Regulation of Fibroblast Growth Factor-inducible 14 (Fn14) Expression Levels via Ligand-independent Lysosomal Degradation

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Background: Fn14 is a highly inducible TNF superfamily cytokine receptor.

Results: Fn14 undergoes rapid, ligand-independent internalization and degradation mediated by the extracellular domain of the receptor.

Conclusion: Fn14 expression is regulated through transcription as described previously and through a novel post-translational mechanism.

Significance: Receptor trafficking may play an important role in regulating receptor availability, cytokine responses, and ligand-independent signaling.

Fibroblast growth factor-inducible 14 (Fn14) is a highly inducible cytokine receptor that engages multiple intracellular signaling pathways, including nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK). Fn14 expression is regulated by several cytokines and growth factors, and Fn14 is transiently up-regulated after injury. In contrast, in states of chronic inflammatory disease and in some solid tumors, Fn14 is persistently up-regulated. However, the post-translational regulation of Fn14 expression has not been directly investigated. Thus, we examined Fn14 proteostasis in the presence and absence of the Fn14 ligand TWEAK-like weak inducer of apoptosis (TWEAK). Similar to other TNF receptor superfamily members, we found that TWEAK induces Fn14 internalization and degradation. Surprisingly, we also observed rapid, TWEAK-independent, constitutive Fn14 internalization and turnover. Fn14 levels are maintained in cell culture by ongoing synthesis and trafficking of the receptor, leading to subsequent down-regulation by lysosomal degradation. Unexpectedly, the extracellular domain of Fn14 is necessary and sufficient for constitutive turnover. Based on these findings, we propose a model in which constitutive down-regulation of Fn14 facilitates dynamic regulation of Fn14 protein levels and prevents spontaneous or inappropriate receptor signaling.

Fn143 is the smallest member of the TNF receptor superfamily and is the only known signaling receptor for TWEAK (1). Fn14 is a multifunctional receptor capable of inducing proinflammatory, angiogenic, proliferative, and cell death responses (2). Fn14 induces expression of cytokines, chemokines, and cell adhesion molecules through the activation of both the canonical and non-canonical NF-κB pathways and MAPK signaling pathways (2, 3).

Fn14 is expressed at low levels in normal tissues but is highly expressed following tissue injury (1, 4–7) and is crucial for wound repair and regeneration (8–10). Although Fn14 appears to play an important physiological role in response to acute injury, Fn14 levels are also dysregulated in models of chronic inflammatory disease (11) as well as in patient samples from a range of human diseases, including rheumatoid arthritis (12), multiple sclerosis (13), inflammatory liver diseases (14), and inflammatory bowel disease (15). Increased Fn14 mRNA and protein levels have also been detected in a number of solid tumors with elevated expression levels correlating with poor patient outcome (16–19). Overall, it appears that Fn14 expression is induced in many chronic disease states and that persistent activation of the TWEAK-Fn14 pathway contributes to the pathology of these diseases.

In cells cultured in vitro, Fn14 expression is induced by a variety of factors, including phorbol 12-myristate 13-acetate, FBS, FGF-1, FGF-2, and PDGF in both murine and human fibroblasts (1, 8, 20), as well as by its own ligand, TWEAK, in glioma cell lines (16). Various cytokines also induce expression of Fn14 (2, 4, 6, 20–23). Increased expression of the receptor

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3 The abbreviations used are: Fn14, fibroblast growth factor-inducible 14; TWEAK, TNF-like weak inducer of apoptosis; MEF, mouse embryonic fibroblast; sulfo-NHS-SS-biotin, sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate; PE, phycoerythrin; CHX, cycloheximide; ICD, intracellular domain; ECD, extracellular domain; TM, transmembrane domain; CD40L, CD40 ligand; TNFR, TNF receptor; TNFRSF, TNF receptor superfamily.
likely enhances sensitivity to ligand and may also increase the probability of ligand-independent signaling by the receptor (16, 24–27).

Although the highly inducible nature of Fn14 and the propensity for Fn14 up-regulation in disease conditions is well established, factors that could have dramatic effects on the sustained activity of the receptor, such as the stability of Fn14 or mechanisms of Fn14 down-regulation, have not been explored. In this study, we report that Fn14 synthesis is not only tightly controlled as reported previously but that cellular mechanisms exist to also regulate Fn14 degradation. The receptor undergoes both ligand-dependent and remarkably rapid ligand-independent receptor loss. In cell culture, steady-state levels of the receptor are maintained by a seemingly dynamic process. Fn14 is constitutively synthesized, trafficked to the cell surface, and then internalized and degraded. Through the use of chimeric receptor constructs, we demonstrate that receptor proteostasis is regulated by the Fn14 extracellular domain and likely occurs through lysosomal degradation. We propose a model in which induction of receptor expression could overcome the steady-state balance between protein synthesis and degradation of Fn14, altering the sensitivity to ligand and raising the possibility of ligand-independent signaling. Our findings reveal a previously unknown facet of Fn14 cell biology that might ultimately alter our understanding of the mechanisms of Fn14 dysregulation during disease progression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa and HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% FBS. The cells were switched to DMEM supplemented with 1% FBS 2 h prior to use. The Fn14 knock-out (KO) MEF cells were cultured as described previously (28). Primary mouse keratinocytes were a gift from Dr. Teruki Dainichi at Columbia University and were maintained in DMEM supplemented with 10% FBS. Primary human keratinocytes were obtained from the Skin Disease Research Center facility at Columbia University and were maintained in EBM-2 medium (Lonza). Human umbilical vein endothelial cells were a gift from Dr. Jan Kitajewski at Columbia University and were 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.

**Antibodies**—The polyclonal anti-Fn14 antibody used for Western blot and immunoprecipitation was purchased from Cell Signaling Technology (catalog number 4403). The polyclonal anti-EGF receptor antibody was purchased from Millipore (catalog number 06-847). The monoclonal anti–GAPDH antibody was purchased from Fitzgerald (10R-G109a). The monoclonal anti-Fn14 antibody (ITEM-4 clone) and isotype control used for flow cytometry and immunofluorescence were purchased from eBioscience. The anti-CD40 antibody was purchased from Santa Cruz Biotechnology. The monoclonal anti–TWEAK antibody (CARL-1 clone) used for neutralization was purchased from eBioscience, and the control IgG was purchased from Pharmingen. The anti-IkBα and anti-phospho-IkBα antibodies were purchased from Cell Signaling Technology.

**Western Blot**—Lysates were harvested in TNT buffer (50 mM Tris, pH 7.5–8, 200 mM NaCl, 1% Triton-X, 0.5 mM EDTA) supplemented with protease and phosphatase inhibitors. Thirty to 50 µg of total protein were run on 10–20% gradient SDS-polyacrylamide gels. After transfer to polyvinylidene difluoride membrane (Millipore), immunoblotting was performed according to the manufacturer’s protocol. Images were captured using the Eastman Kodak Co. X-Omat Developer or the Bio-Rad ChemiDoc MP System. Protein half-life was calculated by densitometry on the Western blot image files using the NIH ImageJ program with subsequent calculation of the ratio of Fn14 band intensity/GAPDH band intensity.

**Biotinylation Assay**—HeLa cells at 90–95% confluence (5 × 10⁶ cells) were washed with ice-cold PBS and incubated with 0.24 mg/ml biotin (EZ-link® sulfo-NHS-biotin, Thermo Scientific) for 30 min at 4°C. Cells were washed with ice-cold TBS (50 mM Tris, pH 7.4, 150 mM NaCl) and then incubated in complete DMEM at 37°C with or without TWEAK 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 14,000 rpm at 4°C. Aliquots were taken of the whole cell extracts, and the remainder of the lysates were incubated with 100 µl of immobilized NeutrAvidin slurry (Thermo Scientific) for 1 h at 4°C. The NeutrAvidin beads were then washed four times with a 50% mixture of TNT and wash buffer (Thermo Scientific) and boiled in SDS sample buffer, and the eluate (biotin pulldown) was subjected to Western blot analysis.

**Flow Cytometry**—For surface staining, HeLa cells and human umbilical vein endothelial cells (10⁶ cells) were incubated with 0.1 µg of PE-conjugated ITEM-4 or isotype control antibody (eBiosciences) for 30 min at 4°C. For total staining, CytOFix/ CytoPerm (BD Biosciences) was used according to the manufacturer’s instructions, and then cells were stained as above. After staining, the cells were analyzed on a LSR II flow cytometer (BD Biosciences), and the data were processed using FlowJo software (Tree Star, Inc.). Statistical analysis was performed using Prism software (GraphPad).

**Inhibition of Protein Synthesis, Trafficking, Degradation, and Shedding**—HeLa cells, starved for 2 h in DMEM supplemented with 1% FBS, were treated with inhibitors for the indicated times by direct addition to the cell culture medium. Protein synthesis was inhibited by cycloheximide (Sigma) treatment at a final concentration of 20 µg/ml. Protein trafficking was inhibited by treatment with brefeldin A (eBiosciences) at a final concentration of 10 µM. Endolysosomal degradation was inhibited by treatment with ammonium chloride at a final concentration of 20 mM, bafilomycin A1 (Sigma) at a final concentration of 100 nM, or leupeptin (Sigma) at a final concentration of 10 µM. Controls were performed by addition of equivalent volumes of DMSO (Sigma).

**Slide Preparation and Immunofluorescence Microscopy**—HeLa cells were grown on coverslips and treated with the indicated agents. Cells were fixed on the coverslips in 4% paraformaldehyde for 5 min at room temperature. They were then washed two times and permeabilized in 0.1% Triton-X in PBS for 10 min at room temperature. The samples were washed two times after permeabilization and incubated overnight with a
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1:300 dilution of anti-Fn14 antibody (ITEM-4) at 4 °C. The following day, the samples were washed four times in PBS and incubated in a 1:500 dilution of Alexa Fluor 546-labeled donkey anti-mouse antibody (Invitrogen) for 1 h at room temperature. For receptor internalization assays, cells were incubated with 1 μg of PE-ITEM-4 or isotype control for the indicated time and then immediately fixed in 4% paraformaldehyde. Fixed coverslips were then washed four times with PBS and mounted on slides using ProLong Gold DAPI solution (Invitrogen). Cells were viewed using either a Zeiss LSM 710 or LSM 510 confocal microscope with a 63× oil immersion objective. Images were acquired using the Zeiss Zen software and analyzed using NIH ImageJ.

Live Cell Imaging—Tnfrsf12a−/− (Fn14 KO) MEF cells stably expressing Fn14-mCherry were grown on 35-mm glass bottom dishes (Mattek). The cells were treated for 120 min with the indicated inhibitors in the presence of 75 nM LysoTracker Red (Invitrogen). The cell culture medium was then changed, and the cells were imaged using a confocal microscope.

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

Plasmid Construction—The plasmid pLent6.2-Fn14ECD/TM-V5 was constructed in the following manner. The Fn14 extracellular and transmembrane domains (Fn14-ΔICD) were amplified from an Fn14 expression vector using the primers 5’-CACCATGGCTCGGGGCTCCTGCT-3’ (forward) and 5’-TCGTCCTCCAGACCAAAAGCAGCAAGGC-3’ (reverse) and subsequently cloned into the pENTR/D-TOPO entry vector (Invitrogen). The Fn14-ΔICD was then recombined into the pLent6.2/C-Lumio/V5-DEST destination vector (Invitrogen) using LR recombination (Invitrogen).

The pLent6.2-Fn14 ECD-C40 TM-ICD (Fn14ΔICD-C40) and CD40 ECD-Fn14 TM-ICD (CD40ΔECD-Fn14) fusions were made by two rounds of PCR amplification. The N-terminal and C-terminal first round products for the Fn14 ECD-C40 TM-ICD fusion were amplified using the following primer pairs, respectively: 5’-CACCATGGCTCGGGGCTCCTGCTG-3’ and 5’-CACCATGGCTCGGGGCTCCTGCTG-3’ (forward) and 5’-CACCATGGCTCGGGGCTCCTGCTG-3’ (reverse) and subsequently cloned into the pENTR/D-TOPO entry vector (Invitrogen). The Fn14-ΔICD was then recombined into the pLent6.2/C-Lumio/V5-DEST destination vector (Invitrogen) using LR recombination (Invitrogen).

RESULTS

Fn14 Undergoes Rapid, TWEAK-induced Turnover—Ligand-induced receptor internalization and degradation is a common feature of several classes of cell surface receptors. A number of TNFR superfamily (TNFRSF) members, including TNFR1 (29, 30), CD40 (31, 32), and Fas (33), undergo ligand-induced down-regulation. Although it is known that TWEAK binding can induce lysosomal degradation of Fn14 pathway signaling components (34), the fate of Fn14 following ligation by TWEAK is unknown.

It has been shown previously that only a portion of Fn14 is present at the cell surface (20). Therefore, to examine the effects of TWEAK binding, we initially limited our analysis to cell surface Fn14. To directly interrogate turnover of cell surface Fn14 in the presence of TWEAK, we performed biotinylation of cell surface proteins using membrane-impermeant sulfo-NHS-SS-biotin (Pierce; Fig. 1A). As indicated by the cell surface biotinylation assay, levels of cell surface Fn14 decreased in response to ligand as early as 5 min post-stimulation (Fig. 1B), suggesting rapid loss of cell surface Fn14 in the presence of TWEAK.

Next we examined the effects of TWEAK stimulation on total cellular Fn14 levels. Given that only a portion of Fn14 is localized to the plasma membrane, we anticipated that TWEAK stimulation would only partially reduce total cellular Fn14 levels. Instead, we observed that treatment of HeLa cells (Fig. 1C) with increasing concentrations of TWEAK for 30 min resulted in a dose-dependent loss of total cellular Fn14 protein. Surprisingly, at high doses of TWEAK stimulation, almost complete loss of total cellular Fn14 was observed (Fig. 1C). The cell surface biotinylation experiments demonstrated loss of cell surface Fn14 at 5 min, whereas detectable changes in total Fn14 were not observed until 15 min of stimulation (Fig. 1B). Taken
together, these results suggest that over time TWEAK gains access to Fn14 that is not initially present at the cell surface. Therefore, we performed a kinetic analysis of total cellular Fn14 levels during continuous TWEAK treatment of HeLa cells. Consistent with Fig. 1B, this revealed loss of Fn14 as early as 15 min post-stimulation (Fig. 1D).

Our results suggest that TWEAK exposure drives rapid internalization and degradation of cell surface Fn14 and that this is sufficient to decrease total cellular Fn14 levels. We wanted to ascertain whether this Fn14 down-regulation, apparent even at 30 min of TWEAK exposure (Fig. 1E), was dependent on the continued presence of TWEAK. To test this, we examined the kinetics of Fn14 recovery following TWEAK withdrawal. Cells were treated with TWEAK for 15 min and then washed repeatedly, returned to fresh medium without TWEAK, and cultured at 37 °C. Using this approach, we found that Fn14 levels were rapidly restored after TWEAK was removed (Fig. 1F). However, we did not observe expression of Fn14 at above basal levels. These results, therefore, suggested that this recovery is likely due to low level basal production of Fn14 rather than TWEAK-induced receptor expression, which has been reported by other groups (16).

Fn14 Undergoes Rapid, Constitutive Turnover—It was surprising that TWEAK would not only induce internalization and degradation of surface Fn14 but also could induce such a dramatic decrease in total cellular Fn14 levels. This suggested that the majority of Fn14 is located at the cell surface or reaches the cell surface within minutes. To address the steady-state stability of the receptor, we next examined the half-life of Fn14 in the absence of added ligand. Protein half-life was determined using the bacterially derived product CHX, which blocks translation elongation and is commonly used to inhibit protein synthesis. Analysis of total Fn14 levels after treatment of HeLa cells with CHX revealed rapid loss of Fn14 with a half-life of 74 min (Fig. 2, A and B). In a proteomics study examining protein stability in HeLa cells, it was shown that the average turnover time for HeLa cell proteins is 20 h with more rapid turnover times for proteins involved in dynamic processes such as mitosis, nucleotide binding, and cytoskeletal reorganization (35). Little is known about the steady-state dynamics of the TNFRSF members in the absence of ligand, but such rapid turnover of a cytokine receptor, as exhibited by Fn14 in HeLa cells, seemed quite remarkable. The rapid turnover of Fn14 also occurred in HEK 293 cells, primary cultured human umbilical vein endothelial cells, and primary cultured keratinocytes (Fig. 2C). These results suggest that although TWEAK accelerates Fn14 turnover rapid Fn14 down-regulation occurs constitutively in the absence of added ligand.

Given our results demonstrating TWEAK-induced (Fig. 1B) dose-dependent loss of total Fn14 (Fig. 1C) as well as recovery of total cellular Fn14 expression levels after TWEAK withdrawal (Fig. 1F), we suspected that Fn14 might be continuously

FIGURE 1. Fn14 undergoes ligand-induced down-regulation. A, cell surface biotinylation was performed on ice using sulfo-NHS-SS-biotin, and cells were then cultured at 37 °C. Whole cell lysates were prepared and either directly (Lysate) or after biotin pulldown (Biotin-PD) using NeutrAvidin beads analyzed by Western blotting. B, HeLa cells were subjected to cell surface biotinylation followed by TWEAK incubation at 37 °C for the indicated times. Whole cell lysates and biotin pulldowns were analyzed by Western blotting for Fn14 and the control proteins EGF receptor (EGFR) and GAPDH. C, HeLa cells were incubated with increasing concentrations of TWEAK for 30 min, and whole cell lysates were prepared and analyzed by Western blotting. D, HeLa cells were treated for the indicated times with 100 ng/ml TWEAK, and Western blotting for the indicated proteins was performed. E, Western blotting and densitometry were used to quantify Fn14 protein levels after continuous TWEAK treatment for the indicated times. Fn14 protein levels are standardized to the level at the 0 timepoint and normalized to GAPDH. Data represent average Fn14 (%) remaining from three independent experiments, and error bars represent S.D. *, p < 0.05; **, p < 0.005. F, HeLa cells were subjected to brief stimulation (15 min) with 100 ng/ml TWEAK and then washed and cultured in fresh media without TWEAK at 37 °C for the indicated times. Lysates were prepared and analyzed by Western blotting as indicated. All data are representative of at least three independent experiments. Stim, stimulation; TWK, TWEAK.
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moving to and from the cell surface. Such a model could explain the ability of TWEAK to access the total cellular pool of Fn14, resulting in dose-dependent receptor down-regulation. Therefore, we wished to ascertain whether cell surface Fn14 was also turning over and being continuously replenished in the absence of TWEAK stimulation. To do this, we analyzed Fn14 levels by flow cytometry. HeLa cells were treated with CHX followed by staining of surface Fn14 and FACS analysis. This analysis revealed loss of surface Fn14 expression by 60 min after inhibition of protein synthesis with CHX, although some receptor loss was often observable at earlier time points (Fig. 2, D and E). This loss could represent either a decrease in the expression of cell surface Fn14 due to the inhibition of protein synthesis or the actual loss of surface receptors by shedding or internalization. To address this issue, we directly assessed turnover of surface Fn14 using the cell surface biotinylation assay (Fig. 1A).

The results of this assay demonstrated that biotinylated cell surface Fn14 indeed exhibits rapid turnover, which is noticeable after 15 min of incubation at 37 °C and more prominent after 30 min. These results indicate that unliganded Fn14 is rapidly trafficking to and lost from the plasma membrane (Fig. 2F).

In steady-state conditions, Fn14 is unstable—The results presented above demonstrated that Fn14 turns over rapidly in the absence of added TWEAK. The loss of surface Fn14 is clearly accelerated upon addition of TWEAK as loss of biotinylated surface Fn14 is apparent at 15 min (Fig. 2F) in the absence of TWEAK but at 5 min in the presence (Fig. 1B) of TWEAK. Although the acceleration of Fn14 turnover upon TWEAK addition suggests that constitutive turnover occurs via a dis-
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chemical inhibitors of cell trafficking pathways. Specifically, we tested brefeldin A, which inhibits the trafficking of newly synthesized proteins from the endoplasmic reticulum by blocking activation of the ARF1p GTPase and formation of transport vesicles (37), and monensin, which is an ionophore that inhibits trafficking within the Golgi by blocking Golgi acidification (38). Treatment of HeLa cells with either inhibitor resulted in accumulation of total cellular Fn14 and protected Fn14 from the turnover apparent upon CHX treatment (Fig. 4A). These results not only suggest that Fn14 is constitutively synthesized and trafficked through the secretory pathway in cell culture but also indicate that trafficking to the plasma membrane precedes constitutive loss of the receptor.

However, because brefeldin A and monensin treatment have also been reported to block endocytic pathways (39, 40), we wanted to determine whether these inhibitors were having the predicted effect on cell surface expression of Fn14 that would result from inhibition of secretory pathway function. FACS analysis demonstrated that although treatment with the secretory pathway inhibitors led to increased levels of total cellular Fn14 surface levels of the receptor were decreased (Fig. 4B). This not only indicates that the inhibitors specifically blocked trafficking through the secretory pathway but also suggests that cell surface expression of Fn14 requires continuous trafficking to the plasma membrane. To confirm the FACS analysis and verify that the observed Fn14 accumulation was occurring intracellularly rather than at the level of cell surface retention, we decided to directly visualize the trafficking of Fn14 upon treatment with brefeldin A or monensin. Therefore, we next examined the effects of treatment with synthesis or trafficking inhibitors on Fn14 levels in HeLa cells by immunofluorescence using an anti-Fn14 antibody. We were able to visualize both the loss of receptor upon inhibition of protein synthesis using CHX and the increase in intracellular receptor levels upon inhibition of trafficking using brefeldin A or monensin (Fig. 4C). Brefeldin A treatment causes complete disassembly of the Golgi structure (37), whereas monensin treatment causes Golgi swelling (38). Accordingly, upon monensin treatment, but not brefeldin A treatment, we saw increased Fn14 levels at a perinuclear structure, suggestive of Golgi accumulation. This observation further implies that the accumulated Fn14 is newly synthesized protein trapped within the secretory pathway. Taken together, our results demonstrate that steady-state cell surface expression of Fn14 in cultured cells is a dynamic process, which is dependent on the constitutive replenishment of receptor that is rapidly lost from the plasma membrane.

**Fn14 Constitutive Turnover Requires Endosomal Acidification**—To determine the fate of Fn14 after trafficking to the plasma membrane, we examined possible mechanisms of receptor loss. Several members of the TNFR superfamily undergo shedding, although this has not been reported for Fn14. Despite using multiple methods, including attempts to immunoprecipitate endogenous and an overexpressed N-terminally 3XFLAG-tagged Fn14 construct from culture supernatants, we were unable to detect extracellular release of Fn14 under any circumstance (data not shown). Given that cell surface Fn14 was rapidly lost (Fig. 2F), the most likely alternative scenario was receptor internalization and lysosomal degrada-

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**FIGURE 3. Fn14 constitutive turnover occurs in the absence of ligand.** A, HeLa cells were pretreated for 30 min with 10 μg/ml anti-TWEAK (TWK) antibody or an IgG isotype control. The cells were then incubated with CHX for 0, 60, or 120 min. Fn14 levels were analyzed by Western blotting. B, HeLa cells were treated with 100 ng/ml TWEAK, 50 μg/ml CHX, or both compounds for the indicated times before subsequent Western blotting analysis. C, densitometry was used to calculate Fn14 half-life using data from triplicate samples. Error bars represent S.D. All data are representative of three independent experiments.
tion. Therefore, we tested the effects of inhibitors of endocytosis and endolysosomal maturation on Fn14 turnover. For this purpose, we used sucrose, an osmotic inhibitor of endocytosis (41), and butanol, which disrupts formation of clathrin-coated pits (42). Pretreatment of HeLa cells (Fig. 5A) and human keratinocytes (data not shown) with either inhibitor prior to inhibition of protein synthesis with CHX resulted in significant stabilization of the receptor (Fig. 5B), thereby implicating endocytosis in receptor loss. It is worth noting, however, that pretreatment of HeLa cells with the endocytic inhibitors also consistently changed the steady-state levels of the protein. The implications of this decrease are unclear but appear to be specific to HeLa cells as the steady-state decrease in Fn14 did not occur in primary keratinocytes (data not shown).

It has been shown previously that overexpressed Fn14 exhibits localization to the plasma membrane and to the trans-Golgi network near the nuclear membrane (20). We verified these findings by overexpression of YFP-tagged cellular markers (43) and subsequent immunofluorescent staining of endogenous Fn14. Fn14 exhibited partial co-localization with a plasma membrane marker, KRAS2, but also substantial intracellular localization (Fig. 5C, upper panels). Intracellular Fn14 exhibited only partial co-localization with the Golgi marker furin (Fig. 5C, lower panels). However, there were also intracellular Fn14 puncta that did not co-localize with KRAS or furin as indicated by the yellow arrows in Fig. 5C, 300× insets.

Based on our biochemical data showing that inhibitors of endocytosis blocked turnover, we speculated that these puncta might represent the endolysosomal system. To evaluate whether Fn14 localizes to the endolysosomal system, cells stably expressing a C-terminally mCherry-tagged Fn14 were generated using Fn14 knock-out MEFs. This fusion protein was
shown to turn over rapidly, although the kinetics were somewhat slower than that of wild-type Fn14 ($t_{1/2}$ = 171 min) (Fig. 5D and data not shown). The Fn14-mCherry-expressing cells were stained with LysoTracker, a marker of acidic organelles, most prominently late endosomes and lysosomes. There were numerous instances of co-localization between Fn14-mCherry and the acidic compartments in the cell as shown clearly in Fig. 5E, inset, suggesting that Fn14 may undergo lysosomal degradation.

To test whether Fn14 was actually subject to lysosomal degradation, we analyzed turnover in the presence of inhibitors of lysosome function. Pretreatment of either Fn14-reconstituted KO MEFs (Fig. 5F) or human keratinocytes (Fig. 5G) with inhibitors of endosomal acidification resulted in receptor accumulation and blocked Fn14 turnover (Fig. 5F). Conversely, pretreatment with epoxomicin, a specific inhibitor of proteasomal degradation (46), had no effect on Fn14 turnover in the presence of CHX (data not shown).

Finally, recent work has explored antibody-mediated targeting of Fn14 as a means of toxin delivery to solid tumors that constitutively overexpress Fn14 (28, 47, 48). Our data suggest that Fn14 might facilitate toxin delivery by constitutive recep-
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The extracellular domain of Fn14 is necessary and sufficient for constitutive turnover. A, schematic of receptor truncations and fusions. B, Fn14 KO MEFs stably expressing wild-type full-length human Fn14 fused to a V5 epitope tag were treated with CHX or increasing concentrations of TWEAK (TWK) as indicated, and total cellular Fn14 levels were assessed by Western blotting with anti-Fn14 and anti-V5 antibodies as indicated. C, Fn14 KO MEFs stably expressing the Fn14-ΔLIQ truncation (Fn14-ΔLIQ) were treated with the indicated concentrations of TWEAK for 30 min or with 50 μg/ml CHX for the indicated times. D, Fn14 KO MEFs stably expressing the Fn14 cytoplasmic domain truncation (Fn14-ΔICD) were treated with CHX for the indicated times. E, Fn14 KO MEFs stably expressing wild-type CD40 were treated with CHX for the indicated times. F, Fn14 KO MEFs stably expressing the indicated fusion receptors were subjected to CHX treatment for the indicated times. G, Fn14 KO MEFs stably expressing the indicated wild-type and fusion receptors were stimulated with the corresponding ligand, either 100 ng/ml TWEAK or 200 ng/ml CD40L, for the indicated times (m, minutes). Signaling was analyzed by Western blotting for phospho (P)-IkBα and total IkBα. Data are representative of three independent experiments. CRD, cysteine-rich domain; stables, stably expressing cells.

The Extracellular Domain of the Receptor Regulates Fn14 Trafficking—To further examine the mechanism of Fn14 constitutive turnover, we constructed a number of receptor truncations and fusions to investigate the regions of the receptor that might be involved in its trafficking (Fig. 6A). Fn14 contains a putative cytoplasmic endocytic motif (LI) at the C terminus (20), which has not yet been evaluated for functional significance. 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. To rule out potential homotypic interactions with endogenous Fn14, we performed these experiments by stably reconstituting Fn14 knock-out MEFs through lentiviral transduction. Fn14 KO MEFs stably expressing wild-type full-length human Fn14 exhibited the expected ligand-independent turnover and TWEAK-induced down-regulation (Fig. 6B). In stably expressing cells, it was shown the ΔLIQ truncation turned over in the presence and absence of ligand with kinetics similar to those observed for wild-type Fn14 (Fig. 6C). Thus, the putative endocytic motif is dispensable for receptor turnover.

As there was not another obvious cytoplasmic motif to interrogate, we next generated a truncation of the receptor lacking the entire cytoplasmic domain (Fn14-ΔICD). This construct was used to stably reconstitute Fn14 knock-out MEFs. In the resulting clonal stably expressing cell line, the cytoplasmic domain truncation turned over with kinetics similar to those of wild-type Fn14 (Fig. 6D). These results suggested that the cytoplasmic domain of the receptor is dispensable for Fn14 constitutive turnover and furthermore that the extracellular and transmembrane domains are sufficient for Fn14 constitutive turnover.

To determine the necessity of the Fn14 ECD for turnover, we generated chimeric receptor fusions between Fn14 and another TNFR family member, CD40, which has a reported half-life of more than 8 h (49) (Fig. 6A). Turnover of CD40 or Fn14 was assessed in reconstituted stable cell lines. Consistent with published results (49), CD40 does not undergo turnover in the same rapid time frame as Fn14 (Fig. 6E). In support of the deletion analysis (Fig. 6D), the fusion construct containing the CD40 ECD in place of the Fn14 ECD (CD40ECDFn14) exhibited stability that was characteristic of wild-type CD40, indicating that the loss of the Fn14 ECD abrogates the rapid constitutive turnover ability of the receptor (Fig. 6F). Conversely, the fusion construct containing the Fn14 ECD in place of the CD40 ECD (Fn14ECDCD40) exhibited rapid turnover kinetics similar to those of wild-type Fn14, indicating that addition of the Fn14...
ECD is sufficient to confer the rapid constitutive turnover ability to another receptor (Fig. 6f). Thus, the Fn14 ECD is both necessary and sufficient for constitutive turnover. Furthermore, transfer of the CD40 ECD onto the Fn14 cytoplasmic tail resulted in a receptor that was not only more stable than wild-type Fn14 but one that exhibited sustained ligand-induced NF-κB pathway signaling as determined by the kinetics of IκBα phosphorylation and degradation (Fig. 6f).

**DISCUSSION**

The TWEAK receptor Fn14 is expressed at low levels in most normal tissue but is rapidly up-regulated during tissue injury and inflammation. Given that the expression of Fn14 is also elevated in a variety of chronic inflammatory conditions (12–15) and in numerous invasive cancers (16–18), we wanted to better understand the post-translational regulation of Fn14.

Through this work, we have identified a previously unrecognized aspect of Fn14 regulation. We found that Fn14 undergoes rapid, constitutive synthesis and degradation and that this degradation is accelerated, rather than triggered, in the presence of TWEAK (Fig. 3). The previously described membrane and Golgi localization of Fn14 (20) represents the steady-state subcellular distribution of a receptor that is being rapidly and constitutively synthesized, trafficked to the plasma membrane, and degraded. The constitutive turnover of the receptor occurred in all cell types tested, suggesting that it is a universal property of Fn14 biology.

It is not entirely surprising that Fn14 is down-regulated in response to ligand (Fig. 1) because this mechanism has been shown for several other TNFRSF members, including TNFR1 (50) and numerous heterologous receptors. The fate of TNFRSF members in the presence of ligand has been well studied; however, little has been done to look at steady-state levels and localization of these receptors in the absence of ligand. These factors may determine the availability of receptors for ligand-induced signaling and furthermore control the quiescence of the signaling pathway in the absence of stimulation.

We examined the possibility that Fn14, like other members of the TNFR superfamily, might be regulated by receptor shedding but were unable to find evidence to support this possibility. Rather, our findings indicated that Fn14 undergoes ligand-dependent (Fig. 1) and ligand-independent (Figs. 2 and 3) receptor internalization and degradation, suggesting that there are previously unrecognized aspects of Fn14 spatiotemporal regulation that require further investigation. It is well established that the unrelated Notch receptor undergoes a mechanism of constitutive turnover similar to the one we are proposing for Fn14 (51). The spatiotemporal regulation of Notch has important implications for its function. In the presence of ligand, Notch requires endocytosis for full activation (51). In the absence of ligand, Notch undergoes continuous endocytosis, thereby preventing spontaneous activation (51). We expect that, as with the Notch receptor, spatiotemporal regulation is very important for prevention of inappropriate, spontaneous activation of Fn14.

Fn14 is known to be tightly controlled at the level of expression (2, 3). Here we show that once expressed the receptor is also under tight post-translational control. There are several potential reasons why Fn14, in particular, might be so tightly regulated. Fn14 is very widely expressed (1, 9, 14, 25, 52–54), and dynamic regulation of receptor function is likely important for tissue homeostasis. Furthermore, the positive feedback loop that leads to amplified receptor expression (16) results in a system that is very sensitive to ligand and potentially to ligand-independent signaling. For comparison, other canonical TNFRSF members, such as TNFR1, are not known to be positively regulated by their own ligand. This positive feedback mechanism may render Fn14 particularly susceptible to dysregulated expression and signaling, leading to excess inflammation and tissue damage. This seems especially probable given that Fn14 is able to self-associate and signal independently of ligand (27). Finally, Fn14 has been shown to directly induce cell death in some cells (2, 55–58). 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 also necessitates tight regulation.

Based on the use of multiple inhibitors of endolysosomal maturation, we concluded that Fn14 constitutive turnover occurs through lysosomal degradation (Fig. 5). Surprisingly, ligand-independent trafficking of Fn14 does not require the described C-terminal LI endocytic motif (20) or the 18-amino acid cytoplasmic motif that mediates TWEAK-independent Fn14 dimerization and signaling (27) (Fig. 6). Instead, the extracellular domain of the receptor is necessary and sufficient for constitutive turnover of Fn14 and is able to confer this ability when fused with a heterologous receptor. Given that the ΔICD Fn14 protein, which lacks the entire cytoplasmic domain, exhibited constitutive turnover in Fn14 knock-out cells, we anticipate that Fn14 levels are regulated through heterotypic binding of the extracellular domain to another transmembrane protein or trafficking partner.

We are currently investigating the biological implications of Fn14 constitutive trafficking. Fn14 levels are elevated in a number of pathological states (12–15), and it is assumed that this is primarily a result of increased Fn14 mRNA expression. Given our findings, however, it is possible that stabilization of the receptor could contribute to pathology as well. Receptor stability can influence the availability of the receptor for both TWEAK-induced and ligand-independent signaling and, therefore, the signaling kinetics, making it an important temporal regulatory factor of receptor activity. Future studies will determine whether persistent Fn14 up-regulation in malignancy and chronic inflammation is related to changes in Fn14 trafficking. Furthermore, trafficking of the receptor may also suggest thus far unknown spatial regulation of Fn14 signaling pathways. A signaling requirement for trafficking-induced compartmentalization has been described previously for other TNFRSF members, notably TNFR1 (59, 60) and CD40 (61, 62).

We have also found that receptor dynamics shape Fn14 signaling outcomes as evidenced by the fact that stabilization of the receptor through fusion with CD40 resulted in extended activation of the NF-κB signaling pathway (Fig. 6G). These results suggest that the receptor extracellular domain not only determines ligand specificity and affinity but also signaling dynamics. Given that the oligomeric status of TWEAK or anti-
Fnn14 agonistic antibodies are crucial determinants of the signaling outcome when Fnn14 is engaged (63), we speculate that regulation of Fnn14 dynamics may likewise influence responses to differentially presented ligand. Future studies will ascertain the contribution of constitutive Fnn14 trafficking and changes in Fnn14 proteostasis to TWEAK-induced signaling outcomes, ligand-independent signaling, and signaling by agonistic anti-Fnn14 antibodies.

The post-translational regulatory mechanism that we have described for Fnn14 is also particularly relevant in light of recent studies that have investigated the use of anti-Fnn14-toxin immunonoconjugates to target Fnn14-expressing tumors (28, 47, 48). One piece of evidence from these studies that supports our model of constitutive receptor endocytosis is the finding by Zhou et al. (28) that binding of an antibody-ribotoxin conjugate to Fnn14 leads to internalization of the ITEM-4 anti-Fnn14 antibody within 2 h. Indeed, we found that ITEM-4 internalization in Fn14 leads to internalization of the ITEM-4 anti-Fn14 antibodies.

Zhou

Post-translational Regulation of Fn14

The potential implications of Fn14 internalization for immunotoxin therapy.

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