Proteolysis is a process crucial to the normal turnover of proteins in the cell. Proteases are not only involved in protein destruction, but also in activation, growth, cell division, differentiation, migration, signalling and other cellular processes. In addition, many viruses use cellular or viral proteases for various purposes, such as activation of their surface proteins or membrane fusion. Consequently, specific protease inhibitors are attractive drug candidates (TABLE 1). Although the different specificities of proteases have been used during recent years to assess their activities in vitro, their actions have been difficult to determine under more-or-less physiological conditions.

Proteases can be divided into a number of classes: the serine, aspartic, cysteine, threonine and metalloproteases. This classification scheme basically refers to the amino acid or metal that catalyses the nucleophilic attack on the substrate’s peptide bonds. Enzymes with proteolytic activities have frequently evolved to carry out important tasks, which has resulted in large and divergent families of proteases. These often unrelated enzymes differ in many respects, but one characteristic unites them: they hydrolyse peptide bonds and thereby cut proteins into small pieces or a few distinct fragments. It is precisely this chemical reaction that allows the design of probes to selectively monitor protease activities.

One generally recognized function of proteases is the recycling of proteins into amino acids. Proteins can be singled out for a number of reasons, including signs of malfunctioning or misfolding. Although the vast majority of proteins face destruction when old and expired, a substantial fraction of newly synthesized proteins undergoes a similar fate, possibly because they fail to adopt a proper conformation. Because misfolded proteins tend to aggregate, the rapid recognition and degradation of such aberrant proteins is crucial for the survival of the cell. Moreover, in many cases the destruction of proteins is crucial for the correct control of cellular regulatory switches. In this case, their degradation is not a result of malfunctioning, and is in fact determined by specific spatially and temporally controlled signalling events.

The route that leads from a full-length intracellular protein to its constituent amino acids is a multistep process (FIG. 1). First, the processive degradation of proteins by the proteasome generates small peptide fragments. This initial cleavage stage is generally sufficient to abolish protein function and activity. Second, a collection of proteases — among which are tripeptidyl peptidase-II (TPP-II), leucine aminopeptidase (LAP) and thimet oligopeptidase (TOP) — trim these small peptide fragments step-wise into single amino acids.
that can be reused in the synthesis of new proteins. Endocytosed proteins do not usually enter the cytoplasm but are instead directed to lysosomal compartments (FIG. 1). In the lysosomes, another group of proteases, the cathepsins14, degrades the internalized protein into pieces. These fragments are subsequently reduced to single amino acids by lysosomal aminopeptidases like TPPI and dipeptidyl peptidase-II (DPP-II). Given the broad substrate specificity of the proteasome and cathepsins, these proteases have to be compartmentalized to prevent the random degradation of ‘innocent bystanders’. This is achieved by placing the active sites of the proteases within large multisubunit complexes, in the case of the proteasome, or within lysosomes, in the case of cathepsins. In addition to these broadly acting proteases, there are other proteases involved in more specific processes, such as blood clotting12, apoptosis13, extracellular matrix degradation14, cleavage of transmembrane proteins15 and precursor processing in the secretory pathway16.

Clearly, proteases not only destroy proteins but also convert proteins into active moieties. In addition, proteolytic cascades regulate important cellular switches. It is therefore not surprising that proteases get a lot of importance in assessing the roles of proteases in diseases (TABLE 1 and FIG. 2) — was approved after successful clinical trials in patients suffering from multiple myeloma29,30.

Notably, two drugs that affect proteasomal degradation have been used in patients for some time without being recognized as such. These drugs are the human immunodeficiency virus (HIV)-1 protease inhibitors ritonavir (Norvir; Abbot Laboratories) and saquinavir (Fortovase; Roche), and have recently been shown to affect proteasome activity as well31. It has been suggested that inhibition of the proteasome by these compounds could account for increased hyperlipidaemia32 and the reduced incidence of Kaposi’s sarcoma33 in patients treated with these drugs. Moreover, HIV-1 inhibitors can radiosensitize tumour cells for ionizing radiation by blocking the proteasome41. Specific protease inhibitors, such as peptide aldehydes, boronic acids, vinyl sulfones, epoxyketones or β-lactones, have been used for many years in the laboratory (FIG. 2). So whereas drugs that target the proteasome are relatively new and unexplored, protease inhibitors and modulators hold great promise as potential drugs for multiple disorders37.

During the past few years several groups have developed fluorescent probes for the ubiquitin–proteasome system that behave as authentic protein substrates (FIG. 3a). Only proteins, which are specifically recruited to the proteasome, and subsequently unfolded and threaded into the core, can be degraded by this complex. The proteasome generally recognizes substrates by the presence of a poly-ubiquitin chain conjugated to an internal lysine residue of the substrate or, less commonly, conjugated to the free amino terminus of the substrate. Proteins are modified by the addition of specific ubiquitylation enzymes41. Given the chemistry of proteases, various fluorescent substrates have been designed during the past few years to test their activities as well as the effects of drug-mediated inhibition of these systems in cells or animals. Understanding the physiology of proteolytic processes inside the living organism is of crucial importance in assessing the roles of proteases in diseases and for developing drugs that target these systems.

**Fluorescent protein substrates**

The ubiquitin–proteasome system is important in regulating the cell cycle and the induction of apoptosis by the degradation of key regulatory molecules. In addition, this proteolytic system is responsible for keeping the cell free of misfolded proteins17. These important functions explain why the ubiquitin–proteasome system is implicated in a large number of diverse pathologies5,17,18. For example, aberrant proteasomal degradation contributes to malignant transformation19–21. The immune system relies on the ubiquitin–proteasome system for the generation of antigenic peptides that are presented by major histocompatibility complex (MHC) class I molecules22,23. The proteasome also has a crucial role in the inflammatory response, as it degrades inhibitors of nuclear factor-κB24. Given these functions, it is not surprising that proteasome inhibitors have antinecancer25, anti-inflammatory26, angiostatic27 and immuno-modulating activity28. Recently, the first proteasome inhibitor, bortezomib (Velcade; Millennium) — a boronic acid inhibitor (TABLE 1 and FIG. 2) — was approved after successful clinical trials in patients suffering from multiple myeloma29,30.

**Table 1 | A few inhibitors and (potential) applications**

| Target               | Inhibitor       | Disease      | References |
|----------------------|-----------------|--------------|------------|
| Matrix metalloproteases | Marinastat      | Cancer       | 86         |
|                      | Pinomastat      |              |            |
|                      | Tanomastat BMS-275291 |            |            |
| Cathepsin S          | Clik60          | Autoimmunity | 85         |
| Proteasome           | Velcade (PS 341) | Cancer       | 30         |
| Caspase-1            | Praincasian     | Arthritis    | 125        |
| Caspase-8            | IDN6556         | Liver cirrhosis | 126       |
| HIV-1 protease       | Ritonavir       | HIV-1 infection | 127       |
|                      | Nelfinavir      |              |            |
|                      | Saquinavir      |              |            |
|                      | Lopinavir       |              |            |
| HCV NS3              | BILN 2016       | HCV hepatitis | 118        |

HCV, hepatitis C virus; HIV, human immunodeficiency virus.

**MAJOR HISTOCOMPATIBILITY COMPLEX (MHC).** A ubiquitously expressed cell-surface glycoprotein that presents a fragment of a protein to the immune system. This fragment, which usually comprises nine amino acids, is generally derived from intracellular proteins and MHC class I molecules, thereby allowing the immune system to assay the intracellular protein content, including pathogens.

**MODULAR DOMAINS**

Domains that fold and function as independent elements. The insertion of modular domains in other proteins changes the behaviour of the protein accordingly.
Another reporter substrate was generated with the help of the CL1 degradation signal. The introduction of this small 16-amino-acid motif converted GFP into a short-lived proteasome substrate (FIG. 4c). It is noteworthy that the CL1 motif was originally identified in a random yeast screen for proteins that are targets for the ubiquitylation enzymes engaged in endoplasmic reticulum (ER)-ASSOCIATED DEGRADATION (ERAD). This ERAD pathway is used by aberrant ER proteins for release from the ER to access the cytosol proteasome for degradation.

MHC class I molecules have been converted into a genuine ER-derived substrate of the ubiquitin–proteasome system. Several viruses frustrate antigen presentation by targeting MHC class I molecules. For example, the human cytomegalovirus (HCMV) protein US11 binds MHC class I molecules in the ER and dislocates them to the cytosol where they are degraded by the ubiquitin–proteasome system.
Huntington’s diseases, Alzheimer’s, Parkinson’s and such disorders include forms insoluble protein deposits. Protein that aggregates and conformationally abnormal nature of the ubiquitin–proteasome system contributes to many neurodegenerative disorders.

The administration of proteasome inhibitors to reporter-expressing cells stabilized the GFP reporters and resulted in a 100–1,000-fold increase in fluorescent intensity. The fact that these fluorescent proteins follow the same pathway as endogenous substrates allows functional analysis of the ubiquitin–proteasome system in cells. Indeed, accumulation of the UFD substrate in response to administration of proteasome inhibitors was shown to correlate with cell-cycle arrest and apoptosis, which is a consequence of general blockade of proteasomal degradation. Parallel evaluation of the individual proteolytic sites of the proteasome (with fluorogenic peptide substrates) and the functional status of the ubiquitin–proteasome system (with the GFP reporters) revealed that cells contain a large excess of proteasomal activity, which is probably required to instantly clear misfolded proteins under stress conditions. The UFD–GFP reporter was also used to study the individual contributions of the proteolytic active sites of the proteasome to protein degradation.

The UFD and N-end rule GFP substrates can be used to analyse the effect of protein domains on proteasomal turnover after simple insertion of the domain in the reporter substrate. This approach has been used for detailed studies on a viral repetitive sequence and the expanded polyglutamine repeats that are the causative factor in a number of neurodegenerative disorders. These two repetitive sequences had previously been shown to affect proteasomal degradation. At present, these reporter substrates are further exploited for the identification of so-called stabilization signals; that is, signals that enable proteins to resist proteasomal degradation (S. Heessen, M. G. Masucci and N. P. D., unpublished results).

Another convenient aspect of using protein substrate reporters, as opposed to peptide substrates, is that they can be readily applied to the generation of transgenic animals. It is notable that the expression of GFP is well tolerated in mice, although some side effects have been reported in specific models. Recently, the first transgenic mouse model of the ubiquitin–proteasome system was generated through ubiquitous expression of the UFD–GFP reporter. As anticipated, the tissues of these mice did not emit GFP fluorescence despite the presence of reporter-encoding transcripts in all tissues analysed. The injection of proteasome inhibitors into these mice resulted in a dramatic increase in fluorescence intensities of affected tissues. In another animal model, a UFD bioluminescence reporter-expressing human cell line was implanted in nude mice. The human cells responded with a dose-dependent increase in bioluminescence after administration of the proteasome inhibitor bortezomib to the mice.

In particular the transgenic mouse model opens a new dimension for the study of the ubiquitin–proteasome system in pathologies because of its ubiquitous expression. It has long been suspected that impairment of the ubiquitin–proteasome system contributes to many neurodegenerative diseases and other conformational disorders. Although it has been confirmed in cellular systems that some disease-associated proteins can indeed affect the ubiquitin–proteasome system, these experimental approaches by no means reflect the complex pathology of such neurodegenerative diseases. This reporter mouse model can now be used, in conjunction with the excellent mouse models available for the study of these disorders, to assess the functionality of the ubiquitin–proteasome system during disease progression. It should be emphasized, however, that impairment of the ubiquitin–proteasome system is just one of the many models put forward as an explanation.
for the pathology observed in conformational disorders. Yet, if this model holds true, crosses between the GFP-reporter mice and disease model mice can also be used to evaluate the effect of drugs that counteract the effects of the impaired ubiquitin–proteasome system in these diseases.

Figure 3 | Fluorescent probes for proteolysis. a | Destruction. Coupling of a degradation signal (DEG) to GFP will result in targeting of the GFP for ubiquitylation followed by proteasomal degradation. Degradation of the GFP substrate results in loss of fluorescence. b | Fluorescence resonance energy transfer (FRET) fluorophore peptide substrate. A FRET peptide substrate contains a donor and acceptor fluorophore with spectral overlap. When the FRET probe is intact and excited with the optimal wavelength for the donor, acceptor fluorescence will be emitted at the expense of donor fluorescence. Cleavage of the FRET probe results in separation of donor and acceptor fluorophores and increased emission from the donor with a concomitant loss of acceptor fluorescence. c | Quenching with fluorophore/quencher pair in peptide substrate. Small peptide fragments containing a fluorophore and a corresponding quencher will be essentially non-fluorescent. After cleavage, when the quencher is spatially separated from the fluorophore, the fluorophore will emit fluorescence. d | Quenching with identical fluorophores in peptide substrate. Two identical fluorophores will quench each other. Principle as in b. e | FRET fluorescent proteins. With CFP and YFP as donor and acceptor fluorophore, respectively. Principle as in b. CFP, cyan fluorescent protein; GFP, green fluorescent protein; YFP, yellow fluorescent protein.
Peptide degradation can be monitored using chemical fluorophores. A strategy for detecting peptidase activity in living cells uses peptides containing a fluorescence resonance energy transfer (FRET) fluorophore pair (FIG. 3b). FRET is only operational with 10–100 Å and not detectable when fluorophores are more than 100 Å apart. Therefore, spatial separation of two fluorophores as the result of protease activity can be easily assayed because this will result in increased emission from the donort fluorophore at the expense of emission from the acceptor fluorophore (BOX 1). This strategy has been used with peptides containing a fluorescein (Fl) group and a tetramethylrhodamine (TMR) group separated by six amino acids. Energy transfer from Fl to TMR occurred with intact peptides but was lost when the peptides were separated as the result of degradation. More dramatic differences were observed when instead of TMR, a quencher molecule was used. The quencher in these peptides very efficiently absorbs the emitted light from the Fl group, unless the two groups are separated (FIG. 3c).

Using more-or-less standard peptide chemistry, substrates can be synthesized for defined questions relating to peptide degradation in living cells. These peptides do not diffuse across membranes and therefore have to be introduced into living cells by microinjections. Detailed analysis of real-time degradation of such peptides in living cells revealed that peptides have a very short half-life of several seconds, solely as a result of aminopeptidase activity. Some of these peptidases are crucial for trimming peptides to the correct size for MHC class I antigen presentation. However, the individual contribution of peptidases to the total intracellular peptidase activities in a cell is unclear. Studies with specific inhibitors showed that the proteasome does not contribute to the digestion of small peptides in living cells even though in vitro peptide degradation can be readily measured and has been used

**Figure 4 | GFP-based proteasome substrates.**

- **a** | N-end rule reporter. A fusion of ubiquitin and GFP is expressed. The endogenous de-ubiquitylation enzymes (DUB) rapidly remove the N-terminal ubiquitin, which generates a GFP with an N-terminal arginine residue. This N-terminal Arg residue is recognized by ubiquitylation enzymes resulting in ubiquitylation close to the N terminus followed by proteasomal degradation.

- **b** | UFD reporter. If the last amino acid of the ubiquitin moiety in the ubiquitin–GFP fusion is changed, the DUBs fail to remove the N-terminal ubiquitin. This results in ubiquitylation of the ubiquitin moiety and proteasomal degradation.

- **c** | CL1 reporter. Insertion of the small amino acid stretch CL1 directly targets GFP for ubiquitylation and proteasomal degradation. The degradation signals in a–c are marked in red.

- **d** | MHC class 1 reporter. Co-expression of the viral US11 and GFP–MHC class I fusion results in rapid dislocation of GFP–MHC class I from the endoplasmic reticulum (ER) to the cytosol followed by proteasomal degradation.
REVIEWS

Box 1 | Fluorescence resonance energy transfer (FRET)

FRET is the physical phenomenon of energy transfer between a donor and an acceptor fluorophore. This will be possible only when the donor/acceptor pair are separated by a very short distance (< 100 Å). This is usually one-quarter or less of the light wavelength used to excite the donor fluorophore. Because it is acting at such a short distance, the principle of energy transfer is not by emission of a photon that is captured by the acceptor fluorophore, but merely electromagnetic energy transfer as a result of dipole moments. FRET efficiency is highly dependent on distance and decays with distance, as formulated in Förster’s law. This makes FRET highly suitable for detecting conformational changes in a protein (when the distance between donor and acceptor changes) or degradation (when the donor and acceptor pair are separated after the cleavage of substrate).

In addition, the relative orientation of the fluorophores determines FRET. This so-called orientation factor is usually unknown and prevents the determination of real distances on the basis of FRET data.

Near-infrared fluorescence (NIRF) probes

Although small quenched peptide substrates have proven to be valuable tools for studying proteases in purified samples in vitro or in cell lines after microinjection, the fact that they cannot cross membranes limits their applications for monitoring intracellular proteolysis in vivo. However, for those proteases active at the cell surface or in the endosomal/lysosomal compartment, this is not a major limitation.

The cysteine, serine and aspartic proteases present in lysosomes have been implicated in many cellular processes. The lysosomal cathepsins are essential to the turnover of proteins that reach the lysosomal compartment from the extracellular space (by endocytosis), the secretory pathway (by sorting) or the cytosol (by autophagy) and have more subtle regulatory functions in MHC class I antigen presentation. Lysosomal proteases are believed to be involved in diseases such as cancer, inflammation, autoimmune disorders, atherosclerosis and several genetic disorders. It has been shown that these proteases can participate in the destruction of the extracellular matrix surrounding tumours, which is an important prerequisite for tumour invasion, metastasis and angiogenesis. However, because most cathepsins have important roles in lysosomal protein turnover, caution should be taken in inhibiting these enzymes, as impairment of cathepsins can result in lysosomal storage diseases. More specialized lysosomal proteases, such as cathepsin S, which is expressed in the immune system and assists in MHC class II antigen presentation, are also important drug targets. In fact, selective inhibitors of cathepsin S have been developed and have been shown to prevent autoimmune responses in a mouse model of Sjögren syndrome (Table 1).

Another important class of drug targets that mediate the breakdown of the extracellular matrix is the extracellular matrix metalloproteases (MMPs), a large family of metalloproteases present at the cell surface and in the extracellular space. MMPs have been recognized as drug targets for cancer treatment because metastatic cells have to degrade the extracellular matrix for angiogenesis and invasion of tissue. Several MMP inhibitors, such as BB-2516 (Marimastat; British Biotech) and BAY12-9566 (Tanomastat; Bayer) (Table 1), have been developed and tested in clinical trials. Although the clinical trials have not been without success, they did not live up to the high expectations for these compounds. Still, there is ample reason to believe that more specific drugs or early interference in tumour progression might improve their efficacy.

Weissleder and co-workers developed a near-infrared fluorescent (NIRF) peptide substrate that can be used for the in vivo monitoring of cathepsin activity. They used a synthetic non-toxic and non-immunogenic co-polymer that had previously been tested in clinical trials and that was composed of a poly-lysine backbone, which was stabilized through the conjugation of multiple polyethylene glycol side chains. Fluorophores were coupled at a high density to these backbones. Identical fluorophores will interfere with each other and increase their relaxation times when excited, similar to normal FRET pairs. Although the mechanism is not fully understood, this results in quenching unless the fluorophores are diluted by degradation. Indeed it was found that the emission signal was efficiently quenched in the intact co-polymer, rendering it almost non-fluorescent, whereas in vitro cleavage of the probe considerably increased emitted light. To measure the fluorescence in living animals a dye was used that is excited with near-infrared light, which because of its long wavelength will hardly be scattered or absorbed by the tissue. This NIRF probe was expected to function as a general molecular probe for the lysosomal proteases that are able to cleave the poly-lysine backbone. Activation of the probe could be followed in cells, and inhibitor experiments confirmed that lysosomal cysteine and serine proteases were primarily responsible for activation of the NIRF reporter in cell lines. Using xenotransplantation of human tumours in nude mice, it was shown that the NIRF probe was predominantly taken up and activated in the tumour tissues, possibly as a consequence of their relatively permeable neovascularure. With the non-invasive near-infrared reflecting imaging technique, small tumours containing cleaved NIRF probes could be identified at a maximal tissue depth of 1 cm.

The same NIRF reporter was also used to study the role of cathepsin B in atherosclerosis when administered intravenously. Cathepsin B activity was found to be upregulated in the lesions in a mouse model for atherosclerosis. NIRF tomographic imaging allowed three-dimensional imaging of the probes in deeper tissue and could localize lesions in the aortic arch in mice solely by the presence of the activated NIRF reporter.
Because the lysine composition of the co-polymer is important for the \textit{in vivo} stability of this probe, the specificity cannot be changed by modifying the amino-acid sequence of the copolymer itself. Instead, a strategy was used in which the fluorophores were connected to the copolymer backbone on peptide stalks conjugated by an isopeptide linkage to the lysine backbone. By changing the amino-acid sequence of the peptide stalk into protease cleavage sites, the probe could be converted into a substrate for specific proteases without losing \textit{in vivo} stability. With this approach, specific NIRF reporters were generated for \textit{cathepsin D} \cite{94,95} and MMP-2 \cite{94,95}. \textit{In vivo} imaging of tumour-bearing mice after probe administration could be used to discriminate between tumours expressing low or high levels of the selected proteases \cite{90,93,94}. More recently, similar probes were used for imaging lymph nodes in living mice \cite{96,97}. Not only can these reporters be helpful for the detailed evaluation of the effect of potential drugs on lysosomal and extracellular proteases \textit{in vivo}, they might also aid early diagnosis of tumours or atherosclerotic lesions, at least when imaging techniques will allow visualization of these probes deep in tissue.

In reality, the substrates of cathepsins or MMPs are not peptides but full-length proteins. It has been shown that protein substrates labelled with high-density fluorophores can also be used to study the activity of lysosomal proteases in cell lines \cite{98}. Although it seems unlikely that this type of protein can be used in animals, important information about the combined action of tumour cells towards extracellular proteins can be deduced in cell culture systems.

\textbf{FRET protein reporters}

Since the introduction of recombinant DNA technology, many reporter substrates for proteases have been generated by the introduction of protease cleavage sites in proteins with easily detectable enzyme activities \cite{99,100}. Proteolytic cleavage of such designed substrates inactivates their enzymatic activity, which forms a reliable and quantitative readout for protease activity. It seems tempting to apply the same approach to GFP or other fluorescent proteins, but unfortunately the presently available fluorescent proteins are not very well suited for this purpose. Although the presently available fluorescent proteins allow the generation of all kinds of N- and C-terminal fusion proteins, their tightly interwoven structure does not allow major modifications within the GFP open reading frame, as this generally results in a complete loss of fluorescence \cite{101}.

Instead, researchers turned towards the FRET technique described above, which only became possible with the development of spectral GFP variants \cite{102}. By positioning a protease cleavage site between two fluorescent protein variants with overlapping emission \textit{from} the donor and excitation spectra \textit{from} the acceptor, a FRET-based probe for proteases can be generated. The use of GFP variants instead of dyes enabled the construction of protease substrates that do not require any \textit{in vitro} modification and which can therefore be directly expressed in target cells or organisms. Several FRET-based probes have been developed for caspases, a family of related cysteine proteases that are involved in the execution of apoptosis \cite{13}. These enzymes are activated in a proteolytic cascade reaction that is tightly controlled. Caspases can be divided into two groups: initiator caspases, which set off the cascade by activating other caspases; and effector caspases, which cleave multiple cellular substrates and destroy the cellular protein content. Apoptosis is a key factor in many disease processes, including neuronal loss by excessive apoptosis in neurodegenerative disorders \cite{102} and escape from apoptosis in cancer \cite{103}. Because caspases are the inducers as well as the executors of cell death, their potential as drug targets is evident \cite{104} (TABLE 1).

Xu and co-workers generated the first functional caspase FRET probe on the basis of fluorescent proteins by inserting a small peptide target sequence for the effector caspase-3 between GFP and the blue fluorescent protein (BFP) \cite{105} (FIG. 5a). Induction of apoptosis in cells expressing this fusion protein resulted in a loss of the FRET signal between the BFP donor and GFP acceptor as a consequence of caspase-3 activation. A major drawback of the BFP–GFP FRET pair is that the BFP donor is excited by ultraviolet light, which causes considerable cellular damage and is therefore far from optimal for studies with living cells. Several new spectral variants, based on modified GFP or fluorescent proteins from other sources, have been generated during the past few years \cite{106}. Ultraviolet excitation is avoided in the more recent caspase-3 FRET probe, in which the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP) are combined \cite{107} (FIG. 5b). By changing the connecting peptide sequence between the CFP–YFP combination, the FRET probe was converted to a probe specific for the initiator caspase-8 \cite{108}. In this study, another advantage of the modular composition of the FRET probes was exploited. Instead of introducing a small cleavage site, larger protein fragments can be inserted because the probe is basically composed of three independent modular domains. In this reporter, the caspase-8 target, BH3-interacting domain death agonist (BID), was used to generate a functional YFP–BID–CFP reporter \cite{109}.

Unfortunately, all FRET probes containing the CFP or BFP variants have limited sensitivities due to the relatively poor quantum yield of these variants. Recently, reporters for caspase-3 have been constructed by combining GFP and YFP, which do not suffer from these limitations \cite{109} (FIG. 5d). Optical filters cannot separate the largely overlapping excitation and emission spectra of these proteins and therefore energy transfer is difficult to quantify by sensitized emission FRET. An alternative quantitative method is fluorescence lifetime imaging microscopy (FLIM), which can measure energy transfer between probes with overlapping or even identical spectra \cite{109}. FLIM measures the effect of two closely located fluorophores on the relaxation time of the donor and acceptor, which is a consequence of FRET. Like FRET, FLIM decays rapidly with distance and is detectable when the fluorophores are within 10–100 Å of one another. Using pulsed light sources, changes in the relaxation time can be determined. Although energy...
The stabilization or destabilization of the GFP reporter as a consequence of caspase activity could be quantified in living cells by flow cytometric or fluorimetric analyses\textsuperscript{110}. This reporter requires only a single fluorescent protein and gives as a readout a simple decrease or increase in GFP fluorescence, two characteristics that make it favourable for use in high-throughput drug screens. However, one inherent problem is that compounds that interfere with the ubiquitin–proteasome system will affect the stability of these reporters as well.

Many infectious pathogens are dependent on proteases for their survival. Viruses often require virally encoded proteases for the processing of precursor proteins, which is important at various steps in the viral life cycle, and so viral proteases have been important targets for the development of antiviral drugs\textsuperscript{111}. The HIV-1 genome encodes an aspartic protease that is essential for the generation of infectious virus particles\textsuperscript{112}. The development of drugs that target this protease has been a successful endeavour, resulting in drugs such as ritonavir, saquinavir, indinavir (Crixivan; Merck), nelfinavir (Viracept; Agouron/Pfizer) and lopinavir (Kaletra; Abbot Laboratories) (TABLE 1). HIV-1 protease inhibitors are now routinely used as part of highly active antiretroviral therapy (HAART), a regime that can efficiently control HIV replication in patients\textsuperscript{113}. Because HAART can successfully suppress but not eradicate the virus in patients, ongoing viral replication often results in the emergence of inhibitor-resistant viral strains\textsuperscript{114}. There is therefore a need for novel HIV-1 protease inhibitors, as well as diagnostic tests to identify and anticipate inhibitor-resistant strains.

HIV-1 protease is generated by an autocatalytic event from a large precursor protein. The presence of HIV-1 protease is toxic for cells, which is not surprising considering that this protease has broad specificity and cleaves many cellular substrates\textsuperscript{115,116}. Two considerations were taken into account when designing a GFP-based reporter for HIV-1 protease. First, the protease must be released from its precursor to become fully active; and second, the presence of fully active protease is toxic for cells. By generating an artificial precursor composed of GFP and the HIV-1 protease, a simple but robust reporter was made based on these two characteristics\textsuperscript{117}. Transient transfection of cells with a plasmid encoding this non-toxic GFP–HIV-1 protease precursor is now a routine procedure in many laboratories. The accumulation of the unprocessed, non-toxic GFP–HIV-1 protease precursor in cells that express this reporter resulted in a dose-dependent increase in GFP fluorescence, due to accumulation of the unprocessed, non-toxic GFP–HIV-1 protease precursor. This non-infectious model is now
being developed as a diagnostic assay for the analysis of inhibitor resistance of virus strains. Indeed, experiments with characterized mutant HIV-1 proteases from patient material showed that this probe can identify inhibitor-resistant HIV-1 proteases (T. Uhlíková, N. P. D., K. Lindsten, M. G. Masucci and J. Konvalinka, unpublished results). The HIV-1 protease inhibitors exemplify the successful approach of targeting viral proteases, and presently much effort is put into the development of similar compounds inhibiting proteases of other life-threatening viruses, such as the proteases of hepatitis C virus and the human corona virus that causes severe acute respiratory syndrome (TABLE 1).

Reits and co-workers used an indirect approach to monitor intracellular protein degradation in living cells. They followed the activity of a peptide transporter that is involved in antigen presentation by MHC class I molecules. They showed that a GFP-tagged transporter associated with antigen processing (TAP) could be used to indirectly monitor the generation of small peptide fragments by intracellular proteases. Using fluorescence recovery after photobleaching (FRAP) techniques to visualize TAP diffusion in the ER membrane, it was shown that TAP diffuses rapidly when inactive, and slowly when in the process of pumping peptides. These differences in mobility probably reflect differences in shape or radius, as predicted by modifications of the Einstein–Stocks formula, in which diffusion is correlated with ln(1/r), where r is the radius of the protein in the membrane.121. Recording of the mobility of TAP in living cells has been used to show that a large portion of peptides are generated from proteins that are immediately degraded after translation, to visualize the contribution of inflammation to the intracellular peptide pool and to visualize the effect of γ-irradiation on intracellular peptide degradation (E. Reits and J. N., unpublished results). The disadvantage of this technique is that it is rather complicated; however, unlike the other reporters, this method visualizes the pool of intracellular peptides rather than the activity of selective proteases.

Conclusions
For understanding the mechanism of proteolysis and the effects of inhibitors in vivo, a variety of fluorescence-based assays have been developed. These assays allow the detection of protease activities in living cells and will help us to elucidate the individual roles of proteases in complex proteolytic systems, such as the living cell. Such assays can be used to monitor the effects of chemical inhibitors of protease activity, or selective downregulation of proteases by RNAi.212.

An interesting difference between following in vivo degradation of designed substrates, as compared with in vitro assays with purified components, is that the designed substrates, which are analogous to genuine substrates, can be subject to multiple cleavage events. For example, it has been shown that degradation of the GFP-based substrates for the ubiquitin–proteasome system can also be influenced by inhibitors of unrelated serine proteases that are apparently directly or indirectly involved in this proteolytic system.123. The FRET caspase reporters and NIRF cathepsin reporters are also subject to cleavage by several caspases. Although this might complicate the interpretation of the cleavage events, it reflects the in vivo situation of substrates and might aid the identification of novel proteases and unanticipated connections between proteolytic systems.

The complexity of proteolysis and the contribution of individual proteases to the total protease activity can only be fully appreciated in living cells or even better, in vivo. The recently generated GFP-reporter mice allow monitoring of protease activities and the effects of proteasome inhibitors in vivo.214. It is to be expected that other transgenic mice expressing fluorescent protein-based reporter systems will follow and aid the development of compounds targeting proteases.

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