Structural transitions in conserved, ordered Beclin 1 domains essential to regulating autophagy

Beclin 1 (BECN1) is a key regulator of autophagy, a critical catabolic homeostasis pathway that involves sequestration of selected cytoplasmic components by multilayered vesicles called autophagosomes, followed by lysosomal fusion and degradation. BECN1 is a core component of class III phosphatidylinositol-3-kinase complexes responsible for autophagosome nucleation. Without heterologous binding partners, BECN1 forms an antiparallel homodimer via its coiled-coil domain (CCD). However, the last 16 CCD residues, composing an “overlap helix” (OH), have been crystallized in two mutually exclusive states: either as part of the CCD or packed against the C-terminal β-α repeated, autophagy-specific domain (BARAD). Here, using CD spectroscopy, isothermal titration calorimetry, and small-angle X-ray scattering, we show that in the homodimeric state, the OH transitions between these two different packing states, with the predominant state comprising the OH packed against the BARAD, contrary to expectations based on known BECN1 interactions with heterologous partners. We confirmed this observation by comparing the impact of mutating four residues that mediate packing of the OH against both the CCD and BARAD on structure and stability of the CCD, the OH+BARAD, and the two-domain CCD–BARAD. Last, we used cellular assays to demonstrate that mutation of these OH-interface residues abrogates starvation-induced up-regulation of autophagy but does not affect basal autophagy. In summary, we have identified a BECN1 helical region that transitions between packing as part of either one of two conserved domains (i.e. the CCD or the BARAD). Our findings have important implications for the relative stability of autophagy-inactive and autophagy-active BECN1 complexes.

Macroautophagy, hereafter called autophagy, is a conserved cellular homeostasis pathway responsible for engulfment of long-lived, misfolded, aggregated, damaged, or mutated proteins, organelles, and pathogens in multilayered vesicles called autophagosomes that fuse with lysosomes to enable degradation of the sequestered contents, facilitating nutrient recycling (1–5). BECN1/Beclin 1/ATG6/VPS30 homologs, first identified as a BCL2-interacting protein in humans (6) and among the first autophagy proteins to be identified in mammals (4), are highly conserved in eukaryotes. As core components of the class III phosphatidylinositol 3-kinase (PI3KC3) complexes essential for autophagosome nucleation and maturation (7–11), BECN1 homologs form a parallel heterodimer with either ATG14 or UVRAG/VPS38 via their coiled-coil domains (CCDs)(12–14). Defective BECN1 or deficient expression levels are linked to various diseases, including neurodegenerative disorders (15–17), cancers (4, 18–21), and infectious diseases (6, 22–24).

BECN1 up-regulates PI3KC3 activity and autophagy in response to diverse signals, although the exact mechanisms by which BECN1 performs this function remain unclear. BECN1 appears to be a major interaction hub for autophagy, interacting with at least 20 different cellular proteins as well as five virus (HIV, influenza virus, α-, β-, and γ-herpesviruses)-encoded proteins that inhibit autophagy to prevent virus degradation (24–26). Although the mechanism(s) by which BECN1 binds so many diverse partners is unknown, conformational flexibility often facilitates multivalent protein interactions, allowing one protein to interact with various binding partners to carry out diverse functions. Thus, understanding BECN1 conformational flexibility is key to understanding varied BECN1 interactions, which, in turn, will improve our understanding of the role of BECN1 in autophagy. Ultimately, this is essential to better understand how eukaryotic organisms respond to and survive cellular stressors.

BECN1 is a conformationally flexible protein that has at least four domains (27): an N-terminal intrinsically disordered region (26, 28), a flexible helical domain (29), a CCD (30, 31), and a β-α repeated, autophagy-specific domain (BARAD) (32, 33). The CCD (residues 175–265 in human BECN1) and the

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This article contains supplemental Tables S1–S3 and Figs. S1–S4.

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The abbreviations used are: CCD, coiled-coil domain; AFM, atomic force microscopy; BARAD, β-α repeated, autophagy-specific domain; D_{max}, maximum particle size; EOM, ensemble optimization method; fringe, scattering intensity; OH, overlap helix, PI3KC3, class III phosphatidylinositol 3-kinase; R_g, radius of gyration; SAXS, small angle X-ray scattering; SEC, size exclusion chromatography; ITC, isothermal titration calorimetry; NES, nuclear export signal; PDB, Protein Data Bank.
BARAD (residues 266–450 in human BECN1) are highly conserved, well-folded domains. The BARAD bears an aromatic finger composed of residues 359–361 (residues 430–432 of yeast VPS30) that is required for BARAD binding to membranes (33). Mass spectroscopy hydrogen–deuterium exchange experiments show that this aromatic finger probably interacts with membranes when part of the PI3KC3 complex (13).

Interestingly, residues 248–264 preceding the BARAD form a helical region that we have named the “overlap helix” (OH), because it has been crystallized in two differently packed states: (i) as part of the anti-parallel CCD homodimer (30, 31), constituting the C-terminal four turns of each CCD, or (ii) packed against the BARAD (33). Notably, in the structure of the quaternary complex of yeast VPS34/PI3KC3, VPS15, VPS30/BECN1, and VPS38/UVRAG, VPS30 residues 304–327 (equivalent to residues 248–271 of human BECN1) are part of the VPS30/BECN1 CCD and pack against the VPS38/UVRAG CCD, stabilizing the parallel CCD heterodimer (13). This led to the assumption that the OH is part of the CCD in the context of full-length BECN1. Notably, mass spectroscopy hydrogen–deuterium exchange experiments indicate that VPS30 residues 312–327 (equivalent to BECN1 residues 256–271), which compose the latter half of the OH, undergo membrane binding–induced conformational changes, although these residues do not directly bind membranes (13). Thus, different conformational states of the BECN1 OH probably play important roles in regulating protein–membrane or protein–protein interactions.

Co-immunoprecipitation assays show that the BECN1 homodimer exists in cells (34). Inclusion of the OH as part of the anti-parallel CCD homodimer would greatly increase stability of the autophagy-inactive homodimer. However, it is unknown whether the BECN1 OH is part of the CCD or the BARAD when BECN1 is not part of PI3KC3 complexes; nor have the factors that regulate this conformational change been established. Therefore, to determine the packing state of the OH in the absence of heterologous binding partners, we investigated the solution structure of a two-domain CCD–BARAD BECN1 fragment using CD spectroscopy and size-exclusion chromatography (SEC) in tandem with small angle X-ray scattering (SAXS). Further, we assessed the impact of mutating OH residues that pack against either the partner helix of the CCD or the BARAD on self-dissociation, using isothermal titration calorimetry (ITC); on structure and stability of the CCD, BARAD, and CCD–BARAD by monitoring thermal denaturation by CD and structural changes by SAXS; and last on cellular autophagy levels by evaluating the change in the number of autophagosomes per cell.

**Results**

The BECN1 OH cannot simultaneously pack against the CCD homodimer partner helix and the BARAD

Superimposition of the human BECN1 OH of the OH+BARAD (PDB entry 4DDP) and one chain of the CCD homodimer (PDB entry 5HHE) reveals that the OH cannot simultaneously be within the CCD and packed against the BARAD, as there are extensive steric conflicts between the BARAD and the partner helix of the CCD (Fig. 1). Further, the same five OH residues (Val250, Met254, Ala257, Leu261, and Leu264) involved in the CCD homodimer interface also pack against the BARAD (Fig. 1). Thus, the OH in the CCD and OH+BARAD crystal structures represent mutually exclusive conformations of BECN1.

Approximately 1359 Å² of surface area are buried upon the OH packing against the BARAD, significantly more than the 937 Å² buried when the OH packs within the CCD. Within the CCD, OH residues are only involved in paired interactions. However, OH residues pack against the first β-sheet and third α-helix of the BARAD (Fig. 1), resulting in more extensive packing.

Each of the OH interface residues is largely conserved from yeast to humans (supplemental Fig. S1A). The position equivalent to human Met254 is least well-conserved,
although all examined vertebrates have a Met or Val at equivalent positions. In comparison with the OH, residues of the partner BECN1 CCD that pack against the OH are not as well-conserved (supplemental Fig. S1B). Based on this preliminary analysis, we hypothesized that simultaneous alanine mutagenesis of four of the common OH interface residues (V250A, M254A, L261A, and L264A (tetrad mutant)) would differently impact structure and stability of the CCD and BARAD. Structural and thermodynamic analyses of these differences would allow us to determine whether the OH is part of the CCD, packs against the BARAD, or transitions between the two states.

**Aromatic finger mutation (AFM) increases solubility of BARAD-containing BECN1 fragments**

We find that the WT OH + BARAD and CCD-BARAD proteins aggregate during purification. Analysis of the OH + BARAD crystal structure (PDB entry 4DDP) indicates that the OH + BARAD molecules are arranged in a head-to-tail manner in the crystal lattice, stabilized by the aromatic finger, consisting of Phe\(^{359}\), Phe\(^{360}\), and Trp\(^{361}\), of one OH + BARAD molecule being buried within a hydrophobic pocket formed partly by the OH in the next OH + BARAD molecule (33) (supplemental Fig. S2). We hypothesized that this interaction may be the cause of protein aggregation during purification of WT OH + BARAD-containing constructs.

The AFM, wherein the aromatic finger residues, Phe\(^{359}\), Phe\(^{360}\), and Trp\(^{361}\), are mutated to Asp, has no effect on the secondary structure content estimated from CD (supplemental Fig. S3 and Table S1) or the SEC-SAXS profiles (supplemental Fig. S4 and Table S2) of the CCD + BARAD constructs. This is consistent with previously published analyses that showed that the AFM does not change the overall secondary structure or thermal stability of the BECN1 OH + BARAD (33). However, we find that the AFM improves the yield of soluble homogeneous protein for both OH + BARAD and CCD + BARAD fragments. Because introduction of the AFM dramatically reduced aggregation and permitted large-scale purification of homogeneous, high-quality protein without affecting structure, all BARAD-containing proteins used for subsequent analysis bear the AFM.

**BECN1 CCD structure is destabilized by the OH tetrad mutation**

The BECN1 CCD interface is composed of 26 interacting pairs comprising 13 unique pairs related by 2-fold homodimer symmetry. Only six of these 13 unique pairs constitute residue pairs that form good hydrophobic interactions, whereas for each of the remaining seven pairs, one residue has a polar and/or bulky side chain, resulting in non-ideal packing (30, 31). At the termini of each set of 13 interacting pairs within the CCD homodimer, each OH contributes to five pairs of interactions: Val\(^{250}\)–Glu\(^{191}\), Met\(^{254}\)–Leu\(^{187}\), Ala\(^{257}\)–Leu\(^{184}\), Leu\(^{261}\)–Leu\(^{180}\), and Leu\(^{264}\)–Ser\(^{177}\) (supplemental Fig. S1). This includes three of the six good hydrophobic interacting pairs. Thus, the OH contributes half of the hydrophobic interactions that stabilize the BECN1 CCD homodimer.

To assess the effect of the tetrad mutation on CCD homodimerization, we compared dimer self-dissociation constants (\(K_d\)) of the CCD and CCD\(^{TETRAD}\), as quantified by ITC. At 20 °C, self-association of the CCD\(^{TETRAD}\) is \(\sim 1.5\)-fold weaker than that of the WT CCD (Table 1). A comparison of the secondary structure content estimated by analysis of CD spectra for the CCD and CCD\(^{TETRAD}\) indicates that the tetrad mutation reduces helicity by \(\sim 9.5\) residues with a concomitant \(\sim 9\)-residue increase in coil content of the CCD but does not impact \(\beta\)-strand content (Fig. 24 and Table 2). Therefore, the tetrad mutation destabilizes CCD secondary structure. The cooperative, reversible melting curves indicate that the melting temperature (\(T_m\)) of \(\sim 33.5\) °C of the WT CCD (Fig. 2B and Table 2) decreases substantially to 22.5 °C for the CCD\(^{TETRAD}\) (Fig. 2C and Table 2), which is also indicative of the tetrad mutation destabilizing the CCD structure.

We used SEC-SAXS to investigate the impact of the tetrad mutation on size, shape, and structure of the CCD. The \(R_g\) of the CCD\(^{TETRAD}\), estimated from the Guinier plot and \(P(r)\) distribution, is \(\sim 39\) Å (Fig. 3 (A and B) and Table 3), which is similar to the \(R_g\) of \(\sim 36\) Å reported for the WT CCD (31). However, the \(D_{\text{max}}\) of the CCD\(^{TETRAD}\) obtained from the \(P(r)\) distribution is 155 Å, which is about 30% longer than the \(D_{\text{max}}\) reported for the CCD (31) (Fig. 3B and Table 3). Further, the Kratky plot indicates that the CCD\(^{TETRAD}\) is largely unfolded (Fig. 3C), in contrast to the CCD, which is well-folded (31). The increase in the \(D_{\text{max}}\) observed in the CCD\(^{TETRAD}\) is consistent with the destabilization of the CCD by the tetrad mutation, observed in secondary structure estimations and melting temperature calculations by CD, because increased disorder would result in polypeptides sampling larger volumes in solution.

The theoretical scattering data calculated from the best fit ensemble optimization method (EOM) (35) model of the CCD\(^{TETRAD}\) fit the experimental SAXS data poorly with a \(\chi^2\) of 7.4 (Fig. 3, D and E), which supports the conclusion that the CCD\(^{TETRAD}\) is highly flexible, and, because the Kratky plot suggests that the CCD\(^{TETRAD}\) is largely unfolded (Fig. 3C), this further suggests that the tetrad mutation destabilizes additional regions of the CCD, such as the N-terminal region of the CCD that it packs against. In contrast, the theoretical scattering calculated from the WT CCD crystal structure fits the WT SAXS data very well (31). Analysis using EOM further confirms that the CCD\(^{TETRAD}\) is highly flexible. EOM generates a random pool of models and selects an ensemble of models from the pool that best fit the scattering data, providing an \(R_g\) and \(D_{\text{max}}\) distribution for both the pool and selected ensemble of models (35). A narrower \(R_g\) and \(D_{\text{max}}\) distribution for the selected ensemble compared with the random pool suggests that the molecule is rigid, whereas an \(R_g\) and \(D_{\text{max}}\) distribution for the ensemble that is as broad as the pool suggests

### Table 1

| BECN1 fragment | \(K_d\) (μM) | \(\Delta H^\circ\) (kcal/mol) | \(\Delta S^\circ\) (cal K\(^{-1}\) mol\(^{-1}\)) |
|----------------|-------------|-----------------|-----------------|
| CCD            | 448 ± 2.0   | 45.0 ± 10.5     | 236.6 ± 36.2    |
| CCD\(^{TETRAD}\) | 707 ± 3.1   | 157.0 ± 8.8     | 615.1 ± 30.0    |
| CCD-BARAD\(^{ATM}\) | 610 ± 8.1   | 207.1 ± 21.5    | 787.1 ± 73.0    |
| CCD-BARAD\(^{TETRAD}\) | 134.2 ± 4.9 | 143.0 ± 10.3    | 561.9 ± 34.9    |

\(\Delta H^\circ\) = change in enthalpy.

\(\Delta S^\circ\) = change in entropy.
that the molecule is flexible (36). The $R_g$ and $D_{max}$ distribution for the CCD\textsuperscript{TETRAD} pool and selected ensemble are equally broad (Fig. 3F), suggesting that the CCD\textsuperscript{TETRAD} is highly flexible.

Taken together, the secondary structure and melting temperature estimations by CD, homodimer association affinity quantified by ITC, solution size and shape parameter determinations by SEC-SAXS, and assessment of flexibility by the Kratky plot and EOM all indicate that CCD secondary, tertiary, and quaternary structure stability is significantly disrupted by the OH tetrad mutation.

The OH tetrad mutation impacts BECN1 OH+BARAD structure less than that of the CCD

The crystal structure of the human BARAD (33) includes the OH packed against the BARAD, but the OH was not included in the yeast VPS30 BARAD crystal structure (32). In each case, the crystal structure as well as solution data indicate that the BARAD is a monomer. Therefore, the OH does not mediate homodimerization of the BARAD; nor does it appear to be indicative of a very good fit.

Next, we used SEC-SAXS to investigate the impact of the tetrad mutation on size, shape, and structure of the OH+BARAD. The $R_g$ of the OH+BARAD\textsuperscript{AFM} estimated from the Guinier plot and $P(r)$ distribution is $\sim 20$ Å (Fig. 5 (A and B) and Table 3). Further, the shapes of the $P(r)$ distribution plot and the Kratky plot indicate that the OH+BARAD\textsuperscript{AFM} is a well-folded, globular domain (Fig. 5, B and C). EOM was used to generate an ensemble of OH+BARAD models that would account for the flexible, N-terminal His$_6$ tag. The best fit of the experimental SAXS data to the theoretical scattering data calculated from an EOM model has a $\chi^2$ of 1.3 (Fig. 5, D and E), indicative of a very good fit.

The OH+BARAD\textsuperscript{AFM,TETRAD} estimated from the Guinier plot and $P(r)$ distribution is $\sim 23$ Å (Fig. 6 (A and B) and Table 3), which is similar to that of the OH+BARAD\textsuperscript{AFM}. Similarly, the shape of the $P(r)$ distribution curve and the Kratky plot reveals that the OH+BARAD\textsuperscript{AFM,TETRAD} is also well-folded and globular (Fig. 6, B and C). However, the 15-Å increase in $D_{max}$ and the extended tail on the $P(r)$ distribution of the OH+BARAD\textsuperscript{AFM,TETRAD} (Fig. 6B) relative to the OH+BARAD\textsuperscript{AFM} (Fig. 5B) suggest the presence of a more flexible region in the OH+BARAD\textsuperscript{AFM,TETRAD}. Indeed, the normalized Kratky (37) shows that although the OH+BARAD\textsuperscript{AFM,TETRAD} is well-folded, it is slightly more flexible than the OH+BARAD\textsuperscript{AFM} (data not shown). This is consistent with the increased coil content estimated by CD.

EOM was used to generate an ensemble of OH+BARAD\textsuperscript{AFM,TETRAD} models to account for the flexible, N-ter-

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**Figure 2. BECN1 CCD CD spectra and melting curves.** A, BECN1 CCD (magenta) and BECN1 CCD\textsuperscript{TETRAD} (purple). B, BECN1 CCD melting curve. C, BECN1 CCD\textsuperscript{TETRAD} melting curve. Boltzmann fits to the melting curves are shown in black.

**Table 2**

CD analysis: Estimated secondary structure content and melting temperatures

| Protein                  | Average estimated secondary structure content | Melting temperature |
|--------------------------|-----------------------------------------------|---------------------|
|                          | Helix | Strand | Coil | Forward | Reverse |
| BECN1 CCD                | 89.8 ± 6.1 | 1.9 ± 1.1 | 8.6 ± 6.4 | 33.9 ± 0.1 | 33.3 ± 0.1 |
| BECN1 CCD\textsuperscript{TETRAD} | 80.4 ± 4.8 | 1.9 ± 1.6 | 17.6 ± 6.8 | 23.2 ± 0.2 | 21.6 ± 0.2 |
| BECN1 OH+BARAD\textsuperscript{AFM} | 46.1 ± 7.9 | 20.2 ± 3.0 | 34.2 ± 9.8 | 47.7 ± 0.1 | NA\textsuperscript{*} |
| BECN1 OH+BARAD\textsuperscript{AFM,TETRAD} | 46.3 ± 3.8 | 10.6 ± 2.6 | 44.0 ± 3.2 | 41.4 ± 0.1 | NA |
| BECN1 CCD-BARAD\textsuperscript{AFM} | 65.2 ± 6.7 | 5.3 ± 1.6 | 29.9 ± 7.4 | 32.9 ± 0.6 | 49.0 ± 0.3 | NA |
| BECN1 CCD-BARAD\textsuperscript{AFM,TETRAD} | 56.2 ± 4.1 | 7.5 ± 0.2 | 36.7 ± 3.7 | 22.1 ± 0.6 | 45.1 ± 0.2 | NA |

\textsuperscript{*} NA, not available; proteins containing the BARAD precipitated, even on heating just to the melting temperature; therefore, reverse $T_m$ could not be determined.
minal His₆ tag of the OH+BARAD fragments. The best fit of the experimental SAXS data to the theoretical scattering data calculated from an EOM model has a $\chi^2$ of 3.2 (Fig. 6, D and E). Last, for both the OH+BARAD⁴⁷ and OH+BARAD⁴⁷,TETRAD, EOM analyses show that the $R_g$ and $D_{\text{max}}$ distribution for the selected ensemble is narrower than that of the initial pool of ensembles (Figs. 5F and 6F), indicating that both constructs are largely conformationally homogeneous in solution.

Taken together, the secondary structure and $T_m$ estimations by CD, solution size and shape parameter determinations by SEC-SAXS analyses, and assessment of flexibility by the Kratky plot and EOM, all demonstrate that the OH tetrad mutation impacts OH+BARAD structure and stability significantly less than it does the CCD.

The OH preferentially packs against the BARAD rather than within the CCD homodimer but transiently samples both conformations

A comparison of the secondary structure content estimated from the CD spectra for the BECN1 CCD–BARAD⁴⁷ and CCD–BARAD⁴⁷,TETRAD indicates that the tetrad mutation decreases helical content by 25 residues and increases strand and coil content by ~4 and ~9 residues, respectively (Fig. 7A)
and Table 2). Thus, secondary structure of the two-domain construct is impacted by the tetrad mutation.

To determine whether the CCD–BARAD also forms a homodimer like the CCD, we used ITC to quantify the dimer $K_d$. At 20 °C, self-association of the CCD–BARAD is 1.4-fold weaker than that of the WT CCD but slightly tighter than that of the CCDTETRAD (Table 1). Notably, self-association of the CCD–BARADTETRAD is ~2-fold weaker than the CCD–BARAD (Table 1). Taken together, these ITC data indicate that the tetrad mutation destabilizes both the CCD and CCD–BARAD homodimer. Equally importantly, compared with the WT CCD homodimer, the CCD–BARAD homodimer...
is substantially dissociated, with a $K_d$ comparable with the CCD$_{TETRAD}$ rather than the CCD, suggesting that the OH helix does not stabilize the homodimer as much in the two-domain fragment.

To assess the thermostability of the CCD–BARAD, we used CD to measure the melting temperature of the two-domain fragment. Although protein precipitation prevented acquisition of reversible curves, melting curves for both the CCD–BARAD$_{AFM}$ and CCD–BARAD$_{AFM,TETRAD}$ contain two well-separated transitions (Fig. 7, B and C), indicating that each construct contains two independently folded domains. The higher $T_m$ of 49 °C for the CCD–BARAD$_{AFM}$ corresponds well
with that of the OH/BARAD (T_m = 48 °C), but notably, the lower T_m of 24 °C corresponds to that of the CCD-TETRAD (T_m = 23 °C) rather than the WT CCD (T_m = 33 °C) (Table 2). This suggests that in the CCD–BARAD construct, the OH is not part of the CCD homodimer but rather packs against the BARAD. This is further corroborated by the melting temperatures of 22 and 45 °C observed for the CCD–BARADAFM, TETRAD (Table 2), as the 22 °C transition temperature is comparable with that of the lower T_m of the CCD–BARAD as well as that of the CCD-TETRAD, whereas the 45 °C transition is a little less than the second T_m of 49 °C for the CCD–BARADAFM or the T_m of 48 °C for the OH+BARADAFM, albeit slightly higher than the T_m of 41 °C for the OH+BARADAFM,TETRAD (Table 2). Therefore, the tetrad mutation does not impact stability of the CCD in the CCD–BARAD construct but rather appears to marginally destabilize the OH+BARAD, consistent with the disruption of packing of the OH against the BARAD in the two-domain construct.

Thus, the melting temperature analysis indicates that the OH packs against the BARAD in the BECN1 homodimer. However, self-dissociation measurements by ITC indicate that the CCD–BARAD homodimerizes slightly better than the CCD-TETRAD and that disruption of the OH packing interface weakens CCD-mediated homodimerization of the CCD–BARAD, although not as much as in the CCD alone. Taken together, these data suggest that in solution, the OH transitions between packing as part of the CCD or against the BARAD, with the predominant state comprising the OH packed against the BARAD.

**SAXS indicates the OH packs against the BARAD in the CCD–BARAD protein fragment**

We used SEC-SAXS to further investigate the conformation of the OH in the CCD–BARADAFM and CCD–BARADAFM,TETRAD. In each case, the R_g estimated from the Guinier plot and P(r) distribution is ~50 Å (Figs. 8 (A and B) and 9 (A and B) and Table 3), and the shape of the P(r) distribution curves and the Kratky plots indicates that both are well-folded, dumbbell-shaped proteins (Figs. 8 (B and C) and 9 (B and C)).

EOM was used to generate the best ensemble of CCD–BARADAFM homodimer models. Two separate sets of ensembles were generated: one with the OH modeled within the CCD and the other with the OH modeled as packed against the BARAD. The average R_g of 53 Å for the ensemble wherein the OH is packed against the BARAD is more similar to the R_g of 50 Å estimated from SAXS data (Table 3) than the R_g of 65 Å for the ensemble wherein the OH is part of the CCD. Further, the experimental SAXS data fit better to the theoretical scattering curve calculated from the best fit EOM model wherein the OH is packed against the BARAD, as indicated by a χ^2 of 6.9 (Fig. 8D), compared with the χ^2 of 11.7 for the fit of the theoretical scattering curve calculated from the best fit EOM model wherein the OH is part of the CCD (Fig. 8E), although neither ensemble fits particularly well. Thus, whereas EOM analysis also suggests that the OH probably packs against the BARAD rather than within the CCD in the BECN1 CCD–BARAD homodimer, the poor fits of both modeled states suggest that different conformations are transiently occupied in solution.

EOM was also used to generate the best ensemble of CCD–BARADAFM,TETRAD homodimer models. Two separate sets of
ensembles were generated: one with the OH modeled packed against the BARAD and the other with the OH modeled as coil. The fit of the experimental CCD–BARADAFM,TETRAD SAXS data was better to the theoretical scattering curve calculated from the best fit EOM model wherein the OH is packed against the BARAD, as indicated by a $\chi^2$ of 14.2 (Fig. 9D), compared with the $\chi^2$ of 41.8 for the fit of the theoretical scattering curves calculated from the best fit EOM model wherein the OH is a coil (Fig. 9E). However, all model fits to the CCD–BARADAFM,TETRAD SAXS data were worse than the fit of the best EOM model with the OH packed against the BARAD to the experimental CCD–BARADAFM SAXS data ($\chi^2 = 6.9$; Fig. 8D). Together, these results suggest that the tetrad mutation destabilizes OH packing in the CCD–BARAD homodimer.

**The tetrad mutant decreases starvation-induced autophagy**

Last, we investigated the impact of destabilization of OH packing on cellular autophagy levels mediated by exogenously expressed full-length BECN1, by quantifying and comparing the impact of either WT or the tetrad mutant (V250A, M254A, L261A, and L264A) BECN1. Because BECN1 is known to be required for autophagosome nucleation, we evaluated cellular autophagy by monitoring and comparing levels of puncta labeled with GFP-tagged LC3, an autophagosome-specific marker, in cells grown in either nutrient-rich or starvation medium. Expression of WT and tetrad mutant BECN1 was comparable in starvation and nutrient-rich conditions (Fig. 10A). We used human breast adenocarcinoma MCF7 cells because they lack detectable endogenous expression of BECN1, resulting in very low basal levels of autophagy (4, 22, 38) even in starvation conditions unless BECN1 is ectopically expressed (Fig. 10, B and C). This allows the effect of BECN1 mutants to be assayed in the absence of endogenous BECN1.

Transient expression of BECN1 in MCF7 cells did not increase autophagy levels in nutrient-rich conditions ($p = 0.951$ for BECN1 expression versus no expression; Fig. 10B) but led to a marked increase in autophagy upon starvation ($p = 0.005$ for starved versus nutrient-rich cells; Fig. 10B). We find that the BECN1 tetrad mutation does not impact autophagy levels in nutrient-rich medium ($p = 0.836$ for mutant versus WT), consistent with the lack of impact of BECN1 expression on basal autophagy levels in nutrient-rich conditions. Strikingly, however, the BECN1 tetrad mutation decreases autophagy levels in starvation medium ($p = 0.004$ for mutant versus WT), indicating that these residues are essential for up-regulating starvation-induced autophagy. This is consistent with the established importance of other conserved regions of BECN1 in starvation-triggered autophagy rather than basal autophagy levels (29, 31).

**Discussion**

Based on the biophysical and structural analyses reported here, we conclude that the BECN1 C-terminal domains, the CCD and BARAD, are linked by a conformationally labile helix. Structural superimposition unambiguously demonstrates that the OH residues present in the crystal structure of both the CCD (30, 31) and OH+BARAD (33) cannot simultaneously pack as part of the CCD and against the BARAD. Our buried surface area calculations identify a larger interface between the OH and BARAD than between the OH and the partner helix within the CCD. Half of the hydrophobic interactions that stabilize the CCD homodimer map to the OH, and alanine mutagenesis of the four hydrophobic interacting OH residues, which are also key components of the interface with the BARAD, substantially weakens the CCD homodimer. Our results show that self-association of the CCD-BARAD is weaker.
than that of the WT CCD alone, but comparable with that of the tetrad mutant CCD, suggesting that in the CCD-BARAD, the OH primarily packs against the BARAD; however, tetrad mutation in the CCD-BARAD further weakens CCD-BARAD homodimerization, indicating that the OH transiently contributes to homodimerization. The comparative melting temperature and secondary structure content assessed by CD spectroscopy together with SEC-SAXS analyses also indicate that the tetrad mutation disrupts CCD structure more than the BARAD. Therefore, contrary to currently established ideas, the OH preferentially packs against the BARAD in the absence of heterologous binding partners but appears to also transiently sample conformations where it forms part of the CCD. Thus, relative to homodimer association reported for the CCD in this and previous reports (30, 31), full-length BECN1 probably homodimerizes more weakly, consistent with that reported here for the CCD–BARAD.

We further show that the BECN1 homodimer is unstable at physiological temperatures. Our melting temperature analyses show that nearly half of the CCD is unfolded in the two-domain CCD–BARAD fragment at 37 °C. Together, the weak self-association and instability of the CCD–BARAD homodimer suggest that BECN1 probably does not exist in a stable homodimeric state at physiological temperatures unless stabilized in that state by BECN1 domains other than the CCD or other protein partners, such as the BCL2 proteins. BECN1 homodimerization probably serves as an important means of regulating autophagy in cells. Notably, this may be a regulatory mechanism that evolved in higher eukaryotes such as vertebrates, as yeast do not encode BCL2 proteins, and it is unknown whether yeast VPS30 homodimerizes.

This is significant because up-regulation of autophagy by BECN1 requires either ATG14 or UVRAG to displace one molecule of the BECN1 homodimer, which would be facilitated by a weak BECN1 homodimer. The 4.4 Å crystal structure (PDB code 5DFZ) of full-length yeast VPS30/BECN1 in complex with VPS34/PI3KC3, VPS15/p150, and VPS38/UVRAG (13), shows that the OH is part of a parallel VPS30–VPS38 CCD heterodimer essential for stabilizing this complex. The 28 Å reconstruction of the quaternary complex of human PI3KC3, p150, BECN1, and ATG14 (12) indicates that BECN1 and ATG14 interact in an analogous manner. A SAXS data–constrained model of the BECN1–ATG14 heterodimer indicates that four of the OH interface residues identified in the homodimer also contribute to the heterodimer coiled-coil interface (Val250–Ile165, Met254–Asn169, Ala257–Leu172, and Leu261–Val176) (31). Notably, these OH residues are largely conserved, especially in vertebrates (supplemental Fig. S1A), and are predicted to pack against ATG14 residues that are also conserved. Indeed among these interacting pairs, the least conserved BECN1 residue, Met254, is paired with Asn169, the least conserved paired residue in ATG14 (31). Thus, the OH contributes critical binding determinants to the parallel CCD heterodimers required for formation of VPS34/PI3KC3 complexes essential for autophagy. Consistent with this, we show that the tetrad mutation of the OH interface residues abrogates the starvation-induced up-regulation of autophagy but has no effect on basal levels of autophagy.

Our experimental evidence showing that the OH preferentially packs against the BARAD in the BECN1 homodimer suggests a mechanism wherein homodimerization via the CCD results in BECN1 conformations that prevent the BARAD aromatic finger from associating with membranes, whereas BECN1 heterodimerization with ATG14 or UVRAG disrupts this inhibitory conformation. The crystal structure and mass spectroscopy hydrogen–deuterium exchange experiments of

**Figure 10. Effect of the OH tetrad mutation on autophagy.** A, Western blotting of MCF7 cell extracts showing comparable expression levels of WT and mutant FLAG-BECN1 in nutrient-rich (R) and starvation (S) conditions, with actin as a loading control. B, light microscopy quantification of discrete GFP-LC3 puncta per cell in GFP-positive MCF7 cells co-transfected with GFP-LC3 and WT or mutant FLAG-BECN1 as indicated below the x axis. Bars, number of puncta per cell. Error bars, S.D. C, representative images of GFP-LC3 (green) and DAPI (blue) staining in cells grown in starvation or nutrient-rich medium and transfected with mutant FLAG-BECN1 as indicated.
the yeast PI3KC3-p150-BECN1-UVrag quaternary complex show that the aromatic finger interacts with membranes when the OH participates in the CCD interface of the heterodimer (13). However, formation of a BECN1 homodimer, wherein the OH preferentially packs against the BARAD, would shorten the CCD and involve reorientation of the BARAD relative to that observed in the yeast PI3KC3-p150-BECN1-UVrag quaternary complex. This would reposition the BARAD such that the aromatic finger is no longer able to interact with membranes, thereby inhibiting BECN1-mediated autophagy.

Another important implication of the OH being preferentially packed against the BARAD in the homodimer is that this would release the N-terminal region of the partner helix within the anti-parallel CCD homodimer. The N-terminal region of the human BECN1 CCD (supplemental Fig. S1B) contains a leucine-rich nuclear export signal (NES) that binds the chromosomal protein 1 (CRM1) to enable nuclear export of BECN1 (39, 40). A functional NES is composed of the sequence motif Lx(L/F)₃Lx(L/F)₃Lx(L/F)₃. NES residues Leu₁₈₄ and Leu₁₈⁷ are essential for BECN1 nuclear export, BECN1-mediated autophagy, and tumor suppression (39, 40). In the CCD homodimer, NES residues Leu₁₈₄ and Leu₁₈⁷ pack against OH interface residues, Ala₂⁵⁷ and Met₂⁵⁴, respectively (31). Therefore, to bind CRM1, the BECN1 NES cannot be packed against the BECN1 OH. Thus, our results showing that the OH preferentially packs against the BARAD in the homodimeric state provide a mechanism of how the NES may be exposed for CRM1 binding to enable nuclear export. Indeed, interaction with CRM1 may stabilize BECN1 conformations wherein the OH is packed against the BARAD. Interestingly, unlike vertebrates, lower eukaryotes do not have a NES located at the N terminus of the CCD (supplemental Fig. S1B). Therefore, this conformational switch is probably not needed for nuclear export in yeast, as the NES is located between yeast VPS30 residues 12 and 21 (39), which is part of the intrinsically disordered region, rather than the CCD.

In summary, previous studies have established the BECN1 CCD and BARAD as structurally well-defined domains, but here we show that these domains include a region, now termed the OH, that can adopt two distinct, mutually exclusive packing states, emphasizing overall BECN1 conformational flexibility. Further, in the absence of heterologous partners, the OH transitions between these states with a preference for packing against the BARAD, rather than the CCD, as has been commonly assumed. This region probably serves to regulate BECN1 function in autophagy and nuclear transport, and interactions with different partners probably stabilize one packing state or the other to promote these different functions. Understanding how different interactions stabilize different BECN1 conformational states is the next critical step toward understanding the regulation of BECN1-mediated autophagy.

Experimental procedures

Structural superimposition and buried surface area calculation

The crystal structures of the human BECN1 CCD homodimer (PDB entry 5HHE) and the human BECN1 BARAD containing the OH (OH + BARAD) (PDB entry 4DDP) were superimposed using the align function of the PyMOL Molecular Graphics System (Schrödinger, LLC) (41). The PDBePISA (42) server (http://www.ebi.ac.uk/pdbe/pisa/) was used to calculate interface buried surface areas.

Plasmid preparation

Human BECN1 residues 175–265 (CCD), residues 175–450 (CCD–BARAD), or residues 248–450 (OH + BARAD) were cloned between the Ncol and NotI restriction enzyme sites of the pMBP-Parallel-1 bacterial expression vector (43). The BECN1 OH + BARAD was also cloned between the Xhol and BamHI restriction enzyme sites of the pET-15b bacterial expression vector encoding an N-terminal His6 tag (supplemental Table S3). The OH tetrad mutation (V250A, M254A, L261A, and L264A) and AFM (F359D, F360D, and W361D) were generated via site-directed mutagenesis. A pcDNA3.1 mammalian expression vector encoding full-length, human WT BECN1 was subjected to site-directed mutagenesis to generate the OH tetrad mutation (V250A, M254A, L261A, and L264A).

Protein expression and purification

For the BECN1 CCD and CCDTETRAD, Escherichia coli BL21(DE3)pLysS cells were transformed and grown in LB medium with 100 μg/ml ampicillin at 37 °C to an A₆₀₀ of ~0.8 before equilibrating the temperature to 20 °C. For WT and mutant BECN1 CCD–BARAD and His₆-BECN1 OH + BARAD, E. coli Arctic Express cells were transformed and grown in LB medium with 100 μg/ml ampicillin at 30 °C to an A₆₀₀ of ~0.6 before equilibrating the temperature to 10 °C. In each case, cells were grown to an A₆₀₀ of 0.8–1.2. Expression of the CCD and CCD–BARAD was induced by the addition of 0.5 mM isopropyl thio-β-D-galactoside. His₆-OH + BARAD constructs were induced with 0.3 mM isopropyl thio-β-D-galactoside. All constructs were expressed overnight.

For all BECN1 CCD and BECN1 CCD–BARAD constructs, soluble maltose-binding protein–tagged fusion protein was purified from clarified crude cell lysate by amylose affinity chromatography (Wash Buffer 1: 0.5 M Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 2 mM DTT), and subjected to overnight, on-column cleavage by GST-to-parallel maltose-binding protein fusion cleavage affinity agarose beads (Wash Buffer 2: 0.5 M Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 2 mM DTT) and subjected to overnight, on-column cleavage by GST-to-parallel maltose-binding protein fusion cleavage affinity agarose beads (Wash Buffer 2: 0.5 M Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 2 mM DTT). The fusion proteins were concentrated in a 10,000 MWCO Amicon Ultra-0.5 centrifugal concentrator (EMD Millipore, Billerica, MA).

For His₆-BECN1 OH + BARAD constructs, fusion protein was purified from clarified crude cell lysate by nickel immobilized ion affinity chromatography (Wash Buffer: 50 mM HEPES acid, pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 25 mM imidazole). Protein was eluted with Wash Buffer containing 350 mM

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imidazole. BECN1 OH+BARAD constructs were subsequently purified to homogeneity by SEC, using a 10/30 HR Superdex 200 column (GE Healthcare) (SEC buffer: 25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM DTT) and concentrated to 5 mg/ml in a 10,000 molecular weight cut-off Amicon Ultra-0.5 centrifugal concentrator (EMD Millipore, Billerica, MA).

At each stage of purification, protein purity was evaluated by SDS-PAGE stained with Coomassie Blue. In each case, the final purified protein was estimated to be >90% pure by Coomassie Blue-stained SDS-PAGE.

## CD spectroscopy

Continuous scanning CD spectra were recorded from 190 to 240 nm at 4 °C in a 300-μl quartz cell (0.1-cm path length) on a Jasco J-815 spectropolarimeter equipped with a Peltier thermoelectric temperature control for each protein sample at concentrations of 3–20 μM in 5 mM sodium phosphate dibasic, 5 mM sodium phosphate monobasic, pH 7.4, 100 mM (NH₄)₂SO₄. Secondary structure content was estimated by analyzing CD data using the SDP48 reference protein database and three programs, SELCON3, CDSSTR, and CONTIN, from the CDpro suite within the Jasco software (44–46). For each spectrum, the average secondary structure was calculated by averaging estimated secondary structure content obtained from the three CD data analysis programs.

Variable temperature CD spectra were recorded at 222 nm at 1 °C intervals, with a ramp rate of 1 °C/min after dilution of protein samples to 3–20 μM in 5 mM sodium phosphate dibasic, 5 mM sodium phosphate monobasic, pH 7.4, 100 mM (NH₄)₂SO₄. Spectra were recorded for BECN1 OH+BARAD and BECN1 CCD–BARAD proteins from 4 to 75 °C and for BECN1 CCD proteins from 4–55 or 55–4 °C for reverse measurements. Data were analyzed using OriginPro version 8 (OriginLab), where mean residue molar ellipticity was plotted against temperature, and the Tm was calculated by fitting the data to the Boltzmann or Double Boltzmann algorithm included in OriginPro version 8.

### ITC

BECN1 CCD, CCD[TETRAD], CCD–BARAD[AFM], and CCD–BARAD[AFM,TETRAD] protein samples were dialyzed against 50 mM HEPES base, pH 8.0, 150 mM NaCl, and 2 mM β-mercaptoethanol. ITC experiments were performed in triplicate at 20 °C using a Low Volume Gold Nano isothermal titration calorimeter (TA Instruments, New Castle, DE). 65 μM CCD–BARAD[AFM], 100 μM CCD–BARAD[AFM,TETRAD], 350 μM CCD, and 100 μM CCD[TETRAD] were separately titrated into dialysis buffer using 20 injections of 2.5 μl each. Data were analyzed with the NanoAnalyze software (TA Instruments), using a dimer dissociation model to calculate the dimer dissociation constants.

### SAXS data collection and analysis

SAXS data were recorded at the BioCAT beamline (ID18) (Advanced Photon Source, Argonne, IL) on a Pilatus 3 1M detector at a sample-to-detector distance of 3.5 meters at a wavelength of 1.03 Å. For all constructs, SEC was performed in tandem with SAXS to ensure that all SAXS data were collected from a homogenous sample eluting from the SEC column (10/30 GL Superdex 200 Increase). SEC-SAXS data were recorded by exposing the column eluate to the X-ray beam for 1 s with a periodicity of 3 s. Scattering data were normalized to the incident X-ray beam intensity, and scattering from buffer was subtracted using the BioCAT beamline pipeline based on the ATSAS suite of programs prior to further analysis. Data were analyzed with the ATSAS program suite (47), including PRI-MUS (48) to calculate Guinier extrapolations to estimate the Rg and Kraty plots to evaluate disorder within the samples. The P(r) function plotted from the Fourier inversion of the scattering intensity, I(q), using GNOM (49) was used to calculate the Rg and Dmax and to reconstruct ab initio envelopes by applying 10 cycles in DAMMIF (50) with subsequent analysis by DAM-SEL, DAMSUP, DAMAVER, and DAMFILT (51) to compare and identify the most probable model, align all models to the most probable model, average aligned models and compute a probability map, and filter the averaged model.

EOM 2.1 (55) was used to generate an ensemble of models for each protein fragment. For the CCD[TETRAD], we modeled the OH and its partner helix (BECN1 residues 175–193) as flexible using PDB entry 5HHE, after removing OH and partner helix residues (ShortCCD), as a rigid body. For the BARAD[AFM] and BARAD[AFM,TETRAD], we modeled the flexible His9 tag using PDB entry 4DDP (OH+BARAD) as a rigid body. For the CCD–BARAD[AFM], we generated two-domain models using (i) the ShortCCD and the OH+BARAD as rigid bodies or (ii) PDB entries 5HHE and 4DDP, after removing OH residues (BARAD), as rigid bodies. For the CCD–BARAD[AFM,TETRAD], we generated two-domain models using (i) the ShortCCD and the OH+BARAD as rigid bodies or (ii) the ShortCCD and BARAD as rigid bodies. Models were obtained using native-like chain options, and P2 symmetry was imposed on CCD-containing fragments by fixing the original coordinates of the PDB ID: 5HHE or ShortCCD. The theoretical scattering data calculated from each model in the ensemble were fitted to the averaged scattering data using CRYSTOL (52). The EOM model that best agreed with the experimentally derived Rg and Dmax and best fit the averaged scattering curves was then fitted into the molecular envelopes using SUPCOMB (53).

### Autophagy assays

Cellular autophagy levels were quantified by monitoring cellular localization of GFP-tagged LC3 protein (54). Each chamber of an 8-well culture slide (Nunc™ Lab-Tek™ chambered coverglass) was seeded with 3.0 × 10⁵ MCF7 cells and cultured overnight in DMEM with 10% FBS until 80–90% confluent. The cells were co-transfected with 500 ng of total plasmid per well, comprising 200 ng of GFP-LC3 and 300 ng of FLAG-BECN1 WT or mutant expression plasmids, using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. After transfection and incubation at 37 °C for 24 h, the cells were cultured in either rich (DMEM, 10% FBS, 2× essential amino acids, and 2× nonessential amino acids) or starvation (Earle’s balanced salt solution) medium for 4 h. Cells were counterstained with DAPI to visualize nuclei and facilitate total cell counts, fixed to slides with 4% (v/v) paraformaldehyde, and then stored in 70% (v/v) glycerol. Cells were washed with PBS between the counterstaining, fixation, and storage stages.
GFP-LC3-positive puncta were observed under a fluorescent microscope (Zeiss AxioObserver Z1) and quantified by counting a minimum of 50 cells for duplicate samples per condition using Imaris software (Bitplan AG, Zurich, Switzerland) in three independent experiments. The significance of alterations in autophagy levels was determined by a two-tailed, heteroscedastic Student’s t test, wherein p ≤ 0.05 is considered significant.

Expression levels of WT FLAG-Beclin1 and mutants were verified by Western blotting using commercial mouse monoclonal anti-FLAG M2-peroxidase antibody (Sigma). As a loading control, actin levels in MCF7 cell lysates were detected with mouse anti-actin (EMD-Millipore).

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