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Discovery of Cell-Type-Specific and Disease-Related Enzymatic Activity Changes via Global Evaluation of Peptide Metabolism

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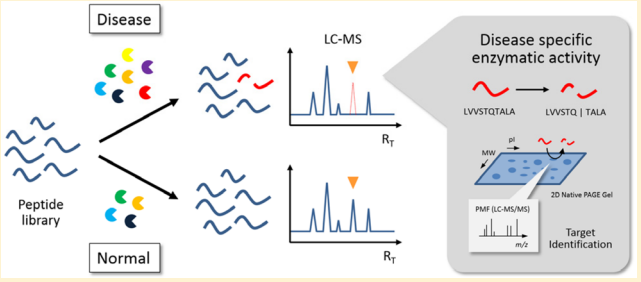
Supporting Information

ABSTRACT: Cellular homeostasis is maintained by a complex network of reactions catalyzed by enormous numbers of enzymatic activities (the enzymome), which serve to determine the phenotypes of cells. Here, we focused on the enzymomics of proteases and peptidases because these enzymes are an important class of disease-related proteins. We describe a system that (A) simultaneously evaluates metabolic activities of peptides using a series of exogenous peptide substrates and (B) identifies the enzymes that metabolize the specified peptide substrate with high throughput. We confirmed that the developed system was able to discover cell-type-specific and disease-related exo- and endopeptidase activities and identify the responsible enzymes. For example, we found that the activity of the endopeptidase neurolysin is highly elevated in human colorectal tumor tissue samples. This simple but powerful enzymomics platform should be widely applicable to uncover cell-type-specific reactions and altered enzymatic functions with potential value as biomarkers or drug targets in various disease states and to investigate the mechanisms of the underlying pathologies.



INTRODUCTION

Cellular functions are mediated by multiple enzymes, and altered enzymatic activities are often observed during the onset and progression of diseases. Therefore, knowledge of altered enzymatic functions not only provides clues to understand pathophysiological mechanisms but also opens up possibilities to diagnose or treat diseases. Although various types of omics studies, including genomics, transcriptomics, and proteomics, are available to characterize the state of cells, they would not necessarily pick up altered enzymatic activities because functional activities are mediated dynamically by many factors, including post-translational modifications, protein−protein interactions, and endogenous inhibitors.[1−4] Therefore, we consider that methodology for enzymomics (comprehensive study of targeted enzymatic activities with direct readout) in biosamples is extremely valuable. Here, we describe a form of enzymomics study that allows us (A) to discover altered enzymatic activities by monitoring the metabolism of a series of exogenous peptide substrates and (B) to identify the protein(s) responsible for a specified peptide-metabolizing activity with high throughput. In the present study, we focused on the activities of proteases and peptidases because these enzymes are an important class of disease-related proteins, regulating various biological phenomena by the activation of signaling proteins, turnover of extracellular matrix components, metabolism of peptidyl hormones, and so on.[5−9] Further, many of them are well-established as drug targets, and altered activities of some hydrolytic enzymes have already been utilized for diagnosis or visual detection of the presence of diseased cells.[10−16] Therefore, global analysis of peptide hydrolases is likely to be a fruitful approach to discover disease-related protein functions. As shown in Figure 1A, our developed methodology consists of two parts: (A) preparation of a series of peptide substrates by the residue-selective hydrolysis of purified proteins and the analysis of their metabolism by means of LC-MS, and (B) identification of enzyme(s) exhibiting activities of interest by using the zymography method termed diced electrophoresis gel (DEG) assay.[17,18]

Here, we describe the developed system and its application to discover cell-type-specific and disease-related enzymatic activ-

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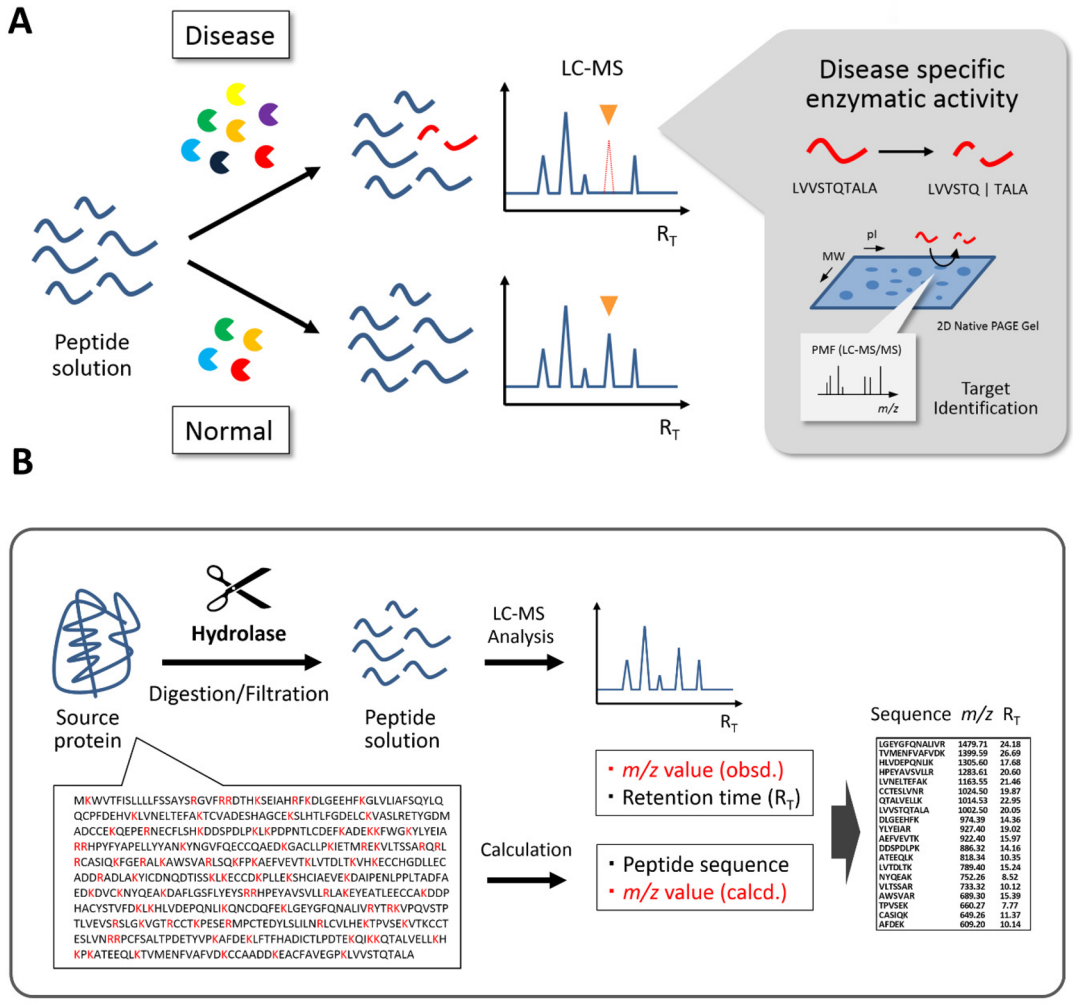

Figure 1. (A) Schematic illustration of analysis principle for identifying altered activities of peptide-metabolizing enzymes in biosamples. (B) Peptide assignment. Observed m/z values in LC-MS analysis and calculated m/z values from the sequence of the source protein were aligned, and matched peaks were assigned using the standard spreadsheet program.

ities, including identification of enzymes whose activities are elevated in surgical specimens of colorectal tumors.

RESULTS

Preparation of Peptides by Residue-Specific Hydrolysis of Source Proteins. Currently, several strategies are available for using a series of peptides to find desired functions by means of binding-and-enrichment strategies such as phage display, on-bead enrichment, and molecular evolution.[19−23] However, affinity-based platforms are not ideal for examining the metabolism of peptides because the required experimental settings are restrictive.[14] We considered that an effective strategy would be to prepare a peptide mixture by cleaving purified proteins with well-characterized enzymes and then to analyze the resulting peptides by means of liquid chromatography coupled with mass spectrometry (LC-MS). Since the sequences of the source proteins and the residue selectivity of the hydrolases are known (Table S1), we can easily assign the observed peaks of hydrolysis products based on m/z values (Figure 1B). By employing this simple way of preparing a series of peptides, we can acquire a mixture of substrates to study enzymatic functions. In this experimental platform, when the peptides are mixed with a biosample, signals of peptides that are metabolized should show decreased mass peak intensities compared with the control. The use of protein fragments for bioanalysis is not a new idea,[24−27] but such a strategy has never been applied to monitor altered enzymatic activities in biosamples that contain multiple enzymes. Our system requires only an HPLC apparatus coupled with a single quadrupole mass spectrometer; such instrumentation is widely available and is suitable for convenient and highly applicable analysis.

An overview of the developed system for preparing and analyzing peptide substrates is shown in Figure 2A (see also Table S2). First, purified proteins are hydrolyzed with residue-specific hydrolases (e.g., trypsin, chymotrypsin, and endopeptidase Glu-C), and the resulting solution is deproteinized by centrifugal filtration to give a mixture of hydrolyzed peptides. The peptides are analyzed by LC-MS, and the peaks of peptides with m/z values matching specific fragments are picked up (peptide assignment) for further analysis. The peptide substrates are then mixed with a biosample, and the resultant solution is analyzed again by LC-MS (metabolism assay). Listed peptides whose peaks are decreased in the treated samples are considered to have been metabolized. An important feature of this assay is that the assay can be run with small amounts of biosamples compared to the substrate peptides because enzyme turnover

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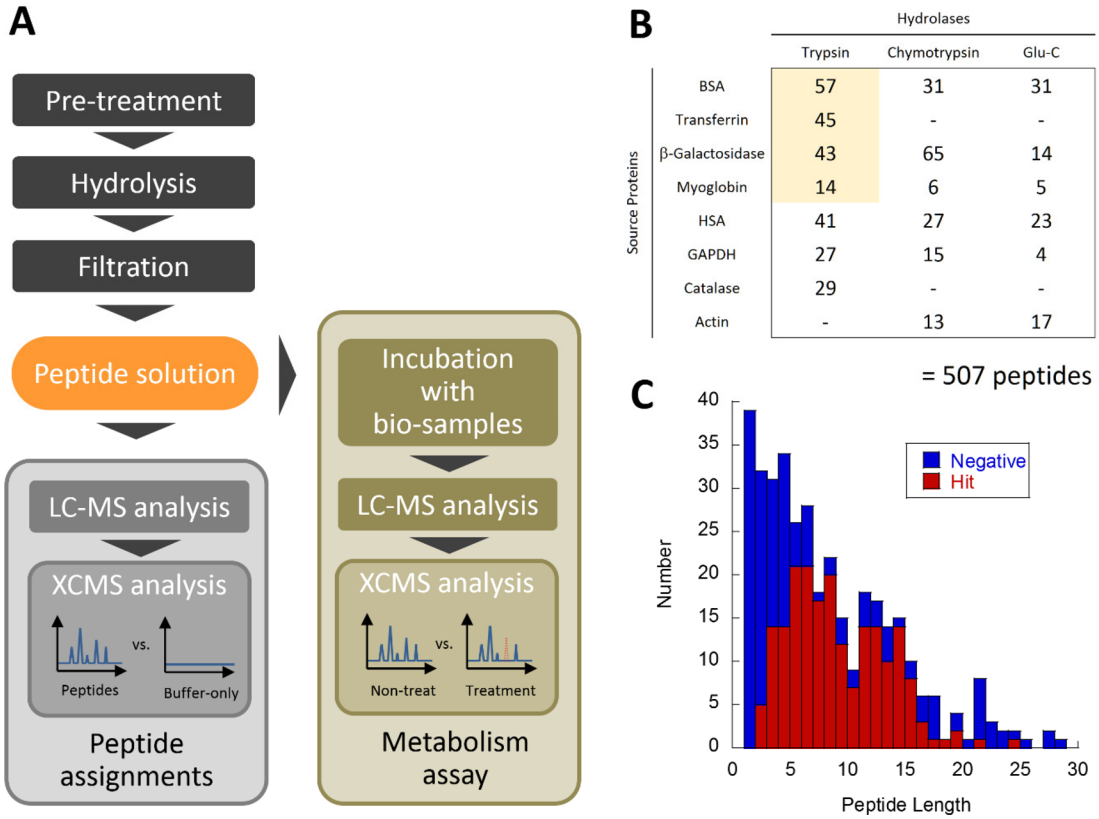

Figure 2. Preparation of peptides by residue-specific hydrolysis of source proteins. (A) Flowchart of the experiments. See Table S2 for the detailed protocols. (B) Numbers of peptides that were assigned using the established protocols and assignment rules. Peptides marked pale orange were used in the following metabolism analysis. (C) Distribution of the lengths (numbers of residues) of assigned peptides by trypsinization. Blue bars represent all peptides predicted from the sequence, and red bars represent detected peptides. The *X*-axis was cut off at 30. Full-range graphs along with those for chymotrypsin and Glu-C are shown in Figure S6.

numbers are expected to be high and, consequently, the changes in mass peaks of metabolized peptides should not be masked by signals arising from the biosamples; this was confirmed in subsequent experiments (Figure S1). The picked-up peptides are then subject to a confirmation process, in which the hit peptide is prepared by solid-phase peptide synthesis, and the activity is evaluated and characterized in a single-peptide metabolism assay. Finally, the enzyme(s) catalyzing the detected activity is characterized by means of biochemistry-based approaches.

In order to maximize the reliability of the assay, we optimized (1) the conditions of hydrolysis (Figure S2), (2) the deproteinization step (Figure S3), (3) the peptide amount used for single analysis (Figure S4), and (4) the LC-MS analysis conditions (Figure S5). For data analysis, we employed the XCMS platform, which is widely used in metabolic analysis.[28−30] In this analysis, LC-MS data are cut into slices of 0.01−0.1 m/z wide and overlapping peaks are generated from correlated slices; the mass uncertainty is usually less than ±0.15 with a standard single quadrupole mass spectrometer.[28]

After the above optimization, we examined peptide solution obtained by using combinations of eight proteins that are commercially available at low cost with good purity (Table S1) and three proteases, trypsin, chymotrypsin, and endopeptidase Glu-C; we obtained more than 500 peaks, corresponding to peptides of various sequences and lengths (Figure 2B,C and Table S3). With the trypsinized peptides as an example, more than 50% of the predicted peptides were detected (Figures 2C and S6). Among the predicted peptides, many that were not detected would have been too short to be retained in the column (1−3 amino acids long) or too long to be well-ionized under our assay conditions (>20 amino acids long), but we were able to detect most peaks from predicted peptides 5−15 amino acids long, which we considered would be most suitable as substrates. To confirm the reliability of the identifications, we randomly selected six peptides and synthesized them; all of the assignments turned out to be correct (Figure S7). Therefore, we next selected 159 peptides (prepared by trypsinization of BSA, transferrin, β-galactosidase, and myoglobin; Figure 2B) for metabolism analysis; the selected peptides would not target enzymes with trypsin-like activities, but other peptidases could be widely targeted. Since each peptide might be subject to multiple metabolic alterations, we considered that this number would be sufficient for discovering cell-type-specific peptide-metabolizing activities.

Search for Cell-Type-Specific Enzymatic Activities. To discover cell-type-specific enzymatic activities, we chose five different cell types (four tumor cell lines and one nontumor cell line) and examined the enzymatic activities in their lysates. Treatment of the peptide solution with each lysate, followed by LC-MS analysis, gave data sets showing the peptide−metabolic signature of each cell line (Figures 3A and S8). We observed many cell-type-specific reactivities, presumably reflecting the different origins of those tumor and nontumor cells.

First, we focused on the activity for hydrolysis of peptide NPDPWAK (one of the trypsin-generated peptide fragments of transferrin), which appeared to be selective to squamous (epithelial) cell carcinoma H226 cells because normal human bronchovascular epithelial (NHBE) cells, epithelial cells, which

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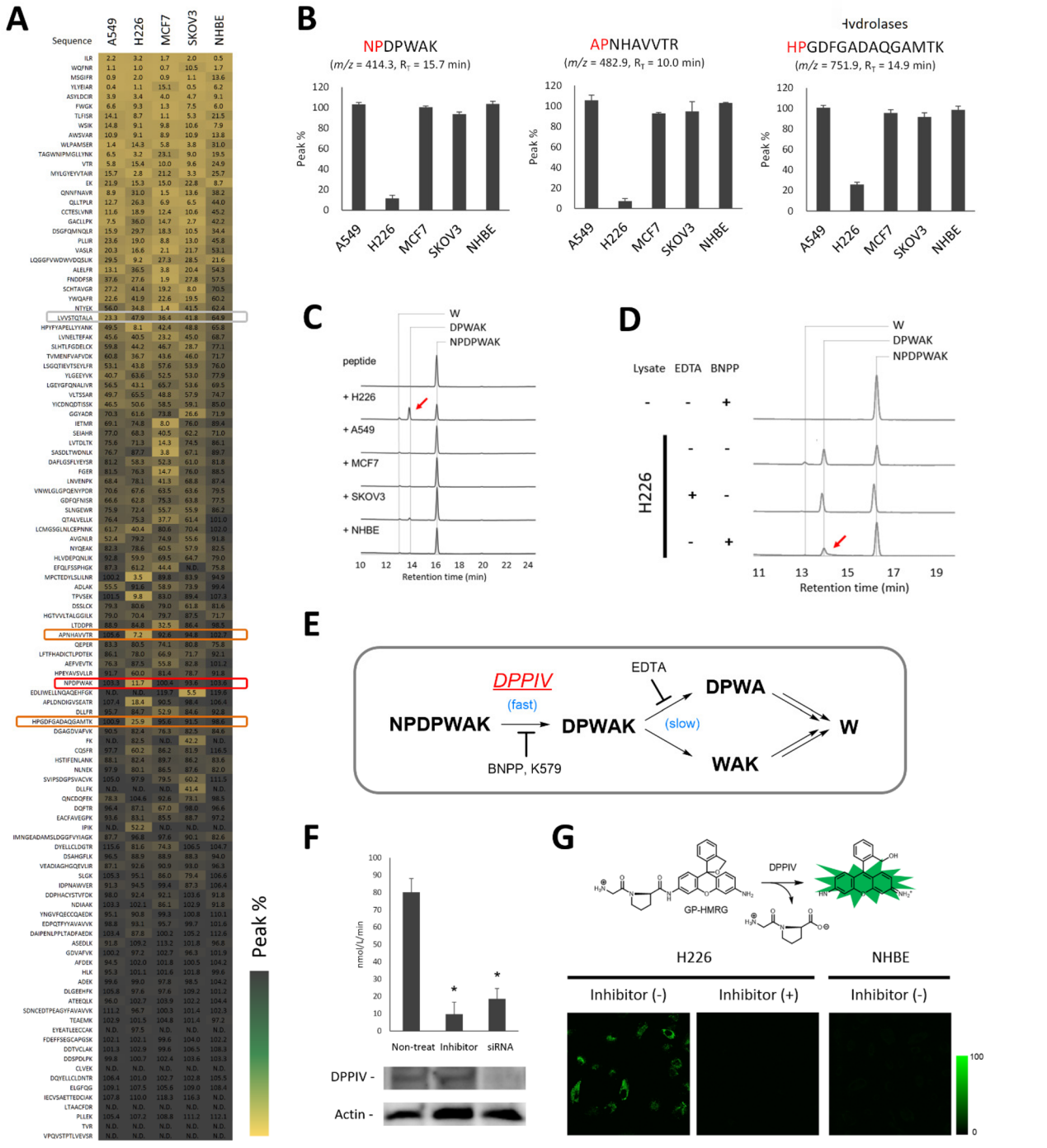

Figure 3. Global analysis of protease and peptidase activities of five different cell types. (A) Reactivity profile. The values indicate % of metabolized peptide compared to nontreated samples. (B) Reactivities of representative peptides NPDPWAK, HPGDFGADAQGAMTK, and APNHAVVTR; n = 3. Error bars represent standard error (SE). ND = not determined. (C) LC-MS analysis of the metabolism of peptide NPDPWAK (7.5 μM) after incubation with cell lysates (0.5 mg protein/mL). The absorbance trace (280 nm) is shown. Peak assignments were done by monitoring corresponding m/z values. (D) LC-MS analysis of the metabolism of peptide NPDPWAK (7.5 μM) after incubation with H226 cell lysates (0.5 mg protein/mL) treated with BNPP (100 μM) or EDTA (10 mM) for 6 h. The absorbance trace (280 nm) is shown. (E) Estimated metabolism of the peptide NPDPWAK in H226 cell lysates. (F) LC-MS analysis of the metabolism of peptide NPDPWAK (7.5 μM) after incubation with H226 cells transfected with DPPIV siRNA or treated with inhibitor K579 (10 nM); n = 3. Error bars represent SE. The results of Western blotting of the lysates are also shown. (G) Fluorescence images of H226 or NHBE cells treated with GP-HMRG (10 μM) with/without K579 (10 nM).

have the same lung origin but are not cancerous, showed little activity (Figure 3B). When we studied the activity in more detail with synthetic NPDPWAK peptide, it was found that the peptide was primarily hydrolyzed at the C-terminus of the second proline (Figure 3C). Although other metabolic activities, such as cleavage of the C-terminal amino acid (presumably mediated by carboxypeptidases), were also observed, the former seemed to be the major pathway of metabolism. Indeed, high activities toward other XP-containing peptides such as APNHAVVTR and HPGDFGADAQGAMTK were also found in the H226 cell

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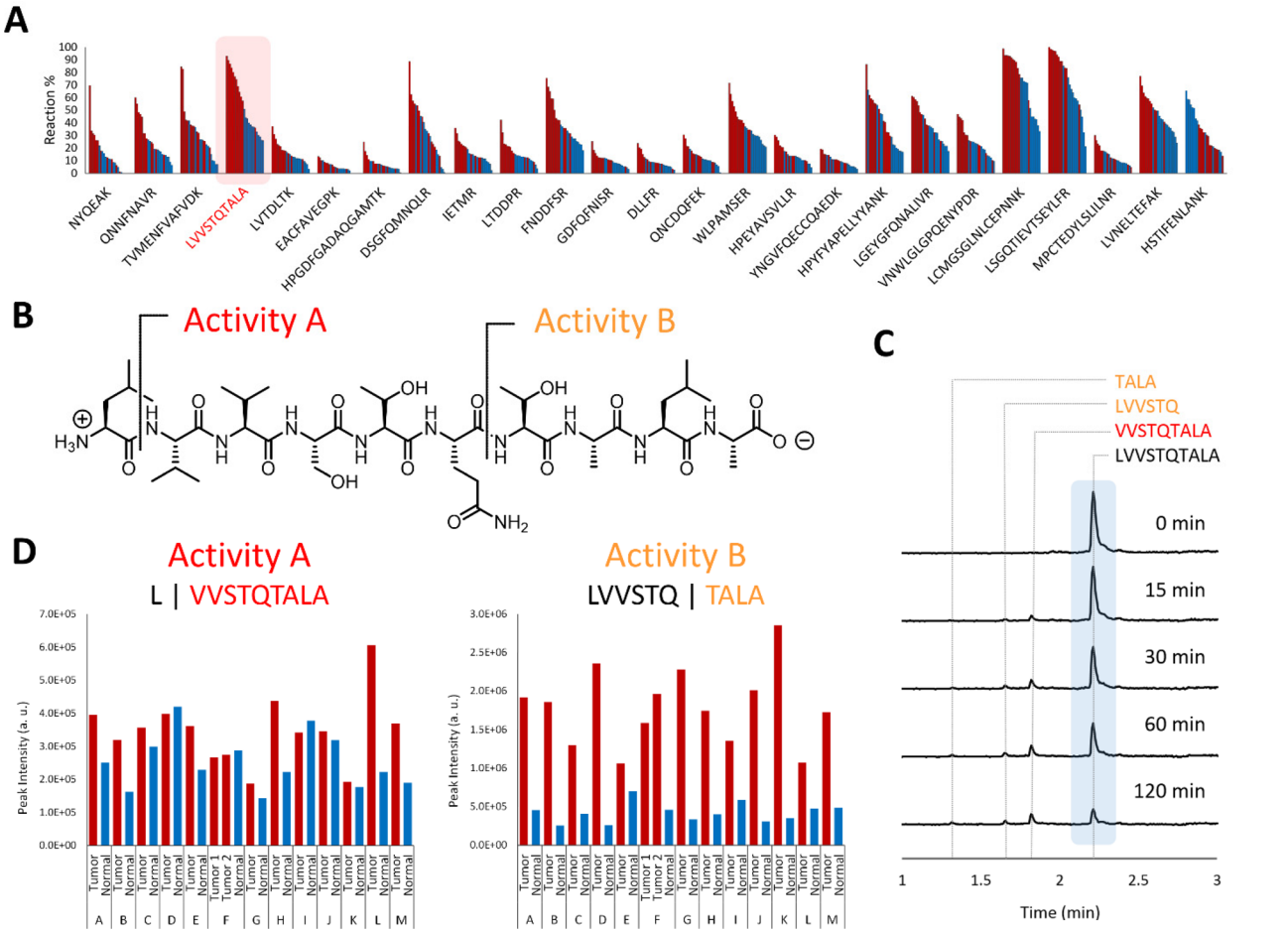

Figure 4. Discovery of colorectal tumor-specific enzymatic activities. (A) Metabolic profiles (reaction %) of peptides that showed altered metabolism (fold > 1.5, $P < 0.05$) between tumor (red bars) and nontumor (blue bars) samples ($n = 12$ each). Reaction % was calculated from the proportion of remaining peptides after the assay. Data are ordered by the reaction %. Tumor samples are shown as red bars, and nontumor samples are shown as blue bars. The whole data set is shown in Figure S13. (B) Metabolism of peptide LVVSTQTALA. (C) Total ion chromatograms of LVVSTQTALA peptide (7.5 μM) mixed with lysates of tumor tissue lysates (50 μg/mL). (D) LC-MS analysis of the consumption of LVVSTQTALA (7.5 μM) and formation of VVSTQTALA and TALA after incubation with lysates of colorectal tumor specimens (0.1 mg/mL) for 2 h. One tumor (red bars) and nontumor (blue) sample each was obtained from patients A–M, and two distinct tumor samples were obtained from patient F.

lysate (Figure 3B), indicating that the same enzyme is contributing to these reactions.

Then, we set out to identify the enzyme mediating the reaction. In this specific case, we were able to identify it on the basis of biochemical knowledge and the effects of class-selective inhibitors on the observed metabolism (Figure 3D). The activity was blocked by the general serine hydrolase inhibitor bis(4-nitrophenyl)phosphate (BNPP), while the metalloenzyme inhibitor EDTA had no effect; however, EDTA affected other downstream reactions that might contribute to conversion of DPWAK to DPWA (Figure S9). Because of the dipeptidyl peptidase-like activity toward XP-containing peptides, we considered that the activity might be mediated by dipeptidyl peptidase IV (DPPIV).[31] Some tumor cells have altered DPPIV activities that contribute to tumor pathogenesis,[11,32−36] so we examined whether or not H226 tumor cells had elevated DPPIV activity compared with nontumor NHBE cells. Using siRNA and the selective DPPIV inhibitor K579,[37] we confirmed that the activity was significantly blocked in both cases (Figures 3E,F and S10), supporting the idea that the primary mediator of NPDPWAK metabolism was indeed DPPIV (Figure S11). Altered DPPIV activity in H226 cells was also confirmed by live cell imaging; a DPPIV-selective fluorogenic substrate[11] generated clear signals in H226 cells but not in inhibitor-treated cells or NHBE cells (Figures 3G and S12). These results confirm the suitability of our system to discover cell-type-specific peptide-metabolizing activities.

Discovery of Elevated Endopeptidase-like Activities in Colorectal Tumors. Next, we applied our peptide enzymomics method to find tumor-specific enzymatic activities in surgical specimens of excised colorectal tumors. Tumor tissues and surrounding nontumor tissues from surgically resected colorectal specimens were lysed separately, and the peptide-metabolizing activities were studied in the same manner as described for the culture cell lysate study (Figures 4A and S13). The activity profiles of tumor and nontumor areas were very similar, but several peptides were preferentially metabolized at tumor sites. One of them was LVVSTQTALA (a trypsin-generated fragment of BSA), for which we detected two distinct hydrolytic activities. One was cleavage after the N-terminal leucine, which seemed to be mediated by leucine aminopeptidase, and the second was an endopeptidase-like activity, affording two fragments, LVVSTQ and TALA (Figure 4B,C). The latter activity was highly elevated in colorectal tumor lysates (Figure 4D). The sequence specificity for amide hydrolysis between Gln and Thr is not an orthodox one, so target estimation based on biochemical knowledge was

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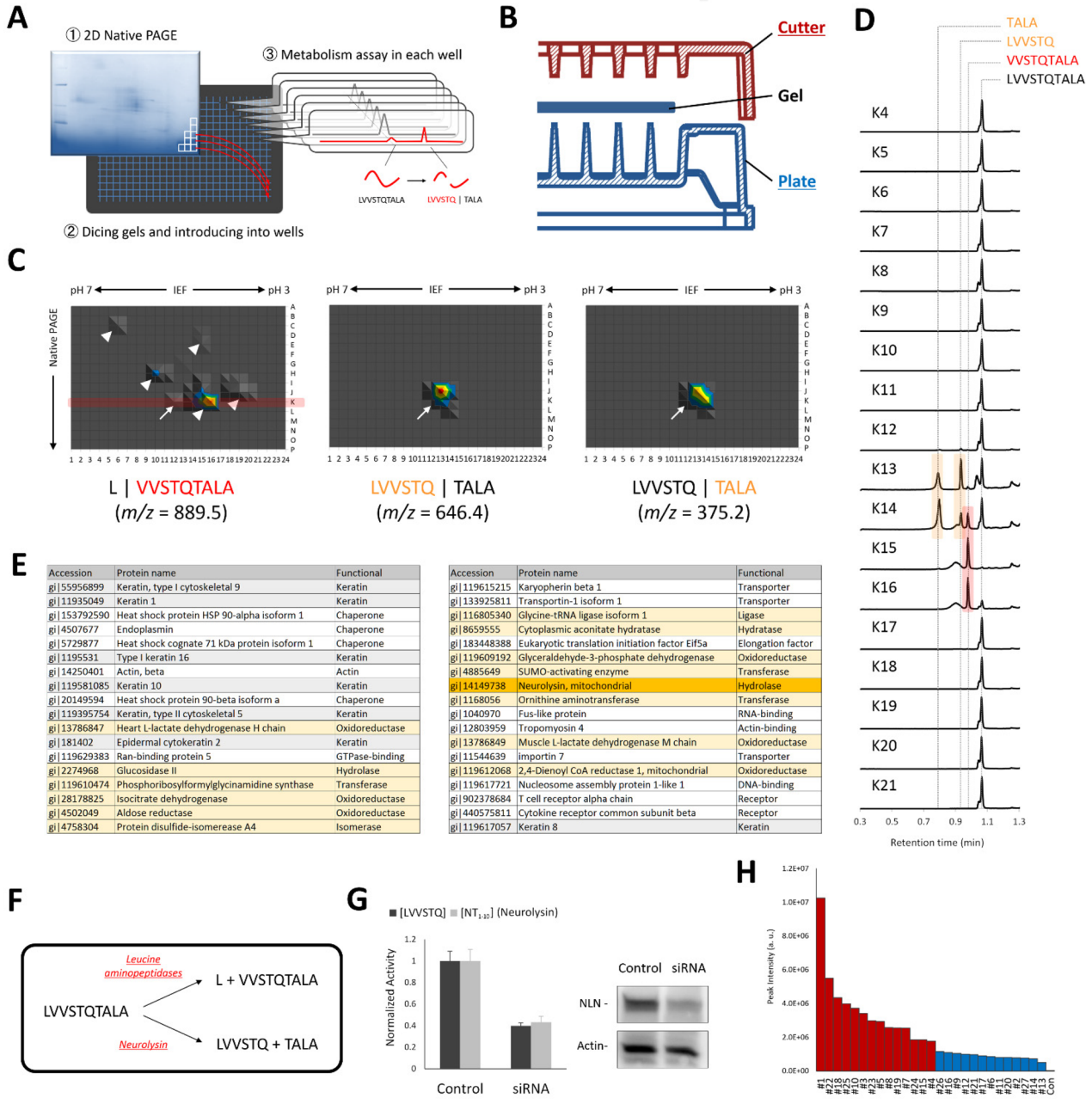

Figure 5. Characterization of novel endopeptidase. (A) Schematic illustration of LC-MS-based analysis applied to DEG assay. (B) Plate and lid used to dice the gel. (C) Result of DEG assay of A549 cell lysate (total 50 μg protein) using peptide LVVSTQTALA (7.5 μM) as a substrate. Formation of VVSTQTALA (white arrowhead, leucine aminopeptidase-like activity) and formation of LVVSTQ and TALA (white arrow, endopeptidase-like activity) are shown. (D) Total ion chromatogram charts of wells in line K. Peak assignments were done by monitoring corresponding m/z values. (E) List of candidate proteins in the well showing endopeptidase-like activity (white arrowhead in (C)). Known functions of hit proteins scanned in protein and enzyme databases such as UniProt and BRENDA are shown, and the table is colored based on the functional categories; keratins as common contaminants in LC-MS analysis are marked in gray, enzymes are marked in pale yellow, and enzymes with peptide-hydrolyzing activities are marked in orange. (F) Estimated metabolic pathway for the LVVSTQTALA peptide in A549 cell lysate. (G) LC-MS analysis of the formation of LVVSTQ peptide from LVVSTQTALA peptide (7.5 μM) and the formation of neurotensin$_{1-10}$ from neurotensin (7.5 μM) in lysates of A549 cells transfected with/without neurolysin siRNA; $n = 3$. Error bars represent SE. (H) LC-MS analysis of the formation of neurotensin$_{1-10}$ from neurotensin (7.5 μM) in lysates of tumor and nontumor areas of colorectal tumor specimen (0.1 mg/mL). Samples containing 14 tumor and 13 nontumor lysates were analyzed blind and ordered by activity (samples 1–27). Red bars show tumor samples, and the blue bars show nontumor samples.

not easy; therefore, we tried another approach to characterize the enzyme mediating this reaction.

Characterization of Colorectal Tumor-Specific Enzyme Activity. In order to identify the target, we established a high-throughput assay platform based on our previously developed zymography method termed diced electrophoresis gel (DEG) assay;[17,18,38] in this method, we separate the proteome by means of polyacrylamide gel electrophoresis (PAGE) under non-denatured conditions; next, the gels are diced and separately loaded into wells of multiwell plates, and the activity assay is performed in them. As previously reported,[17] the method gives sharp separation of the proteome from small amounts of samples

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and is well-suited to high-throughput analysis of enzymatic activities in biosamples. The two-dimensional electrophoresis provides a comprehensive "activity map" from which it is easy to determine on which enzymes to focus.

The original assay employed fluorescence-based reporter substrates, but we thought that LC-MS-based reaction monitoring could be employed in the same experimental setting (Figure 5A). The characterization was performed using tumorigenic A549 cell lysate, which exhibited activity similar to that of colorectal tumor lysate (Figures 3A and S15). First, we separated the A549 cell lysate with two-dimensional native PAGE, and then the gels were diced with specially designed instruments (Figures 5B and S14). Each gel segment was placed in a separate well by centrifugation and incubated with synthetic LVVSTQTALA. Next, the reaction mixture in each well was analyzed by short-column LC-MS to generate an activity map of the peptide metabolism. As a result, we were able to detect both leucine aminopeptidase-like and endopeptidase-like activities in independent locations of the gel (Figure 5C,D). Many wells showed leucine aminopeptidase-like activities, while only a single protein spot seemed to exhibit strong endopeptidase-like activity. Peptide mass fingerprinting analysis of the endopeptidase spot gave a list of candidate proteins (Figure 5E). Among them, we scanned proteins with peptidase activities using protein and enzyme databases and found only a single candidate protein, neurolysin, was reported to exhibit peptidase activities. Neurolysin is an endopeptidase that hydrolyzes neurotensin (pLYENKPRRPYIL) between Pro[10] and Tyr.[11] While this neurotensin-cleaving activity does not resemble the cleavage between Gln and Thr observed here, it is reported that neurolysin recognizes the overall peptidyl structure of substrates rather than P1 and P1′ amino acids specifically,[39] so we considered it a plausible candidate.

Indeed, we were able to confirm that recombinant neurolysin-cleaved LVVSTQTALA peptide exclusively at this site *in vitro* (Figure S16). We also confirmed that siRNA-based knockdown of neurolysin expression in A549 cells resulted in a decrease of the LVVSTQTALA hydrolytic activity in the lysate, and the extent of the activity decrease was similar to that of the protein decrease (Figure 5F,G). Thus, we concluded that the LVVSTQTALA-hydrolyzing endopeptidase is indeed neurolysin (Figure S17). To our surprise, the rate of hydrolysis of LVVSTQTALA by neurolysin was much faster than that of the known substrate, neurotensin (Figure S16). As mentioned above, the substrate preference of neurolysin cannot easily be predicted from the peptide sequence alone,[39−41] and we consider that the present enzyme−substrate relationship would have been difficult to discover without actually monitoring the reaction.

Finally, we confirmed that neurolysin activity was indeed altered in colorectal tumor sites by monitoring the activity toward the established substrate, neurotensin. Indeed, the activity in lysates could almost completely discriminate between nontumor and tumor samples under blinded conditions (Figure 5H and Table S4). Since substrates of neurolysin and their biological roles have not been well-characterized,[42,43] this information may provide useful leads for further research. Further work is ongoing to see whether neurolysin might be a useful disease marker for diagnosis or a target for treatment.

DISCUSSION

In this work, we have established a high-throughput enzymomics platform to globally evaluate peptide-metabolizing activities and to identify enzymes that metabolize specified peptides. We show that the developed system can discover and identify cell-type-specific and disease-related exo- and endopeptidase activities. Specifically, we found that NPDPWAK metabolism is specifically increased in squamous cell carcinoma H226 cells due to increased activity of DPPIV. In addition, LVVSTQTALA metabolism by neurolysin is elevated in colorectal tumor tissues. The relationship of DPPIV activity with tumorigenesis is established,[44−48] but to our knowledge, this is the first study to discover a relationship between altered neurolysin activity and tumorigenesis. While this study alone cannot determine whether the altered enzymatic functions are a cause or a result of the pathogenesis, we believe our technology will provide new opportunities to characterize the phenotypic signatures of diseases.

Thus, we were able to discover tumor-selective enzymatic activities with our peptide-metabolizing enzymomics platform. We believe our system could also be easily applied to study other types of enzymes by suitably biasing the assay conditions; for example, addition of protease inhibitors and specific cofactors to biosamples will increase the chance of discovering enzymes involved in phosphorylation (by adding ATP) or methylation (by adding SAM). Also, adjustment of the assay conditions to acidic pH would favor discovery of alterations in lysosomal proteases, which are already important targets of drugs and tumor-imaging reagents.[6] Our preliminary work has confirmed that reactivity profiles change dramatically in acidic media, providing clues to peptides that might be metabolized preferentially by lysosomal proteases (Figure S18). It is also possible that we could use live cells instead of their lysates as samples. This study was performed with a standard quadrupole mass spectrometer, but the use of other mass spectrometric platforms that might give greater reliability would be also possible. We think this simple but powerful experimental enzymomics strategy has enormous potential for discovering disease-related alterations of protein functions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b11376.

Supplementary methods, characterization data of synthetic peptides, tables, and figures (PDF)

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Notes

The authors declare no competing financial interest.

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