Conformational dynamics control ubiquitin-deubiquitinase interactions and influence in vivo signaling

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Ubiquitin is a highly conserved eukaryotic protein that interacts with a diverse set of partners to act as a cellular signaling hub. Ubiquitin's conformational flexibility has been postulated to underlie its multifaceted recognition. Here we use computational and library-based means to interrogate core mutations that modulate the conformational dynamics of human ubiquitin. These ubiquitin variants exhibit increased affinity for the USP14 deubiquitinase, with concomitantly reduced affinity for other deubiquitinases. Strikingly, the kinetics of conformational motion are dramatically slowed in these variants without a detectable change in either the ground state fold or excited state population. These variants can be ligated into substrate-linked chains in vitro and in vivo but cannot solely support growth in eukaryotic cells. Proteomic analyses reveal nearly identical interaction profiles between WT ubiquitin and the variants but identify a small subset of altered interactions. Taken together, these results show that conformational dynamics are critical for ubiquitin–deubiquitinase interactions and imply that the fine tuning of motion has played a key role in the evolution of ubiquitin as a signaling hub.

computational design | phage display | protein dynamics | protein–protein interactions | ubiquitin signaling

Cellular signaling cascades frequently converge on "hub" proteins, which are recognized by a large number of binding partners. Ubiquitin functions as a eukaryotic signaling hub and is one of the most highly conserved proteins known, with only three amino acid differences between the yeast and human proteins. Ubiquitin is posttranslationally attached to a substrate protein lysine via an isopeptide bond with ubiquitin's carboxyl terminus, with additional ubiquitin molecules added to substrate-conjugated ubiquitin to yield lysine-linked polyubiquitin chains. Each ubiquitin linkage type carries a distinct signal, and the tightly regulated processing of ubiquitin chains is used to convey a wealth of cellular information (1). Indeed, hundreds of proteins are known to bind ubiquitin, and a recent proteomics study has identified ~19,000 ubiquitin conjugation sites in nearly 5,000 human proteins (2). Accordingly, misregulation of ubiquitin processing has been implicated in several disease states, including oncogenesis and neurodegeneration (3, 4).

Ubiquitin is canonically attached to substrates through a three-part E1-E2-E3 enzymatic cascade. The remodeling and removal of conjugated ubiquitin chains is catalyzed by several families of isopeptidases known as deubiquitinases (DUBs). There are ~100 human DUBs, each with distinct substrate specificities and enzymatic properties, implying a largely unexplored wealth of signal regulation. Two prominent DUB families are the ubiquitin C-terminal hydrolase (UCH) and ubiquitin-specific protease (USP) enzymes. UCHs are primarily responsible for recycling ubiquitin by removing small moieties such as intracellular nucleophiles and short peptides from its carboxyl terminus. USPs act typically as signaling modulators, regulating ubiquitin chain length by cleaving the isopeptide bond that connects large moieties (such as whole proteins) to ubiquitin (5).

Because of their role in intracellular signaling, DUBs have emerged as promising new therapeutic targets (6). For example, inhibition of the proteasome-associated USP14 DUB has been shown recently to enhance degradation of proteins involved in amyloidogenic neurodegeneration (7) and to prevent tumor progression (8). The study of USP-type DUB mechanism and regulation is complicated by their relatively poor activity: the catalytic domain of USP-type DUBs typically has an enzyme efficiency of 10^3–10^5 $M^{-1}{\cdot}s^{-1}$ and high micromolar substrate affinities, necessitating the use of covalent suicide "warheads" in structural studies (9). By contrast, UCH-type DUBs are often highly active, with enzyme efficiencies of up to 10^8 $M^{-1}{\cdot}s^{-1}$ and affinity for ubiquitin in the nanomolar range (10, 11).

Recent NMR and computational studies of apo ubiquitin, relying heavily on the thorough analysis of a large set of residual dipolar couplings (RDCs), have suggested that the conformational plasticity of ubiquitin may be fundamental to its recognition by certain partners (12). This work indicated that the β1-β2 loop region is mobile on the low microsecond timescale and that binding partners may select distinct conformations out of this preexisting equilibrium. Other reports suggest that at least some ubiquitin–partner interactions cannot be solely attributed to a conformational selection binding mechanism and that induced fit plays some role in ubiquitin binding (13–15). Intriguingly, single point mutations within ubiquitin's hydrophobic core that decrease overall stability and increase the flexibility of the β-sheet binding interface abrogate recognition by some partner proteins without dramatically altering the ubiquitin fold (16). Although these studies have hinted at the importance of conformational dynamics in ubiquitin recognition, it is currently unknown whether the rates of ubiquitin's motions are functionally important. Indeed, the degree to which conformational kinetics have been fine tuned for function is unclear in any system.

We set out to determine whether DUBs take advantage of ubiquitin dynamics and whether perturbing these conformational changes would have global effects on ubiquitin signaling. Here

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Data deposition: The U14Ub1 coordinates and structural restraints have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2m0x), and chemical shift assignments have been deposited at the BioMagResBank, www.bmrb.wisc.edu (accession no. 18831).

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we show that the USP- and UCH-type DUBs bind distinct conformations of human ubiquitin and use computational and library-based means to introduce core mutations that modulate ubiquitin's conformational dynamics. These ubiquitin variants exhibit increased affinity for USP14, with concomitantly reduced affinity for UCH class DUBs. In contrast to WT ubiquitin, which displays characteristics of both conformational selection and induced-fit binding mechanisms (12–15), these ubiquitin variants bind USP14 via induced fit. NMR measurements show that these variants adopt the native ubiquitin fold but move more slowly within the DUB-binding region centered about the β1-β2 loop. The variants can be ligated into chains both in vitro and in vivo but cannot replace endogenous ubiquitin in vivo, indicating that maintenance of conformational dynamics has played a role in the evolution of the ubiquitin signaling and is critical for in vivo fitness.

Results

DUB Families Bind Distinct Conformations of Ubiquitin. Building on a recent analysis of 16 ubiquitin cocrystal structures (17), we sought to determine whether different DUB families might recognize unique conformational states of ubiquitin. To address this question, we performed pairwise alignments of 56 high-resolution crystal structures of ubiquitin in complex with at least one partner and clustered the results based on the similarity of the β1-β2 conformation (*Methods*). Cluster analysis separates the apparent "smear" of β1-β2 loop conformations into conformational families, with subtle intrafamily differences (Fig. 1 *A* and *B*; Fig. S1). Strikingly, we find that all UCH-type DUBs bind one β1-β2 conformation, and all USPs bind another. These atomic "snapshots" imply that ubiquitin's β1-β2 loop accesses a series of distinct substates that are differentially recognized by UCH- and USP-type DUBs. Computational docking supports this notion, as UCH-type DUBs prefer to bind the UCH state of ubiquitin and USP-type DUBs prefer to bind the USP state (Fig. S2 *A* and *B*). We note that the UCH-bound β1-β2 conformation is similar to that observed for apo ubiquitin, whereas the USP-bound conformation is quite different (Figs. 1 *C* and *D* and 2*A*). This structural distinction is consistent with the reported conformational selection mechanism and correlates with the relative affinities of each DUB family, with UCHs typically possessing nanomolar affinities for ubiquitin and USPs possessing affinities in the high micromolar range. Intriguingly, the USP-bound state is also similar to the conformation adopted by ubiquitin in a recently reported crystal structure of a RING E3-E2–ubiquitin complex (18), suggesting that conformational switching may be important for productive engagement by other classes of ubiquitin interacting proteins.

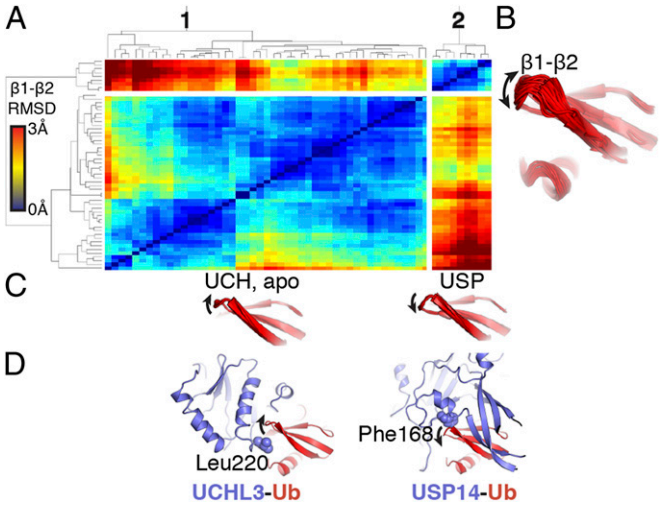

Fig. 1. Cluster analysis of ubiquitin within protein complexes reveals two distinct conformations that influence DUB binding. (*A*) Clustered heat map of the Cα root mean square deviation (RMSD) of ubiquitin's β1-β2 region (residues 6–10) in all high-resolution crystal structures of ubiquitin complexes after pairwise alignment of the remainder of the globular core (residues 1–5 and 11–70) showing two major clusters. (*B*) Without inspection of the clustered RMSD, the conformations adopted by the β1-β2 region appear as a smear of possible conformers. (*C*) The largest cluster (cluster 1) represents an up conformation of the β1-β2 region and contains all ubiquitin structures in complex with UCH-type DUBs, as well as apo ubiquitin (PDB ID codes 1ubi and 1ubq). By contrast, cluster 2 represents a down β1-β2 conformation and contains every USP-type DUB-ubiquitin complex crystallized to date. (*D*) UCH-type DUBs bind to the up conformation of β1-β2 [UCHL3-ubiquitin (Ub) complex PDB ID code 1xd3], whereas USP-type DUBs bind the down state (USP14-Ub complex PDB ID code 2ayo). Ubiquitin is depicted in red; DUBs are shown in blue. DUB residues packing against β1-β2 and apparently restricting its conformation (UCHL3-Leu220, USP14-Phe168) are shown as spheres.

Generation of Ubiquitin Variants with Increased Affinity for USP14. Conformational heterogeneity plays a role in ubiquitin recognition (Fig. 1; Fig. S1) (12), and recent work has shown that ubiquitin binding events possess aspects of both conformational selection and induced-fit binding mechanisms (13, 14). Thus, changing β1-β2's ability to adopt various conformations should alter the energetics of binding a target DUB, possibly altering affinity toward DUBs that recognize a given state. Modulating the energetics of ubiquitin conformational change therefore potentially represents a means to alter both the affinity and selectivity of a protein–protein interaction by modulating interface dynamics.

To explore the conformation and dynamics of ubiquitin's β1-β2 loop, we computationally searched for core positions where mutations are predicted to favor the USP-binding state, using both single-state and multistate RosettaDesign (*Methods*) (19–22). Both types of design experiments identified a consistent set of positions for mutation (Fig. S2 *C* and *D*), and this information was incorporated into phage-displayed libraries of ubiquitin variants, which were panned against a catalytically inactive mutant of the USP domain of USP14 (*Methods*).

Selecting for USP14-binding ubiquitin variants (U14Ubs) yielded several strong sequence preferences, such as the nearly invariant incorporation of a glycine at position 7 (Fig. 2*B*). We cloned, expressed, and purified >40 U14Ubs (designated U14UbXX, where XX denotes the clone number), and screened them for their ability to bind USP14, UCHL1, and UCHL3 via ELISA and, for USP14, via biolayer interferometry (BLI). We focused our analysis on the five clones with the highest apparent affinity for USP14: U14Ub1, 2, 14, 22, and 24 (Fig. 2*C*). Thermal stability measurements show that the U14Ubs are not significantly destabilized with respect to WT ubiquitin (Fig. S2*E*). BLI titrations of these ubiquitin variants reveal that each binds USP14 with 30- to 250-fold improved affinity compared with WT ubiquitin (Table S1). Conversely, ELISA titrations show that each variant binds UCHL1 and UCHL3 more weakly than WT, with a 2- to 200-fold variation in the EC_{50}s for UCHL3 (Fig. 2*C*; Table S1).

U14Ubs Bind USP14 via an Induced Fit Mechanism. Kinetic fits to BLI titrations of USP14 with the U14Ubs revealed that single-parameter fitting of the association kinetics is insufficient to explain the data (Fig. 3*A*; Fig. S3 *A* and *B*). Instead, we observe biphasic association, with only the fast association rate characteristic of k_{on}. Biphasic association kinetics can be observed in cases where conformational changes are commensurate with binding, and in these cases, it is established that the concentration dependence of the slower phase of association distinguishes between conformational selection and induced-fit binding mechanisms (23–25). For each U14Ub variant, we observe a dependence of the slow association rate on the concentration of U14Ub indicative of an induced-fit mechanism of binding (Fig. 3; Fig. S3; Table S2).

If the U14Ubs bound USP14 via a conformational selection mechanism, one would expect that the population of a USP14-binding state would be enriched in the unbound variants. At its most extreme, this could result in a change in the ground-state structure of the U14Ubs, similar to what has been observed recently for a mutant of T4 lysozyme (26). Although our

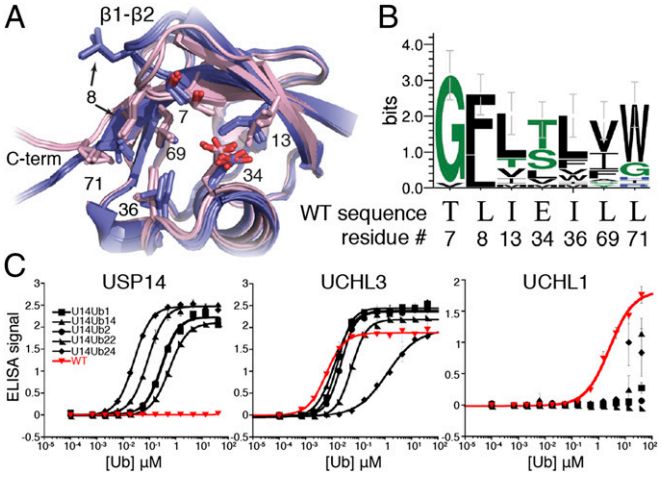

Fig. 2. Mutation of residues in the core of ubiquitin generates U14Ubs with higher affinities for USP14 and lower affinities for UCH-type DUBs. (*A*) Overlay of three ubiquitin structures solved in complex with UCHs (lavender, PDB ID codes 1cmx, 1xd3, and 3ifw) and USPs (pink, PDB ID codes 2ayo, 2hd5, and 2ibi). Comparison of the UCH- and USP-bound ubiquitin structures reveals distinct conformations of the β1-β2, with long-range effects transmitted to the C terminus in a manner that may also influence binding to DUBs. The side chains of residues allowed to mutate in the phage library are shown as sticks. (*B*) Sequence preferences of 23 unique phage clones isolated after five rounds of phage panning against USP14 shown as a logo plot (WebLogo 3.3). Error bars represent 95% Bayesian CIs. The sequences of the five variants with the highest apparent affinity for USP14 are shown in Table S1. (*C*) Protein ELISAs demonstrate that the U14Ubs have an increased affinity for USP14 with a commensurate decrease in the affinity for UCHL3 and UCHL1. WT ubiquitin curves are shown in red; the various U14Ubs are in black. Error bars represent the SD of triplicate measurements.

computational strategy implicitly targeted changes in structure, the incorporation of phage display opened up the possibility of attaining affinity via other mechanisms. Indeed, observing an induced-fit mechanism instead implies formation of an initial, weak U14Ub–USP14 complex that transitions to a fully bound, higher-affinity state.

U14Ubs Adopt the Ubiquitin Fold but Possess Slower Motions. To experimentally investigate whether an induced-fit binding mechanism is linked to changes in the structure or dynamics of the U14Ubs, we assigned the backbone NMR resonances of all five variants and determined the solution structure of U14Ub1 (Fig. S4; Table S3). Both the structure of U14Ub1 and NMR-derived CS-Rosetta models (27) of the other four U14Ubs indicate that the each of the variants adopts the WT ubiquitin fold (Fig. S4 *A* and *B*). However, in the $^{1}H/^{15}N$ HSQC spectra of all of the U14Ubs, many of the backbone amide resonances are broadened relative to WT ubiquitin (Fig. 4*A*; Fig. S4*C*). This broadening is temperature dependent (Fig. 4*B*), and the affected residues are spatially clustered around the β1-β2 loop and in the neighboring β3 and β5 strands, signifying a modulation of conformational dynamics in this region (Fig. 4*C*). Conversely, the $\{^{1}H\}$-^{15}N heteronuclear nuclear overhauser effect (NOE) remains largely unchanged, indicating that fast, subnanosecond motions are unperturbed (Fig. S5*A*) (28). Taken together with the observation that the β1-β2 region of WT ubiquitin is mobile on the sub-50-μs timescale (12), line broadening of the U14Ubs suggests a marked slowing of conformational dynamics of the U14Ubs without a change in fast motions.

To confirm this hypothesis and provide deeper insight into the nature of the U14Ub motions, we performed R_2 dispersion experiments, sensitive to motions on millisecond timescales (millisecond R_{ex}) (28), as well as H_zN_z $R_{1\rho}$ R_{ex} measurements (29), which probe the dynamics of backbone amides on the microsecond timescale (microsecond R_{ex}). In these measurements, higher R_{ex} values imply slower conformational exchange of a mobile segment on the timescale probed by the experiment.

Although the motions present in WT ubiquitin are two to three orders of magnitude too fast to observe by millisecond R_{ex} (30, 31), several of the U14Ubs show significant R_2 dispersion (millsecond R_{ex}) at room temperature, most dramatically in U14Ub14 (Fig. 5; Fig. S5*B*). The R_2 dispersion data fit well to the Carver-Richards model for two-site exchange (28) (*Methods*; Fig. S5*C*); however, most of the U14Ubs are in the fast exchange limit, meaning that the population of an excited state is inseparable from the chemical shift changes and technically limiting our ability to probe the molecular structure of the excited state. The exception is U14Ub2, which has an excited state that is only sparsely populated (0.40 ± 0.05%). Thus, a large increase in the population of an excited state is unlikely to explain the increase in the affinity for USP14. Similarly, the microsecond R_{ex} in the regions in and around the β1-β2 loop in tertiary structure are much more pronounced for the U14Ubs than the corresponding positions in WT ubiquitin, most notably in U14Ub2 (Fig. 5; Fig. S5*B*). In summary, whereas the motion of WT ubiquitin is so fast as to require a complex RDC-based analysis to observe (12), the conformational dynamics of U14Ubs are dominated by much slower microsecond-millisecond timescales.

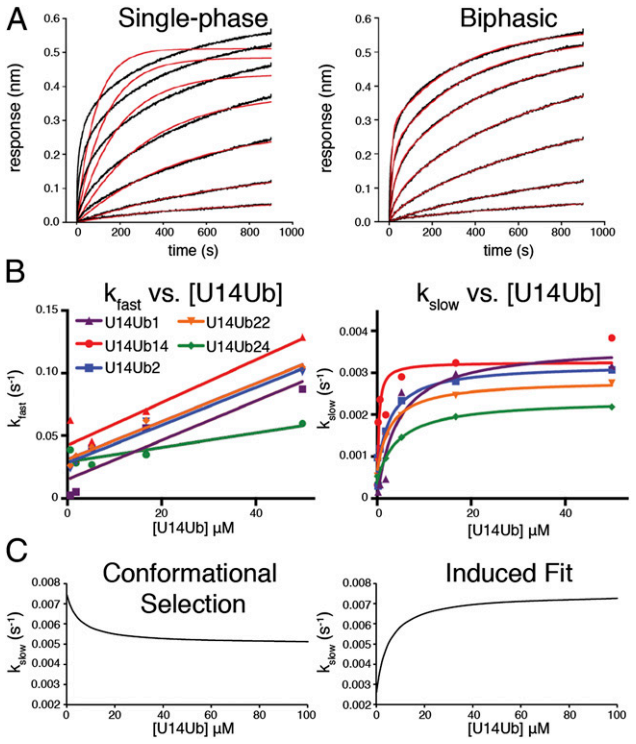

Fig. 3. U14Ubs bind USP14 via an induced-fit mechanism. (*A*) Biolayer interferometry titrations of USP14 with the U14Ub variants show biphasic association kinetics. The raw sensograms are shown in black; the fits are in red. Data are depicted for U14Ub2 (see Fig. S3 for the other variants). (*B*) For each U14Ub variant, the fast component of the biphasic association rate depends linearly on the concentration of U14Ub in a manner that reflects the on rate, whereas the slow component indicates an induced-fit binding mechanism (25). K_Ds determined by steady-state and kinetic fits are in agreement (Fig. S3*D*; Table S2). (*C*) The dependence of k_{slow} on the concentration of a ligand discriminates between conformational selection and induced-fit binding mechanisms. For conformational selection, $k_{slow} = k_f + k_r/(1 + [L]/K_D)$. For induced fit, $k_{slow} = k_r + k_f/(1 + K_D/[L])$. In both cases, k_f and k_r are the forward and reverse rates of the conformational change, respectively, [L] is the concentration of the ligand, and K_D is the dissociation constant. The values used for k_f, k_r, and K_D are 0.005 (1/s), 0.0025 (1/s), and 5 μM, respectively.

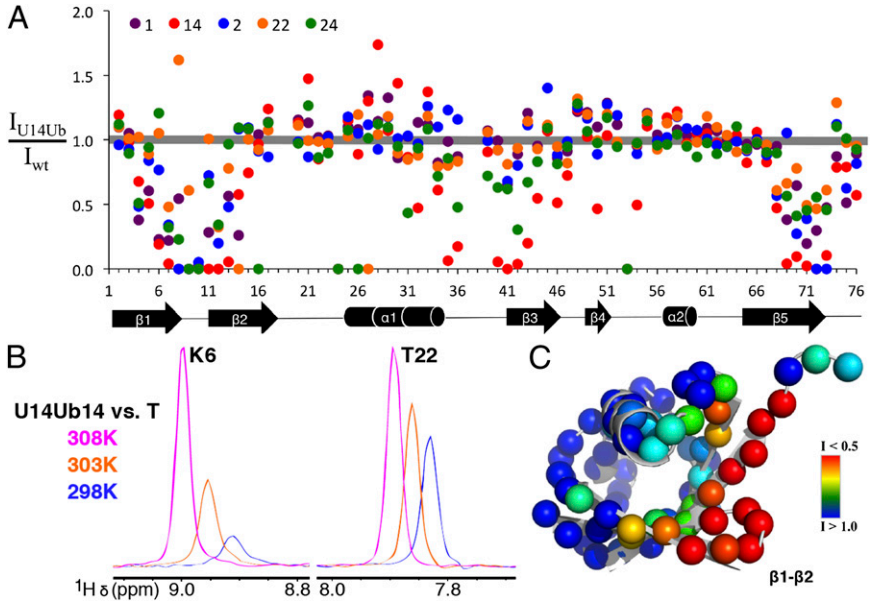

Fig. 4. Significant line broadening of the U14Ubs indicates a modulation of dynamics. (*A*) Ratios of the intensities of amide resonances in ^{1}H-^{15}N HSQC spectra of U14Ubs vs. WT ubiquitin. The ratios were normalized to resonances that showed no exchange. (*B*) Representative rows extracted from ^{1}H-^{15}N HSQC spectra of U14Ub14 recorded at different temperatures demonstrate that the broadening observed is strongly temperature dependent, indicating that it is likely due to conformational exchange. The signals were normalized to the intensity at 308K. (*C*) The average of the intensity ratios plotted onto the structure of WT ubiquitin (PDB ID code 1ubi); the most significant changes occur in the β1-β2 region.

U14Ubs Cannot Replace Endogenous Ubiquitin in Vivo. Most discussions of protein structure–function relationships focus on the ground state or occasionally an excited state. Because the U14Ubs are very similar to WT ubiquitin in ground state structures and excited state populations, but display marked changes in the kinetics of their conformational motion, we sought to determine whether these altered dynamics affect their ability to participate in other aspects of ubiquitin function. Despite their slowed motion, the U14Ubs are efficiently incorporated in vitro into Lys48-linked chains by the human UBE1 and Cdc34 E1 and E2 enzymes to a similar extent as WT ubiquitin (Fig. 6*A*). All five U14Ubs are also effectively incorporated into substrate-linked covalent chains when expressed in *Saccharomyces cerevisiae* together with endogenous WT ubiquitin (Fig. 6 *B* and *C*), suggesting that the ubiquitin ligation machinery is not globally sensitive to changes in the kinetics of ubiquitin motion.

Although the U14Ub variants are incorporated into chains both in vitro and in vivo, their altered dynamics may still negatively affect some aspect of the complex ubiquitin signaling

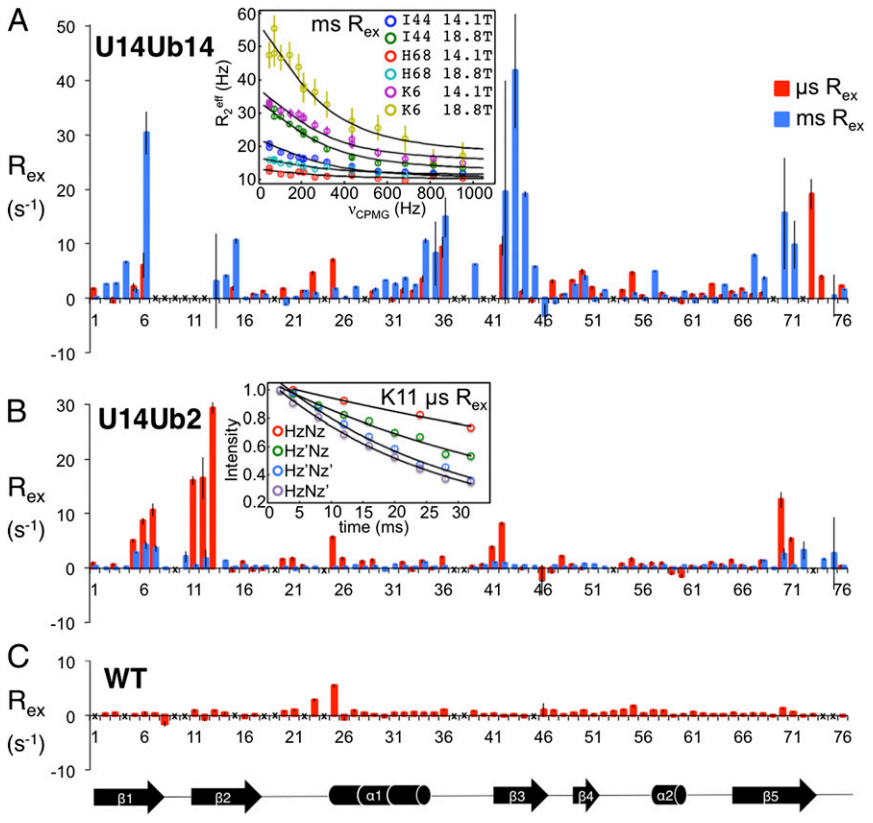

Fig. 5. U14Ub conformational dynamics are slowed relative to WT millisecond (blue) and microsecond (red) motions quantified by R_{ex} measurements for U14Ub14 (*A*), U14Ub2 (*B*), and WT ubiquitin (*C*). Within the timescale probed by each experiment, larger R_{ex} values indicate slower motion. *Insets* in the U14Ub14 and U14Ub2 plots display examples of the raw data for the millisecond and microsecond R_{ex} measurements, respectively. Resonances lacking data due to exchange broadening or spectral overlap are denoted with an X. WT ubiquitin shows no millisecond R_{ex} at these conditions. Errors were determined from the noise level in the raw spectra (*Methods*).

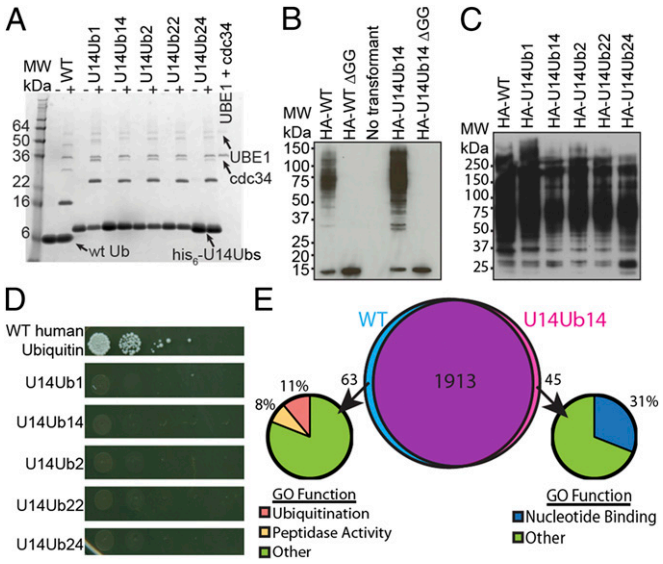

Fig. 6. U14Ubs can form chains in vitro and in vivo but cannot replace endogenous ubiquitin in *S. cerevisiae*. (*A*) An in vitro chain ligation assay shows that the UBE1 and Cdc34 enzymes can incorporate U14Ub variants into Lys48-linked chains to a similar extent as WT ubiquitin. The inclusion of UBE1 and Cdc34 in the reaction mixture is denoted by a +; the absence is denoted by a –. The molecular weight discrepancy between the U14Ubs and WT ubiquitin is due to the presence of the hexahistidine tag, which has not been cleaved from the variants. (*B*) HA-tagged U14Ub14 is incorporated into ubiquitin chains in vivo. HA-tagged ubiquitin, U14Ub14, and C-terminal truncations (ΔGG) were transformed into *S. cerevisiae* and expressed at low levels using a copper promoter (32). Lysates were immunoprecipitated with anti-HA and then visualized with an anti-HA Western blot. (*C*) All five U14Ubs are incorporated into ubiquitin chains in vivo. HA-tagged ubiquitin and U14Ubs were expressed and immunoprecitated as above and visualized with an anti-HA Western blot. (*D*) U14Ubs cannot function as the sole source of endogenous ubiquitin in *S. cerevisiae*, indicating that the rate of conformational motion plays a critical role in some aspect of ubiquitin recognition during the life cycle of the organism. (*E*) MS-identified interactions from HA-tagged pull-downs of WT and U14Ub14 show 95% similarity between the two profiles. Gene Ontology (GO) functional annotation shows significant enrichment in ubiquitination and peptidase pathways in the WT exclusive list. Nucleotide binding proteins were the only significant enrichment in the U14Ub14 exclusive list. For the complete list of interacting proteins identified, see Dataset S1.

network. To investigate how the U14Ubs influence organismal fitness, we used a strain of *S. cerevisiae* that is deficient for endogenous ubiquitin and expresses a single copy of ubiquitin or variant at WT levels from a plasmid (32). Strikingly, although WT human ubiquitin robustly complements cell survival, none of the U14Ub variants are capable of supporting growth in vivo (Fig. 6D). Hence, it appears that some aspect of ubiquitin signaling depends on the exquisitely tuned and profoundly conserved conformational plasticity of WT ubiquitin.

To more fully understand why the U14Ub variants cannot complement WT ubiquitin in vivo, we performed pull-downs of HA-tagged WT ubiquitin and U14Ub14 in a background that contains endogenous WT ubiquitin. Coimmunoprecipitating partners were identified by tandem MS and categorized as similar or different by measuring the significance of pairwise discordance between spectral count data derived from each pull-down (Fig. 6*E*). This approach identifies stark cases in which a protein is only identified with WT ubiquitin or U14Ub14 immunoprecipitation, as well as more subtle changes in the relative abundance of a protein between each sample. We further used C-terminally truncated WT ubiquitin or U14Ub14 (ΔGG) constructs to interrogate whether the proteins identified in these proteomic analyses were covalently linked to the ubiquitin moiety (Dataset S1). WT ubiquitin and U14Ub14 showed nearly identical immunoprecipitation profiles, with 95% of proteins exhibiting no significant preference for either WT ubiquitin or U14Ub14 (Fig. 6*E*). We used functional annotation analysis (33) to classify the outlying proteins and found that 11% of the proteins preferentially enriched by WT ubiquitin were directly involved in ubiquitination pathways, suggesting that a small subset of the conjugation machinery is deficient in engaging U14Ub14. Conversely, a few proteins preferentially immunoprecipitated with U14Ub14, indicating that the variant may be less efficiently cleaved from substrates by DUBs or is inappropriately linked to novel substrates by the endogenous ubiquitination machinery. Repeating these proteomic experiments with U14Ub1, U4Ub14, and WT ubiquitin revealed similarly high overlap between WT and both variants, further underscoring that relatively few components of the ubiquitin signaling network depend on ubiquitin conformational dynamics (Fig. S6; Dataset S1), but these pieces are essential for growth (Fig. 6*D*).

Discussion

We found that the USP- and UCH-type DUBs bind distinct conformations of ubiquitin and rationalized that we would be able to generate variants of ubiquitin with higher affinity for a DUB of interest by altering the relative populations of these states via combined computational and library-based methods. Here we focused on the proteasome-associated USP14, but this approach has recently been used in our laboratory to successfully generate potent inhibitors of USP7, a DUB implicated in destabilizing p53 (22). In the case of USP7, we found ubiquitin variants with dramatic changes in ground state structure, including a β1-β2 loop restricted by a disulfide bond. Others have shown that perturbation of the relative populations of ground and excited states can affect the catalytic efficiency of the enzyme Cyp A (34), change the affinity for a small molecule ligand that binds exclusively to a single state of a T4 lysozyme mutant (26), and produce a more potent IL-2 "superkine" by circumventing the requirement for conformational stabilization of native IL-2 by CD25 (35).

Surprisingly, the U14Ubs we found in this study do not have detectable changes in state but rather have slowed rates of conformational motion, altering the kinetics of a conformational switch at this protein–protein interface. Populations of excited states have recently been implicated in function (12, 34, 35), and our data and others' indicate that the rate at which ubiquitin moves among states also appears to be critical for its biological function (16). Although our data do not definitively assign the conformations between which the U14Ub variants slowly transition, we postulate that their motions may correspond to the same states observed for WT ubiquitin's β1-β2 loop. Further investigation into the atomic details of U14Ub "hidden" substates

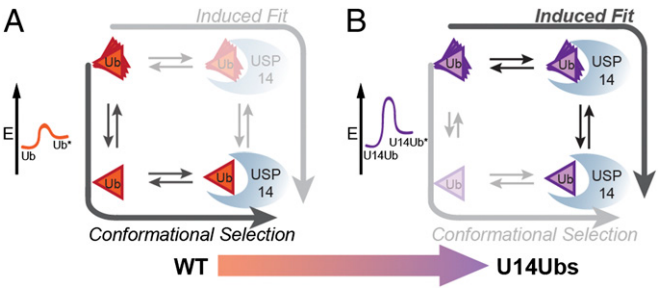

Fig. 7. Perturbation of the conformational energy landscape of ubiquitin alters the mechanism of DUB binding. (*A*) WT ubiquitin binding primarily occurs via conformational selection (12). The rate of conformational changes of WT ubiquitin is relatively fast; therefore, the energy barrier separating substates is modest. (*B*) The motions of the U14Ubs are much slower than WT ubiquitin, suggesting that the energy barrier between states has increased. An increased energy barrier would reduce flux through the conformational selection arm of the pathway and cause the binding mechanism to be dominated by induced fit.

could shed light into the mechanism by which the U14Ub mutations have slowed their dynamics.

Precisely how the slowing of motion in the U14Ubs has increased their affinity for USP14 remains unclear. Point mutations that decrease ubiquitin's overall stability and increase its flexibility over slow, second-minute timescales have been shown to abrogate interactions with ubiquitin interacting motif domains (16), but the U14Ubs appear to be gain-of-function dynamic mutants in that they have enhanced affinities for human USP14. We speculate that an increase in the energy barrier connecting ground and excited states restricts the rate at which the apo U14Ubs access a possible high-affinity excited state, but this energetic barrier can be overcome through binding to the DUB, resulting in the observed shift away from conformational selection and toward an induced-fit binding mode (Fig. 7). The observed affinity increase of the U14Ubs for USP14 would then be due to the difficulty in recrossing this higher energy barrier before dissociation. Taken together, our data indicate that the U14Ubs achieve functional changes by modulating the kinetics of conformational changes rather than the relative populations of states.

Ubiquitin's central role as a hub protein dictates that it must maintain regulated interactions with a wide variety of cellular partners. Likewise, its extreme conservation across all eukaryotes suggests that almost every aspect of its structure and dynamics is under tight evolutionary control. The U14Ubs adopt the WT ubiquitin fold, but unlike WT ubiquitin, which undergoes conformational transitions with a timescale on the order of 10 μs (12, 31), the U14Ubs are dominated by dramatically slower microsecond and millisecond motions. Our data suggest that not all aspects of ubiquitin processing and recognition depend on WT conformational kinetics, as the U14Ubs are incorporated into chains both in vitro and in vivo, and proteomic analysis indicates that ubiquitination of a very small subset of proteins is affected by changes in dynamics. Nonetheless, these changes are sufficient to prevent the U14Ubs from replacing endogenous ubiquitin in yeast, presumably because one or more of their novel or deficient interactions is necessary for survival. This result implies that the rate of conformational dynamics can have dramatic effects on organismal fitness and has therefore been maintained throughout the evolution of the ubiquitin signaling network.

In summary, we identified functional conformations of ubiquitin that govern its interaction with DUBs and have modulated the energy landscape connecting conformational states, yielding increased affinity for USP14 and decreased affinity for other DUBs. Our results give insight into the mechanism of ubiquitin–DUB interactions, provide unique reagents for probing DUB pathways, and represent a significant step toward a better understanding of the means by which conformational dynamics affect macromolecular recognition. We have also shown that the maintenance of fine-tuned conformational dynamics is important in a critical eukaryotic signaling network and thus is necessary for organismal survival. We look forward to future work probing the biophysical properties of the unique U14Ubs variants, as well as further elucidation of how their conformational dynamics dictate their interplay with the complex biological network of ubiquitin signaling.

Methods

Computational design and docking were performed using Rosetta (19–21). A computationally guided ubiquitin library was displayed on the surface of M13 bacteriophage as previously described (22) and selected against the monobiotinylated catalytic domain of USP14 (residues D91-Q494, C114A active site mutation). All protein constructs were cloned into pET vectors, expressed in *Escherichia coli*, and purified via affinity chromatography. The binding affinities of ubiquitin variants to USP14 were measured by biolayer interferometry on an OctetRed 384 instrument. NMR data were collected on Bruker spectrometers at 18.8 and 14.1 T. Detailed methods are available in *SI Appendix*.

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