Novel chemical cross-linker to study protein interactions in living cells

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The dynamics of protein-protein interactions play a central role in various biological processes including the function of multi-protein complexes and the regulation of signal transduction networks. Methods to sense or manipulate such interactions are therefore crucial for studying protein behavior in living cells. The interaction between two proteins can for example be enforced by the use of small cell membrane-permeant molecules (chemical inducers of dimerization), which simultaneously bind to two specific protein domains.[1] While extremely useful for a range of applications, the necessity to fuse the proteins of interest to bulky extraneous domains (usually larger than 18 kDa) frequently affects the proteins’ activities and/or localization.[2] Moreover, the interactions generated between the additional domains are frequently irreversible. Such techniques therefore do not allow the induction of transient linkage events. In order to circumvent these restrictions, we developed a method to perform highly specific cross-linking of two proteins in living cells, each containing only a 12 amino acid peptide tag (4cys-tag). The novel cross-linking approach is based on the formation of stable, covalent complexes between a dimeric biarsenical derivative (xCrAsH) and two proteins containing the unique tetracysteine sequence motif CCPGCC (Figure 1a).[3] In contrast to other methods, the reaction is easily reversible by addition of membrane-permeant dithiols. As an additional feature, the protein pairs become fluorescently labeled upon binding of the cross-linker, which helps to detect and follow target proteins in real time by light microscopy.

Bisarsenic probes in combination with a split tetracysteine tag have been previously used to connect two parts of a protein structure or for monitoring the oligomeric state of proteins.[4] However, application of this approach is very limited because the split motives need to be in very close proximity for cross-linking to occur. To achieve stable dimerization as well as significant flexibility of the cross-linking approach, we synthetically connected two biarsenical molecules with a flexible linker (xCrAsH; Figure 1b) with an overall yield of 53% using carboxyfluorescein as starting material (see supplement for details). We then used xCrAsH to cross-link protein pairs fused to optimized 12 amino acid sequences (FLNCCPGCCMEP, 4cys-tag).[5] The monomeric biarsenical molecule CrAsH (Figure 1b) served as a negative control throughout this work.

As a model protein pair for cross-linking, we first chose FKBP (FK506-binding protein) and FRB (FKBP-rapamycin binding domain of mTor), two proteins that only associate with each other upon binding to the small molecule rapamycin.[6] Their rapamycin-inducible interaction is well characterized in living cells and known to be fairly independent from other cellular events. Based on the X-ray crystal structure of the FRB-FKBP complex,[6b] we decided to introduce the 4cys-tags at the C termini of both proteins.

We initially performed cross-linking with purified His-s-FKBP-4cys and His-s-SUMO-FRB-4cys proteins under standard conditions reported for protein labeling with biarsenicals.[3a] The addition of xCrAsH but not of the CrAsH monomer led to the predominant formation of a cross-linked FRB-FKBP complex in the presence of rapamycin (Figure 2a). In the absence of rapamycin we detected FRB-FKBP heterodimers as well as FRB-FRB and FKBP-FKBP homodimers in approximately equal stoichiometries. As expected, the cross-linked complexes were fluorescent, stable even to denaturing conditions of SDS-PAGE, and sensitive to high concentrations of dithiols (e.g. 5 mM 2,3-dimercapto-1-propanol, BAL) (Figures 2a and S1). The yield of cross-linking after optimization of the assay conditions was 80% as judged by quantification using Deep Purple staining (data not shown). The optimal ratio of cross-linker to protein was between

![Figure 1](image-url)
To test whether xCrAsH can also efficiently cross-links two tagged proteins in living cells, we incubated U2OS (or HeLa) cells transiently expressing FLAG2-FKBP-4cys and ECFP-FRB-4cys with micromolar concentrations of xCrAsH or CrAsH for 2 h. We then measured the efficiency of cross-linking by blotting (Figure 3b and data not shown). As in the experiments with cell lysates, the only cross-link product we detected was the FRB-FKBP heterodimer in cells that had been incubated with rapamycin prior to cross-linker addition. Cross-linking under these conditions reached a yield of up to 50% at optimal cross-linker concentrations of 2.5 µM for U2OS and 10-20 µM for HeLa cells and was significantly reduced when cells were subsequently incubated with a high concentration of BAL (data not shown). xCrAsH can therefore be applied as an inducible, conditional, and reversible cross-linker in living cells.

In order to test whether xCrAsH can be used as a general tool to sense protein-protein interactions and to test their dependence on ligands or stimuli, we applied the cross-linker to monitor the activity state of two heterodimeric kinases that are central to a number of cellular functions, namely protein kinase A (PKA) and death activated protein kinase 1 (DAPK1). In its non-activated state, PKA is a homodimer of regulatory subunits (PKAreg) attached to two catalytic subunits (PKAcat). Upon cooperative binding of four cyclic AMP (cAMP) molecules, the catalytic subunits are released and thereby activated. We hence expect that xCrAsH would be able to cross-link the catalytic to the regulatory subunits before but not after activation of PKA by cAMP. We performed cross-linking reactions in...
lysates of U2OS cells transiently expressing PKAreg-4cys-ECFP and 4cys-PKAcac-mCherry as described above. Without stimulation, we detected heterodimers of regulatory and catalytic subunits as well as both homodimers (Figure 4a and S3). Addition of a cAMP analog (8-Br-cAMP) to lysates prior to but not after the cross-linking reaction reduced the amount of detected heterodimers significantly as predicted. Like FKBP and FRB, the PKA subunits were efficiently cross-linked with xCrAsH not only in cell extracts but also in living cells (Figure 4b).

The binding of DAPK1 to its activator calmodulin depends both on the dephosphorylation of DAPK1 and on intracellular calcium levels. Accordingly, we detected significant cross-linking of DAPK1-calmodulin complexes in lysates of U2OS cells transiently expressing DAPK1-4cys-ECFP (catalytic domain, 334 amino acid residues) and FLAG-4cys-calmodulin only in the presence of calcium ions (Figure S4). Addition of a calcium chelating agent (ethylene glycol tetraacetic acid, EGTA) almost completely abolished protein-protein cross-linking. Thus, we successfully employed the xCrAsH cross-linker to validate the protein-protein interaction status of two kinases in cell lysates. Future optimization of the positions of the 4cys-tags may further increase the final yield of cross-linking between the described protein pairs.

In summary, we established the novel membrane-permeant chemical cross-linker xCrAsH as a tool for studying protein-protein interactions in vitro and in living cells. Three important features distinguish this technique from other approaches: reversibility (by addition of diethiols), conditionality (no forced dimerization) and the use of only a very small peptide tag (4cys-tag). In contrast to the larger domains required for alternative cross-linking methods, the 4cys-tag mimics a protein loop, which significantly reduces the likelihood for disrupting protein function even when inserted as an internal fragment. In order to increase the spectrum of applications, bifunctional cross-linkers based on the combination of one biarsenical moiety with another autoreactive functional group (e.g. benzylguanines or trimethoprim derivatives) could be developed. Such molecules will be especially important for studying multi-protein complexes such as PKA, where homo- and heterodimers may need to be separated from each other. Another beneficial aspect could be the introduction of two different fluorogenic dyes to enable more precise live cell imaging of cross-linked proteins. Finally, the linker region may be modified by the incorporation of a photo-activatable cleavage site to allow the controlled re-opening of the cross-linked interface with cellular or subcellular spatial resolution. Thus, the cross-linking technique presented here is a first step towards the new generation of chemical inducers of dimerization, which may be generally applied to probe and manipulate protein-protein interactions in living cells.

### Experimental Section

All samples from cross-linking assays were analyzed on standard SDS-PAGE using 2xSDS sample buffer containing 100 mM tris(2-carboxyethyl)phosphine (TCEP). Prior to cross-linking, the expression and activity of all constructs was analyzed by live cell imaging. Presented are representative results from at least four independent experiments. Detailed experimental procedures are described in the Supporting Information.

**Keywords:** protein cross-linking · dimerization · biarsenical · protein-protein interactions · small molecules

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FlAsH-based cross-linker
to study protein interactions in living cells

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Supporting information
Reversible and specific in vitro cross-linking: a) Purified proteins (His\textsubscript{6}-FKBP-4cys and His\textsubscript{6}-SUMO-FRB-4cys, 15 µM each) were preincubated with rapamycin and subsequently incubated with xCrAsH (30 µM) at 37°C for 1 h. Where indicated, 2,3-dimercapto-1-propanol (BAL, 5 mM) was added 50 min after the addition of cross-linker. Samples were analyzed by SDS-PAGE and Coomassie staining or an in-gel fluorescence scan. A band representing cross-linked FRB-FKBP heterodimers was visible only in the presence of xCrAsH and was reverted by addition of BAL. Complexes between protein monomers and xCrAsH are indicated by (*). b) Purified His\textsubscript{6}-SUMO-FRB-4cys (30 µM) was incubated with different xCrAsH concentrations (0-75 µM) at 37°C for 1 h. Samples were analyzed SDS-PAGE and Coomassie staining or in-gel fluorescence scan. The fluorescence of cross-linked FRB-FRB homodimers, which reflects the efficiency of cross-linking reaction, was quantified using MultiGauge software. An optimal ratio of cross-linker to protein was around 1:1, which is in the range of the expected theoretical value. c) Lysates of HeLa Kyoto cells transiently co-expressing FLAG\textsubscript{3}-FKBP-4cys and ECFP-FRB-4cys were incubated with different xCrAsH concentrations (0-20 µM) at 37°C for 1 h; where indicated, rapamycin (40 µM) was added 10 min prior to the cross-linking reaction. Samples were analyzed by western blotting using anti-FLAG and anti-GFP antibodies. The yield of cross-linking reaction was quantified as the ratio of cross-linked product to the sum of cross-linked and non cross-linked protein in a given sample using MultiGauge software. The highest efficiency was obtained with xCrAsH concentrations between 0.5 - 5 µM. All results are representative of at least four independent experiments.
Membrane permeability and specific in cell labeling with xCrAsH: a) Confocal images of HeLa Kyoto cells transiently expressing mRFP-FKBP-4cys prior (upper panel) and after 1 h labeling with 1 µM xCrAsH (middle panel) or 1 µM CrAsH monomer (lower panel); CrAsH, excitation 514 nm, emission 530-600 nm. Scale bar, 20 µm. b) Quantification of images presented in Figure 3a. Shown is relative fluorescence ± standard deviation (n=15). Addition of 5 mM BAL completely reversed binding of xCrAsH.
Probing protein-protein interactions of PKA in vitro: a) Western blot of an in vitro cross-linking experiment using lysates from U2OS cell transiently co-expressing PKAreg-4cys-ECFP and 4cys-PKAcat-mCherry (left) or PKAreg-4cys-ECFP and PKAcat-mCherry (right) which were incubated with DMSO or xCrAsH (5 µM) at 37°C for 1 h. Where indicated, 8-Br-cAMP (5 mM) was added 10 min before (B) the addition of xCrAsH. In both lysates PKAreg-PKAreg homodimers were detected, independently of cAMP levels. The holoenzyme complex between PKAcat and PKAreg was observed only when both subunits contained 4cys-tags (left). b) Similar cross-linking experiment performed using lysates from U2OS cell transiently co-expressing PKAreg-4cys-FLAG3 and 4cys-PKAcat-mCherry. The gel running behavior of cross-linked homo- and heterodimers corresponded to their molecular weight. Thus, previously observed aberrant behavior of cross-links containing PKAreg-4cys-ECFP was due to uncompleted denaturation of ECFP (see Figure 4 and Figure S3a).

Probing DAPK1-calmodulin interactions in vitro: Western blot of an in vitro cross-linking experiment using lysates from U2OS cell transiently co-expressing DAPK1-4cys-ECFP (catalytic domain only) and FLAG3-4cys-calmodulin which were incubated with DMSO, CrAsH (5 µM) or xCrAsH (5 µM) at 37°C for 1 h. Where indicated, EGTA (50 mM) was added 10 min before (B) or 50 min after (A) the addition of xCrAsH. Complex formation between DAPK1 and calmodulin was depending on calcium levels and significantly reduced when EGTA was added prior to the cross-linker.
Material and methods

Chemicals and general methods:

Chemicals were purchased from Sigma (21877, 5(6)-carboxyfluorescein), Sigma-Aldrich (213357: mercury (II) oxide; 200077: palladium (II) acetate), Fluka (91725: pentafluorophenyl trifluoroacetate, 03739: 2,2'- (ethylenediox)bis(ethylamine), 82704: pyridine; 02390: 1,2-ethanedithiol), AnaSpec (N-hydroxybenzotriazole) and used without further purification. Dry solvents were purchased from Sigma-Aldrich, Acros, and Fluka, stored over molecular sieves and used as supplied. Solvents for extraction and chromatography were purchased from Fluka, Thermo Fisher Scientific, Merck, and BDH Prolabo (VWR).

Flash column chromatography was performed using Merck silica gel 60 (0.063-0.200 mm particle size) at air pressures around 1-1.5 bar. Analytical thin layer chromatography (TLC) was performed on aluminium-backed, pre-coated silica gel plates (Merck silica gel 60 WF254s). Spots were visualized using a UV lamp (λ = 254, 366 nm) or by staining either with methylamine solution or with ninhydrin solution (250 ml EtOH, 1.5 ml AcOH, 0.5 g ninhydrin) and subsequent heat treatment. Analytical HPLC was performed on a Waters Breeze system with mass-sensitive ESI-MS and UV detector using a RP-18 column (Lichrosorb, 10 μm), a linear gradient from 10% to 90% acetonitrile/water and a flow of 1.5 mL/min. Preparative HPLC was performed with a Knauer Preparative Pump 1800 and a WellChrom Preparative Pump K-1800, a Preparative Dynamic Mixing Chamber, A1054 preparative sample loop 11 mL, UV Detector K-2501 and ABB SE120 chart recorder using a Supercosil LC-18 column (5 μm, 250 x 10 mm, Supelco) and a gradient from 20% to 100% acetonitrile/water with 0.1% TFA for 30 min and a flow of 4.5 ml/min unless stated differently.

NMR spectra were recorded on a Bruker UltraShield™ Advance 400 [400 MHz (1H); 100.6 MHz (13C)] spectrometer. High-resolution (HR) mass spectra were recorded at the University of Heidelberg using electrospray ionization (ESI) MS on a Bruker ApexQe hybrid 9.4 T FT-ICR mass spectrometer.

Products were characterized by HPLC, NMR (1H, 13C) and HR-MS.

Compound synthesis

![Scheme 1 Synthesis of CrAsH (1) and xCrAsH (4)](image)

Reagents: a) HgO, TFA; b) AsCl₃, DIEA, Pd(OAc)₂, NMP; c) EDT, 0.25 M phosphate buffer pH 6.9, RT; d) HOAc-acetic anhydride (1:1), pyridine, 3h, 60 °C; e) Pfp-TFA, THF, pyridine, 18h, 0 °C → RT; f) HOBT, 2,2'- (ethylenediox)bis(ethylamine), DMF, 2h, RT.

RT, room temperature; TFA, trifluoroacetic acid; DIEA, N,N-disopropylethylamine; NMP, N-methyl-2-pyrrolidone; EDT, 1,2-ethanedithiol; Pfp-TFA, pentafluorophenyl trifluoroacetate; THF, tetrahydrofuran; HOBT, hydroxybenzotriazole; DMF, dimethylformamide.
General comment:
High vacuum should be avoided at any step as it might lead to decomposition of compounds.

**CrAsH (1)**
4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-5(6)-carboxylic acid was synthesized and purified as described.[1] The average combined yield for three steps was 60%.

**CrAsH-Ac₂ (2)**
4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-5(6)-carboxylic acid diacetate was synthesized similarly to the published protocol.[2] The average yield was 99%. The solvent was evaporated under reduced pressure and the product was used for the next synthesis step without further purification.

**CrAsH-Pfp-Ac₂ (3)**
4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-5(6)-carboxylic acid diacetate pentafluorophenyl ester.

2 (1eq, 0.2 mmol) was dissolved under argon in 15 ml dry THF and 1.5 ml dry pyridine. The solution was cooled in an ice bath and Pfp-TFA (171 µl, 5eq) was added drop wise. The mixture was slowly warmed up to RT and stirred for 18 h. Afterwards, THF and pyridine were removed by evaporation under reduced pressure and the product was purified by silica gel chromatography using 1:9 and then 2:8 ethyl acetate / cyclohexane with 1% acetic acid to give a white solid when triturated with cyclohexane (yield 90%). Two regioisomers were detected: 5-CrAsH-Pfp-Ac₂ and 6-CrAsH-Pfp-Ac₂ with estimated stoichiometry around 60:40. Purified decomposed under heating (starting at 120 °C).

For analytical purposes (NMR), a sample of the clean 5-CrAsH-Pfp-Ac₂ isomer was isolated from the 5(6) isomer mixture using flash column chromatography (conditions as mentioned above).


**5(6)-CrAsH-Pfp-Ac₂**

1H-NMR (CDCl₃) δ=8.88-8.86 (m, 0.6H, benzoic H-4 (5)), 8.53 (dd, 3J = 8.1, 4J = 1.5, 0.6H, benzoic H-6 (5)), 8.49 (dd, 3J = 8.0, 4J = 1.3, 0.4H, benzoic H-5 (6)), 8.22 (dd, 3J = 8.0, 4J not resolved, 0.4H, benzoic H-4 (6)), 8.1 (m, 0.4H, benzoic H-1 (6)), 7.48 (dd, 3J = 8.1, 4J = 0.4, 0.6H, benzoic H-1 (5)), 6.90-6.76 (m, 4H, xanthene), 3.56-3.44 (m, 4H, S-CH₂-), 3.40-3.29 (m, 4H, S-CH₂-), 2.41 (s, 6H, Ac)

13C-NMR (CDCl₃) δ=169.6, 167.5, 161.1, 157.8, 153.2/153.0, 152.6/152.5, 142.4/140.0/139.3/136.8 (4m, Pfp-ester), 137.2, 133.4, 132.4, 130.5, 129.4, 128.6, 128.2, 126.6, 126.5, 126.1, 125.9, 125.1, 119.9, 116.1, 82.2, 27.1, 21.7

**5-CrAsH-Pfp-Ac₂**

1H-NMR (CDCl₃) δ=8.86-8.83 (m, 1H, benzoic H-4), 8.49 (dd, 3J = 8.1, 4J = 1.5, 1H, benzoic H-6), 7.44 (dd, 3J = 8.1, 4J not resolved, 1H, benzoic H-1), 6.84 (d, 3J = 8.6, 2H, xanthene), 6.74 (d, 3J = 8.6, 2H, xanthene), 3.54-3.41 (m, 4H, S-CH₂-), 3.37-3.25 (m, 4H, S-CH₂-), 2.37 (s, 6H, Ac)

13C-NMR (CDCl₃) δ=169.6, 167.4, 161.0, 157.8, 153.2, 152.5, 142.4/140.0/139.3/136.8 (4m, Pfp-ester), 137.2, 129.4, 128.7/126.6, 128.2, 126.6, 125.8, 125.13, 119.9, 116.1/115.9, 82.0, 26.9, 21.7

![Figure 1 HPLC analysis of purified 5(6)-CrAsH-Pfp-Ac₂](image)

HPLC conditions: 20 min gradient 0-100% of buffer B in buffer A (A: 10% ACN, 90% water, 0.1% TFA, B: 90% ACN, 10% water, 0.1% TFA). Single peaks represent different structural isomers (5 and 6)
**xCrAsH (4)**
diacetylated CrAsH dimer; 3 (10 eq) and HOBT (10 eq) were dissolved in a minimal volume of DMF in a dried sample vial. 2,2’-(ethylenedioxy)bis(ethylamine) (1 eq) was added and the solution was agitated gently for 1-2h at RT. Subsequently, DMF was removed by ethyl acetate/NH₄Cl extraction followed by evaporation of the solvents under reduced pressure. Crude 4 was purified either by preparative HPLC or silica gel chromatography using 6:4, 8:2, 10:0 ethyl acetate/toluene, and then 9:1 ethyl acetate/methanol. Solvent evaporation under reduced pressure gave a light brown oily solid (yield 90%). Purified 4 decomposed progressively under heating (starting at around 220°C).

For analytical purposes (NMR), a compound comprising of only 5-carboxyfluorescein isomers was synthesized (5-xCrAsH).

HR-ESI MS: [M+H]+ calculated: 1696.82304, [M+H]+ found: 1696.82309

5-xCrAsH

**H-NMR** (CDCl₃) δ=8.41 (dd, 3J = 3.6, 5J = 0.7, not resolved completely, 2H, benzoic H-4), 8.23 (dd, 3J = 8.1, 5J = 1.5, 2H, benzoic H-6), 7.29 (dd, 3J = 8.0, 5J = 0.7, partially obscured by solvent, 2H, benzoic H-1), 6.83-6.63 (m, 8H, xanthene), 3.78-3.39 (m, 20H, linker and S-CH₂-, partially obscured by solvent), 3.38-3.23 (m, 8H, S-CH₂-,), 2.36 (s, 12H, Ac)

**13C-NMR** (CDCl₃) δ=169.6, 168.3/168.0 ((5) and (6)), 165.7/165.5 ((5) and (6)), 154.7, 153.0, 152.6, 141.3 (6), 136.9 (6), 135.1 (5), 129.7 (6), 129.2-128.8 (m, (5) and (6)), 128.1 (5), 126.1 (5), 125.6 (5), 125.5/125.4 ((5) and (6)), 124.7 (6), 123.5 (6), 122.7/122.6 (5), 119.8, 116.3/116.2 ((5) and (6)), 109.7, 82.07/81.8 ((5) and (6)), 70.01, 69.4, 39.9, 29.5, 21.7

**Figure 2** HPLC analysis of purified xCrAsH

HPLC conditions: 20 min gradient 0-100% of buffer B in buffer A (A: 10% ACN, 90% water, 0.1% TFA, B: 90% ACN, 10% water, 0.1% TFA).

Single peaks represent different structural isomers (5,5; 5,6; 6,6)

**Cloning:**

Generally, fluorescent fusion constructs for the expression in mammalian cells were derivatives of pECFP-C1/N1 (Clontech) and pmCherry-C1/N1.[3]

pmRFP-FKBP-4cys and pECFP-FRB-4cys were created by amplification of ECFP-FRB and mRFP-FKBP from the plasmids of the lab collection[8] and inserted into pECFP-KCP1-FLAsH[9] using Nhel and BamHI restriction sites. To obtain pmRFP-FKBP, the amplified fragment was inserted into pECFP-C1. pFLAG2-FKBP-4cys was prepared by exchanging of mRFP to pmRFP-FKBP-4cys with a short oligonucleotide encoding for the optimized triple FLAG-tag sequence (3xFLAG, Sigma-Aldrich) using AgeI and Xhol.

pHAT2-FKBP-4cys for His₆-FKBP-4cys protein expression in bacteria was generated by amplification of FKBP-4cys from pmRFP-FKBP-4cys and insertion into the pHAT2 (EMBL Protein Expression and Purification Core Facility) using Ncol and Xhol. pSUMO3-FRBP-4cys for His₆-SUMO-FRBP-4cys protein expression was created by amplification of FRB-4cys from pECFP-FRB-4cys and insertion into the pSUMO3 vector (EMBL Protein Expression and Purification Core Facility) using AgeI and Xhol.

pN1-PKA-RIα-ECFP was prepared by amplification of the human PKA regulatory subunit I alpha from pCMV-XL4-PKA-R1α (OriGene) and insertion into pECFP-N1 using HindIII and BamHI. pN1-PKA-R1α-4cys-ECFP was created by insertion of a short oligonucleotide encoding for the 4cys-tag into pN1-PKA-R1α-4cys-ECFP using BamHI and AgeI. pN1-PKA-R1α-4cys-FLAG3 was generated by exchanging of the ECFP coding sequence with a short oligonucleotide encoding the optimized triple FLAG-tag sequence using AgeI and BsrGI.
pN1-PKA-Ca-Cherry was created by amplification of the human PKA catalytic subunit alpha from pCMV-XL4-PKA-Ca (OriGene) and insertion into pmCherry-N1 using HindIII and BamHI. pN1-4cys-PKA-Ca-mCherry was created by insertion of a short oligonucleotide encoding the 4cys-tag sequence into pN1-PKA-Ca-mCherry using HindIII and SalI.

pN1-DAPK1(334)-ECFP was prepared by inserting the sequence encoding the N-terminal 334 amino acids of DAPK1 into pECPF-N1 using EcoRI and Sall. pN1-DAPK(334)-4cys-ECFP was created by amplification of the 4cys-ECFP fragment from pN1-PKA-Rta-4cys-ECFP and insertion into pN1-DAPK1(334)-ECFP using SalI and BsrGI.

pmPlum-calmodulin was replaced by EFYPF in pEYPFP-C1-calmodulin with mPlum from the pmPlum-C1-annexin A4 vector using Agel and HindIII. pmPlum-4cys-calmodulin was created by inserting a short oligonucleotide encoding the 4cys-tag into pmPlum-calmodulin using Nhel and BbsI. pFLAG-4cys-calmodulin was created by exchanging of the mPlum coding sequence with the triple FLAG-tag sequence using Agel and BgIII.

**Protein expression and purification:**

His6-SUMO-FRB-4cys and His6-FKBP-4cys proteins were purified from the BL21-CodonPlus (DE3)-RIPL E. coli strain (Stratagene).

**FRB:** cell cultures were incubated in Luria Bertani medium supplemented with ampicillin (100 µg/ml) at 37°C. At an OD600 0.7, the temperature was lowered to 25°C and protein expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 5h. Cells were harvested by centrifugation (10 min, 14000 rpm, 4°C). Cell pellets were re-suspended in buffer L1 (50 mM Tris-HCl, 300 mM NaCl, pH 8) supplemented with 2 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonylfluoride (PMSF), and EDTA-free protease inhibitor cocktail (Complete™, Roche), and disrupted twice using a French Press. After centrifugation (20 min, 16000 rpm, 4°C), the cleared supernatant was supplemented with imidazole to a final concentration of 20 mM and subsequently loaded onto a NINeA column (2-2.5 ml, Ni Sepharose™ 6 Fast Flow, GE Healthcare), which had been equilibrated with buffer L1 containing 20 mM imidazole. The column was washed with 40 and 60 mM imidazole in buffer L1 followed by elution with 250mM imidazole in buffer L1. Fractions containing protein (based on A280 absorption) were pooled and concentrated by ultrafiltration (Vivaspin with MWCO of 3000Da). Next, the protein was purified by gel filtration on a HiLoad™ 26/60 Superdex™200 column (GE Healthcare) equilibrated with buffer S1 (20 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM NaN3, 1 mM DTT, and 1 mM PMSF, pH 8). Fractions containing protein were pooled and, if necessary, concentrated as described before. Protein purity was assessed by SDS-PAGE and Coomassie Blue staining. Protein concentrations were determined using a NanoDrop spectrophotometer (ND-1000, Peqlab) or BioRad Protein Assay solution (BioRad). Aliquots were frozen in liquid nitrogen and stored at -80°C.

**FKBP:** cell cultures were incubated in Luria Bertani medium supplemented with kanamycin (40 µg/ml). Expression and purification was performed as described above using buffer L2 (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and buffer S2 (20 mM Tris-HCI, 150 mM NaCl, 1 mM EDTA, 1 mM NaN3, 1 mM DTT, and 1 mM PMSF, pH 8).

**Activity assay for purified FKBP and FRB:**

The proper activity of His6-SUMO-FRB-4cys and His6-FKBP-4cys, based on complex formation upon addition of rapamycin, was confirmed by gel filtration. His6-SUMO-FRB-4cys and His6-FKBP-4cys stocks solutions (60 µM) in buffer C (20 mM Tris-HCl, 100 mM NaCl, 1mM DTT) were used to prepare four samples (200 µl each): (1) FRB in buffer C (1:1, v/v), (2) FKBP in buffer C (1:1, v/v), (3) FRB and FKBP (1:1, v/v) and (4) FRB, FKBP (1:1, v/v) and rapamycin (300 µM end concentration; LC Laboratories, USA). After incubation for 20 min at 25°C, all samples were analyzed on a gel filtration Superdex™ 75HR 10/30 column (GE Healthcare). Fractions containing proteins were analyzed by SDS-PAGE.

**Cross-linking assay with purified proteins:**

Described here are the optimized conditions leading to the highest cross-linking yield. The optimized parameters include: buffer content, concentration of dithiols, temperature and time of the cross-linking reaction, final concentrations of xCrAsH, and conditions of the SDS-PAGE analysis.

**Protein stock solutions (30 µM) in buffer C were mixed 1:1 (v/v), and rapamycin was added to a final concentration of 200µM when indicated. After incubation (10 min, 37°C), 1µl of CrAsH (30 µM, final concentration), xCrAsH (30 µM) or DMSO were added to aliquots of 20 µl. All samples were from now on protected from light and further incubated for 60 min at 37°C. When indicated, 2,3-dimercapto-1-propanol (BAL, Sigma-Aldrich, 10 mM final concentration) was added 50 min after the addition of xCrAsH.

Afterwards, 20 µl of 2× SDS-PAGE sample buffer, containing 100 mM tris(2-carboxyethyl)phosphine (TCEP) as reducing agent, were added and samples were mixed for 2 min at 95°C. Samples were analyzed on SDS-PAGE and proteins detected by in-gel fluorescence (excitation: 489 nm, emission: 506 nm) using a Typhoon™ FLA 7000 molecular imager (GE Healthcare) and subsequently by Coomassie Blue staining. To quantify the cross-linking yield, Deep Purple staining was performed according to the manufacturer's instructions (DeepPurple™, GE Healthcare).

Due to acetylation of xCrAsH molecule, the fluorescence of complexes between xCrAsH and purified 4cys-tag proteins was significantly lower than compared to control using (non-acetylated) CrAsH monomer, which could be observed by in-gel fluorescence (Figure 1a) as well as fluorescence measurements in solution (data not shown).
Cross-linking assay in cell lysates:
U2OS cells (35 mm dishes) transiently expressing the indicated constructs were washed three times with buffer I (20 mM Hepes, pH 7.4, 115 mM NaCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM K₂HPO₄, 2 g L⁻¹ D-glucose) and labeled according to a published protocol. Labeling was performed for 2 h at 37°C with 5% CO₂ in buffer I. When indicated, rapamycin was added 10 min prior to labeling to a final concentration of 20 µM. CrAsH or xCrAsH were used in a final concentration of 1-20 µM in the presence of 12.5-40 µM 1,2-ethanedithiol (EDT) and 0.01 % pluronic (Invitrogen). Free and non-specifically bound CrAsH or xCrAsH were removed by washing with buffer I supplemented with 200 µM EDT (or BAL). When indicated, three washing steps with buffer I supplemented with 5 mM BAL were performed. After cross-linking cells were lysed using the same protocol as for the preparation of cell lysates (see above).

The optimal cross-linker concentration for experiments in living cells was estimated by two different methods: a) the comparison of final cross-linking efficiency for different concentrations of xCrAsH (0.2-25 µM); b) the efficiency of ECFP fluorescence quenching upon addition of different concentration of xCrAsH as described. For the later method, U2OS and Hela Kyoto cells transiently expressing ECFP-FRB-4cys were used.

Cell culture and imaging:
All cell experiments were performed with HeLa Kyoto or U2OS cells, which were passaged and maintained in Dulbecco’s modified Eagle medium (Gibco) supplemented with 10% FBS (Gibco) and 0.1 mg mL⁻¹ primocin (Invitrogen). Cells were plated in 35 mm MatTek dishes (MatTek Corporation) for imaging and in 35 mm or 60 mm dishes (Nunc) for cross-linking experiments. Transfections were performed at 50-70% confluency in Opti-MEM (Gibco) medium with FuGENE 6 reagent (Roche) or Lipofectamine 2000 reagent (Invitrogen) according to the manufacturers’ instructions. For imaging, cells were washed three times with buffer I and labeled as described. The final concentration of CrAsH or xCrAsH was 2 µM in the presence of 12.5 µM EDT. Images were acquired on a Leica TCS SP2 AOBS microscope (Leica Microsystems) with an HCX PL APO Ibd.BL 40.0x 1.25 oil objective at room temperature. Laser power and PMT gain were adjusted from experiment to experiment. Images were taken in 8 bit mode, with 4 lines averaging. The pinhole was usually 2.98 airy. Excitation and emission wavelengths: ECFP excitation 405 nm, emission 460-490 nm, CrAsH 488 / 520-560 nm, RFP 532 / 610-700 nm, mCherry 561 / 580-650 nm, mPlum 594 / 610-700 nm. All image processing and calculations were performed using ImageJ. Background levels were measured outside cells and subtracted globally. Images were smoothed with a median filter (1 pixel radius).