

# Supporting Information

## Site-specific, Reversible and Fluorescent Immobilization of Proteins on CrAsH-modified surfaces

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### General Methods

Unless otherwise specified, all solvents and reagents were purchased from commercial suppliers (Acros, Alfa Aesar, Fluka, Novabiochem, Iris Biotech, Sigma-Aldrich) and used without further purification. Milli-Q grade water was used for all experiments. Analytical thin layer chromatography (TLC) was carried out on Merck pre-coated silica gel plates (60F-254) using ultraviolet light irradiation at 254 nm for detection or a 5% phosphomolybdic acid solution in ethanol as staining reagent. Column chromatography was performed using silica gel from (particle size 0.07 mm – pore size 60 Å). Analytical HPLC-MS data were recorded on a HPLC system with a C4 or a C18 reverse column coupled to an ESI spectrometer, flow rate: 1.0 mL/min; time: 15 min; solvent A: 0.1% HCOOH in water, Solvent B: 0.1% HCOOH acetonitrile; 1 min 10% B, in 10 min to 100% B. High resolution mass spectra (HR-MS) were

measured on a Thermo Orbitrap coupled to a Thermo Accela HPLC machine and using the electron spray ionization technique (ESI). Preparative HPLC separations were carried out using a reversed-phase C4 or C18 column (RP C4, flow 20.0 mL/min, solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile, from 10 % B to 100 % B in 25 min. Optical rotations were measured in a Schmidt & Haensch Polartronic HH8 polarimeter at 589 nm, with values reported in  $10^{-1}$  deg cm<sup>2</sup> g<sup>-1</sup> and concentrations *c* given in g/100mL. NMR spectroscopic data were recorded on Varian Unity Inova 600 (599.8 MHz (<sup>1</sup>H) and 150.8 MHz (<sup>13</sup>C)), Bruker DRX 500 (500.1 MHz (<sup>1</sup>H) and 125.8 MHz (<sup>13</sup>C)), Bruker DRX 400 (400 MHz (<sup>1</sup>H), 100.5 MHz (<sup>13</sup>C) and Varian Mercury VX 400 (400.1 MHz (<sup>1</sup>H) and 100.6 MHz (<sup>13</sup>C)) spectrometers. Chemical shifts are expressed in parts per million (ppm) and the spectra are calibrated to residual solvent signals of DMSO [2.50 ppm (<sup>1</sup>H) and 39.43 ppm (<sup>13</sup>C)]. Coupling constants are given in Hertz (Hz) and the following abbreviations indicate the multiplicity of the signals: s (singlet), d (doublet), t (triplet), q (quartet), qui (quintet), sext (sextet), sept (septet), m (multiplet), app (apparent), br (broad signal). Where possible, structural assignments were attempted using standard 2-D NMR techniques (gCOSY, gHSQC, TOCSY). Peptide synthesis was performed in an automated microwave peptide synthesizer (Liberty, CEM), in a 10 mL closed reaction vessel and temperature was controlled by Fiber optic sensor.

To provide covalent immobilization of amine groups, we purchased glass slides activated with *N*-hydroxysuccinimide (NHS) esters from SCHOTT (NEXTERION® Slide H). The microarray spotting was performed using a non-contact microarray robot (GeSIM NP1.2, Dresden, Germany). Microarrays were scanned using a fluorescence scanner or using a white light lamp and a CCD camera. (GenePix 4000B, Molecular Devices, Typhoon Trio+, General Electric or BioAnalyzer 4F). At maximum the slides parts are illuminated 1 second per scan. Cy5-labeled Streptavidin antibody, Cy3-labeled Ras antibody, Alexa 468-Fluor anti goat antibody, Goat anti PDE $\alpha$  antibody were purchased from Cruz Biotechnology and Invitrogen and used according to the user manual.

H-Ras 1-181<sup>1</sup> and bodipy-labeled farnesylated N-Ras were prepared as previously described.<sup>2</sup>

### Synthesis of CrAsH and Fluorescein derivatives

#### Synthesis of 5(6)-Fluorescein

5(6)-carboxyfluorescein was synthesized according to the reported procedures. Briefly, an homogeneous mixture of 9 g 1,2,4- benzenetricarboxylic anhydride (46.84 mmol), 10.32 g resorcinol (93.69 mmol, 2 eq) and 15.82 g zinc bromide (70.27 mmol, 1.5 eq) was stirred at 180 °C for 80 minutes. The mixture was then cooled down to room temperature and treated with a 1N HCl solution (700 mL). The suspension was refluxed at 110 °C for 1 hour and filtered when still hot. The solid was collected, treated with a 1N HCl solution (700 mL), refluxed for 1 hour and filtered again. The solid that recrystallizes from the two acidic solutions was collected by filtration and dried under high vacuum overnight in the presence of P<sub>2</sub>O<sub>5</sub> to yield 9 g of 5(6)-carboxyfluorescein as a mixture of 2 regioisomers (22.94 mmol; 49 %).

**<sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>):** δ 12.72 (s, 2H, COOH), 10.20 (s, 4H, Ar-OH), 8.40 (s, 1H, CH<sub>Ar</sub>-5-Isomer), 8.30 (dd, *J* = 8.0, 1.5 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 8.23 (dd, *J* = 8.0, 1.2 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 8.11 (d, *J* = 7.5 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 7.65 (s, 1H, CH<sub>Ar</sub>-6-Isomer), 7.39 (d, *J* = 8.3 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 6.70 (d, *J* = 2.4 Hz, 4H, CH<sub>Ar</sub>-5/6-Isomer), 6.61 (dd, *J* = 8.7, 3.8 Hz, 4H, CH<sub>Ar</sub>-5/6-Isomer), 6.55 (dd, *J* = 8.7, 2.4 Hz, 4H, CH<sub>Ar</sub>-5/6-Isomer);

**<sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>):** δ 172.9, 167.85, 167.80, 165.9, 159.6, 151.87, 151.80, 137.3, 136.1, 132.45, 131.2, 129.3, 129.1, 126.7, 125.4, 125.2, 124.6, 124.4, 112.7, 109.0, 108.9, 102.3, 82.9. **LC-ESI-MS:** calcd for C<sub>21</sub>H<sub>13</sub>O<sub>7</sub>: 377.07 [M+H]<sup>+</sup>; found 377.06 [M+H]<sup>+</sup>, R<sub>t</sub> = 7.25 min [C4]; **HR-MS:** *m/z*: calcd for C<sub>21</sub>H<sub>13</sub>O<sub>7</sub>: 377.06558 [M+H]<sup>+</sup>, found 377.06588 [M+H]<sup>+</sup>.

**5(6)-carboxyfluorescein diacetate** 3.92 g 5(6)-carboxyfluorescein (10 mmol) was treated with acetic anhydride (20 mL) and refluxed for 2 hours. The reaction mixture was cooled to room temperature and then water was carefully added until a waxy precipitate was formed. The precipitate was triturated in water and the resulting solid was filtered, washed with more water

and petroleum ether and dried under vacuum to afford 4.3 g of 5(6)-carboxyfluorescein diacetate (8.93 mmol; 89 %) as a yellowish solid that was used without further purification

**<sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>):** δ 12.73 (s, 2H, COOH), 8.46 (s, 1H, CH<sub>Ar</sub>-5-Isomer), 8.32 (dd, *J* = 8.0, 1.4 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 8.28 (dd, *J* = 8.0, 1.1 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 8.18 (d, *J* = 8.2 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 7.85 (s, 1H, CH<sub>Ar</sub>-6-Isomer), 7.54 (d, *J* = 8.0 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 7.30 (d, *J* = 2.1 Hz, 4H, CH<sub>Ar</sub>-5/6-Isomer), 7.05 – 6.87 (m, 8H, CH<sub>Ar</sub>-5/6-Isomer), 2.29 (s, 12H, COCH<sub>3</sub>); **<sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>):** δ 171.9, 170.1, 168.7, 167.5, 165.9, 155.5, 152.2, 152.1, 150.8, 150.7, 136.4, 136.1, 133.3, 129.3, 129.2, 128.9, 126.2, 125.8, 125.6, 124.7, 124.5, 118.6, 118.5, 115.6, 115.5, 110.5, 81.4, 21.0, 20.8; **LC-MS (ESI):** calcd for C<sub>25</sub>H<sub>17</sub>O<sub>9</sub>: 461.09 [M+H]<sup>+</sup>; found 461.08 [M+H]<sup>+</sup>; R<sub>t</sub> 8.34 min. **HR-MS:** *m/z*: calcd for C<sub>25</sub>H<sub>17</sub>O<sub>9</sub>: 461.08671 [M+H]<sup>+</sup>, found 461.08641 [M+H]<sup>+</sup>.

#### **PEG-5(6)-carboxyfluorescein (4)**

5(6)-carboxyfluorescein diacetate (3 g, 6.3 mmol) was dissolved in dry DMF (25 mL) and cooled to 0°C. Triethylamine (1.75 mL, 12.59 mmol, 2 eq) and 1.29 mL of 2,3,4,5,6-pentafluorophenyl trifluoroacetate (PFP-TFA, 7.56 mmol, 1.2 eq) were slowly added. The resulting mixture was then stirred at room temperature for 3 hours. After this time, a solution of 6.05 g *N*-Boc-4,7,10-trioxa-1,13-tridecanediamine<sup>3</sup> (18.89 mmol, 3 eq) and 2.62 mL triethylamine (18.98 mmol, 3 eq) in dry DMF (4 mL) was added and the reaction was stirred at room temperature overnight. The solvent was removed under high vacuum and water was added. The aqueous solution was washed 5 times with DCM. The collected organic phases were then washed with brine, dried over anhydrous sodium sulfate, filtered and evaporated to afford 3.21 g (4.63 mmol) of PEG-5(6)-carboxyfluorescein as an orange solid (yield 74%). **<sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>):** δ 10.13 (s, 4H, Ar-OH), 8.79 (t, *J* = 5.9 Hz, 1H, NHCO<sub>Ar</sub>), 8.64 (t, *J* = 6.2 Hz, 1H, NHCO<sub>Ar</sub>), 8.45 (s, 1H, CH<sub>Ar</sub>-5-Isomer), 8.24 (dd, *J* = 8.1, 1.6 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 8.16 (dd, *J* = 8.1, 1.3 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 8.07 (d, *J* = 8.0 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 7.66 (s, 1H, CH<sub>Ar</sub>-6-Isomer), 7.36 (d, *J* = 8.0 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 6.76 – 6.63 (m, 2H, NHCOO-*t*<sub>bu</sub>),

6.64 – 6.38 (m, 8H, CH<sub>Ar</sub>-5/6-Isomer), 3.64 – 3.13 (m, 26H, CH<sub>2</sub>O, CH<sub>2</sub>NHCO<sub>Ar</sub>), 3.05-2.81 (m, 4H, CH<sub>2</sub>NHCOO<sub>t<sub>bu</sub></sub>), 1.85 – 1.73 (m, 2H, CH<sub>2</sub>-PEG-NHCO<sub>Ar</sub>), 1.75 – 1.62 (m, 2H, CH<sub>2</sub>-PEG-NHCO<sub>Ar</sub>), 1.68 – 1.46 (m, 4H, CH<sub>2</sub>-PEG-NHBoc), 1.36 (s, 18H, *t<sub>bu</sub>*); <sup>13</sup>C NMR (126 MHz, DMSO-*d*6): δ 168.2, 164.7, 164.3, 159.4, 154.6, 152.7, 151.8, 140.8, 136.3, 134.7, 129.3, 129.2, 128.9, 128.2, 126.4, 125.3, 124.2, 122.2, 112.7, 112.6, 108.9, 109.1, 102.2, 83.2, 77.3, 69.8 – 69.7, 68.3 – 68.2, 37.1, 36.9, 36.8, 29.5, 29.3, 29.0, 28.2; LC-MS (ESI): calcd for C<sub>36</sub>H<sub>43</sub>N<sub>2</sub>O<sub>11</sub>: 679.29 [M+H]<sup>+</sup>; found 679.28 [M+H]<sup>+</sup>; R<sub>t</sub> 8.14 min; HR-MS: *m/z*: calcd for C<sub>36</sub>H<sub>43</sub>N<sub>2</sub>O<sub>11</sub>: 679.28614 [M+H]<sup>+</sup>, found 679.28654 [M+H]<sup>+</sup>.

### Boc- PEG-CrAsH

PEG-5(6)-carboxyfluorescein (0.88 g, 1.27 mmol) was dissolved in glacial acetic acid (4 mL) and slowly treated with a solution of 0.55 g mercury oxide (2.53 mmol, 2 eq.) in glacial acetic acid (3 mL – completely dissolved by gentle heating with heat gun). The reaction was stirred at room temperature under inert atmosphere overnight. The next day a red precipitate was observed. The solvent was evaporated under reduced pressure and the residue was treated with water. The solid was filtered, washed with water to eliminate the unreacted mercuric oxide and dried overnight under high vacuum in the presence of P<sub>2</sub>O<sub>5</sub> to afford 1.3 g of red solid that was used for the next step without further purification. The resulting solid was dissolved in dry NMP (8 mL) and the mixture cooled to 0 °C. 1.8 mL of Arsenic trichloride (21.45 mmol, 20 eq) was carefully added, followed by the addition of 1.46 mL of dry DIPEA (8.58 mmol, 8 eq) and of 3 mg of palladium (II) acetate (0.01 mmol). The reaction was moved into an oil bath and stirred at 60°C for 4 hours, protecting the flask from light covering it with aluminum foil. After this time, the reaction mixture was cooled down to room temperature, transferred into an Erlenmeyer flask containing 90 mL of a 1:1 vol/vol mixture of acetone and potassium phosphate buffer (pH 6.9) and treated with 3 mL of ethanedithiol (EDT). Chloroform (50 mL) was then added and the reaction mixture was stirred for 30 minutes at room temperature. The organic phase was collected and the aqueous solution extracted 3 more times with the same volume of chloroform. The collected organic phases were dried over anhydrous sodium sulfate, filtered and evaporated under vacuum at 40°C, without reaching the complete dryness. The residue was left in the flask

overnight under the fume hood. The following day the crude was dissolved with 150 mL of toluene and washed 4 times with water. The organic phase was then dried over anhydrous sodium sulfate and evaporated at 40°C without reaching the complete dryness affording 0.661 g of **10** (0.6 mmol; yield 60 %). **<sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>)**: δ 10.61 (s, 4H, Ar-OH), 8.82 (t, *J* = 5.5 Hz, 1H, NHCO<sub>Ar</sub>), 8.68 (t, *J* = 5.5 Hz, 1H, NHCO<sub>Ar</sub>), 8.44 (s, 1H, CH<sub>Ar</sub>-5-Isomer), 8.24 (dd, *J* = 8.1, *J* = 1.4 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 8.16 (dd, *J* = 8.0, *J* = 1.2 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 8.07 (d, *J* = 8.0 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 7.68 (s, 1H, CH<sub>Ar</sub>-6-Isomer), 7.41 (d, *J* = 8.5 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 6.75 – 6.70 (m, 2H, NHCOO*t*-Bu), 6.58 – 6.56 (m, 8H, CH<sub>Ar</sub>-5/6-Isomer), 3.53 – 3.23 (m, 26H, CH<sub>2</sub>O, CH<sub>2</sub>NHCO<sub>Ar</sub>), 3.05-2.81 (m, 4H, CH<sub>2</sub>NHCOO*t*-Bu), 2.86 – 2.79 (m, 8H, S-CH<sub>2</sub>CH<sub>2</sub>S), 1.78 – 1.62 (m, 4H, CH<sub>2</sub>-PEG-NHCO<sub>Ar</sub>), 1.56 – 1.45 (m, 4H, CH<sub>2</sub>-PEG-NHBoc), 1.36 (s, 18H, *t*-Bu); **<sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>)**: δ 168.1, 164.5, 164.4, 159.4, 154.6, 152.7, 151.8, 140.7, 136.3, 134.7, 129.3, 129.2, 128.9, 128.2, 126.4, 125.3, 124.2, 122.2, 112.7, 112.6, 109.1, 108.7, 102.2, 83.3, 77.3, 69.6 – 69.5, 68.1 – 68.0, 37.2, 36.8 – 36.7, 29.6, 29.2, 29.1, 28.3, 22.1. **LC-MS (ESI)**: calcd for C<sub>40</sub>H<sub>49</sub>As<sub>2</sub>N<sub>2</sub>O<sub>11</sub>S<sub>4</sub>: 1010.91 [M+H]<sup>+</sup>; found 1010.86 [M+H]<sup>+</sup>; R<sub>t</sub> 9.63 min, **HR-MS**: *m/z*: calcd for C<sub>40</sub>H<sub>49</sub>As<sub>2</sub>N<sub>2</sub>O<sub>11</sub>S<sub>4</sub>: 1011.06456 [M+H]<sup>+</sup>, found 1011.0640 [M+H]<sup>+</sup>.

### PEG-5(6)CrAsH (1)

0.25 g (0.24 mmol) of compound Boc-PEG-CrAsH were dissolved in 6 mL of dichloromethane:TFA (4:1). The solution was stirred at room temperature for 3 hours and 30 min and was then coevaporated 4 times with toluene to afford 0.211 g of the final product PEG-CrAsH (1) (0.23 mmol, 94 %). **<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)**: δ 10.32 (s, 4H, Ar-OH), 8.84 (t, *J* = 5.5 Hz, 1H, NHCO<sub>Ar</sub>), 8.69 (t, *J* = 5.4 Hz, 1H, NHCO<sub>Ar</sub>), 8.45 (s, 1H, CH<sub>Ar</sub>-5-Isomer), 8.23 (dd, *J* = 8.1, 1.3 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 8.16 (dd, *J* = 8.1, 1.0 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 8.08 (dd, *J* = 8.1 Hz, 1.4 Hz, 1H, CH<sub>Ar</sub>-6-Isomer), 7.66 (s, 5H, CH<sub>Ar</sub>-6-Isomer, NH<sub>2</sub>), 7.38 (d, *J* = 8.0 Hz, 1H, CH<sub>Ar</sub>-5-Isomer), 6.75-6.70 (m, 2H, NHCOO*t*-Bu), 6.64 – 6.46 (m, 8H, CH<sub>Ar</sub>-5/6-Isomer), 3.58 – 3.41 (m, 26H, CH<sub>2</sub>O, CH<sub>2</sub>NHCO<sub>Ar</sub>), 2.86 – 2.79 (m, 8H, S-CH<sub>2</sub>CH<sub>2</sub>S), 2.62-2.59 (m, 4H, CH<sub>2</sub>NH<sub>2</sub>), 1.87 – 1.60 (m, 12H, CH<sub>2</sub>-PEG-NH<sub>2</sub>, CH<sub>2</sub>-PEG-NHCO<sub>Ar</sub>), 1.26 – 1.22

(m, 4H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>); <sup>13</sup>C NMR (100.5 MHz, DMSO-*d*<sub>6</sub>): δ 168.2, 164.5, 164.4, 154.5, 152.6, 151.8, 140.6, 136.2, 134.7, 129.3 (CH<sub>Ar</sub>), 129.2, 128.9, 128.2, 126.4, 125.3, 124.2, 122.2, 112.7, 112.6, 109.1, 108.8, 102.2, 83.2, 69.7 – 69.5, 68.2 – 68.1, 37.2, 36.9 – 36.8, 29.7, 29.2, 29.1, 22.1. **LC-MS (ESI)**: calcd for C<sub>35</sub>H<sub>41</sub>As<sub>2</sub>N<sub>2</sub>O<sub>9</sub>S<sub>4</sub>: 910.80 [M+H]<sup>+</sup>; found 911.01 [M+H]<sup>+</sup>; R<sub>t</sub> 7.63 min, **HR-MS**: *m/z*: calcd for C<sub>35</sub>H<sub>41</sub>As<sub>2</sub>N<sub>2</sub>O<sub>9</sub>S<sub>4</sub>: 911.01213 [M+H]<sup>+</sup>, found 911.01228 [M+H]<sup>+</sup>.

#### **PEG-5(6)-carboxyfluorescein (4)**

PEG-5(6)-carboxyfluorescein (0.2 mmol) was dissolved in dichloromethane (8 mL) and treated with TFA (2 mL). The solution was stirred at room temperature for 3 hours and coevaporated 3 times with toluene to afford directly the final product in quantitative yield. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 10.22 (s, 4H, OH), 8.84 (t, *J* = 5.5 Hz, 1H, NH), 8.69 (t, *J* = 5.4 Hz, 1H, NH), 8.45 (s, 1H, H4 5-isomer), 8.23 (dd, *J* = 8.1, 1.3 Hz, 1H, H6 5-isomer), 8.16 (dd, *J* = 8.1, 1.0 Hz, 1H, H5 6-isomer), 8.08 (dd, *J* = 8.1 Hz, 1.4 Hz, 1H, H4 6-isomer), 7.66 (s, 5H, H7 6-isomer and NH<sub>2</sub>), 7.38 (dd, *J* = 8.0 Hz, 1.3 Hz, 1H, H7 5-isomer), 6.70 (m, 4H, H1' and H8'), 6.64 – 6.46 (m, 8H, H2', H4', H5' and H7'), 3.58 – 3.41 (m, 28H, CH<sub>2</sub>O and CH<sub>2</sub>NHCOAr), 3.25 (m, 4H, CH<sub>2</sub>NH<sub>2</sub>), 2.84 (m, 4H, CH<sub>2</sub>NHCO), 1.87 – 1.60 (m, 8H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>). <sup>13</sup>C NMR (100.5 MHz, DMSO-*d*<sub>6</sub>) δ 168.2 – 168.1 (C3 5(6)-isomer) 164.5 – 164.4 (NHCOAr 5(6)-isomer), 159.6 (NHCOO*t*-Bu), 154.6 – 152.7, 151.8, 140.8, 136.3, 134.7, 129.3 – 129.2, 128.9, 128.2, 126.4, 125.3, 124.2, 122.2, 112.7 – 112.6, 109.1 – 109.1, 102.2, 83.2, 77.4, 69.7 – 69.5, 68.2 – 68.1, 37.2, 36.9 – 36.8, 29.7, 29.2 – 29.1, 28.2 (C(CH<sub>3</sub>)<sub>3</sub>). **LC-MS (ESI)**: calcd for C<sub>31</sub>H<sub>35</sub>N<sub>2</sub>O<sub>9</sub>: 579.23 [M+H]<sup>+</sup>; found 579.32 [M+H]<sup>+</sup>; R<sub>t</sub> 5.29 min; **HR-MS**: *m/z*: calcd for C<sub>31</sub>H<sub>35</sub>N<sub>2</sub>O<sub>9</sub>: 579.23371 [M+H]<sup>+</sup>, found 579.23406 [M+H]<sup>+</sup>.

## Synthesis of peptides

### General protocol for the synthesis of peptides 2-3

A Rink Amide MBHA resin with an initial loading of  $0.56 \text{ mmol g}^{-1}$  was employed. The loading of the first aminoacid, the peptide elongation and the acetylation of the terminal amino group was performed on an automated microwave peptide synthesizer, on a 0.1 mM scale, using AA/HBTU/DIPEA (4:4:8) for the couplings and 20% piperidine in DMF for the Fmoc removal. The couplings were performed with a single irradiation at an initial power of 40W at  $75^{\circ}\text{C}$  for 10 minutes for all the aminoacids and with a double irradiation with an initial power of 40W at  $50^{\circ}\text{C}$  for 10 minutes for the Cys. Yields are given in regard to the initial loading. The resin loading was measured by UV Fmoc test, measuring the UV absorbance of the sample at  $\lambda = 301 \text{ nm}$  after Fmoc deprotection using a freshly prepared 20% piperidine in DMF solution.

### **Ac-NH-Phe-Leu-Asn-Cys-Cys-Pro-Gly-Cys-Cys-Met-Glu-Pro-Gly-Lys(Ac)-Gly-CONH<sub>2</sub> (2).**

The peptide was synthesized using Fmoc-Lys(Mtt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asn(Trt)-OH and Fmoc-Cys(SiBu)-OH. After the synthesis on automated peptide synthesizer following the general procedure, Lys was selectively deprotected as follows. The resin containing the protected peptide (0.1 mmol) was swelled with 5 mL of DCM for 20 minutes and washed two more times with the same solvent. The resin was treated with 4 mL of a TFA:TES:DCM (1:4:95) solution and it was shaken for 2 minutes. The solution was removed and this cycle was repeated 9 times. After the last deprotecting cycle the resin was washed with DCM and DMF. The acetylation of the resulting free amino group was achieved by treating the resin with 4 mL of a acetic anhydride:Pyridine:DCM (1:1:4) solution and shaking for 30 minutes. The solution was removed and the acylation was repeated a second time. The resin was washed subsequently with DCM, DMF, DCM, MeOH and dried under high vacuum for one hour. The peptide was released from the solid support by treatment of the resin with 6 mL of a TFA:TES:H<sub>2</sub>O (96:2:2) solution for one hour. The solvent was removed and the resin was washed three additional times with 4 mL of TFA for 3 minutes. The collected solutions were coevaporated 3 times with



toluene. The crude (200 mg) was purified by reverse preparative HPLC on a C18 column to afford 20 mg of peptide (0.01 mmol; yield 10%). LC-MS (ESI): calcd for  $C_{83}H_{136}N_{18}O_{20}S_9$ : 997.39  $[M+2H]^{2+}$ , 1014.42  $[M+2NH_4]^{2+}$ ; found 997.72  $[M+2H]^{2+}$ , 1014.88  $[M+2NH_4]^{2+}$ ; Rt 9.16 min. **HR-MS**:  $m/z$ : calcd for  $C_{83}H_{136}N_{18}O_{20}S_9$ : 997.39051  $[M+2H]^{2+}$ ; found 997.39145  $[M+2H]^{2+}$ . The pure protected peptide (0.01 mmol) was dissolved in 6 mL of a 0.1 M  $NH_4HCO_3$  solution (pH 8) and stirred for some minutes, until the bubbling stopped. DTT (5 eq. for each cysteine) was then added and the reaction stirred for 5 hours at room temperature under inert atmosphere. A 10% acetic acid in water solution was added to the reaction until pH 2-3. The aqueous solution was then frozen on liquid nitrogen and lyophilized. The final peptide was purified by reverse preparative HPLC on C18 column to afford 4 mg of peptide **2** (0.002 mmol; yield 20%).  $^1H$  NMR (600 MHz,  $DMSO-d_6$ )  $\delta$  12.12 (s, 1H, COOH Glu), 8.30 (d,  $J = 7.4$  Hz, 1H, NH-Asn), 8.24 – 8.21 (m, 2H, NH-Leu, NH-Glu), 8.13 (t,  $J = 5.8$  Hz, 3H, NH-Gly), 8.10 – 8.07 (m, 3H, NH-Cys, NH-Met), 8.05 (d,  $J = 8.3$  Hz, 1H, NH-Cys), 7.87 (d,  $J = 7.9$  Hz, 1H, NH-Cys), 7.84 (d,  $J = 7.2$  Hz, 1H, NHLys), 7.78 (t,  $J = 5.3$  Hz, 1H, AcNH-Lys), 7.43 (s, 2H,  $NH_2CO$ ), 7.26 – 7.23 (m, 5H, Phe), 7.05 (s, 2H,  $NH_2CO$ ), 4.65 – 4.55 (m, 1H, Asn-H $\alpha$ ), 4.55 – 4.48 (m, 4H, Cys-H $\alpha$ ), 4.42 – 4.36 (m, 2H, Phe-H $\alpha$ , Glu-H $\alpha$ ), 4.36 – 4.28 (m, 4H, Pro-H $\alpha$ 1, Met-H $\alpha$ , Leu-H $\alpha$ ), 4.15 (m, 1H, Lys-H $\alpha$ ), 3.73 – 3.58 (m, 10H, Phe-H $\beta$ , Gly-H $\alpha$ , Pro-H $\delta$ 1 Pro-H $\delta$ 2), 3.01 (m, 2H, Lys-H $\epsilon$ ), 2.87 – 2.70 (m, 9H, Cys-H $\beta$ , Asn-H $\beta$ 1, Met-H $\gamma$ , Glu-H $\gamma$ ), 2.65 – 2.58 (m, 5H, Cys-H $\beta$ , Asn-H $\beta$ 2), 2.47 – 2.35 (m, 2H, Met-H $\beta$ ), 2.31 – 2.28 (m, 2H, Glu-H $\beta$ , Pro-H $\beta$ 1), 2.05 (m, 1H, Pro-H $\beta$ 2), 2.01 (s, 3H, SCH3), 1.94 – 1.91 (m, 1H, Pro-H $\gamma$ 1), 1.83 (m, 1H, Pro-H $\gamma$ 2), 1.79 (s, 3H, acetyl), 1.74 (s, 3H, acetyl), 1.69 – 1.66 (m, 1H, Lys-H $\beta$ 1), 1.59 – 1.53 (m, 3H, Leu-H $\gamma$ , Lys-H $\beta$ 2), 1.50 – 1.45 (m, 2H, Leu-H $\beta$ ), 1.36 (m, 2H, Lys-H $\delta$ ), 1.25 – 1.23 (m, 2H, Lys-H $\gamma$ ), 0.94 – 0.77 (dd,  $J = 6.5$  Hz,  $J = 19.1$  Hz, 6H, CH3 Leu); **LC-MS (ESI)**: calcd for  $C_{67}H_{105}N_{18}O_{20}S_5$ : 1641.64  $[M+H]^+$ , 1658.66  $[M+NH_4]^+$ ; found 1641.03  $[M+H]^+$ , 1658.41  $[M+NH_4]^+$ ; Rt 6.76 min. **HR-MS**:  $m/z$ : calcd for  $C_{67}H_{105}N_{18}O_{20}S_5$ : 1641.63506  $[M+H]^+$ ; 1663.61700  $[M+Na]^+$ ; found 1641.63426  $[M+H]^+$ , 1663.61610  $[M+Na]^+$ .  $[\alpha]_D^{20}$ : -79.94 ( $c = 0.03$ , acetonitrile).

**Ac-NH-Phe-Leu-Asn-Cys-Cys-Pro-Gly-Cys-Cys-Met-Glu-Pro-Gly-Lys(D-biotinyl)-Gly-CONH<sub>2</sub> (3).** The peptide was synthesized using Fmoc-Lys(Mtt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asn(Trt)-OH and Fmoc-Cys(S*t*Bu)-OH. After the synthesis on automated peptide synthesizer following the general procedure, Lys was selectively deprotected. The resin containing the protected peptide (0.1 mmol) was swelled with 5 mL of dichlorometane for 20 minutes and washed two more times with the same solvent. The resin was treated with 4 mL of a TFA:TES:DCM (1:4:95) solution and shaken for 2 minutes. The solution was removed and the cycles was repeated 9 more times. After the last cycle the resin was washed with dichlorometane and DMF. The biotinylation of the resulting free amino group was achieved by treating the resin with a solution of D-biotin (4 eq.), HBTU (4 eq.) and DIPEA (8 eq.) in dry DMF and shaking for 3 hours. The solution was removed and the resin washed subsequently washed with dichlorometane, DMF, dichlorometane, methanol and dried under high vacuum for 1 hour.

The peptide was released from the solid support by treatment of the resin with 6 mL of a TFA:TES:H<sub>2</sub>O (96:2:2) solution for one hour. The solvent was removed and the resin washed 3 additional times with 4 mL of TFA for 3 minutes. The collected solutions were coevaporated 3 times with toluene. The crude (200 mg) was purified by reverse preparative HLPC in C18 column to yield 20 mg of peptide **3** (0.01 mmol; yield 10%). LC-MS (ESI): calcd for C<sub>91</sub>H<sub>148</sub>N<sub>20</sub>O<sub>21</sub>S<sub>10</sub>: 1089.43 [M+2H]<sup>2+</sup>, 1106.45 [M+2NH<sub>4</sub>]<sup>2+</sup>; found 1089.68 [M+H]<sup>2+</sup>, 1106.99 [M+NH<sub>4</sub>]<sup>2+</sup>; R<sub>t</sub> 9.00 min. HR-MS: *m/z*: calcd for C<sub>91</sub>H<sub>148</sub>N<sub>20</sub>O<sub>21</sub>S<sub>10</sub>: 1089.42403 [M+H]<sup>2+</sup>; found 1089.42479 [M+H]<sup>2+</sup>.

The pure peptide (0.01 mmol) was dissolved in 6 mL of a 0.1 M NH<sub>4</sub>HCO<sub>3</sub> solution (pH 8) and stirred for some minutes, until the bubbling stopped. DTT (5 eq. for each cysteine) was added and the reaction stirred for 5 hours at room temperature under inert atmosphere. A 10% acetic acid in water solution was added to the reaction until pH reached 2-3. The aqueous solution was then frozen on liquid nitrogen and lyophilized. The final peptide was purified by reverse preparative HPLC on C18 column to afford 4 mg of product **2** (0.002 mmol; yield 20%).

<sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ 12.12 (s, 1H, COOH Glu), 8.28 (d, *J* = 7.4 Hz, 1H, NH-Asn), 8.21 (m, 2H, NH-Leu, NH-Glu), 8.10 – 8.03 (m, 7H, NH-Gly, NH-Cys, NH Met), 7.87 – 7.82 (m, 2H, NH-Cys, NH-Lys), 7.71 (t, *J* = 5.05 Hz, 1H, biotin-NH-Lys), 7.42 (s, 2H, NH<sub>2</sub>CO), 7.25 (m, 5H, Phe), 7.03 (s, 2H, NH<sub>2</sub>CO), 6.40 (s, 1H, NH biotin), 6.35 (s, 1H, NH biotin), 4.58 – 4.57 (m, 1H, Asn-Hα), 4.53 – 4.51 (m, 4H, Cys-Hα), 4.39 – 4.36 (m, 1H, Phe-Hα), 4.30 – 4.28 (m, 4H, Pro-Hα, Met-Hα, Leu-Hα, Glu-Hα), 4.15 (m, 1H, Lys-Hα), 3.73 – 3.58 (m, 12H, Gly-Hα, Pro-Hδ1, Pro-Hδ2, Phe-Hβ, H3 and H4 biotin), 3.10 (CHS biotin), 3.00 (m, 2H, Lys-He), 2.82 – 2.72 (m, 10H, Cys-Hβ, Asn-Hβ1, Met-Hγ, Glu-Hγ, H5b biotin), 2.60 – 2.52 (m, 6H, Cys-Hβ, Asn-Hβ2, H5a biotin), 2.47 – 2.35 (m, 2H, Met-Hβ), 2.31 – 2.28 (m, 3H, Glu-Hβ, Pro-Hβ1), 2.05 – 2.04 (m, 3H, Pro-Hβ2, CH<sub>2</sub>CO biotin), 2.01 (s, 3H, SCH<sub>3</sub> Met), 1.93 – 1.91 (m, 1H, Pro-Hγ1), 1.83 (m, 1H, Pro-Hγ2), 1.74 (s, 3H, acetyl), 1.74 (s, 3H, acetyl), 1.69 – 1.59 (m, 4H, Lys-Hβ, Leu-Hγ), 1.50 – 1.45 (m, 8H, CH<sub>2</sub> biotin, Lys-Hδ, Leu-Hβ), 1.36 (m, 2H, CH<sub>2</sub> biotin), 1.25 – 1.23 (m, 2H, Lys-Hγ), 0.94 – 0.77 (dd, *J* = 6.5 Hz, *J* = 19.1 Hz, 6H, CH<sub>3</sub> Leu). LC-MS (ESI): calcd for C<sub>75</sub>H<sub>117</sub>N<sub>20</sub>O<sub>21</sub>S<sub>6</sub>: 1825.70 [M+H]<sup>+</sup>, 1842.73 [M+NH<sub>4</sub>]<sup>+</sup>; found 1825.50 [M+H]<sup>+</sup>, 1842.45 [M+NH<sub>4</sub>]<sup>+</sup>; R<sub>t</sub> 6.50 min. HR-MS: *m/z*: calcd for C<sub>75</sub>H<sub>117</sub>N<sub>20</sub>O<sub>21</sub>S<sub>6</sub>: 1825.70209 [M+H]<sup>+</sup>; 1847.68404 [M+Na]<sup>+</sup>; found 1825.70167 [M+H]<sup>+</sup>, 1847.68166 [M+Na]<sup>+</sup>; [α]<sub>D</sub><sup>20</sup>: -39.50 (*c* = 0.02, acetonitrile).

### General protocol for the synthesis of peptides MIC-TC-Peptide

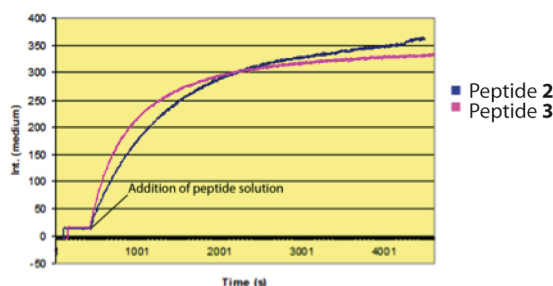
A Rink Amide MBHA resin with an initial loading of 0.56 mmol/g was employed. The loading of the first amino acid and the peptide elongation were performed on an automated microwave peptide synthesizer, on a 0.1 mM scale, using AA/HBTU/DIPEA (4:4:8) for the couplings and 20% piperidine in DMF for the Fmoc removal. The couplings were performed with a single irradiation at an initial power of 40W at 75°C for 10 minutes for all the amino acids and with a double irradiation with an initial power of 40W at 50°C for 10 minutes for the Cys. Yields are given in regard to the initial loading. The resin loading was measured by UV Fmoc test, measuring the UV absorbance of the sample at λ = 301 nm after Fmoc deprotection using a fresh prepared 20% piperidine in DMF solution.

**MIC-TC-Peptide (MIC-Phe-Leu-Asn-Cys(StBu)-Cys(StBu)-Pro-Gly-Cys(StBu)-Cys(StBu)-Met-Glu-Pro-Gly-Lys-Gly-CONH<sub>2</sub>).**

The peptide was synthesized using Fmoc-Lys(Mtt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(*S*tBu)-OH and 6-maleimido-hexanoic acid. After the synthesis on automated peptide synthesizer following the general procedure, the peptide was released from the solid support by treatment of the dry resin with 6 mL of a TFA:TES:H<sub>2</sub>O (96:2:2) solution for 1 hour. The solvent was removed and the resin washed 3 additional times with 4 mL of TFA for 3 minutes. The collected solutions were coevaporated 3 times with toluene. The crude (200 mg) was purified by reverse preparative HPLC employing a C18 column to yield 15 mg of peptide **11** (0.015 mmol; yield 15%). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ 12.10 (s, 1H, COOH Glu), 8.43 (d, *J* = 7.9 Hz, 1H, NH-Asn), 8.28 (d, 1H, *J* = 8.0 Hz, NH-Leu), 8.21 – 8.18 (m, 2H, NH-Glu, NH-Phe), 8.13 (t, *J* = 5.9 Hz, 3H, NH-Gly), 8.03 – 8.00 (m, 3H, NH-Cys, NHMet), 7.96 (d, 1H, *J* = 7.9 Hz, NH-Cys), 7.91 (d, *J* = 7.6 Hz, 1H, NH-Lys), 7.86 (d, *J* = 7.9 Hz, 1H, NH-Cys), 7.64 (s, 2H, NH<sub>2</sub>-Lys), 7.43 (s, 2H, CONH<sub>2</sub>), 7.24 (m, 5H, Phe), 7.05 (s, 2H, CONH<sub>2</sub>), 6.99 (s, 2H, CH=CH MIC), 4.75 (m, 1H, Asn-H $\alpha$ ), 4.53 (m, 5H, Phe-H $\alpha$ , Glu-H $\alpha$ , Cys-H $\alpha$ ), 4.47 (m, 2H, Met-H $\alpha$ , Cys-H $\alpha$ ), 4.34 (m, 1H, Leu-H $\alpha$ ), 4.31 (m, 1H, Pro-H $\alpha$ ), 4.28 (m, 2H, NHCH<sub>2</sub> MIC), 4.21 (m, 1H, Lys-H $\alpha$ ), 3.77 – 3.57 (m, 10H, Pro-H $\delta$ 1, Pro-H $\delta$ 2, Gly-H $\alpha$ , Phe-H $\beta$ ), 3.01 – 2.96 (m, 8H, Cys-H $\beta$ , Asn-H $\beta$ 1, Met-H $\gamma$ 1), 2.81 – 2.71 (m, 6H, Cys-H $\beta$ , Asn-H $\beta$ 2, Met-H $\gamma$ 2, Lys-H $\epsilon$ ), 2.58 (m, 2H, Glu-H $\beta$ ), 2.40 (m, 2H, Glu-H $\gamma$ ), 2.30 (m, 2H, Met-H $\beta$ ), 2.02 (m, 2H, CH<sub>2</sub>CO MIC), 2.00 (s, 3H, SCH<sub>3</sub> Met), 1.90 (m, 2H, Pro-H $\beta$ 1, Pro-H $\beta$ 2), 1.84 (m, 2H, Pro-H $\gamma$ 1, Pro-H $\gamma$ 2), 1.69 (m, 2H, Lys-H $\beta$ ), 1.59 (m, 2H, CH<sub>2</sub> MIC), 1.52 (m, 2H, Lys-H $\delta$ ), 1.45 (m, 2H, Leu-H $\beta$ ), 1.38 (m, 2H, CH<sub>2</sub> MIC), 1.32 (m, 2H, CH<sub>2</sub> MIC), 1.29 (s, 9H, *t*-butyl), 1.28 (s, 9H, *t*-butyl), 1.27 (s, 9H, *t*-butyl), 1.26 (s, 9H, *t*-butyl), 0.87 (dd, *J* = 6.5 Hz, *J* = 19.5 Hz, 6H, CH<sub>3</sub> Leu). LC-MS (ESI): calcd for C<sub>89</sub>H<sub>143</sub>N<sub>19</sub>O<sub>21</sub>S<sub>9</sub>: 1051.92 [M+2H]<sup>2+</sup>; found 1052.39 [M+2H]<sup>2+</sup>; Rt 7.74 min. HR-MS: *m/z*: calcd for C<sub>89</sub>H<sub>143</sub>N<sub>19</sub>O<sub>21</sub>S<sub>9</sub>: 1051.91689 [M+2H]<sup>2+</sup>, found 1051.91811 [M+2H]<sup>2+</sup>; [ $\alpha$ ]<sub>D</sub><sup>20</sup>: -96.39 (*c* = 0.036, acetonitrile).

### Complex formation in solution

The emission intensity of the sample was analyzed in a Jasco P6500 spectrofluorometer at 530 nm after excitation at 508 nm. To a 2.5 ml of a 100 mM MOPS buffer pH 7.2 in a fluorescence cuvette equipped with a magnetic stirring bar, 25  $\mu$ L of a 1 M MesNa solution in water and 2.5  $\mu$ L of a 10 mM EDT solution in DMSO (final concentrations of 10 mM and 10  $\mu$ M respectively) were added. The fluorescence was measured under stirring for some minutes to ensure its stability. After this time, 2.5  $\mu$ L of a 1 mM solution of 5(6)-PEG-CrAsH (**1**) in DMSO were added. The measurement of the fluorescence at the appropriate wavelength was continued for 5–10 minutes and a slight increase in the fluorescence was observed. After 10 minutes, 10  $\mu$ L of a 10 mM solution of the TC-peptide (**2**) in acetonitrile/water 1:1 were added and a rapid increase of the fluorescence was immediately observed. The plateau was reached after approximately one hour. Similar protocol was followed by peptide **3**.



**Figure 1.** Time course for the reaction of 5(6)-PEG-CrAsH (**1**) with TC-containing peptides **2** and **3**.

### Generation of CrAsH-coated glass slides

Nexterion H-barcoded slides were usually separated in five subarrays by applying a 75  $\mu$ L Gene Frame®. If a different CrAsH concentration is not indicated, each square was covered with 100  $\mu$ L of a 1 M solution of biarsenical probe in dry dimethylsulfoxide containing 3 eq. of dry DIPEA. The slide was gently shaken protected from the light at room temperature overnight. The solution was removed and the slide was washed with dry dimethylsulfoxide and blocked with 150  $\mu$ L of a 100 mM solution of ethanolamine in 50 mM of sodium borate buffer pH 8.5 for 3 hours. The solution was removed and the slide was washed with 100 mM MOPS buffer

pH 7.2, rinsed with Millipore water and dried under an argon flow. The modified slides can be stored at -20°C protected from the light for several months.

### **General procedure for peptide and protein spotting and immobilization**

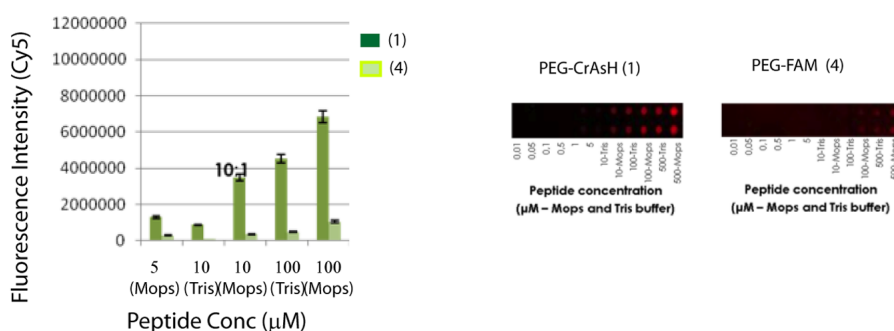
All the microarrays were prepared on Nexterion slides, properly modified according to the above reported protocol. The TC-peptide **2** and **3** were dissolved in acetonitrile/water 1:1 to obtain a 1 M stock solution that was divided in aliquots and stored at -20 °C. Before spotting, peptide stock solutions were diluted to the desired concentration with 100 mM MOPS buffer pH 7.2. Glycerol (10 µL) and 2 % Tween-20 were added to avoid the evaporation of the spots during the incubation time. Moreover, freshly prepared solutions of EDT in DMSO (5 mM) and of MESNa in water (0.5 M) were also added to the diluted peptide solutions to achieve a final concentration of 1 µM and 10 µM respectively.

Peptides were diluted to a final volume of 60 µL in 20 mM Tris pH 7.4, 5 mM MgCl<sub>2</sub>, pH 7.4 (due to the known stability of these proteins in this buffer) containing 10 µL of glycerol and 1.5 % Tween to avoid the evaporation or dehydration of the immobilized proteins and freshly prepared EDT in DMSO and MesNa in water to guarantee the complexation of the thiols with the biarsenic derivatives. Spotting (6 x 12 spots, 5 frames) was performed with a non-contact microarray robot (GeSIM NP1.2). The incubation of the slide for the desired time was performed in a closed, humid environment and protected from the light. After the incubation time, the slide (or the single square) was subsequently washed with Tris buffer, Tris buffer containing 0.05 % Tween-20 and Millipore water. Detection of CrAsH fluorescence enhancement upon complex formation was measured at the following wavelengths (ex. 508 m, em. 530 nm). Microarrays were scanned using a fluorescence scanner or using a white light lamp and a CCD camera. (GenePix 4000B, Molecular Devices, Typhoon Trio+, General Electric or BioAnalyzer 4F).

## Detection of biotinylated peptides

The detection of biotinylated peptides was performed by covering the slide with 1 mL of a 200 nM solution of Streptavidine-Cy5 (Invitrogen) in TETBS buffer (20 mM Tris, 5 mM EDTA, 150 mM NaCl) pH 7.5 for 30 minutes. After that time, the solution was removed and the slide was washed with buffer, rinsed with Milli-Q water and dried with an argon flow. Microarrays were analyzed using a microarray fluorescence scanner (ex. 649 nm, em. 670 nm).

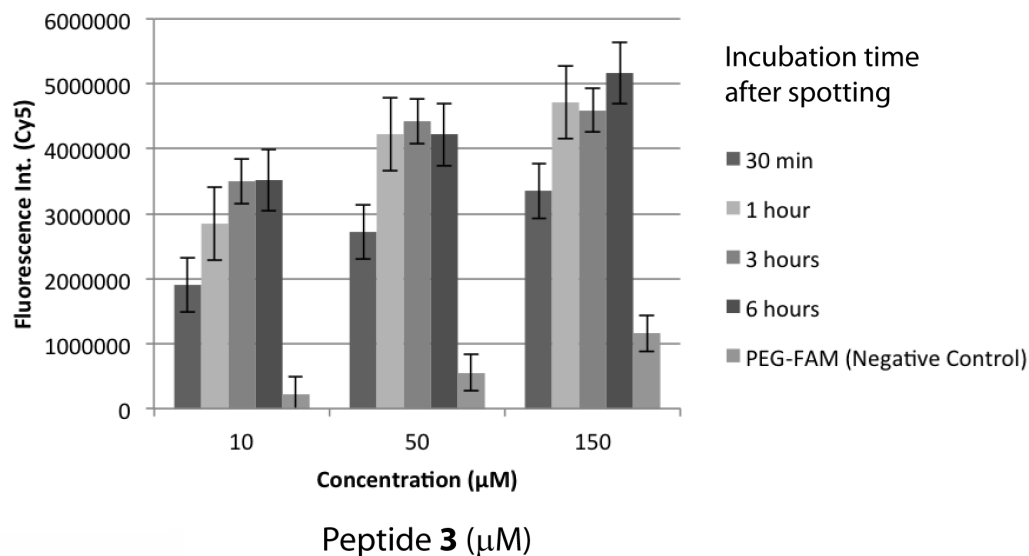
## Peptide immobilization on carboxyfluorescein (4) modified slides vs CrAsH(1) modified slides



**Figure 2.** Peptide **3** was immobilized on CrAsH- and carboxyfluorescein-modified slides and the fluorescence intensity of the resulting spots was quantified as described.

## Time-incubation optimization

A Nexterion slide was modified, according to the general protocol reported above, with a 1 mM solution of compound **1** in DMSO. Samples of peptide **3** in different concentrations (10, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 μM) were prepared and spotted as reported in the general procedure. The slide was divided in subarrays by applying a Gene Frame® and each subarray was incubated for a different time ( $t_i$  = 30 minutes, 1, 3 and 6 hours) at room temperature and protected from the light. After each incubation-time, one of the squares was washed 3 times with 200 μL of Tris buffer containing 0.05 % Tween-20 and 3 times with 200 μL Milli-Q water and quantified as described above using Streptavidine-Cy5.



**Figure 3.** Time-dependent immobilization. Peptide **3** was spotted at different concentrations and incubated for different time courses. Carboxyfluorescein derivative **4** (PEG-FAM) was used as a negative control.

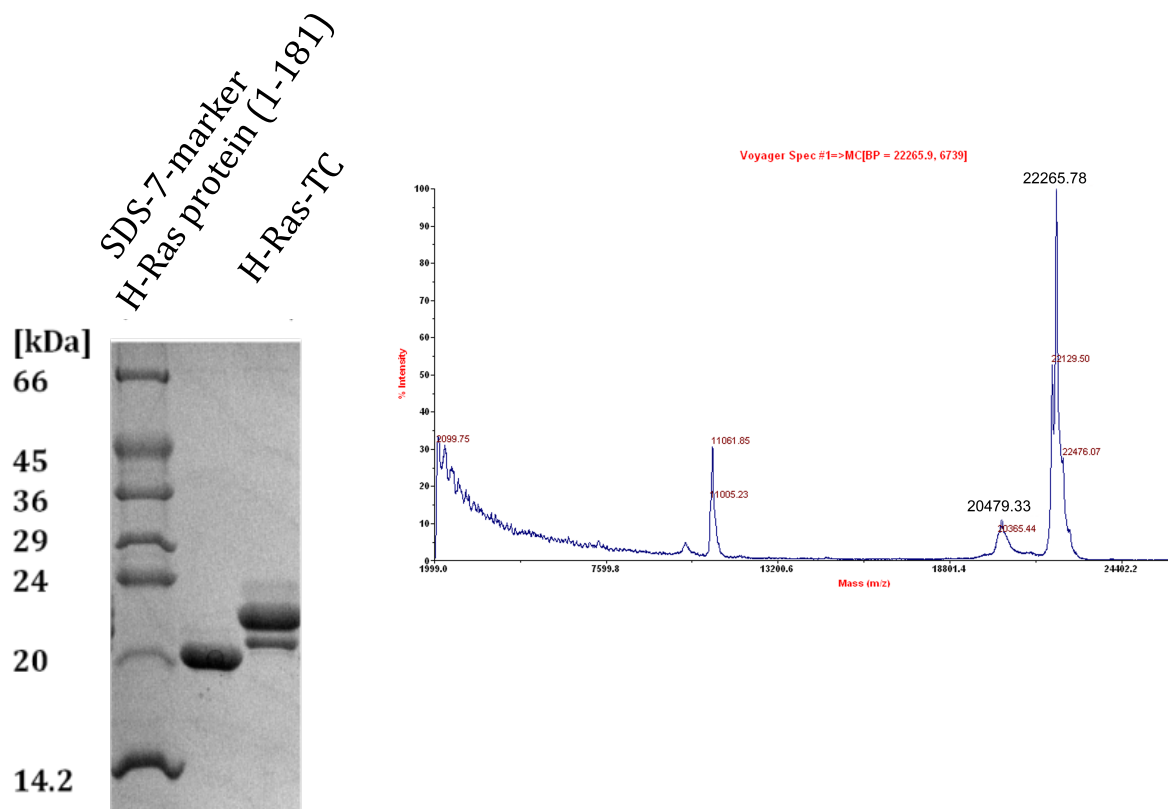
### Slide regeneration

A Nexterion slide was modified, according to the general protocol with a 1 mM solution of compound **1** in DMSO. Samples of peptide **3** in different concentrations (5, 10, 100 and 500 µM) in both Tris (20 mM Tris buffer pH 7.4) and Mops buffer (100 mM MOPS buffer pH 7.2) were prepared and spotted onto the slide. The slide was divided in subarrays by applying of a Gene Frame® and each subarray was covered with a 2 M solution of EDT in dry DMSO and incubated for a different time ( $t_i = 1, 4$ ) at room temperature and protected from the light. After each incubation time, the corresponding array was washed 3 times with 200 µL of DMSO, with 200 µL of Tris buffer containing 0.05 % Tween-20 and 3 times with 200 µL Milli-Q water and quantified as reported above using Streptavidine-Cy5. After detection the slide was used for a second peptide immobilization according to the general procedure described above.



### **Synthesis of TC-H-Ras**

The truncated H-Ras (Ras-wt 1-181) containing a C-terminal cysteine (181) were generated by recombinant expression in *E. coli* as previously described.<sup>1</sup> The purified MIC-TC-Peptide was synthesized and ligated to H-Ras before cysteine deprotection to avoid the intramolecular reaction of the peptide cysteines with the MIC group. Briefly, truncated Ras protein was dissolved in 20 mM Tris buffer (pH 7.5) containing 5 mM MgCl<sub>2</sub>, and was mixed in a 1:2 stoichiometry with the MIC-TC-Peptide (dissolved in 500 μL of water and acetonitrile 1:1). Triton X 114 was added up to 1 mL to avoid the precipitation of the protein and the mixture was put on a rotary wheel at 4°C overnight. For the deprotection of the cysteines, 1,4-dithiothreitol (DTE) was added until a 100 mM concentration and the mixture was put on a rotary wheel at 4°C overnight. This mixture was washed several times with cold Tris buffer and concentrated by centrifugation in an Amicon Ultra membrane with a 10 kDa pore size, to eliminate the unreacted peptide, the DTE and to reduce the amount of Triton X 114. The ligated proteins were purified by gel filtration and eluted with 20 mM Tris buffer (pH 7.5) containing 5 mM MgCl<sub>2</sub> and 50 mM NaCl. The concentrated, ligated protein was obtained in a 20% yield and was characterized by MALDI-MS and SDS-PAGE, finally protein was shock-frozen, aliquoted and stored at -80 °C.

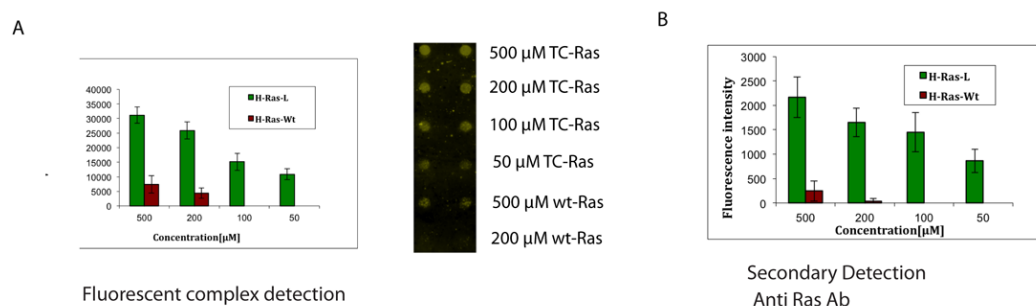


**Figure 4.** Characterization of H-Ras-TC. SDS-PAGE of H-Ras (1-181) and analysis of ligation product after purification.

### TC-H-Ras immobilization

A slide was modified with the biarsenic probe according to the standard protocol. A H-Ras-wt protein lacking the TC tag was employed as negative control. Samples with different concentrations of TC-H-Ras and H-Ras-wt were spotted on five subarrays. All the samples were prepared starting from concentrated solutions of protein by diluting them with 20 mM Tris buffer pH 7.2 containing 5 mM MgCl<sub>2</sub> up to a final volume of 60 μL including 10 μL of glycerol, 15 μL of 2% Tween-20 in water (to avoid the evaporation or dehydration of the immobilized proteins), 12 μL of freshly prepared 5 mM EDT in DMSO and 12 μL of freshly prepared 0.5 M MesNa in water were also added (to guarantee the complexation of the thiols with the biarsenic derivative). Spotting (6 x 12 spots, 5 frames) was performed with a non-

contact microarray robot (GeSIM NP1.2). The incubation of the slide for the desired time was performed in a closed, humid environment and protected from the light. After the spotting the subarrays were incubated at room temperature for 1 hour and washed with 200  $\mu$ L of buffer and Mill-Q water (3 times each). CrAsH fluorescence was analyzed as previously described. After this, the slide was incubated with 1 mL of a 1:4000 solution of specific Cy3-labeled anti-Ras antibody, that specifically recognizes an amino acidic sequence in a correctly folded helix close to the Ras active site and incubated for one hour. After extensive washing with Tris buffer, Tris buffer containing 0.05 % Tween-20, the slide was rinsed with Milli-Q water, dried with argon flow and scanned for the detection of Cy3 (ex.:552 nm, em.:570nm). Microarrays were scanned using a fluorescence scanner or using a white light lamp and a CCD camera. (GenePix 4000B, Molecular Devices, Typhoon Trio+, General Electric or BioAnalyzer 4F).



**Figure 5.** Immobilization of a TC-containing H-Ras protein at concentrations ranging from 500  $\mu$ M to 50  $\mu$ M. A wt Ras lacking the TC motive was used as a negative control. A) Direct detection of the immobilized protein by fluorescence enhancement upon complex formation. B) Secondary detection of the immobilized Ras protein using an anti-Ras antibody.

### pH dependence of Ras immobilization

The immobilization of the purified TC–H-Ras was carried out as described before, but dilution buffers at 9 different pH (varying from pH 4.5 up to 10.5) were prepared and employed. The

solution of TC-H-Ras (150  $\mu$ M) in buffer at different pH was then spotted onto the CrAsH-modified slides. Detection of H-Ras was performed by fluorescence complex formation and after incubation with a Cy3-labeled antibody anti-Ras.

### **Cloning, expression and purification of TC-containing PDE $\delta$ proteins**

To generate TC-fused PDE $\delta$ , the PDE $\delta$  open reading frame (ORF) was amplified using the oligonucleotides 5'- CCGGGCTGCTGCATGGAACCGATGTCAGCCAAGGACGAGCG-3'/5'-CAAACCTGGTCTAGAAAGCTTTAAACATAGAAAAGTCTCACTCTGGATGTGCT -3'. The oligonucleotides 5'- GAAGTTCTGTTTCAGGGTCCCTTTCTGAACTGCTGCCCGGGCTGCTGCATGGAACCG-3'/CGGTTCCATGCAGCAGCCCGGGCAGCAGTTCAGAAAGGGACCCTGAAACAGAACT TC-3' were hybridized. PCR products and hybridized oligonucleotides were inserted into a pOPIN-NHis-3C vectors by means of SLIC cloning (sequence and insert independent cloning).<sup>4</sup> Proteins were expressed in BL21 (DE3) RIL cells in TB medium after induction by IPTG at 20°C or after autoinduction at 20°C overnight. Proteins were purified by means of Ni-sepharose affinity chromatography followed by size-exclusion chromatography and were stored in 25 mM of HEPES, 40 mM NaCl, 1 mM TCEP, pH 7.2. and the immobilization of the purified TC proteins was carried out as described before. Protein samples were spotted at different concentrations and incubated for one h. Then the slide was washed three times for 5 min with Tris buffer, Tris buffer containing 0.05% Tween-20, rinsed with Milli-Q water, dried under argon and protein immobilization was measured by fluorescence scanning (eGFP fluorescence signal (ex.: 484 nm, em.: 507 nm). To verify the site-specific immobilization of proteins, an YFP-tagged Rab7 lacking the TC sequence was also spotted onto the slide as negative control.

### **General procedure for the immobilization of proteins from cell lysates**

**Generation of protein expression lysates.** Proteins were expressed as described above. After harvesting the bacteria, the cells were lysed in buffer (25 mM of HEPES, 40 mM NaCl, 1 mM

TCEP, pH 7.2), 1 g cells/1 mL lysis buffer) by passing them multiple times through a fluidizer (110S, Microfluidics) and subsequent centrifugation. After determination of protein concentration using the Bradford method, proteins were spotted as described previously. To verify unspecific immobilization of proteins from the cell lysate, cell lysate from *E. coli* not expressing TC-tagged proteins were also spotted on the slide at similar concentration as a negative control. The samples were spotted at different concentrations in the 5 chambers of the microarray, after applying a GeneFrame and incubated for one hour. Thereafter, each chamber was washed three times for 5 min with Tris buffer, Tris buffer containing 0.05% Tween-20, rinsed with Millipore water and dried under argon and subsequently scanned.

Immobilized PDE $\delta$  either after protein purification or directly from cell lysates was detected by analysis of the CrAsH-complex formed, by detection of the fluorescent proteins fused to PDE $\delta$ , by detection of bodipy fluorescence (after incubation with a fluorescently bodipy-labeled N-Ras protein or by detection of Alexa-dye after incubation with a PDE $\delta$  antibody.

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