LETTER

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Ubiquitin chain conformation regulates recognition and activity of interacting proteins

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Mechanisms of protein recognition have been extensively studied for single-domain proteins[1], but are less well characterized for dynamic multidomain systems. Ubiquitin chains represent a biologically important multidomain system that requires recognition by structurally diverse ubiquitin-interacting proteins[2,3]. Ubiquitin chain conformations in isolation are often different from conformations observed in ubiquitin-interacting protein complexes, indicating either great dynamic flexibility or extensive chain remodelling upon binding. Using single-molecule fluorescence resonance energy transfer, we show that Lys 63-, Lys 48- and Met 1-linked diubiquitin exist in several distinct conformational states in solution. Lys 63- and Met 1-linked diubiquitin adopt extended 'open' and more compact 'closed' conformations, and ubiquitin-binding domains and deubiquitinases (DUBs) select pre-existing conformations. By contrast, Lys 48-linked diubiquitin adopts predominantly compact conformations. DUBs directly recognize existing conformations, but may also remodel ubiquitin chains to hydrolyse the isopeptide bond. Disruption of the Lys 48–diubiquitin interface changes conformational dynamics and affects DUB activity. Hence, conformational equilibria in ubiquitin chains provide an additional layer of regulation in the ubiquitin system, and distinct conformations observed in differently linked polyubiquitin may contribute to the specificity of ubiquitin-interacting proteins.

Ubiquitin is involved in most aspects of cell biology, as it serves as a post-translational modification of lysine (Lys) residues, regulating many processes including protein degradation, cell signalling, trafficking and the DNA damage response[2]. Most of these functions are mediated by eight structurally and functionally distinct ubiquitin chain types[2], only two of which have been studied extensively. Lys 48-linked ubiquitin chains target proteins for proteasomal degradation[4], whereas Lys 63- and Met 1-linked ubiquitin chains have multiple non-degradative roles in cell signalling[5,6]. Polyubiquitin signals are decoded by ubiquitin-interacting proteins including ubiquitin binding domains[3] and DUBs[7] that bind or hydrolyse ubiquitin chains, respectively. Some ubiquitin-interacting proteins can distinguish between different linkage types, and trigger a specific downstream response to ubiquitination.

The distinct cellular roles of differently linked polyubiquitin have partly been explained by distinct chain conformations. Crystallographic, NMR and small angle X-ray scattering (SAXS) studies have proposed 'compact' conformations for Lys 48-linked diubiquitin[8–10] (Fig. 1a, b). In the prevalent model for Lys 48-linked diubiquitin, ubiquitin moieties interact via a hydrophobic patch on ubiquitin[8] (Fig. 1a), which is also the most common recognition site for ubiquitin-interacting proteins[2]. NMR relaxation and residual dipolar coupling measurements suggested that this conformation is in equilibrium with a second conformation that partly exposes the hydrophobic patch[9]. A recent crystal structure suggested a third compact conformation with exposed hydrophobic patches on both moieties[11] (Fig. 1b). In contrast, Lys 63- and Met 1-linked diubiquitin are thought to adopt 'open' conformations with no interactions between ubiquitin moieties, exposing the hydrophobic patches[10,12,13] (Fig. 1c, d), although a compact crystal structure of Met 1-linked diubiquitin has been reported recently[14] (Fig. 1d).

Notably, the first crystal structures of ubiquitin and diubiquitin bound to ubiquitin-interacting proteins (reviewed in refs 2, 3, 7) indicate that in some cases known diubiquitin structures are incompatible with binding to ubiquitin-interacting proteins. In particular, DUBs must interact with the isopeptide linkage between ubiquitin moieties, which is not accessible in known models of Lys 48-linked polyubiquitin (Fig. 1a, b). This suggests that compact ubiquitin chain types might undergo remodelling ('opening') to be hydrolysed by DUBs. Whether such chain opening is induced by DUB binding, or whether Lys 48-linked diubiquitin pre-exists in open conformations, is unclear.

To understand the principles governing ubiquitin chain recognition, we generated Lys 48-, Lys 63- and Met 1-linked diubiquitin (termed K48NC, K63NC and M1NC, respectively), each containing a fluorescence resonance energy transfer (FRET) compatible dye pair (Alexa488/Alexa647, $R_0 = 5.6$ nm). Linkage-specific assembly reactions and/or selective purification resulted in pure dual-labelled diubiquitin (see Fig. 1e, Supplementary Fig. 1 and Supplementary Methods). Mass-spectrometric and enzymatic analysis, interchanging dye positions, and ensemble measurements of fluorescence lifetime and dye anisotropy confirmed sample quality and dye stability (Supplementary Figs 1 and 2 and Supplementary Methods). Dye photophysics were not significantly altered in labelled diubiquitin (see Supplementary Fig. 2 and Supplementary Methods). All samples showed a FRET signal in ensemble measurements (Fig. 1f).

Distinct ubiquitin chain conformations that underwent FRET were detected at the single molecule level by excitation of the donor fluorophore, with a single laser, and monitoring emission of both donor and acceptor fluorophores. The resulting FRET histograms were fitted to Gaussian functions representing distinct populations of diubiquitin conformations (Fig. 2; see Supplementary Methods). To estimate the proportion of molecules in conformations that gave no detectable FRET (termed 'non-FRET'), two-colour coincidence detection (TCCD; see Supplementary Methods)[15] was used on the same sample. In TCCD, two lasers are used to excite directly the two fluorophores in the diubiquitin independently, allowing direct quantification of the number of molecules with both donor and acceptor fluorophores. Combined use of TCCD and FRET measurements thus enabled estimation of the proportion of molecules that were in non-FRET conformations (see Supplementary Information).

For K48NC, two distinct FRET populations could be resolved (Fig. 2a): a high-FRET population (FRET efficiency $E \approx 0.69$) representing ~90% of all molecules, and a low-FRET population ($E \approx 0.41$) accounting for the remaining ~10%. We were unable to detect any non-FRET populations in these experiments; however, we cannot rule out the existence of a small population of 'hidden'[16] non-FRET K48NC species which would be beyond the detection limit (see Supplementary Methods). Hidden protein conformations may modulate enzyme activity but are not easily detected[16].

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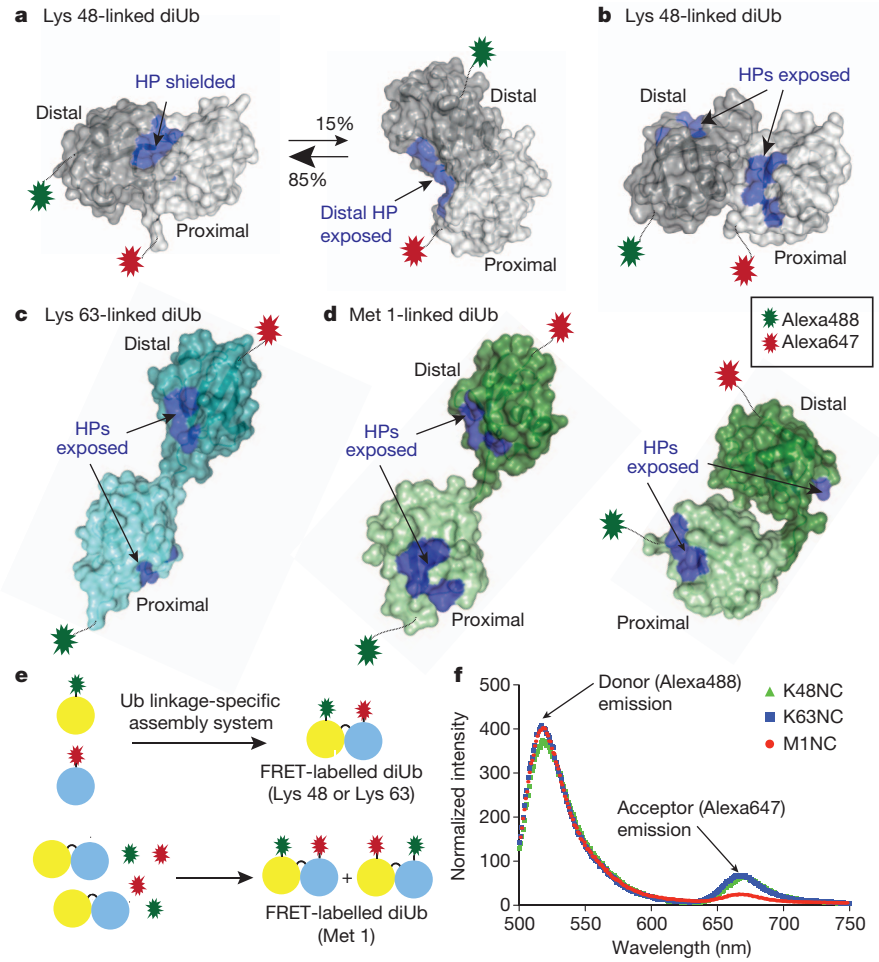

Figure 1 | Ubiquitin chain conformations and ensemble FRET measurements. a–d, Distal (dark) and proximal (light) ubiquitin moieties are shown in surface representation with hydrophobic patch (HP) residues (Leu 8, Ile 44, His 68, Val 70) in blue. N- and C-terminal Alexa dye attachment points are indicated. **a**, Lys 48-linked diubiquitin (diUb) derived from crystallographic and NMR analysis. Protein Data Bank (PDB) accession codes 1AAR (left)[8] and 2PE9 (right)[9]. **b**, Alternative 'compact' Lys 48-linked diubiquitin (PDB 3AUl[11]). **c**, 'Open' Lys 63-linked diubiquitin (PDB 2JF5[13]). **d**, Met 1-linked diubiquitin from crystallography (PDB 2W9N (left)[13] and 3AXC (right)[14]). **e**, Schematic representation of FRET-labelled diubiquitin assembly. See Supplementary Fig. 1 and Supplementary Methods. **f**, Uncorrected ensemble FRET measurements for diubiquitin used in this study (see Supplementary Methods).

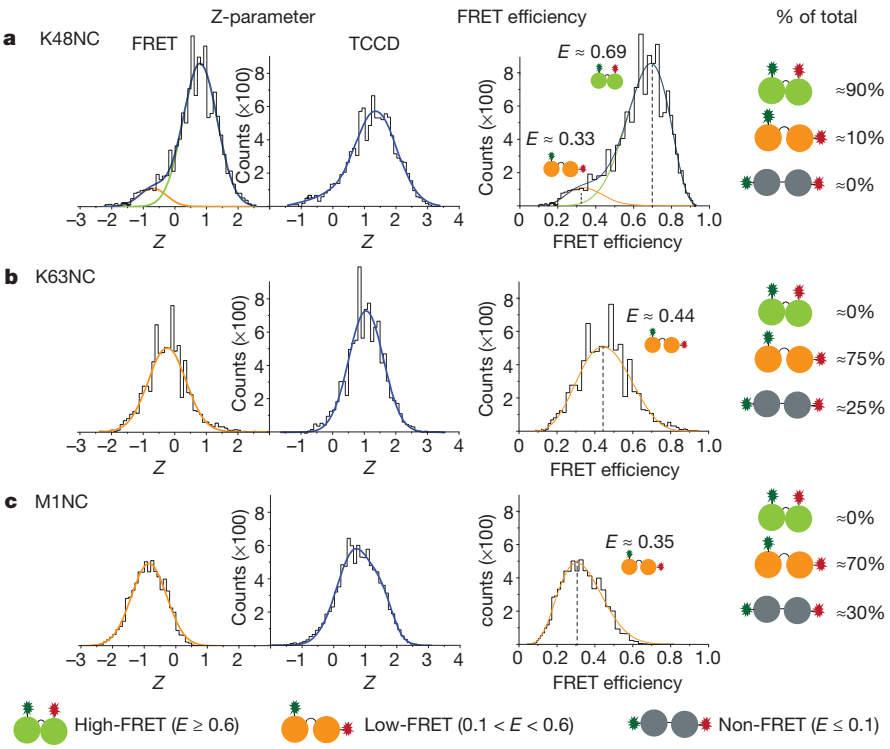

Figure 2 | Single-molecule FRET of K48NC (a), K63NC (b) and M1NC (c). Left: FRET and TCCD signals in histogram counts against the *Z*-parameter ($\ln(I_A/\gamma I_D)$) for fitting to Gaussian functions, normalized to equal area in TCCD. Gaussian functions (see Supplementary Methods) are shown in green (high-FRET), orange (low-FRET) and blue (cumulative fit). Middle: histograms of FRET species plotted against the FRET efficiency. The curves derived from the *Z*-parameter Gaussian fits are shown (see Supplementary Methods). Right: estimate of the relative abundance of each population. Non-FRET refers to dual-labelled molecules (detected by TCCD) without FRET emission.

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Similar to the results for K48NC, multiple conformations were also observed for K63NC and M1NC, with ~70–75% low-FRET and ~25–30% non-FRET populations (Fig. 2b, c). Models of extended Lys 63- or Met 1-linked diubiquitin (Fig. 1c, d) are compatible with non-FRET populations, whereas the compact crystal structure of Met 1-linked diubiquitin[14] (Fig. 1d) may represent a low-FRET M1NC species. The prevalence of compact Lys 63-linked diubiquitin conformations in FRET is surprising, and has not been observed by other methods[10,12,13]. This may be due to multiple compact and semi-compact conformations that collectively result in the observed low-FRET populations.

To test whether the observed diubiquitin conformations are relevant for ubiquitin-interacting protein interaction, single-molecule measurements were performed using pM concentrations of K63NC, K48NC or M1NC mixed with ubiquitin-interacting proteins at concentrations exceeding the dissociation constant (K_d) of the interaction (Supplementary Fig. 7). With our methodology we can follow relative changes in the populations upon addition of ubiquitin-interacting proteins, none of which affected dye photophysics (Supplementary Fig. 8). Several ubiquitin-interacting proteins interacted with pre-existing compact conformations of diubiquitin. A Lys 63 linkage-specific antibody increased the FRET population relative to the non-FRET population of K63NC, in agreement with structural work[17] (Fig. 3a, b). Similarly, the ubiquitin-binding UBAN domain of NEMO[18] enriched the low-FRET population of M1NC, consistent with UBAN binding to a compact conformation of linear diubiquitin[18] (Fig. 3c, d).

Access to the isopeptide bond is essential for DUB activity. AMSH-like protein (AMSH-LP; also called STAMBPL1), a Lys 63-specific JAMM family DUB, binds an open Lys 63-linked diubiquitin conformation[19] (Fig. 3e). Indeed, inactivated AMSH[20] (AMSHi) depleted the FRET and increased the non-FRET population of K63NC in single-molecule measurements (Fig. 3e). Structures of ubiquitin-specific protease (USP) DUBs with diubiquitin bound across the active site are unavailable, but monoubiquitin complexes show that the carboxy terminus of a distal ubiquitin is extended[7] (Fig. 3f), suggesting that USPs also bind open conformations. Indeed, inactivated USP21 (ref. 21; USP21i) enriched non-FRET K63NC conformations in our single-molecule measurements (Fig. 3f). Therefore, whereas the Lys 63 linkage-specific antibody or the UBAN domain of NEMO selects existing compact conformations of K63NC and M1NC, respectively, DUBs select pre-existing open conformations of K63NC (Fig. 3g).

K48NC is in equilibrium between predominantly compact conformations (Fig. 2a). Inactivated OTUB1 (OTUB1i), a Lys 48-specific DUB, enriched the low-FRET and depleted the high-FRET population of K48NC in single-molecule measurements (Fig. 4a, b and Supplementary Fig. 9). This is consistent with recent crystal structures of OTUB1 in complex with E2 and two ubiquitin molecules[22,23] that revealed a relatively compact conformation of the two ubiquitin moieties when bound to OTUB1 (Fig. 4c).

Notably, titration of K48NC with USP21i resulted in depletion of high-FRET and an increase in low-FRET populations, but also gave rise to a non-FRET population of K48NC (Fig. 4d, e). The appearance of open, non-FRET K48NC species can be rationalized structurally, as USP21 stretches the linkage across the active site to form a catalytically competent conformation (Fig. 3f). However, the increase in low-FRET populations indicates that USP21i binds semi-open conformations directly (Fig. 4d, e). Estimation of binding constants for the low- and non-FRET species indicated a slightly higher affinity of USP21i for the open non-FRET conformation (Supplementary Fig. 9).

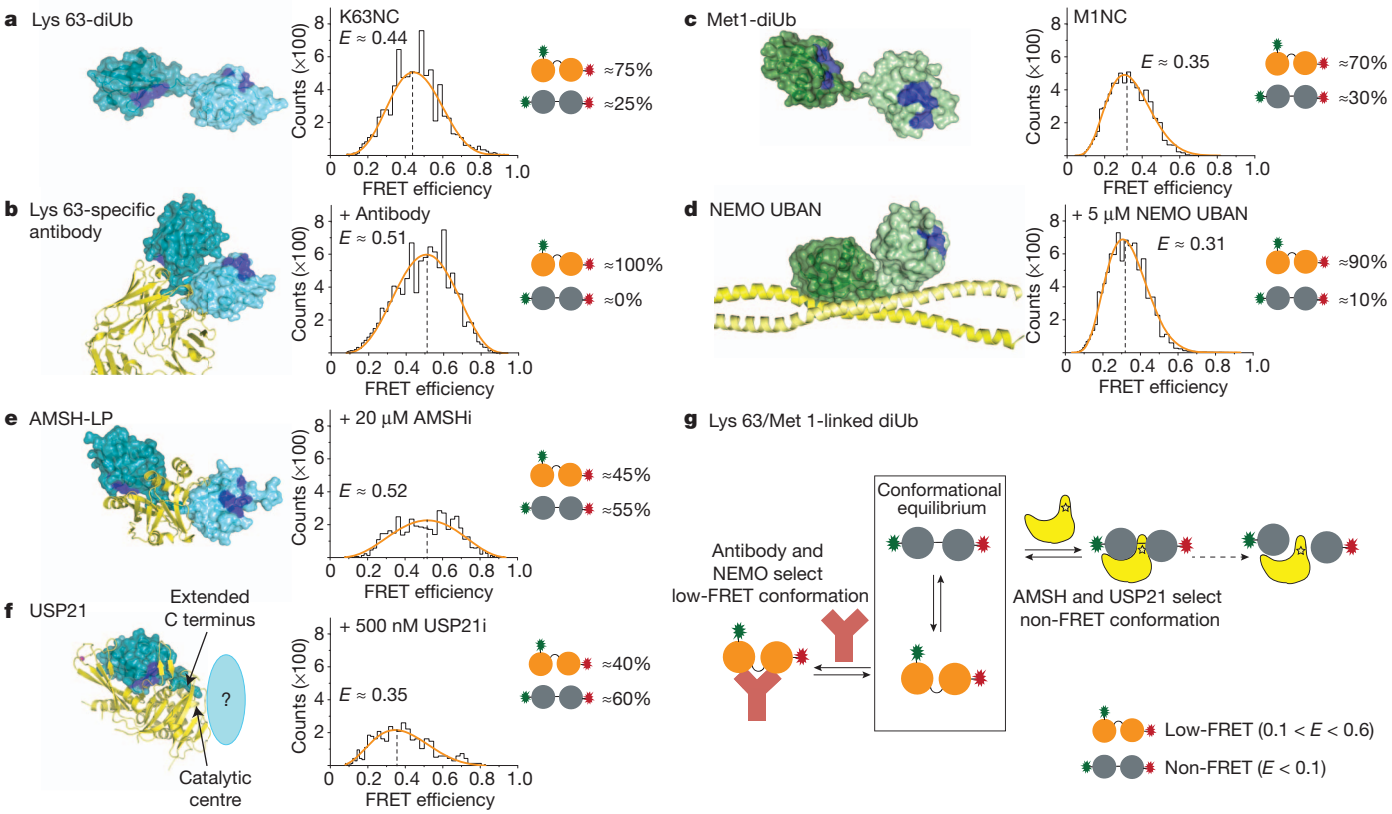

Figure 3 | Ubiquitin-interacting protein binding to Lys 63- and Met 1-linked chains. **a**–**f**, Structural models (coloured as in Fig. 1a with yellow ubiquitin-interacting protein), FRET efficiency histograms and population estimates are shown. All experiments are normalized to equivalent TCCD areas (see Supplementary Fig. 12 for *Z*-parameter plots). **a**, Lys 63-linked diubiquitin (PDB 2JF5[13]). **b**, Complex of Lys 63-linked diubiquitin and Lys 63-linkage-specific antibody (PDB 3DVG[17]). **c**, Met 1-linked diubiquitin (PDB 2W9N[13]). **d**, Complex of met 1-linked diubiquitin and NEMO UBAN (PDB 2ZVO[18], only one diubiquitin shown). **e**, Complex of Lys 63-linked diubiquitin and AMSH-LP (PDB 2ZNV[19]). **f**, Complex of monomeric ubiquitin with USP21 (PDB 2Y5B[21], proximal ubiquitin indicated). **g**, Model for Lys 63- and Met 1-linked diubiquitin interaction with binding partners.

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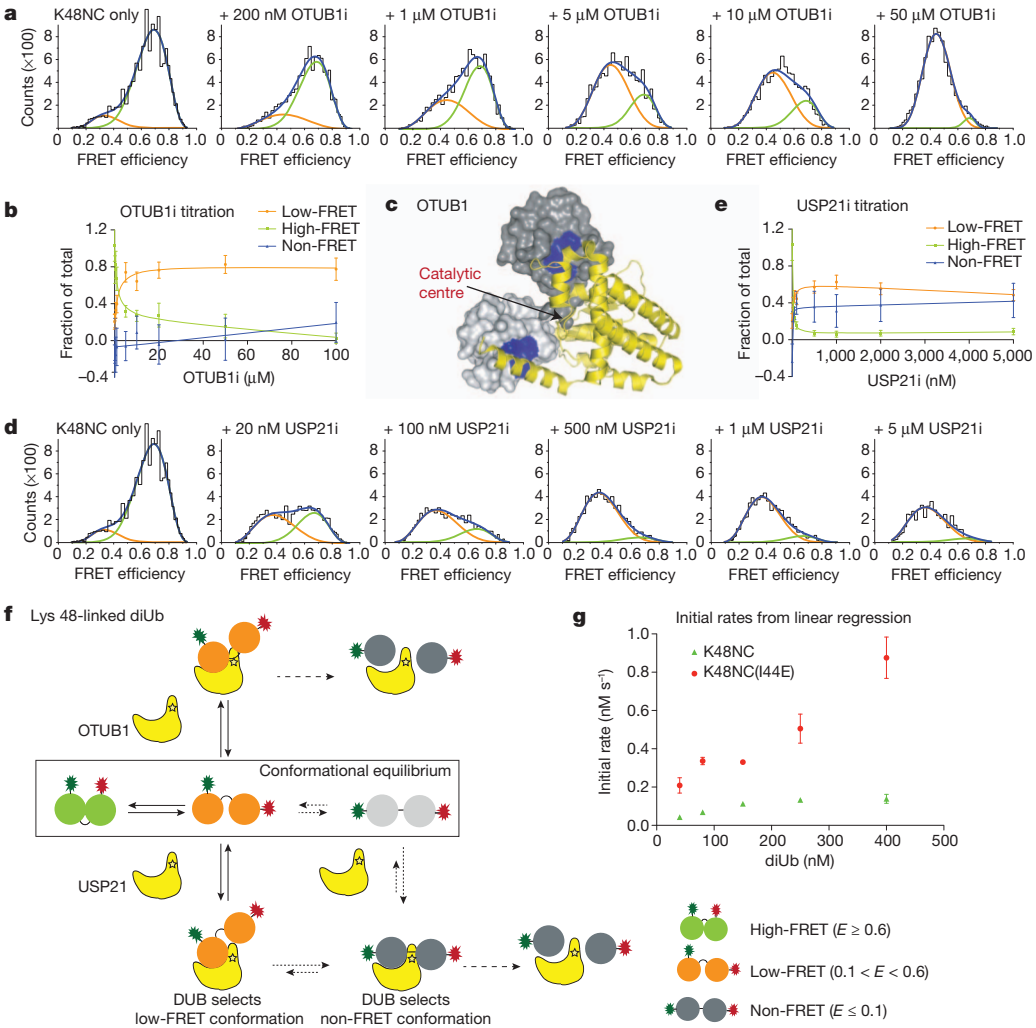

Figure 4 | DUB interaction with Lys48-linked chains. **a**, FRET efficiency histograms of K48NC with increasing OTUB1i concentration. Estimated FRET efficiencies: $E_{\text{high-FRET}} \approx 0.69$, $E_{\text{low-FRET}} \approx 0.45$ (Supplementary Fig. 13 and Supplementary Methods). **b**, Relative changes of K48NC populations from **a**. Errors represent standard deviation in curve fitting. **c**, Structure of OTUB1 (yellow) bound to two ubiquitin molecules (grey) (derived from PDB 4DHZ[22,23]). **d**, FRET efficiency histograms of K48NC with increasing USP21i concentrations as in **a** ($E_{\text{high-FRET}} \approx 0.71$, $E_{\text{low-FRET}} \approx 0.37$). **e**, Relative changes of K48NC populations derived from **d**, shown as in **b**. **f**, Model for interaction of DUBs with Lys 48-linked chains. **g**, Initial rate analysis of USP21-mediated hydrolysis of K48NC (green) and K48NC(I44E) (red). Errors represent standard deviation of triplicate measurements.

Our data result in two models for DUB interactions with Lys 48-linked ubiquitin chains (Fig. 4f). DUBs such as OTUB1 may directly recognize and hydrolyse low-FRET semi-open conformations of Lys 48-linked diubiquitin. Other enzymes such as USP21 recognize semi-open and open (that is, low-FRET and non-FRET) conformations. Binding of semi-open conformations could lead to remodelling to open conformations that are compatible with catalysis (Fig. 4f). Alternatively, dissociation of low-FRET and re-binding of non-FRET diubiquitin could account for generating a catalytically competent enzyme–substrate complex (Fig. 4f). Both models imply that recognition of Lys 48 linkages is governed initially by conformational selection.

Importantly, our data indicate that the compact, high-FRET population of K48NC is not recognized directly. Access to ubiquitin hydrophobic patches is obstructed in the prevalent compact structure of Lys 48-linked diubiquitin (Fig. 1a and refs 8–10), which would correlate with a high-FRET population. Because all known DUBs bind the hydrophobic patch of ubiquitin[7], interconversion from compact high-FRET to semi-open low-FRET conformations could be crucial for DUB activity (Fig. 4f). To test this hypothesis, we mutated Ile 44 in the hydrophobic patch of the proximal ubiquitin of K48NC (K48NC(I44E)) to disrupt a hydrophobic-patch-based interface. We observed a reduction in the high- and low-FRET populations and a shift in their peak positions to lower FRET efficiencies as compared to K48NC (Supplementary Fig. 10), indicating that the mutation changes the conformational equilibria such that open conformations, as well as alternative compact forms of this chain type, may now be populated (for example, Fig. 1b and ref. 11). Importantly, kinetic assessment of USP21-mediated cleavage shows that K48NC(I44E) is hydrolysed significantly faster than wild-type K48NC (Fig. 4g). We propose that ubiquitin chain conformation and dynamics directly affect the rate of DUB hydrolysis.

We show here how FRET/TCCD measurements can be used to probe the complex conformational dynamics of ubiquitin chains in solution, thereby identifying novel conformations not detected using NMR or X-ray crystallography. Our results establish that distinct conformational populations of flexible two-domain ubiquitin chains are recognized by ubiquitin-interacting proteins. In the case of USP21, we suggest that subsequent remodelling may take place to achieve active conformations. Such a combination of 'conformational selection' and remodelling would be consistent with the most recent models of macromolecular recognition[24], and may further extend these models, as the remodelling step here involves significant translation and presumably rotation of flexibly linked domains with respect to each other. Hence, conformational selection is an important mechanism of

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ubiquitin chain recognition. This highlights the importance of understanding the conformational space for the eight different ubiquitin chain types, as this holds the key to linkage-dependent regulation within the ubiquitin system.

Ubiquitin chain recognition further depends on the dynamic interconversion of chain conformations. Interfering with conformational dynamics by mutating ubiquitin can directly affect chain hydrolysis by DUBs, providing the first evidence that conformational rearrangements in the chains may govern the speed at which the chains are hydrolysed. An alternative mechanism to interfere with the conformational dynamics is chain length. Lys 48-tetraubiquitin forms a compact structure[25] in which all ubiquitin moieties interact with each other through their hydrophobic patches and secondary interaction sites (Supplementary Fig. 11). This probably alters chain dynamics ('breathing' of the chains), which we have shown here to be essential for recognition by DUBs. Consistent with this are recent data showing that some DUBs remove mono- and diubiquitin but not tetraubiquitin or longer polymers from substrates[26]. It is tempting to speculate that the reported requirement for Lys 48-linked tetraubiquitin to trigger proteasomal degradation[27] may have originated partly from improved stability of this length/linkage combination towards DUB action. Therefore, factors affecting ubiquitin chain conformation and dynamics (linkage, length, binding partners) may be key regulators of the ubiquitin system.

METHODS SUMMARY

Diubiquitin molecules were assembled with Alexa488 and Alexa647 fluorophores as detailed in Supplementary Fig. 1. Single-molecule measurements were performed at 20 °C in PBST buffer (150 mM NaCl, 18 mM Na_2HPO_4, 7 mM NaH_2PO_4, 0.01% (v/v) Tween20, pH 7.4). Ubiquitin-interacting proteins were incubated with labelled chains for 5 min. The single-molecule instrument was described previously[28]. Recording times depended on the amount of FRET-labelled diubiquitin and the signal-to-noise ratio, and were typically between 30 min and 3 h. Both TCCD and FRET data were collected for all measurements, and were analysed using a coincidence criterion. Coincident events in both channels[15] are only selected when the counts in each channel are above its threshold count value, which was selected automatically as described previously[29]. For experiments in which TCCD data were compared with FRET data, a common threshold was used for the donor channels (determined by maximizing the association quotient in the TCCD experiment), whereas the acceptor channel thresholds were independently determined for FRET and TCCD. This ensured that both the FRET and TCCD measurements of each sample resulted in approximately equal burst rates in the donor channel. Bulk FRET measurements, dye lifetime measurements and periodic acceptor excitation (PAX) experiments were performed as described in Supplementary Methods.

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Supplementary Information is available in the online version of the paper.

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Author Contributions Y.Y., G.B. and M.H.H. designed and performed the experiments, including single-molecule measurements, and analysed the data. Y.Y. and G.B. generated all proteins used in this study. Y.Y. performed kinetic experiments. M.H.H. and S.I. built the PAX instrument and A.A.Z. programmed the control for PAX measurements. S.I. performed single molecule experiments and contributed to data analysis. M.J.R.-R. and A.O. performed lifetime measurements. D.Kl., S.E.J. and D.Ko. directed the research and analysed the results. All authors contributed to the writing of the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.Ko. (dk@mrc-lmb.cam.ac.uk), S.E.J. (sej13@cam.ac.uk) or D.Kl. (dk10012@cam.ac.uk).

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