suggesting that acetic acid–producing ability is an important factor that affects host physiology. Although acetic acid supplementation in casamino acid media did not result in any appreciable effect on host development in the absence of bacteria (i.e., germ-free animals) or in the presence of commensal bacterium other than *A. pomorum* (i.e., germ-free animals monoassociated with *C. intestini*, *G. morbifer*, *L. plantarum*, or *L. brevis*), all disease phenotypes (defects in IIS, development, metabolism and ISCs) found in *P3G5*-monoassociated animals could be effectively reversed by acetic acid supplementation (Fig. 4 and fig. S13). Although the exact mechanism of acetic acid action remains to be elucidated, it is known to affect blood glucose level and insulin signaling in mammals by reducing the digestion rate of complex carbohydrates in the diet (*26*). However, the aforementioned *P3G5*-induced deregulation found when the fly larvae were fed a complex carbohydrate diet (i.e., containing cornmeal) was also observed in fly larvae fed a diet containing simple carbohydrates, such as sucrose or glucose (fig. S13). Furthermore, these defects were reversed by supplementing the simple sugar diet with acetic acid (fig. S13), indicating that acetic acid may influence host IIS and development through a mechanism other than by reducing the digestion rate of complex carbohydrates from the diet. Given that acetic acid can rescue host physiology only in the presence of *P3G5* bacterial metabolic activity, we can conclude that both PQQ-ADH–dependent acetic acid generation and PQQ-ADH–independent acetic acid metabolism are required to promote the effect of *A. pomorum* on host IIS. Further dissection of the *A. pomorum*–controlled gut factor(s) that mediates the effect of acetic acid–producing and –using bacterial metabolic activity on host IIS will provide an important link between gut microbiome activity and host metabolic homeostasis.

In summary, the present study showed that the PQQ-ADH respiratory chain of the *A. pomorum* and IIS of the host interact to maintain the gut-microbe mutualism. Bacterial PQQ-ADH is required, but not sufficient, to explain all of the *A. pomorum*–mediated effects on host physiology, and host signaling pathways, other than IIS, may also be modulated by gut bacteria. Our *Drosophila-Acetobacter* interaction system is a useful genetic model for understanding the mechanistic links between microbiome-modulated host signaling pathways and host physiology.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/334/6056/670/DC1
Materials and Methods
Figs. S1 to S13
Table S1
References (*27*–*37*)

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

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Although many eukaryotic proteins are amino (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, contains a "potentiating neddylation" domain ($Dcn1^P$)

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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 (MS/MS) identified exogenous yeast (y) and human (h), and endogenous human Ubc12 as being N-terminally acetylated on Met[1] (Fig. 1A and 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 Met[1] 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 increased 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 (Figs. 1D and 2A). hUbc12 N-terminal acetylation was

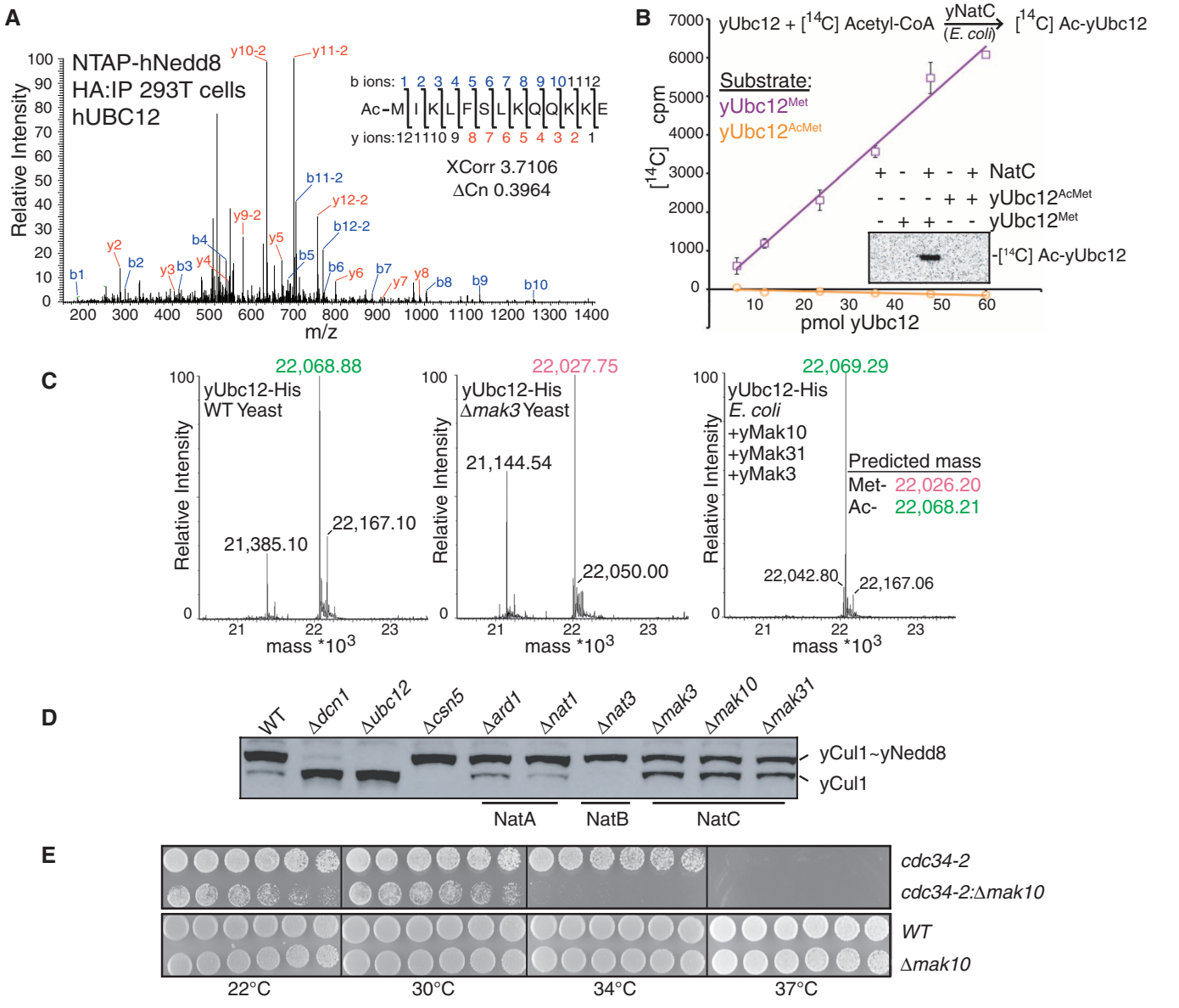


Fig. 1. Ubc12 is N-terminally acetylated in eukaryotic cells. (**A**) Liquid chromatography (LC)–MS/MS spectrum from endogenous hUbc12's N-terminal peptide after Glu-C digestion and desalting, indicating XCorr and ΔCN values, y ions (red), and b ions (blue) used to match peptide sequence. (**B**) In vitro bacterially expressed yNatC reactions with [^{14}C]-acetyl–coenzyme A and Ubc12^{Met} (free N terminus) or Ubc12^{AcMet} (preacetylated negative control). (**C**) MaxEnt LC-TOF (time-of-flight) spectra of yUbc12-His$_6$ purified from wild-type (WT) or Δ*mak3* yeast, or from coexpression with yNatC in *Escherichia coli*. (**D**) Immunoblot of yCul1 (Cdc53p) from indicated yeast strains. (**E**) Genetic interactions between NatC subunit *mak10* and *cdc34-2*.

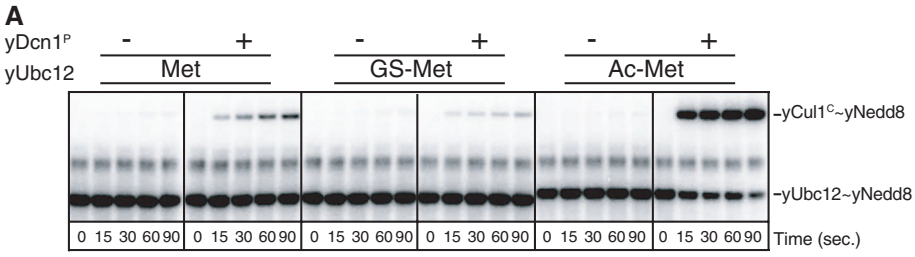
absolutely required for hDcn1^{P}-mediated potentiation of neddylation (Fig. 2B and fig. S2). In all cases, Ubc12 N-terminal acetylation was specific for Dcn1^{P} E3 activity, because Dcn1^{P}-independent Rbx1-mediated transfer of Nedd8 to Cul1 was independent of the state of Ubc12's N terminus (Fig. 2 and 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, we quantified Ubc12-Dcn1^{P} interactions by isothermal titration calorimetry (ITC). N-terminal acetylation increased Ubc12's affinity for Dcn1^{P} by about two orders of magnitude, and this was recapitulated by synthetic Ubc12 N-terminal peptides, which also inhibited neddylation reactions (Fig. 2C and figs. S3 and S4). In contrast, N-terminal acetylation had little effect on Ubc12 peptide binding to E1 (Fig. 2C and 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 and 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 and figs. S7 and 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 Met^{1} 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 dissociation constants (K_d's) 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 Met^{1} was also required to bind Dcn1^{P} (Fig. 3E).



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

Fig. 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 with or without 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.

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, yLeu^{2}/hIle^{2} buries the acetyl. On the other side, Leu^{4} seals the Met^{1} side chain into place. Residues downstream 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 Tyr^{190}/hDcn1-Tyr^{181} clamps between Ubc12's N-acetyl-Met^{1} and yLeu^{2}/hIle^{2}, 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

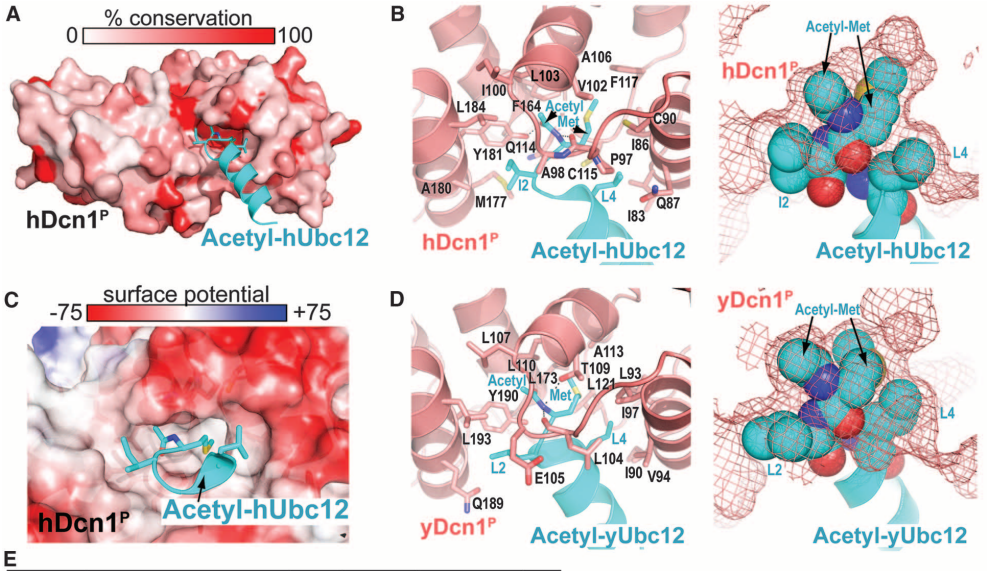


Fig. 3. Dcn1^{P} recognition of Ubc12's N-acetyl-methionine. (**A**) hCul1^{WHB} (not shown)-hDcn1^{P}-AcetylhUbc12$^{1-15}$ structure, with hDcn1^{P} surface colored by conservation among human and yeast orthologs and Acetyl–hUbc12$^{1-15}$ peptide in cyan. (**B**) Close-up of hUbc12's acetylated N terminus (cyan) binding hDcn1^{P} (salmon) in cartoon (left) or hUbc12's N-acetyl-Met^{1} 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 Fig. 2C. NB, no binding. (**F**) Solvent exposure of helical staple in hUbc12 peptide (cyan) bound to hDcn1^{P} (surface, colored by electrostatic potential).

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

F

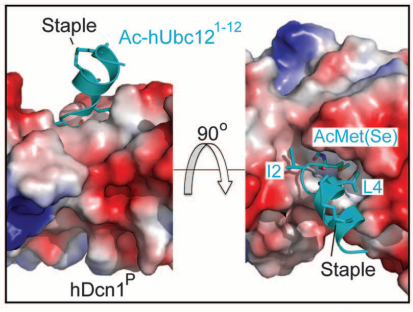
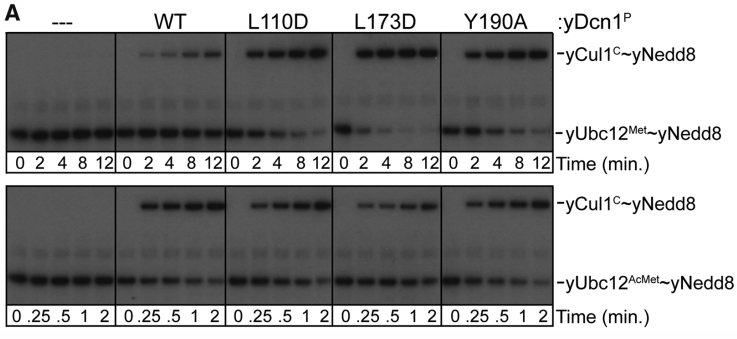


Fig. 4. Structure-based Dcn1 mutant compensation for lack of Ubc12 N-terminal acetylation. (**A**) Pulse-chase [^{32}P]-yNedd8 transfer from yUbc12^{Met} (top) or yUbc12^{AcMet} (bottom) to yCul1^{C}-yRbx1 with structure-based yDcn1^{P} mutants (note different time courses). (**B**) Thermodynamic parameters for binding between yDcn1^{P} or the Tyr^{190}→Ala (Y190A) mutant to unacetylated and acetylated yUbc12. *Reference from Fig. 2C. (**C**) Immunoblots for yCul1 (Cdc53p, top) or HA-tag (bottom) from mid-log whole-cell extracts from *Δdcn1* or *Δdcn1/Δmak10* yeast harboring empty, WT Dcn1-HA, or Y190A Dcn1-HA expression vectors.

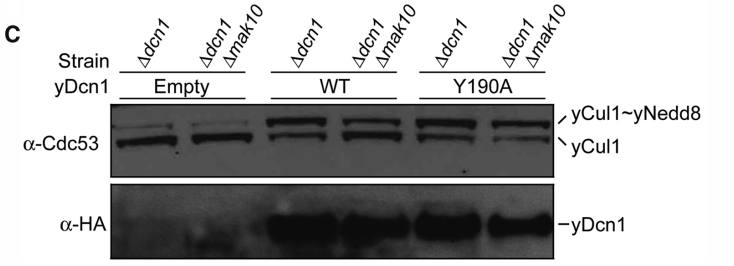
A



B

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	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

C



low-level E3 activity toward yUbc12^{Met}. The structure indicated that Asp substitutions for yDcn1^{P} Leu^{110} or Leu^{173} would approach yUbc12^{Met}'s N terminus to balance the positive charge. Also, an Ala replacement for the Tyr^{190} "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 hemagglutinin (HA)–tagged Dcn1 or the Tyr^{190}→Ala 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 Tyr^{190}→Ala 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-acetylmethionine 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.

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Supporting Online Material

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Materials and Methods
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mTORC1 Senses Lysosomal Amino Acids Through an Inside-Out Mechanism That Requires the Vacuolar H$^+$-ATPase

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The mTOR complex 1 (mTORC1) protein kinase is a master growth regulator that is stimulated by amino acids. Amino acids activate the Rag guanosine triphosphatases (GTPases), which promote the translocation of mTORC1 to the lysosomal surface, the site of mTORC1 activation. We found that the vacuolar H$^+$–adenosine triphosphatase ATPase (v-ATPase) is necessary for amino acids to activate mTORC1. The v-ATPase engages in extensive amino acid–sensitive interactions with the Ragulator, a scaffolding complex that anchors the Rag GTPases to the lysosome. In a cell-free system, ATP hydrolysis by the v-ATPase was necessary for amino acids to regulate the v-ATPase-Ragulator interaction and promote mTORC1 translocation. Results obtained in vitro and in human cells suggest that amino acid signaling begins within the lysosomal lumen. These results identify the v-ATPase as a component of the mTOR pathway and delineate a lysosome-associated machinery for amino acid sensing.

Amino acids are the building blocks of proteins and intermediates in lipid and adenosine triphosphate (ATP) synthesis. They also initiate a signaling cascade that leads to activation of the master growth regulator mTOR complex 1 (mTORC1). This multicomponent protein kinase integrates inputs from growth factors as well as nutrient and energy supplies to control many biosynthetic and catabolic processes (*1*). Most signals upstream of mTORC1 converge on TSC1-TSC2, a heterodimeric tumor suppressor that negatively regulates the Rheb guanosine triphosphatase (GTPase), which is an essential activator of mTORC1 protein kinase activity (*2*, *3*). In contrast, amino acids signal to mTORC1 by promoting its binding to a distinct family of GTPases, the Rag GTPases (*4*, *5*). The Rags form heterodimers consisting of RagA or RagB, which are highly similar to each other, bound to RagC or RagD, which are also highly related. In an amino acid–sensitive fashion, the Rag GTPases recruit mTORC1 to the surface of lysosomes, which also contain Rheb (*5*). The

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