Supporting Information

Oriented Immobilization of Oxyamine-Modified Proteins

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General

Chemicals were purchased from Acros, Sigma-Aldrich or Fluka and used without further purification. All laboratory-reagent grade and analytical grade solvents were purchased from Fisher Scientific. Dry dimethyl formamide (DMF) was obtained from Aldrich and used as received. Dry dichloromethane (DCM) was prepared by distillation from calcium hydride. Ultra pure water was obtained from a Millipore milliQ Advantage system. Primers used in plasmid construction were purchased from Eurofins MWG Operon. The glass slides activated with *N*-hydroxysuccinimide (NHS) esters to provide covalent immobilization of amine groups were purchased from SCHOTT (NEXTERION® Slide H). The microarray spotting was performed using a non-contact microarray robot (GeSIM NP1.2, Dresden, Germany). High resolution mass spectra (HR-MS) were measured using electrospray ionization (ESI). NMR spectroscopic data were recorded on a 400 MHz instrument at room temperature. NMR spectra were calibrated to

the solvent signal of $CDCl_3$ (δ = 7.26 and 77.0 ppm). The following abbreviations are used to indicate the signal multiplicity: s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Microarrays were scanned using a fluorescence scanner (GenePix 4000B, Molecular Devices or Typhoon Trio+, General Electric).

1. Synthesis of 5-amino-2-pentanone ethylene ketal[1] (1)

To a solution of 5-chloro-2-pentanone ethylene ketal (1.5 g, 9.1 mmol) in DMF (20 ml) was added potassium phthalimide (1.7 g, 9.1 mmol). The solution was refluxed for 14 hours. The reaction mixture was added into 1000 ml water, and the resulting white precipitate was filtrated. The white solid was washed with water and ether. Then the solid was dissolved in 95% ethanol and $NH_2NH_2 \cdot H_2O$ (0.4 g, 8 mmol) was added. After refluxing for 1 hour, the reaction mixture was treated with a 2 N KOH solution. Then ten-times volume ether was added. The ether layer was further washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated to yield compound **1** (0.46 g, 3.2 mmol, yield 35%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.90 (m, 4H), 2.69 (t, J = 7.0 Hz, 2H), 2.08 (bs, 2H), 1.64 (m, 2H), 1.53 (m, 2H), 1.29 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 110.04, 64.73, 42.23, 36.49, 27.95, 23.90. ESI MS: calcd for $C_7H_{16}NO_2$ 146.11 $[M+H]^+$, found 146.07 $[M+H]^+$; HR-MS: m/z: calcd for $C_7H_{16}NO_2$: 146.11756, found 146.11713 $[M+H]^+$.

2. Generation of ketone-coated glass slides

A NEXTERION® glass slide stored at -20 ºC was warmed up to room temperature and mounted on a custom-made teflon holder on top of a magnetic stirring bar inside a glass reactor. The slide was soaked with a freshly prepared 20 mM solution of **1** in DMF. DIPEA was subsequently added (600 µL) and the reaction mixture was stirred for 12 h at room temperature under argon.

After washing with DMF for three times, the slide was blocked by reaction with a 100 mM ethanolamine solution in 50 mM sodium borate buffer at pH 8.5 for 3 h. The slide was washed three times with doubly distilled H_2O and dried under an argon stream. The modified slides can be stored at -20 ºC for at least three months for further use. Prior to immobilization, the functionalized slide was treated with a 1% HCl solution at room temperature for 30 minutes to deprotect the ketone. After washing with ddH_2O for three times, the slide was dried using an stream of argon.

3. Preparation of thioester-containing proteins

The protein coding regions were C-terminally fused to an intein-chitin binding domain assembly as implemented in the pTWIN-1 vector (New England Biolabs, Ipswich, MA).[2] The constructed plasmid was transformed in *E.coli* BL21 (DE3) cells and expressed. After harvesting of the bacteria, the cells were lysed in lysis buffer (25 mM sodium phosphate pH 7.5, 500 mM NaCl, 1 mM $MgCl_2$, 10 µM GDP (for GTPases only)) by passing them multiple times through a fluidizer (110S, Microfluidics) and centrifugated. The soluble fraction was applied to chitin affinity beads. The beads were washed extensively with lysis buffer and incubated overnight at room temperature with lysis buffer containing 500 mM 2-mercaptoethanesulfonic acid (MESNA). The resulting thioester-containing proteins were concentrated by size exclusion filtration (Amicon Ultra-15 MWCO 10 kD, Millipore) to a final concentration of 5-10 mg/ml. After aliquotation the protein was shock frozen and stored at -80 ºC.

4. Preparation of oxyamine-containing proteins

Bis(oxyamine).2HCl (**2**) was prepared as previously described.[1] The white solid was dissolved in a 2 M NaOH solution to obtain a 1-2 M stock solution with a pH around 7.5. The resulting

solution was filtrated through a 0.2 µm cellulose acetate sterile membrane. Bis(oxyamine) **2** (final concentration 500 mM) and thioester-containing proteins (5-10 mg/ml) were incubated in sodium phosphate buffer (25 mM, pH 7.5) on ice overnight. Quantitative conversion was observed by ESI-MS, resulting in oxyamine-containing proteins. The protein solution was dialyzed against 1 L of dialysis buffer (30 mM Na-phosphate, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$ and 5 mM DTE) at 4 °C for 2 hours, and subsequently against 1 L of dialysis buffer (30 mM Na-phosphate, pH 7.0, 50 mM NaCl, 1 mM $MgCl_2$ and 5 mM DTE) at 4 °C for 2 hours to remove the excess of bis(oxyamine) **2**. The resulting oxyamine-modified proteins are ready for protein spotting. For GTPases, 10 µM GDP should be added in the dialysis buffer.

5-Generation of protein expression lysates.

Proteins were expressed as described above. After harvesting the bacteria, the cells were lysed in lysis buffer (25 mM sodium phosphate pH 7.5, 500 mM NaCl, 1 mM $MgCl_2$, 10 µM GDP (for GTPases only), 1 g cells/1 mL lysis buffer) by passing them multiple times through a fluidizer (110S, Microfluidics) and subsequently centrifuged. Then the soluble fraction was isolated and treated with a 500 mM MESNA solution (final concentration) at room temperature overnight and with a 300 mM bis(oxyamine) solution (final concentration) on ice for at least 4 h. The resulting solution was dialyzed twice against 1 L of the dialysis buffer at pH 7.5, and against 1 L of dialysis buffer at pH 7.0 to remove the excess of bis(oxyamine) **2**. The resulting cell lysates containing oxyamine-modified proteins are ready for spotting. For control experiments, the cellular lysates were treated with 500 mM MESNA only and then dialyzed again dialysis buffers as described.

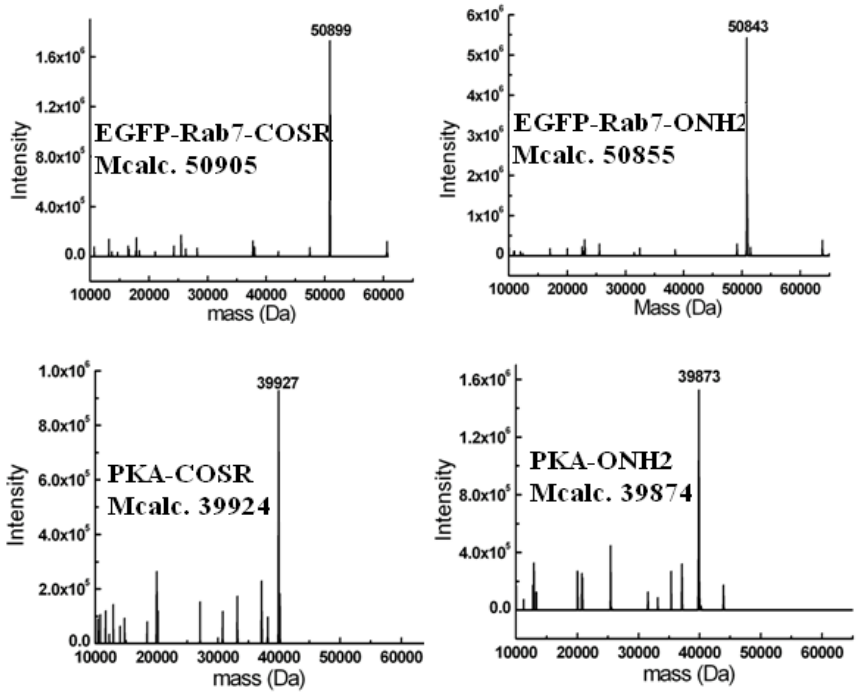

Figure S1. ESI-MS spectra of thioester proteins (left) and their oxyamine proteins (right).

6. General procedure for protein spotting and immobilization

Protein spotting was carried out by a non-contact microarray robot to generate five identical subarrays per slide that can be separated by GeneFrames (ABgene, Thermoscientific) (spot volume: 250 pL; spot size: 400 μm diameter). The spotting buffer contains 20 % glycerol to prevent the drying of protein spots on the slides. After incubating in a humidity chamber at room temperature for several hours, the slides were washed with washing buffer (25 mM HEPES pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM DTE, 0.05% Tween 20) three times (3 × 5 min) to remove unreacted proteins. Then, the slides were blocked with blocking buffer (1% BSA in washing buffer) for 30 min at room temperature. The resulted slides were further washed with washing buffer and rinsed with doubly distilled H_2O and dried under an argon stream for

subsequent fluorescence scanning or interaction with other proteins. The data were analyzed and quantified with the software ImageQuant TL (GE Healthcare).

6.1 Immobilization of EGFP-Rab7-ONH_2 and EGFP-Rab7-thioester on slides

A 35 µM solution of EGFP-Rab7-ONH_2 and EGFP-Rab7 thioester (negative control) in phosphate buffer (30 mM, pH 7.0, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM DTE, 20 µM GDP, 20% glycerol, 2% Tween 20) was spotted on ketone-coated slides to generate protein microarrays, followed by incubation in a humidity chamber for 6 hours at room temperature. After washing with washing buffer and doubly distilled H_2O, the slide was dried under argon. The immobilized proteins were detected with a fluorescence scanner (Typhoon Trio+ scanner, General Electric, Figure S2).

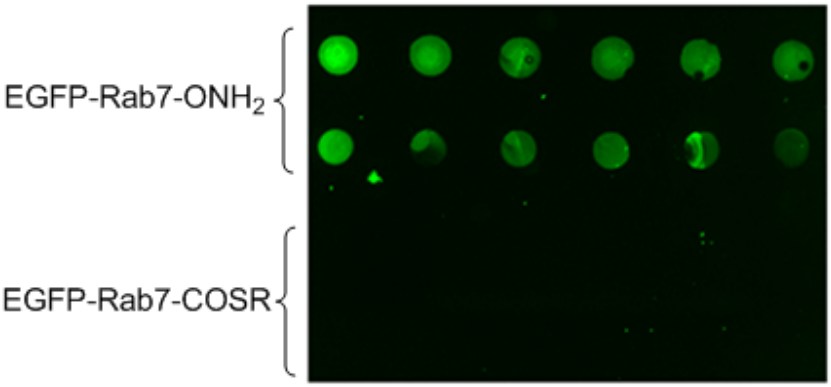

Figure S2. Fluorescence image of a subsection of the EGFP-Rab7 microarray described. The ratio of average fluorescence intensity bewteen the oxyamine-modified protein and the thioester protein is 478:1.

6.2 Time-dependent immobilization of EGFP-Rab7-ONH_2

A 50 μM solution of EGFP-Rab7-ONH_2 in phosphate buffer (30 mM, pH 7.0, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM DTE, 20 μM GDP, 20% glycerol, 2% Tween 20) was spotted on a ketone-coated slide to generate five identical EGFP-Rab7 subarrays that were separated by applying GeneFrames and incubated in a humid chamber. One subarray was washed with washing buffer at each time point to stop the oxime ligation (2, 4, 5, 6, and 7 hours). Finally, after rinsing with water and drying under argon, the microarray was scanned and analyzed as described above.

6.3 pH-dependent immobilization of EGFP-Rab7-ONH_2

Different 45 μM solutions of EGFP-Rab7-ONH_2 in phosphate buffers with different pH values were spotted on the ketone-coated slide to generate five subarrays per slide separated by GeneFrames, followed by incubation in a humid chamber for 6 hours. The microarray was washed, scanned and analyzed as described above.

6.4 Aniline-catalysis of EGFP-Rab7-ONH_2

Considering the fact that aniline is insoluble in water, we prepared a 2 M aniline stock solution in methanol. A 50 μM solution of EGFP-Rab7-ONH_2 containing or not 100 mM of aniline was spotted on ketone-coated slides followed by incubation in a humidity chamber for 6 hours. The slide was washed, scanned and analyzed as described above.

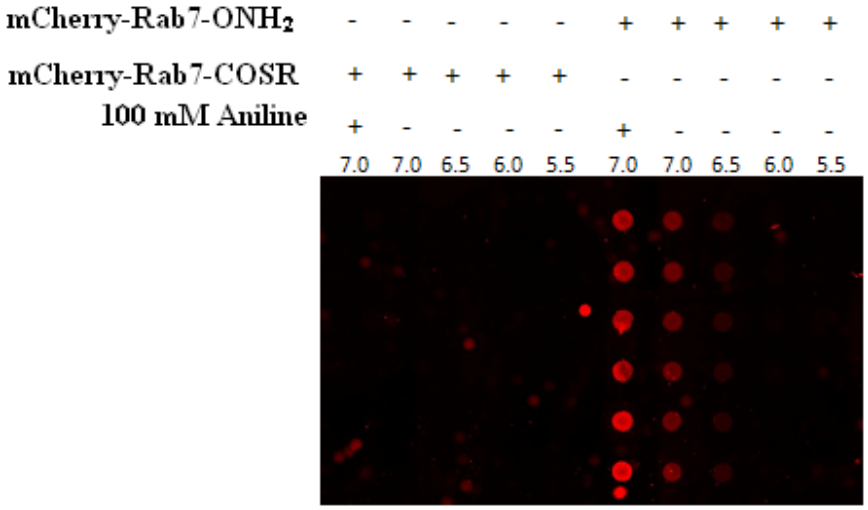

Figure S3. Immobilization of mCherry-Rab7-COSR and mCherry-Rab7-ONH_2 on ketone-coated slides at different pH values with and without aniline catalysis. The slides were incubated with the protein solution at room temperature for 4 hours. The proteins samples were prepared as previously described.[1]

7. Detection of protein-protein interactions

7.1 Preparation of Cy3-REP-1 and Cy3-RabGDI[3]

To a solution of REP-1 (7.0 mg/mL, 50 µL) in labeling buffer (50 mM HEPES, pH 7.2, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM DTE), Cy3-NHS-ester (GE healthcare PA23001) dissolved in 50 µL labeling buffer (0.1 mM) was added and then the reaction mixture was shaken at room temperature for 30 min and one hour on ice. After this, the labelled protein was separated using Hi-Trap desalting columns (Pharmacia Biotech) to obtain the desired Cy3-REP-1 with a final molar dye/protein (D/P) ratio 1.28. Cy3-RabGDI was prepared analogously with a final molar dye/protein (D/P) ratio 1.10.

7.2 Interaction analysis of EGFP-Rab7 microarrays with Cy3-REP-1 and Cy3-RabGDI

The EGFP-Rab7 microarrays were generated as mentioned above. Blocking was done by covering the slide surface with washing buffer containing 1% of BSA for 30 min.

Two subarrays separated by GeneFrames were respectively incubated with 100 µL solution of 200 nM Cy3-REP-1 or 1 µM Cy3-RabGDI for 30 min, followed by washing three times with washing buffer (3x5 min). After rinsing with doubly distilled H_2O and drying under argon, the slide was scanned and quantified as described above.

7.3 Interaction analysis of PKA microarrays with FITC-labeling PKA antibody

The PKA-antibody (sc-365615) was purchased from Santa Cruz Biotechnology. The FITC-labelled antibody was prepared using a FITC protein labeling kit (Invitrogen). Different concentrations of PKA-ONH_2 (5-100 µM) were spotted on ketone-coated slides (pH 7.0) and incubated at room temperature for 6 hours. After washing and blocking, the slide was incubated with 500 nM FITC-antibody for 30 min, followed by washing, drying and fluorescence detection as described above.

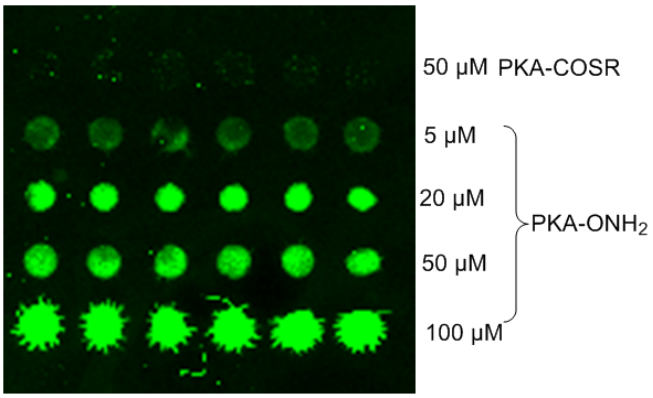

Figure S4. Different concentrations of PKA-ONH_2 were immobilized on ketone-coated microarray and then detected by incubation with a 500 nM solution of FITC-labelled antibody.

7.4 Immobilization of protein expression lysates

Both cellular lysates containing expressed EGFP-Rab7 or PKA were prepared as described above. Cellular lysates containing EGFP-Rab7-ONH_2 (0.5 g and 1 g cell/ml lysis buffer) were mixed with glycerol (final concentration 20%) and Tween 20 (final concentration 2%) and then spotted on a ketone-coated slide to generate three identical EGFP-Rab7 subarrays that were separated by applying GeneFrames and incubated in a humidity chamber for 6 h at room temperature. EGFP-Rab7-COSR cellular lysate (1 g cell/ml lysis buffer) was used as negative control. After washing with washing buffer and doubly distilled H_2O, the slide was dried under an argon stream. The microarray was scanned and analyzed as described above. The PKA-ONH_2 cellular lysate (1 g cell/ml lysis buffer) was immobilized using a similar protocol. After washing and blocking, the slide was incubated with 500 nM FITC-labeled PKA antibody for 30 min., followed by washing, drying and fluorescence detection as described above.

Supporting References

[1] L. Yi, H. Y. Sun, Y. W. Wu, G. Triola, H. Waldmann, R. S. Goody, *Angew. Chem. Int. Ed.* **2010**, *49*, 9417.

[2] T. C. Evans, J. Benner, M. Q. Xu, *Protein Sci.* **1998**, *7*, 2256.

[3] Alexandrov, K.; Simon, I.; Yurchenko, V.; Iakovenko, A.; Rostkova, E.; Scheidig, A. J.; Goody, R. S. *Eur. J. Biochem.* **1999**, *265*, 160.