THESIS

CHARACTERIZATION OF HIV-1 PROTEASE AUTOPROCESSING TRANS-CLEAVAGE MECHANISM

Submitted by

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ABSTRACT

CHARACTERIZATION OF HIV-1 PROTEASE AUTOPROCESSING TRANS-CLEAVAGE MECHANISM

HIV protease is an aspartic acid enzyme responsible for the cleavage reactions essential in the maturation (infectivity) of the viral particle. Protease inhibitors (non-cleavable substrate analogs) have been potent tools in combating HIV infection as well as its result – AIDS. However, the emergence of drug-resistant viruses in patients treated with these inhibitors is an ongoing concern. Thus there is a growing need to find additional therapeutic targets and treatments to supplement the currently available protease inhibitors. A promising new target for drug development is protease autoprocessing which is a virus-specific process responsible for the release of the mature protease from its precursor (Gag-Pol). Unfortunately, structural and mechanistic information pertaining to autoprocessing are yet insufficient. According to the mature protease structure, it is speculated that precursor dimerization is essential for autoprocessing to occur. We have developed a model system to specifically examine the trans-cleavage mechanism mediated by engineered fusion precursors (differentially labeled substrate and enzyme, respectively). Using this system, we demonstrate that *trans*-cleavage happens between fusion precursors both in the presence and absence of a dimer inducing fusion tag (DIFT). *Tran*s-cleavage was also observed when monomeric fusion tags were attached to the fusion precursor. These results hint that autoprocessing mediated by the fusion precursor is independent of dimer-inducing tag in our model system.

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BACKGROUND

Human Immunodeficiency Virus-1 (HIV-1) is the causative agent of Acquired Immune Deficiency Syndrome (AIDS). First discovered in 1983 [1], the virus targets the immune system and leaves the infected vulnerable to otherwise defensible diseases. In order to replicate, the virus inserts its genetic material into the host genome. Then, using the host's own gene expression machinery, synthesizes proteins and genomic RNA for the next generation of viral particles. Treatment of HIV-1 infection must distinguish between reactions which are shared by normal cellular processes and reactions which are unique to viral synthesis. One of these is the viral protease that catalyzes a group of proteolytic reactions.

There are three viral enzymes essential to the survival of HIV. These enzymes are reverse transcriptase, integrase and protease. Each of these enzymes is an excellent target for ant-retroviral therapy. Four classes of drugs are available for use in treatment today. These are fusion inhibitors which block viral entry into its target cells, nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI), and protease inhibitors. Integrase inhibitors are currently in clinical testing. The HIV-1 protease is an aspartic acid protease and the mature HIV-1 protease activity essential for HIV-1 infectivity [2–4]. This has been shown in D25N null mutants of the protease in which viral particles with these mutant proteases are no longer able to mature and thus unable to infect its target cells. The HIV-1 protease is initially synthesized along with capsid proteins, reverse transcriptase, and other viral proteins as part of the Gag-Pol polyprotein [3, 5, 6] (Fig. 1a). Generation of a mature HIV-1 protease relies on a crucial process called *protease autoprocessing* in which the protease domain embedded within the precursor catalyzes the proteolytic cleavage reactions necessary for its release [3, 4, 7]. The released

mature protease exists as homodimers that are essential for at least 10 downstream coordinated cleavage reactions within the Gag and Gag-Pol polyproteins [3]. Due to its central role in the generation of an infectious virion, the structure and activity of the mature protease have been extensively characterized [3, 8–11] and drugs designed to target the active site of the mature protease have been developed. This has led to ten FDA-approved protease inhibitors (PIs), of which nine are currently being used to treat HIV-1 positive patient [12].

However, these PIs fail to be effective in some patients who unfortunately carry or develop drug-resistant HIV-1 strains. First, the HIV-1 genome in much more mutation prone and thus evolves at a rapid rate due to the proofreading capacity of reverse transcriptase – with which 1mutation per 2000 base-pairs occur [13, 14]. Second, the HIV-1 protease is especially plastic because it is adapted to identify with at least ten different cleavage sites which share very little to no sequence homology [12, 15–18]. It is speculated that this low standard of sequence conservancy allows protease to more easily mutate the catalytic sites while retaining enough catalytic activity to survive. Together, these qualities manifest as an ability for proteases (encoded by the HIV-1 genome) to quickly develop drug resistant against PIs just after several generation cycles.

In order to combat this, current treatment uses a multi-target drug treatment in which different drugs with different targets are used together in a cocktail to minimize the chance of a viral strain with a drug resistance proliferating [19, 20]. A weakness of this method is that currently, each target has only one type of drug to overcome. Therefore, the individual genetic barrier for each target is low. In contrast, a stronger method which can also be used to augment the multi-target method is the multi-step, single target method where multiple processes of a single target are inhibited. This requires the availability of various types of drug per target;

representing a growing need for more potent, new drugs to supplement the shortcomings of current therapeutics. In the case of the HIV-1 protease, a promising target for future drug development is the autoprocessing step responsible for the release of the mature protease.

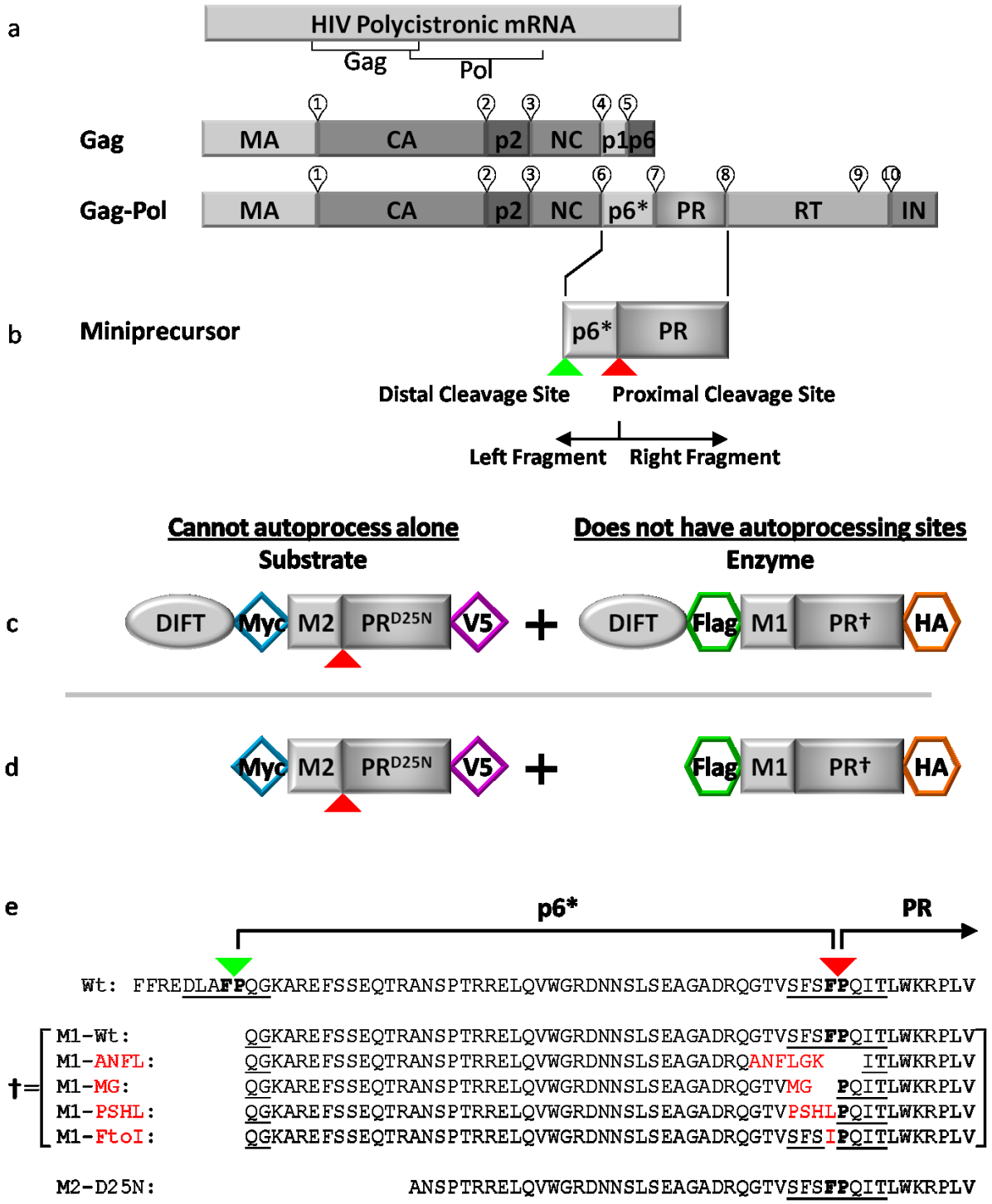

Figure 1. HIV-1 protease and fusion precursors.
a) Diagram of Gag and Pol region within the HIV-1 polycistronic RNA and its polyprotein products. The protease is contained in the Pol region. Gag expression changes to Gag-Pol when the reading frame shifts by -1 at 5-10% frequency in the p1 region. Various cleavage sites observed in wild-type HIV-1 are numbered 1-10. MA=Membrane anchoring protein. CA=Core capsid protein. NC=Nucleocapsid protein. PR=Protease. RT=Reverse transcriptase and RNase H. IN=Integrase. [3, 5, 21]

b) The p6*-PR defined as the miniprecursor. p6* contains two cleavage sites – distal (green arrow) and proximal sites (red arrow). In our study, the proximal cleavage product containing p6* portions is referred to as the left fragment while the proximal cleavage product containing PR is referred to as the right fragment.
c-d) Diagram of fusion precursors used for *trans*-cleavage analysis. Left) D25N mutants lacking proteolytic activity used as the fusion substrates. Right) Enzymatic constructs using various mutations to eliminate the proximal cleavage site used as fusion enzymes. DIFT: dimer-inducing fusion tag.
e) Sequences of wild-type derived fusion precursor and mutants used as the fusion enzyme. The wild-type p6* sequence maintains both distal and proximal cleavage sites while M1 and M2 truncations only contain the proximal cleavage site. Cleavage occurs in the underlined region between the phenylalanine (F) and proline (P) residues.

However, there are several challenges to using autoprocessing as a target. The first of which is that unlike its mature form, the structural details during or the exact mechanism of autoprocessing is largely a mystery [7, 12, 22]. What has been established so far is that the protease precursor molecule functions as both the enzyme and substrate in the autoprocessing reactions [22]. The current model for precursor autoprocessing posits that the precursor needs to dimerize first in order to form an active catalytic site and subsequent proteolytic activity. However, this model borrows heavily from structural data gleaned from the mature protease [23–25] while detailed structural information for the precursor is not available. Thus, whether this is in fact the case is yet to be determined. In this dimerization-requiring model, it is also unknown whether the recognition of the cleavage sites by the catalytic site occurs *in cis* or *in trans* [22]. The *cis*-cleavage model describes the dimer somehow folding back onto its own cleavage site in order to autoprocess. On the other hand, the *trans*-cleavage model describes a formed dimer recognizing cleavage sites on a third, separate precursor molecule. Unfortunately, neither model clarifies how many molecules are involved in order for the reaction to occur.

Another challenge is that a model system with which autoprocessing can be studied has not been previously established. One of the few tools that has been established as a tool for

studying autoprocessing is the *miniprecursor* – which is a simplified system eliminating all but the minimum required fragment of the protease precursor. This peptide sequence, consisting of only the p6*-PR region of the Gag-Pol polyprotein, has been shown to be the smallest essential portion to mediate autoprocessing reactions (Fig. 1b) [3, 23, 26]. Using this system, this thesis focuses on addressing two key questions. 1) Is the miniprecursor able to undergo *trans*-cleavage? 2) Are dimer-inducing sequences upstream of the miniprecursor required for *trans*-cleavage?

In order to distinguish whether the protease precursor is able to undergo *trans*-cleavage, we developed a cell-based assay built upon the miniprecursor system by separating the enzymatic and substrate function between two near-identical *fusion precursors*. These fusion precursors contain either mutations in its cleavage sequences or a null mutation of its catalytic site (Fig. 1c-d). Therefore, with each molecule no longer able to act as both substrate and enzyme, any detected cleavage must have occurred *in trans*.

MATERIALS AND METHODS

Mutagenesis

All fusion precursor constructs used in this study were generated using site-directed mutagenesis as previously described [3, 27–30, 32]. All the constructs were verified by sequencing analysis (Fig. 1e) (Tbl.1).

Table 1. Fusion precursors of this study

mutants	construct	role
DIFT fused		
GST-wt	GST-Flag-M1-PR^{wt}-HA	enzyme
GST-ANFL	GST-Flag-M1-PR^{ANFL}-HA	enzyme
GST-MG	GST-Flag-M1-PR^{MG}-HA	enzyme
GST-PSHL	GST-Flag-M1-PR^{wt}-HA	enzyme
GST-D25N	GST-Myc-M2-PR^{D25N}-V5	substrate
DIFT-lacking		
wt	Flag-M1-PR^{wt}-HA	
ANFL	Flag-M1-PR^{ANFL}-HA	enzyme
MG	Flag-M1-PR^{MG}-HA	enzyme
PSHL	Flag-M1-PR^{wt}-HA	enzyme
FtoI	Flag-M1-PR^{ANFL}-HA	enzyme
D25N	Myc-M2-PR^{D25N}-V5	substrate
Monomeric fusion tagged		
L-MBP	L-MBP-Flag-M4-PR^{wt}-HA	enzyme
C2-MBP	C2-MBP-Flag-M1-PR^{wt}-HA	enzyme

Cell culture, transfection, western blot, and quantification

HEK293T cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium; Invitrogen, Carlsbad, CA) culture media containing penicillin and streptomycin and 10% fetal

bovine serum as previously described [29]. Fusion precursor coding plasmids were transiently transfected using previously described method [27]. In brief, cells were seeded onto 6-well, 12-well or 24-well plates and incubated overnight (approximately 18 hours) the day before transfection. Cell confluency at time of transfection is ideally 50-60%. Shortly before transfection, 1000x chloroquine was added to each well for a final concentration of 25 μM. The total amount of transfected DNA per well was 1 μg (6-well plate), 0.5 μg (12-well plate), or 0.25 μg (24 well plate). Plasmids pcDNA and peGFP (mixed at 19:1) were used to normalize DNA amount to 0.5 per well for a 12-well plate. A DNA ratio of enzyme to substrate was consistently maintained at 1:1 unless otherwise stated. Several experiments used doubled amount of DNA to examine effect of input DNA amount on expression levels. For a 12-well plate, autoclaved H_2O was added to bring the DNA+H_2O volume to 65.7 μL. To this, 9.3 μL 2M $CaCl_2$ and 75 μL 2x HBS (50 mM HEPES, 280 mM NaCl, 10 mM KCl, 12 mM Dextrose, 1.5 mM Na_2HPO_4, pH 7.05) was added drop-wise for a total volume of 150 μL (all volumes or masses were doubled or halved for 6-well and 24-well plates, respectively). The mixture was then applied to plated cells and incubated for 7-11 hours before the media was changed. For experiments involving drug treatment, darunavir was added at this point to the desired concentration. Darunavir (Cat# 8145) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. At 24-30 hours post transfection, cells were washed with PBS and lysed with 80 μL Lysis Buffer/protease inhibitor solution (Lysis Buffer A: 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100 plus 1x protease inhibitor cocktail). Cell debris was removed via pipette tip and lysate was transferred to microcentrifuge tube containing 15 μL 6x SDS Loading Buffer (60% glycerol, 0.6 M DTT powder, 6% SDS, 0.006% Bromophenol blue, 0.35 M Tris-HCl, water). Prior to loading into PAGE, Samples were boiled

for 3-5 minutes and loaded into 13.5% SDS polyacrylamide gel (4% stacking, 13.5% resolving). Blank wells were filled 1x SDS Loading Buffer made with lysis buffer A. Gels were run at 15-22.5 mA per gel for one hour until voltage rose past 200 V, at which point, the gel was run at constant voltage until the 11 kDa marker resolved from the dye front. Samples were transferred onto membrane (Millipore 0.45µ Immobilon P/PVDF) for one hour at 100 V in 1x Transfer Buffer (0.192 M Glycine, 25 mM Tris Base, 20 % HPLC Grade Methanol, and dH_2O) and blocked for 15 minutes (0.25% fetal goat serum, 0.025% fish gelatin, in PBST (2.7 M NaCl, 0.054 M KCl, 0.030 M KH_2PO_4, 0.17 MNa_2HPO_4, 0.8% Tween 20). Primary antibodies used for detection were mouse monoclonal anti-HA (1:20,000), anti-Flag (1:1000), and V5 (1:4000); rabbit monoclonal anti-Myc (1:100). A final concentration of 1:1000 sodium azide was added to allow reuse. Fluorescent secondary antibodies were IR800 goat anti-mouse (Li-COR cat#926-32210, 1:50,000), IR700 goat anti-mouse (Li-COR cat#926-68020, 1:50,000) and IR800 goat anti-rabbit (Li-COR cat#926-32211, 1:50,000). Both primary and secondary antibodies were diluted in blocking solution. Secondary antibodies were a gift from Dr. Carol A Carter. An Odyssey infrared dual laser scanning unit (LI- COR Biotechnology, Lincoln, Nebraska) running imageStudio® was used to visualize western blot images.

Quantification and data analysis

The quantification program offered in imageStudio® determines total intensity for a given area minus background noises. The designation of signal bands are input manually and the area of the bands are determined either automatically or manually. For our calculations, the manual selection method was used. The percent intensity measures of each band were determined relative to the sum total of intensity measures (arbitrary unit) for each sample lane.

Quantified intensity levels of full-length substrate and right fragment were added together. The full-length substrate intensity was divided by this value to determine the raw percent substrate. This number was further normalized to set the highest intensity of raw % full-length substrate seen per panel as 100%. These values were plotted onto graphs with logarithmic horizontal axis of darunavir concentrations (%substrate) and used to determine IC_{50}.

IC_{50} values for individual data sets were derived using data points within the linear range. A logarithmic line of best fit was generated and the IC_{50} value extrapolated by taking the highest and lowest plateau values. The "Cumulative" IC_{50} was determined by plotting the average of all data points generated from our experiments and determining the linear range as well as outlier points. Once again, a logarithmic line of best fit was generated and the IC_{50} value extrapolated by taking the highest and lowest plateau values.

RESULTS AND DISCUSSION

Our lab has previously generated many fusion precursors which contain N-terminal glutathione transferase (GST) fusions [27–31]. Because these constructs where the best characterized, we began testing our model system using these enzymes and substrate. From our experiments, we have gathered that *trans*-cleavage in indeed possible. Furthermore, the reaction was also maintained not only in the presence of dimer-inducing fusion tag (DIFT) but also in the absence of DIFT and in the addition of monomeric tags suggesting independence of autoprocessing from dimerization. Based on results from our lab and others, we propose that the precursor autoprocessing structure is different from the mature protease. Our goal ultimately is to determine the mechanistic details of protease autoprocessing.

Rationale of fusion precursors used in the study

It is our ongoing interest to study the autoprocessing mechanism using our cell-based assay that utilizes fusion precursors expressing the miniprecursor sandwiched between various tags. In our prototype fusion precursor – GST-Flag-p6*-PR-HA, there are two cleavage sites within the p6* region – the proximal and distal site – named in relation to the adjacent PR region [27]. We engineered two classes of fusion precursors in order to specifically examine *trans*-cleavage proteolysis. These fusion precursors separate the enzymatic and substrate role of the precursor into two individual fusion proteins.

The fusion precursor in the first class contains a substitution of the catalytic aspartic acid residue into asparagine (D25N) which has been previously shown to abolish both mature protease and autoprocessing activity [3, 4, 33]. These precursors have the wild-type proximal site

with the distal cleavage site deleted (Fig. 1e, M1 or M2), and are therefore incapable of *cis*-cleavage yet can be processed by other precursors *in trans* (referred to herein as the s*ubstrates*) (Fig. 1c-d left). Additionally, they are tagged with a Myc peptide at the N-terminus and a V5 peptide at the C-terminus of the substrate sequence, which allows specific detection of the substrate and processed products by western blot analysis. These products are referred to as the *left fragment* which contains the p6* region or the *right fragment* which contains the PR region.

Fusion precursors of the second class carry various mutations at the proximal cleavage site carrying the wild-type catalytic site (referred to herein as the *enzymes*) (Fig. 1c-d right). These mutations include various deletions and substitutions upstream of the N-terminus of the PR region which abolishes proteolysis at the proximal site (Fig. 1e). Proper cleavage at this site has been shown to be essential for dimerization in N-terminal extended mature protease [3, 7, 24, 34]. These fusion precursors are also tagged for western blot detection with a Flag peptide at the N-terminus and an HA peptide at the C-terminus. Due to the lack of a cleavage site within the fusion enzyme, separation of the HA and Flag tags are not expected.

The ANFL, MG, and PSHL enzymes are derived from the pseudo wild-type protease and the others are from the NL4-3 sequence. There are six point mutations when comparing the pseudo wild-type and NL4-3 derived protease sequence [27]. The pseudo wild-type PR displays enzymatic activities similar to the wild-type PR and thus has been widely used for structural analysis of mature PR [23, 35, 36]. The ANFL sequence is designed according to a previous publication in which improved proteolysis of the Gag-Pol precursor was observed when the p6* coding sequence was deleted leading to fusion of NC sequence to the PR sequence [37]. In our ANFL construct, the wild-type GTVSFSFP peptide is replaced with ANFLGK sequence leading to mutation and elimination of the proximal site and truncation of the first two amino acids of the

mature protease. The MG enzyme was designed based on another report showing that an extension of MG to the N-terminus of a mature protease sequence abolishes mature protease activity when expressed in *E. coli* [31]. The PSHL sequence was reported by the Wagner group [7]. This mutation introduces four amino acid substitutions to the last four residues of p6* (upstream of the proximal site) while keeping the *gag* reading frame unchanged as the overlapping region of $p6^{gag}$ residues which are involved in virion release from the infected cell [38–40]. A peptide substrate consisting of VPSHL peptide plus the first five amino acids of the mature protease is completely resistant to *in vitro* cleavage with recombinant HIV-1 PR. The FtoI mutant has a single amino acid substitution in the context of NL4-3 derived p6*-PR sequence. Previous studies have demonstrated that a β-branched amino acid at the P1 position of a substrate prevents PR cleavage [41, 42]. Collectively, all these mutations are expected to abolish *cis*-cleavage of the proximal site cleavage and subsequently enabled us to specifically examine their proteolytic activity *in trans*.

Via these mutations, all fusion precursors were shown to have lost the ability to be both the enzyme and substrate simultaneously in the autoprocessing reaction. Therefore, all autoprocessing reaction will require intermolecular interaction between the substrate and enzyme fusion precursors. It is important to note that this trans-cleavage reaction must compete with a self-degradation activity of the protease [43, 44]. This particular property seen with enzymatically active protease restricts our model to be performed *in vivo* as enzymatic fusion precursors cannot be purified. In *vitro* transcription coupled translation also cannot produce a sufficient yield [22, 45].

Optimization of input ratios of constructs used in the study

We first tested *trans*-cleavage processing using GST-fused enzyme and substrate (Fig. 2a). GST is known to form homodimers with low nanomolar dissociation constant [46, 47] and thus is a DIFT. We speculated that GST would facilitate formation of enzyme/substrate dimers as well as enzyme/enzyme and substrate/substrate dimers. Based on the conventional hypothesis that precursor dimerization is essential for protease activity, cleavage products of the substrate should only be detected if dimerization is successful. We first determined the minimum amount of GST-fusion substrate coding plasmid required for detection by transfection of the above mentioned plasmid alone at increasing amounts from 0.05 µg up to 0.5 µg at 0.05 µg increment (Fig. 2b). We found that expression of the substrate could be detected even at 0.05 µg plasmid DNA input in the absence of any processing enzyme. Next, we tested whether a GST-fusion enzyme could process the GST-fusion substrate *in trans* and examined its cleavage efficiency. Therefore, cotransfection of plasmids coding for GST- fusion enzymes and GST-fusion substrate was carried out. Initially, we tested three enzymes (ANFL, MG, and PSHL) at six ratios of plasmid DNA by weight: 20%, 40%, or 80% GST-enzyme to 20% GST-substrate and 10%, 20%, or 40% GST-enzyme to 60% GST-substrate. An empty vector, pcDNA was used to maintain total DNA at 0.5 µg (Fig. 2c). Both the left and right fragments of the GST-fusion substrate were detected in all the tested cell lysates by western blotting using primary antibodies against Myc and V5 which was imaged using fluorescent secondary antibodies. Our data clearly demonstrate that the GST-fused enzyme is capable of processing the GST-fused substrate *in trans*. Assuming that GST is the sole determinant of precursor dimerization, this data could not distinguish whether the *trans*-cleavage seen here resulted from dimeric enzyme acting on dimeric substrate (*trans* between dimers) or from dimeric enzyme/substrate complex (*trans* within the dimer)

although enzymatic activity of dimeric enzyme/substrate is unlikely due to the catalytic reside mutation to one of the dimer subunits. Regardless, these results indicated that *trans*-cleavage indeed occurs as detected with our model. Intriguingly enough, none of the *trans*-cleavage reactions proceeded to 100 % cleavage; and the amount of cleaved product did not noticeably change over a 24-fold difference in enzyme:substrate plasmid ratios. This was rather surprising as this suggests that the enzymatic activity of the fusion precursor is restricted, diminished, consumed, or otherwise lost during the reaction – which, by the definition of an enzyme, should not occur. It is highly unlikely that the reaction is inhibited by unknown cellular components of that the expression condition used was insufficient for complete cleavage as complete depletion of full-length wild-type miniprecursor expressed via transfection of its coding plasmid using the same conditions have been previously shown [27]. Furthermore, cleavage of the substrate must originate from the enzymatically active precursor as transfection of substrate coding plasmid alone does not produce cleavage products. We interpret this phenomenon to indicate that the enzyme dimers are either unconventional or not free-diffused enzymes. It is possible the competitive self-degradation of active enzyme may play a role in decreased cleavage of substrate. However, this does not explain the consistency in the low amount of cleaved substrate across the various ratios. Overall, these results demonstrate that DIFT substrate can be expressed in mammalian cells, *trans*-cleavage occurs in the presence of DIFT, and DIFT enzyme may differ from a conventional, free-diffused enzyme. In light of little to no differences seen between various GST-fusion enzymes to substrate, all later experiments were run using a straightforward 1:1 ratio.

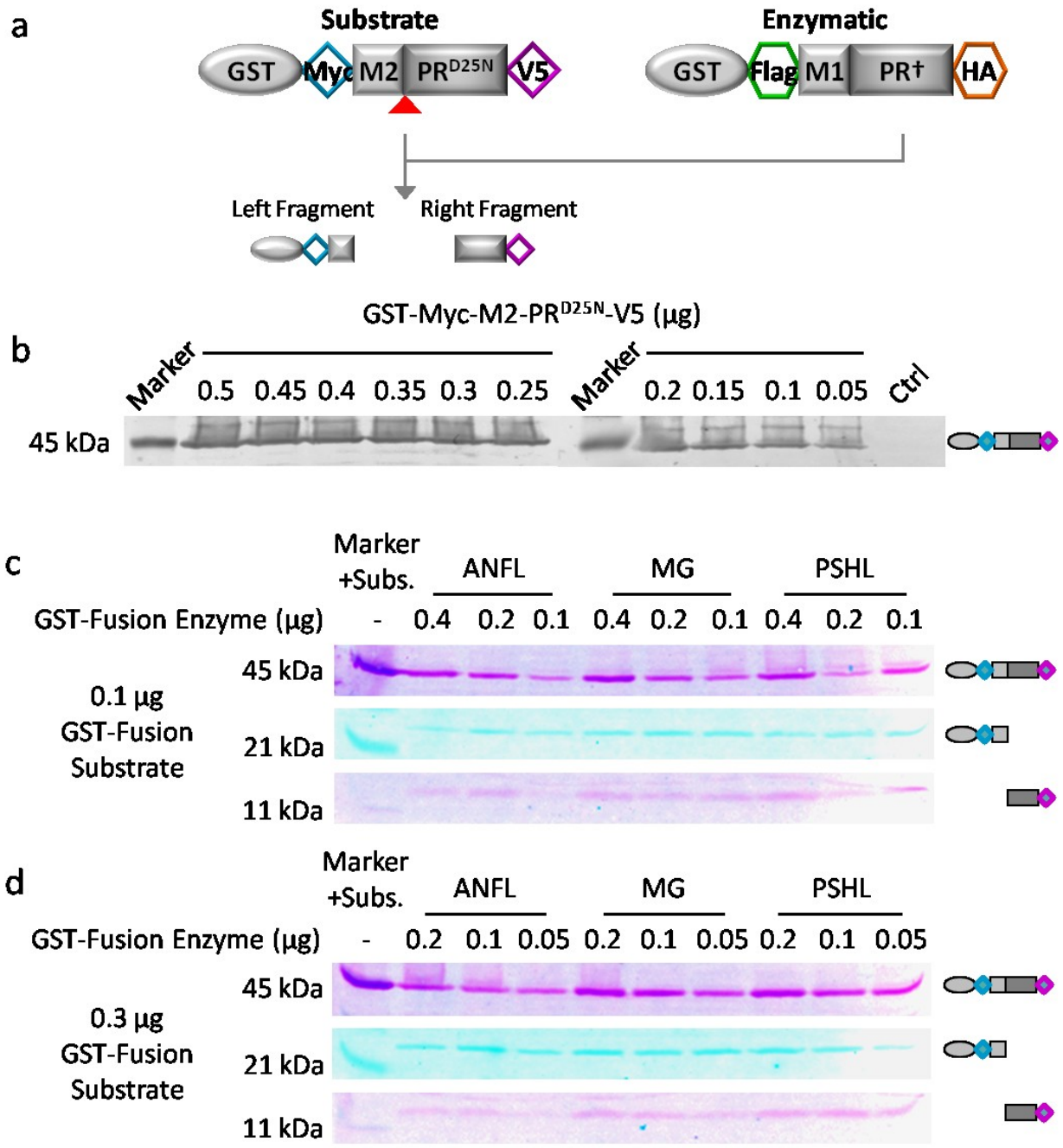

Figure 2. *Trans*-cleavage processing between GST-fused enzyme and substrate at various ratios.

a) Schematics of fusion precursors used in these experiments.

b-d) Western blots visualization of substrate expressed from transfected plasmid detected using mouse anti-Myc and rabbit anti-V5 primary antibodies and visualized using IR700 (red, color inverted to light blue) goat anti-mouse and IR800 (green, color inverted to pink) goat anti-rabbit fluorescent secondary antibodies. DNA amount was maintained constant using pcDNA. Full-length fusion substrate is ~45 kDa, Right fragment ~ 21 kDa, and Left fragment > ~11 kDa.

b) Identification of minimum DNA necessary for detection of GST-fusion substrate. GST-fusion substrate coding plasmid was transfected at various amounts using notated μg of each plasmid per well.
c) Cotransfection of GST-fused enzymes coding plasmids with 0.1 μg GST-fused substrate coding plasmids. Note that size markers and substrate alone were mixed together (M+S).
d) Cotransfection of GST-fused enzymes coding plasmids with 0.3 μg GST-fused substrate coding plasmids.
Samples were collected from HEK 293T cells grown on 12-well plate. Marker+Subs. denotes marker run with sample collected from cells transfected with substrate coding plasmid alone. Plasmid pcDNA was used as control

Trans-cleavage processing between fusion precursors lacking a DIFT

The conventional dimerization hypothesis predicts that precursors which cannot dimerize will fail to catalyze autoprocessing. Therefore, *trans*-cleavage processing is unlikely to be detected between fusion precursors lacking any DIFT. To test this prediction, we engineered a panel of substrate/enzyme precursors that contain no known DIFT (Fig. 3a) (Tbl. 1). Once again, the minimum plasmid DNA for detection was determined and then the plasmids coding for substrate and enzymes lacking DIFT were cotransfected at a 1:1 ratio. Most interestingly, we observed positive *trans*-cleavage processing as indicated by the detection of the right fragment (Fig. 3b); the left fragment was too small (predicted to be ~4kDa) to be detected by the standard Tris-glycine PAGE. All the tested enzymes (ANFL, MG, PSHL, FtoI and the wt) showed ~ 50% processing efficiencies, *i.e.*, approximately equal amounts of processed right fragment and unprocessed substrate detected by the anti-V5 antibody.

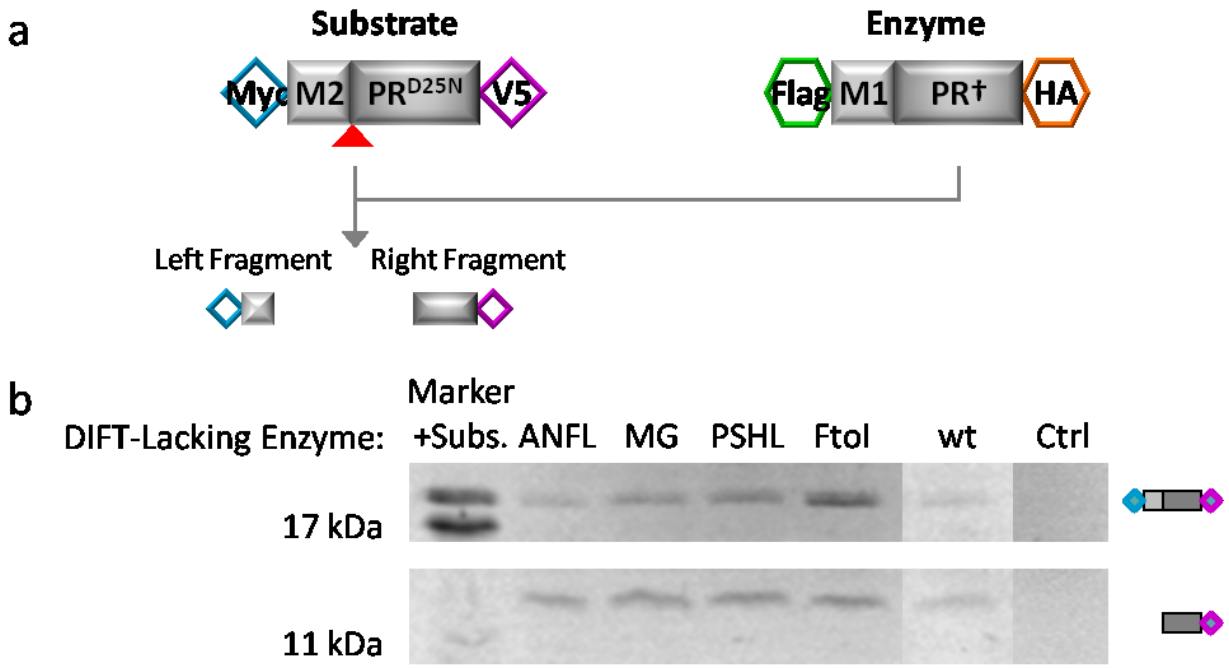

Figure 3. *Trans*-cleavage processing between DIFT-lacking fusion precursors
a) Schematics of fusion precursors used in these experiments.
b) Cotransfection of DIFT-lacking enzymes and substrate coding plasmids. Substrate and its resulting right fragment detected using mouse anti-Myc and rabbit anti-V5 primary antibodies and visualized using IR700 (red, color inverted to light blue) goat anti-mouse and IR800 (green, color inverted to pink) goat anti-rabbit fluorescent secondary antibodies.
Enzymes and substrate coding plasmids were cotransfected using HEK 293T cells grown on a 6-well plate at a 1:1 ratio, using 1 µg of each plasmid per well. Marker+Subs. denotes marker run with sample collected from cells transfected with substrate coding plasmid alone. Plasmid pcDNA was used as control

Effects of darunavir on trans-cleavage processing

In order to determine whether fusion enzymes functioned similar to or unique to mature protease, the activity profiles for each enzyme mutant was compared via a drug gradient panel using protease inhibitor darunavir (DRV) (Fig. 4a). Darunavir was approved in 2006 and is the 10th protease inhibitor approved by the FDA [48, 49]. The drug is a non-cleavable substrate analog and sterically inhibits cleavage by tightly binding to amino acid residues surrounding the protease active site (K_d = 4.5 x 10^{-12} M) [50]. *In vivo* test have shown half maximal inhibitory concentration (IC_{50}) of darunavir against HIV-1 protease is as little as 3 nM. 50% cytotoxicity

concentration has been reported at around 74 μM [48]. The maximum intravenous concentrations attainable by darunavir using the approved dosage are 5 μM which can be doubled to approximately 10 μM when taken together with other drugs to increase absorption [50]. Importantly, detection of mature protease is normally only possible in the presence of darunavir due to the rapid self-degradation of the protease [43, 44].

Plasmids coding for DIFT-lacking enzymes and substrate were transfected in the same way as previous. At time of media change, darunavir was added to reach a six-point gradient ranging from 4.5 μM to 50 nM (Fig. 4b left). We observed an obvious increase in the amount of the full-length substrate with increasing drug concentration for each of the tested enzyme/substrate pairings, suggesting that the *trans*-cleavage processing could be suppressed by darunavir.

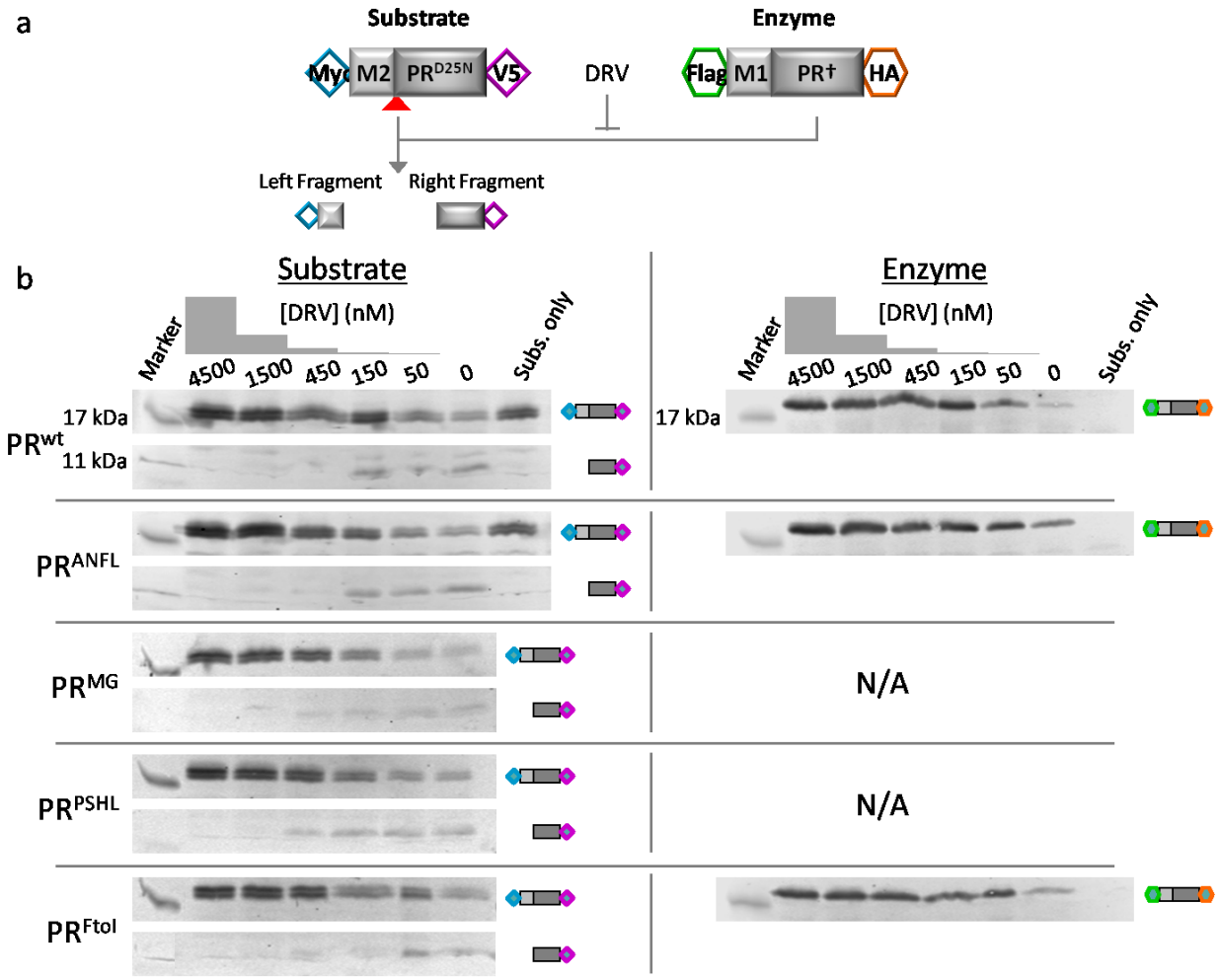

Figure 4. *Trans*-cleavage processing in the presence of darunavir (DRV)
a) Schematics of fusion precursors used in these experiments.
b) Western blotting of cells cotransfected with plasmids coding for DIFT-lacking substrate and enzymes using HEK 293T cells grown on a 12-well plate at a 1:1 ratio, using 0.5 µg of each plasmid per well. Darunavir concentrations are shown at top with scale bars. Substrate detected using mouse anti-Myc and rabbit anti-V5 primary antibodies and visualized using IR700 (red, color inverted to light blue) goat anti-mouse and IR800 (green, color inverted to pink) goat anti-rabbit fluorescent secondary antibodies. Enzyme detected using mouse anti-HA primary antibody and visualized using IR800 (green, color inverted to pink) goat anti-mouse fluorescent secondary antibody. Full-length fusion substrate or enzyme is >17 kDa, Right fragment > 11 kDa. Left half of panel b) Levels of expressed substrate in cell lysate. Right half of panel b) Levels of expressed enzyme in cell lysate.

We determined IC_{50} values as described above. Because all reactions begin at around 50% cleavage, our definition of IC_{50} is the half-way point between the highest and lowest

percentage of full-length substrate detected by western blot (Fig. 5). These values showed that although *trans*- cleavage was inhibited by darunavir, its IC_{50} is 30 to 60 times greater than the amount necessary for mature protease (Tbl. 2). This argues that the method of inhibition by darunavir against fusion precursor *trans*-cleavage and thus the characteristics of the catalytic site are different from the mature protease. Unfortunately, the full validity of this analysis is diminished due to the lack of proper normalization. We also compared the levels of enzyme expression of wild-type, ANLF, FtoI, obtained from a replicate darunavir gradient experiment (Fig. 4b right). The decrease in enzyme level coinciding with decrease in darunavir concentration shows the enzyme fusion precursor underwent self-degradation in the absence of darunavir and increasing darunavir concentrations suppressed such self-degradation.

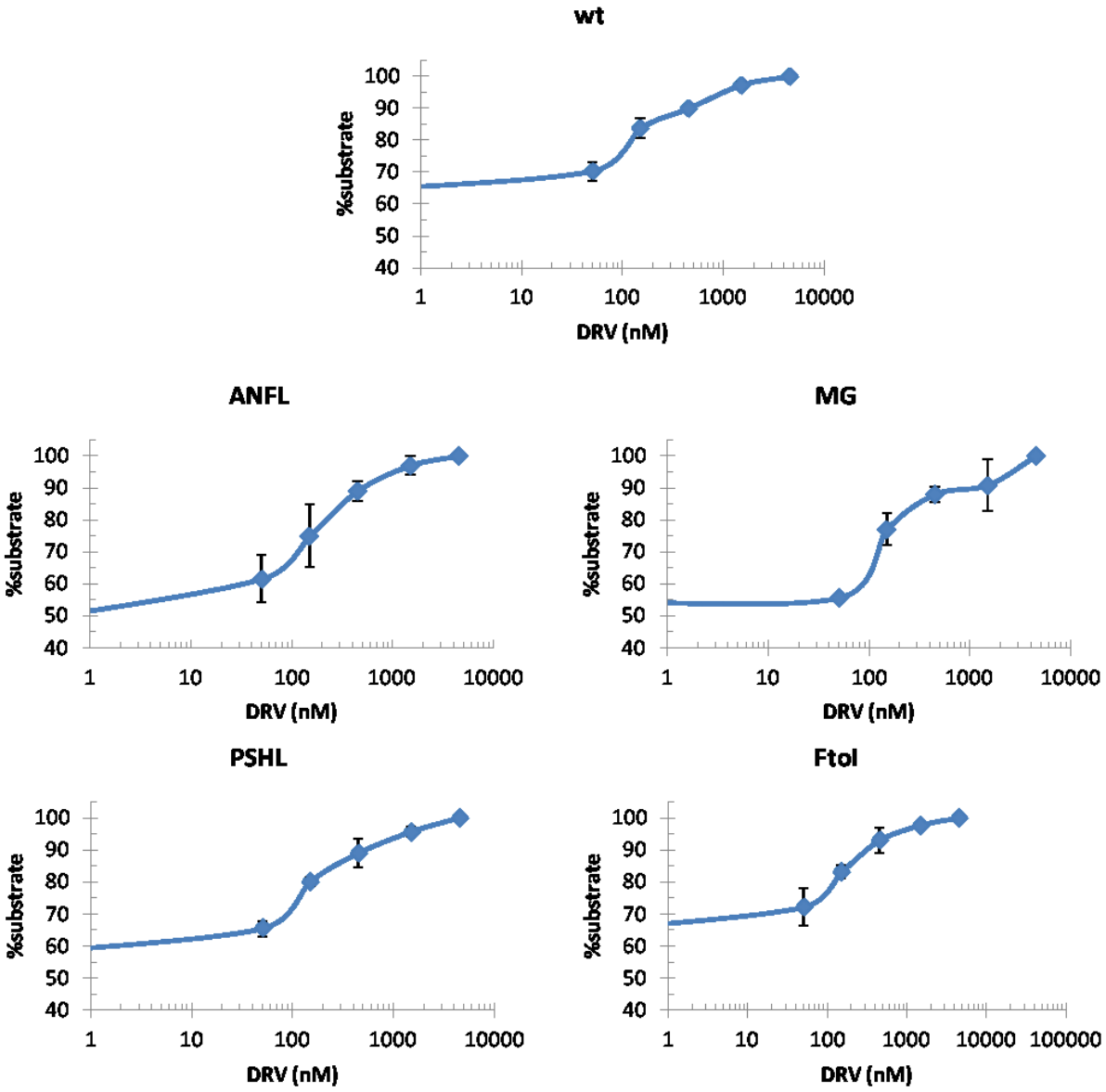

Figure 5. Quantification of *trans*-cleavage processing of different enzymes in the presence of darunavir (DRV)

The %substrate was calculated as percent of full-length substrate over full-length + cleavage product (right fragment). Data points were normalized to set highest level of full-length substrate as 100% within each experiment. Data sets A and B follow darunavir gradient of 50 nM, 150 nM, 450 nM, 1500 nM, and 4500 nM. Data set C follows darunavir gradient of 5 nM, 40 nM, 320 nM, 2500 nM, and 20000 nM. Graphs show average data and error between two experiments. Vertical axis begins at 40%.

Table 2. Darunavir IC_{50} values of mutant enzyme activity.

	IC_{50} Values (nM)				
mutants	wt	ANFL	MG	PSHL	FtoI
Exp. A	134.4	203.3	176.8	133.5	114.9
Exp. B	175.9	262.8	207.7	128.1	175.9
Cumulative	156.2	234.3	192.7	152.9	144.1

Trans-cleavage processing between substrate and enzymes with monomeric fusion tags

Having detected the occurrence of *trans*-cleavage in both DIFT and DIFT-lacking precursor pairings, our final experiment utilized plasmids coding for fusion enzymes carrying Maltose Binding Protein (MBP) cotransfected with plasmids coding for substrate lacking DIFT (Fig. 7a). MBP is a large protein known to be monomeric and acts as a solubilizing agent when fused to other proteins [51–53]. Unfortunately, experimental verification of MBP inhibition of precursor dimerization does not exist at the time. The variation of MBP used was C2-MBP which is has a N-terminal truncation of the MBP signal peptide. The transfected cells were treated with darunavir at concentrations of 1500 nM, 300 nM, and 50 nM. A GST fused wild-type precursor was used as a reference for comparison. Here, we once again saw clear drug concentration dependent cleavage response producing left and right fragments (Fig. 7b-c). This is significant because these constructs are theoretically unlikely to dimerize due to the absence of any DIFT domains and addition of the monomeric fusion tag, which will likely pose steric hindrance to dimerization. Therefore, cleavage of the substrate by this mutant is unlikely to follow the conventional dimerization-requiring model of autoprocessing. However, here we see that not only is *trans*-cleavage detected, its activity and drug response is comparable to a DIFT wild-type protease precursor despite high chance of a very different propensity for dimerization.

This suggests once again that autoprocessing does not depend on the formation of a dimer in order for successful cleavage.

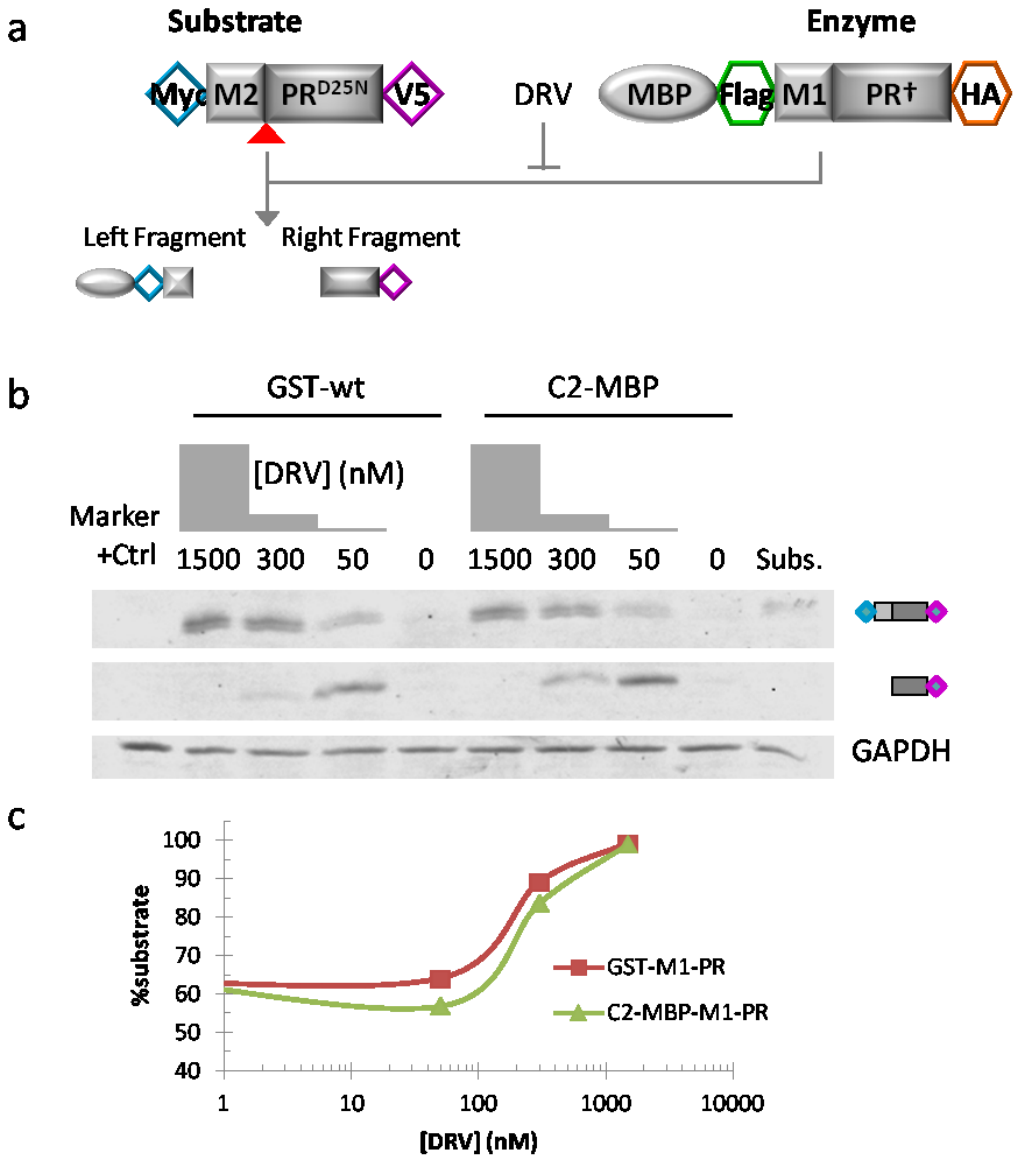

Figure 6. *Trans*-cleavage processing between monomeric fusion tagged enzymes and DIFT-lacking substrate in the presence of darunavir (DRV)
a) Schematics of fusion precursors used in these experiments.
b) Western blotting of cells cotransfected with either DIFT-lacking substrate and GST tagged enzyme or DIFT-lacking substrate and C2-MBP tagged enzyme coding plasmids. Substrate detected using rabbit anti-V5 primary antibody and visualized using IR700 (red, color inverted to light blue) goat anti-mouse fluorescent secondary antibody.
c) Quantification of b. Intensity measures normalized to GAPDH.

CONCLUSION AND FUTURE PERSPECTIVES

Fusion precursors are capable of trans-cleavage reactions

Overall, this study of pairing solely enzymatic or substrate constructs has showed that 1) *Trans*-cleavage occurs between DIFT-containing fusion precursors in the absence of *cis*-cleavage. 2) The *trans*-cleavage processing reaction is maintained in the absence of DIFT. And 3) *Trans*-cleavage is maintained even with the addition of a monomeric tag with a dimerization-inhibitive influence to the enzyme. In summary, effective *trans*-cleavage was detected in all the tested settings and does not require dimer-inducing sequences upstream of the miniprecursor. The autoprocessing mechanism of HIV-1 protease has been assumed to most likely be via *trans*-cleavage mediated by dimerization. Our data showed that fusion precursors are indeed capable of undergoing *trans*-cleavage. This was first confirmed in GST-fused precursors with the detection of both distal and proximal cleavage products. However, whether this indicated an intrinsic ability of the precursor was uncertain due to the dimer forming properties of GST. Therefore, DIFT-lacking fusion precursors were used to test whether the GST conjugate result was replicable and in fact it was. Furthermore, the result from using a monomeric fusion tag, which is sterically unlikely to facilitate dimerization, was still capable of generating cleavage product arguing that cleavage – and therefore autoprocessing – do not require dimerization to occur as is commonly believed. We propose the existence of an autoprocessing pathway that is independent of dimerization characteristic of mature protease (Fig. 8). There is a possibility that this pathway involves the formation of high molecular weight complexes. This is based on unpublished results from our lab showing that D25N miniprecursor exists in high-molecular weight complexes (~200 kDa), apparently higher than would be expected if the miniprecursor was dimeric (~35 kDa).

This would potentially reconcile the inability of enzyme to cleave more than 50% of the available substrate. For example, the formation of an active enzyme and subsequent recognition of the substrate is hindered by structural organization and perhaps these complexes can only reorganize several times before self-degradation of the enzyme abolishes further cleavage of the substrate. As of now, it is impossible to say how these structures are organized or function. Also, it cannot be said that *cis*-cleavage within a dimer is not possible as this could not be tested in this study. Furthermore, the definition of *cis* and *trans*-cleavage begins to change if the individual complexes are not dimers.

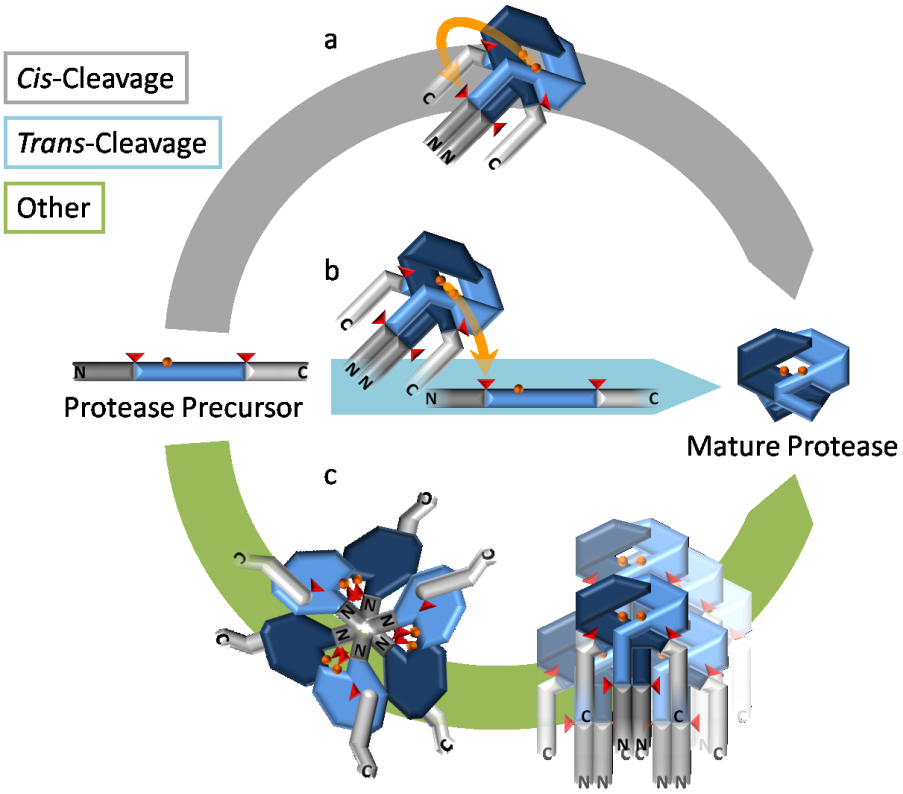

Figure 7. Possible mechanisms of protease autoprocessing
Three possible pathways of protease autoprocessing.
a) *cis*-cleavage mediated autoprocessing. Recognition of the cleavage site is truly intramolecular. However, multiple proteins may participate in achieving catalytic activity. Characteristic dimerization occurs during or after cleavage reaction.
b) *trans*-cleavage mediated by dimerization. Representative of conventional autoprocessing theory where the autoprocessing precursor forms a dimer structure similar or identical to the mature protease in order to achieve catalytic activity.

c) *trans*-cleavage independent of dimerization. Truly speculative representation as is it is yet unknown whether *trans*-cleavage in this model occurs as an ordered multimeric structure, aggregate of dimer-like interactions or other structures.
Red arrow denotes proximal cleavage site. Orange dot denotes catalytic D25 residue. N and C termini do not necessarily terminate as shown in figure except in mature protease.

Our results strongly argue against the dimerization theory for several reasons. In a virus infected cell, the PR region is embedded within the Gag-Pol polyprotein. Although the p6* region has been shown to inhibit dimerization [37, 54], the adjacent proteins regions of MA, CA, NC, RT, and IN form a trimer, dimer, multimer, dimer, and multimer respectively [55, 56]. Therefore, the inhibitory effect of p6* is considered to be overcome by these dimer-inducing domains especially at the late stage of virion assembly and release. However, in our system, which uses the fusion precursors containing only the p6*-PR region, the dimer inhibiting effect of p6* is supposed to be much more pronounced. In light of our results involving monomeric tags versus DIFTs and DIFT-lacking precursors, the likeliness of PR precursor being able to reliably undergo dimerization from monomer is very low. Additional investigations are needed to define precursor autoprocessing mechanism.

REFERENCES

1. AIDS.gov: *30 YEARS OF HIV / AIDS TIMELINE*. 2011(Cdc):1–13.

2. Wiegers K, Rutter G, Kottler H, Tessmer U, Hohenberg H, Kräusslich HG: **Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites.** *J Virol* 1998, **72**:2846–54.

3. Louis JM, Webert IT, Tözsér J, Marius Clore G, Gronenborn AM: **HIV-I protease: Maturation, enzyme specificity, and drug resistance**. In *HIV-1 Mol Biol Pathog. Volume Volume 49*. Edited by Pharmacology K-TJBT-A in. Academic Press; 2000:111–146.

4. Kohl NE, Emini E a, Schleif W a, Davis LJ, Heimbach JC, Dixon R a, Scolnick EM, Sigal IS: **Active human immunodeficiency virus protease is required for viral infectivity.** *Proc Natl Acad Sci U S A* 1988, **85**:4686–90.

5. Myers G, Korber BT FB: *Human Retroviruses and AIDS 1996.* Los Alamos, NM: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory; 1996.

6. Swanstrom R, Wills JW: **Synthesis, Assembly, and Processing of Viral Proteins**. In *Retroviruses*. Edited by Coffin JM, Hughes SH VH. Cold Spring Harbor Laboratory Press; 1997:263–334.

7. Ludwig C, Leiherer A, Wagner R: **Importance of protease cleavage sites within and flanking human immunodeficiency virus type 1 transframe protein p6* for spatiotemporal regulation of protease activation.** *J Virol* 2008, **82**:4573–84.

8. Skalka AM: **Retroviral Proteases : First Glimpses at the Anatomy of a Processing Machine**. *Cell* 1989, **56**:911–913.

9. Wlodawer A, Gustchina A: **Structural and biochemical studies of retroviral proteases.** *Biochim Biophys Acta* 2000, **1477**:16–34.

10. Dunn BM, Goodenow MM, Gustchina A, Wlodawer A: **Retroviral proteases.** *Genome Biol* 2002, **3**:REVIEWS3006.

11. Wlodawer A, Vondrasek J: **Inhibitors of HIV-1 protease: a major success of structure-assisted drug design.** *Annu Rev Biophys Biomol Struct* 1998, **27**:249–284.

12. Huang L, Chen C: **Understanding HIV-1 protease autoprocessing for novel therapeutic development.** *Future Med Chem* 2013, **5**:1215–29.

13. Preston BD, Poiesz BJ, Loeb LA: **Fidelity of HIV-1 reverse transcriptase.** *Science (80-)* 1988, **242**:1168–71.

14. Roberts JD, Bebenek K, Kunkel TA: **The accuracy of reverse transcriptase from HIV-1.** *Science* 1988, **242**:1171–1173.

15. Barrie K a, Perez EE, Lamers SL, Farmerie WG, Dunn BM, Sleasman JW, Goodenow MM: **Natural variation in HIV-1 protease, Gag p7 and p6, and protease cleavage sites within gag/pol polyproteins: amino acid substitutions in the absence of protease inhibitors in mothers and children infected by human immunodeficiency virus type 1.** *Virology* 1996, **219**:407–16.

16. Fehér A, Weber IT, Bagossi P, Boross P, Mahalingam B, Louis JM, Copeland TD, Torshin IY, Harrison RW, Tözsér J: **Effect of sequence polymorphism and drug resistance on two HIV-1 Gag processing sites.** *Eur J Biochem* 2002, **269**:4114–4120.

17. Heaslet H, Rosenfeld R, Giffin M, Lin YC, Tam K, Torbett BE, Elder JH, McRee DE, Stout CD: **Conformational flexibility in the flap domains of ligand-free HIV protease.** *Acta Crystallogr D Biol Crystallogr* 2007, **63**:866–875.

18. Nijhuis M, Van Maarseveen NM, Lastere S, Schipper P, Coakley E, Glass B, Rovenska M, De Jong D, Chappey C, Goedegebuure IW, Heilek-Snyder G, Dulude D, Cammack N, Brakier-Gingras L, Konvalinka J, Parkin N, Kräusslich HG, Brun-Vezinet F, Boucher CAB: **A novel substrate-based HIV-1 protease inhibitor drug resistance mechanism**. *PLoS Med* 2007, **4**:0152–0163.

19. Murphy EL, Collier AC, Kalish LA, Assmann SF, Para MF, Flanigan TP, Kumar PN, Mintz L, Wallach FR, Nemo GJ: *Highly Active Antiretroviral Therapy Decreases Mortality and Morbidity in Patients with Advanced HIV Disease. Volume 135*; 2001:17–26.

20. Thompson MA, Aberg JA, Hoy JF, Telenti A, Benson C, Cahn P, Eron JJ, Günthard HF, Hammer SM, Reiss P, Richman DD, Rizzardini G, Thomas DL, Jacobsen DM, Volberding PA: **Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA panel.** *JAMA* 2012, **308**:387–402.

21. Hung M, Patel P, Davis S, Simon R: **Importance of Ribosomal Frameshifting for Human Immunodeficiency Virus Type 1 Particle Assembly and Replication Importance of Ribosomal Frameshifting for Human Immunodeficiency Virus Type 1 Particle Assembly and Replication**. *Virology* 1998, **72**:4819–4824.

22. Pettit SC, Clemente JC, Jeung JA, Dunn BM, Kaplan AH: **Ordered processing of the human immunodeficiency virus type 1 GagPol precursor is influenced by the context of the embedded viral protease.** *J Virol* 2005, **79**:10601–10607.

23. Louis JM, Clore GM, Gronenborn AM: **Autoprocessing of HIV-1 protease is tightly coupled to protein folding.** *Nat Struct Biol* 1999, **6**:868–875.

24. Tang C, Louis JM, Aniana A, Suh J-Y, Clore GM: **Visualizing transient events in amino-terminal autoprocessing of HIV-1 protease.** *Nature* 2008, **455**:693–696.

25. Agniswamy J, Sayer JM, Weber IT, Louis JM: **Terminal interface conformations modulate dimer stability prior to amino terminal autoprocessing of HIV-1 protease.** *Biochemistry* 2012, **51**:1041–50.

26. Ishima R, Ghirlando R, Tözsér J, Gronenborn AM, Torchia DA, Louis JM: **Folded monomer of HIV-1 protease.** *J Biol Chem* 2001, **276**:49110–49116.

27. Huang L, Chen C: **Autoprocessing of human immunodeficiency virus type 1 protease miniprecursor fusions in mammalian cells.** *AIDS Res Ther* 2010, **7**:27.

28. Huang L, Hall A, Chen C: **Cysteine 95 and other residues influence the regulatory effects of Histidine 69 mutations on Human Immunodeficiency Virus Type 1 protease autoprocessing.** *Retrovirology* 2010, **7**:24.

29. Huang L, Sayer JM, Swinford M, Louis JM, Chen C: **Modulation of human immunodeficiency virus type 1 protease autoprocessing by charge properties of surface residue 69.** *J Virol* 2009, **83**:7789–93.

30. Huang L, Li Y, Chen C: **Flexible catalytic site conformations implicated in modulation of HIV-1 protease autoprocessing reactions**. *Retrovirology* 2011:79.

31. Wan M, Takagi M, Loh BN, Xu XZ, Imanaka T: **Autoprocessing: an essential step for the activation of HIV-1 protease.** *Biochem J* 1996, **316 (Pt 2**:569–573.

32. Huang M, Orenstein JM, Martin MA, Freed EO: **p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease.** *J Virol* 1995, **69**:6810–6818.

33. Loeb DD, Swanstrom R, Everitt L, Manchester M, Stamper SE, Hutchison CA: **Complete mutagenesis of the HIV-1 protease.** *Nature* 1989, **340**:397–400.

34. Tessmer U, Kräusslich H: **Cleavage of Human Immunodeficiency Virus Type 1 Proteinase from the N-Terminally Adjacent p6 * Protein Is Essential for Efficient Gag Polyprotein Processing and Viral Infectivity Cleavage of Human Immunodeficiency Virus Type 1 Proteinase from the N-Termin**. 1998:1–6.

35. Kovalevsky AY, Liu F, Leshchenko S, Ghosh AK, Louis JM, Harrison RW, Weber IT: **Ultra-high Resolution Crystal Structure of HIV-1 Protease Mutant Reveals Two Binding Sites for Clinical Inhibitor TMC114**. *J Mol Biol* 2006, **363**:161–173.

36. Louis JM, Ishima R, Torchia DA, Weber IT: **HIV-1 protease: structure, dynamics, and inhibition.** *Adv Pharmacol* 2007, **55**:261–98.

37. Partin K, Zybarth G, Ehrlich L, DeCrombrugghe M, Wimmer E, Carter C: **Deletion of sequences upstream of the proteinase improves the proteolytic processing of human immunodeficiency virus type 1.** *Proc Natl Acad Sci U S A* 1991, **88**:4776–4780.

38. Chen C, Vincent O, Jin J, Weisz OA, Montelaro RC: **Functions of early (AP-2) and late (AIP1/ALIX) endocytic proteins in equine infectious anemia virus budding.** *J Biol Chem* 2005, **280**:40474–40480.

39. Strack B, Calistri A, Craig S, Popova E, Göttlinger HG: **AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding**. *Cell* 2003, **114**:689–699.

40. Martin-Serrano J, Yarovoy A, Perez-Caballero D, Bieniasz PD: **Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins.** *Proc Natl Acad Sci U S A* 2003, **100**:12414–12419.

41. Pettit SC, Simsic J, Loeb DD, Everitt L, Hutchison CA, Swanstrom R: **Analysis of retroviral protease cleavage sites reveals two types of cleavage sites and the structural requirements of the P1 amino acid.** *J Biol Chem* 1991, **266**:14539–14547.

42. Tözsér J, Weber IT, Gustchina A, Bláha I, Copeland TD, Louis JM, Oroszlan S: **Kinetic and modeling studies of S3-S3' subsites of HIV proteinases.** *Biochemistry* 1992, **31**:4793–4800.

43. Rosé JR, Salto R, Craik CS: **Regulation of autoproteolysis of the HIV-1 and HIV-2 proteases with engineered amino acid substitutions.** *J Biol Chem* 1993, **268**:11939–11945.

44. Mildner AM, Rothrock DJ, Leone JW, Bannow CA, Lull JM, Reardon IM, Sarcich JL, Howe WJ, Tomich CS, Smith CW: **The HIV-1 protease as enzyme and substrate: mutagenesis of autolysis sites and generation of a stable mutant with retained kinetic properties.** *Biochemistry* 1994, **33**:9405–9413.

45. Pettit SC, Everitt LE, Choudhury S, Dunn BM, Kaplan AH: **Initial cleavage of the human immunodeficiency virus type 1 GagPol precursor by its activated protease occurs by an intramolecular mechanism.** *J Virol* 2004, **78**:8477–8485.

46. Huang Y, Misquitta S, Blond SY, Adams E, Colman RF: **Catalytically active monomer of glutathione S-transferase pi and key residues involved in the electrostatic interaction between subunits.** *J Biol Chem* 2008, **283**:32880–32888.

47. Fabrini R, De Luca A, Stella L, Mei G, Orioni B, Ciccone S, Federici G, Lo Bello M, Ricci G: **Monomer-dimer equilibrium in glutathione transferases: a critical re-examination.** *Biochemistry* 2009, **48**:10473–10482.

48. Koh Y, Nakata H, Maeda K, Ogata H, Bilcer G, Devasamudram T, Kincaid JF, Boross P, Wang Y-F, Tie Y, Volarath P, Gaddis L, Harrison RW, Weber IT, Ghosh AK, Mitsuya H: **Novel bis-tetrahydrofuranylurethane-containing nonpeptidic protease inhibitor (PI) UIC-94017 (TMC114) with potent activity against multi-PI-resistant human immunodeficiency virus in vitro.** *Antimicrob Agents Chemother* 2003, **47**:3123–3129.

49. Busse KHS, Penzak SR: **Darunavir: a second-generation protease inhibitor.** *Am J Health Syst Pharm* 2007, **64**:1593–1602.

50. Vermeir M, Lachau-Durand S, Mannens G, Cuyckens F, van Hoof B, Raoof A: **Absorption, metabolism, and excretion of darunavir, a new protease inhibitor, administered alone and with low-dose ritonavir in healthy subjects.** *Drug Metab Dispos* 2009, **37**:809–820.

51. Raran-Kurussi S, Waugh DS: **The Ability to Enhance the Solubility of Its Fusion Partners Is an Intrinsic Property of Maltose-Binding Protein but Their Folding Is Either Spontaneous or Chaperone-Mediated**. *PLoS One* 2012, **7**.

52. Sun P, Tropea JE, Waugh DS: **Enhancing the solubility of recombinant proteins in Escherichia coli by using hexahistidine-tagged maltose-binding protein as a fusion partner.** *Methods Mol Biol* 2011, **705**:259–274.

53. Fox JD, Waugh DS: **Maltose-binding protein as a solubility enhancer.** *Methods Mol Biol* 2003, **205**:99–117.

54. Chatterjee A, Mridula P, Mishra RK, Mittal R, Hosur R V: **Folding regulates autoprocessing of HIV-1 protease precursor.** *J Biol Chem* 2005, **280**:11369–11378.

55. Buxton P, Tachedjian G, Mak J: **Analysis of the contribution of reverse transcriptase and integrase proteins to retroviral RNA dimer conformation.** *J Virol* 2005, **79**:6338–6348.

56. Tsiang M, Niedziela-Majka A, Hung M, Jin D, Hu E, Yant S, Samuel D, Liu X, Sakowicz R: **A trimer of dimers is the basic building block for human immunodeficiency virus-1 capsid assembly.** *Biochemistry* 2012, **51**:4416–28.