ChemPubSoc Europe

DOI: 10.1002/cbic.201600017

CHEMBIOCHEM Communications

Development of Diubiquitin-Based FRET Probes To Quantify Ubiquitin Linkage Specificity of Deubiquitinating Enzymes

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Deubiquitinating enzymes (DUBs) are proteases that fulfill crucial roles in the ubiquitin (Ub) system, by deconjugation of Ub from its targets and disassembly of polyUb chains. The specificity of a DUB towards one of the polyUb chain linkages largely determines the ultimate signaling function. We present a novel set of diubiquitin FRET probes, comprising all seven isopeptide linkages, for the absolute quantification of chain cleavage specificity of DUBs by means of Michaelis–Menten kinetics. Each probe is equipped with a FRET pair consisting of Rhodamine110 and tetramethylrhodamine to allow the fully synthetic preparation of the probes by SPPS and NCL. Our synthetic strategy includes the introduction of *N*,*N*′-Boc-protected 5-carboxyrhodamine as a convenient building block in peptide chemistry. We demonstrate the value of our probes by quantifying the linkage specificities of a panel of nine DUBs in a high-throughput manner.

Ubiquitin (Ub), a 76 amino acid protein, is a post-translational modifier that is crucial for a wide range of cellular processes, including protein degradation, trafficking, and signaling.[1] Ub is generally attached via its C-terminal carboxylate to the side-chain amine of a lysine residue in the target protein, thereby forming an isopeptide bond. Target proteins are frequently modified with a polyUb chain, in which multiple Ub modules are successively linked at the N terminus (linear polyUb) or any of the seven internal lysine residues (isopeptide-linked polyUb: K6, K11, K27, K29, K33, K48, and K63). The type of polyUb chain largely determines its signaling function.[1]

Ubiquitination is mediated by the concerted action of three enzymes, E1 (activating), E2 (conjugating), and E3 (ligase), the particular combination of which provides specificity for the protein target or polyUb chain topology. Removal of Ub from its targets and disassembly of polyUb chains are catalyzed by deubiquitinating enzymes (DUBs). About 100 human DUBs have been identified;[2] some exhibit Ub linkage specificity. DUB action can rescue proteins from proteasomal degradation and alter Ub signaling functions through chain remodeling in a linkage-specific manner.[1] The synthesis of diubiquitin (diUb) has made it possible to study processing by DUBs.[3] In order to determine specificity, a DUB can be incubated with either a native diUb molecule[4] or with a diUb activity-based probe[5] of a given linkage. However current methods do not allow fast and absolute quantification of DUB linkage specificity, and furthermore cannot separate this specificity into binding affinity and catalytic turnover rate (K_M and k_{cat}, respectively, in Michaelis–Menten kinetics).

The application of FRET pairs has proved useful in the study of DUB activity, Ub chain conformation, and Ub-interacting proteins.[6] In order to investigate chain cleavage specificity across all isopeptide linkages, we developed a full chemical synthesis of all seven isopeptide-linked diUb FRET pairs. These pairs carry a novel dye-pair suitable for FRET and compatible with solid phase peptide synthesis (SPPS). We determined K_M and k_{cat} values of linkage-specific DUBs that are used in Ub chain restriction analysis,[7] in order to obtain insight into their catalytic action.

In the FRET-based assay (Figure 1) the reagents consist of two Ub modules, one equipped with a donor fluorophore and the other with an acceptor; these are specifically linked by a native isopeptide bond to each of the seven lysine residues. We reasoned that the best position for fluorophore attachment would be the N termini of both Ub modules, because the distance between the N termini ranges from 30 to 50 Å, based on available crystallographic data (Table S1 in the Supporting Information), an ideal distance for FRET. Because the fluorophores need to be compatible with all synthetic steps (see below), we developed a new FRET pair by using 5-carboxyrhodamine110 (Rho) as the donor and 5-carboxytetramethylrhodamine (TAMRA) as the acceptor. Fluorescein, the more commonly used FRET donor, was initially tried but proved incompatible with the desulfurization step in the final synthesis step (see below) and was therefore replaced by Rho. Upon addition of a DUB, the diUb FRET pair is cleaved, thereby resulting in loss of the FRET signal and hence an increase in donor emission.

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Supporting information for this article can be found under http://dx.doi.org/10.1002/cbic.201600017.

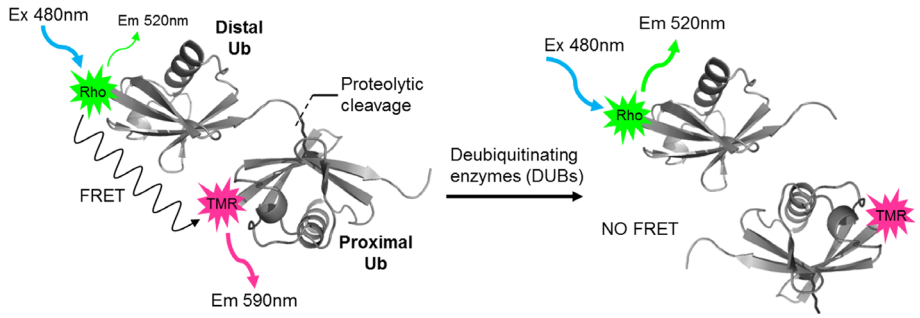
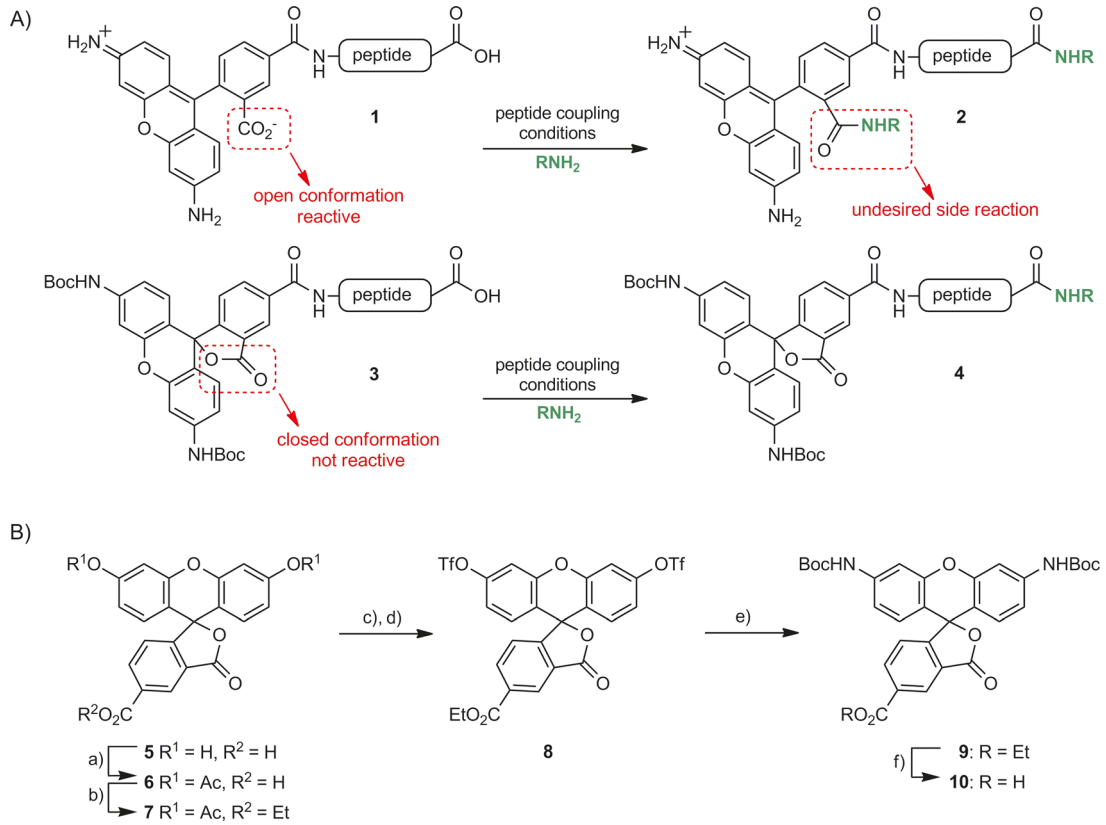


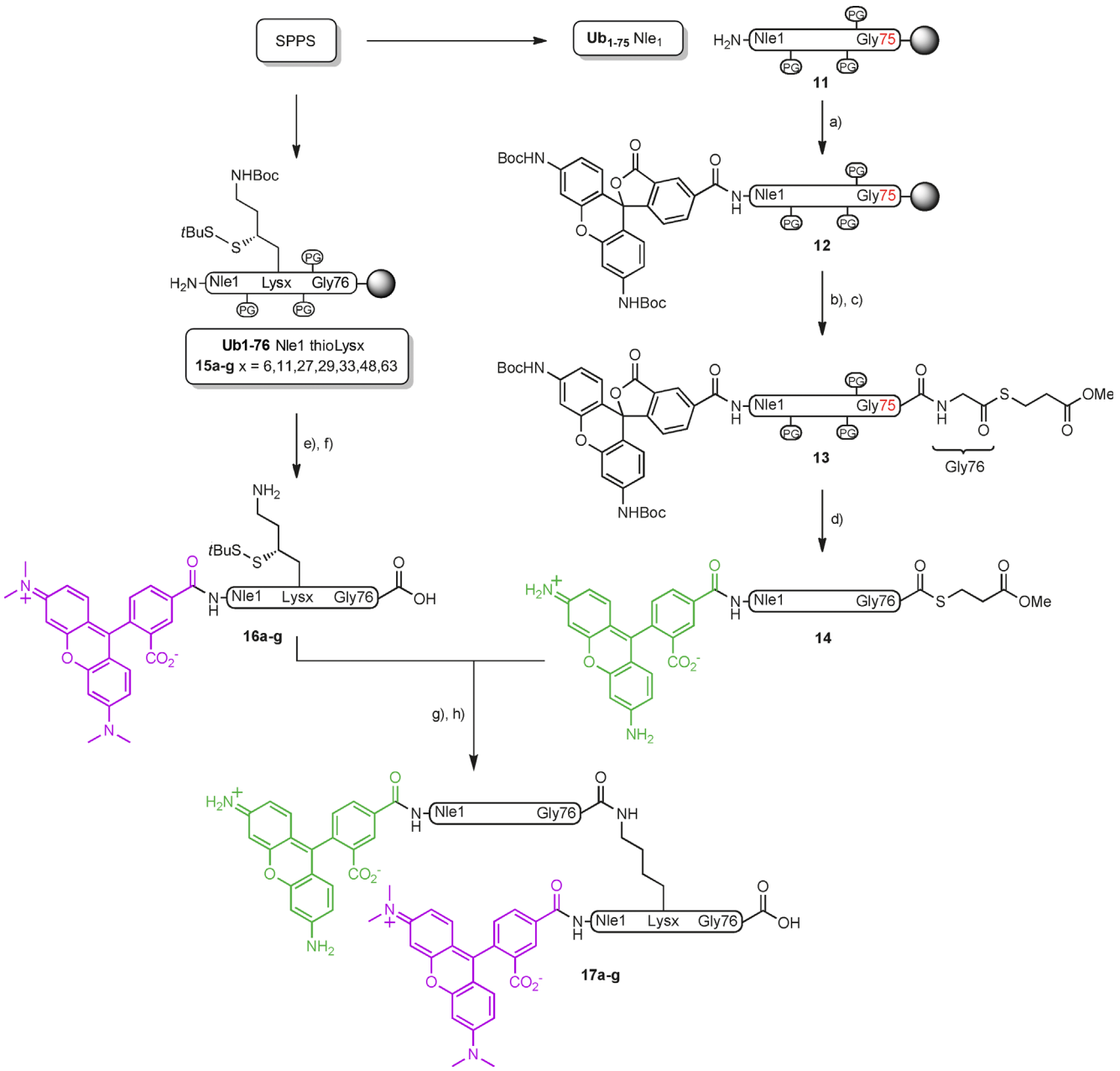
Figure 1. Principle of the FRET-based diUb cleavage assay. Upon cleavage of the diUb FRET pair by a DUB, the FRET signal is lost.



Scheme 1. A) Problems encountered with unprotected Rho in peptide chemistry. B) Synthesis of *N,N'*-Boc-protected Rho. a) Ac_2O, H_2SO_4, 120 °C (99%); b) EtOH, EDC, CH_2Cl_2 (94%); c) NaOEt, EtOH; d) Tf_2O, pyridine, CH_2Cl_2 (57%); e) $BocNH_2$, Pd_2dba_3, Xantphos, Cs_2CO_3, dioxane, 100 °C (82%); f) NaOH, THF (95%).

A major problem in the use of Rho (but also TAMRA) in SPPS (Scheme 1 A) is that when Rho is attached to an amine in a globally side-chain-protected peptide, the 1-carboxylate moiety of Rho is in an open conformation and can react upon further extension of the peptide chain (Scheme 1 A, **1**→**2**). In addition, the coupling of Rho is generally difficult because of the poor solubility and intrinsic reactivity of the aniline moieties. We therefore prepared *N,N'*-Boc-protected Rho. When this molecule is coupled to a peptide, the dual Boc protection locks the 1-carboxylate in the closed lactone form, thus making it unreactive (Scheme 1 A, **3**→**4**). We modified the method reported by Grimm and Lavis[8] to prepare *N,N'*-Boc-protected Rho **10** (Scheme 1). 5-Carboxyfluorescein (**5**) was converted in four steps into ditriflate **8**. Buchwald–Hartwig coupling with $BocNH_2$ resulted in the formation of *N,N'*-Boc-protected Rho (**9**). Use of ethyl ester protection of the 5-carboxylate allowed selective liberation of the 5-carboxylate without affecting the Boc groups, thereby resulting in **10**. In contrast to unprotected Rho, **10** is very soluble in organic solvents, can easily be coupled under standard peptide coupling conditions, and can be prepared on a multi-gram scale.

The seven diUb FRET pairs **17a–g** were constructed by native chemical ligation (NCL) between Rho-Ub-thioester **14** and TAMRA-Ub containing a γ-thioLys building block[3,9] **16** (Scheme 2). The individual Ub modules where synthesized by linear SPPS on hyper-acid-labile trityl resin.[3] DiBoc-protected Rho (**10**) was coupled to Ub_{1-75} (**11**) on resin to result in **12**, which was subsequently cleaved from the resin under mild



Scheme 2. Synthesis of the seven isopeptide-linked diUb FRET pairs. a) **10**, PyBOP, DIPEA, NMP; b) 20% HFIP/CH_2Cl_2; c) HCl·H-Gly-S$(CH_2)_2CO_2$Me, EDC, HOBt, CH_2Cl_2; d) TFA/H_2O/phenol/iPr_3SiH (90.5:5:2.5:2); e) TAMRA, PyBOP, DIPEA, NMP; f) TFA/H_2O/phenol/iPr_3SiH (90.5:5:2.5:2); g) MPAA, 6 M Gnd·HCl, pH 7.2; h) TCEP, GSH, VA-044, 6 M Gnd·HCl, pH 7.0.

acidic conditions without affecting the global protection scheme. Methyl-3-(glycylthio)-propionate was coupled to the liberated C-terminal carboxylate to give **13**. Global deprotection under strong acidic conditions followed by cation exchange and RP-HPLC purification gave Rho-Ub-thioester **14**. It is of note that the Boc groups on Rho are concomitantly removed during the global deprotection, thereby restoring its fluorescent properties. TAMRA-Ub modules containing γ-thioLys on each of the respective lysine positions (**16a–g**) were prepared by coupling the 5-carboxy isomer of TAMRA to the Ub_{1-76} polypeptides **15a–g**, followed by global deprotection and purification (Figure S1 in the Supporting Information). Methionine-1 was replaced by the isostere norleucine to prevent oxidation of the thioether moiety. NCL reactions between **14** and **16a–g**, followed by desulfurization under radical conditions,[10] purification by RP-HPLC, and gel filtration gave the final seven diUb FRET pairs **17a–g** in good yield and purity.

The purities of **17a–g** were confirmed by LCMS analysis (Figure 2A, B, and Supporting Information) and gel analysis (Figure 2C). Upon excitation at 466 nm, the emission spectra of the diUb FRET pairs and Rho-Ub and TAMRA-Ub revealed that all seven molecules show a clear FRET signal (Figure 2D). We performed fluorescence lifetime imaging microscopy (FLIM) to determine FRET efficiencies of all the FRET pairs (Figure 2E,

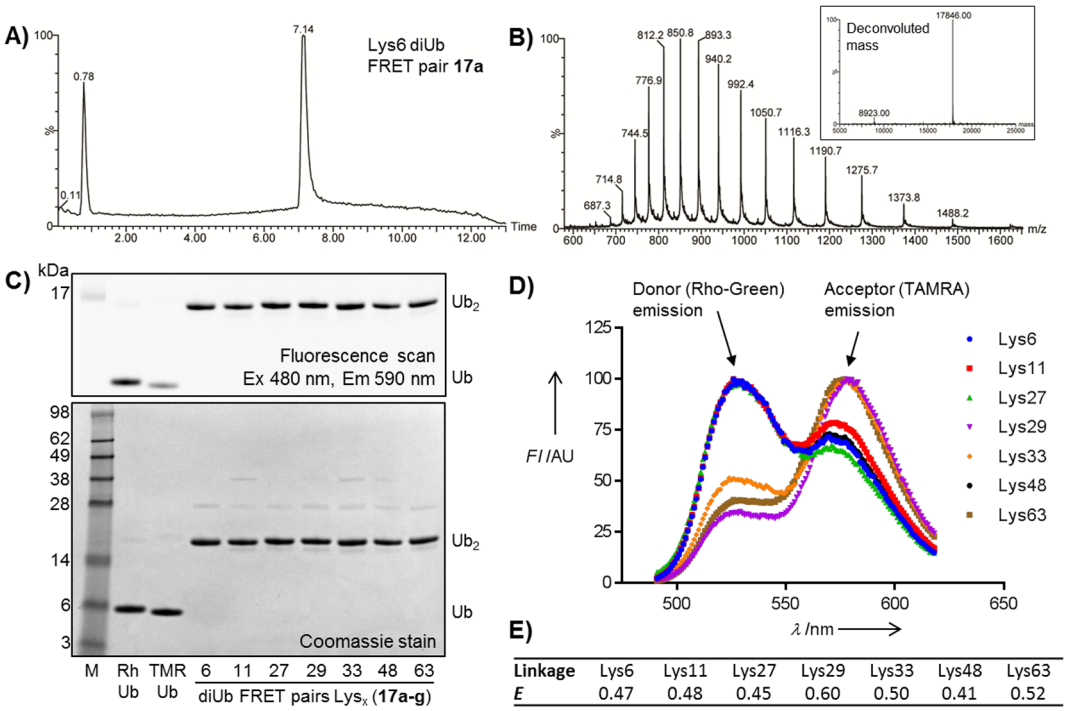


Figure 2. Characterization of diUb FRET pairs **17a–g**. A) Analytical HPLC and B) MS of Lys6-linked diUb FRET pair **17a**. C) SDS-PAGE analysis. D) Emission spectra recorded at λ_{ex} = 466 nm. E) FRET efficiencies (*E*) determined by FLIM.

Table S3); these were found to be 0.45–0.60, depending on the linkage, thus demonstrating efficient FRET in all these molecules.

DUB-mediated cleavage of our new diUb FRET pairs was first assessed by incubation with USP7 and OTUD2, two well-studied DUBs from the two largest DUB families and for which cleavage of unlabeled diUbs has been reported. Reactions were analyzed by SDS-PAGE (Figures S2 and S3) which showed that the diUb selectivity of both DUBs was in good agreement with reported data.[4a,b] We then incubated OTUD2 with Lys11- and Lys27-linked diUb FRET pairs (**17b** and **17c**, respectively) and the corresponding unlabeled diUbs. We also included a 1:1 mixture of the FRET pair and the unlabeled diUb for both linkages. SDS-PAGE analysis (Figures 3A, S4 and S5) showed that both the FRET pair and the unlabeled diUb substrates were equally processed, thus we concluded that the attached fluorophores do not affect DUB activity.

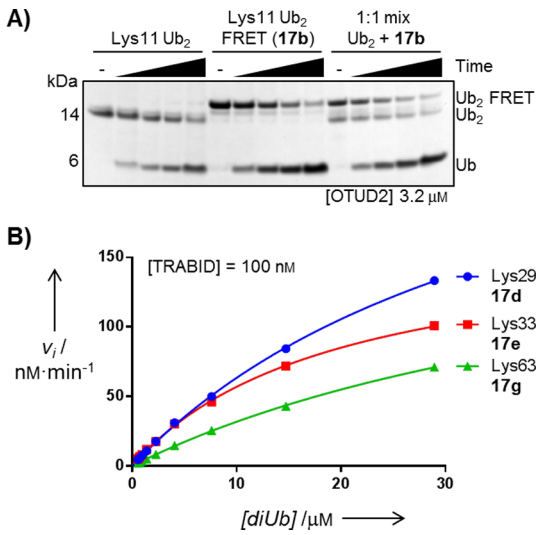


Figure 3. A) SDS-PAGE analysis of Lys11-linked diUb cleavage. OTUD2 was incubated with unlabeled diUb, FRET pair **17b**, or a 1:1 mixture; samples were taken after 10, 30, 60, and 180 min. B) Michaelis–Menten kinetics of TRABID for Lys29-, Lys33- and Lys63-linked diUb, as determined by the FRET assay.

We next applied our FRET reagents for the quantification of diUb linkage-specificity for nine DUBs derived from three different DUB families; each was shown to display a distinct specificity (Table 1).[4b] We incubated the DUBs with all diUb FRET pairs at a fixed concentration (0.5 μM, to keep initial fluorescence constant for all samples) with an increasing concentration of the unlabeled diUb (0–28 μM). The enzyme concentration was chosen such that the reaction proceeded linearly for at least 20 min (Supporting Information). The total amount of processed diUb was then determined by monitoring the increase in donor fluorescence over time; from this the rates of initial velocity were calculated and fitted to the Michaelis–Menten equation (Figure 3B and Supporting Information), from which K_M and, k_{cat} were determined.

Table 1 shows the data for all combinations of DUB and diUb substrate for which activity could be measured. Overall, the individual diUb linkage types cleaved by each DUB were consistent with published qualitative data.[4] As expected from earlier findings, the unspecific DUB USP21 showed similar activity towards most linkages.[4a] The virus-derived DUB vOTU, which is also considered to be unspecific, showed some interesting results: the catalytic efficiency (k_{cat}/K_M) for Lys6 was two times higher than for Lys48, and four times higher than for

Table 1. Kinetic characterization of DUBs that are used in Ub chain restriction analysis[7] for the diUb FRET pairs **17a–g**.[a]

DUB	Linkage	K_M [μM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$M^{-1}s^{-1}$]
AMSH	Lys63	*45.4*	0.0027	59
AMSH*[b]	Lys63	2.4	0.17	70048
Cezanne	Lys11	19.4	1.5	78818
OTUB1	Lys48	≫50	n.d.	
OTUB1*[b]	Lys48	*38.6*	0.66	17158
OTUD1	Lys63	≫50	n.d.	4020
OTUD3	Lys6	≫50	n.d.	
	Lys11	*52.4*	0.0085	162
	Lys63	*57.9*	0.0061	105
TRABID	Lys29	*40.1*	0.053	1317
	Lys33	19.6	0.028	1431
	Lys63	*54.0*	0.034	627
OTUD2	Lys11	*87.9*	4.4	50253
	Lys27	≫50	n.d.	
	Lys29	≫50	n.d.	
*v*OTU	Lys6	3.6	0.87	242031
	Lys11	3.4	0.22	63110
	Lys48	8.4	1.0	119952
	Lys63	1.4	0.091	66266
USP21	Lys6	2.1	0.12	60633
	Lys11	1.4	0.087	62735
	Lys33	2.1	0.13	60814
	Lys48	1.7	0.067	39181
	Lys63	2.7	0.16	60558

[a] Values in italics were obtained by extrapolation beyond the highest substrate concentration. [b] Activated versions of AMSH and OTUB1 were created by fusing them to their activators (STAM and UBE2D2, respectively).[11]

Lys11 and Lys63 diUbs; this can largely be attributed to differences in k_{cat} rather than K_M. Another interesting result was for AMSH. In agreement with earlier findings, this DUB had absolute specificity for Lys63 diUb,[4c] although the overall efficiency was rather low. Remarkably, the recently reported fusion of AMSH with its natural activator STAM2[11] resulted in a more than 1000-fold increase in catalytic efficiency, which can be attributed to increases in both affinity and catalytic turnover. Taken together, these data show that our quantitative assessment of the DUB linkage specificity is in accordance with reported data and that new insights can be obtained from the kinetic parameters.

In summary, the set of all seven isopeptide-linked diUb FRET pairs allows absolute quantification of DUB linkage specificity. Our synthetic strategy, which includes a convenient *N*,*N*′-Boc-protected Rho building block, allows efficient preparation of these reagents in large quantities. The assay requires low amounts of material, can easily be automated, and can be used in high-throughput small-molecule screening or for the assessment of (di)Ub binding domains.[6c] Overall, we believe that our diUb FRET probes will be of great value in ongoing efforts to crack the ubiquitin code and that the FRET pair presented here will facilitate FRET-pair synthesis in general.

Acknowledgements

We thank Dris El Atmioui and Henk Hilkman for SPPS and Prof. Dr. Kees Jalink for help with FLIM. This work was supported by the European Research Council (ERC grant no. 281699) and the Netherlands Organization for Scientific Research, (NWO VICI grant no. 724.013.002) to H.O. Work in the D.K. lab was funded by the Medical Research Council (U105192732), the European Research Council (Grant no. 309756), and the Lister Institute for Preventive Medicine, as well as the Marie Curie Initial Training Network "UPStream" (T.E.T.M.) and an EMBO Long-Term Fellowship (J.N.P.).

Keywords: deubiquitinating enzymes · FRET · native chemical ligation · solid-phase synthesis · ubiquitin conjugates

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Manuscript received: January 12, 2016
Final article published: ■■ ▮▮, 0000

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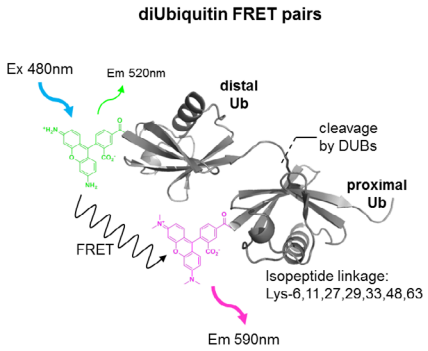

The magnificent seven: All seven isopeptide-linked diubiquitin conjugates equipped with a Rhodamine-TAMRA FRET pair were prepared. The synthesis includes the use of a highly convenient *N*,*N*′-Boc-protected Rhodamine building block. These probes enable the absolute quantification of the ubiquitin linkage specificity of deubiquitinating enzymes by means of Michaelis–Menten kinetics.

