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# Towards the identification of unknown neuropeptide precursor-processing enzymes: Design and synthesis of a new family of dipeptidyl phosphonate activity probes for substrate-based protease identification

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ABSTRACT

Specific proteolytic processing of inactive precursors is an exquisite cellular mechanism that triggers the activation of numerous physiologic peptides and proteins. This process ensures the generation of biologically active peptides, such as many neuropeptides and peptide hormones, in the appropriate cellular compartments at the right time, and its failure leads to several pathological conditions. Identification of the proteases involved in this limited proteolysis is, therefore, an essential step for the subsequent establishment of new therapeutic targets. As a first effort along this line, we synthesized eight new dipeptidyl phosphonate activity-based probes and used them to explore the soluble proteome from mouse brain and pituitary gland for substrate-based protease identification both by in-gel analysis and mass spectrometry.



## 1. Introduction

Many bioactive peptides and proteins (e.g., neuropeptides, cell surface glycoproteins and proteolytic enzymes) are synthesized as large inactive precursors, which require specific proteolytic processing to yield the final active peptides.[1,2] The synthesis of large precursors and the need to undergo limited proteolysis to gain biological activity is a key cellular mechanism for the generation of biologically active peptides in the appropriate cellular compartments and at the pertinent moment. Many cellular processes, such as cell–cell communication, immunity, and programmed cell death, are regulated by the specific proteolysis of precursor proteins, thus, converting proteases involved in these processes into attractive targets for drug discovery.[3]

Neuropeptides and peptide hormones are among the most intensively studied proteolytic-activated peptides, not only because of their involvement in a great number of physiological processes,[4,5] but also because many neuropeptide-processing peptidases remain largely unknown. Classical biosynthetic cleavage of neuropeptide precursors occurs at basic residues within the consensus sequence Lys/Arg-$(Xxx)_n$-Lys/Arg↓ with $n = 0, 2, 4$ or 6. This cleavage is performed by a relatively small number of well-known peptidases, including prohormone convertases and cathepsin L.[6,7] However, the discovery and characterization of new neuropeptides has led to the description of a new non-classical pathway with cleavage occurring at tryptophan, leucine and other amino acids.[8] The neuropeptide-processing peptidases involved in this new non-classical pathway remain largely unknown, although a few have recently been reported, such as proteins SKI-1/S1P, NARC-1, PPE/ADAM-10 and endothelin-converting enzymes.[9–13]

Identification of these unknown proteases is an essential requirement for the subsequent establishment of new therapeutic targets that could restore physiological neuropeptide levels in pathological situations. Several approaches have used broad-spectrum activity-based probes for the functional characterization of serine and cysteine proteases.[14,15] However, the development of new highly selective probes[16] based on natural substrates facilitates the identification of proteases and can, thus, lead to the establishment of new therapeutic targets using substrate-based protein identification. Recently, we demonstrated the possibility of using substrate-based dipeptidyl phosphonates to selectively monitor endogenous post-proline protease activity by mass spectrometry and in-gel analysis.[17] Here, we expand on this initial approach by synthesizing eight new dipeptidyl phosphonate probes and use them to explore mouse brain and pituitary gland proteomes for substrate-based protease identification.

## 2. Results and discussion

### 2.1. Design and synthesis of activity-based probes

Probes were designed based on chromogranin B-derived neuropeptides originated by proteolytic cleavage at non-canonical

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sites.[18–20] Among the several neuropeptide sequences reported from chromogranin B, we selected those sequences with an unknown related protease and with a conserved cleavage site among different mammalian species ( Fig. 1). Thus, the phosphonate probes synthesized here included a dipeptidyl moiety containing these amino acids placed in positions P1 and P2 of the selected neuropeptide sequences (Fig. 2). The presence of this dipeptidyl moiety should enrich samples with proteases that recognize a certain amino acid combination and this should facilitate the identification of the proteases involved in the processing of the selected sequences.

In addition to the dipeptidyl moiety, the synthesized probes also included a phosphonate functional group in the C-terminus to bind the active site of serine proteases, and an alkyne functional group introduced in the N-terminus to allow fluorescent $TriN_3$-tag conjugation[21] by Cu(I)-catalyzed Huisgen [3+2] cycloaddition.[22,23]

To synthesize the dipeptidyl phosphonate probes, leucine- and tryptophan-derived diphenyl 1-aminoalkanephosphonates **4** and **9** were previously synthesized as outlined in Scheme 1. Briefly, compound **4** was obtained by an α-amidoalkylation in acetic acid as described previously[24] (Scheme 1A) while **9** was synthesized through a Michaelis–Arbuzov reaction and an α-carbon alkylation (Scheme 1B). Initially, previously synthesized ethyl diphenyl phosphite[25] was used in a 5-day Michaelis–Arbuzov reaction to afford **5** (35%). The yield was reduced with shorter reaction times and no significant increase was detected with longer reaction times. Deprotection of the amine-protecting group with hydrazine gave **6** in good yield (74%) and the subsequent imine formation afforded **7** in 45% yield. The tryptophan analog **9** was finally prepared by alkylation of **7** with KHMDS, HMPA and 3-indolylmethyl bromide (**8**, 27%) followed by a final α-nitrogen deprotection with TFA and $H_2O$ in DCM as a dark oil (**9**, 90%).

Probe **1d** was synthesized from **4** by direct peptide elongation in solution whereas a convergent strategy was used for synthesizing probes **1a–c** (Scheme 2). In the synthesis of probes **1a–c**, compounds **12a–c** were obtained by solid-phase peptide synthesis and subsequently coupled in solution to **4** or **9** using a polymer-bound *N*,*N*′-dicyclohexylcarbodiimide to give **13a–c**. The removal of the side chain protecting groups of compounds **13a–c** resulted in probes **1a–c** whereas for the synthesis of probes **2a–c** transesterification (**14a–c**) was introduced before deprotection. Probe **2d** was directly obtained by transesterification of probe **1d**.

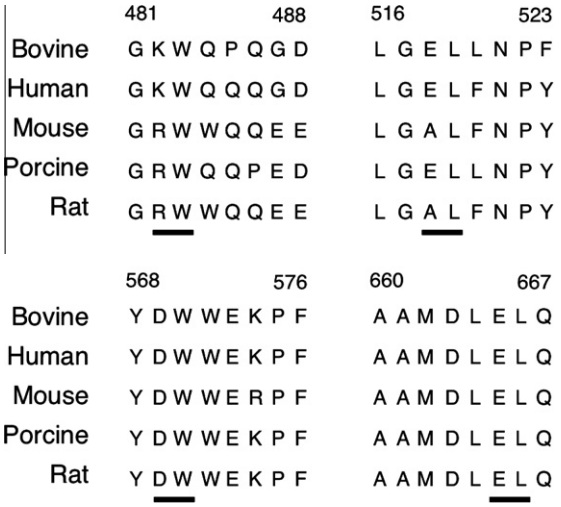



**Figure 1.** Multiple alignment of diverse chromogranin B-derived peptides. The black bars highlight the amino acids in positions P1 and P2 of the cleavage site while numbers refer to amino acid residues in the mouse chromogranin B sequence.

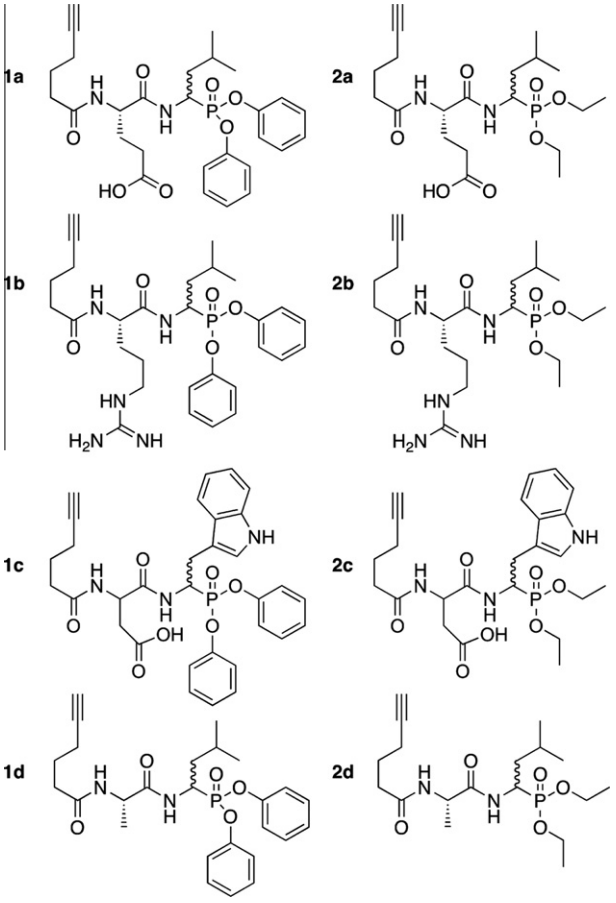
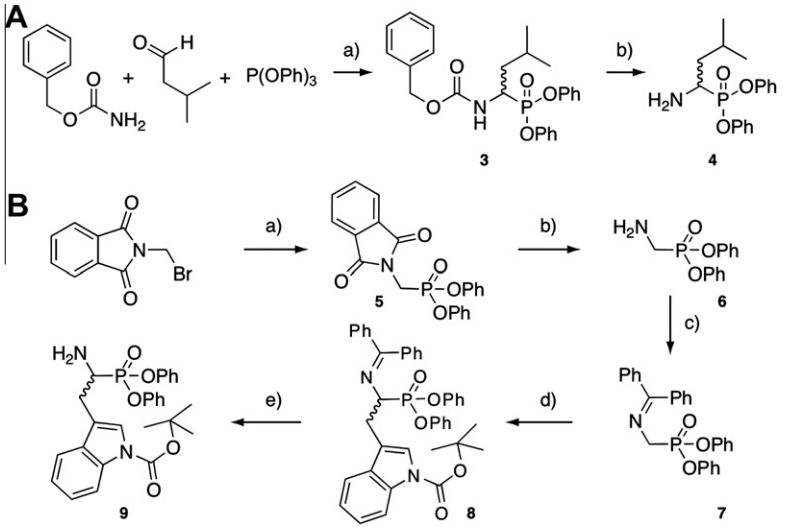



**Figure 2.** Structure of the synthesized dipeptidyl phosphonate probes Aha-Glu-Leu$^P$(OPh)$_2$ (**1a**), Aha-Glu-Leu$^P$(OEt)$_2$ (**2a**), Aha-Arg-Leu$^P$(OPh)$_2$ (**1b**), Aha-Arg-Leu$^P$(OEt)$_2$ (**2b**), Aha-Asp-Trp$^P$(OPh)$_2$ (**1c**), Aha-Asp-Trp$^P$(OEt)$_2$ (**2c**), Aha-Ala-Leu$^P$(OPh)$_2$ (**1d**), and Aha-Ala-Leu$^P$(OEt)$_2$ (**2d**).

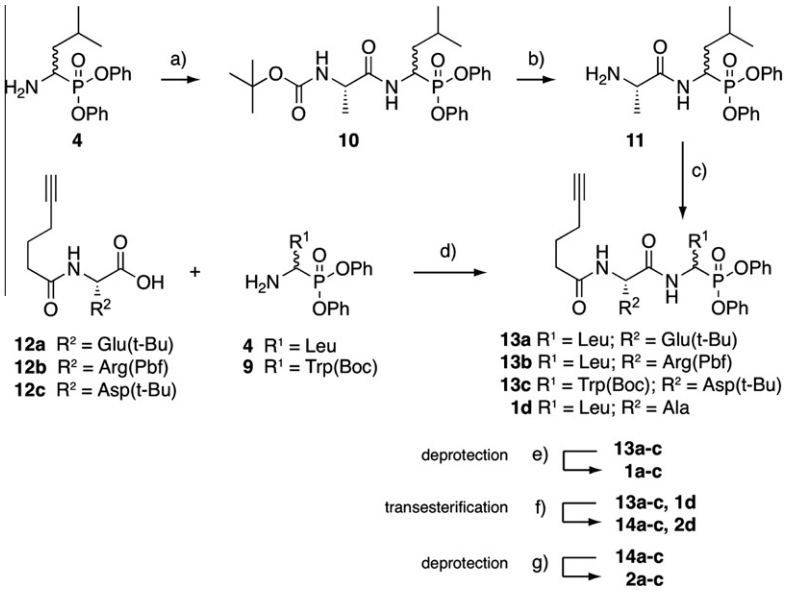
## 2.2. Proteome exploration

The synthesized phosphonate probes were used for the functional characterization of mouse soluble brain and pituitary gland proteomes to detect those serine proteases that specifically recognize the dipeptidyl moiety of the probes. First, both active and heat-denatured proteomes were incubated with probes **1a–d** and **2a–d** (25 μM and 100 μM, 1 h at 25 °C). Samples were labeled using the trifunctional fluorescent $TriN_3$-tag (Supplementary Fig. 1), and were analyzed by SDS–PAGE and visualized with a fluorescence scanner. This in-gel approach allowed the analysis of multiple tissue-probe combinations in short amounts of time and revealed the detection of several activity-based bands for the different phosphonate probes (Fig. 3 and Supplementary Figs. 2–9).

The most promising tissue–probe combinations from the in-gel analyses were therefore selected for further characterization by MudPIT analysis in order to identify those proteins specifically labeled by the dipeptidyl phosphonate probes. After incubating (1 h at 25 °C) brain and pituitary gland homogenates with each phosphonate probe (25 μM), probe-protein complexes were labeled with the $TriN_3$-tag, and removed from the sample by means of avidin beads. The pulled-down proteins from both replicates were analyzed by reverse-phase chromatography and subsequently identified by MS/MS. The resulting data set was analyzed with the SEQUEST software[26] using the mouse IPI database.[27] Protein abundance was estimated from spectral counts[28] and the maximum false-positive rate was set to 1% using a random coiled database and the DTASelect software.[29] A heat-denatured sample was



**Scheme 1.** Reagents and conditions: (A) synthesis of H-Leu$^{P}$(OPh)$_2$ (**4**): (a) acetic acid, 1 h at 25 °C and 16 h at 50 °C; (b) HBr in acetic acid, 1 h at 25 °C. Neutralized with triethylamine. (B) Synthesis of H-Trp$^{P}$(OPh)$_2$ (**9**): (a) POEt(OPh)$_2$, xylene, reflux, 5 days; (b) hydrazine in THF, 72 h at 70 °C; (c) benzophenone in toluene, 12 h, reflux (Dean Stark); (d) HMDA, KHMDS in THF, 30 min at −78 °C; (e) *t*-butyl-3-bromomethylindole-1-carboxylate in THF, 4 h at −78 °C; and (f) $H_2O$ (1%), TFA (3%) in DCM, 1 h at 25 °C.



**Scheme 2.** Reagents and conditions: (a) HATU, DIEA and Boc-Ala-OH in DMF, 16 h at 25 °C; (b) 4 M HCl in 1,4-dioxane, 30 min from 0 to 25 °C. Neutralization with triethylamine; (c) HATU, DIEA and 5-hexynoic acid in DMF, 16 h at 25 °C; (d) polymer-bound *N*,*N*′-dicyclohexylcarbodiimide in DMF, 24 h at 25 °C; (e) removal of side-chain-protecting groups with TFA–$H_2O$ (19:1), 1.5 h at 25 °C; (f) transesterification with KF, 18-crown-6 ether in ethanol, 10 min at 80 °C, 16 h at 25 °C; and (g) Removal of side-chain-protecting groups with TFA–$H_2O$ (19:1), 1.5 h at 25 °C.

used as negative control to identify non activity-dependent probe-protein complexes. A second control consisting of samples incubated without any probe was also performed.

The MudPIT analysis resulting from incubation of the pituitary gland homogenates with the phosphonate probe **1d** led to the identification of the liver carboxylesterase N precursor, a 60 kDa serine hydrolase (IPI00138342; P23953; ESTN_MOUSE) and its predicted counterparts (Table 1). In these assays, the liver carboxylesterase N precursor entries were, together with a phosphoglycerate kinase (IPI00230002, IPI00555069), the only proteins detected with an over eight-fold sample/control ratio and present in both sample replicates. All the other proteins detected were either present in the sample and at least one control (<eight-fold) or had less than five spectral counts (Supplementary Table). The liver carboxylesterase N precursor was identified with ten peptides (>25% of the sequence coverage), of which five were unique peptides, that is, peptides belonging only to that protein: AISESGVVINTNVGK, SFNTVPYIVGFNK, EGASEEETNLSK, HSLLPPVVDTTQGK, NPPETDP TEHTEH (Supplementary Fig. 10). The 60 kDa serine hydrolase observed cleaved both amide and ester bonds and has already been described as a surfactant convertase and a carboxylesterase.[30,31] However, the putative involvement of the identified serine hydrolase in neuropeptide processing needs to be experimentally confirmed. Further work is required for the identification of the other bands observed during in-gel analyses.

These results constitute not only a further step towards the identification of unknown neuropeptide-processing enzymes, but also confirmation that dipeptidyl phosphonate probes are valuable

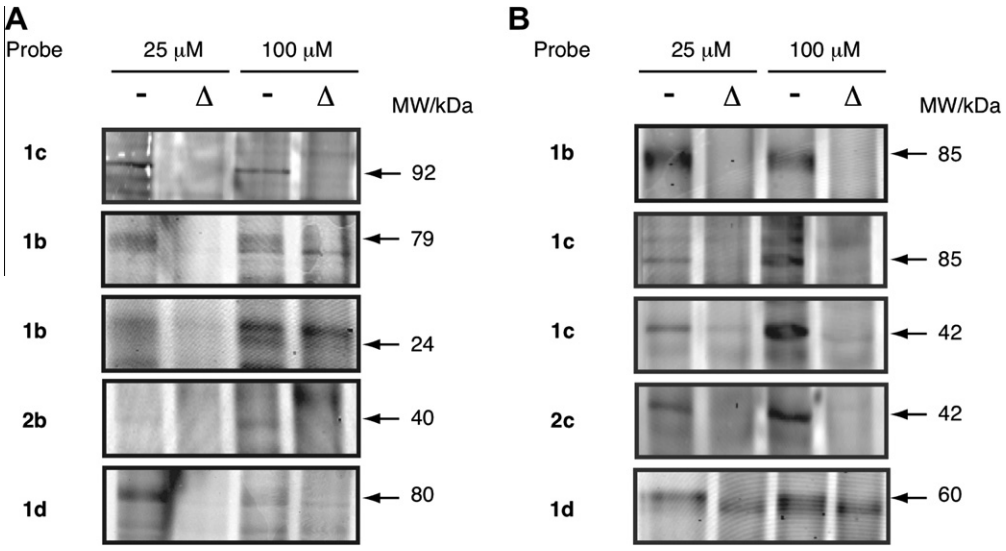



**Figure 3.** (A) Specific bands detected in an activity-dependent manner when exploring the brain soluble proteome with probes **1a–d** and **2a–d**. Both active and heat-denatured soluble proteomes were incubated with probes **1a–d** and **2a–d** (25 and 100 μM, 1 h at 25 °C) and labeled using the trifunctional fluorescent $TriN_3$-tag. Samples were analyzed by SDS–PAGE and visualized with a fluorescence scanner. (B) Specific bands detected in an activity-dependent manner when exploring the pituitary gland soluble proteome with probes **1a–d** and **2a–d**. Samples were prepared and analyzed as described for A).

**Table 1**
Spectral counts of proteins identified by MudPIT with an over eight-fold sample/control ratio and more than five spectral counts

| IPI number[a] (Mouse) | Sample | Control A (Boiled sample) | Control B (No. probe) | Sample/controls ratio |
|---|---|---|---|---|
| IPI00553586 | 12.0 ± 2.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | n.a. |
| IPI00677642 | | | | |
| IPI00675322 | | | | |
| IPI00678863 | | | | |
| IPI00672511 | | | | |
| IPI00671100 | | | | |
| IPI00667966 | | | | |
| IPI00664458 | | | | |
| IPI00662318 | | | | |
| IPI00138342 | 8.0 ± 1.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | n.a. |
| IPI00230002 | 6.0 ± 1.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | n.a. |
| IPI00555069 | | | | |

[a] IPI00138342: liver carboxylesterase N precursor. IPI00555069, IPI00230002: phosphoglycerate kinase. IPI00553586, IPI00677642, IPI00675322, IPI00678863, IPI00672511, IPI00671100, IPI00667966, IPI00664458, IPI00662318: PREDICTED—Similar to liver carboxylesterase.

tools for substrate-based protease identification in complex samples and, eventually, for the identification of the proteases involved in the proteolytic processing of chromogranin B-derived peptides.

## 3. Conclusion

A new family of dipeptidyl phosphonate activity-based probes for substrate-based identification of new proteases is reported. These probes were designed based on chromogranin B-derived neuropeptides originated by proteolytic cleavage at non-canonical sites and used for exploring the soluble proteome from mouse brain and pituitary gland. An efficient synthetic methodology for the preparation of these probes was developed. The in-gel analysis revealed several putative candidates that were detected by the synthetic probes in an activity-dependent manner. Preliminary mass spectrometry analysis of these candidates allowed the identification of a 60 kDa serine hydrolase (IPI00138342; P23953; ESTN_MOUSE) as a protease putatively involved in the proteolytic processing of chromogranin B-derived peptides. These results render dipeptidyl phosphonate probes effective tools for substrate-based protease identification, which can be used to screen complex proteomes and to pinpoint unknown neuropeptide-processing enzymes.

## 4. Experimental section

### 4.1. Synthesis

For detailed probe synthesis and characterization the reader is referred to the Supplementary data.

### 4.2. In-gel analysis

#### 4.2.1. Preparation of soluble brain and pituitary gland proteomes

Brain and pituitary gland homogenates were obtained from adult male mice (BALB/c, 8 weeks old). Both tissues were frozen after extraction and homogenized using 8 ml (brain) and 2 ml (pituitary gland) of PBS (pH 7.4) and a tight douncer homogenizer

from Wheaton Science Products (Millville, NJ USA). Homogenates were centrifuged on a bench centrifuge (5 min, 4 °C, 2000 rpm) and on an ultracentrifuge (1 h, 4 °C, 100,000g). Pellets were discarded and the supernatant was collected to obtain the soluble proteome. The total protein content was quantified with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as standard. Aliquots of the brain and pituitary gland homogenates were immediately prepared and stored at −80 °C.

#### 4.2.2. Activity-dependent labeling experiments

Probes **1a–d** and **2a–d** (25 and 100 μM final) were added to either pituitary gland (0.5 mg ml$^{-1}$) or brain homogenates (1 mg ml$^{-1}$) or a mixture of both, and samples were gently mixed during 1 h at room temperature (total volume = 50 μl). One microliter of the $TriN_3$-tag (5 mM in DMSO) was then added, followed by 1 μl of freshly prepared tris-(2-carboxyethyl)-phosphine (50 mM in $H_2O$), 3 μl of tris(triazolyl)amine (1.7 mM in DMSO/*t*-butanol (1:4)) and 1 μl of $CuSO_4$ (50 mM in $H_2O$). Samples were incubated at 25 °C for 1 h and were intermittently vortex-mixed. Reaction products were analyzed by SDS–PAGE (20 × 20 cm, 1.5 mm, Dual Gel P10DS-1 Emperor Penguin from Thermo Fisher Scientific Inc., Waltham, MA USA) and visualized on a fluorescence scanner. Heat-denatured samples (Δ) were inactivated (5 min at 95 °C) before adding the phosphonate probe to be used as a negative control.

### 4.3. Mass spectrometry assays

#### 4.3.1. Sample preparation

Brain homogenates (1 mg ml$^{-1}$) were incubated (1 h at 25 °C) with peptidyl phosphonate probes **1a–d** and **2a–d** (25 μM) (total volume = 500 μl). $TriN_3$-tag (11.6 μl, 5 mM in DMSO) was subsequently added, followed by freshly prepared tris-(2-carboxyethyl)-phosphine (11.6 μl, 50 mM in $H_2O$), tris-(triazolyl)amine (35 μl, 1.7 mM in DMSO/*t*-butanol (1:4)) and $CuSO_4$ (11.6 μl, 50 mM in $H_2O$). After another 1 h incubation at 25 °C, samples were centrifuged (4 min, 25 °C, 2000g) and the supernatant was removed. Cold methanol (0.5 ml) was added to the pellet and the samples were sonicated (0.5 Hz, 50%), shaken (10 min, 4 °C) and centrifuged (4 min, 4 °C, 2000g). This washing procedure with methanol was repeated twice. 1.2% SDS in PBS (1 ml) were added to the pellet and samples were sonicated briefly prior to sample heating (5 min at 95 °C) and dilution with 0.2% SDS in PBS (5 ml). Previously (PBS) washed avidin beads (50 μl, Avidin-Agarose from egg-white from Aldrich, Milwaukee, WI, USA) were added to the samples, which were then incubated for 1 h at 25 °C. The supernatant was subsequently removed by centrifugation (3 min, 25 °C, 1400g) and the avidin beads were successively washed with 0.2% SDS in PBS (1 × 10 ml, 3 min), PBS (3 × 10 ml, 1 min) and $H_2O$ (3 × 10 ml, 1 min). Samples were denatured and reduced (30 min, 25 °C) with 6 M urea in PBS and 10 mM tris-(2-carboxyethyl)-phosphine (final volume = 500 μl) and alkylated in the dark (30 min) with iodoacetamide (25 μl, 400 mM). Urea (200 μl, 2 M) in PBS and trypsin (4 μl, 0.5 mg/ml) were then added and samples were digested overnight at 37 °C. Finally, avidin beads were removed by centrifugation (3 min, 25 °C, 1400g) and samples were analyzed by mass-spectrometry with no further manipulation. Heat-denatured homogenates (5 min, 95 °C) and homogenates incubated without any probe were used as controls. All assays were done in duplicate.

#### 4.3.2. Multidimensional protein identification technology (MudPIT)

Each sample was subjected to MudPIT analysis in a Thermo Finnigan LTQ mass spectrometer equipped with a nano-LC electrospray ionization source. Samples were loaded into a NanoEase pre-column (C18 Symmetry 300, 5 μm, Waters) and analyzed in a 10 cm Biphasic PicoFrit column (PF7515-04CMSCX-06CMPP2300, New Objective) packed with C18 reverse-phase material and strong cation exchange material. An automated five-step chromatography was performed for each sample. The first step of each run consisted of a 55 min gradient from 0% to 45% buffer B, 10 min gradient from 45% to 100% buffer B and a 20 min hold at 100% buffer B. The following steps were 112 min each with the following profile: 3 min of 100% buffer A, 2 min of *X*% buffer C, 1 min of 95% buffer A, a 10 min gradient from 5% to 15% buffer B, a 45 min gradient from 15% to 25% buffer B, and a 52 min gradient from 25% to 55% buffer B. The 2 min buffer C percentages (*X*) in steps 2–5 were as follows: 25%, 50%, 80% and 100%. Buffer A: 95% $H_2O$, 5% MeCN and 0.1% formic acid. Buffer B: 20% $H_2O$, 80% MeCN and 0.1% formic acid. Buffer C: 500 mM ammonium acetate, 95% $H_2O$, 5% MeCN and 0.1% formic acid.

MS/MS data were analyzed with the SEQUEST software[26] using the mouse IPI database version 3.23[27] from the European Bioinformatics Institute and protein abundance was estimated from spectral counts.[28] The maximum false-positive rate was set to 1% using a random coiled database and the DTASelect software.[29]

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.066.

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