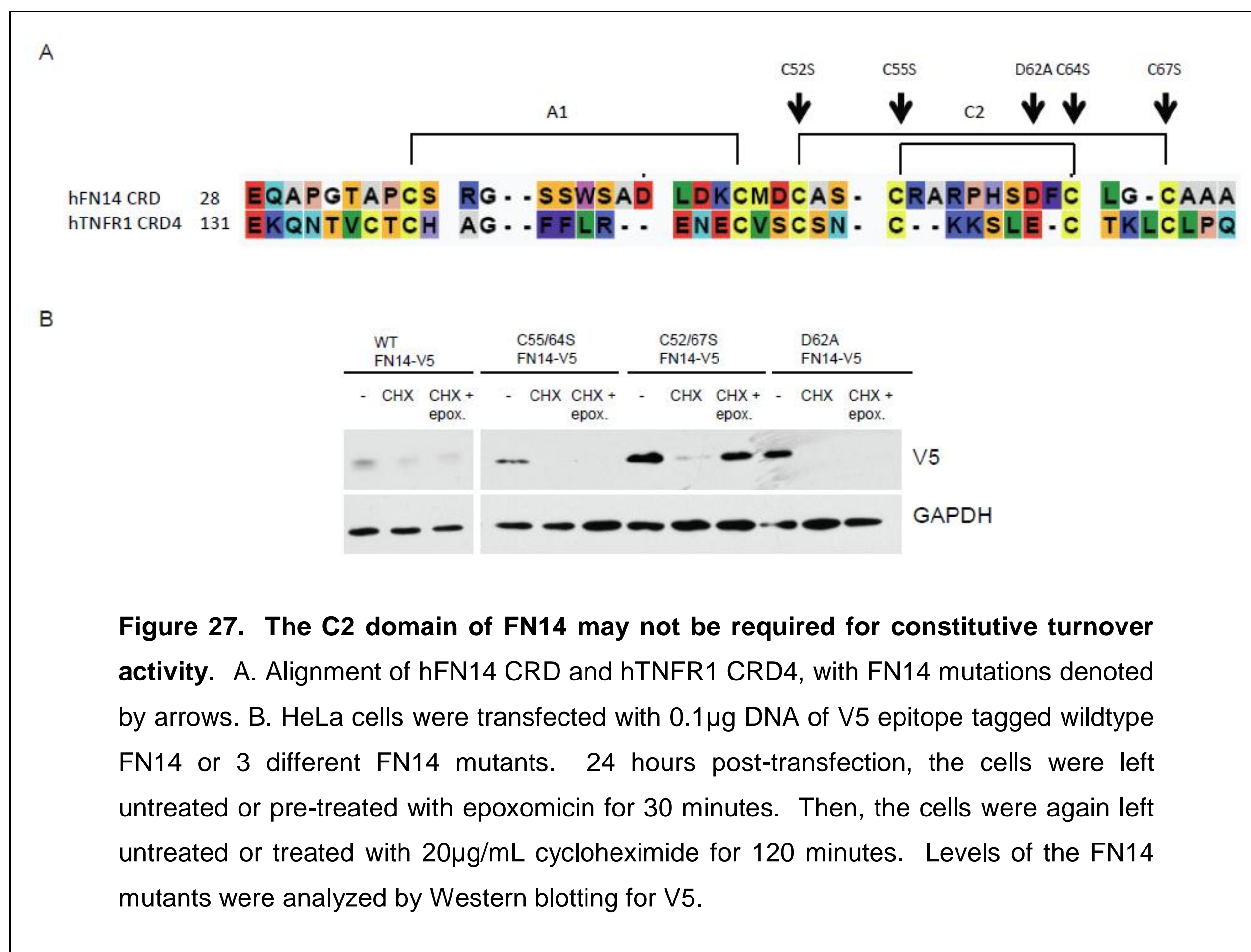

Figure 27. The C2 domain of FN14 may not be required for constitutive turnover activity. A. Alignment of hFN14 CRD and hTNFR1 CRD4, with FN14 mutations denoted by arrows. B. HeLa cells were transfected with 0.1µg DNA of V5 epitope tagged wildtype FN14 or 3 different FN14 mutants. 24 hours post-transfection, the cells were left untreated or pre-treated with epoxomicin for 30 minutes. Then, the cells were again left untreated or treated with 20µg/mL cycloheximide for 120 minutes. Levels of the FN14 mutants were analyzed by Western blotting for V5.

The FN14 ECD-V5 truncation experiment indicated that the extracellular and transmembrane domains of FN14 are sufficient to undergo turnover. However, there was a concern that the truncation might be poorly anchored to the membrane, and therefore lost through shedding. Thus, it was necessary to use another method to verify the initial results. It was previously shown in our lab that the TNF receptor family member, CD40, does not turn over constitutively. To verify this, FN14 KO MEFs were transduced with virus either expressing CD40 or FN14, and were selected for cells stably expressing the receptors. Turnover of CD40 or FN14 was assessed in these cells, and it was verified that CD40 does not undergo turnover in the same rapid time frame as FN14 (Figure 28A). Given this result, three different fusion constructs were made between FN14 and CD40 to evaluate the contribution of different regions of FN14 to the constitutive turnover ability. The FN14 ECD was fused to the CD40 TM/ICD, the CD40 ECD was fused to the FN14 TM/ICD, and finally, the CD40 CRD4 B1 domain was swapped into FN14 in place of the FN14 C2 domain (Figure 28B). It was difficult to evaluate turnover of these constructs by transfection because overexpression masked changes in protein levels due to turnover. We hypothesize that this occurs due to saturation of the turnover machinery in situations of highly elevated receptor expression, thereby leading to decreased turnover ability and increased receptor accumulation. This idea will be discussed in more detail later. To look at turnover under more moderate expression conditions, cells stably expressing the three FN14-CD40 fusions were cultivated and turnover was evaluated in these cells by cycloheximide treatment and subsequent Western blotting. In concordance with the previous results from the FN14 ECD-V5 truncation, the construct with FN14 ECD and CD40 TM/ICD turned over within 120 minutes (Figure 28C). This suggests that it is truly the FN14 ECD, and not the transmembrane domain, that is responsible for protein turnover. As expected given the previous result, the construct with the CD40 ECD and FN14 TM/ICD did not turnover within 120 minutes (Figure 28C). Thus, the FN14 ECD is not only sufficient, but it is also necessary for the constitutive turnover ability of the receptor. The possible role of the FN14 C2 domain in constitutive turnover was further examined using cells stably expressing the FN14-CD40 B1 construct, which lacks the C2 domain. This construct turned over normally, through a mechanism that was not mediated by instability leading to proteasomal degradation (Figure 28D). This result corresponds with the results obtained using the FN14

C2 mutants, confirming that the C2 region is not important for the constitutive turnover ability of the receptor.

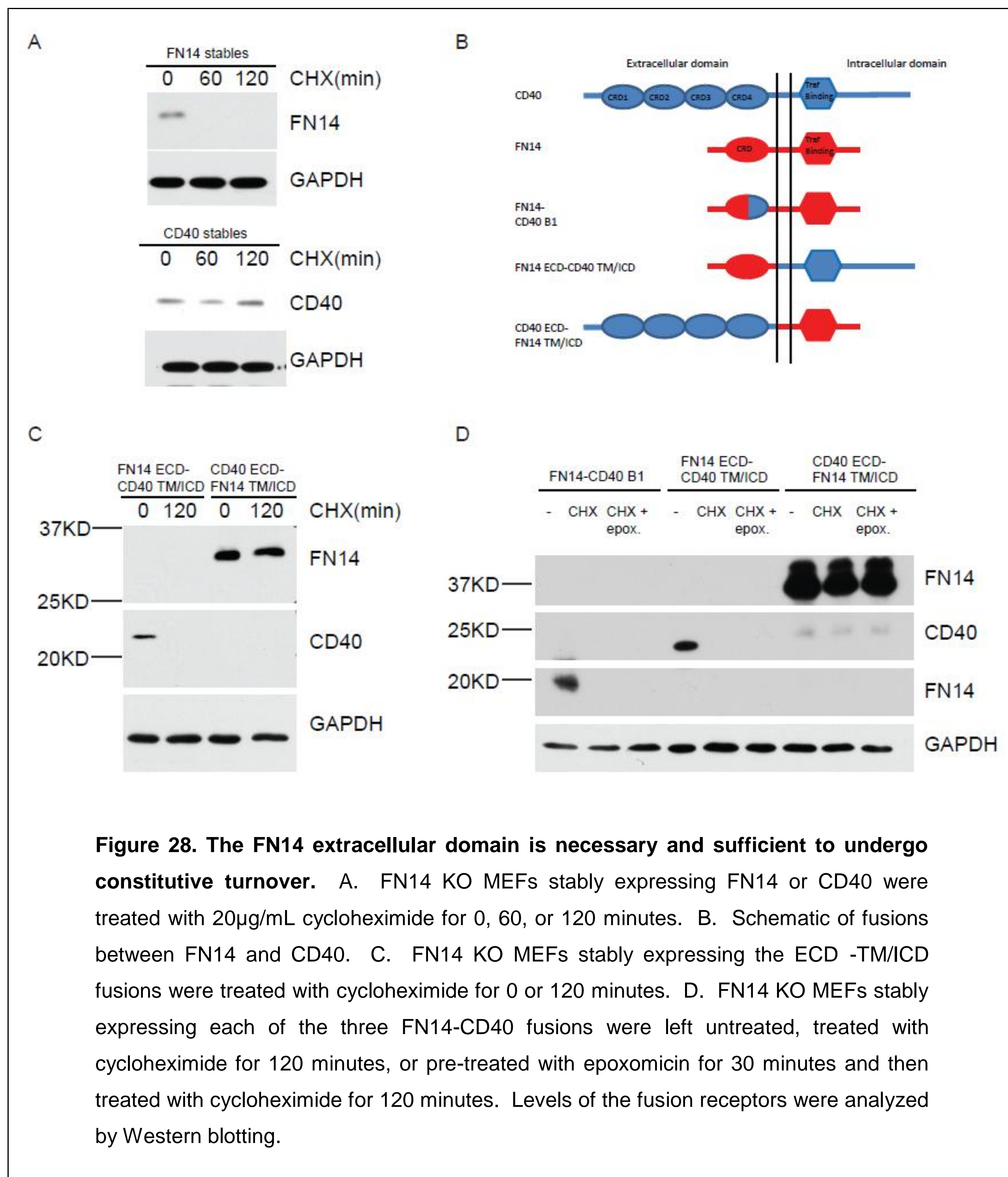

Figure 28. The FN14 extracellular domain is necessary and sufficient to undergo constitutive turnover. A. FN14 KO MEFs stably expressing FN14 or CD40 were treated with 20µg/mL cycloheximide for 0, 60, or 120 minutes. B. Schematic of fusions between FN14 and CD40. C. FN14 KO MEFs stably expressing the ECD -TM/ICD fusions were treated with cycloheximide for 0 or 120 minutes. D. FN14 KO MEFs stably expressing each of the three FN14-CD40 fusions were left untreated, treated with cycloheximide for 120 minutes, or pre-treated with epoxomicin for 30 minutes and then treated with cycloheximide for 120 minutes. Levels of the fusion receptors were analyzed by Western blotting.

Since the C2 domain does not seem to be important for constitutive turnover of FN14, it is necessary to examine other domains of the receptor to further define the motif that confers this ability. The only remaining domains to examine in the extracellular region of FN14 are the membrane proximal domain of the receptor and the A1 domain of the CRD. To assess the importance of the membrane proximal domain in constitutive turnover, a fusion receptor, comprised of full-length FN14 containing the CD40 extracellular membrane proximal domain, was constructed (Figure 29A). Cells stably expressing this fusion, denoted FN14-mpCD40, were cultivated, and both ligand-dependent and constitutive turnover were assessed in these cells. The stability of the fusion receptor was also examined by pre-treatment with proteasome inhibitors. The Western blot results indicate that the membrane proximal domain is not required for either ligand-dependent or constitutive turnover of FN14 (Figure 29B). In addition, the fact that epoxomicin does not block turnover while MG-132 does block turnover, indicates that the fusion receptor likely undergoes constitutive turnover by the same mechanism as previously described for wildtype FN14.

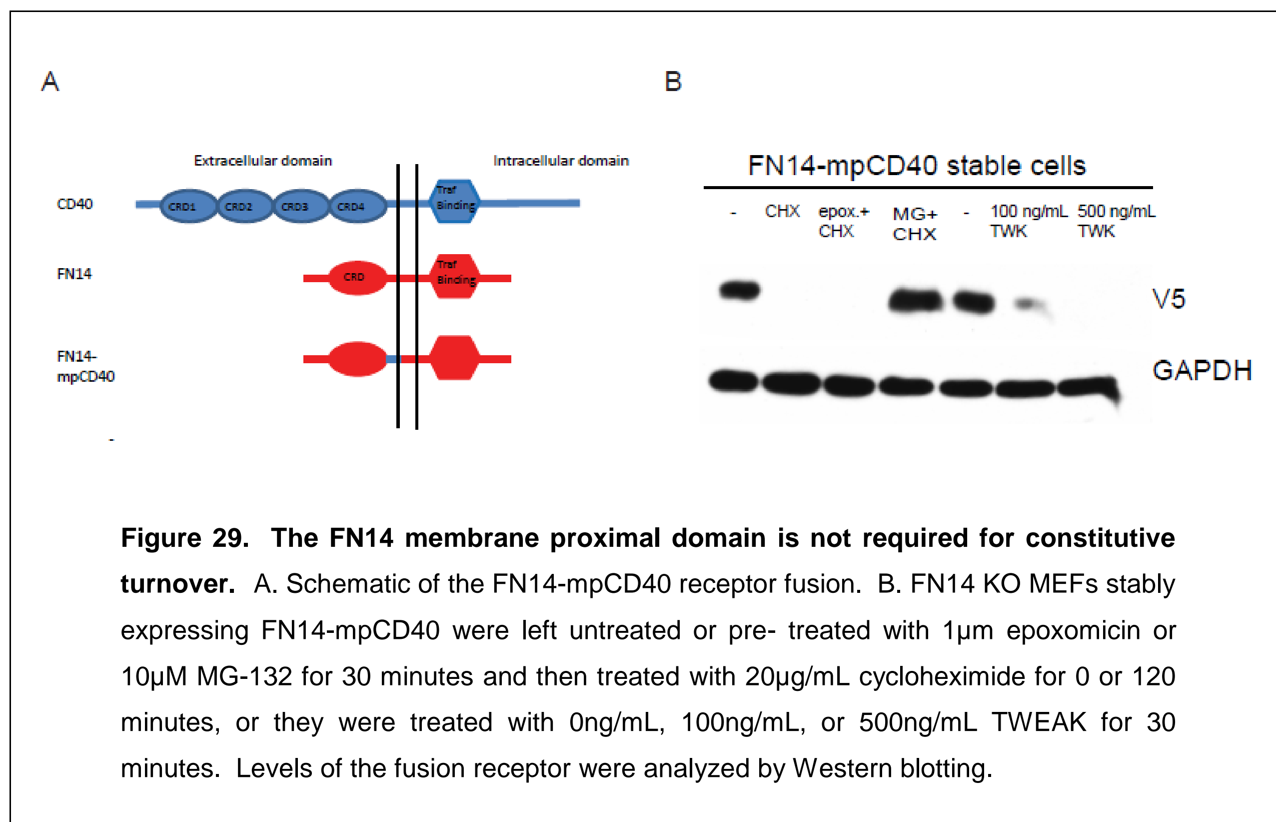

Figure 29. The FN14 membrane proximal domain is not required for constitutive turnover. A. Schematic of the FN14-mpCD40 receptor fusion. B. FN14 KO MEFs stably expressing FN14-mpCD40 were left untreated or pre- treated with 1μm epoxomicin or 10μM MG-132 for 30 minutes and then treated with 20μg/mL cycloheximide for 0 or 120 minutes, or they were treated with 0ng/mL, 100ng/mL, or 500ng/mL TWEAK for 30 minutes. Levels of the fusion receptor were analyzed by Western blotting.

Discussion

A number of truncations and receptor fusions were constructed during the course of this project in order to specifically define the region of FN14 which confers the constitutive turnover ability. It is clear that the extracellular domain of the receptor is responsible for this function, though we were not able to determine the exact region or residues involved. Through a process of elimination, it seems likely that the A1 domain of the FN14 CRD may be the required region. However, this hypothesis remains to be tested directly. The fact that the extracellular region of the receptor is required for constitutive turnover implies that the FN14 ectodomain may bind to a trafficking partner, thereby facilitating internalization and degradation. By determining the exact region of FN14 that interacts with the trafficking partner or trafficking machinery, it might be possible to design a peptide that inhibits trafficking and induces FN14 accumulation. Manipulating FN14 levels in this way would provide further evidence that FN14 truly undergoes constitutive turnover in a manner dependent on the extracellular domain of the receptor.

Another interesting possibility that was evoked by the receptor fusion experiments is the idea that TNFR1 and FN14 may traffic constitutively through the same mechanism. The facts that both receptors undergo rapid constitutive turnover and that various fusions between these two receptors retain the rapid turnover ability suggests that they share a functional motif important for constitutive turnover. It is known that there is cross-talk between the TWEAK-FN14 and TNF-TNFR1 signaling pathways. Perhaps competition for a trafficking receptor could represent another mechanism of cross-talk. If this is the case, overexpression of one receptor could affect surface expression of the other receptor. This can easily be tested *in vitro*. Alternatively, the two receptors could be regulated by different members of a trafficking family, thereby resulting in more specific regulation of each receptor. In order to determine the identity of potential trafficking partners, an shRNA screen can be performed to look for proteins whose loss leads to increased receptor levels.

Taken together, the results from this chapter demonstrate that a predicted endocytic motif is dispensable for ligand-induced and constitutive turnover of FN14. On the other hand, the extracellular domain of FN14 is necessary and sufficient for constitutive turnover. FN14 and TNFR1 have

interchangeable domains for constitutive turnover ability, and may undergo trafficking through a common mechanism. In the following chapter, the biological significance of constitutive turnover will be explored.

Chapter 4: Biological Significance of FN14 Turnover

Introduction

Ligand-induced signaling

FN14 signaling can activate a variety of downstream pathways, and can lead to diverse outcomes such as cellular proliferation, survival, migration, and death. Spatiotemporal regulation of the receptor could presumably mediate the sensitivity, strength, and specificity of response upon receptor stimulation.

FN14 can activate both the canonical and non-canonical NF-κB pathway, likely through TRAF2 and TRAF5. Soluble and trans-membrane TWEAK have been proposed to signal with different strengths to the canonical and non-canonical NF-κB pathways (120). Specifically, membrane-bound TWEAK has been suggested to be a better stimulator of the canonical pathway (120). A role for the kinase activity of TAK1 in TWEAK signaling has also been suggested since the activation of NF-κB was significantly inhibited in TAK1-deficient MEFs in comparison to wild-type MEFs (121). TWEAK binding to FN14 activates IκBα phosphorylation as early as 3 minutes after stimulation, leading to the generation of NF-κB DNA-binding complexes containing p50 and RelA (120,122). FN14 also has the capability for long-lasting NF-κB signaling, which seems to require p100 processing and a transition from RelA DNA binding complexes to RelB DNA binding complexes by 8 hours post-stimulation, lasting until at least 24 hours post stimulation (122). In glioma cells, which express high levels of FN14, IκBα phosphorylation seems to be sustained at early time points, unlike in other cell lines in which there is phosphorylation around 10 minutes, and then a second weaker phosphorylation event at 2 hours (51,74,120). *In vitro*, TWEAK treatment has been shown to induce numerous NF-κB target genes, including the secreted matrix metalloproteinase *MMP9*, the anti-apoptotic proteins A20, cIAP2, BCL-2, and BCL-XL, and the signaling molecules TRAF1 and TRAF3 (2). TWEAK treatment also induces expression of many pro-inflammatory molecules, including cytokines such as IL-6, granulocyte macrophage-colony stimulating factor (GM-CSF), chemokines such as IL-8, MCP-1, and Rantes, and cell-adhesion molecules such as ICAM1 and VCAM1 (2).

Stimulation through FN14 can also activate the MAPK pathway. Specifically, TWEAK treatment of HUVEC has been shown to induce phosphorylation of ERK and JNK (69). TWEAK treatment of the mouse osteoblastic line MC3T3-E1 has also been shown to induce phosphorylation of ERK p42/44, which is important for RANKL expression in these cells (65). In C2C12 myoblasts, TWEAK activates NF-κB and AP-1 and phosphorylation of p44-42 MAPK (63).

Several groups have shown that FN14 may signal through the small GTPases RhoA and Rac1. The Rho-family GTPases, which include Rho, Rac, and Cdc42, regulate cytoskeletal rearrangement and cell adhesion, and are implicated in polarization and motility in a number of cell types (123,124). It is thought that, during processes such as cell motility, integrin binding leads to regulated endocytosis, which facilitates the recruitment and retention of the GTPases to high affinity binding sites in plasma membrane lipid rafts (125). Then, the activated Rho GTPases capture and stabilize microtubules through effector molecules at the cell cortex, thereby leading to polarized cell morphology and directional cell migration (123,124). In the T98G glioma cell line, FN14 has been shown to coimmunoprecipitate with Rac1, independent of Rac1 activation state but in a manner dependent on the FN14 cytoplasmic TRAF binding site (51). Furthermore, TWEAK treatment or FN14 overexpression induced activation of Rac1 in these cells (51). Conversely, TWEAK treatment or FN14 overexpression decreased RhoA activation, suggesting that TWEAK-FN14 pathway has opposite effects on Rac1 and RhoA GTPase activity in glioma cells (51). It was then shown using Rac1 siRNA-mediated knockdown that Rac1 regulates FN14-induced glioma cell migration (51). It was further demonstrated that TWEAK treatment of T98G cells induces phosphorylation of IKKβ and IκBα in a manner that is dependent on Rac1 activity (51). RhoA and Rac1 have also been shown to interact with FN14 in C2C12 myoblasts, and it has been shown that FN14 knockdown inhibits the levels of activated RhoA in these cells (63). It is thought that RhoA might be an important component in the FN14 signaling pathway during myogenic differentiation (63). Given that FN14 has been shown to interact with RhoA and Rac1 is a number of systems, and that Rho and Rac have been implicated to relocalize to lipid rafts during events of cell motility, it is possible that FN14 requires lipid raft localization for the interaction with these GTPases. The link between FN14, Rac1 activity, and tumor cell migration support this hypothesis. FN14 localization to lipid rafts or membrane retention of the receptor could play an important role in its signaling capacity.

FN14 does not have a canonical death-domain, but under certain conditions can induce cell death. This cell death activity seems to occur only in certain cells lines and transformed cells, often requires long incubation periods with TWEAK, and in most cell types requires pre-treatment with cycloheximide to block anti-apoptotic signaling. The TWEAK-induced cell death has been shown to occur through both caspase-dependent apoptosis and, in the absence of caspase activity, through necrosis (91,126,127). There have been instances of both direct and indirect TWEAK-mediated activation of cell death in different cell lines (127). Specifically, TWEAK-induced cell death in HSC3 cells and IFN-γ treated HT-29 cells is not inhibited by cycloheximide or anti-TNF treatment, suggesting a direct mechanism of action in these cells (127). It seems likely that indirect cell death happens through TNF-TNFR1 signaling. TWEAK-induced cell death in Kym-1 cells is mediated by TNF and requires new protein synthesis (127). In concordance with this finding, it has been shown that TWEAK treatment that causes apoptosis induces TNF-α production in various sensitive cell lines (128). TWEAK treatment has also been shown to inhibit the TNFR1-mediated activation of NF-κB and JNK, which are important for resistance to apoptosis, and to enhance TNF-induced cell death (126,128). This occurs at least partly because TWEAK-FN14 signaling depletes the cells of cIAP1 and TRAF2 (128). Two mechanisms have been proposed for this depletion. It has been shown that TWEAK treatment leads to a translocation of TRAF2 to Triton-X insoluble fractions (126). Another group has demonstrated lysosomal degradation of a cIAP1-TRAF2 complex (128). This discrepancy is thought to be due to the use of different stimuli; one group used soluble TWEAK trimers, while the other used oligomerized TWEAK. The depletion of TRAF2 and cIAP1 activates non-canonical NF-κB signaling and sensitizes transformed cells to TNF-mediated cell death (128). It has also been seen that TWEAK pre-treatment results not only in reduced association of TRAF2 with TNFR1, but also in the absence of modified RIP associating with TNFR1 upon TNF stimulation (126). This could indicate that TWEAK also interferes with the composition and function of the TNFR1 signaling complex.

It has also been reported that TWEAK signals through the (PI3K)/AKT and Wnt/GSK3β pathways. In MC3T3-E1 cells, TWEAK treatment induces phosphorylation of AKT, which is a downstream target of PI3K, and it is this pathway that is responsible for TWEAK-induced RANTES production in the osteoblastic cell line (65). A study in which C2C12 cells were transfected with a

dominant negative Akt protein indicates that TWEAK-mediated activation of NF-κB and the expression of MMP-9 may involve the upstream activation of Akt kinase (121). However, an independent study suggests that in C2C12 myoblasts, TWEAK activates the NF-κB and MAPK pathways, but may inhibit phosphorylation of AKT (63). In human primary osteoblasts, TWEAK treatment altered levels of phosphorylated and total GSK3β and active and total levels of β-catenin, implying that the Wnt signaling pathway is also affected by TWEAK (66).

The major signaling pathways downstream of TWEAK ligation of FN14 are depicted in Figure 30.

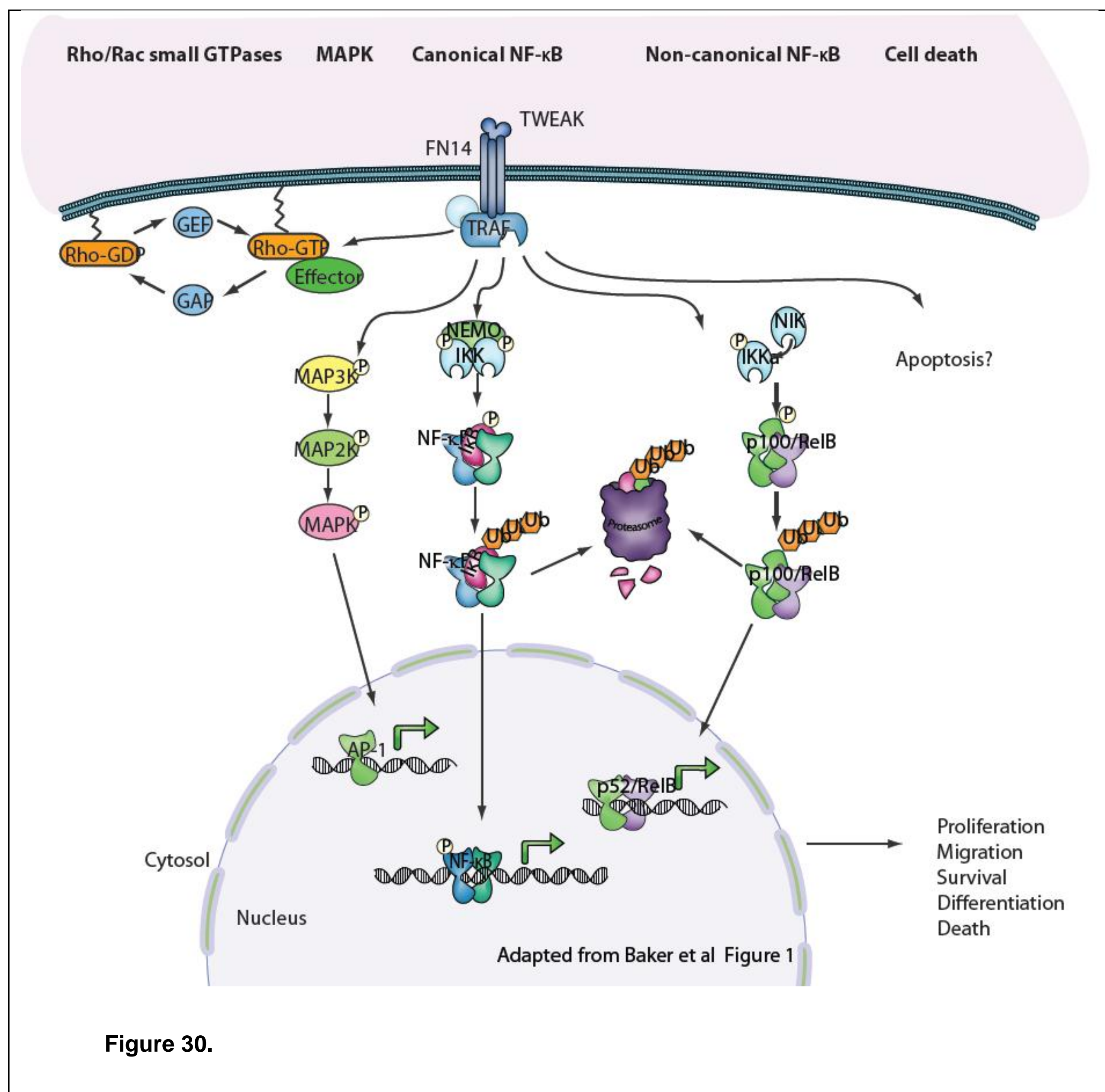

Figure 30.

Figure 30. Major signaling pathways and cellular outcomes downstream of FN14 ligation. TWEAK ligation of FN14 can activate the canonical and non-canonical NF-κB pathways, the MAPK pathway, the RhoA/Rac1 pathway, as well as cell death pathways. Activation of these pathways results in diverse cellular outcomes.

Ligand-independent signaling

A number of groups have shown that overexpression of FN14 can lead to ligand-independent signaling. FN14 overexpression is reported to activate NF-κB signaling, in a manner dependent on the TRAF binding site (116,117). In addition, ectopic FN14 expression has also been shown to initiate numerous cellular responses. It has been reported that overexpression of FN14 in glioma cells can induce cell migration through a Rac1 dependent mechanism (51). Overexpression of FN14 in T98G and SF767 glioma cells can also induce resistance against TRAIL or camptothecin-induced death by NF-κB dependent upregulation of the anti-apoptotic factor BCL-X_L and BCL-W (74). FN14 overexpression in PC12 cells also promotes neurite extension and filopodial and growth cone formation, in a manner that is demonstrated to be independent of TWEAK (129). This FN14-induced neurite outgrowth was shown to require Rac1 function (129). However, FN14 seems to coimmunoprecipitate with Rac1 independent of Rac1 activation state and FN14 overexpression does not seem to alter the amount of GTP-bound Rac1 (129). Thus, the relationship between FN14 expression and the Rac1 signal cascades is not yet fully defined. Finally, overexpression of FN14 can induce rat aortic smooth muscle cell migration in a manner dependent on a functional TRAF binding site (117). It has also been shown that overexpression of FN14 in esophageal adenocarcinoma cell lines increases invasiveness in a Matrigel assay (72). Taken together, these results suggest that ligand-independent signaling of FN14 can lead to cellular proliferation, migration, and survival, among other outcomes.

Presumably, high levels of cell surface FN14 expression can induce receptor trimerization and oligomerization, which in turn can promote TRAF binding and activation of an intracellular signal cascade. Although the self-association domain of FN14 has not yet been identified, the receptor is proposed to trimerize since TWEAK is predicted to be a trimeric ligand (53). It is not known if receptor oligomerization

and ligand-independent signaling occurs *in vivo*, but the fact that FN14 is highly expressed in solid tumors, in tissues after injury, and during progression of chronic inflammatory states makes this a compelling question. It has been hypothesized that situations *in vivo* in which FN14 is highly expressed and TWEAK is lowly expressed could lead to ligand-independent signaling. There is little known about regulation of the receptor that keeps inappropriate signaling from occurring.

Regulation at the level of protein stability

Thus far, our data has indicated that constitutive trafficking is a previously unknown property of FN14 cell biology, which is mediated by the extracellular domain of the receptor. The significance of trafficking for FN14 activity, however, has not been elucidated. At the start of this project, it became clear that FN14 levels are highly inducible by serum factors, and that protein level changes are perceptible only in low serum conditions. This suggests that potential mechanisms of downregulation might become saturated when FN14 levels are elevated. It has been shown that growth factor treatment induces mRNA expression of FN14. FN14 mRNA and protein levels have also shown to be induced in various injury models and models of chronic disease, as discussed in Chapter 1. However, there have also been discrepancies in the kinetics of mRNA and protein induction of FN14 in some of the models. For example, in tubular epithelial cells, cytokine induction of FN14 mRNA peaks at 2 hours post-stimulation, while protein expression also peaks at 2 hours post-stimulation (80). This begs the question whether the enhanced the protein expression might result from a mechanism other than transcriptional upregulation. We hypothesize in this chapter that FN14 constitutive turnover is just one example of FN14 regulation at the protein level, and that there may be other mechanisms in place to regulate FN14 protein stability.

The second focus of this chapter is to define the biological significance of the regulatory mechanism we have elucidated in this thesis, namely constitutive turnover. Since FN14 overexpression can induce ligand-independent signaling, it seems likely that stabilization of the receptor or retention at the cell surface might influence receptor oligomerization and ligand-independent signaling. Alternatively, stabilized FN14 levels could also increase the cellular sensitivity to ligand. These possibilities will be explored in this chapter using the FN14-CD40 fusions described in Chapter 3.

Results

In Chapter 1, it was shown that FN14 undergoes constitutive turnover in a number of cell types, including HUVEC. It is important to note that these cells were grown under minimal growth factor and low-serum conditions prior to the evaluation of turnover. The complete medium normally used for HUVEC growth and maintenance contains various growth factors including EGF and VEGF. When grown in these conditions, the expression of FN14 is highly elevated, and turnover is not detectable by Western blotting (Figure 31). This suggests that increased expression of FN14 may saturate the turnover machinery. As mentioned previously in Chapter 3, overexpression of the various receptor constructs also precludes constitutive turnover, even though the constructs undergo constitutive turnover when stably expressed. Thus, overexpression and growth factor induction both seem to mask constitutive turnover, potentially by overwhelming the turnover machinery. The implication of this idea is that in situations of increased FN14 expression, such as in glioma cells or in ischemic brains, the inability of the unliganded receptor to be downregulated at the appropriate rate could contribute to further amplification of the receptor. Ultimately, this could lead to increased sensitivity to ligand or oligomerization of the receptor resulting in increased ligand-independent signaling.

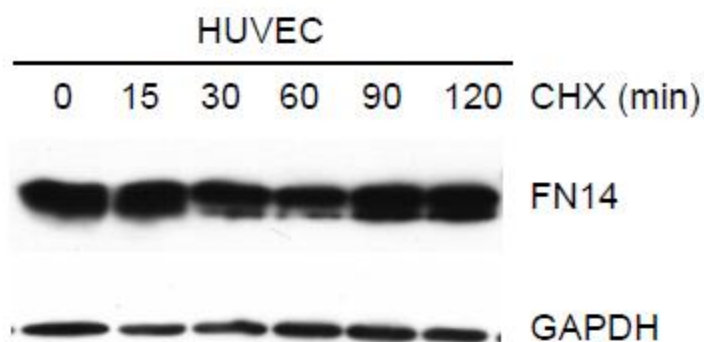

Figure 31. Saturating levels of FN14 inhibit constitutive turnover. HUVEC were grown in complete growth media and treated with 20µg/mL cycloheximide for the indicated times. FN14 levels were analyzed by Western blotting.

It is clear that growth factor treatment can induce expression of FN14. However, given that the kinetics of FN14 mRNA and protein expression after stimulation do not always correlate in the literature, it seems possible that FN14 levels might also be modulated through a non-transcriptional mechanism. In order to test this, FN14 KO MEFs stably reconstituted with V5-tagged FN14 were treated with 10ng/mL EGF for increasing amounts of time. These cells were chosen because FN14 is under the control of a CMV promoter instead of the endogenous promoter. While the endogenous promoter may contain growth factor response elements, the CMV promoter is less likely to be responsive to growth factors. Surprisingly, treatment with EGF resulted in the accumulation of FN14, beginning at 2 hours and continuing until at least 6 hours post-stimulation (Figure 32A). Since CMV promoters may be responsive to transcription factors which are downstream of the EGF receptor, mRNA was also harvested after EGF treatment to look at transcriptional upregulation of FN14. RT-PCR analysis showed that FN14 message was not upregulated in the reconstituted cells upon EGF treatment (Figure 32B). These results demonstrate that FN14 protein levels are regulated by EGF in a manner independent of transcription.

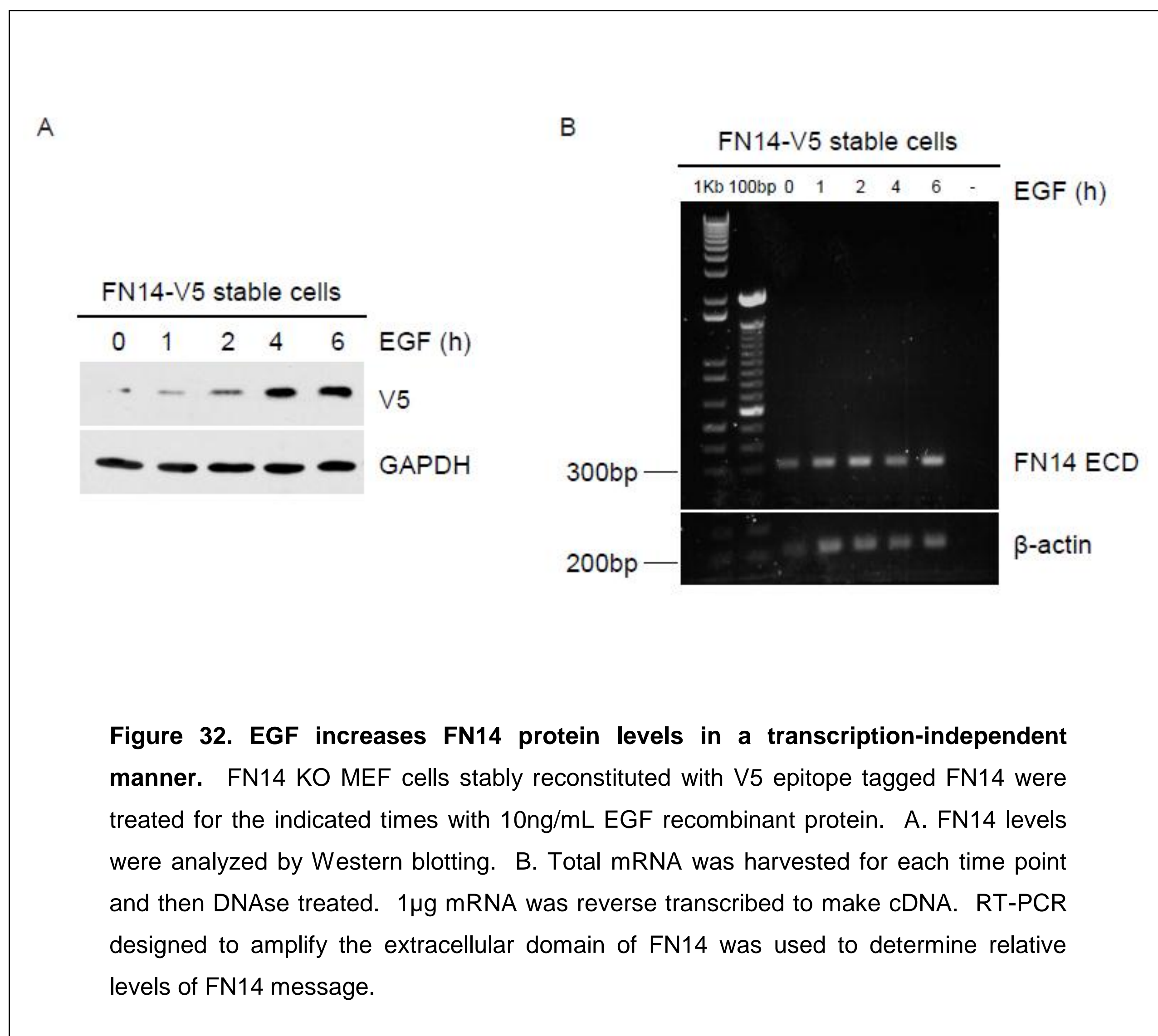

Figure 32. EGF increases FN14 protein levels in a transcription-independent manner. FN14 KO MEF cells stably reconstituted with V5 epitope tagged FN14 were treated for the indicated times with 10ng/mL EGF recombinant protein. A. FN14 levels were analyzed by Western blotting. B. Total mRNA was harvested for each time point and then DNAse treated. 1μg mRNA was reverse transcribed to make cDNA. RT-PCR designed to amplify the extracellular domain of FN14 was used to determine relative levels of FN14 message.

It is known that FN14 levels are tightly controlled at the level of expression, and our results suggest that FN14 levels are also regulated at the protein level. We have found that FN14 undergoes constitutive trafficking, and that this likely represents a novel regulatory mechanism of the receptor. However, the exact biological significance of constitutive trafficking is not yet clear. Since the CD40 ECD-FN14 TM/ICD fusion, described in Chapter 3, does not undergo constitutive turnover, and is thus more stable than wild-type FN14, it seems possible that the signaling kinetics of the fusion receptor might be altered in respect to FN14 signaling. Before testing this hypothesis, it was necessary to verify that the main defect of the fusion receptor was in the mechanism of constitutive turnover. CD40 is known to

undergo ligand-dependent downregulation, and our results indicate that FN14 also turns over in response to ligand. To test whether the CD40 ECD-FN14 TM/ICD fusion undergoes normal ligand-dependent turnover similar to wildtype FN14, cell lines stably expressing FN14 and CD40 ECD-FN14 TM/ICD were treated with either TWEAK or CD40L for 30 minutes, and then turnover was assessed. The Western blots indicate that the fusion receptor is competent for ligand-induced turnover, suggesting either that the ligand-dependent turnover ability results from a motif in the FN14 cytoplasmic tail or that FN14 and CD40 have interchangeable motifs which contribute to the ligand-dependent turnover ability (Figure 33).

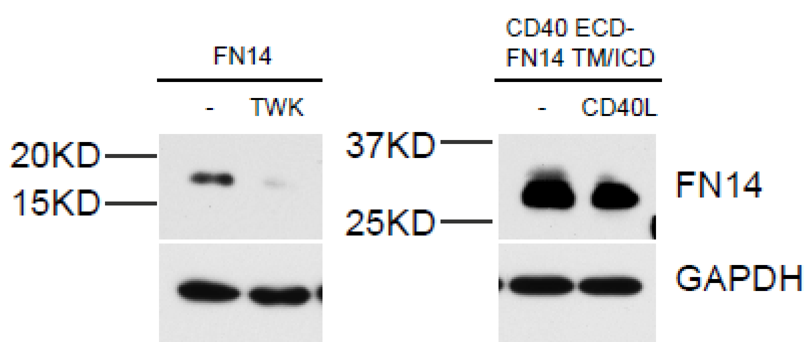

Figure 33. The CD40 ECD-FN14 TM/ICD fusion receptor is competent for ligand-dependent turnover. FN14 KO MEF cells stably reconstituted with FN14 or the CD40 ECD-FN14 TM/ICD fusion receptor were treated with their corresponding ligand, either TWEAK or CD40L, for 30 minutes. Receptor levels were analyzed by Western blotting.

The hypothesis that altered stabilization of the CD40 ECD-FN14 TM/ICD fusion in comparison to wildtype FN14 may lead to altered signaling strength or kinetics was tested by first examining ligand-independent signaling of the two receptors. It might be expected that overexpression of CD40 ECD-FN14 TM/ICD would activate a stronger NF-κB response in comparison to overexpression of FN14 due to the increased stabilization of the fusion receptor. To test this possibility, increasing amounts of the receptor constructs were transfected into HEK 293 or FN14 KO MEFs along with an NF-κB luciferase reporter and a control Renilla luciferase plasmid. 24 hours after transfection, a luciferase assay was performed on the cell lysates. While the highest dose of the transfected plasmids did induce NF-κB activation, there did not

seem to be a significant difference between the ligand-independent signaling activities of the two receptors (Figures 34A and 34B). However, these results are difficult to interpret since the experiment requires expression levels of the two receptors that are high enough to induce ligand-independent signaling, but are not so high as to overwhelm the turnover machinery and obscure differences due to constitutive turnover. Another method is obviously needed to evaluate the effects of alterations in constitutive turnover on FN14 ligand-independent signaling. Since FN14 ligand-dependent and ligand-independent signaling require TRAF association, it seems likely that stabilization of FN14 might affect TRAF2 recruitment to the receptor. Thus, TRAF2 association with the receptor upon treatment with trafficking inhibitors is currently being evaluated.

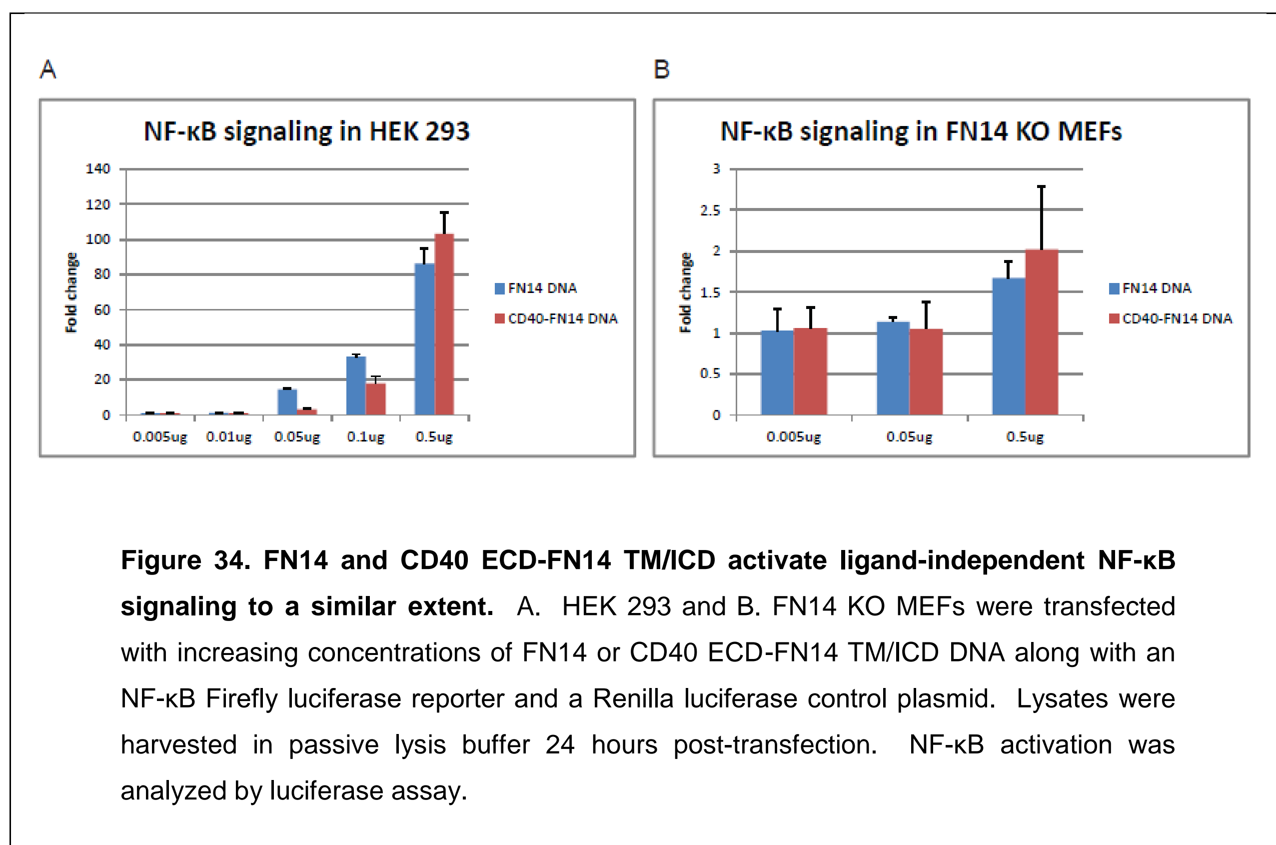

Figure 34. FN14 and CD40 ECD-FN14 TM/ICD activate ligand-independent NF-κB signaling to a similar extent. A. HEK 293 and B. FN14 KO MEFs were transfected with increasing concentrations of FN14 or CD40 ECD-FN14 TM/ICD DNA along with an NF-κB Firefly luciferase reporter and a Renilla luciferase control plasmid. Lysates were harvested in passive lysis buffer 24 hours post-transfection. NF-κB activation was analyzed by luciferase assay.

In order to examine if the increased stability of CD40 ECD-FN14 TM/ICD in comparison to wild-type FN14 changes the pattern of ligand-dependent signaling, stimulations of the cell lines stably expressing the fusion receptors were performed. The cell lines were plated at an equal density, and then

stimulated with either TWEAK or CD40L, in correspondence with the receptor that they expressed. Then, canonical NF-κB signaling was analyzed by IκBα phosphorylation and degradation by Western blotting analysis. Stimulation of the CD40 ECD-FN14 TM/ICD fusion receptor activated the canonical NF-κB pathway more strongly than stimulation of either FN14 or CD40, as evidenced by increased degradation of IκBα (Figure 35). Interestingly, stimulation of the FN14 ECD-CD40 TM/ICD resulted in similar kinetics to stimulation of wild-type FN14 (Figure 35). These results suggest that the extracellular domain contributes greatly to the sensitivity to ligand, and influences the kinetics of downstream signaling. The reason for the increased signaling activity seen by the CD40 ECD-FN14 TM/ICD receptor, however, is not entirely clear. We speculate that the decreased turnover rate of the fusion receptor may contribute to the increased strength and duration of the signaling activity. However, it is also possible that the signaling differences are a result of multiple factors, including the differences in multimerization ability of the extracellular domains of the two receptors, differences in the affinity for the two ligands, and intrinsic signaling differences between the cytoplasmic tails of the receptors. By further defining the region involved in constitutive turnover, it may be possible to make more targeted fusions in order to retain ligand binding and receptor self-association, while disrupting turnover ability.

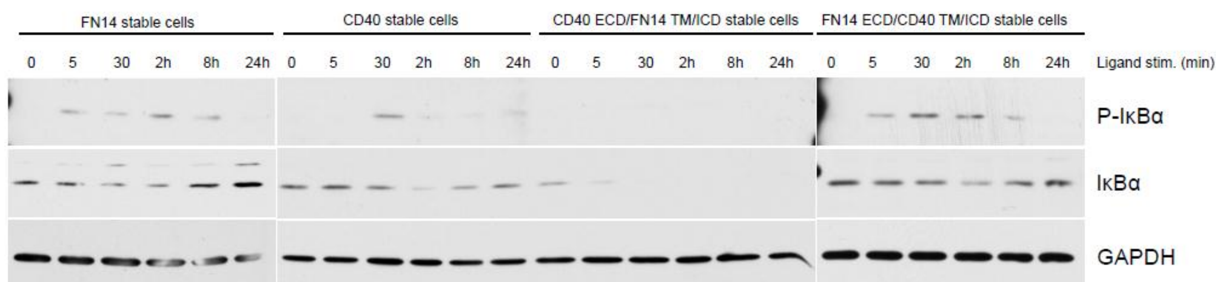

Figure 35. The extracellular domain influences ligand-dependent signaling kinetics. A. FN14 KO MEFs stably expressing FN14, CD40, or the receptor fusions were stimulated with the appropriate ligand, either 100ng/mL TWEAK or a stimulating concentration of CD40L, for 0m, 5m, 30m, 2h, 8h, or 24h. NF-κB signaling was analyzed by Western blotting for P-IκBα and IκBα.

Discussion

Taken together, our findings suggest that protein stability of FN14 is tightly regulated and that the stabilization of the protein affects signaling outcomes. FN14 undergoes constitutive turnover, but this mechanism can become overwhelmed when the receptor is highly expressed. In situations of chronic inflammation in which FN14 expression is upregulated, perhaps this inability to remove surface receptor at the appropriate rate could contribute to the positive feedback loop that amplifies FN14 expression and activity. This idea is supported by the finding that the CD40 ECD-FN14 TM/ICD fusion, which is stabilized in comparison to wildtype FN14, exhibits sustained canonical NF-κB signaling.

Although FN14 seems to undergo constitutive turnover under steady-state conditions, our findings suggest that growth factor treatments increase FN14 levels in a transcription-independent manner. This could potentially occur through divergence of FN14 from the degradation pathway to a recycling pathway. This possibility will be further explored through the following experiments. The ability of EGF treatment to stabilize receptor levels upon cycloheximide treatment will be assessed. In addition, the ability of EGF treatment to modulate levels of endogenous FN14 in HeLa cells in the absence or presence of a transcription inhibitor will also be evaluated. Finally, visualization of FN14 and localization to recycling vesicles will be assessed using confocal microscopy. The finding that EGF may regulate FN14 protein stability has implications for cancer cells in which growth factor and FN14 signaling seem to play a role. For example, FN14 is highly expressed in HER2 (EGF receptor 2) positive/estrogen receptor negative breast cancer patient samples, and the increased expression correlates with enhanced invasiveness (73). It is possible that EGF stimulation in these cells stabilizes FN14 levels, thereby promoting invasiveness through the TWEAK-FN14 pathway.

A future use of the FN14-CD40 fusions is the exploration of whether different regions of the receptors influence their signaling ability and outcomes. FN14 shares similar signaling intermediates with other members of the TNFRSF. For example, both FN14 and CD40 can activate canonical and non-canonical NF-κB signaling through utilization of some of the same TRAF molecules. It seems likely that the specific properties of the different receptor domains contribute to the signaling diversity that occurs between the TNFRSF members. We attempted to test if the FN14 specific regulatory mechanism of

constitutive turnover could explain differences in downstream signaling outcomes between CD40 and FN14. However, to do this properly, it is necessary to create fusions that can be stimulated by the same ligand, but differ in other key regions. A more easily interpretable use of the fusions would be to compare signaling outcomes between FN14 and FN14 ECD-CD40 TM/ICD because this would distinguish between the signaling outcomes that result from the FN14 cytoplasmic tail versus those that result from the CD40 cytoplasmic tail. For example, both receptors are known to activate non-canonical NF-κB signaling, but FN14 is thought to induce extended activation of this pathway, evidenced by p50/RelB DNA binding 24 hours after stimulation (122). The fusions could be utilized to determine which receptor domain influences the extended signaling kinetics exhibited by FN14.

Ultimately, our findings have demonstrated that FN14 is regulated at the protein level in a number of ways, which were previously unrecognized and therefore require further investigation. Various tools have been designed to analyze the contribution of receptor dynamics and structural domains of the receptor on signaling outcomes. Future directions for this project will be discussed in the following chapter.

Chapter 5: Conclusions, Perspectives, and Future Directions

Findings

The goal of this work was to better understand the regulation of FN14, a cytokine receptor that is expressed at low levels in normal tissue, but whose expression is dysregulated in a variety of autoimmune conditions and in a number of invasive cancers. This work demonstrates a previously unrecognized property of the receptor. The membrane localization of FN14 represents the steady-state of a receptor that is being rapidly and constitutively synthesized, trafficked, and degraded. FN14 can undergo both ligand-induced and ligand-independent turnover. The constitutive turnover of the receptor seems to be a property that occurs in various tumor and primary cell types. The turnover occurs through endocytosis and then degradation by a lysosomal mechanism. γ-secretase cleavage may also contribute to constitutive turnover of FN14. Surprisingly, a described C-terminal LI endocytic motif is dispensable for both TWEAK-induced and constitutive turnover. In contrast, the constitutive turnover requires the extracellular domain of the receptor. It is possible that this domain of FN14 interacts with an unknown trafficking receptor which shuttles FN14 to the lysosome. Interestingly, the FN14 ECD is interchangeable with the TNFR1 ECD for this constitutive turnover ability, but is not interchangeable with the CD40 ECD. Transfer of the CD40 ECD to FN14 abolishes constitutive turnover, and alters the kinetics of canonical NF-κB signaling in response to ligand.

FN14 regulation is tightly controlled at the level of expression. However, we show in this thesis that once expressed, FN14 levels are also controlled at the protein level. Although FN14 is constitutively undergoing trafficking to the cell surface, endocytosis, and turnover, it seems that EGF treatment can stabilize receptor levels. We hypothesize that EGF treatment results in a divergence of the FN14 degradation route perhaps to a recycling pathway. This model would allow for a highly sensitive and rapid response to ligand in the appropriate environment, while preventing cell surface accumulation of receptor in the steady-state.

It is surprising that FN14 undergoes constitutive trafficking and turnover since this process is likely metabolically costly for the cell. Yet, given our data, it seems that constitutive trafficking must

provide a regulatory benefit for the signaling pathway. There are several potential reasons why FN14 in particular might be so tightly regulated. FN14 is very widely expressed, and thus regulation of receptor function is important for homeostasis in most tissues. Furthermore, the positive feedback loop that leads to amplified receptor expression results in a system that is very sensitive to ligand and to ligand-independent signaling, but also has the potential to become dysregulated, leading to excess inflammation and tissue damage. Constitutive expression and then downregulation of receptor from the plasma membrane likely prevents receptor accumulation and aggregation as well as exposure to ligand in steady-state conditions, while retaining a rapid response to ligand under stimulating conditions. Finally, FN14 has been shown to directly induce cell death in some cells. Although the contribution of FN14-mediated apoptosis and necrosis *in vivo* is not understood, the potential role of the receptor in cell death pathways likely necessitates tight regulation.

It is not yet clear what biological purpose is served by constitutive trafficking of FN14. FN14 levels are dysregulated in a number of pathological states. It is assumed that this is primarily a result of amplified receptor expression. Given our findings, however, it is possible that impairment of the constitutive turnover ability of the receptor could contribute to pathology as well. Stabilization of FN14 could lead to increased surface availability of the receptor, and thereby increased sensitivity to ligand. This could result in an increase of ligand-dependent signaling, and subsequently inappropriate pro-inflammatory activity, enhanced cell migration and invasiveness, or increased proliferative ability. In addition, since FN14 overexpression can lead to ligand-independent signaling, changes in the surface density of the receptor could also lead to inappropriate, spontaneous signaling by the receptor. Presumably even a small increase in surface levels of the receptor could lead to a noticeable increase in FN14-mediated inflammation since TWEAK-FN14 signaling itself induces FN14 expression. The fact that turnover of FN14 upon cycloheximide treatment is undetectable in HUVEC grown in growth factor containing, high serum conditions gives credence to this model. *In vitro*, increased expression of FN14 seems to overwhelm the turnover machinery, thereby leading to accumulation of the receptor. Along these lines, it would be interesting to determine whether FN14 constitutive turnover is detectable in glioma cells. These cells have high levels of FN14 expression and respond to TWEAK treatment with sustained phosphorylation of IκBα at early time points, unlike other cells in which there are multiple

rounds of phosphorylation around 10 minutes and then again at 2 hours (120). Given our results, it seems possible that saturation of the turnover mechanism could contribute to the increased FN14 levels and activity seen in glioma cells.

A model for how FN14 might be regulated at the protein level in various situations is depicted in Figure 36.

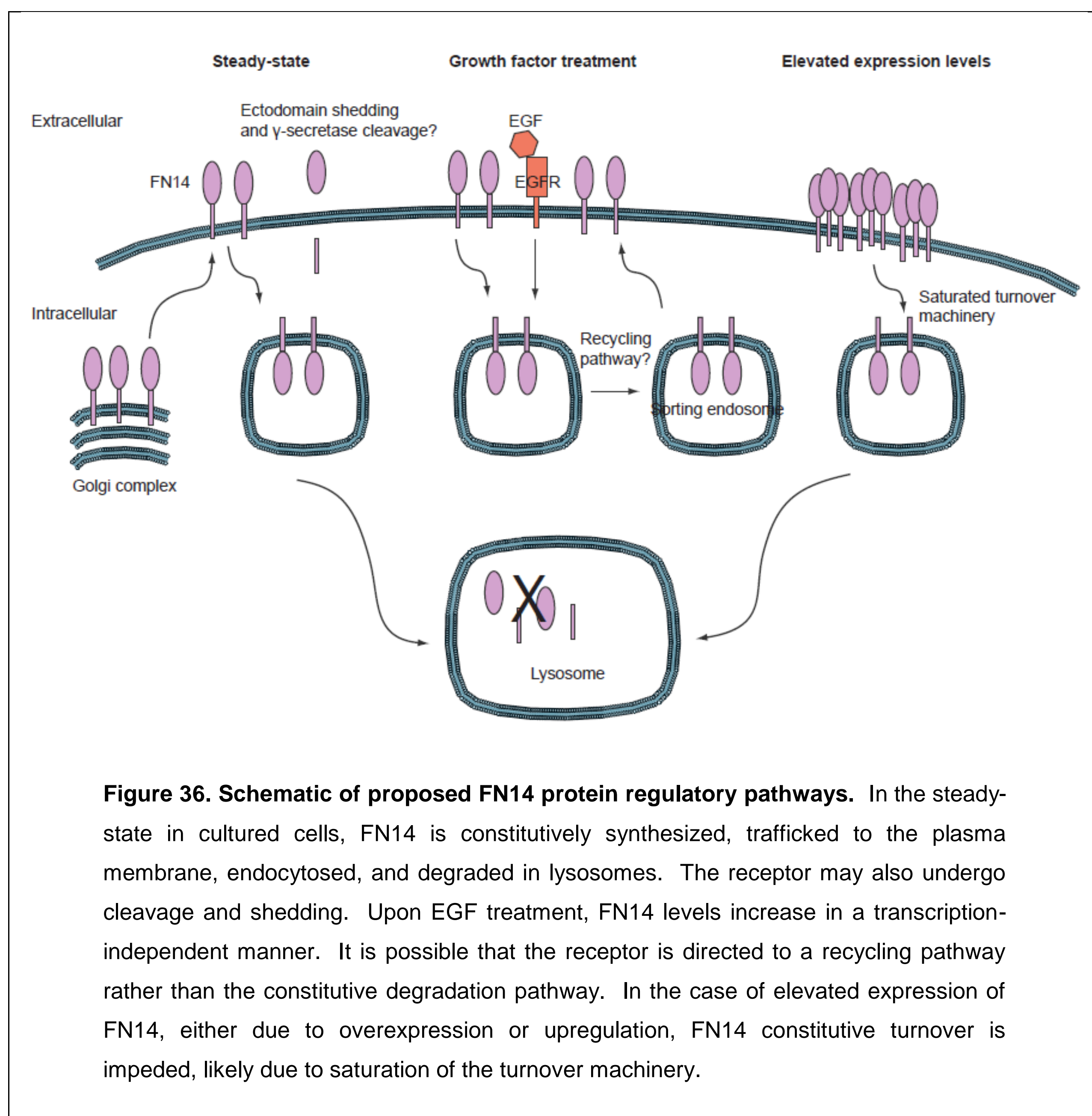

Figure 36. Schematic of proposed FN14 protein regulatory pathways. In the steady-state in cultured cells, FN14 is constitutively synthesized, trafficked to the plasma membrane, endocytosed, and degraded in lysosomes. The receptor may also undergo cleavage and shedding. Upon EGF treatment, FN14 levels increase in a transcription-independent manner. It is possible that the receptor is directed to a recycling pathway rather than the constitutive degradation pathway. In the case of elevated expression of FN14, either due to overexpression or upregulation, FN14 constitutive turnover is impeded, likely due to saturation of the turnover machinery.

Future Directions

A facet of FN14 biology that is not yet understood is how TWEAK stimulation can activate different downstream signaling pathways and induce such diverse biological effects. It is known that internalization or compartmentalization of other TNFRSF members can influence their signaling outcomes. Given our results that FN14 undergoes ligand-dependent internalization, it would be interesting to see whether inhibition of endocytosis affects TWEAK-FN14 signaling to downstream pathways. FN14 can activate the NF-κB and MAPK pathways, and under specific circumstances can induce cell death. It is possible that internalization or localization of the receptor to endocytic vesicles contributes to one or more of these divergent signaling pathways.

Given our preliminary results with the γ-secretase inhibitor, it would be interesting to determine if the extracellular and cytoplasmic portions of the receptor have any signaling functions after γ-secretase cleavage. Notch is an established substrate of γ-secretase, and it is known that, after cleavage, the Notch intracellular domain translocates to the nucleus and initiates gene expression (130). It is possible that the FN14 intracellular domain is also competent for transcription initiation. This is not a transcriptional mechanism common to cytokine receptors, and is thus an intriguing possibility. As for the extracellular domain of the receptor, it is known that the extracellular domains of many TNFRSF members are subject to shedding, thereby neutralizing or buffering activity of the TNF ligands. It is possible that shed FN14 might be competent to neutralize or buffer TWEAK activity.

In light of our results demonstrating EGF modulation of FN14 protein levels, the possibility of FN14 receptor recycling requires further examination. In migrating cells, endocytosis seems to be important for shuttling receptors and signaling molecules to specific regions of the plasma membrane. Specifically, studies of the GTPases Rac and Rho, which are involved in cell motility, suggest that differential regulation of endocytic pathways upon integrin binding may control recruitment and retention of signaling molecules to the plasma membrane (125). Since FN14 is involved in cell migration in glioma cells, and has been implicated to signal through Rac and Rho, it would be interesting to examine whether recycling of FN14 to rafts could play a role in its function. We hypothesize that FN14 localization to or retention in rafts may allow for interaction with the Rho/Rac pathway, and would eventually like to

examine this pathway further. In addition, it has been reported that disruption of lipid rafts switches TNF signaling from NF-κB activation to apoptosis. FN14 stimulation leads to TRAF2 re-localization to lipid rafts, but the membrane localization of FN14 itself has not been thoroughly examined. It would be interesting to test whether disruption of rafts affects the diverse FN14 signaling outcomes.

There are various methods of internalization that can occur from the plasma membrane, specifically from lipid rafts, and the different methods of internalization contribute to the diversity of signaling outcomes. A future goal for this project would be to determine the mechanism of internalization of FN14. Along these lines, it is known that ubiquitination plays a role in endocytosis of cell-surface receptors. For example, Notch is a receptor that undergoes ligand dependent and ligand independent internalization, and ubiquitination is thought to be involved in the divergence from the Notch degradation/recycling routes that occurs upon receptor activation (130). An examination of the ubiquitination status of steady-state and ligand-activated FN14 would likely provide insight into the molecular mechanisms of endocytosis and trafficking of the receptor.

Therapeutic applications

Although the FN14 mutants and fusions were insufficient to define the region involved in constitutive turnover, they may serve another purpose. It has been shown that residues in the A1 and C2 of the FN14 CRD are necessary for ligand binding (115). However, it is not known if these residues are needed for contact with ligand or if they are important for FN14 self-association, a factor that could also influence ligand binding. It might be possible to utilize our FN14 mutants or fusions to define the FN14 self-association domain, as has already been done for TNFR1. Soluble PLAD has been used therapeutically to disrupt TNFR1 binding to TNF, thereby inhibiting pathological signaling (131). Defining the FN14 PLAD may have similar therapeutic applications.

In the case of TNFR1, there are known mutations that result in altered trafficking and accumulation of the receptor. Furthermore, these mutations have been reported to play a role in the inflammatory disease TRAPS (103). To date, mutations in FN14 have not been linked to known human diseases, but it would be interesting to identify potential binding partners for FN14. Cells deficient in LRP-

1 exhibit increased inflammation, and it has been proposed that this is a consequence of the loss of LRP-1 regulation on TNFR1 cell surface levels (102). If FN14 receptor is indeed trafficked to lysosomes by an associated trafficking receptor, then dysfunction of this binding partner could influence the availability of cell-surface FN14. The correlation of FN14 trafficking to disease would help establish biological relevance for the constitutive trafficking that we have described.

Conclusion

In this thesis, we have described a novel regulatory mechanism of the FN14 cytokine receptor. The finding that FN14 undergoes ligand-dependent and ligand-independent turnover is a significant contribution to the understanding of FN14 cell biology which ultimately opens a number of avenues for further investigation. We demonstrate that FN14 is tightly regulated at the protein level by constitutive turnover, and suggest that growth factor stimulation may induce stabilization of the receptor. This method of regulation likely allows for a signaling system that is both sensitive and rapid in its response, while also guarding against receptor aggregation and inappropriate spontaneous signaling. Given the pleotropic and multi-functional nature of FN14, it seems fitting that receptor levels are controlled at both the level of expression, as has previously been described, as well as through this newly discovered mechanism of regulated protein stability.

Chapter 6: Materials and Methods

Cell Culture – HeLa, HEK 293, and MEF cells were maintained in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 5% FBS. The cells were switched to DMEM supplemented with 1% FBS 18 h prior to use. Primary mouse keratinocytes were maintained in DMEM supplemented with 10% FBS. HUVEC were a gift from the Kitajewski Lab and maintained in basal Endothelial Growth Medium (EGM-2) supplemented with 2% FBS and manufacturer recommended growth factors, cytokines and supplements (Lonza, CC-4176). The cells were switched to basal EGM-2 supplemented only with 2% FBS 24 h prior to use. The FN14 KO MEF cells were a gift from the Winkles Lab.

Antibodies -- The polyclonal anti-FN14 (#4403) used for Western blot and IP was purchased from Cell Signaling Technology. The polyclonal anti-EGFR (#06-847) was purchased from Millipore. The monoclonal anti-GAPDH (10R-G109a) was purchased from Fitzgerald. The monoclonal anti-FN14 (ITEM-4 clone, 14-9018-80) used for flow cytometry was purchased from eBioscience. The anti-CD40 was purchased from Santa Cruz Biotechnology. The monoclonal anti-TWEAK (CARL-1 clone, 14-9915) used for neutralization was purchased from eBioscience, while the control IgG was from PharMingen. The polyclonal anti-TRAF2 (#4724) was purchased from Cell Signaling Technology.

Western Blot – Lysates were harvested in TNT buffer (50mM Tris pH 7.5-8, 200mM NaCl, 1% Triton-X, 0.5mM EDTA) supplemented with protease and phosphatase inhibitors. Thirty to fifty micrograms of total protein were run on 10-20% gradient SDS–polyacrylamide gel electrophoresis gels. After transfer to polyvinylidene fluoride membrane (Millipore), immunoblotting was performed according to the manufacturer's protocol.

Flow Cytometry -- For surface staining, HeLa cells and HUVEC (10^6 cells) were incubated with 0.1μg ITEM-4 for 30 min at 4 ^{0}C, followed by Alexa 546-labeled donkey anti-mouse (Invitrogen). For total staining, the cells were fixed in 4% paraformaldehyde, permeabilized in PBS containing 1% BSA and

0.5% saponin, and then stained as above. After staining, the cells were analyzed on a LSRII (BD Biosciences), and the data were processed using the FlowJo software (Tree Star, Inc.).

Inhibition of protein synthesis, trafficking, degradation, and shedding -- HeLa cells, cultured overnight in DMEM supplemented with 1% FBS, were treated with inhibitors for the indicated times by direct addition to the cell culture media. Protein synthesis was inhibited by cycloheximide (Sigma) treatment at a final concentration of 20μg/mL. Protein trafficking was inhibited by treatment with Brefeldin A (eBiosciences) at a final concentration of 3μg/mL or of Monensin (Sigma) at a final concentration of 10μM. Proteasomal degradation was inhibited by treatment with epoxomicin at a final concentration of 1μM or of MG-132 (Sigma) at a final concentration of 10μM. Lysosomal degradation was inhibited by treatment with ammonium chloride at a final concentration of 20mM or of chloroquine at a final concentration of 1X. TACE cleavage was inhibited by treated with TAPI-1 at a final concentration of 25μM. Controls were performed by addition of equivalent volumes of DMSO (Sigma).

Slide preparation and Immunofluorescence Microscopy -- HeLa cells were grown on coverslips, and given the indicated treatments. Cells were fixed on the coverslips in 4% PFA for 5 minutes at room temperature. They were then washed 2X and permeabilized in 0.1% Triton-X in PBS for 10 minutes at room temperature. The samples were washed 2X after permeabilization and incubated overnight with a 1:300 dilution of anti-FN14 (ITEM-4) at 4 ^{o}C. The following day, the samples were washed 4X in PBS, and incubated in a 1:500 dilution of Alexa 546-labeled donkey anti-mouse (Invitrogen) for 1 h at room temperature. The samples were then washed 4X, and were mounted on slides using ProLong Gold DAPI solution (Invitrogen). Cells were viewed with the Zeiss confocal microscope using the X63 lens. Images were acquired using the Zeiss camera and software, and analyzed using ImageJ (NIH).

Live cell imaging -- FN14 KO MEF cells stably expressing FN14-mCherry were grown on 35mm glass-bottom dishes purchased from Mattek. The cells were treated for 0 or 120 minutes with the indicated inhibitors, in the presence of 75nM lysotracker, purchased from Invitrogen. The cell culture medium was then changed, and the cells were viewed using the confocal microscope.

Biotinylation Assay -- HeLa cells at a confluency of 90-95% (5 x 10^6 cells) were washed with ice-cold PBS and incubated with 0.24 mg/ml biotin (EZ-link® Sulfo-NHS-SS-Biotin, Thermo Scientific) for 30 min at 4 ^{0}C. Cells were washed with ice-cold TBS (50mM Tris pH 7.4, 150mM NaCl), and then incubated at 37 ^{0}C for the indicated times. The cells were then washed in PBS and lysed in TNT buffer, and the lysates were cleared by centrifugation for 15 min at 14,000rpm at 4 ^{0}C. Aliquots were taken of the whole cell extracts, and the remainder of the lysates were incubated with 100μl of Immobilized NeutrAvidin Gel bead slurry (Thermo Scientific) for 1 h at 4 ^{0}C. The samples were then spun down, washed four times with a 50% mix of TNT and wash buffer (Thermo Scientific), and subjected to Western blot analysis.

TWEAK Neutralization -- HeLa cells, cultured overnight in DMEM supplemented with 1% FBS, were pre-treated for 30 minutes with 10μg/mL of the anti-TWEAK mAb CARL-1 or with 10μg/mL control IgG. The cells were then treated with 20μg/mL cycloheximide for the indicated times. The treated cells were washed in PBS, lysed in TNT buffer, and the lysates were subjected to SDS-PAGE and Western blot analysis.

Plasmid Construction -- The plasmid pLenti6.2-FN14ECD-V5 was constructed in the following manner. The FN14 extracellular domain (ECD) was amplified from an FN14-expression vector using the following primers: 5'-CACCATGGCTCGGGGCTCGCTG-3' (forward) and 5'-TCGTCTCCAGACCAAAAAGCCAGAAAGC-3' (reverse), and was subsequently cloned into the pENTR/D-TOPO entry vector (Invitrogen). The FN14 ECD was then recombined into the pLenti6.2/C-Lumio/V5-DEST destination vector (Invitrogen) using LR recombination (Invitrogen).

The pLenti6.2-FN14-CD40 B1 fusion was made by two rounds of PCR amplification. The N-terminal and C-terminal fragments of FN14 were amplified using the following sets of primers, respectively: 5'- CACCATGGCTCGGGGCTCGCTG -3' (forward) and 5'-TGCTGCAGCACAGACAACATCAGTCTT-3' (reverse) and 5'-GTTGTCTGTGCTGCAGCACCTCCTGCC-3' (forward) and 5'-TTACTGGATCAGCGCCACAGCTGGG-3' (reverse). The CD40 B1 fragment was amplified using the following primers: 5'-TGCATGGACTGTGAGACCAAAGACCTGGTT-3' (forward) and

5'-GGTCTCACAGTCCATGCACTTGTCCAG-3' (reverse). The final FN14-CD40 B1 fusion was assembled by PCR amplification using the 3 first round products as the template, and using only the outer primers for amplification. The fusion was then cloned as described above for the FN14 ECD construct. The FN14 ECD-CD40 TM/ICD and CD40 ECD-FN14 TM/ICD fusions were amplified and cloned in a similar manner. The N-terminal and C-terminal first round products for the FN14 ECD- CD40 TM/ICD fusion were amplified using the following primers, respectively: 5'- CACCATGGCTCGGGGCTCGCTG -3' (forward) and 5'-CACCAGGGCGGGCCAAAGCAGCCGGAAG-3' (reverse) and 5'-CTTTGGCCCGCCCTGGTGGTGATCCCC-3' (forward) and 5'-CTATCACTGTCTCTCCTGCACTGAGATGCG-3' (reverse). The N-terminal and C-terminal first round products for the CD40 ECD-FN14 TM/ICD fusion were amplified using the following primers, respectively: 5'-CACCATGGTTCGTCTGCCTCTGCAG-3' (forward) and 5'-CCCAAGGATGATAAAGACCAGCACCAA-3' (reverse) and 5'-CGGCTGAGAATCCTTGGGGGCGCTCTG-3' (forward) and 5'-TTACTGGATCAGCGCCACAGCTGGG -3' (reverse). The final FN14-CD40 fusions were assembled by PCR amplification using the 2 first round products as the template, and using only the outer primers for amplification. These fusions were then cloned as described above.

The TNFR1-FN14 fusions and the FN14-mpCD40 fusion were cloned as described previously for the FN14-CD40 fusions using two rounds of PCR amplification and subsequent directional cloning and recombination into a lentiviral vector.

The ΔLIQ FN14 truncation was made using the following primers: 5'-CACCATGGCTCGGGGCTCGCTG -3' (forward) and 5'-CGCCACAGCTGGGCAGCCCTC-3' (reverse) and cloned as described previously.

The FN14 mutants were made by PCR amplification using primers containing single base pair mutations and unique restriction sites also found in full-length FN14. The mutated, digested fragments were subsequently cloned into the digested FN14-pENTR entry vector using T4 ligation (New England BioLabs), and subsequently recombined into a lentiviral vector.

Generation of stable cell lines — Lentivirus was generated by transfection of 293FT cells (Invitrogen) using the traditional Lipofectamine 2000 protocol (Invitrogen) with the pLenti-6.2-V5-Dest-C-Lumio

vectors created above and the ViraPower packaging plasmids (Invitrogen). Supernatant was harvested at 48-72 h post-transfection, centrifuged at 100g for 5 minutes, and filtered through a 0.22uM filter. FN14 KO MEF cells were infected with 10mL of the virus-containing supernatant for 24 h. The cells were then switched to 10µg/mL blasticidin- (Invitrogen) containing media for selection of infected cells. Clonal lines were isolated by serial dilution and subsequent expansion of clonal cells. Individual clones were screened for expression of the introduced gene by Western blotting.

Immunoprecipitation -- HeLa cell lysates were incubated with the anti-FN14 (Cell Signaling Technology) antibody overnight at 4˚C. Protein G-Sepharose beads were added the following day for 1 hour at 4˚C. The beads were pelleted and washed 4 times with PBS, and then boiled in reducing SDS sample buffer. The samples were subjected to SDS-PAGE and Western blot analysis using the anti-FN14 or anti-TRAF2 antibody.

Cell transfections — HEK 293, HeLa, or FN14 KO MEFs were transfected using the reverse transfection protocol described in the Lipofectamine 2000 (Invitrogen) manual. DNA amounts were standardized using pCDNA3.1v(+) (Invitrogen).

Luciferase Reporter Assay -- HEK293 or FN14 KO MEFs were transfected in triplicate with FN14 or CD40 ECD-FN14 TM/ICD DNA, the pBIIXLuc NF-κB firefly luciferase reporter, and a Renilla luciferase control plasmid using Lipofectamine 2000 (Invitrogen) as described above. 24 hours post-transfection, lysates were harvested in 1X passive lysis buffer (Promega). Luciferase activity was measured using the Dual Luciferase Assay (Promega) according to the manufacturer’s protocol.

mRNA extraction, Reverse Transcription, and RT-PCR -- RNA was isolated from EGF treated FN14 KO MEFs stably expressing FN14-V5 using the RNeasy kit purchased from Qiagen. The samples were DNase treated using the recommended on-column protocol (Qiagen). 1µg of RNA from each sample was reverse transcribed using SuperScript III (Invitrogen). The cDNA was used in RT-PCR reactions to amplify β-actin and the FN14 extracellular domain using GoTaq Green Master Mix (Promega). The RT-

PCR primers for amplification of the FN14 ECD cDNA were the primers previously used to clone the FN14 ECD.

CD40L and TWEAK Stimulation — HeLa cells, cultured overnight in DMEM supplemented with 1% FBS, were stimulated with recombinant TWEAK (R&D Systems) or a purified membrane preparation of CD40L from baculovirus-infected SF9 cells (gifted from M. Shlomchik) by direct addition to the media (132). Unless otherwise indicated, TWEAK was used at a final concentration of 100ng/mL for 30 minutes and CD40L was used at a 1:200 dilution.

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