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N-Terminal Acetylation Acts as an Avidity Enhancer Within an Interconnected Multiprotein Complex*

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Abstract

Although many eukaryotic proteins are N-terminally acetylated, structural mechanisms by which N-terminal acetylation mediates protein interactions are largely unknown. Here we found that N-terminal acetylation of the E2 enzyme, Ubc12, dictates distinctive E3-dependent ligation of the ubiquitin-like protein, Nedd8, to Cul1. Structural, biochemical, biophysical, and genetic analyses revealed how complete burial of Ubc12's N-acetyl-methionine in a hydrophobic pocket in the E3, Dcn1, promotes cullin neddylation. The results suggest that the N-terminal acetyl both directs Ubc12's interactions with Dcn1, and prevents repulsion of a charged N-terminus. Our data provide a link between acetylation and ubiquitin-like protein conjugation, and define a mechanism for N-terminal acetylation-dependent recognition.

Many eukaryotic proteins are N-terminally acetylated (1–4). Genetic data underscore the importance of N-terminal methionine acetylation (1, 5–10), although specific interactions mediated by N-acetyl-methionine are largely unknown. We examined how N-acetyl-methionine can direct protein interactions by studying an E2 enzyme. E2s play central roles in E1>E2>E3 ubiquitin-like protein (UBL) conjugation cascades. First, an E2 transiently binds E1 for generation of a thioester-linked E2~UBL intermediate, which then interacts with an E3. For RING E3s, the UBL is transferred from E2 to an E3-associated target's lysine, producing an isopeptide-bonded target~UBL complex. E2 core domains are sufficient for binding E1s and RING E3s (11). Contacts beyond E2 cores often mediate pathway-specific interactions. A unique N-terminal extension on Nedd8's E2, Ubc12, binds both E1 and E3 (12–16). Nedd8 transfer from Ubc12 to cullins involves a "dual E3" mechanism (16): a RING E3, Rbx1, is essential for cullin neddylation; a co-E3, Dcn1,

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Author contributions: DCS, JKM, EJB designed, performed, analyzed experiments; DCS and BAS wrote the manuscript with all authors contributing; JWH and BAS advised and assisted on all aspects.

Supporting Online Material
www.sciencemag.org, Materials and Methods, Figs. S1–S10, Table S1, References (26–44)

contains a "potentiating neddylation" domain ($Dcn1^P$) thought to bind different Ubc12 and cullin surfaces (15–18). Notably, human Dcn1 acts as an oncogene (19).

Because Dcn1's E3 activity was elusive with bacterially-expressed Ubc12 (14, 17), we asked whether in eukaryotes Ubc12 might be modified. Tandem mass spectrometry identified exogenous yeast (y) and human (h), and endogenous human Ubc12 as being N-terminally acetylated on Met1 (Fig 1A, Fig S1). Although mammalian N-terminal acetyltransferases (Nats) appear partially redundant (3), yNat specificities are well-defined (20). yUbc12's N-terminal Met-Leu sequence was predicted to retain Met1 and be acetylated by the Mak3p-Mak10p-Mak31p complex comprising yNatC (21). Indeed, *Mak3* gene deletion prevented yUbc12 N-terminal acetylation in yeast, and bacterially-expressed yNatC catalyzed yUbc12 N-terminal acetylation (Fig 1). Thus, yNatC performs yUbc12 N-terminal acetylation.

To address whether Ubc12 N-terminal acetylation influences Nedd8 ligation, we examined yNedd8~yCul1 steady-state levels in yeast with Nat gene deletions. Only yeast lacking NatC components displayed decreased yNedd8~yCul1 (Fig 1D). Furthermore, loss of NatC activity was synthetically lethal in combination with the *cdc34-2* temperature-sensitive allele (Fig 1E) - a hallmark for Nedd8 pathway components due to roles in yCul1/SCF-regulated cell division (14, 22). Thus, yUbc12 N-terminal acetylation is important for yCul1 neddylation and function in vivo.

In vitro, Ubc12 N-terminal acetylation dictated $Dcn1^P$-mediated Nedd8 transfer to Cul1 in pulse-chase assays comparing N-terminally acetylated $Ubc12^{AcMet}$, $Ubc12^{Met}$ (identical sequence but not N-terminally acetylated), and $Ubc12^{GSMet}$ (unacetylated with Gly-Ser-Met at the N-terminus) (16). $yDcn1^P$ E3 activity was substantially magnified for $yUbc12^{AcMet}$, with lower $yDcn1^P$-enhancement of yNedd8 transfer to yCul1 from $yUbc12^{Met}$ or $yUbc12^{GSMet}$ consistent with the residual neddylation in NatC null yeast (Fig 1D, 2A). hUbc12 N-terminal acetylation was absolutely required for $hDcn1^P$-mediated potentiation of neddylation (Fig 2B, Fig S2). In all cases, Ubc12 N-terminal acetylation was specific for $Dcn1^P$ E3 activity, because $Dcn1^P$-independent Rbx1-mediated transfer of Nedd8 to Cul1 was independent of the state of Ubc12's N-terminus (Fig 2, Fig S2). Nonetheless, even in the presence of $Dcn1^P$ and Ubc12 N-terminal acetylation, Cul1 neddylation required Rbx1's RING E3 activity and was blocked by the CAND1 inhibitor (Fig S2). Thus, in addition to roles of the acetylated Ubc12-Dcn1 E2–E3 complex, in vivo steady-state Cul1~Nedd8 levels may also reflect Dcn1- and acetylation-independent regulation.

To obtain mechanistic insights, Ubc12-$Dcn1^P$ interactions were quantified by isothermal titration calorimetry (ITC). N-terminal acetylation increased Ubc12's affinity for $Dcn1^P$ by ~2 orders-of-magnitude, and this was recapitulated by synthetic Ubc12 N-terminal peptides, which also inhibited neddylation reactions (Fig. 2C, Fig S3, S4). In contrast, N-terminal acetylation had little effect on Ubc12 peptide binding to E1 (Fig. 2C, Fig S5).

To understand how N-acetyl-methionine mediates interactions, we determined crystal structures of yeast and human $Dcn1^P$ bound to acetylated Ubc12 peptides (Table S1, Fig S6). As in prior structures, $Dcn1^P$ forms a helical domain containing two EF-hand-like folds (14, 16, 23). The Ubc12 N-terminal peptides are α-helical, as in full-length $yUbc12^{GSMet}$ (16). A $Dcn1^P$ groove at the junction between the two EF-hand-like subdomains cradles Ubc12's helix, culminating in Ubc12's N-acetyl-methionine burial in a conserved, deep, hydrophobic pocket in $Dcn1^P$ (Fig 3, Fig S7, S8).

N-acetyl-methionine recognition consists of three major components (Fig 3). First, the methyl portion of the acetyl group fits snuggly in a hydrophobic pocket. Second, the amide

makes a hydrogen bond with a structurally conserved carbonyl oxygen from Dcn1. Third, the Met1 side-chain is also fully enwrapped by the hydrophobic pocket.

The structures suggest two mechanisms by which Ubc12's N-terminal acetylation dictates binding to Dcn1^{P}. First, the acetyl group interacts directly with Dcn1^{P}. Second, acetylation eliminates an N-terminal positive charge, which would impede burial in the hydrophobic pocket. These concepts were substantiated by assaying Dcn1^{P} binding to N-terminally formylated Ubc12 peptides, which lack the acetyl methyl but retain the amide and are uncharged. N-terminal capping via formylation did improve binding ~10-fold in comparison to unacetylated peptides, although the K_ds were decreased ~9- and ~17-fold compared with the acetylated human and yeast peptides, respectively, highlighting the importance of the acetyl methyl (Fig 3E). Further agreeing with the structures, Ubc12's N-terminal Met1 was also required to bind Dcn1^{P} (Fig 3E).

Ubc12's N-acetyl-methionine is sealed into Dcn1^{P}'s hydrophobic pocket by Ubc12's N-terminal helix positioning hydrophobic residues 2 and 4 (Fig 3). On one side, yLeu2/hIle2 buries the acetyl. On the other side, Leu4 seals the Met1 side-chain into place. Downstream residues from Ubc12's helix also interact with Dcn1^{P} (Fig S9).

Although yUbc12^{GSMet}'s N-terminal extension is helical (16), we wished to test the structurally-observed role for the helix with human proteins because hUbc12^{Met}'s N-terminal region forms an extended structure in complex with E1 (12, 13). A 2.0 Å resolution structure with a stapled-helix (24) peptide superimposed with unstapled hUbc12^{AcMet}-hDcn1^{P}, confirming solvent exposure of the staple (Fig 3F). Helical stapling improved binding to hDcn1^{P} ~14-fold, largely due to decreasing the entropic cost (Fig 3E). Moreover, helical stapling eliminated E1-binding. Thus, locking the flexible hUbc12 N-terminal region into a helix contributes to the hDcn1^{P} interaction.

Additional Dcn1^{P} elements secure Ubc12's N-acetyl-methionine in place. First, yDcn1's Tyr190/hDcn1-Tyr181 clamps between Ubc12's N-acetyl-Met1 and yLeu2/hIle2, pressing Ubc12's N-acetyl-methionine into Dcn1^{P}'s hydrophobic pocket. Second, the loop between Dcn1^{P}'s E- and F-α-helices closes down on Ubc12's N-acetyl-methionine. In prior yDcn1^{P} structures lacking Ubc12(14, 16, 23) these elements are repositioned to occlude the hydrophobic pocket (Fig S10). yDcn1^{P} apparently initially engages Ubc12's acetylated N-terminus, and subsequently clamps it down. Conformational flexibility may account for yDcn1^{P}'s low-level activity toward yUbc12 even without N-terminal acetylation.

Given yDcn1^{P}'s structural malleability, we reasoned that mutations alleviating repulsion of an N-terminal charge might enhance yDcn1^{P}'s low-level E3 activity toward yUbc12^{Met}. The structure indicated that Asp substitutions for yDcn1^{P} Leu110 or Leu173 would approach yUbc12^{Met}'s N-terminus to balance the positive charge. Also, an Ala replacement for the Tyr190 "clamp" would not force a charged yUbc12^{Met}'s N-terminus directly into the hydrophobic pocket. Indeed, the three Dcn1^{P} mutants showed enhanced E3 activity specifically toward unacetylated yUbc12 (Fig 4A).

We asked whether a structure-based mutation could compensate for in vivo defects in cullin neddylation resulting from loss of NatC-mediated yUbc12 acetylation. Thus, we expressed HA-tagged Dcn1 or the Tyr190Ala mutant (we could not express comparable levels of the other mutants in yeast), in strains deleted for *Dcn1* alone, or both *Dcn1* and the NatC subunit *Mak10*. As with the in vitro enzymology and improved binding, the Tyr190Ala mutant rescued the defect in yCul1~yNedd8 conjugate formation that resulted from lack of NatC activity (Fig 4).

We showed N-terminal acetylation of Ubc12 to be an avidity enhancer, contributing a critical interaction within a highly interconnected neddylation complex. As only part of molecular recognition within large multicomponent complexes, many interactions depending on N-terminal acetylation likely remain unknown, and may be auxiliary (2, 20, 25). Our study raises the question of whether rules dictating N-terminal acetylation determined evolution of interactions controlling functions of N-terminally acetylated proteins. Specificity may also involve proximal elements, such as Ubc12's N-terminal helix. Because N-acetyl-methionine can be completely enwrapped in a hydrophobic environment where it would be unfavorable to bury the positive charge masked by acetylation, we propose that N-acetyl-methionine can serve as a distinctive residue type allowing burial of protein N-termini into hydrophobic pockets of interacting proteins. Such N-acetyl-methionine binding sites may serve as targets for small molecules disrupting these critical interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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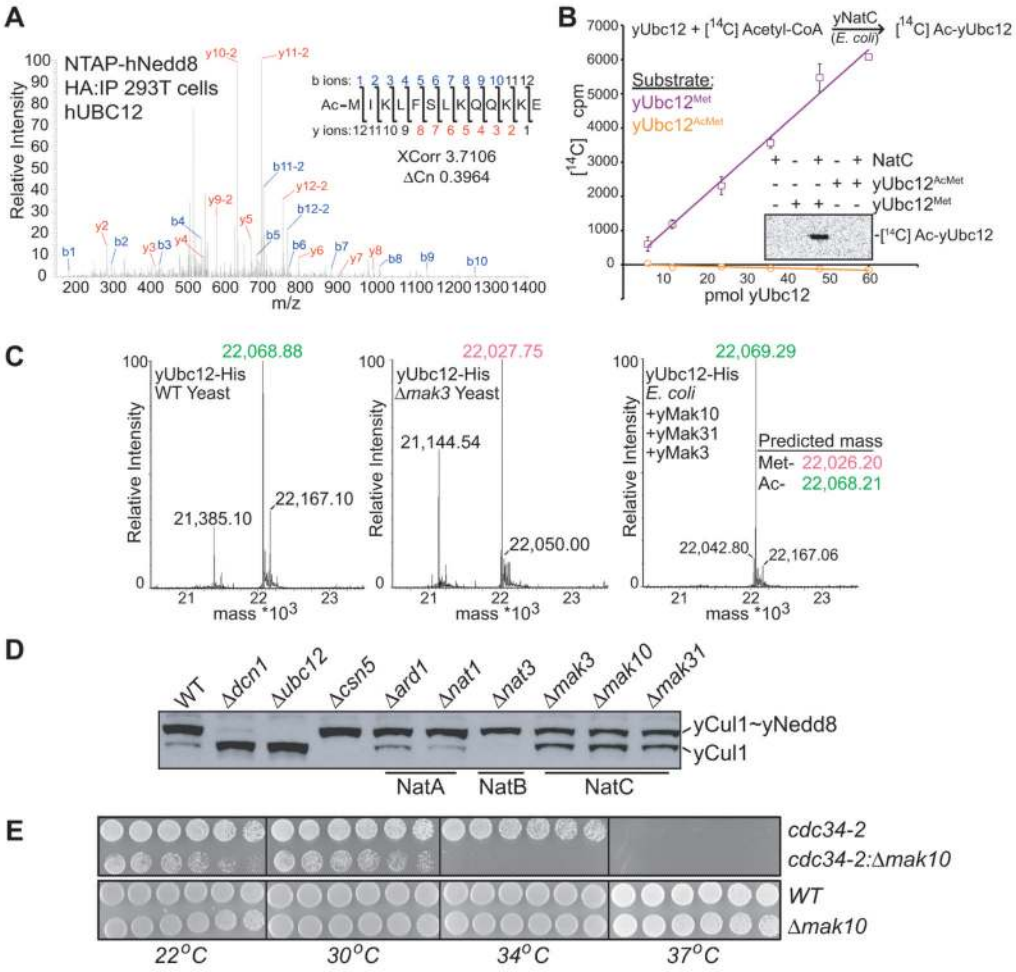
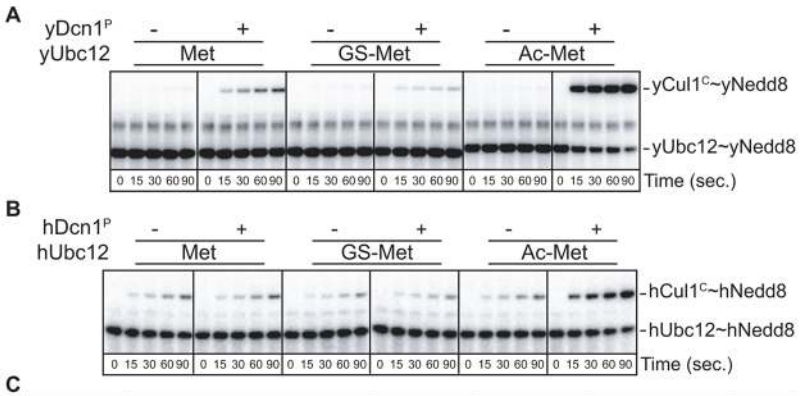


Figure 1. Ubc12 is N-terminally acetylated in eukaryotic cells

A, LC-MS/MS spectrum from endogenous hUbc12's N-terminal peptide after Glu-C digestion/desalting, indicating XCorr and ΔCN values, y (red) and b (blue) ions used to match peptide sequence. **B,** In vitro bacterially-expressed yNatC reactions with [^{14}C]-Acetyl-CoA and Ubc12^{Met} (free N-terminus) or Ubc12^{AcMet} (pre-acetylated negative control). **C,** MaxEnt LC-TOF spectra of yUbc12-His$_6$ purified from WT or Δ*mak3* yeast, or from coexpression with yNatC in *E. coli*. **D,** Immunoblot of yCul1 (Cdc53p) from indicated yeast strains. **E,** Genetic interactions between NatC subunit *mak10* and *cdc34-2*.

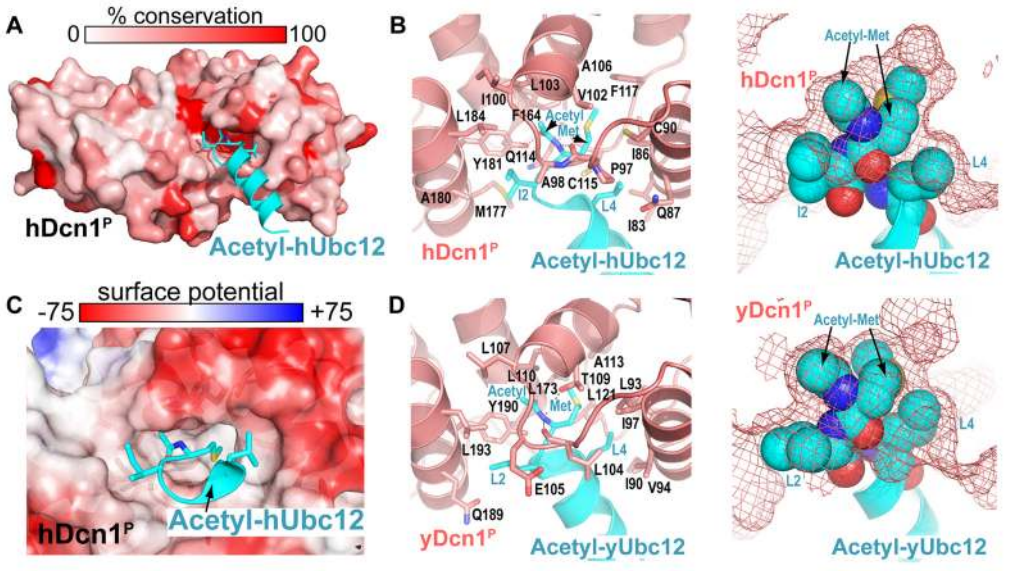


C

N-Term	Titrant : Protein	K_d (μM)	ΔH (cal/mol)	ΔS (cal/mol/deg)	N
$^{+}NH_3$-Met	yDcn1^{P} : yUbc12	>36	6,005	41.4	0.35
CH_3-C(=O)-Met	yDcn1^{P} : Ac-yUbc12	0.375	3,840	42.9	0.70
$^{+}NH_3$-Met	yUbc12$^{1-24}$: yDcn1^{P}	>40	3,777	33.4	1.16
CH_3-C(=O)-Met	Ac-yUbc12$^{1-24}$: yDcn1^{P}	0.444	4,145	43.6	0.99
$^{+}NH_3$-Met	hDcn1^{P} : hUbc12	NB	NB	NB	NB
CH_3-C(=O)-Met	hDcn1^{P} : Ac-hUbc12	0.970	-6,931	3.21	0.76
$^{+}NH_3$-Met	hUbc12$^{1-26}$: hDcn1^{P}	>130	1,259	22.2	0.75
CH_3-C(=O)-Met	Ac-hUbc12$^{1-26}$: hDcn1^{P}	1.11	-6,107	5.84	0.92
$^{+}NH_3$-Met	yUbc12$^{1-24}$: yE1	0.070	-8,571	2.65	1.05
CH_3-C(=O)-Met	Ac-yUbc12$^{1-24}$: yE1	0.016	-11,260	-3.80	0.99
$^{+}NH_3$-Met	hUbc12$^{1-26}$: hE1	0.585	-8,881	-1.99	1.29
CH_3-C(=O)-Met	Ac-hUbc12$^{1-26}$: hE1	0.943	-9,425	-4.79	1.14

Figure 2. Dependence of Dcn1 co-E3 activity on Ubc12 N-acetylation

A, Pulse-chase [^{32}P]~yNedd8 transfer from yUbc12 variants to yCul1 C-terminal domain (yCul1^{C}) complexed with Rbx1 −/+ yDcn1^{P}. **B,** Same as **A**, but with human proteins. **C,** Thermodynamic parameters for Ubc12 or its peptides binding to Dcn1^{P} and E1 by ITC. NB = no binding.

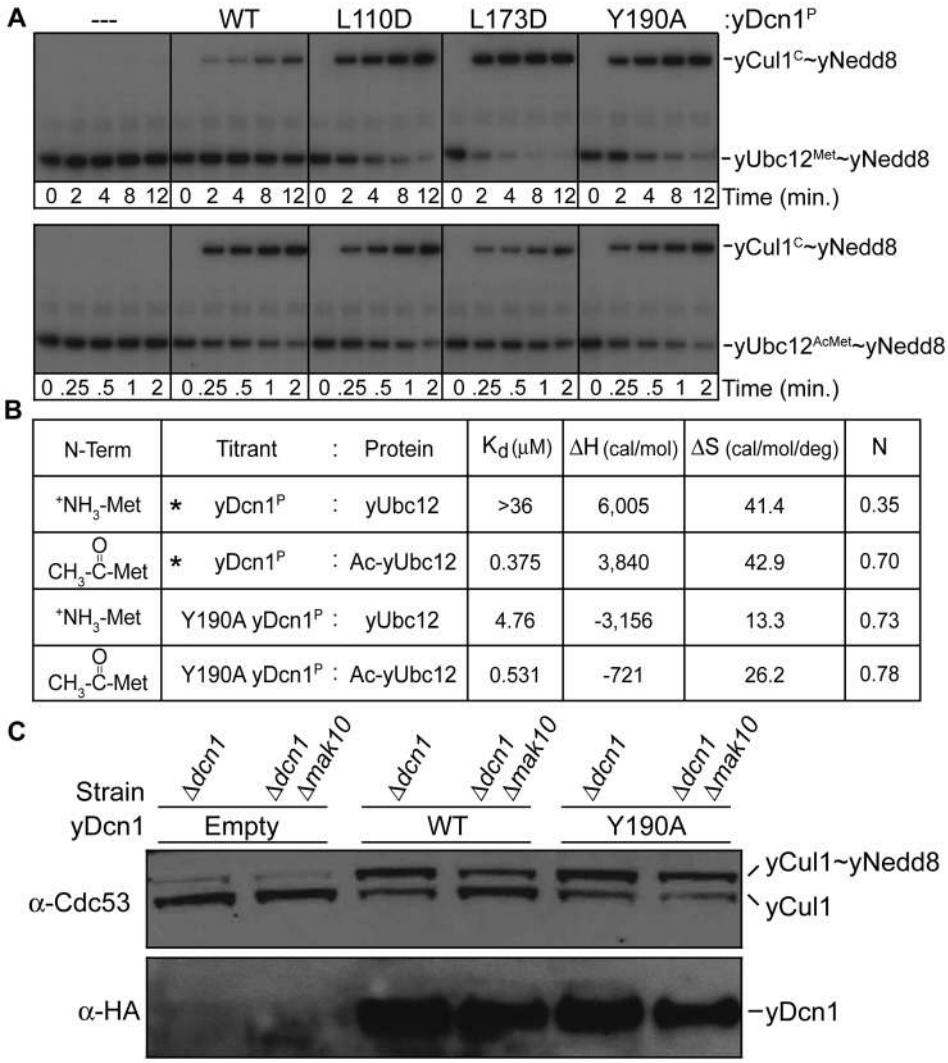


E

N-Term	Titrant : Protein	K_d(μM)	ΔH(cal/mol)	ΔS(cal/mol/deg)	N
CH_3-C(=O)-Met	* Ac-yUbc12$^{1-24}$: yDcn1^P	0.44	4,145	43.6	0.99
H-C(=O)-Met	Form-yUbc12$^{1-24}$: yDcn1^P	7.46	2,824	33.4	0.90
CH_3-C(=O)-Leu	Ac-yUbc12$^{2-24}$: yDcn1^P	>37	3,761	33.4	0.92
CH_3-C(=O)-Met	* Ac-hUbc12$^{1-26}$: hDcn1^P	1.11	-6,107	5.84	0.92
H-C(=O)-Met	Form-hUbc12$^{1-26}$: hDcn1^P	9.71	-4,893	5.79	1.00
CH_3-C(=O)-Ile	Ac-hUbc12$^{2-26}$: hDcn1^P	>100	1,719	24.1	0.52
CH_3-C(=O)-Met	Ac-hUbc12$^{1-12}$: hDcn1^P	2.15	-4,992	8.79	0.99
CH_3-C(=O)-Met	Ac-hUbc12$^{1-12}$(5:9S): hDcn1^P	0.15	-3,386	19.3	1.18
CH_3-C(=O)-Met	Ac-hUbc12$^{1-12}$: hE1	0.63	-11,490	-11.0	0.78
CH_3-C(=O)-Met	Ac-hUbc12$^{1-12}$(5:9S): hE1	NB	NB	NB	NB

Figure 3. Dcn1^P recognition of Ubc12's N-acetyl-methionine

A, hCul1^{WHB} (not shown)-hDcn1^P-AcetylhUbc12$^{1–15}$ structure, with hDcn1^P surface colored by conservation among human and yeast orthologs and Acetyl–hUbc12$^{1–15}$ peptide in cyan. **B,** Close-up of hUbc12's acetylated N-terminus (cyan) binding hDcn1^P (salmon) in cartoon (left) or hUbc12's N-acetyl-Met1 and residues 2 and 4 as spheres in a mesh view of hDcn1^P (right). **C,** Close-up of hUbc12's acetylated N-terminus (cyan) binding hDcn1^P surface colored by electrostatic potential. **D,** Same as **B,** but with yeast proteins. **E,** Thermodynamic parameters for Ubc12 peptide binding to Dcn1^P by ITC. 5:9S refers to helical staple. *reference from **2C**. NB = no binding. **F,** Solvent-exposure of helical staple in hUbc12 peptide (cyan) bound to hDcn1^P (surface, colored by electrostatic potential).



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$^{+}NH_3$-Met	* yDcn1^P : yUbc12	>36	6,005	41.4	0.35
CH_3-C(=O)-Met	* yDcn1^P : Ac-yUbc12	0.375	3,840	42.9	0.70
$^{+}NH_3$-Met	Y190A yDcn1^P : yUbc12	4.76	-3,156	13.3	0.73
CH_3-C(=O)-Met	Y190A yDcn1^P : Ac-yUbc12	0.531	-721	26.2	0.78

Figure 4. Structure-based Dcn1 mutant compensation for lack of Ubc12 N-terminal acetylation

A, Pulse-chase [^{32}P]-yNedd8 transfer from yUbc12^{Met} (top) or yUbc12^{AcMet} (bottom) to yCul1^C-yRbx1 with structure-based yDcn1^P mutants (note different time-courses). **B,** Thermodynamic parameters for binding between yDcn1^P or the Tyr190Ala mutant to unacetylated and acetylated yUbc12. *reference from **2C. C,** Immunoblots for yCul1 (Cdc53p, top) or HA-tag (bottom) from mid-log whole cell extracts from Δ*dcn1* or Δ*dcn1*/Δ*mak10* yeast harboring empty, WT Dcn1-HA, or Y190A Dcn1-HA expression vectors.