SUPPORTING INFORMATION

Click Synthesis of Ubiquitin Dimer Analogs to Interrogate Linkage-Specific UBA Domain Binding

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Contents:

1. SUPPORTING TABLE

Table S1: Calculated and observed masses in the MALDI-TOF MS spectra of trypsin digested triazole-linked diUbs.

diUb	$[M+H]^+_{calc.}$ / Da	$[M+H]^+_{obs.}$ / Da
*t*6 (**3a**)	2586.24	n.o.
*t*11 (**3b**)	2461.27	2460.66
*t*27 (**3c**)	2160.10	2159.77
*t*29 (**3d**)	875.46	n.d.
*t*33 (**3e**)	1696.82	1696.30
*t*48 (**3f**)	1519.79	1519.51
*t*63 (**3g**)	2303.20	2302.96

n.d. = not determined; n.o. not observed

2. SUPPORTING FIGURES

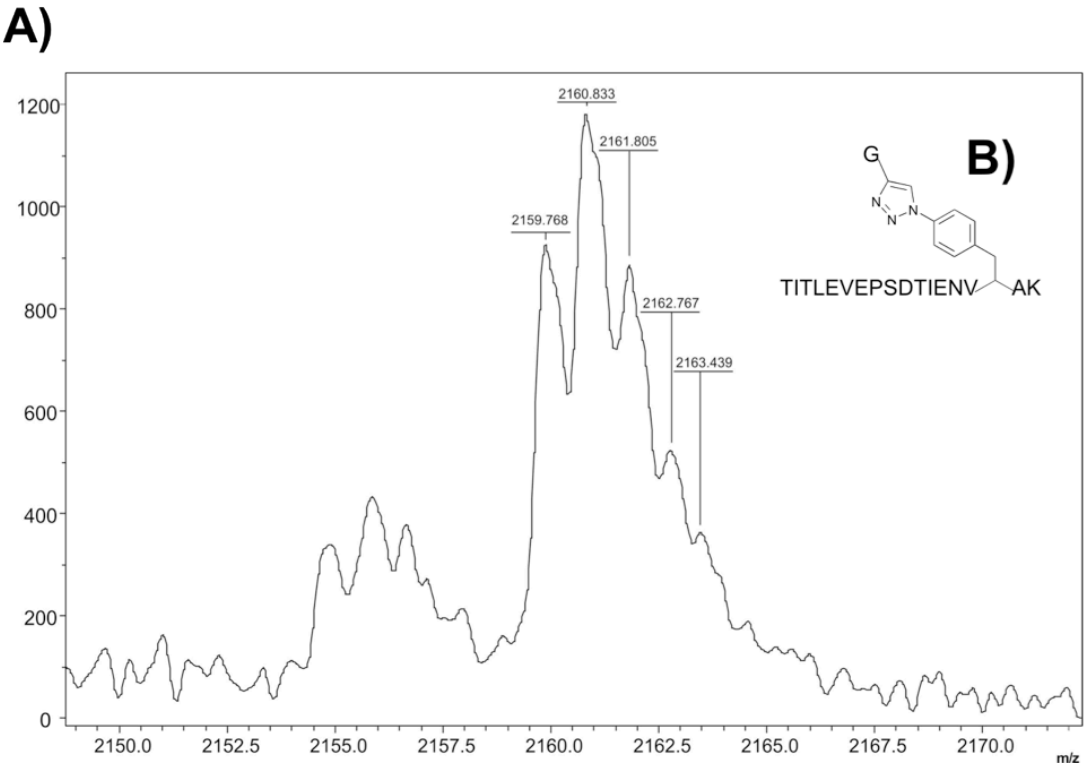

Figure S1: MALDI-TOF MS analysis of *t*27-diUb (3c). Panel A: Mass spectrum of a trypsin-digested sample ($[M+H]^+_{calc.}$ = 2160.10 Da, $[M+H]^+_{obs.}$ = 2159.77 Da). Panel B: Chemical structure of the trypsin fragment.

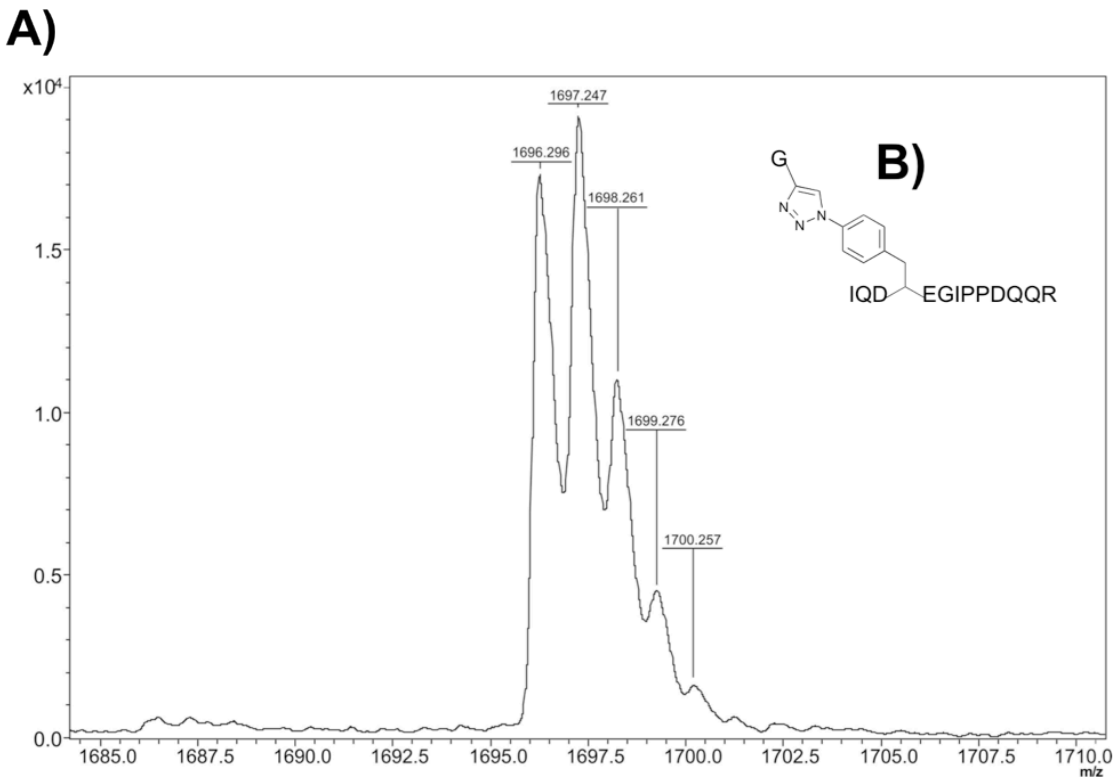

Figure S2: MALDI-TOF MS analysis of *t*33-diUb (3e). Panel A: Shown is the mass spectrum of a trypsin-digested sample ($[M+H]^+_{calc.}$ = 1696.82 Da, $[M+H]^+_{obs.}$ = 1696.30 Da). Panel B: Chemical structure of the fragment generated by trypsin digest.

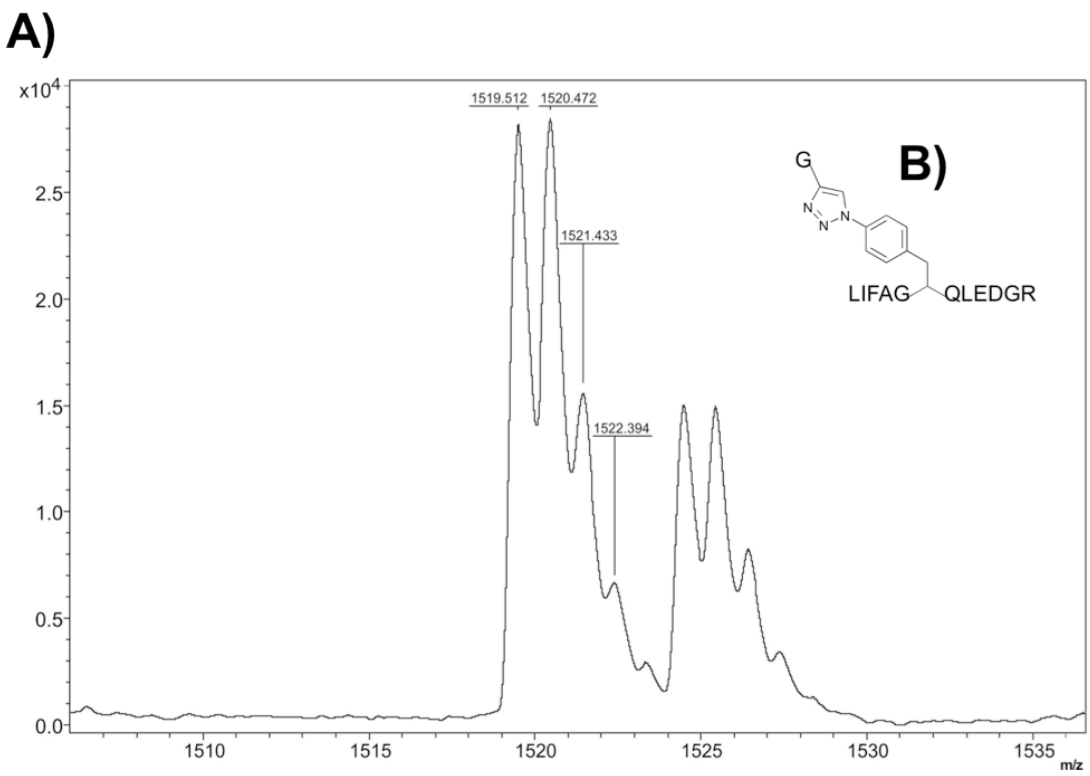

Figure S3: MALDI-TOF MS analysis of *t*48-diUb (3f). Panel A: Mass spectrum of a trypsin-digested sample ($[M+H]^+_{calc.}$ = 1519.79 Da, $[M+H]^+_{obs.}$ = 1519.51 Da). Panel B: Chemical structure of the trypsin fragment.

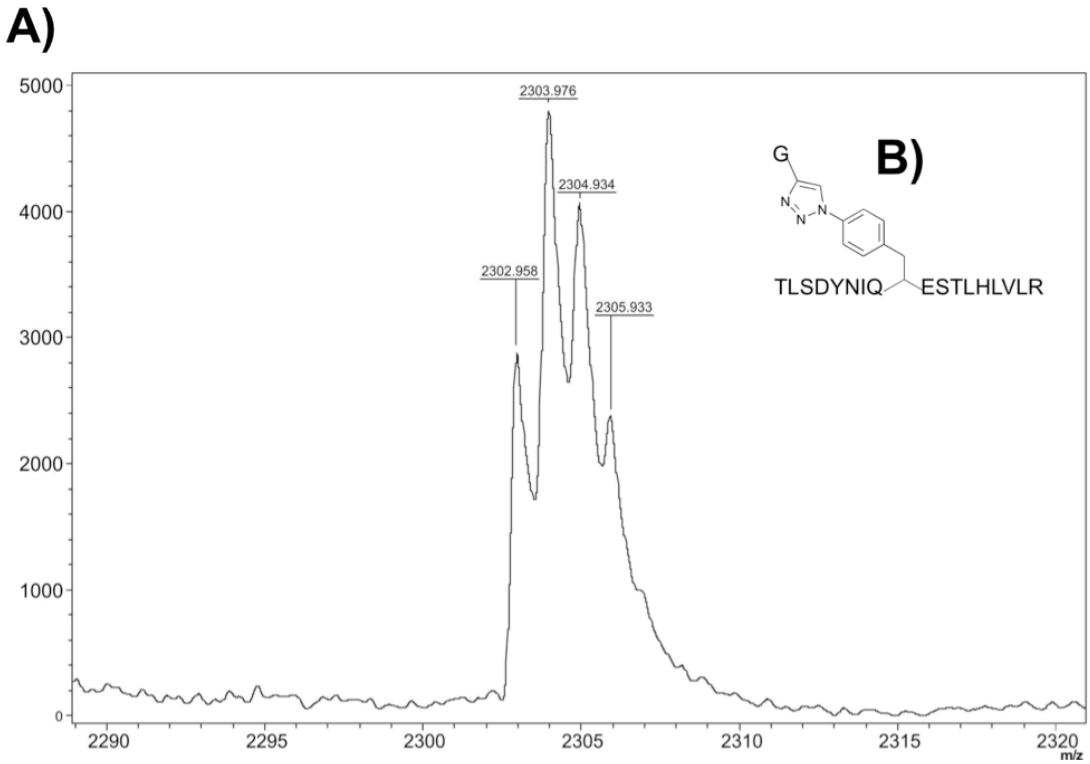

Figure S4: MALDI-TOF MS analysis of *t*63-diUb (3g). Panel A: Shown is the mass spectrum of a trypsin-digested sample ($[M+H]^+_{calc.}$ = 2303.20 Da, $[M+H]^+_{obs.}$ = 2302.96 Da). Panel B: Chemical structure of the trypsin fragment.

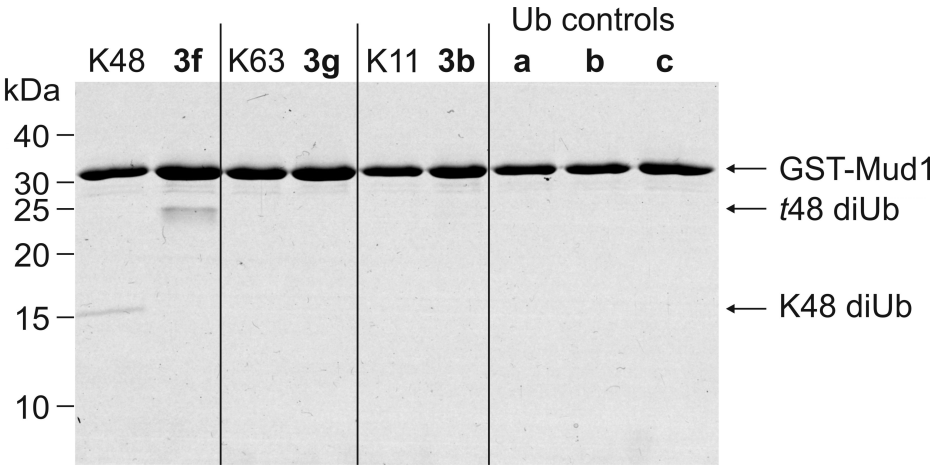

Figure S5: Linkage-specific binding of diUb analogs to the Mud1 UBA domain. Shown is the same experiment as in Figure 2a of the main article, however, analysis was performed on a Coomassie-stained SDS-PAGE gel instead of immuno blotting. Therefore the protein GST-Mud1_UBA is visible and of the diUbs only the K48- and *t*48-variants that exhibited the strongest binding. Purified GST-Mud1_UBA was incubated with equal amounts of each of the native or triazole-linked diUbs and loaded on a resin with immobilized glutathione. After washing, proteins were eluted with glutathione and analyzed by Coomassie staining. Lane a: Ub; lane b: His7-Ub; lane c: SBP-Ub. Note that the difference in molecular weight between the native and the synthetic diUbs stems from the SBP and His_7 tags that are only fused to the latter set of proteins.

3. MATERIALS AND METHODS

General

Standard molecular biology procedures were applied for DNA cloning and expression. Antibiotics were used at 100 µg/mL (ampicillin) and 30 µg/ml (chloramphenicol). Synthetic DNA oligonucleotides were purchased from Biolegio (Nijmegen, the Netherlands). All DNA vectors were confirmed by DNA sequencing (GATC Biotech, Konstanz, Germany). Site-directed mutagenesis was performed according to the QuickChange protocol from Stratagene. Native Ub, K48- and K63-linked diUbs were purchased from Enzo Life Sciences (Lörrach, Germany) and K11-linked diUb from Biomol (Hamburg, Germany). A pSUPAR-plasmid encoding the AzF-tRNA synthetase and tRNA was kindly provided by Peter G. Schultz. The plasmid encoding GST-Mud1_UBA was kindly provided by Jane A. Endicott and the plasmid encoding His_6-Usp5 was kindly provided by Frauke Melchior.

Cloning of expression plasmids

pNW31 for expression of SBP-Ub(ΔG)-GyrA*-CBD: pNW31 was cloned as previously described.[1]

pAU13 for expression of His_7-Ub: The gene encoding for human ubiquitin was ordered as a synthetic fragment from Mr. Gene (Regensburg, Germany) and ligated into a pET16b vector by *Nco*I and *Hind*III restriction sites.

pAU14 for expression of His_7-Ub(K6AzF): pAU13 served as a template for site directed mutagenesis to insert a *TAG* codon at position K6 to allow for unnatural amino acid incorporation using primers 5'-ATGCAGATTTTTGTGTAGACCCTGACCGGCAAAAC-3' (forward; stop codon underlined) and 5'-GTTTTGCCGGTCAGGGTCTACACAAAAATCT-GCAT-3' (reverse).

pAU15 for expression of His_7-Ub(K29AzF): Site-directed mutagenesis was used as above with oligonucleotides 5'-GAAAACGTGAAAGCGTAGATTCAGGATAAAGAAG-3' (forward) and 5'-CTTCTTTATCCTGAATCTACGCTTTCACGTTTTC-3' (reverse).

pNW28 for expression of His_7-Ub(K48AzF): Site-directed mutagenesis was used as above with oligonucleotides 5'-CTGATCTTTGCCGGTTAGCAGCTGGAAGATGG-3' (forward) and 5'-CCATCTTCCAGCTGCTAACCGGCAAAGATCAG-3' (reverse).

pNW29 for expression of His_7-Ub(K63AzF): Site-directed mutagenesis was used as above with oligonucleotides 5'-CGACTATAACATCCAGTAGGAATCCACCCTGCACC-3' (forward) and 5'-GGTGCAGGGTGGATTCCTACTGGATGTTATAGTCG-3' (reverse).

pNW32 for expression of His_7-Ub(K11AzF): Site-directed mutagenesis was used as above with oligonucleotides 5'-CTTCCAGTGTAATGGTTAGACCGGTCAGGGTTTTC-3' (forward) and 5'-CTTCCAGTGTAATGGTCTAACCGGTCAGGGTTTTC-3' (reverse).
pNW33 for expression of His_7-Ub(K27AzF): Site-directed mutagenesis was used as above with oligonucleotides 5'-CGATTGAAAACGTGTAGGCCAAAATCCAGGAC-3' (forward) and 5'-GTCCTGGATTTTGGCCTACACGTTTTCAATCG (reverse).
pNW34 for expression of His_7-Ub(K33AzF): Site-directed mutagenesis was used as above with oligonucleotides 5'-GCCAAAATCCAGGACTAGGAGGGTATTCCGCCT-3' (forward) and 5'-AGGCGGAATACCCTCCTAGTCCTGGATTTTGGC-3' (reverse).

Protein expression and purification

Protein expression was performed in *E. coli* BL21(DE3) cells which were transformed with the suitable plasmids. Cells were grown in LB media supplemented with ampicillin at 37°C to an OD_{600} of 0.6. Expression was induced with 0.4 mM IPTG and carried out for 4 - 5 h at 28°C.

For expression of proteins with *p*-Azidophenylalanine (AzF)[2], *E. coli* BL21(DE3) cells were cotransformed with the plasmid encoding the target gene containing the amber stop codon and the vector encoding the orthogonal *p*AzFRS/$tRNA_{CUA}$ pair. Cells were grown in M9-minimal media (45 mM Na_2HPO_4, 25 mM KH_2PO_4, 8.5 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 22 nM $FeCl_3$, 0.03 mg/ml thiamine, 0.1 % NH_4Cl, 0.2 % glucose) supplemented with 30 µg/ml chloramphenicol and 100 µg/ml ampicillin at 37 °C. When an an OD_{600} of 0.6 was reached, AzF (purchased from Bachem) was added to a final concentration of 1 mM and protein expression was induced with 0.4 mM IPTG. Cultures were then incubated for another 4 h at 37 °C. All cells were harvested by centrifugation, resuspended in the respective buffer, and lysed by two passages through an Avestin C5 emulsifier.

Purification of CBD-tagged Ub was carried out on chitin beads (New England Biolabs, Inc.) equilibrated with chitin column buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, pH 8.0). The clarified cell extract was applied to chitin beads and incubated for 1 h at 4°C under slight agitation. Then the beads were extensively washed with 20 column volumes (CV) chitin column buffer. On-column cleavage was performed with chitin column buffer containing 250 mM MESNA and 250 mM propargylamine, pH 8.0 for 48 h at 4°C under gentle agitation. Elution fractions were checked for purity via SDS-PAGE. In some cases uncleaved and unbound starting material was still present, which was then removed by

passing the solution over fresh chitin beads. Purified proteins were dialyzed against dialysis buffer (20 mM HEPES, 150 mM NaCl, pH 8.0).

His-tagged Ubs were purified on Ni-NTA agarose (Invitrogen, Darmstadt, Germany) equilibrated in Ni-NTA buffer A (50 mM Tris, 300 mM NaCl, pH 8.0) supplemented with 10 mM imidazole. After washing with Ni-NTA buffer A supplemented with 20 mM imidazole a gradient was run ranging from 20 to 500 mM imidazole using an ÄKTA purifier (GE Healthcare, Munich, Germany). Purified proteins were dialyzed against dialysis buffer and stored at – 80°C.

Purification of SBP-tagged conjugates was carried out on StrepTactin® sepharose (IBA BioTAGnology, Göttingen, Germany) equilibrated in Strep buffer W (100 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0). After washing with 20 CV buffer W the proteins were eluted with Strep buffer E (buffer W supplemented with 2 mM desthiobiotin). Purified diUb conjugates were dialyzed against transfer buffer (20 mM HEPES, 110 mM KAc, 2 mM MgAc, 1 mM EGTA, 1 mM DTT, pH 7.3) and stored at -80°C.

GST-tagged Mud1_UBA was purified on GST–bind resin (Novagen, Darmstadt, Germany) in GST-bind/wash buffer (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3) and washed with 10 CV GST-bind/wash buffer prior to elution with four times 1 CV GST elution buffer (GST-bind/wash buffer containing 100 mg red. glutathione). Purified protein was then dialyzed against transfer buffer and stored at -80°C.

All protein concentrations were determined using the calculated absorbances at 280 nm.

Cu(I) catalyzed azide-alkyne cycloaddition (CuAAC)

For diUb conjugation, proteins were mixed in equimolar amounts of 50 µM each and treated with 500 µM TCEP. Then TBTA at 50 µM and $CuSO_4$ at 500 µM final concentrations were added to the solution. All CuAAC reactions were carried out for 30 min at room temperature in a total reaction volume of 1.5 – 3 ml. Following the CuAAC reaction EDTA (5 mM) was added and the solution was dialyzed against Ni-NTA buffer A (300 mM NaCl, 50 mM Tris, pH 8.0) for further purification. Stock solutions were prepared as follows: 50 mM TCEP, 100 mM $CuSO_4$, 500 mM EDTA in ddH_2O each and 1.7 mM TBTA in 1:4 (v/v) DMSO/tBuOH.

Mud1_UBA pulldown assays

For Mud1_UBA domain interaction studies with the diUb constructs, GST-Mud1_UBA was incubated with the respective interaction partners in a molar ratio of 2:1 (1.3 nmol GST-Mud1_UBA; 0.65 nmol diUb-construct) on ice for 1 h. The mixture was then applied to GST–bind resin equilibrated with transfer buffer supplemented with 0.05% Tween-20 (TBT) and incubated for another hour at RT under gentle agitation. The beads were then washed three times with TBT before the proteins were eluted with GST elution buffer. Samples were analyzed by SDS-PAGE and western blotting using antibody "ubiquitin pAb" (Enzo Life Sciences, Lörrach, Germany).

DUB assay

His_6-Usp5 was diluted prior to use to 0.2 mg/ml with DUB dilution buffer (150 mM NaCl, 25 mM Tris, 10 mM DTT, pH 7.5) and incubated for 10 min at 23°C. 10 µl of this solution were added to 5 µg K48- or *t*48-linked diUb and 3 µl 10x DUB buffer (500 mM NaCl, 500 mM Tris, 50 mM DTT, pH 7.5) in a total volume of 30 µl.[3] Samples were taken after the indicated time points and diUb hydrolysis was analyzed by SDS-PAGE.

4. SUPPORTING REFERENCES

1. S. Sommer, N. D. Weikart, A. Brockmeyer, P. Janning and H. D. Mootz, *Angew Chem Int Ed*, 2011, **50**, 9888-9892.
2. J. W. Chin, S. W. Santoro, A. B. Martin, D. S. King, L. Wang and P. G. Schultz, *J Am Chem Soc*, 2002, **124**, 9026-9027.
3. A. Bremm, S. M. Freund and D. Komander, *Nat Struct Mol Biol*, 2010, **17**, 939-947.