

TECHNIQUES

A biosensor for the activity of the “sheddease” TACE (ADAM17) reveals novel and cell type-specific mechanisms of TACE activation

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Diverse environmental conditions stimulate protein “shedding” from the cell surface through proteolytic cleavage. The protease TACE [tumor necrosis factor- α (TNF α)-converting enzyme, encoded by *ADAM17*] mediates protein shedding, thereby regulating the maturation and release of various extracellular substrates, such as growth factors and cytokines, that induce diverse cellular responses. We developed a FRET (fluorescence resonance energy transfer)-based biosensor called TSen that quantitatively reports the kinetics of TACE activity in live cells. In combination with chemical biology approaches, we used TSen to probe the dependence of TACE activation on the induction of the kinases p38 and ERK (extracellular signal-regulated kinase) in various epithelial cell lines. Using TSen, we found that disruption of the actin cytoskeleton in keratinocytes induced rapid and robust TSen cleavage and the accumulation of TACE at the plasma membrane. Cytoskeletal disruption also increased the cleavage of endogenous TACE substrates, including transforming growth factor- α . Thus, TSen is a useful tool for unraveling the mechanisms underlying the spatiotemporal activation of TACE in live cells.

INTRODUCTION

The tumor necrosis factor- α (TNF α)-converting enzyme (TACE), encoded by the *ADAM17* gene, is a transmembrane protease that is implicated in numerous physiological processes, including inflammation (1, 2), wound healing (3), development (4), and cancer progression (5, 6). In these diverse processes, TACE plays a common role as an extracellular sheddase that cleaves the pro-/transmembrane form of a wide variety of ligands and receptors (6). For example, TACE activity mediates autocrine and paracrine signaling mediated by TNF α during the immune response by cleaving pro-TNF α , thereby enabling the release of soluble TNF α , which binds to and activates the TNF receptor (2). Similarly, TACE activity mediates autocrine and paracrine signaling mediated by TGF α (transforming growth factor- α) and amphiregulin (7), both of which are ligands of EGFR (epidermal growth factor receptor) that regulate cellular motility during development, wound healing, and metastasis (8–10). EGFR is also cleaved by TACE, enabling complex feedback mechanisms in the regulation of cellular motility (11). Although ligand shedding has gained increased attention as a major posttranslational modification mechanism and significant research has been conducted in an effort to understand the consequences of the release of TACE substrates on autocrine and paracrine cell signaling, relatively less is known about how TACE activity is itself regulated.

Several lines of evidence suggest that TACE activity is spatially and temporally regulated within a cell or a tissue (12–14). TACE activation has been proposed to require the proteolytic cleavage of the autoinhibited pro-TACE form (15), mediated by the protease furin (6); however, furin-deficient cells display a clear ability to mature and activate TACE (16). Thus, the mechanism mediating the proteolytic activation of TACE is incompletely understood. It has been suggested that the trafficking of TACE to the plasma membrane is the primary means of TACE regulation in cells (17–21). Direct phosphorylation of TACE at Thr⁷³⁵ by either p38 MAPK (mitogen-

activated protein kinase) or ERK (extracellular signal-regulated kinase) has been proposed to facilitate the trafficking of TACE to the plasma membrane and, hence, its subsequent extracellular activity (17–21). Other studies point to the importance of the exit of TACE from the endoplasmic reticulum (ER), which is mediated by the inactive rhomboid protease iRhom2 (13). Additionally, pathophysiological mutants of the kinase Src have been shown to regulate plasma membrane display of TACE, and there is some evidence that wild-type Src may also mediate this process (22, 23).

How the TACE protease is regulated such that it is able to cleave 76 substrates in a cell type-dependent manner (6) and mediate diverse physiological and pathophysiological processes is an outstanding question. The current mechanistic understanding of TACE regulation is hampered by a lack of effective tools to measure the spatiotemporal regulation of TACE activity in live cells. Here, we developed a novel FRET (fluorescence resonance energy transfer)-based biosensor for TACE activity called TSen, and its use in live cells reveals that rapid TACE activation can be achieved not only through the activity of p38 and ERK but also through changes in the subcellular trafficking of TACE. Using TSen, we identified novel chemical modulators that either activate or repress TACE, which may also prove to be of further use as research tools to study the functional regulation and consequences of TACE activity.

RESULTS

The TSen FRET biosensor was designed using a mixture of domains from TGF α and TNF α

Using a chimeric protein design approach, we developed a novel, genetically encoded FRET biosensor, which we call TSen, to measure TACE activity in live cells by fluorescence microscopy. As displayed schematically in Fig. 1A, TSen has an N-terminal leader sequence derived from TGF α , a TACE cleavage site from TNF α flanked by linker regions, a PDGFR (platelet-derived growth factor receptor) transmembrane domain, an eCFP (enhanced cyan fluorescent protein) FRET donor, and a YPET [a yellow fluorescent protein (YFP) variant] FRET acceptor in a similar manner as to what has been used for the MMP14 (matrix metalloproteinase

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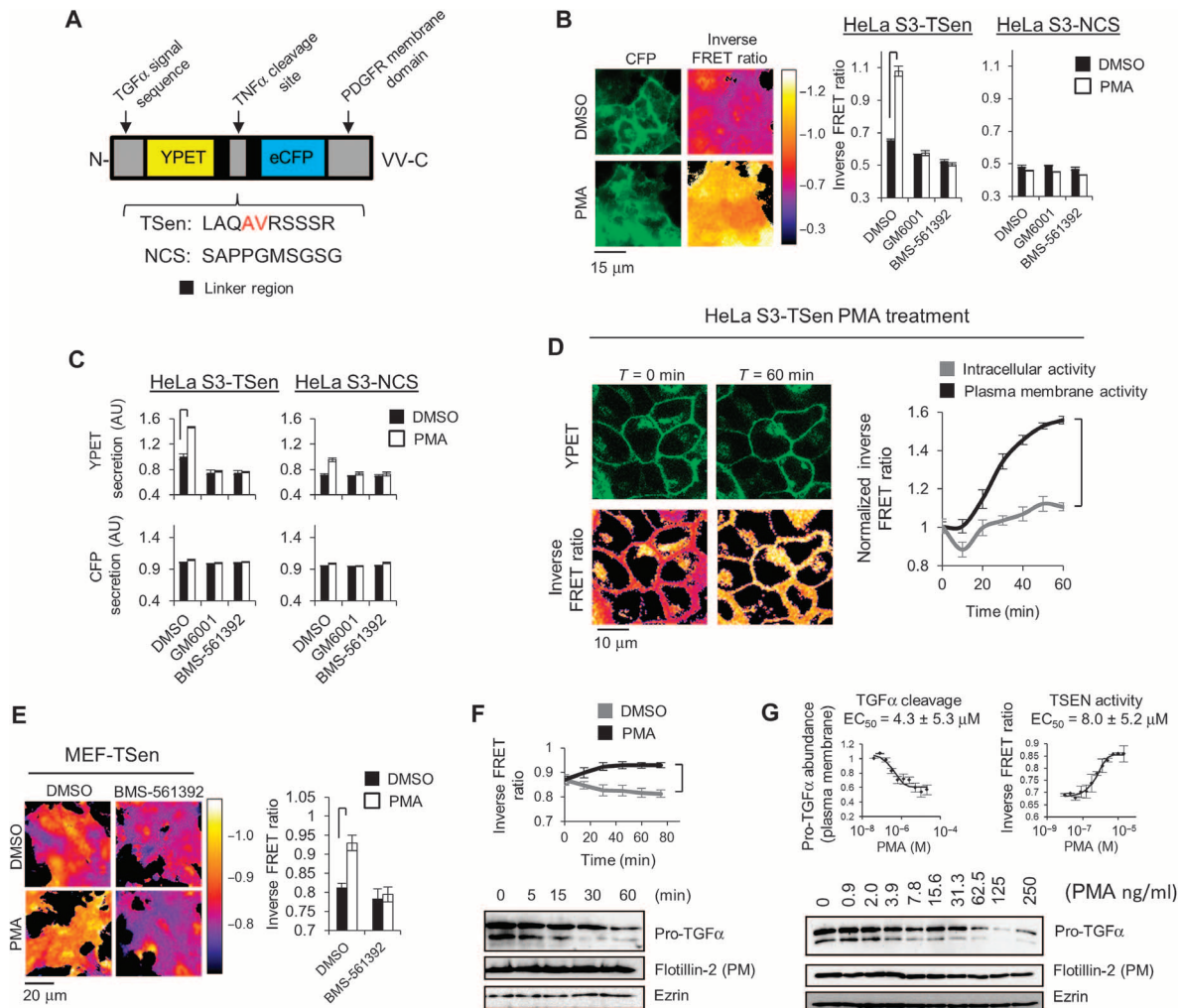


Fig. 1. TSen measures the catalytic activity of TACE in live cells. (A) Schematic of the TSen FRET biosensor and the noncleavable control sensor (NCS). (B) Activity of TACE assessed by the cleavage of the sensor (inverse FRET ratio) in HeLa S3 cells stably expressing TSen and cultured with either PMA or vehicle for 1 hour. (C) Abundance of YPET with either PMA or vehicle for 3 hours in the presence of TACE inhibitors. AU, arbitrary units. (D) Confocal microscopy of TACE activity assessed by FRET analysis of TSen in HeLa S3-TSen cells cultured with PMA. Images are of the same

cells at both time points. (E) Activity of TACE by FRET analysis in MEF-TSen cells cultured with PMA and TACE inhibitor for 1 hour. (F) Time course of TACE activity by FRET analysis (top) in live cells or Western blotting for pro-TGF α (bottom) in plasma membrane (PM) fractions of MEF-TSen cells treated with PMA. (G) Relative abundance of pro-TGF α at the plasma membrane (left) and the inverse FRET ratio of Tsen (right) in MEF-TSen cells cultured with PMA for 1 hour. Microscopy data are means $\pm$ SEM from three trials, >500 cells each. Brackets: $P < 0.01$ by t test. All data are representative of at least two independent experiments.

14) FRET biosensor (24). A key difference between TSen and other protease cleavage biosensor designs is that two tandem valines make up the immediate C-terminal end of the sensor, which mimic the C-terminal valines that are required for TGF α maturation in live cells (25). The use of a TACE cleavage site derived from TNF α in TSen is the result of extensive research that identified a small peptide region within TNF α as an efficient and specific substrate of TACE over other metalloproteinases (26, 27). Although a TACE-specific cleavage site derived from TNF α is incorporated into TSen, TSen differs from the TNF α and TGF α proteins in the distance between the cleavage site and the transmembrane domain along the polypeptide chain (about 245, 18, and 7 amino acids, respectively). However, in TSen, the TACE cleavage site is separated from the PDGFR trans-

membrane domain with an eCFP domain, whose N and C termini are positioned within 22 ± 1.8 Å (corresponding to a distance of about six amino acids) of each other, according to the eCFP structure in Protein Data Bank (identifier 2WSN) (28). Thus, although the TACE cleavage site in TSen is fairly distant from the transmembrane domain in the primary structure, these two domains are likely positioned near each other in the tertiary structure. Additionally, TSen differs from TNF α in that TSen is a type I transmembrane protein, whereas TNF α is a type II. Because TGF α , another TACE substrate, is a type I transmembrane protein, it appears that TACE does not take into account the type of transmembrane protein as a parameter for selectivity. Despite the highly chimeric structure of TSen relative to the structures of endogenous TACE substrates, we found that

this chimeric nature did not interfere with TSen being a highly suitable substrate for TACE.

TSen measures TACE activity in live cells

TSen measures TACE activity as a function of inverse FRET ratio (CFP/FRET), meaning that the FRET signal decreases relative to CFP when TSen is cleaved, presumably by TACE, and the CFP and YPET fluorophores are separated, where YPET is then released as a soluble protein into the surrounding medium. In HeLa S3 cells stably expressing TSen, the inverse FRET ratio rapidly increased when PMA (phorbol 12-myristate 13-acetate), a previously reported activator of TACE (18), was added to the culture medium (Fig. 1B and fig. S1A). This PMA-induced change in the FRET signal was blocked by the addition of TACE inhibitors BMS-561392 (DPC-333) or GM6001. BMS-561392 is a partially selective TACE inhibitor ($K_i = 180$ pM) and has 100-fold selectivity over several other MMPs and ADAM family members [MMP12: IC_{50} (half maximal inhibitory concentration) = 2 nM; MMP13: $IC_{50} = 12$ nM; ADAMTS4: $IC_{50} = 10$ nM], whereas GM6001 is a nonselective MMP/TACE inhibitor (29). Similar results were found in human embryonic kidney 293T cells transfected with TSen (herein called 293T-TSen cells) (fig. S1B). To determine whether PMA-dependent TACE activity, as measured by our sensor, is contingent on the specific sequence of the TNF α cleavage site, we constructed a noncleavable sensor, in which the TACE cleavage site no longer resembled an ideal TACE substrate (Fig. 1A). The noncleavable sensor sequence was predicted to be a poor TACE substrate, according to a previous study (30). PMA-dependent activation was not observed in HeLa S3 cells stably expressing the noncleavable sensor compared to those stably expressing TSen (HeLa S3-TSen cells) (Fig. 1B), validating that the TACE cleavage site is the primary and required feature of TSen to show PMA-dependent TACE activity.

Because TSen was designed to display reduced FRET efficiency through proteolytic cleavage, we validated that cleavage was indeed occurring. To this end, we additionally measured the release of YPET and CFP from HeLa S3-TSen cells after a 3-hour period of PMA stimulation in the presence or absence of either BMS-561392 or GM6001. PMA enhanced YPET, but not CFP, release into the medium, and YPET release was inhibited by either pharmacological TACE inhibitor (Fig. 1C). When the same experiment was conducted in HeLa S3 cells transfected with the noncleavable sensor, PMA-stimulated YPET release was greatly reduced but was not completely absent (Fig. 1C). This residual PMA-dependent cleavage of the “non-cleavable” sensor is likely still dependent on TACE because both GM6001 and BMS-561392 inhibited its activation. The remaining small portion of TACE activity detected through YPET release is likely to be attributed to cleavage at a cryptic site located between CFP and YPET in the non-cleavable sensor that is independent of the TNF α cleavage site. These YPET secretion experiments, in conjunction with the microscopy experiments, demonstrate the versatility of TSen. Thus, TSen can be used in either a secretion-based fluorescent spectroscopy assay, in a similar manner to the available TACE bioassays (31), or in a FRET-based live cell microscopy assay.

The use of TSen in live cells also enabled the determination of the intracellular location at which TACE is active. Using confocal microscopy, we repeated our experiments with HeLa S3-TSen cells in the presence of PMA to determine whether cleaved TSen localizes to intracellular vesicles or to the plasma membrane. We found that TSen cleavage localizes to a large degree to the plasma membrane and to a far lesser degree to intracellular vesicles under these conditions (Fig. 1D). This may be the result of selectively localized TACE activity, or more likely, it is a reflection of the selective localization of TSen. Therefore, TSen is not expected to show all possible subcellular locations where TACE may be active, nor does it eluci-

date where TACE itself is activated within a cell. Rather, the observations suggest that TSen is primarily a sensor of TACE activity at the plasma membrane.

TSen reports TACE activity with similar dose and kinetic profiles to that of pro-TGF α cleavage at the plasma membrane

Mouse embryonic fibroblasts (MEFs) have been used previously to study TACE-dependent cellular responses, wherein TACE mediates the cleavage of EGFR ligands (4). MEFs stably expressing TSen (MEF-TSen) responded to PMA stimulation with TACE activation, assessed by an increase in inverse FRET ratio (Fig. 1E), similar to that observed for both HeLa S3-TSen and 293T-TSen cells (Fig. 1B and fig. S1B). We aimed to determine whether the time and dose profiles of TACE activity in response to PMA were similar between TSen-measured activation of TACE and endogenous TGF α cleavage assessed by Western blot analysis. Although we could not detect cleavage of endogenous pro-TGF α in whole-cell lysates (fig. S1C), we could detect the time-dependent disappearance of pro-TGF α in subcellular fractions enriched for plasma membrane (Fig. 1F). The time- and dose-dependent amount of pro-TGF α at the plasma membrane closely resembled the TACE activity as reported by TSen in fluorescence microscopy experiments (Fig. 1, F and G). These similarities between the PMA-induced dose- and time-response profiles for TSen and TGF α cleavage suggest that TSen may be a faithful reporter of TACE activity in general at the plasma membrane.

TSen reports TACE activity with high specificity

To determine the specificity of TSen for measuring TACE activity, we stably expressed short hairpin RNA (shRNA) against *ADAM17* (the mRNA encoding TACE) in HeLa S3-TSen cells. Knocking down TACE (Fig. 2A) showed that the PMA-induced change in FRET ratio reported by TSen was dependent on TACE (Fig. 2B). Similarly, *Adam17*^{-/-} MEFs transfected with TSen had no measurable changes in the TSen-reported inverse FRET ratio in response to PMA, in contrast to wild-type MEF-TSen cells, which display a significant increase in inverse FRET ratio in response to PMA (Fig. 2C). Previous studies show that TNF α can be cleaved not only by TACE but also by the protease PR3 (proteinase 3, encoded by *PRTN3*) (32). Additionally, the peptidomimetic gelatinase B inhibitor Regasepin-1 is capable of inhibiting not only gelatinase B but also MMP8 and TACE to the same degree, suggesting that these proteases may share overlapping substrate specificity (33). However, knocking down either MMP8 or the protease PR3 in HeLa S3-TSen cells (Fig. 2D) was insufficient to alter either the basal or PMA-stimulated inverse FRET ratio of TSen (Fig. 2E). Together, these series of experiments show that TSen is a biosensor that faithfully reports TACE activity with specificity for TACE over other similar proteases.

TACE activation by stimulating EGFR is p38- and ERK-dependent

TACE has been reported to mediate the proteolytic activation of several EGFR ligands (2, 7). We determined that PMA-dependent TACE activation measured through TSen depends partially on the kinases p38 and ERK (Fig. 3A), which is consistent with the findings in other studies (17, 18). Because p38 and ERK are both activated upon stimulation of EGFR, and there is uncertainty whether this can enhance TACE activation (34), we aimed to determine the breadth of the phenomenon of EGFR-mediated activation of TACE in several established cell lines. To this end, we created three additional stable epithelial cell lines expressing TSen (HaCaT keratinocytes, SCC13 squamous carcinoma cells, and VMCUB1 bladder cancer cells) and tested their responses to EGF. Although TACE was not activated by exogenous EGF in HeLa S3-TSen cells (fig. S2A), EGF efficiently activated

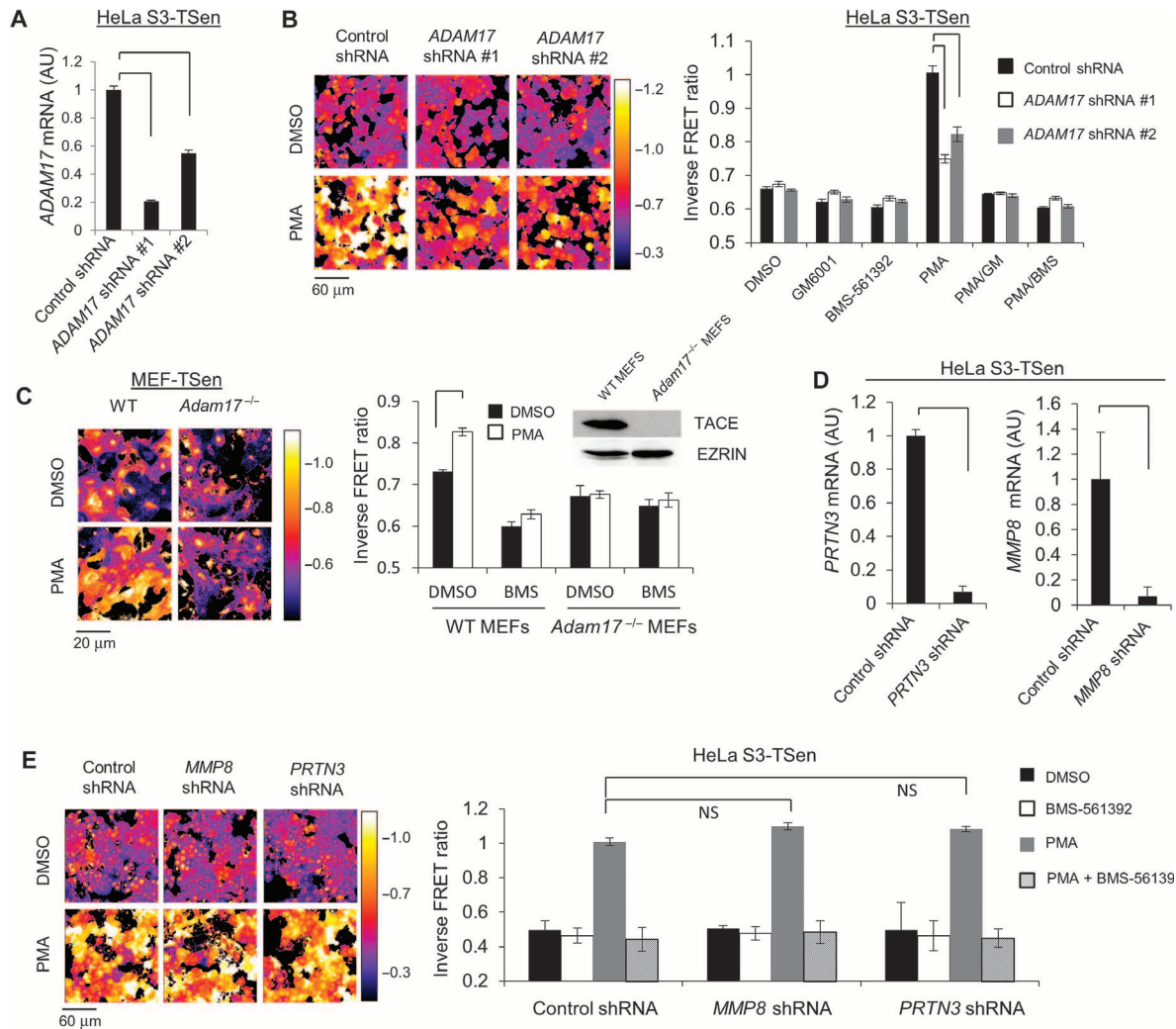


Fig. 2. TSen reports specifically TACE activity. (A) Reverse transcription polymerase chain reaction (RT-PCR) for *ADAM17* mRNA (encoding TACE) in HeLa S3-TSen cells stably expressing TACE shRNAs. (B and C) FRET analysis in (B) shRNA-expressing HeLa S3-TSen cells or (C) TSen-expressing wild-type (WT) or *Adam17*^{-/-} MEFs cultured with PMA and either vehicle or TACE inhibitor (BMS-561392) for 1 hour. (D) *PR3* and

MMP8 mRNA abundance in shRNA-transfected HeLa S3-TSen cells. (E) Activity of TACE by FRET analysis in shRNA-transfected HeLa S3-TSen cells cultured with PMA, TACE inhibitor, or both for 1 hour. Microscopy data are means $\pm$ SEM from three trials, >500 cells each. Brackets: $P < 0.01$ by t test, except in (E). NS, not significant. All data are representative of at least two independent experiments.

TACE in HaCaT, SCC13, and VMCUB1 cells (Fig. 3, B to D). The kinetics of activation of TACE was faster in PMA-stimulated cells (1 hour to saturation, fig. S1A), compared to EGF-stimulated cells (3 hours to saturation, fig. S2B). EGF-dependent TACE activation was blocked in all cell lines by pharmacological EGFR inhibition using gefitinib, which is consistent with the theory that the activation of TACE by EGF occurs through EGFR. As expected, the TACE inhibitor BMS-561392 diminished both basal and EGF-dependent TACE activation, revealing that only a portion of the total TACE activity in all three of these cell types was dependent on EGFR. Additionally, the effects of the inhibitors SB-203580 and CI-1040, which target p38 and MEK1 (MAPK kinase 1, which phosphorylates ERK), respectively, consistently showed the importance of both of these kinases in EGF-dependent TACE activation. Thus, EGF-dependent activation of TACE in these three cell lines was similar to that of PMA-dependent activation of TACE in both HeLa S3 and 293T cells (Fig. 1E and fig. S1B), in

which both chemical and biochemical activation of TACE were p38- and ERK-dependent.

Chemical compound screening reveals novel activators and inhibitors of TACE

To further understand the cellular regulation of TACE, we conducted an unbiased screen of small-molecule compounds in HaCaT-TSen cells to find those that are capable of modifying TACE activity. We screened 81 compounds that affect diverse cellular functions (table S1), from a library of small molecules that either have been widely used research tools or are approved by the Food and Drug Administration for clinical applications. Thus, each compound has a known mechanism of action. Our screen revealed two candidate compounds that activate TACE [cytochalasin D (CytoD) and quinacrine] and two candidate compounds that repress TACE (doxorubicin and sunitinib) (Fig. 4A). All four candidates were

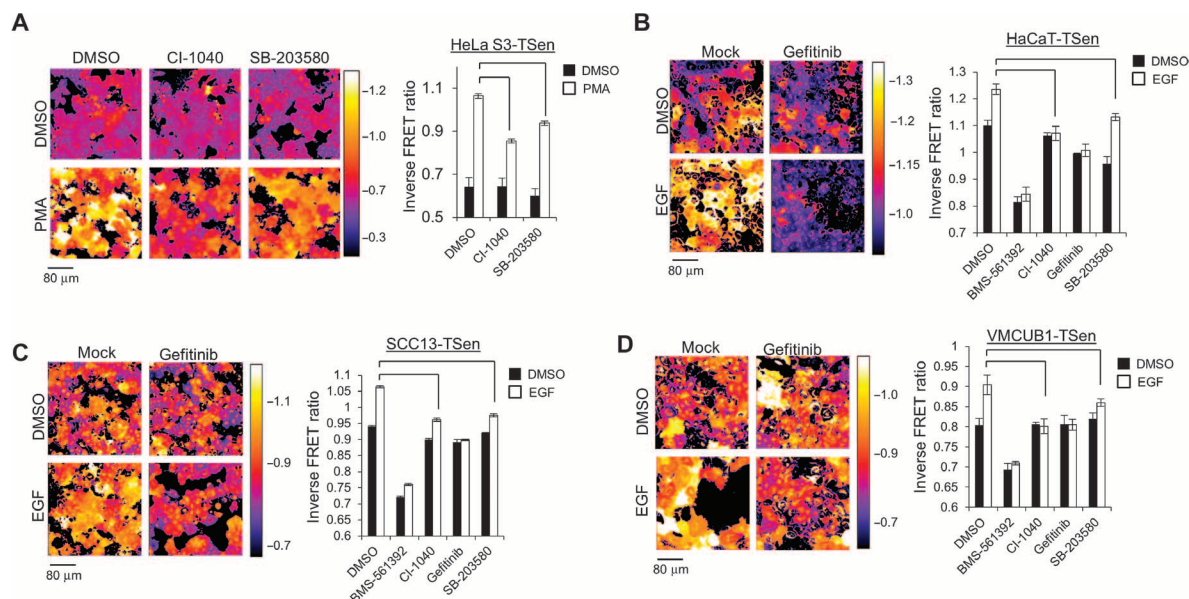


Fig. 3. ERK and p38 mediate PMA- and EGF-induced activation of TACE. (A) Activity of TACE in HeLa S3-TSen cells cultured with PMA for 1 hour in the presence of vehicle or inhibitors of MEK (CI-1040) or p38 (SB-203580). (B to D) Activity of TACE in (B) HaCaT-TSen cells, (C) SCC13-TSen cells, and (D)

VMCUB1-TSen cells cultured with EGF for 3 hours in the presence of vehicle or inhibitors of TACE (BMS-561392), MEK, EGFR (gefitinib), or p38. Microscopy data are means $\pm$ SEM from three trials, >500 cells each. Brackets: $P < 0.01$ by t test. All data are representative of at least two independent experiments.

validated in independent titration experiments (Fig. 4B and fig. S3, A to C). Doxorubicin is a DNA-damaging agent that can activate an apoptosis response in cells through the activation of caspase-8, a protease capable of activating TACE (35). Although we observed doxorubicin to be an inhibitor of TACE activity, we investigated whether this suppression was an indirect effect as a result of its activation of apoptosis. We found that doxorubicin did not activate apoptosis as assessed by caspase-3 cleavage under the experimental conditions we used, but it can cause the accumulation of pro-TGF α in whole-cell lysates, supporting the claim that doxorubicin inhibits TACE (fig. S3D). Presumably, this inability of doxorubicin to activate apoptosis in these cells may be attributed to the high density (1200 cells/mm²) at which cells were screened, which leads to density-dependent inhibition of cellular proliferation (fig. S3E) and hence to decreased DNA replication and a decreased amount of doxorubicin-associated DNA intercalating damage.

Actin cytoskeleton-dependent activation of TACE relies on the accumulation of TACE at the plasma membrane

Because CytoD, an inhibitor of actin polymerization, emerged as the strongest potential activator of TACE, we investigated the possible regulatory connection between the actin cytoskeleton and TACE activity. We conducted a dose-response experiment in HaCaT-TSen cells using latrunculin B (LatB), which is another actin-depolymerizing agent with a mechanism of action that is different from that of CytoD. We found that LatB had a similar ability to increase the inverse FRET ratio (fig. S3F and Fig. 4, C to E) with faster kinetics than that of EGF (half maximum at ~30 min compared with ~120 min, respectively) (figs. S2B and S3G). We confirmed that these actin-targeting drugs were capable of modulating the actin cytoskeletal structure at their respective EC₅₀ (half maximal effective concentration) concentrations by fixing HaCaT cells in the presence or absence of either CytoD or LatB and staining for F-actin using phalloidin-rhodamine B (fig. S3H). Furthermore, the actin depolymerization-induced increase of the inverse FRET ratio in TSen-expressing cells did not require

the kinase activity of ERK or p38 in HaCaT and SCC13 cells (Fig. 4, C and D), but it did in VMCUB1 cells and MEFs (Fig. 4, E and F). Although this ERK and p38 dependence was cell type-specific, the fact that actin disruption can occur independently of these kinase activities suggests that actin disruption is capable of regulating TACE activity through a mechanism that is distinct from TACE phosphorylation.

Three observations suggested that actin cytoskeletal disruption does indeed cause endogenous activation of TACE. First, CytoD-dependent induction of an inverse FRET ratio in MEF-TSen cells was completely blocked upon deletion of the *Adam17* gene (Fig. 4F). Second, actin-depolymerizing drugs efficiently induced the release of YPET into the surrounding medium, which was blocked by pharmacological inhibition of TACE with BMS-561392 (Fig. 4G). Third, actin-depolymerizing agents induced EGFR-dependent ERK activation in HaCaT, SCC13, and VMCUB1 cells stably expressing a well-known ERK FRET biosensor, EKAR (36), which was blocked by pretreating cells with either BMS-561392 or gefitinib (Fig. 4H and fig. S3, I and J). These experiments provide additional support that TSen enabled the detection of a previously unknown mechanism of TACE activation: perturbation of the actin cytoskeleton.

Because cytoskeletal disruption is variably mediated by p38 and ERK activities across the many cell lines investigated, we sought to explain how actin-depolymerizing drugs were capable of activating TACE independently of p38 and ERK. The actin cytoskeleton is important for a myriad of cellular processes, including endocytosis through clathrin-coated pits (37). Thus, we performed subcellular fractionation of MEFs by differential centrifugation to separate the plasma membrane in cell lysates from the vesicle and soluble components of the cytosol. TACE accumulated at the plasma membrane in cells exposed to CytoD but not PMA (Fig. 4I). However, pro-TGF α abundance was decreased in cells exposed to either PMA or CytoD, the latter eliciting a greater magnitude change (Fig. 4I), which was consistent with our fluorescence microscopy experiments (Figs. 2C and 4I). Upon either PMA or CytoD treatments, the abundance of the TACE sensor TSen at the plasma membrane remained unchanged. The

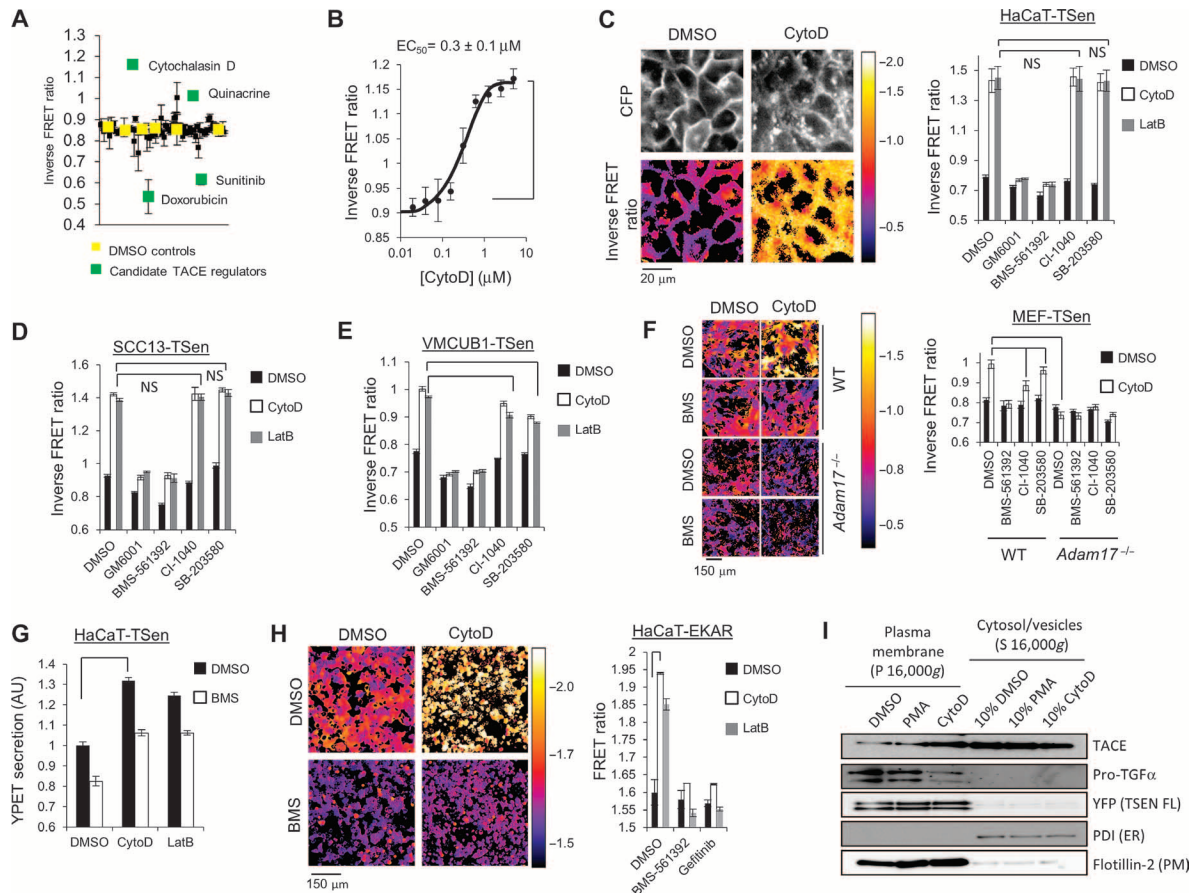


Fig. 4. Actin depolymerization activates TACE. (A) FRET analysis in HaCaT-TSen cells cultured with chemicals from an unbiased chemical library. Dose information and raw data are in table S1. (B) Dose response in HaCaT-TSen cells cultured with CytoD for 1 hour. (C to E) FRET analysis of TSen indicating TACE activity in (C) HaCaT-TSen cells, (D) SCC13-TSen cells, or (E) VMCUB1-TSen cells cultured with CytoD or LatB for 1 hour. (F) FRET analysis of TSen in WT or *Adam17*^{-/-} MEF-TSen cells cultured with CytoD in the presence of vehicle or the indicated inhibitor for 1 hour. (G) Abundance of YPET in the medium of HaCaT-TSen cells cultured with CytoD or LatB in the presence of vehicle or TACE

inhibitor (BMS) for 1 hour. (H) FRET analysis of the ERK activity reporter EKAR in HaCaT cells cultured with CytoD and LatB in the presence of vehicle or the indicated inhibitor for 1 hour. (I) Western blot analysis for TACE, pro-TGF α , and TSen in plasma membrane (PM) or cytosolic fractions of MEF-TSen cells cultured with either PMA or CytoD for 1 hour. Flotillin-2 and the ER-resident PDI (protein disulfide isomerase) served as subcellular fractionation markers. Microscopy data are means $\pm$ SEM from three trials, >500 cells each. Brackets: $P < 0.01$ by t test, except in (E). All data are representative of at least two independent experiments.

accumulation of TACE at the plasma membrane is likely to be independent of the kinase activity of p38 or ERK, because PMA-dependent activation of TACE consistently relies on the activity of these kinases, but it did not lead to an accumulation of TACE at the plasma membrane. Although cleavage of the full-length sensor was expected to mirror the cleavage of pro-TGF α , we were unable to detect a substantial decrease of the uncleaved TSen by Western blot, although the cleaved product (YPET) accumulated in the medium (Fig. 4G). To explain this observation, we performed fluorescence recovery after photobleaching (FRAP) in HaCaT-TSen cells, which revealed that TSen turnover at the plasma membrane occurred with a half-life of 1 min, independently of TACE activity (fig. S3K). In contrast, the activation of TSen measured by fluorescence microscopy occurred about 30 times more slowly (fig. S3G). These data suggest that the combination of a relatively small portion of plasma membrane-localized TSen being cleaved by TACE and the high turnover of TSen at the plasma membrane may preclude the ability to detect TSen cleavage by

Western blotting. Nevertheless, our subcellular fractionation data suggest that only actin cytoskeleton disruption, not PMA stimulation, alters the subcellular trafficking of TACE to and from the plasma membrane.

DISCUSSION

Our study illustrates not only a novel technique for measuring plasma membrane-localized TACE activity with high specificity in live cells but also how the TSen sensor can successfully be used to shed light on the complexity of TACE activation in cells. The development and use of TSen enabled us to discover a previously unknown actin cytoskeleton-dependent mechanism of TACE activation by regulating its accumulation at the plasma membrane. We propose that this mechanism for TACE activation highlights a role for the actin cytoskeleton as a sensory structure, whose integrity is functionally linked to TACE activation, EGFR ligand shedding, and subsequent ERK activation. ERK regulates cytoskeletal remodeling through

contactin activation (38–41), suggesting that cells may use TACE-mediated shedding of EGFR ligands as a means to induce ERK-mediated actin remodeling and repair in response to actin damage.

In addition, TSen enabled us to determine that EGF-dependent activation of TACE was driven by a combination of p38- and ERK-dependent mechanisms ubiquitously, but to varying degrees, across the many cell lines investigated. This observation suggests that cells may engage a positive feedback system for TACE-dependent shedding of EGFR ligands, where TACE activity can promote subsequent increases in TACE activity through EGFR and p38 and/or ERK activation. Such positive feedback has been proposed in previous studies (11). One physiologically relevant occurrence of such a feedback loop may be in the skin to promote wound healing. In wound healing model systems for the skin, epithelial sheets of keratinocytes undergo sustained motility upon wounding, which depends on sustained EGFR activity (42). Initiation of epithelial sheet migration may rely on a wide range of possible initial stimuli, such as reactive oxygen species (43), that activate p38 and/or ERK in response to direct wounding. Our study suggests that TACE-mediated EGFR ligand shedding likely continues after the initial activation of p38 or ERK subsides, even when the initial stimulus has been removed. Using TSen may provide insight into not only how individual cells respond to environmental stimuli, as shown here, but also how groups of cells respond during a physiological process like wound healing. This TACE biosensor should facilitate the investigation of mechanisms regulating TACE activation and the development of new pharmacological agents to control its activity.

MATERIALS AND METHODS

Cell culture and pharmacological inhibitors

All cells were cultured using Dulbecco's modified Eagle's medium as previously described (42). Wild-type and *Adam17* null MEFs were gifts from C. Blobel. Unless explicitly stated in the figures, the following doses were used for cell treatments: EGF, 100 nM; BMS-561392, 2.5 μ M; GM6001, 10 μ M; CytoD, 1 μ M; LatB, 2.5 μ M; PMA, 200 nM; gefitinib, 1 μ M; CI-1040, 500 nM; SB-203580, 10 μ M; doxorubicin, 5 μ M.

Construction of the TACE sensor

The parental vector for TSen, EKAREV, is described by Komatsu *et al.* (44) and provided by K. Aoki and M. Matsuda. A cassette encoding a TGF α signal peptide (MVPSAGQLALFALGIVLAACQALENSTSPLSDPPVAAAVVSH), hemagglutinin (HA) tag (YPYDVPDYA), PDGFR transmembrane domain, and FLAG tag VV (DYKDDDDKVV) was synthesized by GenScript (sequence is available upon request) and subcloned into the Eco RI–Sal I site of EKAREV to produce the pBBSR-TGFA-HA-PDGF-FLAG plasmid. Oligonucleotides encoding the TACE cleavage site (sense strand: 5'-TCGAGAGCGGCCTGAGATCTAGCGGCCTGGCCAGGCCGTGATCCAGCTCCAGAGCGCGGACGGATCCACAGC-3', and antisense strand: 5'-GGCCGCTGGTGGATCCGCTGCCGCTCTGGAGCTGGA-TCTCACGGCCTGGGCCAGGCCGCTAGATCTCAGGCCGCTC-3') or a noncleavable site (sense strand: 5'-TCGAGAGCGGCAGCGGCAGCGGCAGCGCTCCCCGGGCATGAGCGGCAGCGCGCGGCAGCGGCAC-3', and antisense strand: 5'-GGCCGCTGCCGCTGCCGCGCGCGCTGCCGCTCATGCCCCGGGGAGCGCTGCCGCTGCTGCCGCTGCCGCTC-3') were inserted in Xho I–Not I site of the EKAREV plasmid to generate TACE-REV and NCS-REV plasmids, respectively. The fragments encoding YPET-TACE-REV-ECFP or YPET-NCS-REV-ECFP were excised by digestion with Eco RI–Xba I and inserted in between the HA and PDGFR domains (Eco RI–Avr II) of pBBSR-TGFA-HA-PDGF-FLAG. The resultant vectors were named as TSen and NCS (Fig. 1).

FRET data analysis and fluorescence spectroscopy

Live cell microscopy was conducted as previously described (42). Filters used for FRET measurements were the following: FRET excitation 438/24–25, dichroic 520LP, emission 542/27–25 (Semrock MOLE-0189); CFP excitation 438/24–25, dichroic 458LP, emission 483/32–25 (Semrock CFP-2432B-NTE-Zero). Time lapse microscopy images were analyzed, and FRET calculations were performed using MATLAB (data file S1). Briefly, images were background-corrected through subtraction using images acquired from samples of cell-free media. Pixels representing cells were identified as having an intensity 1000 units above background. FRET ratio or inverse FRET ratio was calculated as either FRET intensity against CFP intensity or CFP intensity against FRET intensity, respectively, and all relevant pixels were averaged. Each image was acquired at $\times 4$ magnification for calculations, which encompasses data from at least 1000 cells per measurement. Measurements were done in triplicate. For images displayed, $\times 40$ magnification was used. A similar method of inverse FRET ratio calculations was used in confocal microscopy experiments, where a z plane of 1- μ m height at $\times 100$ magnification was measured, and segmentation of membrane and vesicle fractions was performed in MATLAB.

Fluorescent protein secretion experiments

YPET media secretion was quantified by transfer of media supernatant to 96-well plates, followed by measurement of YPET fluorescence using a Tecan Microplate reader (excitation 500/20, emission 550/20). CFP media secretion was performed in the same manner with different parameters (excitation 438/20, emission 485/20).

Stable shRNA expression

Stable shRNA expression was achieved using TRC Lentiviral shRNA (Thermo) with the following shRNA constructs: *ADAM17* (TACE), TRCN0000052172 and TRCN0000052168; *PR3*, TRCN0000418696; *MMP8*, TRCN0000373060; and SHC016 (a control construct). Stable shRNA knockdown cell lines were selected in the presence of puromycin (2 μ g/ml). Lentiviral manufacturing was done in 293T cells, using pHCMV-VSVg, pMDLg, and pREV vectors.

Western blotting

Whole-cell lysates were prepared in radioimmunoprecipitation assay buffer [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Subcellular fractionation was achieved by lysis in fractionation buffer [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 0.1% Tween 20], with a buffer-to-cell ratio (by volume) of 10:1, for 10 min at 4°C, followed by seven volume strokes with a 1-ml syringe/25G needle. Lysate was centrifuged at 3000g to pellet nuclei. Supernatant was then centrifuged at 16,000g. The resulting pellet was used as the plasma membrane fraction, whereas the supernatant was used as the cytosol/vesicle fraction. Antibodies used for Western blots were against pro-TGF α (3715S, Cell Signaling Technology), TACE (Ab39162, Abcam), GAPDH (SC47724, Santa Cruz Biotechnology), caspase-3 (9665p, Cell Signaling Technology), flotillin-2 (3436s, Cell Signaling Technology), PDI (3501p, Cell Signaling Technology), and YPET (632376, Clontech Laboratories).

Reverse transcription polymerase chain reaction

For RT-PCR, the following primers were used: *ADAM17* (TACE), 5'-GAGCCACTTTGGAGATTGTTAATG-3' (forward) and 5'-GTTCCGATAGATGTCATCAACTCTGTC-3' (reverse); *PR3*, 5'-ATGGCCTCCCTGCAGATGCGGGG-3' (forward) and 5'-GCCAGCCAACCTCAGTGCCTCCG-3' (reverse); and *MMP8*, 5'-AGCTGTCTCAGAGGCTGAGGTAGAAA-3' (forward) and 5'-CCTGAAAGCATAGTTGGGATACAT-3' (reverse).

Small-molecule compound library

All drugs in the screen were used at 10 μ M, and measurements were made after 1 hour. The small-molecule compound libraries included selected compounds from the Approved Oncology Drugs Set and the Structural Diversity Set, which were provided by the National Cancer Institute Developmental Therapeutics Program (http://dtp.nci.nih.gov/branches/dscb/repo_open.html).

FRAP experiments

TSen-expressing HaCaT cells were imaged using a Nikon A1R LSM confocal microscope. Measurements for CFP were achieved using a 405 laser with a 450/50 emission filter. A 100 $\times$ oil immersion objective was used. A 2 μ m $\times$ 2 μ m square was selected over both the cellular junctions and an unoccupied extracellular space, where photobleaching was performed for 4 s at 100% laser power. n = 20 cells in each of two independent experiments. Image analysis was performed in MATLAB.

Cell proliferation assay

The Click-IT EdU Alexa Fluor 488 Imaging Kit (Life Technologies) was used to assay the percentage of cells dividing in populations of cells plated at different density, according to the manufacturer's instructions. Cells were exposed to EdU (5-ethynyl-2'-deoxy-uridine) for 8 hours.

Statistical analysis

All comparisons indicated with brackets were the result of a two-tailed t test, from which P values were obtained, using Microsoft Excel. Two-tailed t tests were conducted between data collected from triplicate trial measurements in individual experiments.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/8/365/rs1/DC1

Fig. S1. TSen reports TACE activity in HeLa S3 and 293T cells, whereas measuring pro-TGF α abundance in whole-cell lysate does not.

Fig. S2. TACE activity measured with TSen in HeLa S3 and HaCaT cells.

Fig. S3. Validation of CytoD-dependent activation of TACE.

Table S1. A complete list of compounds used to screen for TACE regulators.

Data file S1. The MATLAB script used to perform calculations of inverse FRET ratio in TSen-transfected cells.

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