**Electronic Supplementary Information (ESI)**

# Linkage-specific conformational ensembles of noncanonical polyubiquitin chains

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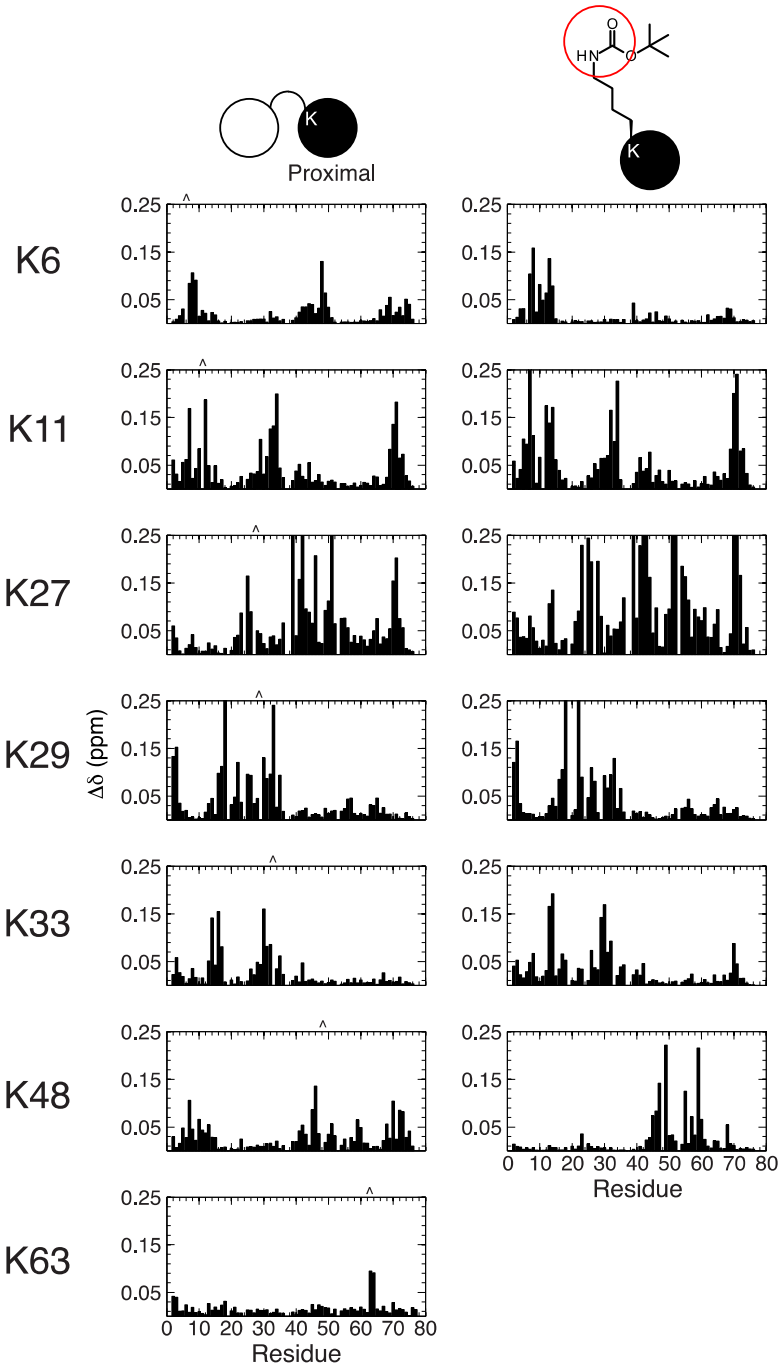


**Figure S1**. Comparison of amide CSPs between the proximal Ub vs. monoUb (left) for each $Ub_2$ (as indicated) and the CSPs for the corresponding Lys(Boc) Ub variant vs. monoUb (right). The linkage lysine is indicated on the left. Shown on the top of the right panels is the chemical structure of Lys(Boc), with the circle indicating the isopeptide-bond mimic.

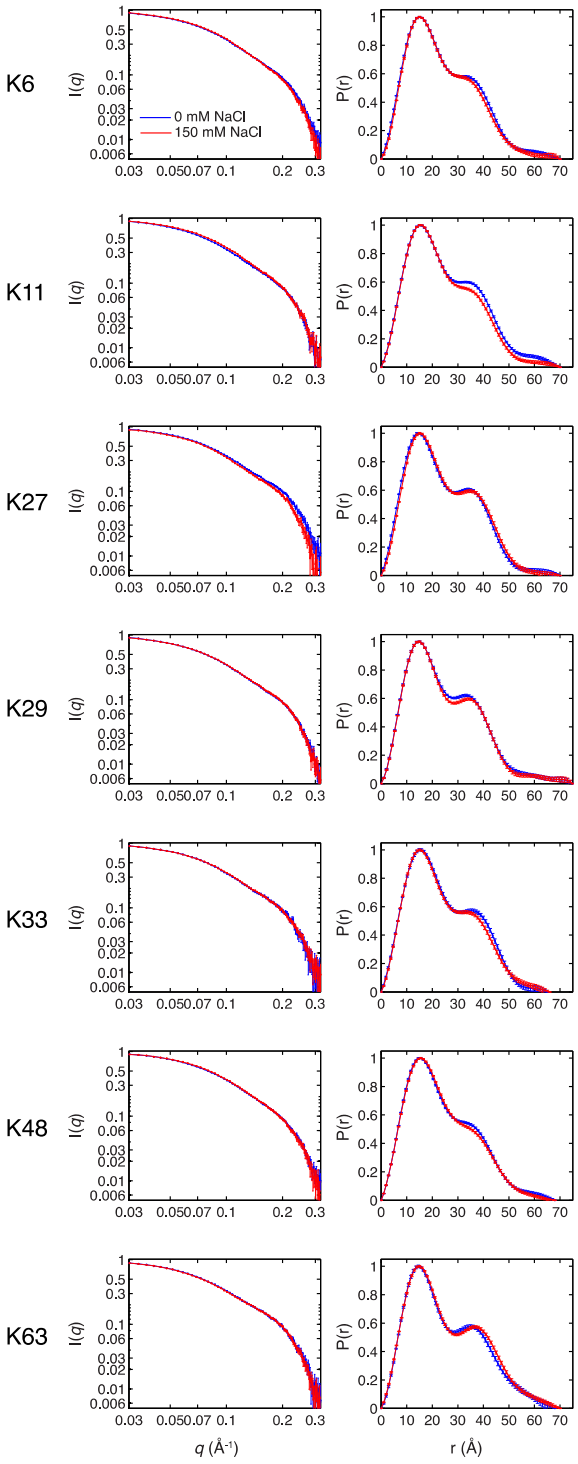


**Figure S2.** Experimental I(*q*) and P(r) profiles for each $Ub_2$. P(r) distributions were calculated using GNOM[1]. For each $Ub_2$, data were collected at pD 6.8 in the absence of NaCl (blue) and in presence of 150 mM NaCl (red).

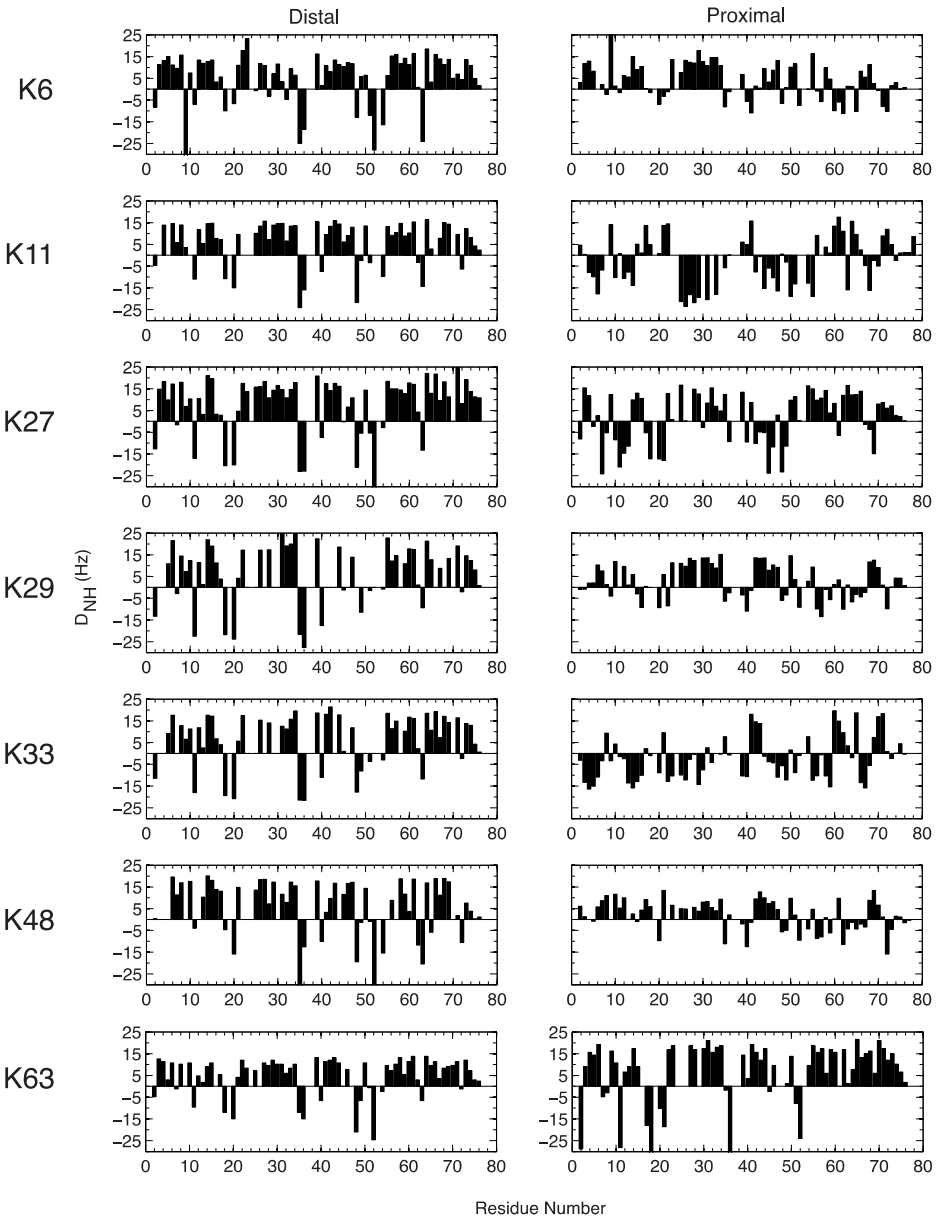


**Figure S3.** Residue-specific $^{15}$N-$^{1}$H RDCs for the distal (left) and proximal (right) Ub in each $Ub_2$.

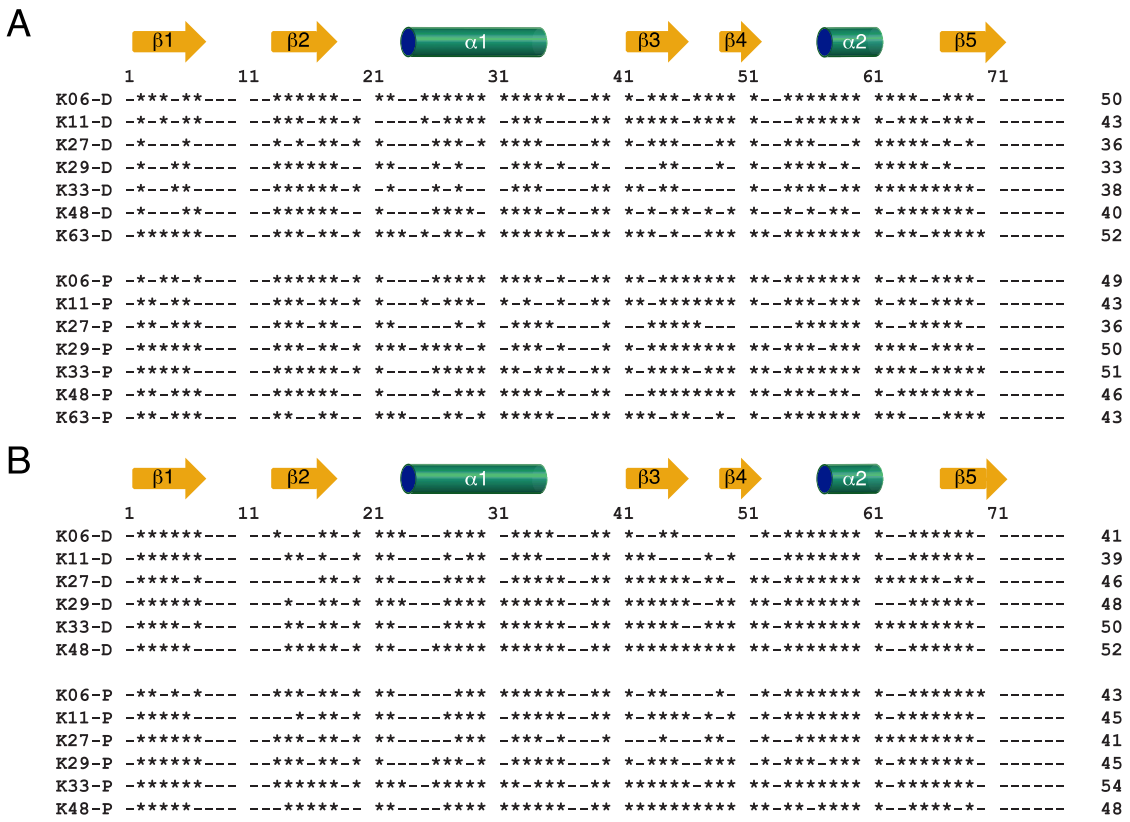


**Figure S4.** Marked residues (*) indicate residues that were used for determination of alignment tensors (A) and diffusion tensors (B) for each Ub unit in $Ub_2$s. For consistency, only RDC data for these marked residues were used in the SES conformational ensemble analyses.

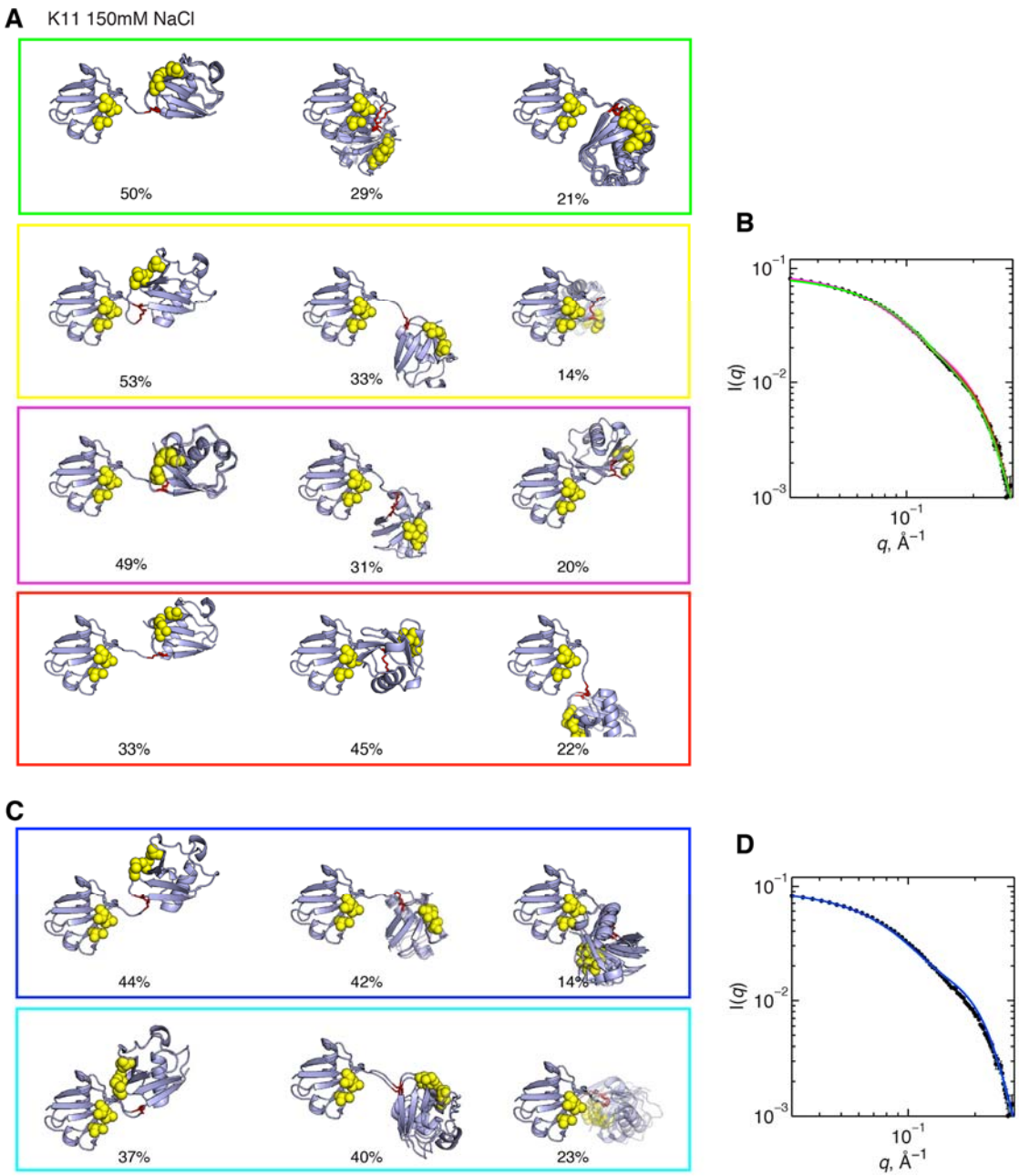


**Figure S5.** 3-conformer ensembles for K11-$Ub_2$ in the presence of 150 mM NaCl. (A, B) Shown are 3-conformer ensembles that are in good agreement with experimental SANS data. (C, D) These 3-conformer ensembles show poorer agreement with SANS data. Structure rendering is the same as in Figure 1 (main text).

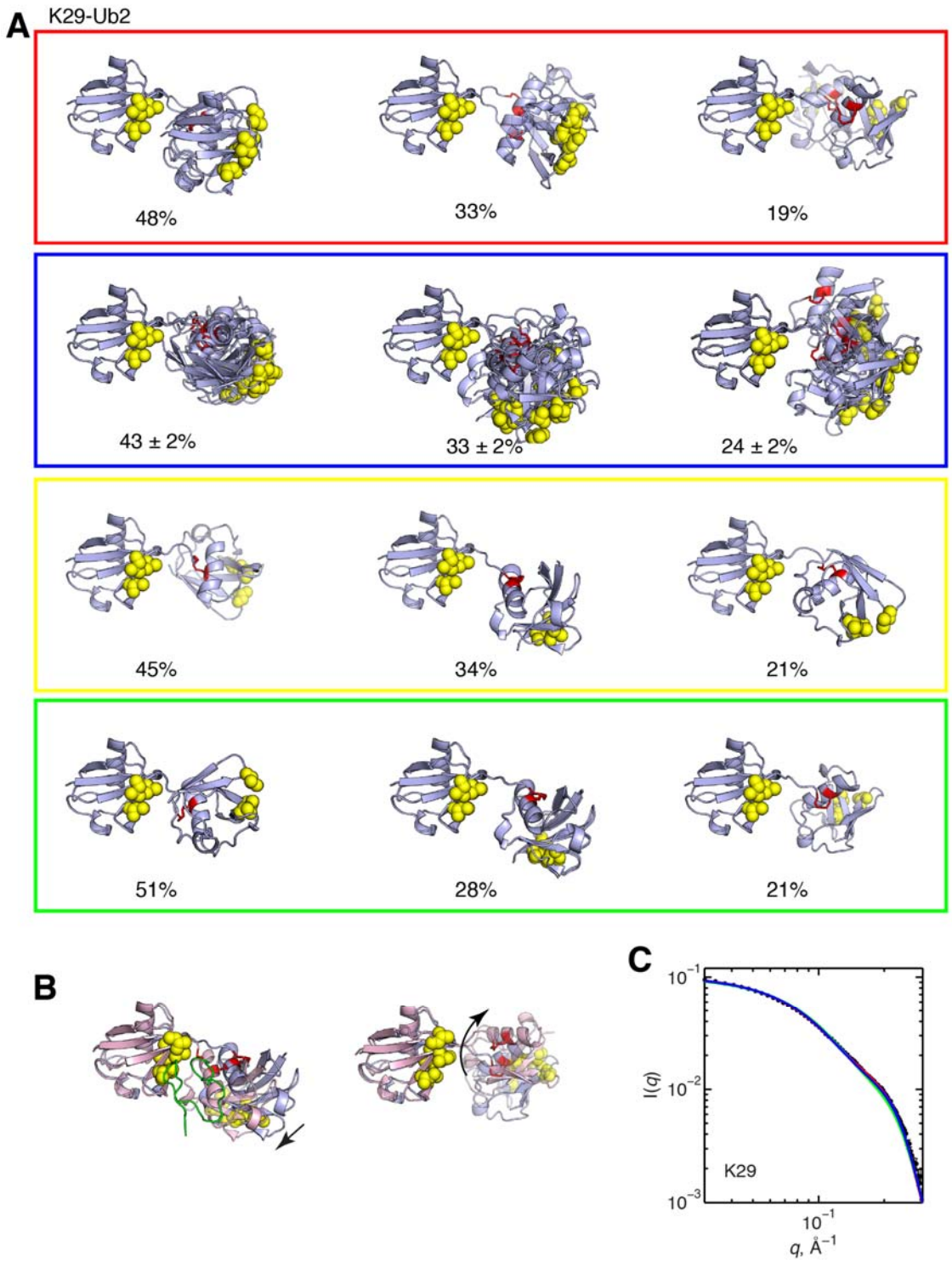


**Figure S6.** (A) Additional 3-conformer ensembles for K29-$Ub_2$ that are in good agreement with experimental SANS data. (B) Overlay between select ensemble conformers (blue) and crystal structures (pink), 4S1Z (left) and 4S22 (right). Green ribbon represents TRABID NZF1 protein in 4S1Z. (C) Predicted SANS profiles for the population-weighted ensembles (colored according to panel A) are all in good agreement with experimental data. Structure rendering is the same as in Figure 1 (main text).

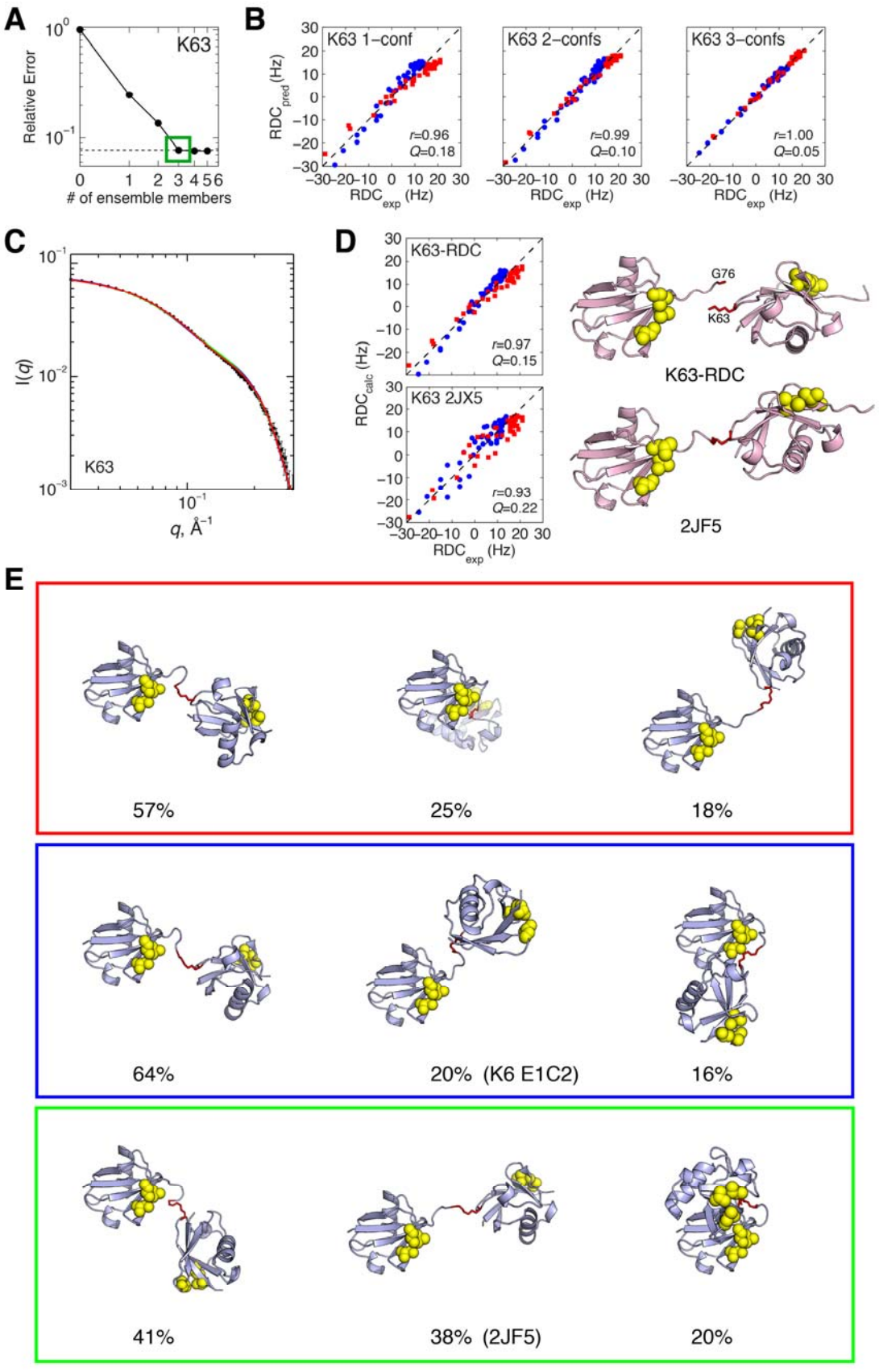
A
Relative Error
K63
# of ensemble members
B
K63 1–conf
K63 2–confs
K63 3–confs
r=0.96
Q=0.18
r=0.99
Q=0.10
r=1.00
Q=0.05
RDC_pred (Hz)
RDC_exp (Hz)
C
I(q)
K63
q, Å^-1
D
K63-RDC
K63 2JX5
r=0.97
Q=0.15
r=0.93
Q=0.22
RDC_calc (Hz)
RDC_exp (Hz)
G76
K63
K63-RDC
2JF5
E
57%
25%
18%
64%
20% (K6 E1C2)
16%
41%
38% (2JF5)
20%

**Figure S7.** SES analysis of the conformational ensembles for K63-$Ub_2$. (A) *l*-curve analysis revealed that three is the optimal number of conformers (indicated by green square) for K63-$Ub_2$. The dashed line represents the error for the best possible ensemble solution of size > 0. (B) Agreement between experimental RDCs for both Ubs taken together and the RDCs predicted from 1-conformer, 2-conformer, and 3-conformer ensembles. Data for the distal and proximal Ubs are colored blue and red, respectively. Pearson's correlation coefficient (*r*) and quality factor (*Q*) values are indicated inside each plot. (C) Agreement between experimental (black circles) and predicted SANS I(*q*) profiles for three representative 3-conformer ensembles. The I(*q*) curve for each ensemble is color-coded according to panel E. (D) Agreement between experimental RDCs for both Ubs taken together and the RDCs back-calculated from the RDC-derived structure in Varadan et al.[2] (top) and from the crystal structure of K63-$Ub_2$ (PDB ID 2JF5) (bottom). Data are colored as in panel B. Structure rendering is the same as in Figure 1 (main text). Note that only one of the two symmetry-related RDC-derived structures is shown here. The other structure differs by a 180° rotation of the proximal Ub about the horizontal axis, as in Figure 6 (main text). (E) Three representative 3-conformer ensembles that are in good agreement both with RDCs and SANS data for K63-$Ub_2$. Numbers below the structures indicate population weight of each conformer. For those conformers that exhibit similarity to other structures, the PDB ID is noted. One conformer is similar to a conformer identified in a conformational ensemble of K6-$Ub_2$. The code refers to ensemble number, E, and conformer number, C (in order of their appearance in Figure 8 (main text)).

**Table S1.** Predicted radius of gyration, $R_g$, from crystal structures of the indicated $Ub_2$.

| $Ub_2$ Linkage | PDB ID | $R_g$ (Å) |
|---|---|---|
| K6 | 2XK5 | 16.1 |
| K11 | 2XEW | 16.5 |
| K11 | 3NOB | 16.5 |
| K29 | 4S22 | 18.7 |
| K29 | 4S1Z* | 17.2 |
| K33 | 5AF4 | 16.3 |
| K33 | 5AF6* | 17.1 |
| K48 | 1AAR | 15.4 |
| K48 | 3NS8 | 16.6 |
| K63 | 2JF5 | 22.3 |
| K63 | 3A1Q* | 22.8 |

* For these crystal structures, ligand was removed so that $R_g$ reflects the $Ub_2$ conformation only.

**Table S2.** Correlation of the distal-Ub RDCs between $Ub_2$s of different linkages.

| | K6 | K11 | K27 | K29 | K33 | K48 | K63 |
|---|---|---|---|---|---|---|---|
| **K6** | 1.00 | 0.86 | 0.82 | 0.69 | 0.79 | 0.85 | 0.79 |
| **K11** | | 1.00 | 0.91 | 0.91 | 0.94 | 0.94 | 0.94 |
| **K27** | | | 1.00 | 0.94 | 0.96 | 0.79 | 0.95 |
| **K29** | | | | 1.00 | 0.98 | 0.75 | 0.96 |
| **K33** | | | | | 1.00 | 0.80 | 0.97 |
| **K48** | | | | | | 1.00 | 0.83 |
| **K63** | | | | | | | 1.00 |

Shown here and in Table S3 are Pearson's correlation coefficients (*r*) calculated between the indicated $Ub_2$s. Colors indicate degree of correlation, with black the most, and white the least.

**Table S3.** Correlation of the proximal-Ub RDCs between $Ub_2$s of different linkages. See footnotes to Table S2 for explanations.

| | K6 | K11 | K27 | K29 | K33 | K48 | K63 |
|---|---|---|---|---|---|---|---|
| **K6** | 1.00 | -0.72 | 0.23 | 0.59 | -0.63 | 0.46 | 0.28 |
| **K11** | | 1.00 | -0.08 | -0.69 | 0.33 | -0.33 | -0.24 |
| **K27** | | | 1.00 | -0.13 | -0.06 | -0.30 | 0.55 |
| **K29** | | | | 1.00 | 0.13 | 0.83 | 0.26 |
| **K33** | | | | | 1.00 | 0.17 | -0.02 |
| **K48** | | | | | | 1.00 | -0.06 |
| **K63** | | | | | | | 1.00 |

**Table S4.** $Ub_2$ conformers in the SASSIE[3] input ensembles that show similarities to the known crystal structures.

| Crystal structure (PDB ID) | $Ub_2$ linkage | Best corresponding conformer in SASSIE Ensemble | $C_\alpha$ RMSD (Å)[a] |
|---|---|---|---|
| 2XK5 | K6 | 10664 | 1.91 |
| 3NOB | K11 | 10425 | 2.12 |
| 2XEW | K11 | 10563 | 1.85 |
| 4S1Z | K29 | 2409 | 1.69 |
| 4S22 | K29 | 7768 | 2.14 |
| 5AF4 | K33 | 7813 | 4.25 |
| 5AF6 | K33 | 20750 | 2.99 |

[a] $C_\alpha$ RMSD was calculated using residues 1-70 of both distal and proximal Ubs.

References for Electronic Supplementary Information


1 A. V. Semenyuk and D. I. Svergun, *J. Appl. Crystallogr.*, 1991, **24**, 537–540.
2 R. Varadan, N. Assfalg, A. Haririnia, S. Raasi, C. Pickart and D. Fushman, *J. Biol. Chem.*, 2004, **279**, 7055–7063.
3 J. E. Curtis, S. Raghunandan, H. Nanda and S. Krueger, *Comput. Phys. Commun.*, 2012, **183**, 382–389.