Protease-based synthetic sensing and signal amplification

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The bottom-up design of protein-based signaling networks is a key goal of synthetic biology; yet, it remains elusive due to our inability to tailor-make signal transducers and receptors that can be readily compiled into defined signaling networks. Here, we report a generic approach for the construction of protein-based molecular switches based on artificially autoinhibited proteases. Using structure-guided design and directed protein evolution, we created signal transducers based on artificially autoinhibited proteases that can be activated following site-specific proteolysis and also demonstrate the modular design of an allosterically regulated protease receptor following recombination with an affinity clamp peptide receptor. Notably, the receptor's mode of action can be varied from >5-fold switch-OFF to >30-fold switch-ON solely by changing the length of the connecting linkers, demonstrating a high functional plasticity not previously observed in naturally occurring receptor systems. We also create an integrated signaling circuit based on two orthogonal autoinhibited protease units that can propagate and amplify molecular queues generated by the protease receptor. Finally, we present a generic two-component receptor architecture based on proximity-based activation of two autoinhibited proteases. Overall, the approach allows the design of protease-based signaling networks that, in principle, can be connected to any biological process.

Significance

Synthetic biology envisages the creation of custom-based signaling by means of modular plug-and-play. This concept has primarily been realized in the construction of synthetic gene circuits. However, all real-time events in biology are processed by protein-based sensing and signal transducing systems; yet, the systematic bottom-up design of protein-based signaling systems remains elusive to date. Here we report a strategy for construction of modular protein switches based on artificially autoinhibited proteases whose activity can be modulated by specific proteolysis, ligand binding, or protein–protein interactions. We demonstrate that such protease-based ligand receptors or signal transducers can be assembled into different types of integrated signal sensing and amplification circuits that, in principle, can be connected to any biological process.

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Conflict of interest statement: V.S. and K.A. are coinventors on a Patent Cooperation Treaty (PCT) patent application that covers aspects of the protease-based biosensor technology described in this publication.

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Thus far, all synthetic signal recognition or relay units represent single stage architectures, which imposes limits on their sensitivity and the potential for subsequent propagation and processing of the signal. Similarly, reporter systems only incorporate limited types of readouts that, to date, have largely been constrained by the availability of functional modulators (i.e., notably inhibitors) for the reporter enzymes. To overcome these limitations, we developed a generic approach for the engineering of protease-based signal transducers and receptors based on artificially engineered autoinhibited proteases. We demonstrate that protease-based signal transducers and receptors can be assembled into signal sensing and amplification circuits. Importantly, we derive and discuss key theoretical and empirical design guidelines that should greatly facilitate the engineering of synthetic protease-based signaling systems for molecular diagnostics or cellular engineering.

Results

Rationale of Synthetic Protease-Based Signaling. To create a generic approach for the bottom-up construction of protein-based signaling systems to custom specification, we chose proteases as elementary signal transducing units. The reason for this choice is threefold. First, proteases comprise a large family of structurally related enzymes with specific and frequently nonoverlapping substrate specificities, which provides a rich repertoire of potentially orthogonal signal transducers. By virtue of their catalytic activity, proteases are also able to amplify a biomolecular response that is not dependent on cofactors or external sources of energy, and thus significantly reduces the complexity of protease-based signaling systems. In addition, peptide bond cleavage causes drastic changes in the physical properties of a protein that can be detected rapidly in multiple ways. Peptide bond cleavage is also a very common modulator of protein function that facilitates the development of protease-responsive effectors.

Although protease-based signaling systems are common in nature (e.g., caspases in apoptosis and clotting factors in the blood coagulation cascade), elements of these cascades cannot be readily adopted for the construction of synthetic signaling systems. First and foremost, the substrate specificities of naturally occurring transducer proteases are deeply intertwined with the regulation and actuation of their endogenous signaling networks. At the same time, changing their substrate specificity to orthogonalize them provides a formidable protein engineering challenge. The same applies to the complex structural transitions that underlie the allosteric activation of transducer proteases. Therefore, we devised a strategy for the de novo creation of artificially autoinhibited proteases. In this approach, a protease of choice is extended with a competitive autoinhibitor (AI) that blocks its active site in the basal state (Fig. 1A). Depending on the functional elements in the connecting linker, the activity of the autoinhibited protease can be allosterically regulated by modulating access of the competitive inhibitor to the active site by, for instance, (i) incorporating into the linker cleavage sites for an activating protease, thus creating a protease-inducible transducer; or (ii) replacing a linker with a ligand-binding domain capable of a conformational transition, thus creating a reversibly regulated, ligand-responsive receptor protease.

Developing Protease-Based Signal Transducers. To create a protease-based elementary signal transducing unit, we set out to develop a protease-inducible autoinhibited version of the Nia protease from tobacco vein mottling virus (TVMV). Members of the Nia protease family have naturally evolved to operate in the complex environment of the eukaryotic cytosol and display stringent substrate specificities toward their native seven-α-amino-acid-long cleavage sites in the viral polyprotein (23). This stringent substrate specificity fulfills the requirement for a high degree of orthogonality, which is crucial for making the behavior of synthetic signaling systems predictable in cellular context (4). In addition, Nia proteases including TVMV are well characterized biochemically (24) and structurally (25).

As genetically encodable site inhibitors of Nia proteases are not available, the AI domains were developed de novo. Briefly, the N-terminal cleavage product of the optimal TVMV peptide substrate ETVRFQ (24) served as a lead sequence for the creation of a competitive active site inhibitor. The lead peptide was appended to the C terminus of TVMV by a connecting linker encoding a cleavage site for thrombin flanked by Gly- and Ser-rich sequences. The linker covered a distance of 18 Å between the N terminus of the active site-bound peptide product and the last structured residue E217 in TVMV (25). The resulting mutant was recombinantly produced in Escherichia coli, and its proteolytic activity could be induced approximately fivefold following thrombin cleavage (Figs. S1 and S24). To improve the performance of the module, an activity-based optimization screen was performed to improve binding of the AI domain to the protease. To this end, additional hydrophobic interactions between P5 and P6 in the AI domain and T214 and W216 in the adjacent β-sheet β13 (25) of TVMV were screened (SI Materials and Methods). The activity of the resulting mutant slightly improved to ~13-fold (Fig. S28); yet, kinetic analysis suggested that residual background activity primarily arose from proteases lacking an AI domain as opposed to insufficiently strong competitive inhibition (Fig. S2C). A second screen was therefore devised to identify dipeptide motifs that were capable of binding across the P1-P1’ junction of the TVMV active site without being cleaved and enable the introduction of a C-terminal His6 affinity purification tag to purify full-length proteins (SI Materials and Methods). This screen yielded an autoinhibited mutant of TVMV with an AI domain EYVRFAP whose activity was induced ~68-fold following thrombin cleavage (Fig. 1C), whereas, on its own,
the AI domain inhibited TVMV with a $K_i$ of 196 ± 27 μM, indicating that even a weak inhibitor could function as an effective AI domain (Fig. S2E). This result can be rationalized with a simple mathematical model that guides the design and configuration of autoinhibited protease modules (SI Materials and Methods and Fig. S3). Notably, the induction ratio is linearly proportional to the effective, intramolecular concentration of the inhibitor, which, in practice, depends on the proximity of the N and C termini to the active site, as well as the structure and length of the connecting linkers. At the same time, an AI domain must not bind its transducer protease too tightly to minimize continuing competitive inhibition after cleavage. As a rule of thumb, the $K_i$ of a potential inhibitor should be at least two- to fivefold greater than the operating concentration of the transducer protease to achieve at least 60–83% of the maximum induction ratio (Fig. S3). Both criteria are satisfied in the case of the newly developed TVMV-based signal transducer.

To confirm the general applicability of the approach and to create another autoinhibited protease module that can be used to relay and amplify protease-based signals, we developed a protease-based signal transducer from hepatitis C virus (HCV) NS3 serine protease. Conveniently, noncleavable peptides capable of bridging the P1-P1′ junction of the HCV active site have been reported previously (26). Applying the design principles used for developing TVMV-based signal transducers, we engineered a TVMV-inducible autoinhibited signal transducer based on HCV (Fig. 1D). The HCV activity of the resulting transducer could be induced >75-fold following cleavage by TVMV (Fig. 1D). Crucially, TVMV and HCV proteases are naturally orthogonal (Fig. S4) and can be readily assembled into functional signaling cascades without undesirable cross-activation. At the same time, we would like to highlight that reaction conditions were chosen to favor high induction ratios by applying below $K_M$ substrate concentrations and comparatively low concentrations of the protease-based signal transducer; these are easily controlled in vitro, but in cells may ultimately require optimization e.g., either by using stronger inhibitors or fine-tuning expression levels to regulate the concentration of the substrate or the protease-based signal transducers.

### Protease-Based Ligand Sensors

Allosterically regulated receptors are ubiquitous in biology and key to the function of signaling systems. We hence set out to create a ligand activated allosteric protease receptor based on our TVMV signal transducer. To this end, we sought to incorporate a receptor scaffold that undergoes a large conformational transition on ligand binding between the TVMV’s catalytic and AI domains. As a model receptor, we chose an “affinity clamp”—an artificial two-domain receptor composed of a circularly permuted Erbin PDZ domain connected by a flexible serine-glycine linker to an engineered fibronectin type III (FN3) domain (27, 28). Peptide-based ligands RGSIDTWV (B1) and PQPSDTWV (B2) bind the affinity clamp with $K_I$ values of 0.6 and 5 nM, respectively, by inducing association of the PDZ and FN3 domains and structuring of the connecting linker (27, 28). To create a peptide-responsive version of TVMV, we replaced the protease-cleavable linker in the AI-TVMV module with the ePDZ-b1 version of the affinity clamp (27). As shown in Fig. 2A, this arrangement of functional domains allows in principle four major conformational states, two of which would switch TVMV-based protease activity either ON or OFF on ligand binding. We hypothesized that these four conformational states can be selectively accessed by varying the length and the structure of the linkers L1, L2, and L3 that connect the more rigid functional domains (Fig. 2B). To test this idea, we generated a range of linker truncations and elongations: as a starting point, a short GPG motif was chosen for linker L3 (connecting FN3 to the AI domain) while progressively shortening linker L1 (connecting TVMV to PDZ). This truncation screen yielded a series of allosteric protease receptors including mutant TVMV221-PDZ81-FN3GPG-AI whose activity was suppressed by ligand binding (Fig. 2C). Further shortening

![Fig. 2. Construction of protease-based peptide receptors.](image-url)
linker L1 by three amino acids yielded a protease TVMV\textsuperscript{E217,PDZ}\textsuperscript{G37}FN3GPG-AI that was unresponsive to either ligand B1 or B2 (Fig. 2D). A single amino acid deletion in L1 then yielded a mutant TVMV\textsuperscript{E217,PDZ}\textsuperscript{G37}FN3GPG-AI that responded with 30-fold induction of activity to the addition of ligand B1 (Fig. S5A). Inserting an additional glycine residue in L2 yielded the most effective switch-ON mutant TVMV\textsuperscript{E217,PDZ}\textsuperscript{G37}FN3GPG-AI that responded to the addition of ligand B1 with a 37-fold activity induction (Fig. 2D) while reaching ~34% activity compared with the TVMV-based signal transducer, suggesting that the affinity clamp remains in a partially autoinhibited state (Fig. S5D). The affinity of the chimeric receptor for B1 and B2 was reduced compared with the parental ePDZ-b1 binder ~20- and 200-fold, respectively (Table S1; mutant TVMV\textsuperscript{E217,PDZ}\textsuperscript{G37}FN3GPG-AI), suggesting that binding of the AI domain to TVMV directly competes with binding of the FN3 domain to the ligand–PDZ complex (27). At the same time, different linker compositions only had a negligible effect on induction ratios and apparent $K_d$ for switch-ON receptors, giving further support to this assumption (Fig. S5 and Table S1). Here, increasing L2 linker length likely balances stereric constraints with the entropic penalty associated with linker structuring for the two competing binding interactions in a similar fashion. Furthermore, increasing L2 has a smaller effect on the apparent $K_d$ in the protease-based receptor compared with in the affinity clamp on its own where a GGSGG insertion has previously been shown to result in a fivefold reduction in binding affinity (28). This reduced reduction in binding affinity is likely because binding of TVMV to the AI domain constrains the affinity clamp in a conformation that is unfavorable for PDZ ligand binding; thus, increasing the length of linker L2 is not associated with the same entropic penalty in the protease-based receptor as to the original affinity clamp.

**An Integrated Signal Sensing and Amplification Circuit.** We next sought to connect the TVMV-based allosteric receptor with an HCV-based signal amplifier to form an integrated signal sensing and amplification circuit (Fig. 3A). To ensure efficient signal relay between the TVMV-based signal sensor and the HCV-based signal transducer, the first SH3 domain of the Crk adaptor protein (29) was added to the N terminus of a TVMV-based ligand sensor, whereas its cognate peptide ligand PPPLPKRRR (30) was fused to the C terminus of the HCV-based signal transducer. In this way, the sensitivity of the assay could be improved between one and two orders of magnitude as equivalent responses could be recorded in a shorter period using less protein compared with the TVMV-based ligand sensor on its own (Fig. 3C) or the TVMV-based ligand sensor in an untagged, two-stage amplification system (Fig. S6A).

In addition, the relative responsiveness to ligand binding changed in the two-stage system: first, no differences in the induction ratio of the TVMV-based sensor to different strength ligands were evident in the two-stage system as the weaker binding ligand B2 activated the HCV-based signal amplifier slightly faster compared with the stronger binding ligand B1 (Fig. 3B and Fig. S6B). Considering the ligand binding kinetics differ only by an eightfold faster off-rate for ligand B2 (28), it appears that processing of a TVMV cleavage site within an HCV-based signal amplifier (as opposed to a much smaller TVMV-based peptide substrate) imposes additional kinetic constraints. Those may include displacement of the AI domain from the TVMV active site and isomerization of the affinity clamp following dissociation of the FN3-domain or the peptide ligand to enable access of the TVMV-based ligand sensor to the cleavage site in the HCV-based signal amplifier. Furthermore, nonspecific activation was observed even in the absence of ligand in both the SH3-tagged (Fig. 3B) and the untagged two-stage systems (Fig. S6A). As noise propagation is an inherent feature of signal amplification, further optimization and modeling of the system to obtain optimal signal-to-noise ratios will be required.

To test the linearity of the response in the integrated signal sensing and amplification circuit, we titrated it with increasing concentrations of the ligand peptides (Fig. 3D). Plotting the activity of HCV in the exponential phase against the ligand concentration showed a dose-dependent response that could be

Fig. 3. (A) Construction of an integrated signal sensing and amplification circuit from an allosterically regulated protease sensor TVMV\textsuperscript{E217,PDZ}\textsuperscript{G37}FN3GPG-AI and an HCV-based signal transducer. Efficient signal transmission between the sensor and the transducer is ensured by protein–peptide interactions between an SH3 domain and its cognate peptide ligand fused to the modules. (B) Activation of HCV protease activity in response to affinity clamp ligand peptides B1 and B2 in an integrated signaling circuit. (C) A control experiment where the protease activity of the TVMV affinity clamp chimera was measured directly in the presence or absence of the two peptide ligands B1 and B2. (D) Determination of apparent $K_d$ values for B1 and B2 using experimental setup shown in B with different ligand concentrations. The rate of HCV activity in the exponential phase (normalized over the concentration of TVMV) was plotted against the concentration of the ligand peptide and fitted to Eq. S2. (E) As in D but directly measuring the activity of the TVMV-based receptor from experiment shown in C. RFU units generated through the cleavage of TVMV- and HCV-specific peptide substrate proteases in the two different systems scale by a factor of 1.8. Protease activities in D and E are normalized over the concentration of the ligand sensing TVMV protease. Proteases were assayed with 5 μM quenched fluorescent substrate peptide.
fit to the value of 11 ± 1.0 nM for B1 and 921 ± 187 nM for B2 (Fig. 3D and Fig. S7A–D). These affinities are comparable to the apparent $K_d$ determined for the stand-alone sensor (Fig. 3E). To obtain a quantitative measure for signal amplification, we compared the protease signal generated (output) at the different ligand concentrations (input) in the single and two-stage system while normalizing over the concentration of the primary TVMV-based ligand sensor and taking into account 1.8-fold lower quantum yield of the HCV substrate peptide (Fig. S4C). Regarding absolute signal gain, we estimate signal amplification factors of >40- and >150-fold for the B1 and B2 ligands, respectively, over time scales where the signal is proportional to the bound ligand (Fig. 3D, Fig. S7 E and F, and SI Materials and Methods). Conversely, regarding signal-to-noise ratios, signal amplification drops to 0.3 for ligand B1 while remaining constant at 1.3 for ligand B2 (Fig. S7 G and H). Over prolonged time periods, signal-to-noise ratios are further reduced as all of the HCV-based signal transducer is eventually cleaved even in the absence of ligand, leading to continuous generation of an HCV-based protease signal.

Protease-Based Proximity Sensors. The observed PPI-enhanced coupling of autoinhibited protease modules in a signal amplification cascade (Fig. 3A) prompted us to explore further utilities of this architecture. We conjectured that ligand-induced molecular proximity can drive proteolytic activation of autoinhibited protease modules and thus be exploited to create protease-based receptors. Activation of such receptors would be governed by changes in intermolecular distances rather than a conformational change. To validate this concept, we fused our autoinhibited TVMV- and HCV-based signal transducers to FKBP12 and FRB domains, which selectively associate with each other in the presence of rapamycin (31) (Fig. 4A). We tested a configuration where HCV was connected to AI via a TVMV cleavable linker, whereas TVMV was connected to AI via a thrombin cleavage sequence. The latter enabled us to test the role of TVMV’s AI domain in the activation of the rapamycin receptor. We then monitored the activity of HCV in the absence and presence of rapamycin and in the presence and the absence of thrombin. As can be seen in Fig. 4B and C, the addition of rapamycin induced HCV activity both in the presence and the absence of thrombin, indicating that molecular proximity was the main driving force in the system’s activation. However, the preactivation with thrombin resulted in higher background activity at the chosen concentrations (Fig. 4B and C). Titration experiments demonstrated that we could confidently detect concentrations of rapamycin below 0.5 pM using 200 nM of the TVMV- and HCV-based signal transducers each (Fig. 4D). Furthermore, we could faithfully reproduce the $K_d$ for rapamycin binding to FKBP12 with 107 ± 2 pM and $K_d = 117 ± 3$ pM for the autoinhibited and uninhibited TVMV-based signal transducers, respectively (Fig. 4E and F and Fig. S7 I and J). Although the detection of rapamycin at subpicomolar concentrations required an extended measurement (Fig. 4E), we expect that connecting the receptor system to further signal amplification modules will significantly reduce the response time. Overall, we successfully created a two-component receptor architecture that does not rely on conformational changes but uses molecular clustering following ligand binding. From an engineering perspective, this greatly facilitates sensor design as it does not involve optimization of intramolecular interactions or linker compositions, potentially allowing the detection of any molecule for which specific binders can be obtained.

Discussion

We report a generally applicable approach for construction of signal sensors, transducers, and amplifiers based on highly specific proteases. We developed a modular design strategy where transducer proteases and AI domains are connected through linkers containing different types of functional units modulating the activity of the resulting autoinhibited enzyme. This approach enables rapid mix-and-match development of functional modules as evidenced by the sensors developed in this study. Demonstration that AI domains require only weak affinities for the active site and can be easily derived from the protease’s substrate peptides makes construction of additional autoinhibited protease modules straightforward. The same AI domains were used to create an allosterically regulated receptor protease based on an artificial
peptide receptor known as an affinity clamp. We demonstrate that, through adjustments of linker length, both ON- and OFF-switches could be generated using this approach. Notably, affinity-clamping is proposed to be a modular and generic architecture for the creation of allosteric protein binders (27) that can be used for the development of allosterically regulated protease receptors. The high degree of orthogonality of the developed modules combined with the simplicity of engineering connectivity also allowed us to create two component receptors and signal relays. The system's modularity, tunable substrate $K_{\text{d}}$, and control over the kinetic parameters via local concentrations enabled rapid, sensitive and tunable reactions can also be introduced, for example, by exploiting kinase, phosphatase, or other modifying enzyme. Efficient re-enzyme or incorporation of appropriate cleavage sites into the modular, tunable and sensitive system allows for the construction of protein or peptide binders are required for the construction of artificialzymogens, their development is likely to be straightforward, and neither depends on specific structural features (32) or on significant structural rearrangements (33). In vivo artificialzymogens or incorporation of appropriate cleavage sites into cellular proteins could enable actuation of such artificial sig- naling circuits through potentially any protein-mediated activity. Such a molecular queue can be a binding event or a chemical modification mediated by the enzymatic activity of a protein kinase, phosphatase, or other modifying enzyme. Efficient re-verse reactions can also be introduced, for example, by exploiting


**Supporting Information**

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**SI Materials and Methods**

**Cloning Procedures.**

**General.** Single-stranded oligonucleotides and gBlock DNA fragments were obtained commercially from Integrated DNA Technologies. Melting temperatures for PCR reactions were calculated with the Sigma Genosys Oligocalculator. All PCR, restriction enzyme digests, USER Enzyme, and T4 DNA ligase-mediated multifragment DNA assembly reactions were purified using the QiAquick PCR purification kit before use. PCR conditions were performed according to standard conditions using polymerase PfuX7 (1) with 1 μM forward and reverse primers, 250 nM dNTPs each, 20 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM (NH₄)₂SO₄, 10 mM KCl, 100 μM of the quenched metal solution (6). Autolysis was stopped by the addition of 1 mM of the protease assay buffer supplemented with 40 μM of the fluorescent peptide substrate for TVMV (Table S2). The protease assay buffer consisted of 50 mM Tris·HCl (pH 8.8 at 25 °C), 10 mM (NH₄)₂SO₄, 10 mM KCl, 100 μM of the quenched metal solution (6), 0.1% (vol/vol) Triton X-100, and 2 mM MgSO₄.

**Extension times were 30 s/kbp DNA and the melting temperature was chosen as 2 °C lower than the lesser melting temperature for the DISC primer pair**

**Summary of protein coding sequences.** A list of protein-coding sequences of all of the different protease-based biosensors is given below. The amino acid sequence of FRB and VPR are underlined. The amino acid sequence of the autoinhibitor sequence of the protease is underlined, the amino acid sequence of all of the different protease-based biosensors is given below. The nontarget expression of NIa proteases in E. coli acts as a molecular chaperone enabling more efficient recombinant protein expression. The nontarget expression of NIa proteases in E. coli was grown in shaking incubator (Infors HT Multitron) at the rpm as indicated.

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**Bacterial growth media and protease assay buffers.**

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**Library Screening.**

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Plasmids were transformed into chemically competent BL21(DE3)-RIL cells hosting the autolysis plasmid pS665 (Fig. S1B) and plated onto LB agar plates supplemented with 100 μg/mL carbenicillin, 50 μg/mL kanamycin, and 34 μg/mL chloramphenicol. Following overnight incubation at 37 °C, single colonies were inoculated into 96 deep-well plates filled with 1 mL minimal autoinduction medium supplemented with 100 μg/mL carbenicillin, 50 μg/mL kanamycin, and 34 μg/mL chloramphenicol. Cells were grown for 72 h at 30 °C at 320 rpm and typically reached OD values of ~10. To lyse cells, 100-μL aliquots of cell cultures from half a 96-well plate (48 samples) were diluted twofold into autoinduction medium (100 μL) and incubated for 90 min at 30 °C with agitation at 200 rpm. Aliquots of cell lysates (10 μL) were transferred in duplicates into separate halves of a black 96-well plate (Corning) filled with protease assay buffer (140 μL). Half of the duplicates were supplemented with 1 U thrombin to cleave the linker connecting TVMV to its autoinduction domain. The reaction was initiated by addition of 50 μL substrate solution, which gave rise to 10 μM TVMV substrate peptide in the final reaction. The reaction was monitored using a plate reader (Biotek Synergy 4) by measuring the release of a quenched fluorophore (7-methoxycoumarin-4-acetyl) at 405 nm following excitation at 330 nm. The induction of TVMV protease activities was evaluated by comparing the time course of the reactions in the presence and absence of thrombin. The plasmids encoding for mutants with high induction ratios were subsequently isolated from single colonies and subjected to sequencing.

Engineered Artificially Autoinhibited TVMV Modules.

Primary design. To create a TVMV-based signal transducer, the N-terminal cleavage product ETVRFQ (separated by a thrombin cleavage site flanked by additional glycine and serine residues) was appended to the C-terminal end of TVMV: GSFLVYDDAPEDDFMSGLVPVRGVTQFRGQ. Starting mutant: C-terminal tail of TVMVWT (underlined) and the N-terminal cleavage product (double underlined) acting as an AI-domain separated by a thrombin cleavage site (bold).

Library screen 1. To improve the performance of the TVMV-based module, the first library screen aimed to enhance the affinity between the product-based inhibitor and the TVMV protease through additional intercalating interactions between P5 and P6 in the N-terminal cleavage product and T214 and V216 in P13 of TVMV.

Library screen 2. The second library aimed to identify di-peptide motives that could bind across the P1-P1' junction, but could not be cleaved by TVMV. In this way, it became possible to move the His6 affinity purification tag from the N to the C terminus and enable purification of full-length proteins only. To this end, a small library with a proline in the P1’ position and a fully randomized P1 position was screened enabling us to identify a di-peptide motives covering the P1-P1’ junction that bound to the active site of TVMV but that were not cleaved. The design of the library was based on the notion that substrate mapping data for the related Naα protease from TEV suggests that proline in the P1’ position prevents cleavage (7). It is, however, unclear whether sequences with a proline in the P1’ position cannot bind or can bind but cannot be cleaved by TEV protease. Similarly, it is not clear to what extent this holds true for other members of the Naα potyvirus protease family including the Naα protease from TVMV.

Library 2: C-terminal tail of mutant TVMVVT149L, V216W (underlined) and the AI domain (double underlined) separated by the thrombin cleavage site (bold). The diversified position X coding for all 20 amino acids and the proline residue in the P1’ position is highlighted in red.

Protein Expression and Purification.

Bacterial growth media and buffers. Terrific broth (TB) consisted of 1.2% (wt/vol) tryptone, 2.4% (wt/vol) yeast extract, 0.04% glycerol, 0.17 M KH2PO4, and 0.72 M K2HPO4. PBS consisted of 10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4. Washing and binding buffer consisted of 20 mM NaH2PO4 and 20 mM imidazole, pH 8.0, which was supplemented with 300 mM NaCl for TVMV-based signal transducers, 500 mM NaCl for HCV-based signal transducers, and 1 M NaCl for TVMV-based affinity clamp receptors. Elution buffer was composed as washing and binding buffer except that it contained 50 mM NaCl rather than 20 mM imidazole. Protein storage buffer contained 50 mM Tris-HCl and 10% (vol/vol) glycerol, pH 8.0, which was supplemented with 1 mM EDTA and 2 mM DTT for TVMV-based signal transducers, 1 M NaCl, 1 mM EDTA, and 2 mM DTT for TVMV-based allospecific receptors, and 500 mM NaCl and 2 mM β-mercaptoethanol for HCV-based signal transducers.

Protein expression. Plasmids were transformed into chemically competent BL21(DE3)-RIL cells and plated onto Luria Bertani (LB) agar plates supplemented with 100 μg/mL carbenicillin and 34 μg/mL chloramphenicol and incubated overnight at 37 °C. A single colony was used to inoculate TB medium (250 mL) supplemented with 0.2% lactose, 0.05% glucose, 2 mM MgCl2, 100 μg/mL carbenicillin, and 34 μg/mL chloramphenicol. Cells were grown over the course of 48 h at 30 °C at 200 rpm and the protein expressed by autoinduction. Cells usually reached OD600 values of ~14 before being harvested by centrifugation at 4,500 × g, washed once with PBS (500 mL), and stored at ~20 °C.

Protein purification. To purify the protease-based signal transducers and allospecific receptors, cells were resuspended in washing and binding buffer and lysed using a one shot cell disruptor (Constant Systems). Samples were subsequently centrifuged at 25,000 × g, and the supernatant was passed through a 0.25-μm nitrocellulose filter. The His6-tagged proteins were purified using the AKTA-Express FPLC system. Briefly, the lysate was loaded onto Ni-NTA columns (5 mL HisTrap FF Crude; GE Healthcare) equilibrated with the binding buffer, and the column was washed with 200 mL of the washing buffer. The protein was eluted with an imidazole gradient from 20 to 500 mM in 40-column volumes at a flow rate of 5 mL/min. The protein typically eluted around 100 mM imidazole. Fractions containing protein were subsequently pooled and concentrated with 10-kDa cutoff centrifugal filters (Amicon Ultra) and transferred into the storage buffer by gel filtration on disposable PD-10 desalting columns according to manufacturer’s instructions (GE Healthcare). Proteins were generally stored at ~80 °C.

Protease Assays.

Fluorogenic protease substrate peptides and affinity clamp peptide ligands. Protease substrates (Table S2) were obtained commercially (Mimotopes) and dissolved in DMSO to a final concentration of 8 mM and stored at ~80 °C.

Assaying TVMV- and HCV-based signal transducers. To measure induction ratios under saturating reaction conditions, 500 nM TVMV- and HCV-based signal transducers were preincubated for 10 min in 150 μL protease assay buffer (50 mM Tris-HCl, 100 mM NaCl, 50 μg/mL BSA, and 2 mM DTT) in the presence and absence of the inducing protease; i.e., 1 U thrombin for TVMV-based signal transducers and 500 nM TVMV for HCV-based signal transducers. The reaction was initiated following the addition of 50 μL
substrate solution (Table S2) to a final concentration of 5 μM peptide substrate (Table S2) in a final reaction volume of 200 μL. The reaction was monitored using a fluorescent plate reader (Biotek Synergy 4) by measuring the release of 7-methoxycoumarinyl-4-acetyl from the quenched substrate peptide with λex/em of 330 and 405 nm. The induction ratio of TVMV-based protease activities in the inhibited and uninhibited states was calculated by dividing the initial rate in the presence of the activating protease over the initial rate in the absence of the activating protease.

**Enzyme kinetics of TVMV-based signal transducers.** To determine the kinetic parameters, the proteolytic activity of different protease biosensors was measured in protease assay buffer over varying concentrations of the substrate TVMV-DD (Table S2) using the protease concentrations indicated in the figure captions. Measurements were performed in duplicate for each substrate concentration. Initial rates were extracted from the change in fluorescence (after subtracting the background fluorescence with no enzyme) and plotted against the substrate concentration. Kinetic parameters were subsequently obtained by a nonlinear regression fit of the curve to Eq. S1

$$Y = V_{max} \times \frac{[\text{Substrate}]}{[\text{Substrate}] + K_M}$$  \hspace{1cm} [S1]

To estimate the $K_i$ of the inhibitory peptide EYVRAP, 500 nM of the TVMVV14 to V216W mutant was assayed with 5 μM of the TVMV-DD substrate in the presence of varying concentrations of the inhibitory peptide EYVRAPGST. Measurements were performed in duplicate for each inhibitor concentration, and the initial rates were extracted as described above. The $K_i$ was subsequently determined by a nonlinear regression fit of the curve to Eq. S2 with the $K_M$ and the substrate concentration set to 65 and 5 μM, respectively.

$$Y = V_{max} \times \frac{[\text{Substrate}]}{[\text{Substrate}] + K_M \times \left(1 + \frac{\text{Inhibitor}}{K_i}\right)}$$  \hspace{1cm} [S2]

**Assaying TVMV-based allosteric receptors.** The maximum induction ratios and apparent $K_{i}$ of TVMV-based allosteric receptors were measured in ligand titration experiments. The maximum induction ratios were measured using 250 nM TVMV affinity clamp chimera, whereas the apparent $K_{i}$ for the stronger and weaker binding affinity clamp ligands B1 and B2 were measured using 10 and 100 nM TVMV affinity clamp chimera, respectively. Initial rates were extracted from the change in fluorescence (after subtracting the background fluorescence with no enzyme) and plotted against the ligand concentration. The apparent $K_{i}$ of the different types of receptors were determined by a nonlinear regression fit of the curve to Eq. S3

$$Y = V_0 + \left(V_{max} - V_0\right) \times \frac{[\text{Sensor}] + [\text{Ligand}] + K_d}{\left([\text{Sensor}] + [\text{Ligand}] + K_d\right)^2 - 4 \times [\text{Sensor}] \times [\text{Ligand}] - 2 \times [\text{Sensor}]}$$  \hspace{1cm} [S3]

**Protease cascades.** TVMV- and HCV-based signaling cascades were measured as described above except that the reaction was initiated by the addition of the HCV-based signal amplifier. The concentration of the two different ligands B1 and B2, the HCV-based signal transducers, and the TVMV-based allosteric receptors are indicated in the figure legends. The HCV-based substrate was included at 5 μM (Table S2). Initial rates were extracted from the change in fluorescence (after subtracting the background fluorescence with no enzyme) and plotted against the ligand concentration. The apparent $K_{i}$ were determined by a nonlinear regression fit of the curve to Eq. S3.

**Protease-based proximity sensors.** Protease-based proximity sensors tagged with FRB and FKBP were measured as described above except that the reaction was initiated by the addition of the FRB-tagged HCV-based signal amplifier. The concentration of rapamycin, the FRB-tagged HCV-based signal transducer, and the FKBP12-tagged TVMV-based signal transducer are indicated in the figure annotations. The HCV-based substrate was included at 5 μM (Table S2). Initial rates were extracted from the change in fluorescence (after subtracting the background fluorescence with no enzyme) and plotted against the concentration of rapamycin. The apparent $K_{i}$ of for rapamycin was determined by a nonlinear regression fit of the curve to Eq. S3.

**Rationale of Protease-Based Signal Transducers.** In the simpler case of a protease-inducible transducer protease, we formulated a mathematical model based on Michaelis–Menten Kinetics defining the parameters modulating the signal output (Fig. S3). For any given autoinhibited transducer protease, the induction of activity is defined as the ratio of enzyme velocities in the cleaved and uncleaved states, which are subject to intra- and intermolecular inhibition, respectively, as defined by Eq. S4. Residual inhibition by the cleaved product peptides that remains associated with the protease is not taken into account in the model

$$\text{Induction} = \frac{V_{Cleaved}}{V_{Uncleaved}} = \frac{V_{max} \times [S]}{K^c_{i} + [S]}$$

Assume competitive inhibition model

$$\text{Induction} = \frac{V_{Cleaved}}{V_{Uncleaved}} = \frac{V_{max} \times [S]}{K_{M} \left(1 + \frac{[I]}{K_i}\right) + [S]}$$

After cleavage $[I]_{Cleaved} = [E]$

$$\text{Induction} = \frac{V_{Cleaved}}{V_{Uncleaved}} = \frac{K_{M} \left(1 + \frac{[E]}{K_i}\right) + [S]}{K_{M} \left(1 + \frac{[E]}{K_i}\right) + [S]}$$

Simplify

$$\text{Induction} = \frac{V_{Cleaved}}{V_{Uncleaved}} = \frac{1 + \frac{[I]}{K_i} + [S]}{1 + \frac{[E]}{K_i} + [S]}$$  \hspace{1cm} [S4]

A plot of Eq. S1 illustrates that the induction of activity is linearly proportional to the intramolecular concentration of the AI
domain in the uncleaved state \([I]\) (Fig. S3). In practice, this effectively depends on the proximity of the N and C termini to the active site, as well as the structure and length of the connecting linker. The strength of binding of the AI domain \(K_i\) also favorably affects the induction ratio, but must not significantly exceed the rate determining step of the transducer to prevent continuing intermolecular inhibition after cleavage. Maximum induction is achieved under saturating reaction conditions at low substrate \([S]\) and protease transducer concentrations \([E]\) where competitive inhibition in the intra- and intermolecular states are minimized, and \([S] = K_M\) and \([E] = K_c\).

**Note on Estimating Signal Amplification Factors.** To obtain an estimate of signal amplification between the one- and two-stage systems, we estimate and compare signal amplification factors both in terms of absolute signal gain and in terms of signal-to-noise ratios. In the former case, protease signals were directly compared with each other, whereas in the latter case, protease signals were additionally normalized over the background signal in the absence of ligand (as denoted by the red traces in Fig. 3B and C). Moreover, to estimate signal amplification factors, we compare the gradient in the exponential phase (Fig. S7A and C) as an approximate estimation of increasing concentrations of active HCV, which can thus be more directly compared with the signal generated by TVMV in a single-stage setup. At the same time, we emphasize that it is difficult to compare signal amplification between first- and second-order processes based on one- and two-stage signal amplification systems. However, the fact that we obtain similar \(K_d\) values analyzing one- and two-stage amplification data indicates that the experimental setup and data analysis used are appropriate. Sequences for linkers L1, L2, and L3 encoding for different affinity clamp mutants are summarized in Table S1.

Engineering TVMV-Based Signal Transducers

(A) To create an artificially autoinhibited version of TVMV, its N-terminal cleavage product ETVRFQ was fused to its C terminus via a linker encoding a thrombin cleavage site, yielding a TVMV-based signal transducer (Starting Mutant), which activity could be induced by approximately fivefold following thrombin cleavage. Protease activities were measured using 892 nM TVMV and 5 μM TVMV substrate. (B) To improve the performance of the module, a library screen was devised to enhance the interactions affinity between TVMV and its AI domain. This screen yielded a mutant protease, TVMV^{T214I, V216W}, and an altered N-terminal cleavage product EYVRFQ serving as an AI domain (screen 1). Although the induction ratio improved slightly, to ~13-fold, this was still considered insufficient for practical applications. Protease activities were measured using 892 nM TVMV and 10 μM TVMV substrate. (C) Notably, equivalent $K_m$ values were determined for the TVMV-based protease activities before and after thrombin mediated activation, which suggests that the majority of background activity can be attributed to proteases that lack an AI domain as a result of premature termination of translation or proteolytic degradation. In the autoinhibited state, kinetic parameters were determined using 1.78 μM TVMV. In the activated state following thrombin cleavage, the kinetic parameters were determined using 178 nM TVMV. (D) Michaelis–Menten kinetics of mutant TVMV^{V214I, T216W} in its activated (at 62.5 nM) and autoinhibited from (at 10 μM) assayed with varying concentrations of protease substrate TVMV-DD (Table S2). The kinetic parameters were determined by plotting the initial rates against the substrate concentration and the curve fit by nonlinear regression to Eq. S1. The $K_m$ value for the thrombin activated form is in agreement with previously measured values for an equivalent substrate of WT TVMV using an HPLC-based assay (1). For the autoinhibited form, the $K_m$ value associated with the residual activity is ~70-fold weaker but still comparable to the uninhibited enzyme, indicating that this activity is still predominantly due to a population of uninhibited proteases. (E) Titration of 500 nM solution of the TVMV^{T214I, V216W} mutant with increasing concentrations of the peptide derived from the autoinhibitor EYVRFAPGST. The $K_i$ value was determined by plotting the initial rates against the ligand concentration and the curve fit by nonlinear regression to Eq. S2.

Fig. S3. Plot of the theoretical induction of activity of a protease-based signal transducer as a function of normalized substrate [S], enzyme [E], and effective, intramolecular concentration of the inhibitor [I] according to Eq. S4.

Fig. S4. TVMV- and HCV-based protease activities are mutually orthogonal. (A) TVMV$^{T214I, V216W}$ (at 500 nM) and HCV protease (at 500 nM) was incubated with TVMV substrate (at 5 μM). No appreciable cleavage of the TVMV substrate is achieved by HCV. (B) TVMV$^{T214I, V216W}$ (at 500 nM) and HCV protease (at 500 nM) were incubated with HCV substrate (at 5 μM). No appreciable cleavage is achieved by TVMV. (C) Comparison of RFUs generated by the cleavage of TVMV- and HCV-specific protease substrates. Over a range of 1–5 μM of cleaved protein substrates, RFUs are generated linearly for both TVMV- and HCV-specific cleavage products. RFU units generated through the cleavage of TVMV- and HCV-specific peptide protease substrates scale by a factor of 1.8.
Fig. S5. Engineering TVMV-based ligand receptors. (A) The starting mutant TVMV<sup>E221</sup>-PDZ<sup>G38</sup>-FN3<sup>GPG</sup>-AI responded to the addition of either ligand B1 or B2 in a switch-OFF fashion. Further shortening L1 by three amino acids yielded a ligand sensor TVMV<sup>D218</sup>-PDZ<sup>B1</sup>-FN3<sup>GPG</sup>-AI that exhibited relatively low activities, but was largely unresponsive to the addition of either ligand B1 or B2. A single amino acid truncation then induced a sharp transition in the switching behavior yielding a ligand sensor TVMV<sup>E217</sup>-PDZ<sup>G37</sup>-FN3<sup>GPG</sup>-AI that responded to the addition of the two different ligands B1 and B2 in 30- and 4-fold switch-ON fashions, respectively. (B) Elongation of linker L2 (connecting the PDZ domain with the FN3 domain) based on switch-ON mutant TVMV<sup>E217</sup>-PDZ<sup>G37</sup>-FN3<sup>GPG</sup>-AI by a single glycine residue yielded mutant TVMV<sup>E217</sup>-PDZ<sup>G37</sup>-FN3<sup>GPG</sup>-AI with a 37-fold induction ratio for ligand B2. Inserting up to four additional amino acids reduced the induction ratio down to 30-fold. (C) Further truncation of the GPG motif in linker L3 (connecting the FN3 domain with the AI domain) based on switch-ON mutant TVMV<sup>E217</sup>-PDZ<sup>G37</sup>-FN3<sup>GPG</sup>-AI down to two or a single glycine residue did not appreciably alter the induction ratio. (D) Comparing the activities of TVMV-based signal transducer and TVMV-based allosteric protease receptor. Under full induction conditions, the TVMV-based allosteric protease receptor achieves 34% activity of the TVMV-based signal transducer (as judged by the concentration normalized initial rates over the first 12 min).
Fig. S6. Integrated signal sensing and signal amplification. (A) Signal transmission between a TVMV affinity clamp chimera and an HCV-based amplifier in the absence of scaffolding interactions. Time-resolved trace of HCV protease activities in signal sensing and amplification circuit based on a TVMV affinity clamp chimera (50 nM TVMV\textsuperscript{E217-PDZ\textsuperscript{G38-FN3\textsuperscript{GPG}}-AI}) and an HCV-based signal amplifier (500 nM HCV\textsuperscript{TVMV-AI}). Here, the TVMV-specific cleavage site in the HCV-based signal amplifier constitutes the only molecular recognition element that mediates signal transmission between the sensor and the amplifier. (B) Signal transmission between a TVMV affinity clamp chimera and an HCV-based amplifier in the presence of SH3-dependent scaffolding interactions based on an unscaffolded TVMV affinity clamp chimera (50 nM TVMV\textsuperscript{E217-PDZ\textsuperscript{G38-FN3\textsuperscript{GPG}}-AI}) and an HCV-based signal amplifier (500 nM HCV\textsuperscript{TVMV-AI}). (C) Comparing the time resolved traces of protease activities at high sensor concentrations in an integrated ligand sensing and amplification circuit based on an unscaffolded TVMV-based ligand sensor (100 nM TVMV\textsuperscript{E217-PDZ\textsuperscript{G38-FN3\textsuperscript{GPG}}-AI}) and an HCV-based signal amplifier (200 nM SH3-HCV\textsuperscript{TVMV-AI}) and (D) the TVMV-based ligand sensor (100 nM TVMV\textsuperscript{E217-PDZ\textsuperscript{G38-FN3\textsuperscript{GPG}}-AI}) on its own; here, scaffolding interactions are necessary for efficient signal transmission. RFU units generated through the cleavage of TVMV- and HCV-specific peptide protease substrates in the two different systems scale by a factor 1.8–1 (Fig. S4C).
Fig. S7. Two-component signaling systems based on autoinhibited TVMV and HCV proteases. (A–D) Determination of the apparent $K_d$ in the two-stage amplification system. In the two-stage signal sensing and amplification system, the apparent $K_d$ of the receptor protease (SH3-TVMV$^{E217}$,PDZ$^{G38}$-FN$^{GPG}$-AI) for ligands B1 and B2 were determined by plotting the observed change in fluorescence between (A) 24–38 min and (C) 14–28 min (as denoted by the vertical lines). The background fluorescence in the absence of enzyme was subtracted and the resulting values plotted against the ligand concentration and (B and D) fitted by the nonlinear regression to Eq. S3. Considering the irreversibility of HCV-based signal amplifier activation by the TVMV-based ligand sensor, the time window for recording the change in fluorescence was deliberately chosen before full activation of the HCV-based signal amplifier has occurred. (E and F) Legend continued on following page
Determining amplification factors for absolute signals in the one- and two-stage amplification system across different concentrations of ligands B1 and B2: The concentration normalized change in fluorescence in the two-stage system (measured as HCV activity in the exponential phase) was divided by the change in fluorescence in the one stage system (measured through TVMV activity). (G and H) Determining amplification factors over signal-to-noise in the one- and two-stage amplification system across different concentrations of ligands B1 and B2: As in E and F, but this time the concentration normalized change in fluorescence for HCV- and TVMV-based signals was additionally normalized over the background signal for [Ligand] = 0 as derived from the curve fits in Fig. 3 D and E. (I and J) The apparent $K_d$ for rapamycin binding to FKBP12:FRB in the two-component system using an autoinhibited TVMV transducer is comparable to the $K_d$ obtained with uninhibited TVMV (Fig. 4 F). To derive the apparent $K_d$ the observed change in fluorescence between 300 and 400 min (as denoted on the plot by the vertical lines) was corrected for the background fluorescence in the absence of enzyme and plotted against the ligand concentration and the data were fitted by nonlinear regression to the Eq. S3.

### Table S1. Summary of $K_d$s for TVMV-based allosteric receptor proteases with switch-ON behavior

<table>
<thead>
<tr>
<th>Mutant name</th>
<th>Link sequences</th>
<th>Apparent $K_d$s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linker L1</td>
<td>Linker L2</td>
</tr>
<tr>
<td><strong>Receptor proteases with switch-OFF or neutral behavior (L1 truncations)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVMV$^{E217,PDZ}^{G^{221}},FN^{3}^{3}^{GPG}$,AI</td>
<td>EDAPESG</td>
<td>GSGGG</td>
</tr>
<tr>
<td>TVMV$^{E217,PDZ}^{G^{220}},FN^{3}^{3}^{GPG}$,AI</td>
<td>EDAPSG</td>
<td>GSGGG</td>
</tr>
<tr>
<td>TVMV$^{E217,PDZ}^{A^{219}},FN^{3}^{3}^{GPG}$,AI</td>
<td>EDASG</td>
<td>GSGGG</td>
</tr>
<tr>
<td>TVMV$^{E217,PDZ}^{A^{218}},FN^{3}^{3}^{GPG}$,AI</td>
<td>EDSG</td>
<td>GSGGG</td>
</tr>
<tr>
<td><strong>Receptor proteases with switch-ON behavior (L2 elongation + L3 truncation)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVMV$^{E217,PDZ}^{G^{37}},FN^{3}^{3}^{GPG}$,AI</td>
<td>ESG</td>
<td>GSGGG</td>
</tr>
<tr>
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<td>GSGGGG</td>
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<tr>
<td>SH3-TVMV$^{E217,PDZ}^{G^{38}},FN^{3}^{3}^{GPG}$,AI</td>
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<td>GSGGGGG</td>
</tr>
<tr>
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<td>GSGGGG</td>
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<tr>
<td>TVMV$^{E217,PDZ}^{G^{38}},FN^{3}^{3}^{G}$,AI</td>
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### Table S2. Summary of protease fluorogenic peptide substrates and peptide receptor ligands

<table>
<thead>
<tr>
<th>Protease</th>
<th>Substrate sequence</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVMV</td>
<td>ANA-GETVQFSQT(164)-NH$_2$</td>
<td>Used in library screening and protease activity assays</td>
</tr>
<tr>
<td>HCV</td>
<td>ANA-DDVTPCSMS(164)-NH$_2$</td>
<td>Used in library screening and protease activity assays</td>
</tr>
<tr>
<td>TVMV-DD</td>
<td>ANA-GETVRQFSQT(164)DD-NH$_2$</td>
<td>Used for measuring enzyme kinetics; contains additional Asp residues for improved water solubility</td>
</tr>
<tr>
<td>B1 ligand (strong)</td>
<td>NH$_2$-RGSIDTWV-COOH</td>
<td>$K_d = 0.6$ nM for the native affinity clamp ePDZ-b1</td>
</tr>
<tr>
<td>B2 ligand (weak)</td>
<td>NH$_2$-POPVDWV-COOH</td>
<td>$K_d = 5$ nM for the native affinity clamp ePDZ-b1</td>
</tr>
</tbody>
</table>

ANA, 5-amino-2-nitrobenzoyl group; 164, Mimotopes-specific code for lysine coupled to a 7-methoxycoumarinyl-4-acetyl group.