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Engineering Caspase 7 as an Affinity Reagent to Capture Proteolytic Products

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Abstract

Many proteases recognize their substrates with high specificities, with this in mind, it should theoretically be possible to utilize the substrate binding cleft of a protease as a scaffold to engineer an affinity reagent. In this study, we sought to develop reagents that would differentiate between substrates and products of proteolysis, based on a caspase 7 scaffold. Firstly, we engineered a form of caspase 7 that can undergo conversion to a substrate binding conformation without catalysis. Seeking to generate a product-only trap, we further engineered this construct by incorporating mutations that compensate for the generation of a negative charge in the neo C-terminus of a newly generated product. This was accomplished with only three substitutions within the substrate binding cleft. Moreover, the affinity of the product trap for peptides was comparable to the affinity of caspase 7 to parental substrates. Finally, generation of a hybrid fluorescent protein with the product trap provided a reagent that specifically recognized apoptotic cells and highlights the versatility of such an approach in developing affinity and imaging agents for a variety of cysteine and serine proteases..

Keywords

apoptosis; mutagenesis; Protease trap; proteolysis imaging; protein engineering

Introduction

Proteases are generally expressed in an inactive pro-form, requiring further processing to free the active site for substrate access. When a protease is processed into its catalytically active form, its activity can be further modulated by the presence or absence of endogenous protease inhibitors. Furthermore, proteases require the presence of substrates at sufficient concentrations to perform catalysis, adding another layer of complexity in protease

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characterization. Therefore, three questions are presented to properly characterize a protease: is the protease in its active conformation, is the protease in an uninhibited state, and is there a sufficient concentration of substrates for proteolysis to occur?

To date, shortcomings exist in technologies that can reliably answer these questions. For this study, instead of developing strategies to answer each of these questions separately, we hypothesized that if we could detect the end-result of proteolysis, or a protease product, then we could answer all three questions. For example, if a reagent existed that identified the presence of proteolytic products of a specific protease, one could infer that said protease must have been active, uninhibited, and in the presence of sufficient substrates to perform proteolysis.

Strategies to understand protease substrates can take advantage of enzyme traps. For example, an engineered, catalytically inactive version of ClpXP protease was used to enrich and characterize its cellular substrates, uncovering important recognition signals in the regulation of protein degradation (1). While the substrate trap is useful to identify what substrates may be targeted, it does not yield information about which substrates are actually being processed. To identify which substrates are actually processed, one would need to develop technologies that look at the presence of products. Such technologies could include focused proteomic techniques such as N-terminomics and terminal amine isotopic labeling of substrates (TAILS) (2)(3). However, these technologies do not reveal the identity of the protease responsible for cleavage. To expand these technologies, it would be useful to utilize the scaffold of a protease to identify not only their substrates, but also their products. Hence, we set out to develop a specific protease product trap that would identify the products of proteolysis by that protease.

Caspases are well characterized proteases with well-defined substrate repertoires, and therefore, act as an appropriate scaffold to develop such a reagent. Accordingly, we aimed to harness the endogenous affinity of caspase 7 towards its proteolytic products, and couple it to an engineered active site capable of distinguishing between substrates and products. A major goal of the study was to increase the affinity towards all of caspase 7's N-terminal products, with affinities paralleled by the Km values of their parental substrates. This product trap should be capable of capturing N-terminal protease products from solution, while showing minimal interaction with a parental substrate.

Results

Principles of the product trap.

The product trap is an affinity reagent that is applied after proteolysis has taken place. Cysteine and serine proteases undergo a two-step catalytic process (4). First, the substrate enters the active site of the protease and is cleaved between the P1 and P1' residues. After this first step, a C-terminal product is released, and the protease is left covalently linked to the N-terminal product as an acyl intermediate. Next, a hydrolysis reaction results in the release of the N-terminal product, and recycling of the protease. We designed the product trap as an exogenously added reagent that can selectively interact with N-terminal products

of specific proteolysis. Such a reagent could also be used to label the end-result of proteolysis for imaging. (Figure 1)

Engineering of caspase 7 scaffold competent for binding product.

Caspase 7 is expressed as a single chain in its inactive, pro-form. Cleavage of an inter-subunit peptide linker frees caspase 7 to change conformation and open its active site to accept substrates (5). As a scaffold for the protease trap, we needed to adopt a similar mechanism of action to generate a functional active site conformation. We replaced the endogenous inter-subunit linker with a linker amenable to cleavage by TEV protease (Figure 2a)(6). This substitution was incorporated to utilize TEV protease's substrate selectivity and proteolytic activity in a variety of buffers.

To validate the proposed scaffold, wild-type caspase 7, with a substitution within its inter-subunit linker to create a TEV protease site, was cloned and expressed in *E. coli* with an N-terminal Myc tag C-terminal 6xHis purification tag. To determine the optimal concentration of TEV protease, serial dilutions were performed and visualized by SDS-PAGE (Figure 2b). Each fraction from the TEV protease titration was subjected to a caspase 7 activity assay to ensure this scaffold forms a functional active site upon TEV protease cleavage (Figure 2c). The activity of caspase 7 was highest at the highest concentrations of TEV protease while no activity was detected without TEV protease treatment, correlating with the SDS-PAGE data, and importantly confirming that treatment of this construct with TEV protease promotes an open active site.

Development of a product trap.

By definition, the difference between a substrate and an N-terminal product is that the product contains a neo-carboxy terminus and lacks prime side residues. Accordingly, our strategy was to create an active site that lacked catalytic activity, blocked access of prime side residues, while also accommodating the negative charge of the neo-carboxy terminus. Based on the structure of caspase-7 recorded in PDB: 1F1J we identified E146, C186, and Y230 within caspase 7 as candidate residues to fulfill these criteria and form the basis for a product trap (Figure 3A)(7). Three separate constructs were generated, a catalytic mutant C186A, C7RAK, and C7RRA (Figure 3B). Each of these constructs was expressed in *E. coli* and purified by their 6x-HIS tag. Following purification, the constructs were cleaved by TEV protease to ensure cleavage of the inter subunit linker and the generation of a free active site. All three constructs were dose-dependently cleaved by TEV protease as visualized by SDS-PAGE. A final concentration of 200nM TEV protease was chosen for subsequent studies.

Validation of the product trap.

The caspase 7 mutants were first tested for their ability to specifically interact with N-terminal products in solution. A consensus caspase 7 substrate would have a sequence P5-DEVD-P1', and the product would be P5-DEVD-C', where C' is the neo-carboxy terminus (8). To simulate these, synthetic versions of a caspase 7 substrate and a caspase 7 product were generated by fusing them to the C-terminus of a GST-SUMO scaffold (Figure 4). GST-SUMO-DEVD represents an N-terminal product, and GST-SUMO-DEVDGVD represents a

substrate sequence. Each construct was expressed in *E. coli* and purified by their GST-tag. Following purification, the caspase 7 trap constructs were tested for their ability to capture the substrate and product mimetics from solution. No capture of the product mimetic was observed with C186A or C7RRA. In contrast, C7RAK specifically pulled down the product mimetic and C186A specifically pulled down the substrate mimetic. In an additional specificity test, we found that C7RAK specifically labeled GST-SUMO-DEVD when visualized in a western blot (Figure 5). Therefore, C7RAK was chosen for further characterization as a product trap. In examining different buffer compositions, we identified 100mM HEPES, 250mM NaCl, 0.2% CHAPS, pH 7.3, as optimal.

Within the caspase 7 consensus sequence, the P1-P4 residues define affinity for substrates (9). We hypothesized that affinities of the N-terminal products to C7RAK should correlate with the Km of parental substrates (uncleaved product) with wild-type caspase 7. To measure this, we synthesized divergent peptide sequences and determined their binding affinity to C7RAK by isothermal titration calorimetry (ITC) and their Km values as substrates (Figure 6a). IETD is a good control because it is predominantly a caspase-8 substrate, which is very poorly cleaved by caspase-7. Similarly, VEID is a good control because it is predominantly a caspase-6 substrate, which is very poorly cleaved by caspase-7 (10). Affinities between C7RAK and the product peptides trended with affinities between wild-type caspase 7 and the parental substrates, supporting our hypothesis (Figure 6b). We conclude that the geometry of the C7RAK active site is essentially the same as wild type caspase 7.

Fluorescent C7RAK labels apoptotic cells.

An additional goal of this study was to determine if a protease could be engineered as an imaging reagent. We constructed a fluorescent fusion protein with N-terminal citrine fused to C7RAK (Figure 7a). Prior to employing citRAK in imaging studies, it was validated in a pull-down experiment to ensure it interacted with N-terminal products in the same manner as C7RAK (Figure 7b). This fluorescent version of C7RAK, citRAK, was tested for whether it could identify apoptotic cells. MDA-MB-231 cells were treated with TRAIL to induce apoptosis, fixed, permeabilized and stained with both citRAK and anti-cleaved caspase 3, and imaged on a fluorescent microscope (Figure 8a). citRAK staining overlaid well with cleaved caspase 3 staining, and signal intensities between cells in various stages of apoptosis were similar with both treatments.

To validate the utility of the product trap in identifying the victims of executioner caspase proteolysis, we examined labeling by the product trap within a heterogeneous cell population of viable and dying cells. Accordingly, citRAK was tested for its ability to specifically identify a naturally dying cell within an untreated population of MDA-MB-231 cells. MDA-MB-231s were cultured under normal conditions until confluent. The cells were then fixed, permeabilized and stained with citRAK and anti-cleaved caspase 3. Both citRAK and the anti-cleaved caspase 3 antibody were able to identify apoptotic cells within a population of healthy cells (Figure 8b). This experiment reveals the specificity of citRAK towards apoptotic cells (defined by cleaved caspase 3 labeling), with viable cells showing no citRAK labeling.

Discussion

Proteases represent close to 2% of the human proteome (11) and are the subject of therapeutic intervention. Transcriptomics technologies afford us the ability to understand RNA expression of a protease at single cell level and identify the physical presence of a protease in a pathological condition, however, technologies that characterize protease activity are still developing. In order to confidently validate a protease's catalytic activity as a therapeutic target, it is important to characterize the protease of interest as catalytically active, and to determine the identity of its target substrates. We propose that the development of a reagent that can identify the products of proteolysis would be an essential step in identifying the biological function of a specific protease. Towards this goal, caspase 7 was engineered as an affinity reagent to its proteolytic products (a product trap). In principle, caspase activity can be visualized with a synthetic substrate, however, this would not indicate whether a caspase has completed proteolysis on endogenous target substrates. A product trap overcomes this shortcoming. Importantly, we are not trying to find every caspase 7 substrate, we are trying to reveal the signature of caspase 7 activity.

Protease traps are not a novel technology, with multiple reports of protease traps being developed as proteomic tools to identify the substrates of specific proteases (12)(1). However, the traps previously developed were substrate traps designed to help researchers uncover substrates of specific proteases. We aimed to build off of these principles to generate a trap specific to products of proteolysis. To distinguish product trap from a substrate trap, we needed to create a reagent that, first, facilitates product binding, and second, blocks substrate access. The trap would need to take advantage of the presence of a negatively charged carboxylate at the neo-carboxy terminus of an N-terminal product. A glutamic acid residue within the caspase 7 active site, E146, sits in close proximity to the product's newly developed C-terminus. We hypothesized this could lead to a repulsive interaction, ultimately leading to expulsion of N-terminal products. Therefore, our first strategy focused on engineering a positively charged environment, on the background of a catalytic mutant, surrounding the neo-carboxy terminus to stabilize the presence of an N-terminal product within the caspase 7 active site. Another differentiating feature of a protease product is the absence of prime side residues present in substrates. To occlude substrate binding, we engineered bulky residues into the prime side of caspase 7.

In total, three mutants were expressed and characterized for their trapping potential. The first mutant, C186A, was developed as a potential substrate trap based off previously reported studies indicating the utility of catalytic mutants as substrate traps (13)(14)(15)(12)(1). The next two traps, C7RAK and C7RRA, were engineered with the goal of developing a product trap. C7RAK, with mutations E146R, C186A, Y230K, was engineered to have positively charged residues surrounding the neo-carboxy terminus of N-terminal products, while also providing space for the N-terminal product to occupy the active site by mutating C186 to an alanine. C7RRA, with mutations E146R, C186R and Y230A, replaced the catalytic cysteine with an arginine. The C186R mutation was designed with the goal of achieving a more stringent exclusion of substrates through the incorporation of an arginine in close proximity to the neo-carboxy terminus. In summary, the C186A catalytic mutant behaved as a substrate trap in pull-down experiments, preferentially pulling down substrates, C7RAK interacted

specifically with N-terminal products and C7RRA was unable to interact with either products or substrates. Consequently, C7RAK fulfilled the criteria of a product trap, and was chosen for further development as an imaging reagent.

The design of the product trap was based on the structure of the active enzyme bound to an inhibitor (7). The logic of our design principles predicted that the mechanism of binding of product to the product trap would be essentially the same as substrates binding to active protease. Indeed, the order of binding affinities of product peptides generally paralleled affinities of substrates towards active caspase 7. This suggests that the binding of product to the product trap is as envisaged in the design of the affinity reagent.

One goal of our study was to develop an affinity reagent capable of visualizing proteolysis. We designed an N-terminal fusion of citrine (a modified yellow fluorescent protein) with C7RAK as a fluorescent fusion protein, creating citRAK. By labeling cells with citRAK, caspase 7 and its close paralogue caspase 3 have almost identical substrate preferences. Thus, it would be very difficult, if not impossible, to distinguish caspase 7 and caspase 3 products. Nevertheless, since they are both implicated in apoptotic execution, we expected to stain the products of caspase 7 and caspase 3 proteolysis, thus identifying apoptotic cells. We compared citRAK to a cleaved caspase 3 antibody, a well-validated positive control of apoptosis (16)(17). In separate experimental conditions, citRAK labeling of apoptotic cells correlated with staining of the cleaved caspase 3 antibody. citRAK's ability to label apoptotic cells highlights the utility of this approach to visualize apoptosis. The underlying principles behind the development of this product trap could potentially be applied to a variety of cysteine and serine proteases to develop affinity and imaging agents.

Methods

Generation of TEV protease-activatable caspase 7.

wtC7TEV: A caspase 7 construct containing a TEV protease linker (18), was amplified by incorporating a 5' myc tag, and NdeI and XhoI restriction sites for introduction into pET21b vector and expressed in *E coli* as previously described for wt caspase 7. (18)

Measurement of TEV protease inducible caspase 7 activity.

To test activity of the wtC7TEV construct, TEV protease (expressed in-house) was titrated onto 1μM wtC7TEV in the presence of 5mM DTT and left to incubate overnight at 4°C. The following day, each point from the titration was analyzed by SDS-PAGE and each point was also subjected to an activity assay. For the activity assay, each point in the titration was measured for caspase 7 activity using Ac-DEVD-AFC (19).

Cloning, expression and TEV protease treatment of C7RAK, C7RRA and C186A.

The three protease trap prototypes were cloned into pET21b by using the pET21b-Myc-C7P20-TEV-C7P10–6xHIS as a template. Overlapping primer mutagenesis was performed to create the following mutants: C186A, C7RRA (E146R, C186R and Y230A), C7RAK (E146R, C186A, Y230K), which were expressed in *E. coli* (18).

TEV protease was titrated onto a constant amount of each mutant with a starting ratio of 1:1 C7:TEV protease ratio, with the TEV protease being sequentially diluted at each point. Cleavages were left to incubate overnight at 4°C and visualized the following day by SDS-PAGE.

Cloning and expression of GST-tagged substrate and product mimetics.

Product and substrate mimetics were created by fusing a GST domain to a SUMO2 domain containing a C-terminal peptide sequence corresponding to consensus substrate and product sequences for caspase 7. All constructs were inserted into the BamHI and XhoI cloning sites of pGEX-4T-1. This vector contains a GST fusion protein to aid with purification and detection. In addition, SUMO2 was inserted resulting in a multidomain protein which mimics a protease substrate or product depending on what residues are added C-terminal to the SUMO2 sequence. Four synthetic protein mimetics were generated: GST-SUMO-DEVD, GST-SUMO-IETD, GST-SUMO-DEVDGVD, and GST-SUMO-DEVDGVDEVA. All constructs were expressed in BL21(DE3) by induction with 0.2mM IPTG at 25°C for 4 hours. Bacterial pellets were lysed by sonication in Trap Assay Buffer (100mM HEPES, 250mM NaCl, 0.2% CHAPS, pH 7.3), cleared, allowed to bind overnight to Glutathione Sepharose 4b at 4°C, extensively washed with Trap Assay Buffer, and eluted in the same buffer containing 50mM reduced glutathione. Proteins were buffer exchanged by dialysis into Trap Assay Buffer and purity was assessed by SDS-PAGE.

Pull-down experiments.

For the pull-down experiments, 25μg of TEV protease treated caspase 7 mutants were incubated with 25μg of each of the GST-SUMO-peptide constructs. 10μl of anti-Myc bead slurry (ThermoFisher catalog# 88842) were added to each tube and reaction volumes were brought up to 800μl in Trap Assay Buffer. Samples were incubated at 4°C, and the following day, and the beads were recovered by centrifugation and washed twice with assay buffer via aspiration and centrifugation. Samples were analyzed by SDS-PAGE.

Blotting with product trap.

5μg of each of the proteins GST-SUMO-DEVD, GST-SUMO- DEVDGVD and GST-SUMO-IETD were loaded onto an SDS-PAGE gel with four different gels having the three samples plus a size standard. Gels were transferred to a nitrocellulose membrane through use of the Invitrogen blotting module at 10V for 60 minutes. The membrane was then treated with ponceau stain and molecular weight standards were marked with a marker. The membrane was then blocked in 100mM HEPES, 250mM NaCl, 0.2% CHAPs, 3% w/v BSA overnight at 4°C. The following day, the blots were each treated separately with C7RAK at concentrations of 240nM, 24nM 2.4nM and 0nM in traps buffer and incubated at room temperature for 2 hours. Following incubation, blots were washed 3x for five minutes in traps buffer and 10μg/mL anti-myc 9E10 antibody was added and incubated for 30 minutes in traps buffer. The membrane was then washed 3x for five minutes with traps buffer and then subjected to treatment with an IR800 conjugated anti-mouse IgG H+L antibody. After a fifteen-minute incubation, blots were washed three times in traps buffer and imaged in a LiCor Odyssey western blot device.

Determination of Michaelis-Menten constant (Km) for individual fluorogenic substrates.

Caspase 7 was analyzed for activity using four tetrapeptide substrates with a C-terminal 7-amino-4-carbamoylmethylcoumarin (ACC) fluorogenic reporter group (Ac-DEVD-ACC, Ac-AEVD-ACC, Ac-VEID-ACC, Ac-IETD-ACC) (20) at 37°C in caspase buffer (10% w/v sucrose, 20mM Pipes, 10mM NaCl, 1mM EDTA, 10mM DTT, pH = 7.2). assay conditions were as follows: 100μL total reaction volume, eight different substrate concentrations (10μM-1000μM), and caspase 7 at 20nM. Substrate hydrolysis was monitored for up to 30 min, and most linear segment of a kinetic curves were captured. K_M values were determined as described elsewhere (10).

Determination of peptide binding affinities.

C7RAK was measured for its affinity towards substrate or product mimicking peptides CGSGSIETD, CGSGSDEVD, CGSGSAEVD and CGSGSVEID (obtained from A & A Labs in San Diego, CA). Isothermal Titration Calorimetry (ITC) was performed in the Sanford Burnham Prebys Protein Analysis Core, using an ITC200 calorimeter from Microcal (Northampton, MA). 2.0μl aliquots of solution containing between 0.5 and 2mM peptides were injected into the cell containing 0.05 to 0.12mM protein. The experiments were performed at 23ºC in buffer containing 100mM Tris pH 7.3, 250mM NaCl, 0.2% CHAPS. ITC data were analyzed using Origin software provided by Microcal.

Cloning and expression of fluorescent product trap citRAK.

To develop an imaging agent, we coupled citrine to the N-terminus of C7RAK to generate citRAK (purchased from IDT, Coralville, Iowa, as a gBlock fragment). The synthesis included flanking sequences that encoded for NdeI on the 5’ end and a restriction side for XhoI on the 3’ end for insertion into the pet21b vector, encoding a 6xhis C-terminal purification tag. The construct was cleaved with the restriction enzymes NdeI and XhoI and cloned into pET21b and expressed in *E coli.*

Validation of fluorescent product trap.

citRAK was subject to a pull-down experiment. 25μg of citRAK was incubated with 25μg of GST-SUMO-DEVD, GST-SUMO-IETD, GST-SUMO-DEVDGVD and GST-SUMO-DEVDGVDEVA and 10μl GSH beads in a volume of 1mL with traps buffer for two hours at 4°C with end over end rotation. Samples were then boiled in reducing SDS and visualized by SDS-PAGE.

citRAK overlay with cleaved caspase 3 antibody.

MDA-MB-231 cells were seeded into polyL lysine treated wells of an 8-chamber slide at 20K cells per well in complete DMEM growth medium (DMEM + 10% Fetal Bovine Serum + Pen/Strep). Following seeding, cells were allowed to adhere overnight at 37°C, 5%CO2. To induce apoptosis, cells were treated with 5μM of the IAP antagonist, birinipant (LCLabs) and 5nM TRAIL(BioLegend) dissolved in complete DMEM growth medium and were incubated for 6 hours at 37°C 5% CO2. After incubation, cells were washed once with 1x dPBS and fixed with formalin, blocked with 0.5% TritonX-100 v/v dissolved in 100mM HEPES, 250mM NaCl, 0.2% CHAPs, 3% BSA w/v, pH 7.3 (traps-b buffer). The next day,

the slide was treated with 2μg/mL of anti-cleaved caspase 3 antibody (Cell Signaling Technologies Cat# 9664S) dissolved in traps-b buffer, and incubated at room temperature for one hour, washed twice with traps-b buffer, and treated simultaneously with 10μM pre-cleaved citRAK and 16μg/mL of an alexafluor-647 conjugated anti-rabbit antibody (JacksonImmunoresearch, West Grove, PA) suspended in traps-B buffer. After a two-hour incubation, and then washed with traps-b buffer. The slide was dried, mounted with DAPI, sealed with nail polish, and imaged with both 20x objective and 60x oil immersion imaging on an LSM 710 NLO Zeiss Multiphoton Confocal Microscope at the Sanford Burnham Prebys cell imaging core.

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Abbreviations:

GST-SUMO	a fusion of glutathione S-transferase and small ubiquitin-like modifier protein
ITC	isothermal calorimetry
K_m	Michaelis constant
TEV	tobacco etch virus
TRAIL	TNF-related apoptosis-inducing ligand

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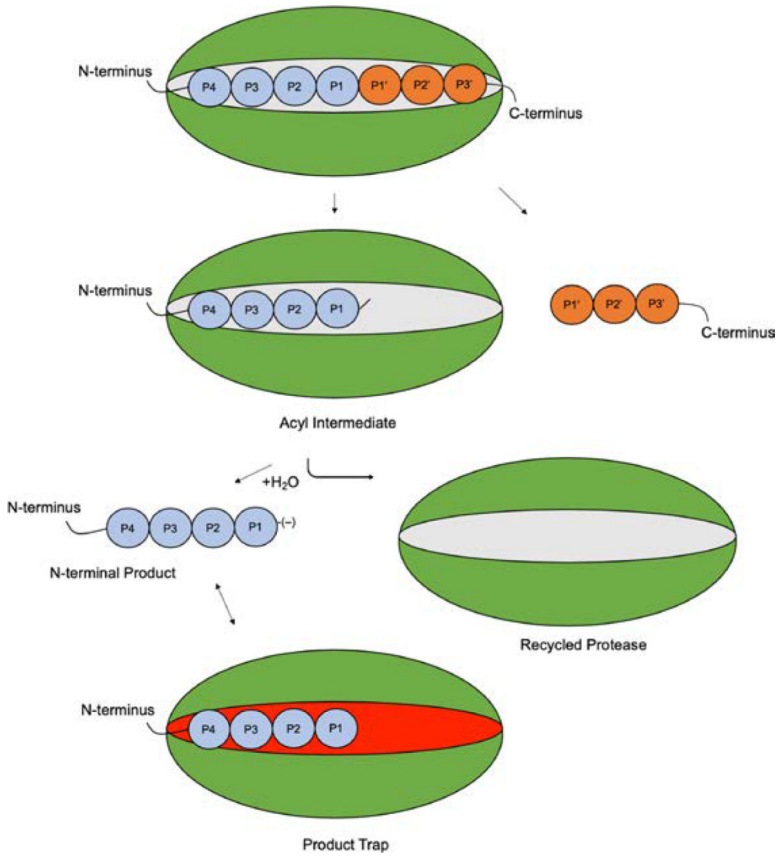


Figure 1. Mechanisms of Proteolysis by Cysteine and Serine Proteases.

A substrate (blue and orange circles) enters the active site of a protease (grey shading) and the protease cleaves between the P1(blue circles) and P1' residues (orange circles), leading to the generation of a C-terminal product (orange circles) and the protease (green oval) covalently linked to the N-terminal product (blue circles). In the second step, a hydrolysis reaction releases the N-terminal product (blue circles), and the protease (green oval) is recycled. The product trap (green oval) is a protease containing mutated substrate-binding residues (indicated by the red shading) added after proteolysis as an affinity reagent to detect the presence of product (blue circles).

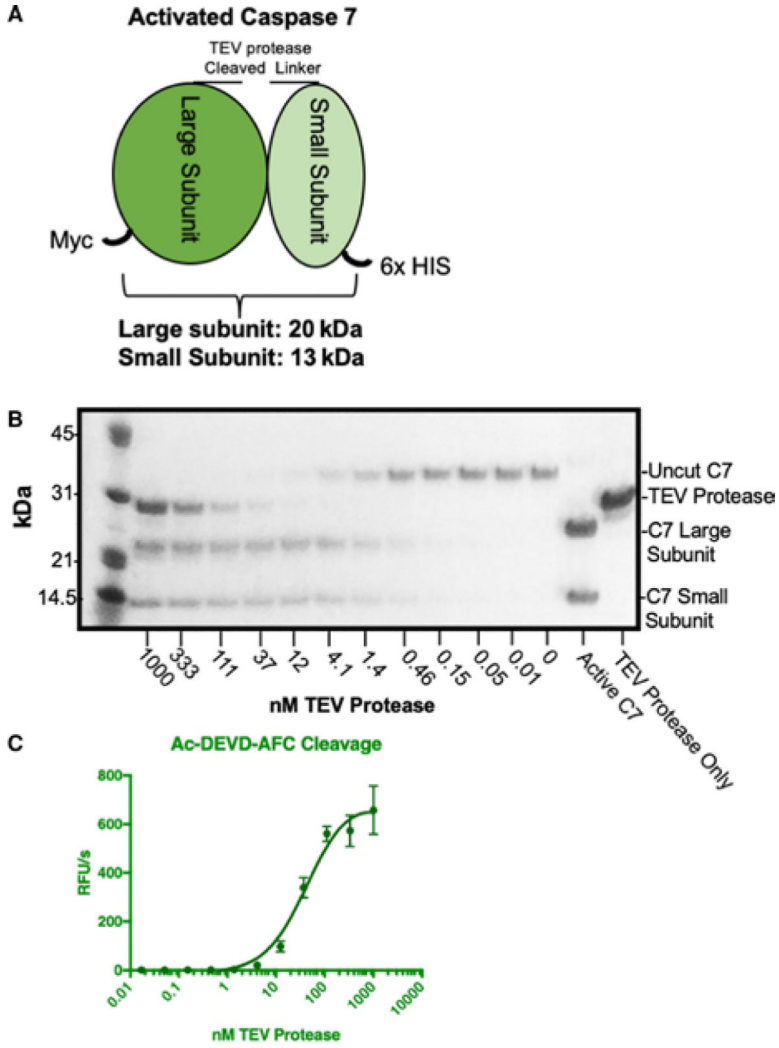
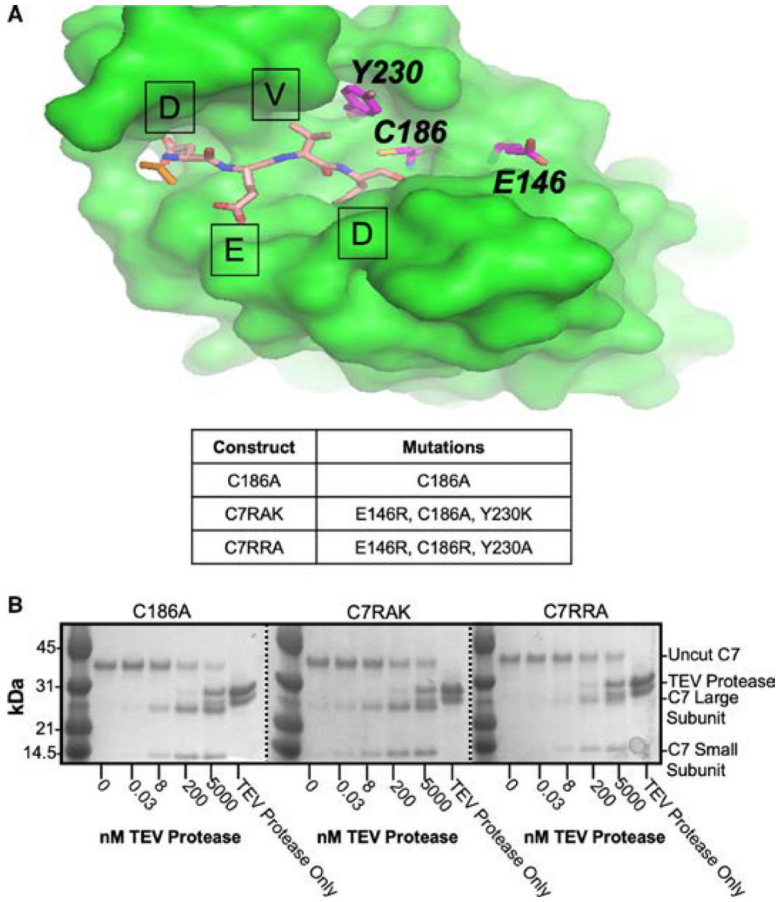


Figure 2. Scaffold Development

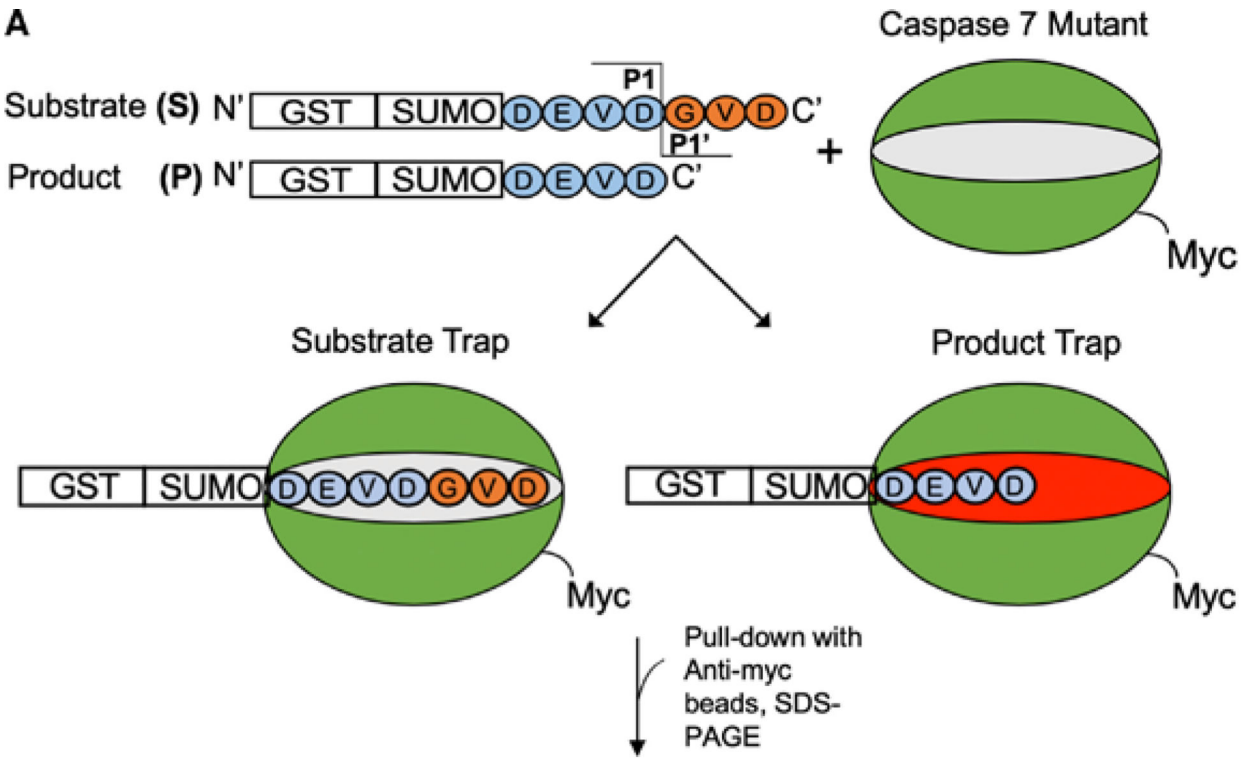
(A) Schematic representing the scaffold used to engineer the product trap (B) SDS-PAGE of a serial dilution of TEV protease on caspase 7 (C) Activity assay showing Ac-DEVD-AFC cleavage with each point from the TEV protease titration. Error bars represent standard error of the mean from three replicates and SDS-PAGE representative of three independent experiments.

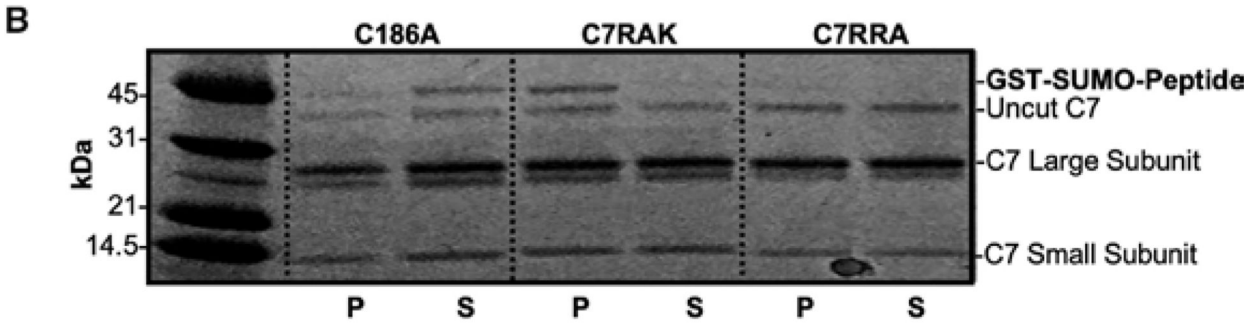


Construct	Mutations
C186A	C186A
C7RAK	E146R, C186A, Y230K
C7RRA	E146R, C186R, Y230A

Figure 3. Rational Design and Expression of Product Traps

(A) Structure of caspase 7 active site with a peptide forming an acyl-intermediate with caspase 7, with P4-P1 residues highlighted (DEVD). Three caspase 7 residues bolded, E146, C186 and Y230, were focused on to engineer the product trap. Three mutants were generated on the scaffold described in Figure 2. A catalytic mutant, C186A, and two potential product traps, C7RAK and C7RRA. (PyMOL PDB: 1F1J.) (B) SDS-PAGE of *E. coli* derived caspase 7 (C7) mutants treated with a serial dilution of TEV protease (representive gel of two independent experiments).





C7 Mutant	Binding Characteristics
C186A	Substrate Trap
C7RAK	Product Trap
C7RRA	No Binding

Figure 4. Capture of Caspase 7 Products and Substrates with Caspase 7 Mutants

(A) A mimetic version of a preferred substrate of caspase 7, or a preferred N-terminal product of caspase 7, was separately incubated with the three caspase 7 mutants. The caspase 7 constructs were then captured with anti-Myc beads targeting their N-terminal Myc tag and (B-*top*) visualized by SDS-PAGE for the presence of mimetic substrate or mimetic product and (B-*bottom)* summarized for their status as a product trap or a substrate trap. Data are representative of three separate experiments.

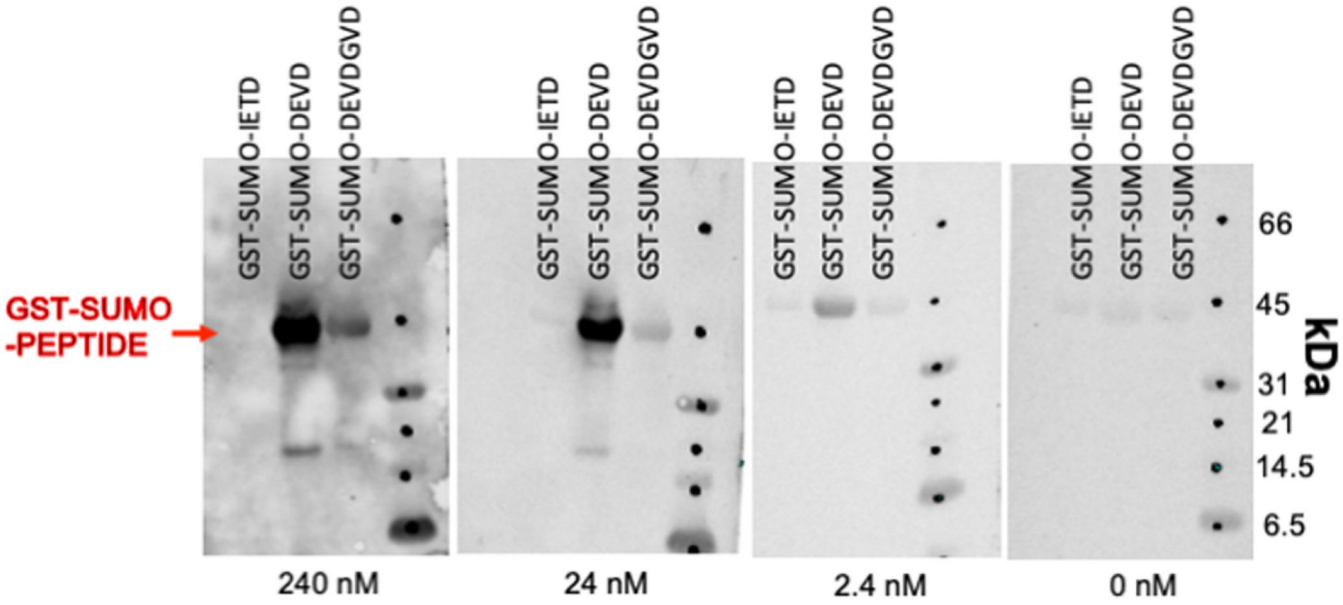
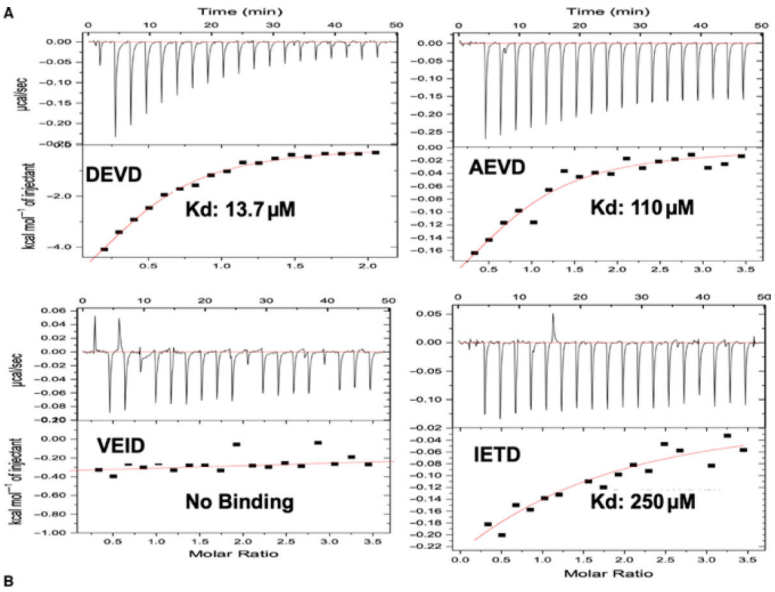


Figure 5. Blotting with C7RAK.

The indicated GST-SUMO conjugated peptides were run in SDS- PAGE and transferred to a nitrocellulose membrane and detected by indicated concentrations of C7RAK. Data are representative of three separate experiments.

A



B

Peptide:	DEVD	AEVD	VEID	IETD
Km (μM) Caspase 7	49.6 ± 2.7	143 ± 8	285 ± 23.2	401 ± 29
Kd (μM) C7RAK	14 ± 3	110 ± 50	No Binding	250 ± 10

Figure 6. Binding Affinities of C7RAK to Caspase 7 Products

(A) ITC of synthetic peptides interacting with C7RAK. (B) Comparison of C7RAK Kd values compared to caspase 7 Km values of equivalent substrate peptides towards products from ITC and active caspase 7 Km values of substrates from kinetic experiments. Data are means of duplicate separate experiments ± SEM.

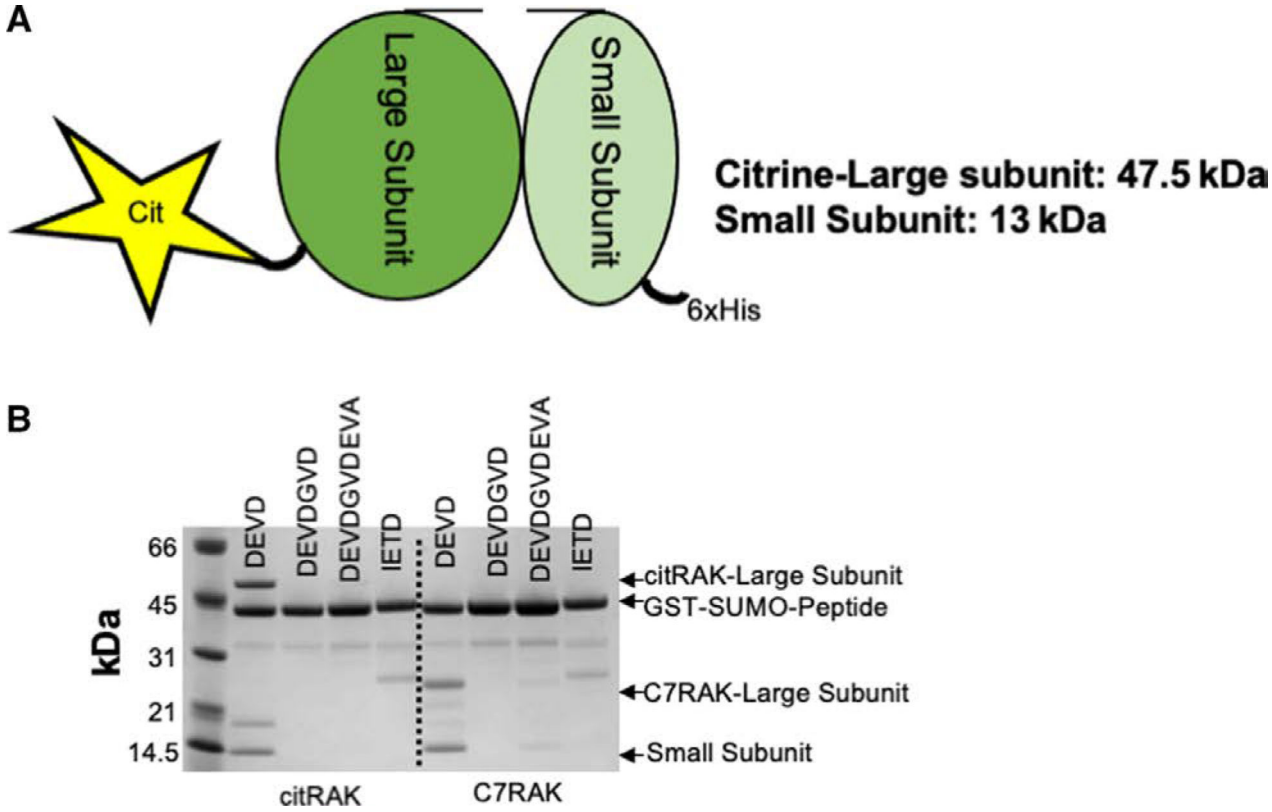


Figure 7. citRAK Characterization

(A) Schematic representing citRAK (B) The ability of citRAK to bind product was validated by interaction with substrate- and product-based SUMO hybrid mimetics. This was accomplished by incubating either citRAK or C7RAK with either GST-SUMO-DEVD (product), GST-SUMO- DEVDGVD (substrate), GST-SUMO-DEVDGVDEVA (substrate), GST-SUMO-IETD (negative control product) and GSH beads. Pull-downs were visualized by SDS-PAGE (representive gel of two separate experiments).

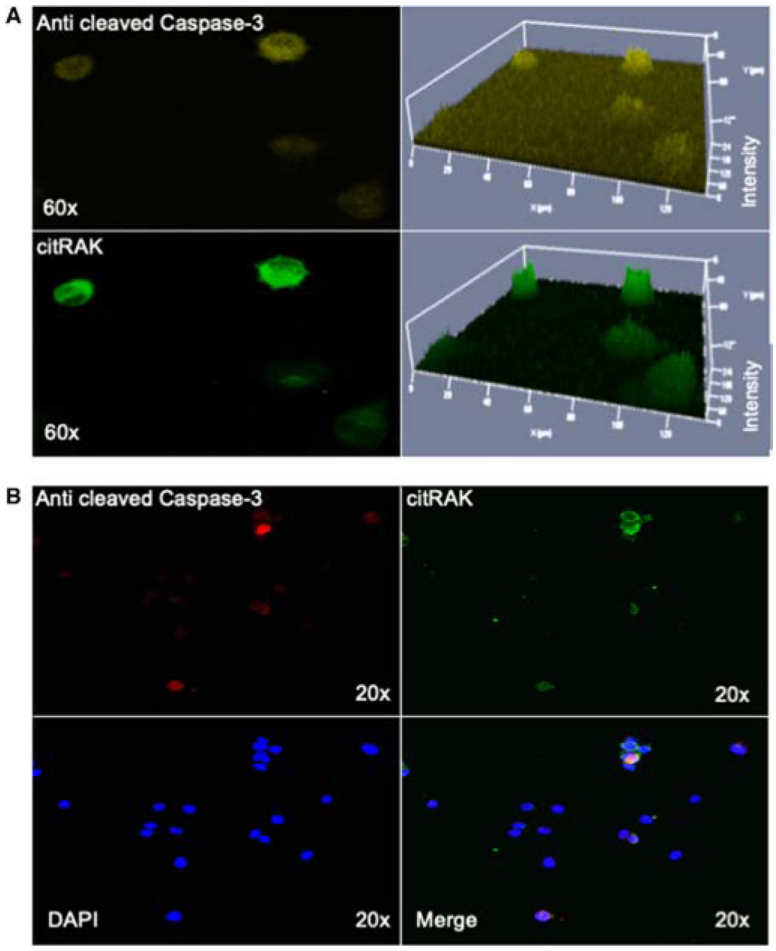


Figure 8. Imaging with citRAK

(A) MDA-MB-231 cells were plated and treated with TRAIL to induce apoptosis. Following incubation, cells were permeabilized and stained with anti-cleaved caspase 3 and citRAK and imaged on a Zeiss microscope. Left panels are images at 60x magnification and right panels show fluorescence intensity of each cell. Data are representative of three separate experiments. (B) Identification of apoptotic cells in a background of proliferating cells. MDA-MB-231 cells were cultured and stained with anti-cleaved caspase 3, citRAK and DAPI, and imaged at 20x magnification. Data are representative of three separate experiments.