Supporting Information

Cell-Permeable Nanobodies Allow Dual-Color Super-Resolution Microscopy in Untransfected Living Cells

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**Experimental Procedures**

**General materials and methods**

**Solvents and chemicals**

Solvents (DMF, DCM) were purchased from Thermo Fisher Scientific (USA). Amino acids, ring amide resin and coupling reagents were purchased from Iris Biotech (Germany). 5(6)-Carboxytetramethylrhodamine (TAMRA) was purchased from Merck (Germany). HATU was purchased from Bachem (Switzerland). DIEA and TFA were purchased from Carl Roth (Germany).

Salts, LB medium, antibiotics and other buffer components were purchased from Carl Roth (Germany). Mammalian cell culture media and fetal bovine serum were purchased from VWR (USA).

**Analytical UPLC-MS**

UPLC-UV traces were obtained on a Waters H-class instrument equipped with a Quaternary Solvent Manager, a Waters autosampler and a Waters TUV detector with an Acquity UPLC-BEH C18 1.7 μm, 2.1x 50 mm RP column. The following gradient was used: A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA 5-95% B 0-5 min, flow rate 0.6 mL/min. UPLC-UV chromatograms were recorded at 220 nm.

**Preparative HPLC**

Preparative HPLC of peptides was done on a Gilson PLC 2020 system using a Nucleodur C18 Htec Spum column (Macherey-Nagel, 100 A, 5 m, 250 mm x 32 mm, 30 mL/min). The following gradient was used in all purifications: A = H_2O + 0.1% TFA, B = MeCN + 0.1% TFA 5% B 0-10 min, 5-50% B 10-60 min, 50-99% 60-80 min.

**High resolution mass spectrometry (HRMS)**

High resolution mass spectra were measured on a Xevo G2-XS QTof (Waters) mass spectrometer coupled to an acquity UPLC system running on water and acetonitrile, both with 0.01% formic acid. Protein spectra were devonvoluted using the MaxEnt 1 tool.

**Size exclusion chromatography of proteins**

Size exclusion chromatography was done on an AKTA Purifier system (GE Healthcare) on a Superdex S75 increase 10/300 column (GE Healthcare).

**SDS-PAGE**

Proteins were mixed with 4x reducing (containing β-mercaptoethanol) or non-reducing Laemmli buffer (Bio-Rad) and boiled at 95° C for 5 minutes before separation on 15% SDS-PAGE gels. In-gel fluorescence was imaged first, followed by Coomassie staining and imaging. Gels were imaged on a ChemiDoc XRS+ system (Bio-Rad).

**Software**

Microscopy pictures were processed with ImageJ including the FIJI package. Graphing and statistics were done using Graphpad Prism 8.

**Peptide synthesis**

All peptides were synthesized by standard Fluorenylmethoxycarbonyl (Fmoc)-solid-phase peptide synthesis (SPPS) on Rink amide resin (0.05 mmol scale, 0.22 mmol/g). Amino acid couplings were done using five equivalents of amino acid with five equivalents of HCTU (O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and four equivalents of Oxyma (Ethyl cyanohydroxyiminoacetate) with ten equivalents of DIEA (N,N-Diisopropylethylamine) in DMF (Dimethylformamide). Fmoc removal was accomplished by incubating the resin three times for five minutes with a 20% solution of piperidine in DMF. Arginine was incorporated with Pbf protection, cysteine was incorporated on the N-termini with Boc and Trityl protection.

In the synthesis of the Trolox-containing peptide, the linear synthesis was completed first with a lysine that was orthogonally protected with 4,4-dimethyl-2,6-dioxoclohex-1-ylidene (Dde). After completion of the linear synthesis, Dde was removed using 2% hydrazine in DMF, five times for five minutes, followed by washing with DMF and DCM. Then, Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was subsequently coupled with two equivalents of the carboxylic acid with two equivalents of HATU and four equivalents of DIEA.

The Ellman’s reagent (thionitrobenzoic acid, TNB)-activated peptide was generated by reacting the cysteine-containing decaarginine (Cys-R10) at a 5 mM concentration with 10 equivalents of Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid)) for 1 hour at room temperature. The resulting peptide (TNB-R10) was purified by reverse phase HPLC.

Peptides were cleaved from the solid support using 95% TFA with 2.5% trisopropylsilanil (TIS) and 2.5% dithiotretiol (DTT) for 2 hours at room temperature, increased to 4 hours for the R10 peptide to ensure complete removal of the Pbf protecting groups. The linear sequences of all peptides used in this study is found in supplementary table 1, the final structure and analytical data in supplementary figure 1.

**Supplementary table 1 Linear sequences of peptides used in this study.** PEG* corresponds to 8-amino-3,6-dioxaoctanoic acid.

| Entry | Peptide | Sequence |
|-------|---------|----------|
| 1     | Cys-K(N3) | Boc-C(Trt)-PEG*-PEG-K(N3)-Amide |
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|   | Compound | Synthesis  |
|---|----------|-----------|
| 2 | Cys-[K(N\textsubscript{2})\textsubscript{3}] | Boc-C(Trt)-PEG-K(N\textsubscript{2})\textsubscript{3}-PEG-K(N\textsubscript{2})\textsubscript{3}-PEG-K(N\textsubscript{2})\textsubscript{3}-Amide |
| 3 | Cys-K(N\textsubscript{2})-Trolox | Boc-C(Trt)-PEG-K(N\textsubscript{2})-K(Dde)-Amide |
| 4 | Cys-R10 | Boc-C(Trt)-PEG-RRRRRRRRRR-Amide |

Synthesis of Abberior Star Red alkyne

Abberior star red NHS-ester (1 mg, as obtained from abberior GmbH, Germany) was dissolved in 100 µl of anhydrous dimethylformamide and propargylamine (10 equivalents, 0.6 µl) was added. The solution was stirred at room temperature for one hour. The solution was diluted to 5 mL with water and the product was purified using reverse-phase HPLC using a gradient from: 10% B 0-10 min, 10-60% B 10-60 min, 60-99% 60-80 with A = H\textsubscript{2}O + 0.1% TFA, B = MeCN + 0.1% TFA. The product was lyophilized to give a blue solid (0.8 mg, 85%).

Cloning, nanobody expression and expressed protein ligation

GBP1 Nanobody:

The GFP-binding nanobody GBP1 was expressed and labelled through expressed protein ligation (EPL), similarly to a previously published protocol. Briefly, the nanobody was expressed in BL21 cells as a fusion protein with the DnaE intein and a chitin binding domain (pTXB1 vector system). Protein sequence (Nanobody sequence after intein cleavage underlined):

\[
\text{MAADVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYQAPKEREWVAGMSAGDSSYEDSVKGFTRTIARSDARNTYVLQMNLSLKPEDTAVVYCNVNFEYWGGQTQVTSSAACITGTDALVPEGESVRADIVPGARPNSDNAIDLKVLDRHGNPMLADRLFSGEHYPYVTQVEGLRTGVTANHPLLCLVDVAGPTLLKWLDIEKPKDGYAVIQRSAFSDCAGFKEPFTPVTGVPGLVRFLEAHHRDPDAQIAADELTDGRFYAKVAVSVDAGQPYYSLRVDTDAHFTINGFVSHATGLTGLNSGTLPNGVSAQWNTAYTAGQLVTRYNGTYYKLCQHTLSLWEPNSVPLWQLQ}\]

For the expression, BL21 T7 express cells (New England Biolabs) were transformed with the plasmid and grown overnight at 37°C in 5 mL of LB medium with 100 µg/mL ampicillin. The next day, the expression culture in 250 mL LB medium with ampicillin was inoculated with 1 mL of the starter culture. The culture was incubated at 37°C until it reached an OD\textsubscript{600} of 0.6. Protein expression was then induced using 1 mM IPTG and the culture was incubated for 16 hours at 18°C. Cells were collected by centrifugation at 4000xg for 15 minutes. The cells were washed once in PBS, then resuspended in lysis buffer (20 mM Tris-HCl pH 8.5, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 100 µg/mL lysozyme and 25 µg/mL DNAse I), lysed using sonication (3x 2 min, 30% Amplitude), followed by debris centrifugation at 25'000xg for 30 min.

For the purification, the clear lysate was loaded on 2 mL of chitin-agarose, equilibrated in EPL buffer (20 mM Tris-HCl pH 8.5, 500 mM NaCl). The chitin beads were washed with 20 column volumes of EPL buffer. Then, the cysteine- and azide containing peptides (see table 1, entries 1-3) were coupled to the C-terminus of the protein using EPL. For this, the protein was reacted on the chitin column with 1 mM peptide in 20 mM Tris-HCl pH 8.5, 500 mM NaCl and 100 mM sodium 2-mercaptoethanesulfonate for 16 hours while shaking at room temperature. The next day, the protein was washed off the column using 5 mL of EPL buffer. The protein was concentrated down to 500 µl and further purified from the reaction mixture using size exclusion chromatography on a Superdex 75 10/300 column in 20 mM HEPES at pH 8.0, 300 mM NaCl. Peak fractions were pooled, and protein aliquots were shock-frozen and stored at -80 °C.

LaM4 Nanobody:

A plasmid encoding the mCherry nanobody LaM4 was obtained from addgene (plasmid #70696). The nanobody-encoding sequence was cloned into the pTXB1 expression vector using restriction cloning with the Ndel and SapI cleavage sites. During the PCR amplification of the nanobody, a 15 amino acid-long helical linker ((EAAAK)\textsubscript{3}) was appended to the C-terminus of the protein. Protein sequence (Nanobody sequence after intein cleavage underlined):

\[
\text{MAQVQLVESGGSLVQPGGSLLRSLCAASGSRFAESSSMGWFWROAPKEREFVAISWSSGATNYADSASKGRFTLSRDNTKYTVYLMNSLKPDDTAVYYCAANLGNYSQRQLYGYWGGGTQTVSSPPTAEEAAKEAAKEAAKEAAACITGTDALVPEGESVRADIVPGARPNSDNAIDLKVLDRHGNPMLADRLFSGEHYPYVTQVEGLRTGVTANHPLLCLVDVAGPTLLKWLDIEKPKDGYAVIQRSAFSDCAGFARKTCPAPTYYTVGVPGLVRFLEAHHRDPDAQIAADELTDGRFYAKVAVSVDAGQPYYSLRVDTDAHFTINGFVSHATGLTGLNSGTLPNGVSAQWNTAYTAGQLVTRYNGTYYKLCQHTLSLWEPNSVPLWQLQ}\]

The expression of the nanobody, and the subsequent purification and expressed protein ligation were done exactly as with the GBP1 nanobody. The Cys-K(N\textsubscript{2})-Trolox peptide was used in the EPL.

Lamin Nanobody:

The nanobody-encoding sequence was cloned into the pTXB1 expression vector using restriction cloning with the Ndel and SapI cleavage sites. During the PCR amplification of the nanobody, a 15 amino acid-long helical linker ((EAAAK)\textsubscript{3}) was appended to the C-terminus of the protein. Protein sequence (Nanobody sequence after intein cleavage underlined):

\[
\text{MAOVLVESGGSLVQPGGSLLRSLCAASGSRFAESSSMGWFWROAPKEREFVAISWSSGATNYADSASKGRFTLSRDNTKYTVYLMNSLKPDDTAVYYCAANLGNYSQRQLYGYWGGGTQTVSSPPTAEEAAKEAAKEAAKEAAACITGTDALVPEGESVRADIVPGARPNSDNAIDLKVLDRHGNPMLADRLFSGEHYPYVTQVEGLRTGVTANHPLLCLVDVAGPTLLKWLDIEKPKDGYAVIQRSAFSDCAGFARKTCPAPTYYTVGVPGLVRFLEAHHRDPDAQIAADELTDGRFYAKVAVSVDAGQPYYSLRVDTDAHFTINGFVSHATGLTGLNSGTLPNGVSAQWNTAYTAGQLVTRYNGTYYKLCQHTLSLWEPNSVPLWQLQ}\]

The expression of the nanobody, and the subsequent purification and expressed protein ligation were done exactly as with the GBP1 nanobody. The Cys-K(N\textsubscript{2})-Trolox peptide was used in the EPL.
PCNA Nanobody:
The nanobody-encoding sequence was cloned into the pTXB1 expression vector using restriction cloning with the NdeI and SapI cleavage sites. During the PCR amplification of the nanobody, a 15 amino acid-long helical linker ((EAAAK)₃) was appended to the C-terminus of the protein. Later, due to the apparent intracellular instability of the protein, an additional valine residue was added to the N-terminus of the protein after the initiating methionine to increase intracellular half-life of the protein. The expression of the nanobody, and the subsequent purification and expressed protein ligation were done exactly as with the GBP1 nanobody. The Cys-K(N₃)-Trolox peptide was used in the EPL.

Plasmids for transfection:
The mCherry-Vimentin-N-18 plasmid was obtained from addgene (Plasmid #55158).

Fluorophore Conjugation via Copper Catalyzed Click Chemistry and Conjugation of the R10-Peptide
To conjugate fluorophores to the nanobodies after expressed protein ligation, copper catalyzed click chemistry was employed, based on a previously published protocol[1]. The proteins were diluted to a 30 µM concentration in 20 mM HEPES at pH 8.0, 300 mM NaCl. To the protein solution were added 1.5 equivalents of fluorophore-alkyne (Atto 594 alkyne, Atto 647N alkyne (Atto-Tec GmbH)) or Abberior Star Red (Abberior GmbH) per azide and 5 mM Aminoguanidine hydrochloride. Then, 100 µM CuSO₄, 500 µM Tris(3-hydroxypropyltriazolylmethyl)-amine (THPTA, Sigma Aldrich) and finally 5 mM sodium ascorbate were added to the reaction. They were incubated at room temperature for 1 hour while shaking. For the conjugation, subsequent purification, and storage of nanobodies with the hydrophobic Atto 647N fluorophore, 10% glycerol (v/v) were added to the nanobody solution. The fluorescent nanobodies were purified on a Superdex 75 10/300 column (GE Healthcare) into 20 mM HEPES at pH 8.0, 300 mM NaCl. The nanobodies as obtained after the fluorophore conjugation (typically 5-20 µM concentration) were treated with 5 equivalents of the TNB-R10 peptide. They were then incubated overnight at 4°C and used within a week in cellular uptake experiments or frozen in liquid nitrogen and stored at -80°C for longer periods of time.

Mammalian cell culture and cellular uptake experiments
Cell lines were grown at 37°C in a humidified atmosphere with 5% CO₂. A list of cell lines with their corresponding media can be found in supplementary table 1.

Supplementary Table 1 Cell lines used in this study.

| Cell line      | Medium                                      |
|----------------|---------------------------------------------|
| HeLa Kyoto     | DMEM 4.5 g/L Glucose + 10% fetal bovine serum (FBS) |
| HeLa Kyoto GFP-PCNA | DMEM 4.5 g/L Glucose + 10% FBS               |
| HeLa Kyoto EGFP-H2B[2] | DMEM 4.5 g/L Glucose + 10% FBS, 0.5 mg/mL G418 |

For microscopy experiments, 10’000 cells were seeded into the wells of a 96-well glass bottom plate (Cellvis). The cells were left to adhere and grow for 24 hours at 37°C with 5% CO₂. The cells were washed once with DMEM before addition of the protein samples in DMEM. Nanobodies were generally used between 1 to 2 µM (depending on the experiment). The cells were incubated for 1 hour at 37°C. The cells were then washed twice with DMEM with 10% fetal bovine serum (FBS). In some experiments SiR-DNA (Spirochrome) was added at a final concentration of 500 nM. Cells were generally imaged live with incubation at 37°C and 5% CO₂.

Microscopy
Confocal microscopy images were acquired on a Nikon-CSU spinning disc microscope with an CSU-X1 (Andor) and a live cell incubation chamber (OKOlab). Confocal images were acquired using a PlanApo 40x NA 1.4 air objective (Nikon) and an EMCCD (AU888, Andor). Brightfield images were acquired along with fluorescence images. Standard laser, a quad Dicroic (400-410,486-491, 560-570, 633-647, AHF) and Emission filters were used in the acquisition of confocal fluorescence images (BFP (Hoechst 33342), ex.: 405 nm em.:450/50, GFP (EGFP), ex.: 488 em.:525/50, RFP (mCherry, Alexa 594), ex.: 561 em.:600/50 nm and iRFP (Abberior Star Red, Atto 647N, SiR-Hoechst), ex.: 640 em.:685/50 nm.

Super resolution confocal microscopy was performed with a Leica SP8 microscope (Leica Microsystems, Germany). A 100× 1.4 NA oil objective was used. Images were acquired using the LAS X software (Leica Microsystems, Germany).

For the quantification of replications sites, images of several nuclei were acquired in living cells treated with the fluorescent PCNA-nanobody. Replication sites were counted manually in both the confocal and STED images.

Pearson correlation coefficients to determine colocalization were calculated with the “Coloc 2” plugin for ImageJ. The correlation coefficient was calculated from unprocessed images, over the entire image area.
Results and Discussion

Figure S1. Structures and UV-purity of peptides used in this study. A, Cys-N$_3$, HRMS: Calc.: 622.2977 [M+H], exp.: 622.3035. B, Cys-(N$_3$)$_2$, HRMS: Calc.: 1075.5425 [M+H], exp.: 1075.5326. C, Cys-N$_3$-Trolox, HRMS: Calc.: 982.5026 [M+H], exp.: 982.4997. D, TNB-R10, HRMS: Calc.: 543.3013 [M+4H], exp.: 543.3025.
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Figure S2. Characterization of anti-GFP-nanobody GBP1 and its CPP conjugates. A, SDS-PAGE gel, stained with Coomassie and fluorescence imaging of Atto 594 on the bottom. Shown are the variants of the GBP1-nanobody after expressed protein ligation to the azide peptides, the conjugation of the atto 594 fluorophore and finally the conjugation of the R10 peptide via a disulfide bond, that is removed under reducing conditions (left side of the gel). 1: GBP1 with azide-containing peptide (Cys-N$_3$); 2: GBP1 with peptide containing three azides (Cys-(N$_3$)$_3$); 3: GBP1 with peptide containing azide and trolox (Cys-N$_2$-Trolox). B, High resolution mass spectrum of GBP1-Atto594-R10 (1). Calc.: 16404 [M+H]; Exp.: 16403. C, High resolution mass spectrum of GBP1-(Atto594)$_2$-R10 (2). Calc.: 18547 [M+H]; Exp.: 18542. D, High resolution mass spectrum of GBP1-Atto594-Trolox-R10 (3). Calc.: 16766 [M+H]; Exp.: 16763.

Figure S3. Bleaching curves of GBP1-Atto594-Trolox-R10. After cellular uptake of the nanobody-fluorophore conjugates into cells, 30 consecutive images were taken at several different positions for each nanobody construct. The fluorescence intensity was quantified for each image. The data was then normalized and plotted. Shown are A, bleaching curves from individual imaged areas and B, bleaching curves from the averages of the two different constructs with non-linear decay fit.
Figure S4. Characterization of anti-GFP-nanobody GBP1 conjugated to Cy5 and R10-CPP. A, Structure of DBCO-Sulfo-Cy5 (Jena Bioscience). B, SDS-PAGE gel, stained with Coomassie and fluorescence imaging of Cy5 on the bottom. 1: GBP1 with azide-containing peptide (Cys-N₃) and DBCO-Sulfo-Cy5; 2: GBP1 with peptide containing azide and trolox (Cys-N₃-Trolox) and DBCO-Sulfo-Cy5. C, High resolution mass spectrum of GBP1-Sulfo-Cy5-R10. Calc.: 16569 [M+H]; Exp.: 16570. D, High resolution mass spectrum of GBP1-Trolox-Sulfo-Cy5-R10. Calc.: 16931 [M+H]; Exp.: 16931.
Figure S5. Bleaching curves of GBP1-SulfoCy5 conjugates. A. Images of the bleaching test in live and fixed cells. GBP1-Sulfo-Cy5 conjugates including trolox or the fluorophore alone were taken up into HeLa Kyoto cells expressing nuclear EGFP fused to histone H2B. 30 consecutive images were taken at several different positions for each nanobody construct. Shown are the first 5 images at select positions. Scale bars 5 µm. B. The fluorescence intensity was quantified for each image. The data was then normalized and plotted. In fixed cells (graph on the right), the difference between the trolox-stabilized measurements and the unstabilized measurements is more pronounced.
Figure S6. Characterization of Abberior Star Red alkyne. UV-trace of the purified fluorophore after HPLC. HRMS: Calc.: 947.2378 [M+H+Na], exp.: 947.1923.

Figure S7. Characterization of LaM4 anti-mCherry nanobody conjugate. A, SDS-PAGE gel, stained with Coomassie and fluorescence imaging on the bottom. Shown is the LaM4-nanobody conjugate after expressed protein ligation to the Cys-N₃-Trolox peptide, the conjugation of the Abberior Star red fluorophore and finally the conjugation of the R10 peptide via a disulfide bond. B, High resolution mass spectrum of LaM4-nanobody-abberior Star Red-Trolox-R10 construct. Calc.: 15321 (Unmodified nanobody) [M+H], 19182 (Nanobody-conjugate) [M+H], 19182 (Nanobody-conjugate) [M+H], 19226 (Nanobody conjugate) [M+2Na]; Exp.: 15320, 19223.
Figure S8. Characterization of anti-Lamin nanobody conjugates. a-b, SDS-PAGE gels, stained with Coomassie and fluorescence imaging on the bottom. Shown are the Lamin-nanobody conjugates after expressed protein ligation to the Cys-N$_3$-Trolox peptide, the conjugation of the Atto 594 (a) or Atto 647N (b) fluorophore and finally the conjugation of the R10 peptide via a disulfide bond. c, High resolution mass spectrum of Lamin-nanobody-Atto594-Trolox-R10. Calc.: 19278 [M+H]; Exp.: 19275. d, High resolution mass spectrum of Lamin-nanobody-Atto647N-Trolox-R10. Calc.: 19118 [M+H]; Exp.: 19113.
Figure S9. Live-cell confocal microscopy of HeLa Kyoto cells replicating after uptake of the anti-Lamin nanobody. A. After cellular uptake of 1 µM anti-Lamin nanobody conjugated to trolox and atto 647N, confocal images of HeLa Kyoto cells were taken every 30 minutes at 37°C. Scale bar 10 µm. B. Overview images of cells at the starting point after cellular uptake and after 20 hours. Scale bars 20 µm.
Figure S10. Characterization of anti-PCNA nanobody conjugates. a-b, SDS-PAGE gels, stained with Coomassie and fluorescence imaging on the bottom. Shown are the PCNA-nanobody conjugates after expressed protein ligation to the Cys-N3-Trolox peptide, the conjugation of the Atto 594 (a) or Abberior Star Red (b) fluorophore and finally the conjugation of the R10 peptide via a disulfide bond. c, High resolution mass spectrum of PCNA-nanobody-Atto594-Trolox-R10. Calc.: 18314 [M+H]; Exp.: 18310.

d, High resolution mass spectrum of PCNA-nanobody-Abberior Star Red-Trolox-R10. Calc.: 18396 [M+H], 18440 [M+2Na]; Exp.: 18437.
**Figure S11. Confocal microscopy with fluorescent PCNA-nanobody.**

A. Live cell microscopy of HeLa Kyoto cells expressing GFP-PCNA treated with 2 µM of the cell-permeable R10-CPP-containing PCNA nanobody. The nanobody shows colocalization with the nuclear GFP.

B. HeLa Kyoto cells expressing GFP-PCNA were fixed and permeabilized with cold methanol. The cells were then stained with the PC10 antibody (abcam) and with 100 nM of the PCNA nanobody conjugated to atto 594. Two replication foci were marked with yellow arrowheads. Both the antibody and the nanobody seem to stain the same replication foci. The nanobody also shows a cytoskeletal background staining which we do not observe in live cells. Scale bars 20 µm.

**References**

[1] V. Hong, S. I. Presolski, C. Ma, M. G. Finn, *Angewandte Chemie* 2009, 48, 9879-9883.

[2] T. Walter, M. Held, B. Neumann, J. K. Heriche, C. Conrad, R. Pepperkok, J. Ellenberg, *J Struct Biol* 2010, 170, 1-9.

**Author Contributions**

A.F.L.S. cloned, expressed, and modified proteins, synthesized peptides and performed uptake and microscopy experiments. L.B. cloned, expressed, and modified the LaM4 nanobody. The manuscript was written through contributions of all authors.