

## MIT Open Access Articles

*Harnessing protease activity to improve cancer care*

The MIT Faculty has made this article openly available. **Please share** how this access benefits you. Your story matters.

**Citation:** Dudani, Jaideep S., Andrew D. Warren, and Sangeeta N. Bhatia, "Harnessing protease activity to improve cancer care." Annual review of cancer biology 2 (2018): p. 353-76 doi 10.1146/ANNUREV-CANCERBIO-030617-050549 ©2018 Author(s)

**Published Version:** 10.1146/ANNUREV-CANCERBIO-030617-050549

**Publisher:** Annual Reviews

**Permanent Link:** <https://hdl.handle.net/1721.1/124760>

**Version:** Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

**Terms of use:** <http://creativecommons.org/licenses/by-nc-sa/4.0/>





*Annual Review of Cancer Biology*

# Harnessing Protease Activity to Improve Cancer Care

Jaideep S. Dudani,<sup>1,2</sup> Andrew D. Warren,<sup>1,3</sup>  
and Sangeeta N. Bhatia<sup>1,3,4,5,6,7</sup>

<sup>1</sup>Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA; email: jdudani@mit.edu, andrew.warren11@gmail.com, sbhatia@mit.edu

<sup>2</sup>Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

<sup>3</sup>Harvard-MIT Division of Health Sciences and Technology, Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

<sup>4</sup>Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

<sup>5</sup>Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA

<sup>6</sup>Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts 02139, USA

<sup>7</sup>Howard Hughes Medical Institute, Cambridge, Massachusetts 02139, USA

Annu. Rev. Cancer Biol. 2018. 2:353–76

The *Annual Review of Cancer Biology* is online at [cancerbio.annualreviews.org](http://cancerbio.annualreviews.org)

<https://doi.org/10.1146/annurev-cancerbio-030617-050549>

Copyright © 2018 by Annual Reviews.  
All rights reserved

## Keywords

protease activity, degradomics, activity-based biomarkers, responsive nanomaterials, protease-targeted therapeutics, protease-triggered therapeutics

## Abstract

Proteolysis plays critical roles in normal and pathologic physiology; these enzymes are intricately involved in cancer progression and spread. Our understanding of protease function has advanced from nonspecific degrading enzymes to a modern appreciation of their diverse roles in posttranslational modification and signaling in a complex microenvironment. This new understanding has led to next-generation diagnostics and therapeutics that exploit protease activity in cancer. For diagnostics, protease activity may be measured as a biomarker of cancer, with wide-ranging utility from early detection to monitoring therapeutic response. Therapeutically, while broad inhibition of protease activity proved disappointing, new approaches that more specifically modulate proteases in concert with secondary targets might enable



potent combination therapies. In addition, clinical evaluation is underway for tools that leverage protease activity to activate therapeutics, ranging from imaging agents that monitor surgical margins to immunotherapies with improved specificity. Technologies that interact with, measure, or modulate proteases are poised to improve cancer management on diagnostic and therapeutic fronts to realize the promise of precision medicine.

## TOWARD IMPROVED CANCER MANAGEMENT BY LEVERAGING PROTEASE ACTIVITY

Management of cancer will benefit from the integration of biomarkers and precision therapies. Detection of tumors at early stages when resection is especially effective significantly improves outcomes both for patients and the healthcare system (Etzioni et al. 2003), highlighting the need for specific and sensitive biomarkers that can inform clinical decisions (Sawyers 2008). Therapeutics guided by these biomarkers to precisely target the hallmarks of cancer can then significantly impact patient outcomes. Affordability and scalability of these new tools are especially important, as global cancer incidence has reached over 10 million new cases a year and is rising (Kanavos 2006, WHO 2017).

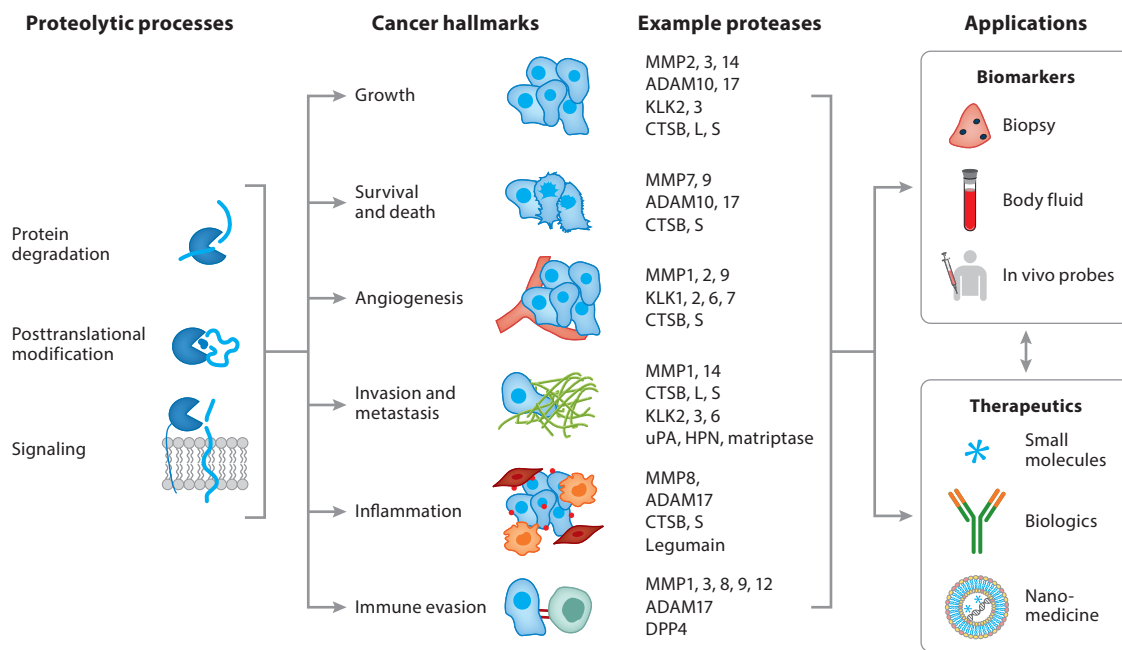
Modern diagnostics and therapeutics have made significant headway with the development of genetic biomarkers, targeted therapies, and immune modulators to enable precision medicine. However, there are fundamental limitations in detection of cancer using blood biomarkers due to nonspecificity, dilution, and degradation of analytes (Hori & Gambhir 2011). An additional complication is our incomplete understanding of genotype/phenotype correlation, limiting the broad application of genetic variants as biomarkers for precision medicine (Friedman et al. 2015). As many biomarkers are discovered through associational studies, their biological roles may not be fully understood. For example, the physiological role of HE4, a blood biomarker for ovarian cancer, was not well understood when it was approved by the US Food and Drug Administration (FDA) (Li et al. 2009). In contrast, functional biomarkers like HER2 and PD-L1 status inform clinical decisions involving treatments with therapeutic antibodies (Topalian et al. 2012, Wolff et al. 2007). With respect to therapeutics, novel approaches that increase tumor-specific activation or rationally target putative resistance pathways will improve the management of cancer. This review aims to highlight new technologies that target or leverage protease activity to tackle some of the challenges posed above and to improve cancer management.

There are over 500 human proteases—around 2% of human genes—that play critical roles in biology by degrading proteins (both intra- and extracellularly), activating zymogens, and regulating signaling (López-Otín & Bond 2008, Rawlings & Salvesen 2013) (**Figure 1**). Proteases are categorized by catalytic type (metallo, cysteine, aspartic, threonine, or serine) and, together with their inhibitors and substrates, compose the degradome (López-Otín & Overall 2002, Pérez-Silva et al. 2016, Rawlings et al. 2016). Proteases play critical roles in almost every hallmark of cancer, and numerous processes regulated by proteases are broadly dysregulated and functionally distinct in tumors (Hanahan & Weinberg 2011). Because of their biological importance, protease activity is normally tightly regulated by the integration of complex signaling pathways (Overall & Blobel 2007).

### Protease Biology in Cancer

While protease dysregulation in cancer has been appreciated since the 1940s, new understanding has paved the way for technologies that use protease activity to improve cancer management (López-Otín & Overall 2002). The many and diverse roles of proteases in the tumor





**Figure 1**

Leveraging protease biology for improved biomarkers and therapeutics. (*Left*) Proteolytic enzymes play critical, fundamental roles in numerous biological processes. (*Middle*) Aberrant protease function (amplified, diminished activity, or altered localization) can contribute to numerous hallmarks of cancer in a complex manner. (*Right*) Technologies that assay protease activity can be used as biomarkers in cancer; therapeutics that can perturb or leverage proteases can be developed for improved efficacy. Abbreviation: ADAM, a disintegrin and metalloproteinase; CTS, cathepsin; DPP, dipeptidyl peptidase; HPN, hepsin; KLK, kallikrein-related peptidase; MMP, matrix metalloproteinase; uPA, urokinase plasminogen activator.

microenvironment have been extensively reviewed, including analysis of the roles of metalloproteinases (Egeblad & Werb 2002, Kessenbrock et al. 2010), cathepsins (Olson & Joyce 2015), and tissue kallikreins (Borgoño & Diamandis 2004). Additionally, proteases are important beyond promoting tumorigenesis, and they play diverse roles in signaling pathways or tumor suppression (López-Otín & Matrisian 2007). We briefly discuss proteases identified in various facets of cancer (Figure 1) but also refer the reader to the references above for a more extensive overview.

**Growth.** Cell growth is controlled through signaling pathways and growth factors, which are affected by protease activity. Several matrix metalloproteinases (MMPs), including MMP2, MMP9, and MMP14, affect the bioactivity of TGF- $\beta$ , which could enhance tumor growth (Kessenbrock et al. 2010). Other proteases like ADAM10 (a disintegrin and metalloproteinase) and ADAM17 can modulate the bioactivity and availability of EGFR, resulting in tumor cell proliferation (Kessenbrock et al. 2010). These sheddases are critical for numerous signaling processes as regulators of cell surface ligands (Peschon et al. 1998). Kallikrein-related peptidases (KLKs), like KLK2, also regulate growth factors (Borgoño & Diamandis 2004). Additionally, cathepsin B (CTSB), CTSL, and CTSS play critical roles in lysosome catabolism necessary for cellular proliferation (Olson & Joyce 2015). These cathepsins seem to have different roles depending on tumor type: For example, *Ctsb* knockout mice have severely delayed tumor growth across several cancers, but the knockout has no apparent effect in a model of squamous cell carcinoma. Additionally, deletion

of *Ctsb* in a mouse model of breast cancer increased *Ctsz* expression, which may mask phenotypic changes (Olson & Joyce 2015). Similar compensatory mechanisms have been uncovered by combinatorial deletion of cathepsins (Akkari et al. 2016). This complex interplay of several proteases and their substrates in cancer growth is a cautionary lesson against targeting a single protease or nonspecifically targeting a family of proteases.

**Survival and death.** Cancer cells upregulate mechanisms to prevent cell death, often by protease-mediated signaling. For example, modulation and degradation of the Fas ligand by MMP7 and ADAM10 may suppress caspase-mediated apoptosis (Kessenbrock et al. 2010, Mitsiades et al. 2001). Similarly, CTSS is upregulated following ionizing radiation, resulting in increased cell survival and reduced therapeutic efficacy (Olson & Joyce 2015).

**Angiogenesis.** The angiogenic switch is necessary for tumors to progress from occult lesions at 1–2 mm in diameter, ensuring appropriate exchange of metabolites and nutrients for growth (Bergers & Benjamin 2003). MMP9 is a potent initiator of angiogenesis through the regulation of VEGF bioavailability (Kessenbrock et al. 2010). Like MMP9, CTSS can generate proangiogenic fragments, but other cathepsins have antiangiogenic properties. The interplay is complicated through degradation of tissue inhibitors of metalloproteinases (TIMPs) by cathepsins and, in turn, degradation of cystatins (cysteine protease inhibitors) by MMPs (Olson & Joyce 2015). Similarly, KLKs regulate angiogenesis through degradation of the extracellular matrix (ECM) and activation of MMPs, with both pro- and antiangiogenic effects (Borgoño & Diamandis 2004).

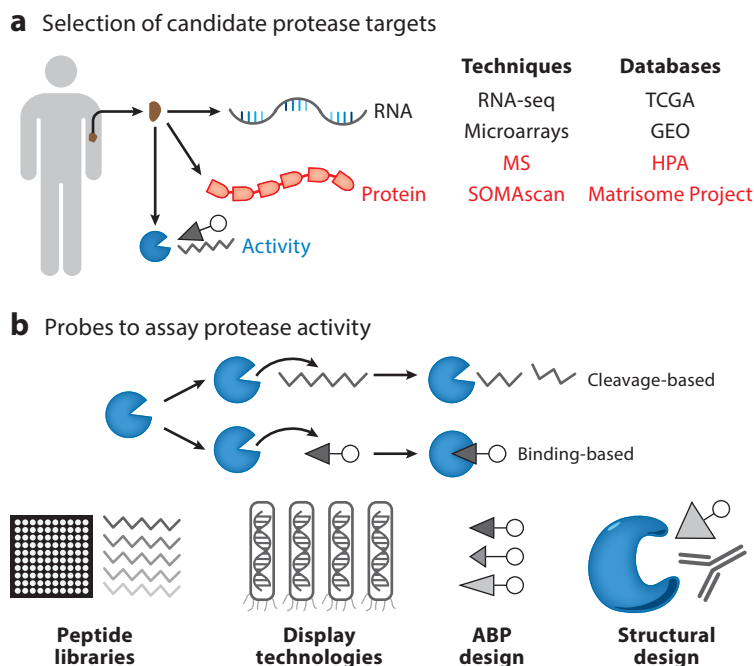
**Invasion and metastasis.** The spread of cancer to distant organs is enabled by the breakdown of the ECM, which is mediated by numerous proteases, including MMPs (e.g., MMP1, MMP14), cathepsins (e.g., CTSB, CTSL, CTSS), kallikreins (e.g., KLK3, KLK6) and other serine proteases [e.g., hepsin, matriptase, urokinase plasminogen activator (uPA)] (Borgoño & Diamandis 2004, Gocheva & Joyce 2007, Kessenbrock et al. 2010, Klezovitch et al. 2004, Uhlund 2006). uPA plays a critical role in ECM degradation in concert with its receptor and plasminogen in part by activating MMPs (Borgoño & Diamandis 2004). These roles in controlling invasion can occur through several mechanisms: KLK1 activates MMP2 and MMP9, while KLK2, KLK4, and KLK15 activate uPA (Borgoño & Diamandis 2004).

**Inflammation.** Sustained inflammation can be protumorigenic through signaling with stromal and immune cells. Activity of TNF- $\alpha$ , a proinflammatory cytokine, is reliant on activation by ADAM17. Additionally, MMP8 activity increases inflammation by generating PGP (N-acetyl Pro-Gly-Pro) and recruiting neutrophils to sites of inflammation (Kessenbrock et al. 2010). Cathepsins and legumain have also been implicated in tissue inflammation when expressed by tumor-associated macrophages (TAMs) (Luo et al. 2006, Olson & Joyce 2015).

**Immune evasion.** Proteases play several roles in protecting tumors from immune surveillance and destruction. Processing of cytokines like CCL8 (by MMP1 and MMP3) and CXCL11 (by MMP8, MMP9, and MMP12) modulates immune cell recruitment. ADAM17 can suppress natural killer (NK) cytotoxicity against tumor cells by shedding major histocompatibility complex class I-related surface proteins (Kessenbrock et al. 2010). Additionally, dipeptidyl peptidase 4 (DPP4) truncates the chemokine CXCL10 and decreases lymphocyte trafficking to tumors (Barreira da Silva et al. 2015).

Clearly, protease contributions to tumor pathogenesis are highly complex (Fortelny et al. 2014, Overall & Blobel 2007). The layers of regulation are further affected by the various cell





**Figure 2**

Protease discovery and analysis tools. (a) Candidate proteases can be selected by assaying cancer samples for transcripts, protein, or activity of proteases. (b) Approaches for measuring protease activity either focus on measuring the cleavage of defined peptide substrates or by binding probes to active proteases. Abbreviations: ABP, activity-based probe; GEO, Gene Expression Omnibus; HPA, Human Protein Atlas; MS, mass spectrometry; RNA-seq, RNA sequencing; TCGA, The Cancer Genome Atlas.

types present in the tumor site, as well as by proteolytic remodeling of the microenvironment (Kessenbrock et al. 2010, Quail & Joyce 2013). The fact that multiple proteases can play a role in the same biological process demonstrates redundancy, suggesting that not all cancers will share a common proteolytic profile. Therefore, selection of target proteases to develop biomarker or therapeutic technologies requires careful consideration and suggests the importance of identifying signatures that incorporate multiple proteases.

## TOOLS TO IDENTIFY AND MEASURE PROTEASE ACTIVITY

The dynamic *in vivo* control of protease function necessitates new technologies that can tackle the problem of dissecting protease activity in disease. Furthermore, a shortfall of sufficiently specific probes for each protease contributes to the challenge of targeting a given enzyme to locally detect or perturb its activity. Here, we describe protease discovery strategies ranging from transcriptomic to activity-based analyses. We additionally discuss approaches for developing better probes for protease activity.

### Selection of Candidate Proteases Involved in Human Cancers

A variety of approaches can be applied to identify proteases as candidate biomarkers or therapeutic targets (Figure 2a; see the sidebar titled Considerations in the Selection of Target Proteases).

## CONSIDERATIONS IN THE SELECTION OF TARGET PROTEASES

The complexity of proteolytic regulation requires significant thought in the development of useful diagnostic or therapeutic approaches. For example, early detection biomarkers should be designed around proteases that play critical roles in tumor establishment and angiogenesis. However, proteases that play roles in metastasis may be ideal for monitoring for metastatic recurrence after therapy. An additional avenue to explore is the noncanonical localization of proteases in cancer. For example, several proteases with typically lysosomal localization have been shown to be secreted by cancer cells (Olson & Joyce 2015); exploitation of these properties may further increase diagnostic or therapeutic specificity. The broadly important nature of proteases indicates that putative targets may also be highly expressed in other tissues elsewhere in the body (e.g., in comorbidities and healthy tissues). These false positives can be mitigated using other 'omic tools and datasets to downselect promiscuously expressed proteases (Table 1). For broadly expressed targets, specificity can be achieved by improving the localization of the diagnostic or therapeutic agent (e.g., through nanotechnology) (Kwon et al. 2015) or by multiplexing. Lastly, it is important to consider the expression of modulators of protease activity, most notably the expression of any relevant endogenous protease inhibitors.

**RNA.** While protease activity is regulated by several mechanisms beyond transcription, transcriptomic analyses (e.g., RNA sequencing, microarrays, NanoString) can be performed with relative ease and low cost, creating an abundance of data available for mining. Combining transcriptomic data with thorough clinical annotation enables rapid hypothesis testing and identification of protease expression patterns that correlate with clinical outcomes (Table 1). For example, PRSS3 (serine protease 3) was identified as upregulated in metastatic prostate cancer through analysis of microarray data, and this relationship was validated experimentally in prostate cancer cell lines (Hockla et al. 2012).

**Protein.** Proteomic analysis of tissues, biological fluids, cell lines, and other samples is a powerful approach to identifying proteases that may be relevant to biological processes of interest (Hanash et al. 2008, Uhlén et al. 2015). Proteomics (by immunohistochemistry or mass spectrometry) have yielded insight into protease abundance (Table 1) (Naba et al. 2012, Uhlén et al. 2015): Several proteases (including ADAMs and MMPs) and their inhibitors are more abundant in metastatic cell lines (Naba et al. 2014). New technologies that improve protein-level quantification such as SOMAscan, a highly multiplexed aptamer-based approach for measuring protein abundance, could improve protease target discovery. SOMAscan applied to non-small-cell lung cancer patient samples identified several proteases that play critical roles in invasion and inflammation, such as uPA, MMP7, and MMP12 (Mehan et al. 2012).

**Activity.** Activity-based analyses ensure that the discovered protein is functional and therefore more likely to play a causal role in the disease. Importantly, this approach precludes the selection of candidate proteases whose activity is blocked by locally expressed inhibitors. One embodiment of this category of assays is a class of molecules known as activity-based probes (ABPs) that covalently react with the active site of a protease using a chemical warhead and that can incorporate various recognition moieties. ABPs can profile serine hydrolases in cancer cell lines of varying invasiveness to identify candidate enzymes (Jessani et al. 2002). They have also been applied to validate the activity of cysteine cathepsins identified through gene expression analysis of pancreatic islet tumors from a transgenic mouse (Joyce et al. 2004). However, as discussed below, these tools



**Table 1** Useful publicly available resources for protease selection and understanding

Database	URL	Description	Reference
cutDB	<a href="http://cutdb.burnham.org/">http://cutdb.burnham.org/</a>	Contains endogenous substrate information of proteases and can be queried by disease type.	Igarashi et al. 2007
Degradome database	<a href="http://degradome.uniovi.es/dindex.html">http://degradome.uniovi.es/dindex.html</a>	Contains information on proteases, inhibitors, and their orthologs.	Pérez-Silva et al. 2016
Gene Expression Omnibus (GEO)	<a href="https://www.ncbi.nlm.nih.gov/geo/">https://www.ncbi.nlm.nih.gov/geo/</a>	Repository that can be queried for expression datasets of interest.	Barrett et al. 2013
Genotype-Tissue Expression (GTEx) project data set	<a href="https://www.gtexportal.org/home/">https://www.gtexportal.org/home/</a>	Multi-organ expression data from normal samples, which may be useful for downselecting promiscuously expressed proteases.	GTEx Consort. 2013
Human Protein Atlas	<a href="https://www.proteinatlas.org/">https://www.proteinatlas.org/</a>	Contains immunohistochemistry staining of many proteases as well as analyses in cell lines. These data provide useful tissue-based analysis, enabling removal of promiscuously expressed protease candidates.	Uhlén et al. 2015
MEROPS	<a href="https://www.ebi.ac.uk/merops/">https://www.ebi.ac.uk/merops/</a>	Contains information on proteases and inhibitors, including putative cleavage specificities for proteases and information on potential disease roles.	Rawlings et al. 2016
The Cancer Genome Atlas (TCGA)	<a href="https://cancergenome.nih.gov/">https://cancergenome.nih.gov/</a>	Contains expression data on many cancers and patient samples with extensive clinical annotation.	
The Matrisome Project	<a href="http://matrisomeproject.mit.edu/">http://matrisomeproject.mit.edu/</a>	Contains data from mass spectrometry analysis of human and mouse primary and metastatic tumors compared to normal samples.	Naba et al. 2012

are limited by the catalytic mechanism of the protease. Other techniques such as substrate cleavage assays, in conjunction with alternative analytical approaches, can enable pan-protease discovery (O'Donoghue et al. 2012).

### Probe Development for Protease Activity

Two primary approaches for measuring protease activity are (*a*) substrates that result in signal generation after proteolytic cleavage and (*b*) probes that bind active proteases (**Figure 2b**) (Edgington et al. 2011, Sanman & Bogyo 2014). A key feature of substrate-based probes is the diffusion of the cleavage fragment away from the protease, which enables unique detection modalities but hampers identification/localization of the target protease. Alternatively, binding-based approaches enable the specific identification of target proteases by downstream biochemical analysis, but they directly modify the enzyme and therefore offer less flexibility in assay design and implementation. The large number of techniques to develop these types of probes is indicative of the challenges inherent to assaying protease activity.

**Cleavage-based assays.** When developing substrate probes, it can be helpful to start with the natural protein cleavage site of the protease of interest. Powerful approaches can be used to

understand the substrate specificities of proteases in complex mixtures, such as in cellular lysates (Agard et al. 2012, Kleifeld et al. 2010). For example, the terminal amine isotopic labeling of substrates approach, which identifies peptide N termini generated after proteolysis, identified over 200 cleavage sites for MMP2 in 150 substrates using fibroblast protein homogenates (Kleifeld et al. 2010).

However, natural substrate recognition sequences may not be ideal for use as probes due to their presentation, specificity, or overall cleavage rate. Several peptide library-based approaches can be used to develop probes, including positional scanning libraries (Schneider & Craik 2009), peptide microarray technologies (Salisbury et al. 2002), fluorogenic peptides (Harris et al. 2000, Kwong et al. 2013, Miller et al. 2011), and other mixture-based peptide libraries (Schilling & Overall 2008, Turk et al. 2001). Each approach has its own limitations; for example, positional scanning libraries often only provide non-prime-site specificity of the substrates (i.e., P1–P4 amino acid positions) and do not provide information on subsite cooperativity, while peptide microarrays may exhibit altered substrate presentation compared to untethered substrates. A recently developed multiplexed substrate library for global identification of peptidase specificity with mass spectrometry readouts has shown promise for identifying substrates to assay protease activity in biological samples and for providing substrate specificity across protease types (O'Donoghue et al. 2012). This tool enables rapid screening for specific probes using cancer biopsy tissues. Additionally, unnatural amino acids have been incorporated into peptide libraries and often result in more selective probes, as was recently demonstrated for neutrophil elastase (Kasperkiewicz et al. 2014).

Evolution-based display technologies have also been used to identify better substrates for proteases and may become more powerful with higher-throughput sequencing and improved bioinformatics (Deng et al. 2000, Ratnikov et al. 2009). For example, phage display can be applied to complex samples to find probes that are selectively activated in the tumor by performing parallel *in vitro* and *in vivo* selection (Whitney et al. 2010). However, it is difficult to assay the kinetics of substrate cleavage with these approaches due to the single timepoint measurement, which may result in the selection of specific probes with poor cleavage rates. Another screening approach using cellular libraries of peptide substrates (CLiPS) involves tethered protease substrates on the surface of *Escherichia coli*, where cleavage can be monitored by flow cytometry (Boulware & Daugherty 2006).

Understanding of protease binding pockets can further improve hit substrates. For example, the use of a traditional Förster resonance energy transfer substrate that subsequently incorporated unique analogs that fit into the substrate pocket of MMP9 resulted in a markedly improved probe for the activity of MMP9 that was not cleaved by other MMPs (Tranchant et al. 2014).

However, substrate promiscuity for small peptides is common, highlighting the importance of multiplexing. This can be achieved by profiling many proteases simultaneously to provide specificity maps and to potentially deconvolve protease signals (Kwong et al. 2013, Miller et al. 2011, O'Donoghue et al. 2012). Ultimately, as tumor-specific protease profiles are generated, multiplexed substrate-based technologies could be developed to target proteolytic signatures.

**Binding-based assays.** Alternatively, probes that bind active proteases serve as a valuable tool in assaying function by enabling direct identification of the bound enzyme (Edgington et al. 2011). The primary class of these are ABPs that covalently bind into the active site of proteases, typically by reacting with a nucleophile on the protease or using a photocrosslinking step for metalloproteinases and aspartyl proteases (Cravatt et al. 2008, Sanman & Bogyo 2014). The design and development of numerous ABPs have been reviewed recently and readers are referred to Sanman & Bogyo (2014) as a useful resource (see the sidebar titled Activity-Based Probes). As a separate approach, several antibodies have been generated to recognize only active proteases by



## ACTIVITY-BASED PROBES

As discussed in the text, ABPs have wide-ranging utility, from discovery of candidate proteases to *in vivo* imaging. The key feature that enables covalent attachment of ABPs is the reactive functional group that binds to the protease of interest. Several variant reactive groups have been developed to profile different classes of proteases, but significant challenges remain in the design of such probes for aspartyl proteases and metalloproteinases (Sanman & Bogyo 2014). Approaches for ABPs for MMPs typically require a photocrosslinking step to lock in the probe near the protease (Saghatelian et al. 2004). Target specificity for proteases can be enhanced by using a recognition domain, for example, by using protease inhibitors or substrates as the reactive warhead (Cravatt et al. 2008, Sanman & Bogyo 2014). Many of the approaches for making peptide substrates discussed in the main text can also be used to select the recognition sequence in the ABP.

binding either the active site or other epitopes veiled in zymogens, enabling their use as probes (Darragh et al. 2010). Binding-based probes can be engineered to be highly specific for a single protease, which can obviate multiplexing for well-validated targets.

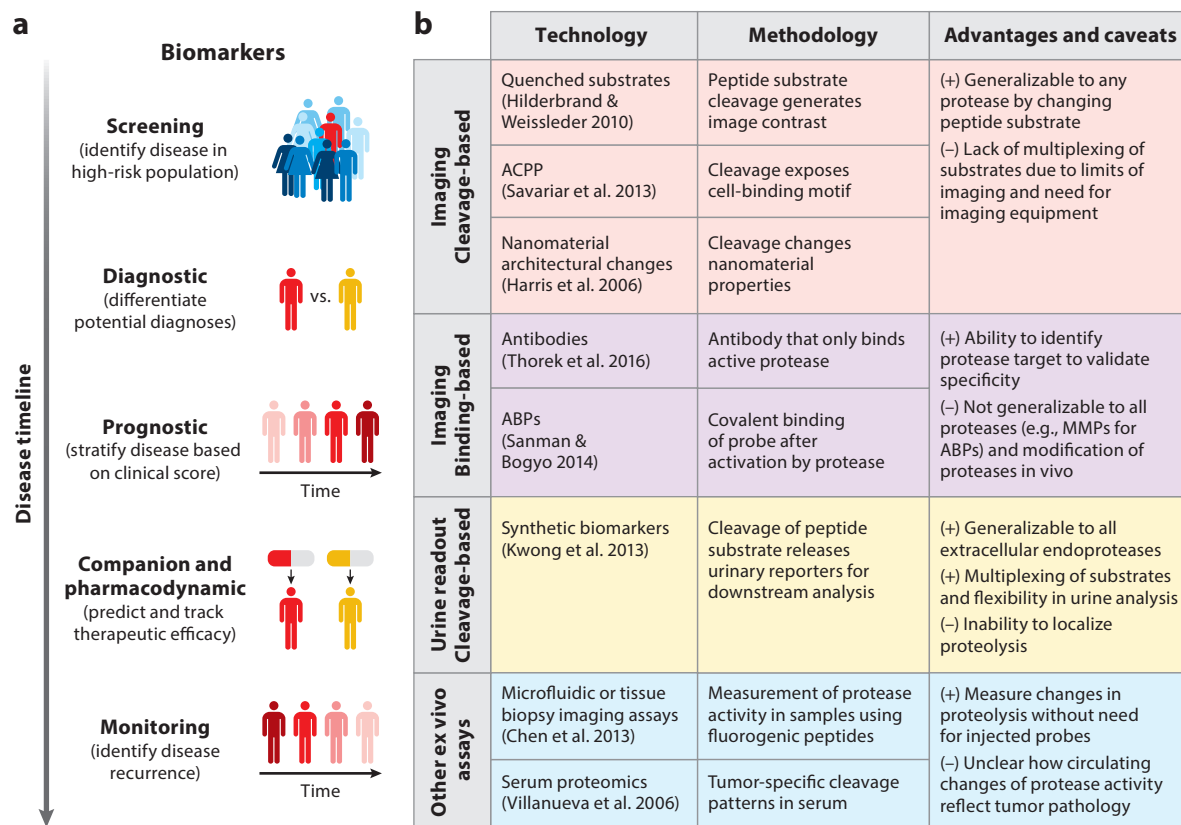
## PROTEASE ACTIVITY AS A FUNCTIONAL BIOMARKER OF CANCER

Biomarkers are objectively measured indicators of pathological processes or of response to treatment and have an incredible potential to impact disease outcomes (**Figure 3a**) (Inst. Med. 2007, La Thangue & Kerr 2011, Sawyers 2008). However, many biomarkers are disease by-products and are only correlated (or associated) with the disease; therefore, their clinical use is often plagued by insufficient sensitivity and specificity (Prensner et al. 2012). In contrast, assaying for biomarkers that are causal in disease progression may improve patient care by being more predictive. As proteases play critical roles in cancer progression, protease activity measurements may serve as robust biomarkers (**Figure 3b**). Additionally, protease-based biomarkers have the potential to outperform clinically approved blood biomarkers, which often suffer from low sensitivity (Hori & Gambhir 2011), by leveraging the catalytic nature of proteases for signal amplification (Kwong et al. 2015).

Biomarkers can be deployed for screening and detection, prognosis, prediction of therapeutic efficacy, and monitoring of therapeutic response (La Thangue & Kerr 2011, Prensner et al. 2012, Sawyers 2008). The design criteria for a good biomarker will necessarily be informed by the type of biomarker needed (**Figure 3a**; see the sidebar titled Considerations in the Selection of Target Proteases). Additionally, the need for biomarkers varies across cancer type: For example, prostate cancer care could be most improved by prognostic biomarkers that stratify aggressive disease at the time of diagnosis (Prensner et al. 2012), while ovarian cancer care may be most impacted by achieving early detection, as a majority of patients are diagnosed at late stages (Brown & Palmer 2009, Natl. Cancer Inst. 2017).

**Early detection and diagnosis.** The first steps in enabling therapeutic intervention are accurate detection and diagnosis, either from screening efforts or upon clinical presentation. Numerous detection approaches function by imaging the proteases involved in the early stages of cancer progression. The most common tools are substrate-based probes that fluoresce, typically in the near-infrared spectrum, upon proteolytic cleavage in the tumor microenvironment (Grimm et al. 2005, Hilderbrand & Wiessler 2010, Wiessler 2006, Wiessler et al. 1999). For example, the ProSense<sup>®</sup> probe (from PerkinElmer), which generates a signal upon cathepsin cleavage,





**Figure 3**

Protease activity measurements as biomarkers. (a) Biomarkers can guide management throughout disease progression, ranging from screening platforms to measuring efficacy of therapeutics. (b) Technologies being developed to assay protease activity as biomarkers in cancer. Thematically, these approaches image proteolysis in vivo, rely on renal clearance of cleavage fragments in vivo to achieve urine readouts, or measure proteolysis ex vivo. Abbreviations: ABP, activity-based probe; ACPP, activatable cell-penetrating peptide; MMPs, matrix metalloproteinases.

resolved millimeter-sized tumors in a mouse model of lung cancer using three-dimensional fluorescence molecular tomography (Grimm et al. 2005). A newly developed analogous technique called magnetic resonance tuning involves separating a paramagnetic enhancer molecule from a superparamagnetic quencher using a protease cleavable substrate. Upon proteolysis, the  $T_1$  signal is increased (Choi et al. 2017). This new technology could overcome many of the limitations of optical imaging protease activity in vivo.

Other approaches involve material changes to probes that can be imaged upon proteolysis of a substrate. One technique releases a cell-penetrating peptide, termed activatable cell-penetrating peptide (ACPP), to image cancer cells after proteolysis, typically by MMPs (Jiang et al. 2004). This technology has been improved with ratiometric dye ACPPs (RACPPs), where two dyes are placed on the same ACPP (Savariar et al. 2013), or with nanoparticles for dual fluorescence imaging and magnetic resonance imaging (MRI) (Olson et al. 2010). Similarly, hydrophobic molecules containing a positron-emission tomography (PET) agent can be released from a hydrophilic domain after proteolysis to enhance tumor-specific PET signals (Chuang et al. 2012). Substrate cleavage methods have also been used to drive iron oxide nanoparticle self-assembly to alter  $T_2$  measured by MRI

(Harris et al. 2006, von Maltzahn et al. 2007) or to unveil bioactive motifs presented on the particle surface (Harris et al. 2008). Nanoparticle systems offer numerous multimodal advantages that have been used for various applications in protease detection (Kwon et al. 2015, Welsler et al. 2011).

Alternatively, binding-based imaging probes have been developed for protease detection. Examples include antibodies developed to detect tumors expressing active matriptase, which is up-regulated in many cancers (Darragh et al. 2010, LeBeau et al. 2013). Additionally, quenched ABPs (qABPs) have been used to image tumors in vivo (Blum et al. 2007, Edgington et al. 2013, Verdoes et al. 2013). qABPs can also query features of tumors such as inflammatory state, as demonstrated by a probe specific for legumain (Edgington et al. 2013), which is highly expressed in TAMs (Luo et al. 2006). An important strength of ABPs in comparison to antibodies is their capacity to enter cells to image intracellular proteases. These probes have also shown promise in imaging proteolytic activity in excised tissues, which may prove valuable in rapidly analyzing surgical samples (Withana et al. 2016).

In contrast to imaging approaches that limit multiplexing, our lab has developed protease-responsive nanoparticles termed synthetic biomarkers, where proteolytic cleavage liberates small reporters that are cleared by the kidney and detected in the urine (Kwong et al. 2013). Synthetic biomarkers offer sensitivity advantages in tumor detection due to enzymatic amplification and renal concentration of reporter fragments. Detection of the urinary fragments can be highly multiplexed with heavy isotope-encoded peptide barcodes detected by mass spectrometry (Kwong et al. 2013) or ligand-encoded barcodes detected by ELISA (Warren et al. 2014b), digital ELISA (Warren et al. 2014a), and point-of-care paper-based assays (Dudani et al. 2016, Warren et al. 2014b). Ten-plex synthetic biomarkers that were responsive broadly to MMPs outperformed a clinically approved blood biomarker, CEA, in a mouse model of colorectal cancer (Kwong et al. 2013). A recently built mathematical framework for synthetic biomarker performance in vivo identified strategies for ultrasensitive tumor detection for diameters below 5 mm, which is the current limit for many imaging modalities (Kwong et al. 2015). Leveraging the insights from this mathematical model, researchers engineered a more sensitive synthetic biomarker, using a single substrate for MMP9, to detect small tumor lesions by combining improved substrate presentation with molecular targeting techniques, resulting in an order-of-magnitude improvement in the limit of detection (Kwon et al. 2017).

**Prognostic.** Identifying patients that are likely to progress rapidly and need therapeutic intervention is essential. There are numerous proteases with apparent upregulation in patients with recurrent cancer. For example, breast cancer patients with elevated MMP9 have decreased survival and increased relapse rates, possibly due to the increased invasive potential of tumors (Dufour et al. 2011).

Several of the approaches discussed above have shown promise as prognostic biomarkers. For example, RACPPs identified invaded lymph node metastases, with fluorescent signal correlating with percent lymph node invasion (Savariar et al. 2013). Sustained fibrosis and inflammation can often lead to cancer, so classifying advancing or resolving fibrosis may provide insight into tumor progression; when administered to mice with resolving liver fibrosis, synthetic biomarkers were able to identify mice with resolving disease at earlier timepoints than histopathological slide analysis (Kwong et al. 2013). The activity of proteases that play a causal role in cancer pathogenesis could predict pathology prior to current gold standards, although these approaches need to be evaluated in a clinical setting.

**Companion and pharmacodynamic.** Pairing diagnostic and therapeutic strategies is essential for precision medicine. Novel protease-activated therapeutics could be paired with technologies to assay tumor proteolytic signatures or predict patient response for improved potency and



therapeutic development. Recently, our lab has developed a pair of technologies that can be selectively activated in the tumor microenvironment *in vivo* to report on local protease activity. One approach uses a photolabile protecting group to unmask a protease substrate only upon treatment by light at the site of disease (Dudani et al. 2015), while another approach releases protease substrates into the microenvironment from a thermosensitive liposome after triggering with alternating magnetic fields (Schuerle et al. 2016). These approaches can be more readily multiplexed with urinary readouts to assess multiple substrates across a range of tumor models. Alternative techniques discussed above could be applied similarly.

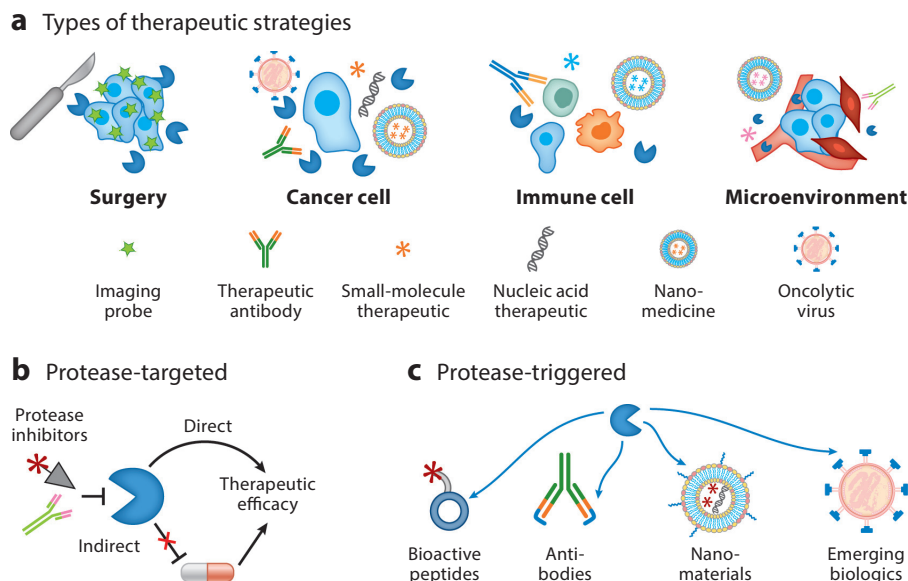
Pharmacodynamic monitoring enables the rapid evaluation of drug efficacy. Many techniques can monitor caspase activity as pharmacodynamic biomarkers of apoptosis, including caspase-targeted ABPs to monitor the efficacy of a monoclonal antibody mimic of TRAIL (Edgington et al. 2009) and a caspase-triggered assembly of nanostructures to image the dynamics of tumor response to doxorubicin (Ye et al. 2014). Similarly, a quenched MMP substrate was able to report on the efficacy of the MMP inhibitor, prinomastat, *in vivo* (Bremer et al. 2001). These approaches may also become useful as new protease-targeted therapeutics advance.

**Disease kinetics and monitoring.** Serial monitoring of protease activity in patients following treatment may provide early indicators of relapse. These assays often need to be simple and performed at the point of care, which is more feasible with *ex vivo* protease assays such as zymography or some of the imaging assays mentioned above (Vandooren et al. 2013). Active proteases have been found in numerous biological samples, including urine and blood (Roy et al. 2009), which might serve as a useful proving ground for the development of biomarkers that measure protease activity. Other approaches for rapid, multiplex profiling of clinical samples for protease activity include microfluidic sampling and measuring cleavage of fluorogenic peptide substrates in droplets (Chen et al. 2013). One risk of these approaches is that protease activity detected in the circulation may not accurately reflect the local pathology, as the enzymes largely act in the tumor microenvironment. Alternatively, measuring endogenous protease cleavage fragments could be useful as a monitoring paradigm; studies have found differential exoprotease activity between cancer types that generated unique peptide fragments in serum, which provided unique signatures for prostate, bladder, and breast cancer (Villanueva et al. 2006).

A different approach to monitoring therapeutic response *in vivo* used a radiolabeled antibody probe for active KLK2 to image the androgen receptor (AR) pathway in prostate cancer, which is an important therapeutic target (Thorek et al. 2016). KLK2 is regulated by the AR pathway, and the radiolabeled antibody was able to monitor the response to castration in xenograft cancer models and genetically engineered mouse models. This probe showed improved specificity of measuring KLK2 versus standard-of-care measurements of PSA (KLK3) in serum and imaging using  $^{18}\text{F}$ -sodium fluoride, demonstrating the critical importance of protease biomarker selection.

**Emerging approaches.** Many exciting new techniques to assay protease activity are under development and may find unique biomarker applications. One example uses endogenous proteases in samples to activate circuits built using principles of synthetic biology (Stein & Alexandrov 2014). Additionally, approaches that provide information on multiple aspects of tumor biology beyond protease expression (e.g., hypoxia, pH, metabolism) may become valuable for more complete understanding of tumor pathology. In one example, labeled sugar molecules were caged by a peptide to prevent incorporation unless cleaved by KLK3/PSA; this approach could be expanded to provide information on both protease expression and glycan biosynthesis in cancer (Chang et al. 2010).





**Figure 4**

Protease therapeutic approaches. (a) Types of therapeutic strategies to harness protease activity that have shown promise. These include image-guided surgery, cancer cell targeting with various therapeutics, immune cell targeting (e.g., T cells, macrophages, natural killer cells), and tumor microenvironment targeting. (b) Protease inhibition can be therapeutic directly (by blocking cancer-promoting functions) or indirectly (by modulating the efficacy of other drugs). (c) Proteases can act as triggers to activate various types of therapies, such as antibodies and nanomaterials.

## THERAPEUTIC TARGETING OF PROTEASE ACTIVITY

Proteases have been attractive therapeutic targets due to their critical roles in cancer (Figure 4). While these discussions focus on the signaling and extracellular matrix functionalities of proteases, there are also diverse roles for proteases in the proteasome and elsewhere that can be therapeutically targeted (reviewed by Manasanch & Orlowski 2017, among others). Studies in the 1990s and 2000s targeted MMPs with inhibitors with the simple hypothesis that broad inhibition would hinder cancer progression; these treatments were ineffective due to an underdeveloped understanding of the disease process and the broad-spectrum nature of these therapeutics (Coussens et al. 2002). More nuanced understanding of protease biology and more selective therapies have renewed interest in proteases as therapeutic targets. Novel protease-targeting strategies potentiate efficacy of or minimize resistance to other therapies (Figure 4b). Analogous to prodrugs, another strategy uses protease activity to trigger therapeutic release without directly modulating the enzyme itself (Figure 4c).

### Therapeutic Inhibition of Active Proteases

Past failures in broad-spectrum protease inhibitors demonstrated the importance of target selection and pharmacologic specificity (Coussens et al. 2002, Vandenbroucke & Libert 2014). Increased specificity can be achieved by new screens that use ABPs as the readout, enabling simultaneous testing of a compound on both on- and off-target enzymes (Bachovchin et al. 2014). Alternatively, targeting unique structural sites such as the hemopexin domain of MMP9, which is

not highly conserved across MMPs, has the potential for improved selectivity and efficacy (Dufour et al. 2011).

Biologics enable new applications and targets typically inaccessible to small molecule approaches. For example, therapeutic antibodies specific to activated proteases have been evaluated in preclinical tumor models (Devy et al. 2009, Marshall et al. 2015). An allosterically binding MMP9 antibody significantly reduced tumor burden and metastasis in an orthotopic colorectal cancer mouse model without inducing toxic effects seen in broad-spectrum inhibitors (Marshall et al. 2015). Antibody variants (e.g., camelids) and antibody-like molecules (e.g., knottins) could further improve therapeutic targeting of proteases (Kromann-Hansen et al. 2016). In one example, camelid-like features were incorporated onto a human antibody scaffold to identify potent inhibitors of MMP14 (Nam et al. 2016). Therapeutics like these could uniquely target extracellular signaling and processing.

### Modulating Protease Biology to Enhance Therapies

Beyond simple inhibition, proteases can modulate immune function and mediate therapeutic resistance; many research groups have investigated combination therapies to thwart resistance or potentiate existing treatment. Proteolytic pathways that elicit therapeutic resistance are enhanced in doxorubicin-treated cancer cells; in one example, MMP7 cleaved Fas ligand off the cell surface, resulting in decreased cell death (Mitsiades et al. 2001). In similar studies, MMP9 had a tumor-protective effect and elimination MMP9 improved treatment efficacy (Nakasone et al. 2012). Cathepsins also promote therapeutic resistance, which can be counteracted by cathepsin inhibitors (Olson & Joyce 2015). Counter to the idea that increased protease activity supports resistance, MAPK inhibition decreases receptor shedding by proteases like ADAM10 and ADAM17, increasing cell signaling and growth; neutralizing the cognate inhibitor TIMP1 rescued therapeutic efficacy (Miller et al. 2016).

Proteolytic involvement in immune system evasion by tumors constitutes an additional route to potentiate immunotherapies. For example, DPP4 modifies chemokines to prevent lymphocyte trafficking to tumors, and DPP4 inhibition with sitagliptin (used clinically for type II diabetics) improved tumor control alone and in combination with adoptive T cell therapy or checkpoint inhibitors (Barreira da Silva et al. 2015). Additionally, NK cells, which engage in antibody-dependent cellular cytotoxicity, express CD16, which can bind the Fc portion of therapeutic antibodies to kill tumor cells. The shedding of this receptor is partially regulated by metalloproteinases; in combination with therapeutic antibodies or bispecific NK cell engagers, metalloproteinase inhibition potentiated NK cell function by sustaining CD16-mediated signaling (Romee et al. 2013, Wiernik et al. 2013, Zhou et al. 2013). These therapeutic strategies provide exciting options for next-generation and combination immunotherapies.

### Active Proteases as Therapeutic Triggers

Protease-activated therapeutics have existed since the 1980s as protease-cleavable moieties coupled to chemotherapies (Choi et al. 2012). Novel protease-triggered therapeutics incorporate more sophisticated activation strategies in the tumor microenvironment, which is particularly useful for therapeutics with dose-limiting off-target toxicity (**Figure 4c**).

**Bioactive peptides.** Peptides are small and may be synthetically manufactured with relative ease. More than 60 FDA-approved peptide therapeutics are on the market, with a multitude of



mechanisms of action; at least 140 additional peptide therapeutics are in various stages of clinical development (Fosgerau & Hoffmann 2015). One class is ACPPs. ACPP-guided surgery improved survival in two xenograft mouse models and is currently being evaluated by Avelas Biosciences in a phase I trial for breast cancer surgery (Nguyen et al. 2010). Another ACPP approach uses a cyclic RGD to first bind  $\alpha_v\beta_3$  integrins; following proteolysis by MMP2, the peptide delivers chemotherapeutics intracellularly (Crisp et al. 2014). In a related approach, protease-masking domains cleaved by PSA or PSMA were coupled to pore-forming peptides (LeBeau & Denmeade 2015). This approach is compatible with exoproteases like PSMA, in contrast with ACCPs, which have typically required endoprotease cleavage.

Beyond enhanced cellular accumulation, another class of protease-activated cyclized peptides potentiate tumor penetration. Identified by phage display, these tumor-penetrating peptides bind to a primary tumor-associated receptor (e.g., integrins) and are cleaved by a protease to expose a secondary binding domain for neuropilin-1, which initiates tumor penetration and potentiation of drug delivery (Ruoslahti et al. 2010). It is possible to engineer different cleavage domains to modify protease specificity; for example, a uPA consensus cleavage domain was incorporated into tumor-penetrating peptide domains and combined with RGD for integrin binding (Braun et al. 2016). This approach allows therapeutics to more specifically match the protease profiles of patients as identified by companion diagnostics.

**Antibodies.** Therapeutic antibodies account for a significant fraction of cancer therapeutics and are now driving the immune oncology revolution with checkpoint blockade drugs. However, many antibodies and antibody-drug conjugates (ADCs) have significant toxicity. To mitigate off-target release of drug molecules from antibodies, researchers have engineered cleavable linkers sensitive to lysosomal proteases (Doronina et al. 2003). Brentuximab vedotin, an FDA-approved ADC developed by Seattle Genetics, targets CD30 overexpressed in lymphomas and is conjugated to an antimetabolic drug (vedotin) by a cathepsin-cleavable linker. This allows for selective drug delivery to tissues with high protease activity (both extracellular and intracellular).

Another exciting technique masks antibody binding using linkers cleaved by extracellular tumor-associated proteases (Desnoyers et al. 2013). This so-called probody technology, developed by CytomX, improves specificity based on tumor protease expression and therefore can more broadly target expressed antigens of interest. One example is a modified EGFR antibody with a masking substrate identified through the CLiPs approach described above; the resultant probody only binds EGFR following cleavage by matriptase, uPA, or legumain. Another probody targeting PD-L1 is under evaluation in phase I studies. Similarly, MMP1-cleavable masking domains have been applied to Affibodies (pro-Affibody), a type of antibody mimic (Sandersjö et al. 2015). As next-generation protein therapeutics are engineered, improving specificity by leveraging protease activity will be an attractive approach.

**Nanomaterials and polymeric conjugates.** Nanomaterials are promising anticancer agents, in part due to their ability to carry various therapeutic cargos and to engineer emergent function into nanosystems, including protease-responsive elements (Kwon et al. 2015). One approach releases therapeutic drugs upon disruption by protease cleavage, such as polymer-caged liposomes that become highly unstable after cleavage by uPA to enable burst release of therapeutics (Basel et al. 2011). Another approach for rapid drug release used silica nanoparticles with MMP-degradable polymeric coatings (Singh et al. 2011).

Similar to probodies, an alternative approach unveils responsive elements on nanoparticles following proteolysis. Lipid nanoparticles generally suffer from nonspecific liver accumulation;



protease-based approaches may enable nonliver targeting. For example, incorporating polyethylene glycol (PEG)-ylated lipids with an MMP-cleavable linker into lipid nanoparticles improves on-target delivery of small interfering RNA by increasing nanoparticle binding to tumor cells (Hatakeyama et al. 2011). Others have harnessed nanomaterials with proteolytically unveiled cell-penetrating motifs (e.g., TAT peptide) to internalize paclitaxel (Zhu et al. 2013). These approaches should be generalizable to various cargo and material types (Harris et al. 2006, 2008) such as combination therapies with short hairpin RNA and small molecules (Huang et al. 2013) or intracellular delivery of proteins using nanocapsules (Biswas et al. 2011).

An additional approach under clinical evaluation is imaging of protease activity using a quenched fluorophore peptide-polymer (PEG) conjugate that detects cathepsin activity (Whitley et al. 2016). Here, the protease imaging agent is combined with a handheld imaging device that provides real-time, high-resolution imaging during debulking surgery to improve surgical margins. The probe is strongly activated in mouse xenograft tumors and in human patients with soft tissue sarcoma or breast cancer; this technology is being developed by Lumicell and is under evaluation in phase II trials. Protease responsive materials can also be integrated into carriers: An emerging alternative to PEG is XTEN, a biologically produced repetitive polypeptide under development by Ammunix, which extends the half-life of proteins like PEGylation does but with greater homogeneity and biological functionality (Schellenberger et al. 2009). In one example of this approach, XTEN was synthesized with an ACPP domain and a cytostatic/cytotoxic peptide agent on a single plasmid (Haeckel et al. 2016).

**Emerging biologics (toxins, viruses, and cellular therapies).** With the success of therapeutic antibodies, next-generation biologics with diverse functions are being explored. Bacterial toxins (e.g., anthrax and diphtheria toxin) are biologically inspired technologies that are being leveraged for tumor treatment and therapeutic cargo delivery. The protective antigen (PA) on anthrax toxin is normally processed by furin-like proteases to oligomerize and provide binding sites for other toxin components. These PA cleavage sites can be modified to target cancer cells expressing MMPs (Liu et al. 2000) or uPA (Liu et al. 2003). Such approaches, combined with altering lethal factor binding sites, have shown exceptional specificity and low toxicity in mouse models of melanoma (Liu et al. 2005). Toxins can also be linked to antibody fragments to form immunotoxins, which may benefit from protease specificity redirection (Pastan et al. 2007).

Oncolytic viruses represent another class of emerging biologics. These viruses can directly kill tumor cells and subsequently activate antitumor immunity (Kaufman et al. 2015). Reprogramming viral specificity through protease activity may improve their efficacy; one example is the engineering of proteolytic activation into a Sendai virus by replacing a tryptic cleavage domain in the fusion glycoprotein with an MMP-cleavable linkage (Kinoh et al. 2004). The resulting oncolytic virus spread in a xenograft tumor that expressed active MMPs and significantly inhibited tumor growth. Measles virus may be similarly engineered to be activated by MMPs and uPA for the treatment of hepatocellular carcinoma (Mühlebach et al. 2010). Bacterial therapeutics are also under development with advances in synthetic biology circuits to encode advanced functions, such as releasing or detecting enzymes based on quorum sensing (Danino et al. 2015, Din et al. 2016). These could be developed to sense and respond to proteolysis in the tumor microenvironment.

With advances in manufacturing and improved vectors, cell therapies are poised for clinical impact. Chimeric antigen receptor (CAR) T cells are engineered *ex vivo* to specifically retarget cytotoxic T cells to tumor antigens and have shown significant clinical promise but are often limited by systemic toxicity. An approach to mitigate off-target recognition is the addition of protease-cleavable masks (similar in nature to the proboddy) that are removed by uPA, matriptase, and legumain (Han et al. 2017). In this study, the masked CAR T cell had improved specificity while



retaining similar efficacy to unmasked variants in a lung xenograft, although clinical evaluation is pending.

### SUMMARY POINTS

1. Proteases are involved in every hallmark of cancer but often have complex roles. Redundancy in protease action and variation in protease expression by organ microenvironment and tumor type underline the importance of validating protease levels and activity in the human cancers of interest.
2. Several approaches have been developed to identify candidate proteases. Using probes that directly measure protease activity is ideal, but this is balanced by the lack of available data and tools compared to more standardized approaches, such as RNA sequencing.
3. Protease activity is often promiscuous and designing selective probes can be challenging. Multiplexing probes may enable specific protease activity measurements.
4. Functional biomarkers are likely more predictive for tumor progression than associated biomarkers. Activity-based biomarkers that can amplify protease signals might enable more sensitive cancer detection.
5. Numerous platform technologies have been developed to measure protease activity as biomarkers of cancer, but these must be evaluated in patients.
6. The complexity of protease function in cancer suggests that it is important to target them in combination with other therapies; for example, inhibiting proteases to potentiate therapies that target another aspect of cancer biology could improve outcomes.
7. Protease-triggered therapies can benefit from improved pharmacokinetics by tumor microenvironment activation. This consideration is especially important when applied to potentially toxic therapeutic modalities (e.g., potent immunotherapies).
8. New approaches like protease-activatable toxins, oncolytic viruses and cellular therapies may similarly benefit from selective activation in the tumor microenvironment.

### FUTURE ISSUES

1. It will be necessary to understand differences between protease-level changes and cleavage-level changes caused by protease dysregulation (e.g., to understand compensatory changes due to protease modulation).
2. What is the patient-to-patient variance in protease activity for a given cancer type and site? How does this compare to variation between different tumor types?
3. Global identification of protease activity in human samples that is integrated with other 'omic data will enable the rapid development of future protease-targeted technologies.
4. Development of exquisitely sensitive probes for all proteases in the human genome will more readily enable technologies for clinical translation.
5. Emerging therapeutics and diagnostics should leverage improved basic knowledge of protease biology and use improved substrate designs.



6. Using protease activity, is it possible to develop signatures for pan-cancer detection? How many simultaneous in vivo protease activity measurements are feasible or necessary?
7. Can protease activity measurements and therapeutics be integrated to improve patient care?

## DISCLOSURE STATEMENT

J.S.D., A.D.W., and S.N.B. are listed as inventors on patents and patent applications related to protease-based technologies. A.D.W. is currently an employee of Glympse Bio. S.N.B. is a shareholder of and consultant to Glympse Bio.

## ACKNOWLEDGMENTS

The authors apologize for being unable to cite many pieces of primary literature due to length considerations. The authors thank Drs. H. Fleming, A. Schepers, and E. Kwon (MIT) for critical editing of the manuscript and helpful discussion. J.S.D. and A.D.W. acknowledge support from the Ludwig Center for Molecular Oncology fellowship and the National Science Foundation Graduate Research Fellowship Program. S.N.B. is a Howard Hughes Medical Institute Investigator.

## LITERATURE CITED

- Agard NJ, Mahrus S, Trinidad JC, Lynn A, Burlingame AL, Wells JA. 2012. Global kinetic analysis of proteolysis via quantitative targeted proteomics. *PNAS* 109(6):1913–18
- Akkari L, Gocheva V, Quick ML, Kester JC, Spencer AK, et al. 2016. Combined deletion of cathepsin protease family members reveals compensatory mechanisms in cancer. *Genes Dev.* 30(2):220–32
- Bachovchin DA, Koblan LW, Wu W, Liu Y, Li Y, et al. 2014. A high-throughput, multiplexed assay for superfamily-wide profiling of enzyme activity. *Nat. Chem. Biol.* 10:656–63
- Barreira da Silva R, Laird ME, Yatim N, Fiette L, Ingersoll MA, Albert ML. 2015. Dipeptidylpeptidase 4 inhibition enhances lymphocyte trafficking, improving both naturally occurring tumor immunity and immunotherapy. *Nat. Immunol.* 16(8):850–58
- Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, et al. 2013. NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Res.* 41:D991–95
- Basel MT, Shrestha TB, Troyer DL, Bossmann SH. 2011. Protease-sensitive, polymer-caged liposomes: a method for making highly targeted liposomes using triggered release. *ACS Nano* 5(3):2162–75
- Bergers G, Benjamin LE. 2003. Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* 3(6):401–10
- Biswas A, Joo K-I, Liu J, Zhao M, Fan G, et al. 2011. Endoprotease-mediated intracellular protein delivery using nanocapsules. *ACS Nano* 5(2):1385–94
- Blum G, von Degenfeld G, Merchant MJ, Blau HM, Bogoy M. 2007. Noninvasive optical imaging of cysteine protease activity using fluorescently quenched activity-based probes. *Nat. Chem. Biol.* 3(10):668–77
- Borgoño CA, Diamandis EP. 2004. The emerging roles of human tissue kallikreins in cancer. *Nat. Rev. Cancer* 4(11):876–90
- Boulware KT, Daugherty PS. 2006. Protease specificity determination by using cellular libraries of peptide substrates (CLiPS). *PNAS* 103(20):7583–88
- Braun GB, Sugahara KN, Yu OM, Kotamraju VR, Mölder T, et al. 2016. Urokinase-controlled tumor penetrating peptide. *J. Control. Release* 232:188–95
- Bremer C, Tung CH, Weissleder R. 2001. *In vivo* molecular target assessment of matrix metalloproteinase inhibition. *Nat. Med.* 7(6):743–48
- Brown PO, Palmer C. 2009. The preclinical natural history of serous ovarian cancer: defining the target for early detection. *PLOS Med.* 6(7):e1000114



- Chang PV, Dube DH, Sletten EM, Bertozzi CR. 2010. A strategy for the selective imaging of glycans using caged metabolic precursors. *J. Am. Chem. Soc.* 132(28):9516–18
- Chen C-H, Miller MA, Sarkar A, Beste MT, Isaacson KB, et al. 2013. Multiplexed protease activity assay for low-volume clinical samples using droplet-based microfluidics and its application to endometriosis. *J. Am. Chem. Soc.* 135(5):1645–48
- Choi J, Kim S, Yoo D, Shin T-H, Kim H, et al. 2017. Distance-dependent magnetic resonance tuning as a versatile MRI sensing platform for biological targets. *Nat. Mater.* 18:537–42
- Choi KY, Swierczewska M, Lee S, Chen X. 2012. Protease-activated drug development. *Theranostics* 2(2):156–78
- Chuang C-H, Chuang K-H, Wang H-E, Roffler SR, Shiea J, et al. 2012. *In vivo* positron emission tomography imaging of protease activity by generation of a hydrophobic product from a noninhibitory protease substrate. *Clin. Cancer Res.* 18(1):238–47
- Coussens LM, Fingleton B, Matrisian LM. 2002. Matrix metalloproteinase inhibitors and cancer—trials and tribulations. *Science* 295(5564):2387–92
- Cravatt BF, Wright AT, Kozarich JW. 2008. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu. Rev. Biochem.* 77:383–414
- Crisp JL, Savariar EN, Glasgow HL, Ellies LG, Whitney MA, Tsien RY. 2014. Dual targeting of integrin  $\alpha_v\beta_3$  and matrix metalloproteinase-2 for optical imaging of tumors and chemotherapeutic delivery. *Mol. Cancer Ther.* 13(6):1514–25
- Danino T, Prindle A, Kwong GA, Skalak M, Li H, et al. 2015. Programmable probiotics for detection of cancer in urine. *Sci. Transl. Med.* 7(289):289ra84
- Darragh MR, Schneider EL, Lou J, Phojanakong PJ, Farady CJ, et al. 2010. Tumor detection by imaging proteolytic activity. *Cancer Res.* 70(4):1505–12
- Deng SJ, Bickett DM, Mitchell JL, Lambert MH, Blackburn RK, et al. 2000. Substrate specificity of human collagenase 3 assessed using a phage-displayed peptide library. *J. Biol. Chem.* 275(40):31422–27
- Desnoyers LR, Vasiljeva O, Richardson JH, Yang A, Menendez EEM, et al. 2013. Tumor-specific activation of an EGFR-targeting probody enhances therapeutic index. *Sci. Transl. Med.* 5(207):207ra144
- Devy L, Huang L, Naa L, Yanamandra N, Pieters H, et al. 2009. Selective inhibition of matrix metalloproteinase-14 blocks tumor growth, invasion, and angiogenesis. *Cancer Res.* 69(4):1517–26
- Din MO, Danino T, Prindle A, Skalak M, Selimkhanov J, et al. 2016. Synchronized cycles of bacterial lysis for *in vivo* delivery. *Nature* 536(7614):81–85
- Doronina SO, Toki BE, Torgov MY, Mendelsohn BA, Cerveny CG, et al. 2003. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol.* 21(7):778–84
- Dudani JS, Buss CG, Akana RTK, Kwong GA, Bhatia SN. 2016. Sustained-release synthetic biomarkers for monitoring thrombosis and inflammation using point-of-care compatible readouts. *Adv. Funct. Mater.* 26:2919–28
- Dudani JS, Jain PK, Kwong GA, Stevens KR, Bhatia SN. 2015. Photoactivated spatiotemporally-responsive nanosensors of *in vivo* protease activity. *ACS Nano* 9(12):11708–17
- Dufour A, Sampson NS, Li J, Kuscus C, Rizzo RC, et al. 2011. Small-molecule anticancer compounds selectively target the hemopexin domain of matrix metalloproteinase-9. *Cancer Res.* 71(14):4977–88
- Edgington LE, Berger AB, Blum G, Albrow VE, Paulick MG, et al. 2009. Noninvasive optical imaging of apoptosis by caspase-targeted activity-based probes. *Nat. Med.* 15(8):967–73
- Edgington LE, Verdoes M, Bogyo M. 2011. Functional imaging of proteases: recent advances in the design and application of substrate-based and activity-based probes. *Curr. Opin. Chem. Biol.* 15(6):798–805
- Edgington LE, Verdoes M, Ortega A, Withana NP, Lee J, et al. 2013. Functional imaging of legumain in cancer using a new quenched activity-based probe. *J. Am. Chem. Soc.* 135(1):174–82
- Egeblad M, Werb Z. 2002. New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* 2(3):161–74
- Etzioni R, Urban N, Ramsey S, McIntosh M, Schwartz S, et al. 2003. The case for early detection. *Nat. Rev. Cancer* 3(4):243–52
- Fortelny N, Cox JH, Kappelhoff R, Starr AE, Lange PF, et al. 2014. Network analyses reveal pervasive functional regulation between proteases in the human protease web. *PLOS Biol.* 12(5):e1001869



- Fosgerau K, Hoffmann T. 2015. Peptide therapeutics: current status and future directions. *Drug Discov. Today* 20(1):122–28
- Friedman AA, Letai A, Fisher DE, Flaherty KT. 2015. Precision medicine for cancer with next-generation functional diagnostics. *Nat. Rev. Cancer*. 15(12):747–56
- Gocheva V, Joyce JA. 2007. Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle* 6(1):60–64
- Grimm J, Kirsch DG, Windsor SD, Kim CFB, Santiago PM, et al. 2005. Use of gene expression profiling to direct *in vivo* molecular imaging of lung cancer. *PNAS* 102(40):14404–9
- GTE Consortium. 2013. The Genotype-Tissue Expression (GTEx) project. *Nat. Genet.* 45(6):580–85
- Haeckel A, Appler F, Ariza de Schellenberger A, Schellenberger E. 2016. XTEN as biological alternative to PEGylation allows complete expression of a protease-activatable killin-based cytostatic. *PLOS ONE* 11(6):e0157193
- Han X, Bryson PD, Zhao Y, Cinay GE, Li S, et al. 2017. Masked chimeric antigen receptor for tumor-specific activation. *Mol. Ther.* 25(1):274–84
- Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. *Cell* 144(5):646–74
- Hanash SM, Pitteri SJ, Faca VM. 2008. Mining the plasma proteome for cancer biomarkers. *Nature* 452(7187):571–79
- Harris JL, Backes BJ, Leonetti F, Mahrus S, Ellman JA, Craik CS. 2000. Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. *PNAS* 97(14):7754–59
- Harris TJ, von Maltzahn G, Derfus AM, Ruoslahti E, Bhatia SN. 2006. Proteolytic actuation of nanoparticle self-assembly. *Angew. Chem. Int. Ed.* 45(19):3161–65
- Harris TJ, von Maltzahn G, Lord ME, Park J-H, Agrawal A, et al. 2008. Protease-triggered unveiling of bioactive nanoparticles. *Small* 4(9):1307–12
- Hatakeyama H, Akita H, Ito E, Hayashi Y, Oishi M, et al. 2011. Systemic delivery of siRNA to tumors using a lipid nanoparticle containing a tumor-specific cleavable PEG-lipid. *Biomaterials* 32(18):4306–16
- Hilderbrand SA, Wiessler R. 2010. Near-infrared fluorescence: application to *in vivo* molecular imaging. *Curr. Opin. Chem. Biol.* 14(1):71–79
- Hockla A, Miller E, Salameh MA, Copland JA, Radisky DC, Radisky ES. 2012. PRSS3/mesotrypsin is a therapeutic target for metastatic prostate cancer. *Mol. Cancer Res.* 10(12):1555–66
- Hori SS, Gambhir SS. 2011. Mathematical model identifies blood biomarker-based early cancer detection strategies and limitations. *Sci. Transl. Med.* 3(109):109ra116
- Huang S, Shao K, Liu Y, Kuang Y, Li J, et al. 2013. Tumor-targeting and microenvironment-responsive smart nanoparticles for combination therapy of antiangiogenesis and apoptosis. *ACS Nano* 7(3):2860–71
- Igarashi Y, Eroshkin A, Gramatikova S, Gramatikoff K, Zhang Y, et al. 2007. CutDB: a proteolytic event database. *Nucleic Acids Res.* 35:D546–49
- Inst. Med. 2007. *Cancer Biomarkers: The Promises and Challenges of Improving Detection and Treatment*. Washington, DC: Natl. Acad. Press
- Jessani N, Liu Y, Humphrey M, Cravatt BF. 2002. Enzyme activity profiles of the secreted and membrane proteome that depict cancer cell invasiveness. *PNAS* 99(16):10335–40
- Jiang T, Olson ES, Nguyen QT, Roy M, Jennings PA, Tsien RY. 2004. Tumor imaging by means of proteolytic activation of cell-penetrating peptides. *PNAS* 101(51):17867–72
- Joyce JA, Baruch A, Chehade K, Meyer-Morse N, Giraudo E, et al. 2004. Cathepsin cysteine proteases are effectors of invasive growth and angiogenesis during multistage tumorigenesis. *Cancer Cell* 5(5):443–53
- Kanavos P. 2006. The rising burden of cancer in the developing world. *Ann. Oncol.* 17(Suppl. 8):viii15–23
- Kasperkiewicz P, Poreba M, Snipas SJ, Parker H, Winterbourn CC, et al. 2014. Design of ultrasensitive probes for human neutrophil elastase through hybrid combinatorial substrate library profiling. *PNAS* 111(7):2518–23
- Kaufman HL, Kohlhapp FJ, Zloza A. 2015. Oncolytic viruses: a new class of immunotherapy drugs. *Nat. Rev. Drug Discov.* 14(9):642–62
- Kessenbrock K, Plaks V, Werb Z. 2010. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 141(1):52–67
- Kinoh H, Inoue M, Washizawa K, Yamamoto T, Fujikawa S, et al. 2004. Generation of a recombinant Sendai virus that is selectively activated and lyses human tumor cells expressing matrix metalloproteinases. *Gene Ther.* 11(14):1137–45



- Kleifeld O, Doucet A, auf dem Keller U, Prudova A, Schilling O, et al. 2010. Isotopic labeling of terminal amines in complex samples identifies protein N-termini and protease cleavage products. *Nat. Biotechnol.* 28(3):281–88
- Klezovitch O, Chevillet J, Mirosevich J, Roberts RL, Matusik RJ, Vasioukhin V. 2004. Hepsin promotes prostate cancer progression and metastasis. *Cancer Cell* 6(2):185–95
- Kromann-Hansen T, Oldenburg E, Yung KWY, Ghassabeh GH, Muylderms S, et al. 2016. A camelid-derived antibody fragment targeting the active site of a serine protease balances between inhibitor and substrate behavior. *J. Biol. Chem.* 291(29):15156–68
- Kwon EJ, Dudani JS, Bhatia SN. 2017. Ultrasensitive tumour-penetrating nanosensors of protease activity. *Nat. Biomed. Eng.* 1:0054
- Kwon EJ, Lo JH, Bhatia SN. 2015. Smart nanosystems: bio-inspired technologies that interact with the host environment. *PNAS* 112(47):14460–66
- Kwong GA, Dudani JS, Carrodegus E, Mazumdar EV, Zekavat SM, Bhatia SN. 2015. Mathematical framework for activity-based cancer biomarkers. *PNAS* 112(41):12627–32
- Kwong GA, von Maltzahn G, Murugappan G, Abudayyeh O, Mo S, et al. 2013. Mass-encoded synthetic biomarkers for multiplexed urinary monitoring of disease. *Nat. Biotechnol.* 31(1):63–70
- La Thangue NB, Kerr DJ. 2011. Predictive biomarkers: a paradigm shift towards personalized cancer medicine. *Nat. Rev. Clin. Oncol.* 8(10):587–96
- LeBeau AM, Denmeade SR. 2015. Protease-activated pore-forming peptides for the treatment and imaging of prostate cancer. *Mol. Cancer Ther.* 14(3):659–68
- LeBeau AM, Lee M, Murphy ST, Hann BC, Warren RS, et al. 2013. Imaging a functional tumorigenic biomarker in the transformed epithelium. *PNAS* 110(1):93–98
- Li J, Dowdy S, Tipton T, Podratz K, Lu W-G, et al. 2009. HE4 as a biomarker for ovarian and endometrial cancer management. *Expert Rev. Mol. Diagn.* 9(6):555–66
- Liu S, Aaronson H, Mitola DJ, Leppla SH, Bugge TH. 2003. Potent antitumor activity of a urokinase-activated engineered anthrax toxin. *PNAS* 100(2):657–62
- Liu S, Netzel-Arnett S, Birkedal-Hansen H, Leppla SH. 2000. Tumor cell-selective cytotoxicity of matrix metalloproteinase-activated anthrax toxin. *Cancer Res.* 60(21):6061–67
- Liu S, Redeye V, Kuremsky JG, Kuhnen M, Molinolo A, et al. 2005. Intermolecular complementation achieves high-specificity tumor targeting by anthrax toxin. *Nat. Biotechnol.* 23(6):725–30
- López-Otín C, Bond JS. 2008. Proteases: multifunctional enzymes in life and disease. *J. Biol. Chem.* 283(45):30433–37
- López-Otín C, Matrisian LM. 2007. Emerging roles of proteases in tumour suppression. *Nat. Rev. Cancer* 7(10):800–8
- López-Otín C, Overall CM. 2002. Protease degradomics: a new challenge for proteomics. *Nat. Rev. Mol. Cell Biol.* 3(7):509–19
- Luo Y, Zhou H, Krueger J, Kaplan C, Lee S-H, et al. 2006. Targeting tumor-associated macrophages as a novel strategy against breast cancer. *J. Clin. Invest.* 116(8):2132–41
- Manasanch EE, Orłowski RZ. 2017. Proteasome inhibitors in cancer therapy. *Nat. Rev. Clin. Oncol.* 14:417–33
- Marshall DC, Lyman SK, McCauley S, Kovalenko M, Spangler R, et al. 2015. Selective allosteric inhibition of MMP9 is efficacious in preclinical models of ulcerative colitis and colorectal cancer. *PLOS ONE* 10(5):e0127063
- Mehan MR, Ayers D, Thirstrup D, Xiong W, Ostroff RM, et al. 2012. Protein signature of lung cancer tissues. *PLOS ONE* 7(4):e35157
- Miller MA, Barkal L, Jeng K, Herrlich A, Moss M, et al. 2011. Proteolytic Activity Matrix Analysis (PrAMA) for simultaneous determination of multiple protease activities. *Integr. Biol.* 3(4):422–38
- Miller MA, Oudin MJ, Sullivan RJ, Wang SJ, Meyer AS, et al. 2016. Reduced proteolytic shedding of receptor tyrosine kinases is a post-translational mechanism of kinase inhibitor resistance. *Cancer Discov.* 6(4):382–99
- Mitsiades N, Yu W, Poulaki V, Tsokos M, Stamenkovic I. 2001. Matrix metalloproteinase-7-mediated cleavage of Fas ligand protects tumor cells from chemotherapeutic drug cytotoxicity. *Cancer Res.* 61(2):577–81
- Mühlebach MD, Schaser T, Zimmermann M, Armeanu S, Hanschmann K-MO, et al. 2010. Liver cancer protease activity profiles support therapeutic options with matrix metalloproteinase-activatable oncolytic measles virus. *Cancer Res.* 70(19):7620–29



- Naba A, Clauser KR, Hoersch S, Liu H, Carr SA, Hynes RO. 2012. The matrisome: *in silico* definition and *in vivo* characterization by proteomics of normal and tumor extracellular matrices. *Mol. Cell. Proteom.* 11(4):M111.014647
- Naba A, Clauser KR, Lamar JM, Carr SA, Hynes RO. 2014. Extracellular matrix signatures of human mammary carcinoma identify novel metastasis promoters. *eLife* 3:e01308
- Nakasone ES, Askautrud HA, Kees T, Park J-H, Plaks V, et al. 2012. Imaging tumor-stroma interactions during chemotherapy reveals contributions of the microenvironment to resistance. *Cancer Cell* 21(4):488–503
- Nam DH, Rodriguez C, Remacle AG, Strongin AY, Ge X. 2016. Active-site MMP-selective antibody inhibitors discovered from convex paratope synthetic libraries. *PNAS* 113(52):14970–75
- Natl. Cancer Inst. 2017. *Cancer stat facts: ovarian cancer*. SEER (Surveill. Epidemiol. End Results) cancer stat facts. <https://seer.cancer.gov/statfacts/html/ovary.html>
- Nguyen QT, Olson ES, Aguilera TA, Jiang T, Scadeng M, et al. 2010. Surgery with molecular fluorescence imaging using activatable cell-penetrating peptides decreases residual cancer and improves survival. *PNAS* 107(9):4317–22
- O'Donoghue AJ, Eroy-Reveles AA, Knudsen GM, Ingram J, Zhou M, et al. 2012. Global identification of peptidase specificity by multiplex substrate profiling. *Nat. Methods* 9(11):1095–100
- Olson ES, Jiang T, Aguilera TA, Nguyen QT, Ellies LG, et al. 2010. Activatable cell penetrating peptides linked to nanoparticles as dual probes for *in vivo* fluorescence and MR imaging of proteases. *PNAS* 107(9):4311–16
- Olson OC, Joyce JA. 2015. Cysteine cathepsin proteases: regulators of cancer progression and therapeutic response. *Nat. Rev. Cancer* 15(12):712–29
- Overall CM, Blobel CP. 2007. In search of partners: linking extracellular proteases to substrates. *Nat. Rev. Mol. Cell Biol.* 8(3):245–57
- Pastan I, Hassan R, FitzGerald DJ, Kreitman RJ. 2007. Immunotoxin treatment of cancer. *Annu. Rev. Med.* 58:221–37
- Pérez-Silva JG, Español Y, Velasco G, Quesada V. 2016. The Degradome database: expanding roles of mammalian proteases in life and disease. *Nucleic Acids Res.* 44(D1):D351–55
- Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, et al. 1998. An essential role for ectodomain shedding in mammalian development. *Science* 282(5392):1281–84
- Prensner JR, Rubin MA, Wei JT, Chinnaiyan AM. 2012. Beyond PSA: the next generation of prostate cancer biomarkers. *Sci. Transl. Med.* 4(127):127rv3
- Quail DF, Joyce JA. 2013. Microenvironmental regulation of tumor progression and metastasis. *Nat. Med.* 19(11):1423–37
- Ratnikov B, Cieplak P, Smith JW. 2009. High throughput substrate phage display for protease profiling. In *Proteases and Cancer*, ed. TH Bugge, TM Antalis, pp. 93–114. New York: Humana
- Rawlings ND, Barrett AJ, Finn R. 2016. Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* 44:D343–50
- Rawlings ND, Salvesen G. 2013. *Handbook of Proteolytic Enzymes*. London: Academic. 3rd ed.
- Romee R, Foley B, Lenvik T, Wang Y, Zhang B, et al. 2013. NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). *Blood* 121(18):3599–608
- Roy R, Yang J, Moses MA. 2009. Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer. *J. Clin. Oncol.* 27(31):5287–97
- Ruoslahti E, Bhatia SN, Sailor MJ. 2010. Targeting of drugs and nanoparticles to tumors. *J. Cell Biol.* 188(6):759–68
- Saghatelian A, Jessani N, Joseph A, Humphrey M, Cravatt BF. 2004. Activity-based probes for the proteomic profiling of metalloproteases. *PNAS* 101(27):10000–5
- Salisbury CM, Maly DJ, Ellman JA. 2002. Peptide microarrays for the determination of protease substrate specificity. *J. Am. Chem. Soc.* 124(50):14868–70
- Sandersjö L, Jonsson A, Löfblom J. 2015. A new prodrug form of Affibody molecules (pro-Affibody) is selectively activated by cancer-associated proteases. *Cell. Mol. Life Sci.* 72(7):1405–15
- Sanman LE, Bogoy M. 2014. Activity-based profiling of proteases. *Annu. Rev. Biochem.* 83:249–73



- Savariar EN, Felsen CN, Nashi N, Jiang T, Ellies LG, et al. 2013. Real-time *in vivo* molecular detection of primary tumors and metastases with ratiometric activatable cell-penetrating peptides. *Cancer Res.* 73(2):855–64
- Sawyers CL. 2008. The cancer biomarker problem. *Nature* 452(7187):548–52
- Schellenberger V, Wang C, Geething NC, Spink BJ, Campbell A, et al. 2009. A recombinant polypeptide extends the *in vivo* half-life of peptides and proteins in a tunable manner. *Nat. Biotechnol.* 27(12):1186–90
- Schilling O, Overall CM. 2008. Proteome-derived, database-searchable peptide libraries for identifying protease cleavage sites. *Nat. Biotechnol.* 26(6):685–94
- Schneider EL, Craik CS. 2009. Positional scanning synthetic combinatorial libraries for substrate profiling. In *Proteases and Cancer*, ed. TH Bugge, TM Antalis, pp. 59–78. New York: Humana
- Schuerle S, Dudani JS, Christiansen MG, Anikeeva P, Bhatia SN. 2016. Magnetically actuated protease sensors for *in vivo* tumor profiling. *Nano Lett.* 16(10):6303–10
- Singh N, Karambelkar A, Gu L, Lin K, Miller JS, et al. 2011. Bioresponsive mesoporous silica nanoparticles for triggered drug release. *J. Am. Chem. Soc.* 133(49):19582–85
- Stein V, Alexandrov K. 2014. Protease-based synthetic sensing and signal amplification. *PNAS* 111(45):15934–39
- Thorek DLJ, Watson PA, Lee S-G, Ku AT, Bournazos S, et al. 2016. Internalization of secreted antigen-targeted antibodies by the neonatal Fc receptor for precision imaging of the androgen receptor axis. *Sci. Transl. Med.* 8(367):367ra167
- Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, et al. 2012. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* 366(26):2443–54
- Tranchant I, Vera L, Czarny B, Amoura M, Cassar E, et al. 2014. Halogen bonding controls selectivity of FRET substrate probes for MMP-9. *Chem. Biol.* 21(3):408–13
- Turk BE, Huang LL, Piro ET, Cantley LC. 2001. Determination of protease cleavage site motifs using mixture-based oriented peptide libraries. *Nat. Biotechnol.* 19(7):661–67
- Uhland K. 2006. Matriptase and its putative role in cancer. *Cell. Mol. Life Sci.* 63(24):2968–78
- Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, et al. 2015. Tissue-based map of the human proteome. *Science* 347(6220):1260419
- Vandenbroucke RE, Libert C. 2014. Is there new hope for therapeutic matrix metalloproteinase inhibition? *Nat. Rev. Drug Discov.* 13(12):904–27
- Vandooren J, Geurts N, Martens E, Van den Steen PE, Opdenakker G. 2013. Zymography methods for visualizing hydrolytic enzymes. *Nat. Methods* 10(3):211–20
- Verdoes M, Oresic Bender K, Segal E, van der Linden WA, Syed S, et al. 2013. Improved quenched fluorescent probe for imaging of cysteine cathepsin activity. *J. Am. Chem. Soc.* 135(39):14726–30
- Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, et al. 2006. Differential exoprotease activities confer tumor-specific serum peptidome patterns. *J. Clin. Investig.* 116(1):271–84
- von Maltzahn G, Harris TJ, Park J-H, Min D-H, Schmidt AJ, et al. 2007. Nanoparticle self-assembly gated by logical proteolytic triggers. *J. Am. Chem. Soc.* 129(19):6064–65
- Warren AD, Gaylord ST, Ngan KC, Dumont Milutinovic M, Kwong GA, et al. 2014a. Disease detection by ultrasensitive quantification of microdosed synthetic urinary biomarkers. *J. Am. Chem. Soc.* 136:13709–14
- Warren AD, Kwong GA, Wood DK, Lin KY, Bhatia SN. 2014b. Point-of-care diagnostics for noncommunicable diseases using synthetic urinary biomarkers and paper microfluidics. *PNAS* 111(10):3671–76
- Weissleder R. 2006. Molecular imaging in cancer. *Science* 312(5777):1168–71
- Weissleder R, Tung C-H, Mahmood U, Bogdanov A. 1999. *In vivo* imaging of tumors with protease-activated near-infrared fluorescent probes. *Nat. Biotechnol.* 17(4):375–78
- Welsler K, Adsley R, Moore BM, Chan WC, Aylott JW. 2011. Protease sensing with nanoparticle based platforms. *Analyst* 136(1):29–41
- Whitley MJ, Cardona DM, Lazarides AL, Spasojevic I, Ferrer JM, et al. 2016. A mouse-human phase 1 clinical trial of a protease-activated fluorescent probe for imaging cancer. *Sci. Transl. Med.* 8(320):320ra4
- Whitney M, Crisp JL, Olson ES, Aguilera TA, Gross LA, et al. 2010. Parallel *in vivo* and *in vitro* selection using phage display identifies protease-dependent tumor-targeting peptides. *J. Biol. Chem.* 285(29):22532–41
- WHO (World Health Organ.). 2017. *Cancer*. Fact sheet, Feb. 2017. Geneva: WHO. <http://www.who.int/mediacentre/factsheets/fs297/en/>



- Wiernik A, Foley B, Zhang B, Verneris MR, Warlick E, et al. 2013. Targeting natural killer cells to acute myeloid leukemia *in vitro* with a CD16 × 33 bispecific killer cell engager and ADAM17 inhibition. *Clin. Cancer Res.* 19(14):3844–55
- Withana NP, Garland M, Verdoes M, Ofori LO, Segal E, Bogyo M. 2016. Labeling of active proteases in fresh-frozen tissues by topical application of quenched activity-based probes. *Nat. Protoc.* 11(1):184–91
- Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, et al. 2007. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch. Pathol. Lab. Med.* 131(1):18–43
- Ye D, Shuhendler AJ, Cui L, Tong L, Tee SS, et al. 2014. Bioorthogonal cyclization-mediated *in situ* self-assembly of small-molecule probes for imaging caspase activity *in vivo*. *Nat. Chem.* 6(6):519–26
- Zhou Q, Gil-Krzewska A, Peruzzi G, Borrego F. 2013. Matrix metalloproteinases inhibition promotes the polyfunctionality of human natural killer cells in therapeutic antibody-based anti-tumour immunotherapy. *Clin. Exp. Immunol.* 173(1):131–39
- Zhu L, Wang T, Perche F, Taigind A, Torchilin VP. 2013. Enhanced anticancer activity of nanopreparation containing an MMP2-sensitive PEG-drug conjugate and cell-penetrating moiety. *PNAS* 110(42):17047–52

