

The Essential Ubc4/Ubc5 Function in Yeast Is HECT E3-dependent, and RING E3-dependent Pathways Require Only Monoubiquitin Transfer by Ubc4*

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The ubiquitin (Ub)-conjugating enzymes Ubc4 and Ubc5 are involved in a variety of ubiquitination pathways in yeast, including Rsp5- and anaphase-promoting complex (APC)-mediated pathways. We have found the double deletion of *UBC4* and *UBC5* genes in yeast to be lethal. To investigate the essential pathway disrupted by the *ubc4/ubc5* deletion, several point mutations were inserted in Ubc4. The Ubc4 active site mutation C86A and the E3-binding mutations A97D and F63A were both unable to rescue the lethal phenotype, indicating that an active E3/E2~Ub complex is required for the essential function of Ubc4/Ubc5. A mutation that specifically eliminates RING E3-catalyzed isopeptide formation but not HECT E3 transthiolation (N78S-Ubc4) rescued the lethal phenotype. Thus, the essential redundant function performed by Ubc4 and Ubc5 in yeast is with a HECT-type E3, likely the only essential HECT in yeast, Rsp5. Our results also suggest that Ubc1 can weakly replace Ubc4 to transfer mono-Ub with APC, but Ubc4 cannot replace Ubc1 for poly-Ub chain extension on APC substrates. Finally, the backside Ub-binding mutant S23R-Ubc4 has no observable effect in yeast. Together, our results are consistent with a model in which Ubc4 and Ubc5 are 1) the primary E2s for Rsp5 in yeast and 2) act as monoubiquitinating E2s in RING E3-catalyzed pathways, in contrast to the processive human ortholog UbcH5.

Ubiquitination, the process by which proteins are covalently modified by the small protein ubiquitin (Ub),² is a versatile method of regulation used in all eukaryotes from yeast to humans. Many cellular pathways utilize ubiquitination as a regulatory mechanism, including DNA repair, endocytosis, transcription, protein trafficking, and protein quality control (1). Ubiquitination involves a multienzyme reaction cascade in which the C terminus of Ub is first activated in an ATP-dependent reaction by the ubiquitin-activating enzyme (E1) and then transferred to the active site cysteine of a ubiquitin-conjugating enzyme (E2) to form an E2~Ub conjugate (Fig. 1A). Ultimately, E2~Ub transfers the Ub to a lysine

side chain of a substrate in concert with a ubiquitin ligase (E3) (2). Additional Ub may be transferred to a lysine on the first Ub to form poly-Ub chains on a substrate.

Protein modification by a single Ub signals different cellular fates than modification by poly-Ub chains (1). However, the determining factors for whether a substrate will be mono- or polyubiquitinated are not well understood. It is becoming increasingly clear that the E2 enzyme itself can be the determining factor for product formation catalyzed by RING (really interesting new gene)-type E3 ligases (3). For example, the human RING E3 BRCA1/BARD1 can function with 10 different E2s, and the identity of the E2 determines whether mono- or polyubiquitination is observed (4). In contrast, it is the E3 that determines the nature of the product in HECT (homologous to E6AP carboxyl-terminal ligase) E3-catalyzed Ub transfer (5).

The Ubc4/UbcH5 E2 enzymes compose the largest E2 subfamily. Most species have more than one member: there are four members of this subfamily in humans (Ube2D1–4), four in nematodes, 10 in *Arabidopsis thaliana*, and two in yeast (Ubc4 and Ubc5) (6). Even between species, individual members of this subfamily are highly related. For example, UbcH5 shares 80% sequence identity with yeast Ubc4 and Ubc5. Ubc4 and Ubc5 in yeast are 94% identical and are thought to have predominantly redundant cellular functions (7). The Ubc4/UbcH5 E2s are active with many E3s and show robust Ub transfer activity *in vitro*, often making them the E2 of choice for *in vitro* assays with known or putative E3 ligases (8–11). However, the identity of E3s with which these E2s function *in vivo* is largely unknown. Yeast Ubc4 is reported to function with many E3s in yeast, including Rsp5 (12), the anaphase-promoting complex (APC) (13), Pex10 (14), SCF (Skp1/cullin/E-box) (15), and Not4p (16). It is likely that the pathways in which both UbcH5 and Ubc4 participate are widespread and numerous in both yeast and humans.

Structural and mechanistic studies of UbcH5c have revealed that a noncovalent interaction between Ub and the β -sheet surface of UbcH5 (named the “backside” because of its location on the opposite side from the active site) is necessary for poly-Ub chain formation. A backside surface mutation, S22R, specifically disrupts the noncovalent interaction between UbcH5 and Ub and eliminates polyubiquitination but maintains mono-Ub transfer with RING-type E3 ligases (17). The same mutation has no effect on the ability of UbcH5 to transfer Ub with HECT-

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² The abbreviations used are: Ub, ubiquitin; APC, anaphase-promoting complex; TROSY-HSQC, transverse relaxation-optimized spectroscopy-heteronuclear single quantum coherence.

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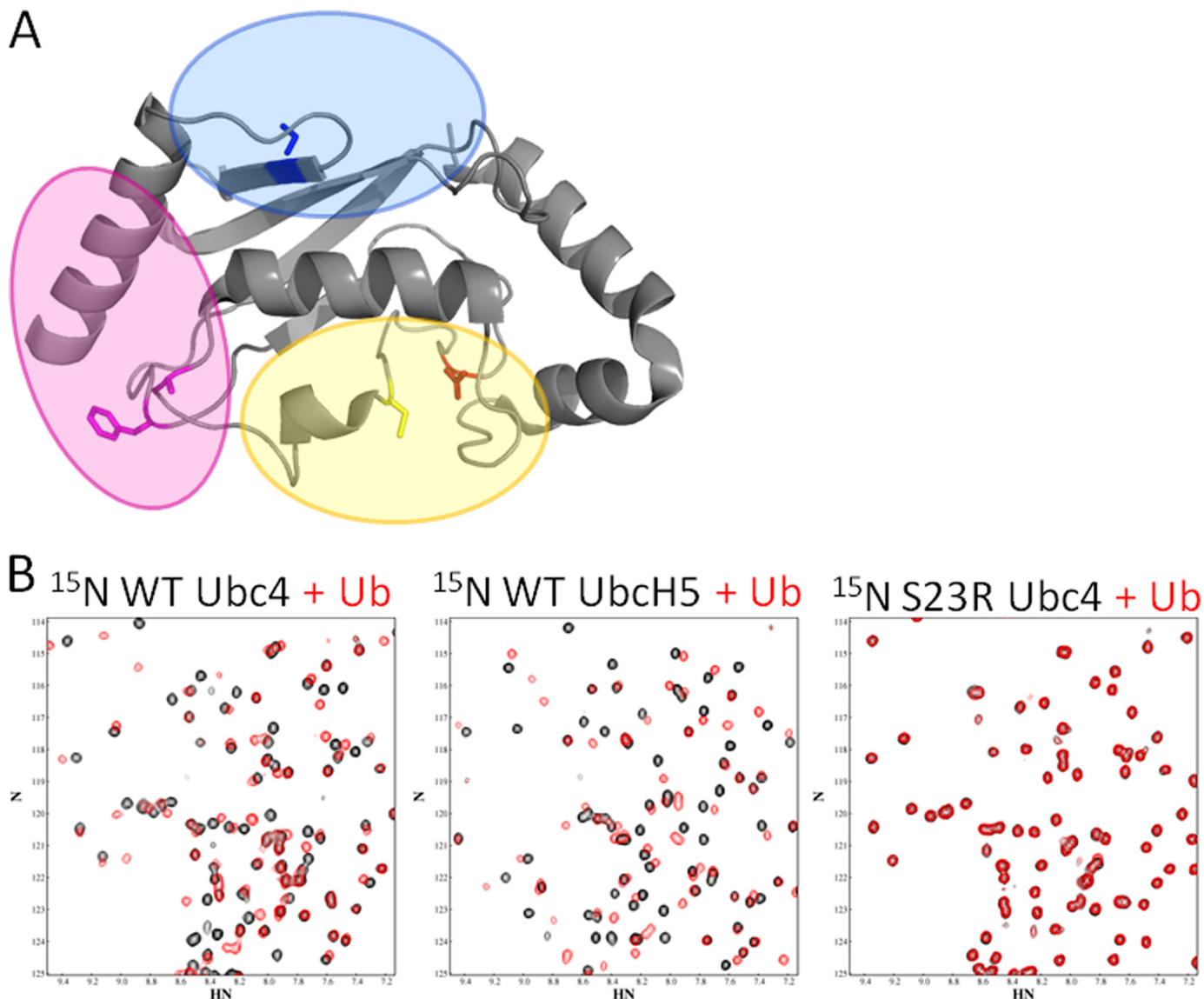


FIGURE 1. *A*, structure of Ubc4 (Protein Data Bank code 1QCQ) (27). The active site is indicated in *yellow*, with the essential active site cysteine Cys-86 in *yellow* stick representation and the conserved Asn-78 in *green* stick representation. The Ub interaction site, or backside, is indicated in *magenta*, with Ser-23 in *magenta* stick representation. The E3-binding surface is indicated in *blue*, with E3-binding residues Ala-97 and Phe-63 shown in *blue* stick representations. *B*, ^{15}N two-dimensional TROSY-HSQC NMR. *Left panel*, ^{15}N -labeled Ubc4 spectrum (*black*) overlaid with a ^{15}N -labeled Ubc4 spectrum in the presence of excess Ub (*red*). *Middle panel*, ^{15}N -labeled Ubc5c spectrum (*black*) overlaid with a spectrum of ^{15}N -labeled Ubc5c in the presence of excess Ub (*red*). *Right panel*, ^{15}N -labeled S23R-Ubc4 spectrum (*black*) overlaid with a ^{15}N -labeled S23R-Ubc4 spectrum in the presence of Ub (*red*). All E2 concentrations were 250 μM .

type E3s.³ This property affords an opportunity to parse out the important functional properties of the Ubc4/Ubc5 family *in vivo* through exploration of the effects of disrupting the Ub backside binding by Ubc4 in a genetically tractable system, *Saccharomyces cerevisiae*.

While investigating the specific functions of Ubc4 *in vivo*, we found that double deletion of yeast *UBC4* and *UBC5* is lethal, contrary to previous reports (7). Upon further investigation of the essential pathway requiring Ubc4/Ubc5, we found that the ability of Ubc4 to form a productive E3/E2~Ub complex is essential. Because the essential pathway catalyzed by Ubc4 and Ubc5 requires the E2 activity, we were able to ask mechanistic questions about Ubc4-mediated Ub transfer in both RING and

HECT E3-catalyzed pathways. Here, we demonstrate that the ability of Ubc4 to noncovalently bind Ub is not essential and provide evidence that the role of Ubc4 is to transfer mono-Ub with RING E3s. Furthermore, we have isolated the essential Ubc4/Ubc5 function to be one catalyzed by a HECT E3, likely the essential HECT E3, Rsp5.

EXPERIMENTAL PROCEDURES

Strains Used in This Study—Yeast strains are listed in Table 1. The exact open reading frames in the *UBC4* and *UBC5* genes were deleted in BSY9 (W303) yeast by PCR product-mediated gene replacement and verified by PCR analysis. A haploid MAT α *ubc4* Δ ::*hphMX4* strain was mated with a haploid MAT α *ubc5* Δ ::*kanMX6* strain to create the heterozygous double deletion *UBC4/ubc4* Δ ,*ubc5* Δ /*UBC5* strain. The diploid strain was

³ C. M. Pickart, P. S. Brzovic, and R. E. Klevit, unpublished data.

TABLE 1

***S. cerevisiae* strains used in this study**

All strains are derived from strain BSY9 with the following genotype: MAT α /MAT α ade2-1oc/ade2-1oc ADE3/ade3 Δ 100 can1-100/can1-100 CYH2s/cyh2r his3-11,15/his3-11,15 leu2-3, 112/leu2-3, 112 trp1-1/trp1-1 ura3-1/ura3-1.

Strain	Relevant genotype	Source
KSY1-2C	MAT α <i>ubc4</i> Δ :: <i>hphMX4</i>	This study
KSY7-1C	MAT α <i>ubc5</i> Δ :: <i>kanMX6</i>	This study
KSY12-6D	URA3:: <i>ubc4-S23R ubc4</i> Δ :: <i>hphMX4 ubc5</i> Δ :: <i>kanMX6</i>	This study
KSY16-3B	URA3:: <i>UBC4 ubc4</i> Δ :: <i>hphMX4 ubc5</i> Δ :: <i>kanMX6</i>	This study
KSY26-2D	URA3:: <i>UBCH5c ubc4</i> Δ :: <i>hphMX4 ubc5</i> Δ :: <i>kanMX6</i>	This study
KSY27-5A	URA3:: <i>ubcH5c-S22R ubc4</i> Δ :: <i>hphMX4 ubc5</i> Δ :: <i>kanMX6</i>	This study
KSY28-8D	URA3:: <i>ubc4-N78S ubc4</i> Δ :: <i>hphMX4 ubc5</i> Δ :: <i>kanMX6</i>	This study

sporulated, and the ability of the meiotic products to grow was assessed. *ubc4* mutants S23R, C86A, N78S, and A97D/F63A and the wild-type *UBC4* gene with its native promoter were cloned into the integrating vector pRS306. The mutants were integrated at *URA3* in a *ubc4* Δ haploid by linearization at *URA3* with restriction enzyme BstBI. The successful *ubc4* transformants were mated with the *ubc5* Δ deletion strain to create a new strain with the following genotype: *UBC4/ubc4* Δ *ubc5* Δ /*UBC5 URA3::ubc4/ura3-1*. The diploid strain was sporulated, and the growth of the meiotic products was assessed.

Growth Assay—Starter cultures of freshly dissected haploid *URA3::ubc4-N78S ubc4* Δ *ubc5* Δ and *URA3::UBC4 ubc4* Δ *ubc5* Δ were grown overnight in YPD at 30 °C. The cultures were used to inoculate YPD cultures in duplicate to an initial reading of ~20 Klett units. Cultures were incubated at 30 °C with shaking, and readings were taken every 30 min until the end of the exponential phase (~125 Klett units). The doubling time was calculated.

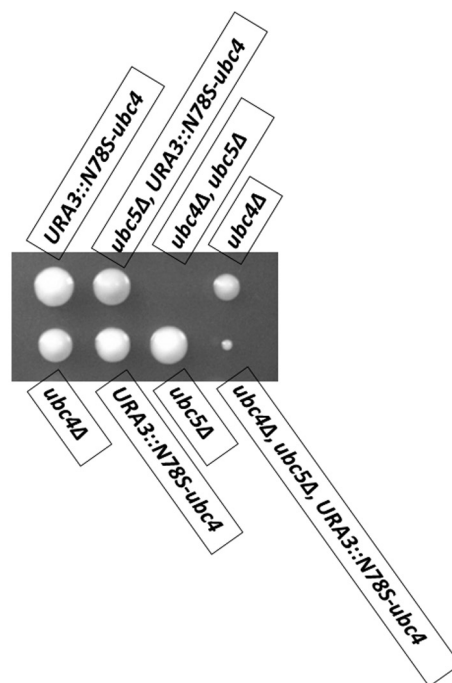
Stress Conditions—Yeast were grown on synthetic minimal medium plus dextrose with various chemical stressors or elevated temperatures to test phenotypic differences between wild-type and S23R-Ubc4 mutant yeast, including heat shock at 37 and 42 °C (7), treatment with 50 μ g/ml proline analog azetidine 2-carboxylate, or treatment with 50 μ M CdCl₂ (18). Yeast cultures were serially diluted from 0 to 1:1000 to grow single colonies sufficient for comparison between wild-type and S23R mutant strains.

NMR Spectroscopy—All NMR samples were prepared in 25 mM sodium phosphate and 150 mM NaCl (pH 7.0), and data were collected at 25 °C with a Bruker 500-MHz magnet. To test Ub binding, 250 μ M ¹⁵N-labeled wild-type E2s UbH5 and Ubc4 were observed in the absence and presence of >10 molar eq of unlabeled Ub. The presence of chemical shift perturbations indicative of a binding event was observed by two-dimensional TROSY-HSQC NMR. To test Ub-binding disruption by the point mutant S23R, two-dimensional TROSY-HSQC NMR spectra of 250 μ M ¹⁵N-labeled S23R-Ubc4 were collected in the absence and presence of 1 molar eq of Ub.

RESULTS

Ubc4 and Ubc5 Perform an Essential Redundant Function—To investigate the role of noncovalent Ub binding in the determination of product formation by the UbcH5 family of E2s, we first deleted both *UBC4* and *UBC5* in yeast to make a strain into which we could reintroduce E2 mutants. Each single deletion was viable with only a minor growth phenotype in the case of *ubc4* Δ (haploid colonies originating from

A



B

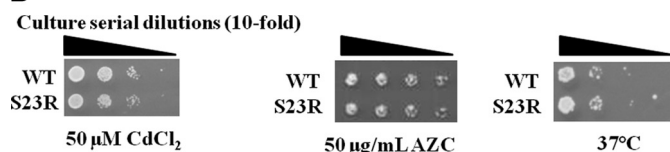


FIGURE 2. *A*, meiotic products produced from tetrad dissection of MAT α *URA3::ubc4-N78S ubc4* Δ ::*hphMX4* mated with MAT α *ubc5* Δ ::*kanMX6*. The *ubc4ubc5* Δ deletion was lethal but rescued by the insertion of *URA3::ubc4-N78S*. N78S-Ubc4 rescued the lethal phenotype, but the yeast cells were severely slow growing as seen when comparing the size of the colonies grown from single spores. *B*, wild-type Ubc4- and S23R-Ubc4-containing yeast. Strains were spotted in 10-fold dilutions from left to right onto synthetic minimal medium plus dextrose containing 50 μ M CdCl₂ or 50 μ g/ml azetidine 2-carboxylate (AZC) or on plates incubated at 37 °C for 48 h and then allowed to recover at 30 °C for 48 h.

a single spore were consistently smaller than the *ubc5* Δ colonies), but the double *ubc4* Δ *ubc5* Δ deletion is lethal (Fig. 2A). The deletions of *UBC4* and *UBC5* were confirmed by PCR. Thus, Ubc4 and Ubc5 act redundantly and perform an essential function in yeast.

Yeast Ubc4 Binds Ub Noncovalently—Although yeast Ubc4 and human UbcH5 are highly similar in both sequence and structure, there are several amino acid differences within the backside Ub-binding site defined in the structure of a complex of UbcH5 and Ub (17). We therefore verified the ability of Ubc4 to bind Ub directly by two-dimensional NMR. ¹H, ¹⁵N TROSY HSQC spectra of ¹⁵N-labeled Ubc4 collected in the absence and presence of excess Ub (>10 molar eq) confirmed an interaction as indicated by the chemical shift perturbations between the two spectra (Fig. 1B, left panel). Furthermore, we confirmed that the backside S23R-Ubc4 mutation fully disrupts the noncovalent interaction between Ub and yeast Ubc4 (*red* and *black* spectra overlay perfectly) as predicted based on its homology to UbcH5 (Fig. 1B, right panel).

S23R-Ubc4 Rescues the Lethal Phenotype of *ubc4* Δ *ubc5* Δ —The lethal phenotype of the double deletion *ubc4* Δ *ubc5* Δ pro-

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TABLE 2

Phenotypes of *S. cerevisiae* strains

NA, not applicable.

Description	Effect of mutation	Phenotype
<i>ubc4</i> Δ	NA	Slight growth retardation
<i>ubc5</i> Δ	NA	Normal
<i>ubc4</i> Δ <i>ubc5</i> Δ	NA	Inviabile
<i>URA3::UBC4 ubc4</i> Δ <i>ubc5</i> Δ	NA	Normal
<i>URA3::ubc4-S23R ubc4</i> Δ <i>ubc5</i> Δ	Eliminate Ub noncovalent binding	Normal
<i>URA3::UBCH5c ubc4</i> Δ <i>ubc5</i> Δ	Human homolog	Normal
<i>URA3::UBCH5c-S22R ubc4</i> Δ <i>ubc5</i> Δ	Eliminate Ub noncovalent binding of human homolog	Normal
<i>URA3::ubc4-C86A ubc4</i> Δ <i>ubc5</i> Δ	Eliminate conjugation of Ub at active site	Inviabile
<i>URA3::ubc4-A97D,F63A ubc4</i> Δ <i>ubc5</i> Δ	Eliminate RING and HECT E3 binding	Inviabile
<i>URA3::ubc4-N78S ubc4</i> Δ <i>ubc5</i> Δ	Eliminate isopeptide formation	Growth retardation

vides a straightforward strategy to investigate the importance of the specific properties of Ubc4 (Fig. 1A and Table 2). Reintroduction of wild-type *UBC4* rescues lethality. Reintroduction of the backside mutant S23R-Ubc4 rescues the lethal phenotype as well. Therefore, the ability of Ubc4 to bind Ub noncovalently at its backside surface is not essential in yeast.

Ubc4 and Ubc5 are reported to play a role in the yeast cellular stress response (7). To further investigate possible effects of eliminating Ub noncovalent binding to Ubc4 *in vivo*, we tested *ubc4*Δ*ubc5*Δ yeast rescued with either wild-type Ubc4 or S23R-Ubc4 under several cellular stress conditions. Stress tests included heat shock at 37 and 42 °C (7), sensitivity to the proline analog azetidine 2-carboxylate, and CdCl₂ sensitivity (18). There were no significant differences under any condition, indicating that the ability of yeast Ubc4 to bind Ub does not contribute to the enzyme's function under stress conditions at a detectable level (Fig. 2B).

Human wild-type UbcH5c and S22R-UbcH5c were introduced into the double deletion strain under the control of the *UBC4* promoter. Both forms of UbcH5 rescued the lethal phenotype, indicating functional complementarity between the E2 orthologs in yeast.

The Essential Function Requires an Active E3/E2~Ub Complex—To further investigate the specific function of Ubc4/Ubc5 that is essential for yeast viability, additional Ubc4 mutations were reintroduced. Insertion of the active site mutant C86A-Ubc4, which is unable to conjugate Ub at its active site, did not rescue the lethal phenotype, indicating that the E2 enzymatic activity of Ubc4 is essential. The double mutant A97D/F63A, which abrogates interactions with both RING and HECT E3s (4, 19), did not rescue the lethal phenotype, indicating that the essential function of Ubc4 involves formation of a productive E2-E3 complex.

The Essential Function of Ubc4 and Ubc5 Requires a HECT-type E3—Ubc4 is active with several E3 ligases, including the HECT E3 Rsp5 (5, 12, 20) and the RING E3 complex APC (13). Although several RING E3s are essential in yeast, only one of the five HECT E3s is essential, Rsp5 (21). To identify the class of E3(s) involved in the essential function of Ubc4/Ubc5, we took advantage of the mechanistic difference between the two. In a RING E3-catalyzed reaction, the E2~Ub thioester bond is directly attacked by the nucleophilic lysine of the substrate to form an isopeptide bond. In a HECT-catalyzed reaction, the E3/E2~Ub pair performs a transthiolation reaction in which the Ub attached to the E2 transfers to the active site cysteine of the HECT E3, which then catalyzes the formation of the isopep-

ptide bond to the lysine of a substrate. To distinguish between a RING- or HECT-dependent essential Ubc4 pathway, we used a mutation that abolishes activity with RING-type E3s but does not affect the transfer of Ub between an E2 and HECT-type E3s. Mutation of the highly conserved near-active site residue Asn-77 (UbcH5 numbering) in all E2s tested eliminates RING-catalyzed Ub transfer (22–24) but does not affect HECT-catalyzed activity, providing a direct way to differentiate between RING- and HECT-catalyzed reactions *in vivo* (22, 23).

Introduction of an N78S-Ubc4 insert rescued the lethal phenotype (Fig. 2A), indicating that the essential function performed by Ubc4/Ubc5 involves a HECT-type E3. Rsp5 is the only essential HECT in yeast and is therefore the most likely E3 candidate for the essential Ubc4/Ubc5 pathway. Additionally, the ability of S23R-Ubc4 to rescue lethality as reported above is consistent with the essential function of Ubc4/Ubc5 being HECT-mediated, as HECT-type E3s are not sensitive to the S22R mutation in UbcH5.³

Rsp5 has been shown to be active with three E2s *in vitro*: Ubc4, Ubc5, and Ubc1 (5). The ability of N78S-Ubc4 to rescue the lethal phenotype also reveals that Ubc1 is not redundant with Ubc4/Ubc5 for the essential Rsp5-mediated pathway *in vivo* because the nonviable *ubc4*Δ*ubc5*Δ strain contains endogenous Ubc1.

Nonessential Functions of Ubc4 with RING E3s Involve Transfer of Mono-Ub but Not Poly-Ub Chain Elongation—Although N78S-Ubc4 rescued the double deletion, it resulted in a severe growth phenotype (haploid colonies originating from a single spore were consistently much smaller than all other colonies) (Fig. 2A). In liquid culture, yeast cells relying on N78S-Ubc4 grew half as fast as a strain relying on wild-type Ubc4 (doubling time of 195 min compared with 104 min) (Fig. 3). Thus, restoration of the essential Ubc4 function (with Rsp5) unmasks a second critical but nonessential Ubc4 function, which must involve a RING-type E3. APC has been shown to require both Ubc4 and Ubc1 for its function in yeast, and of the 13 yeast E2s, only Ubc4, Ubc5, and Ubc1 are active with the APC (13). Ubc4 serves as the “priming” E2 with APC, transferring mono-Ub to substrates that are subsequently polyubiquitinated by APC and another yeast E2, Ubc1 (13). Therefore, the inability of N78S-Ubc4 to perform the first priming Ub transfer to APC substrates could result in slower growth rates, as shown in Figs. 2A and 3.

Ubc1 was shown to be essential for APC function, and the *ubc1*Δ deletion is inviable (13, 25). This result, taken together with the slow growth phenotype of N78S-Ubc4, indicates that

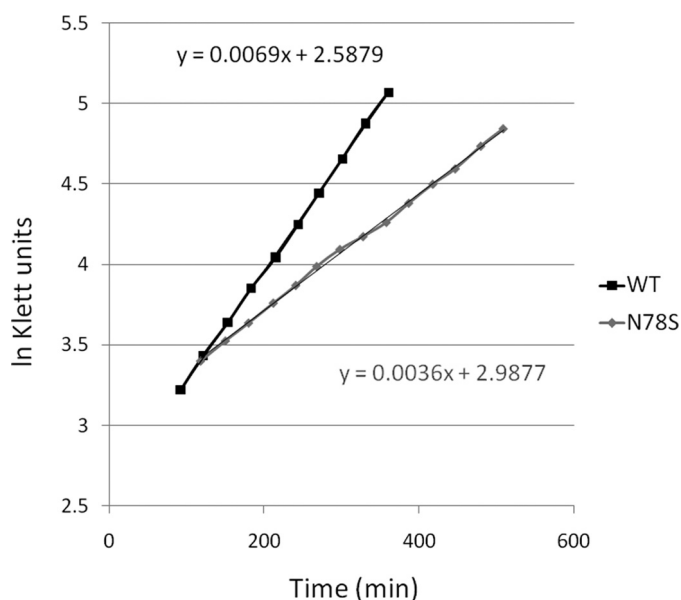


FIGURE 3. Growth curves of wild-type Ubc4 and N78S-Ubc4 yeast demonstrate a nearly 2-fold decrease in doubling time for the mutant. Natural log Klett units were plotted against time. The slope of the data gives the growth constant, k , which can be used to calculate the doubling time by $g = \ln 2/k$. The Ubc4 yeast doubling time averaged over two replicates was 104 min. The N78S-Ubc4 yeast doubling time averaged over two replicates was 195 min.

although Ubc1 can weakly function in place of Ubc4, Ubc4 cannot replace the role of Ubc1 as a chain-elongating E2 in the essential APC pathway. Finally, the lack of an S23R-Ubc4 phenotype is consistent with a model in which Ubc4 transfers only mono-Ub with RING E3s, a function that does not require the ability to bind Ub on the backside site of the E2.

DISCUSSION

Our initial investigation into the role of noncovalent Ub binding by the UbcH5 family of E2s in determining product formation *in vivo* led to several important findings concerning the functionality of Ubc4 with both HECT and RING-type E3s in yeast. First, the E2s Ubc4 and Ubc5 perform an essential redundant function. In a previous study, deletion of both genes in yeast was reported to yield only a heat sensitivity phenotype (7), although another recent study found the double deletion to be lethal (26). We cannot rule out the possibility that the lethal phenotype is strain-specific. However, *ubc4Δubc5Δ* lethality is not surprising given that known Ubc4 pathways include the essential HECT E3 Rsp5- and RING E3 APC-mediated pathways.

Second, the essential Ubc4/Ubc5 pathway requires the ability to form a productive E3/E2~Ub complex. Third, the use of a mechanism-specific E2 mutation, N78S-Ubc4, allowed us to determine that the required E3 belongs to the HECT class of E3 ligases. As the only essential HECT in yeast, Rsp5 is likely the essential HECT E3 pathway catalyzed by Ubc4. Our results suggest that the E2 Ubc1, which has reported activity with Rsp5 *in vitro* (5), cannot rescue the lethal phenotype when the *UBC4* and *UBC5* genes are deleted, indicating that endogenous Ubc1 is not able to participate in the essential Rsp5 pathway *in vivo*.

Fourth, the ability of S23R-Ubc4 to rescue the lethal phenotype indicates that the essential function of Ubc4/Ubc5 does

not require the E2 to bind a second Ub on its backside, a property required for poly-Ub chain elongation with the human homolog UbcH5 and RING E3s. The lack of an S23R phenotype is consistent with a model in which Ubc4 transfers mono-Ub with RING E3s but does not extend poly-Ub chains. For example, Ubc4 and Ubc1 play a critical role with the essential RING-type E3 APC in a sequential mechanism by which Ubc4 transfers the first Ub, and Ubc1 elongates poly-Ub chains onto APC substrates (13). The RING-specific N78S-Ubc4 mutant demonstrates that in the absence of a RING-active Ubc4 or Ubc5, Ubc1 is able to weakly replace the role of Ubc4 to transfer the first Ub onto APC substrates: the yeast cells survive, but are slow growing. However, the *ubc1Δ* deletion is lethal (13, 25), so endogenous Ubc4 cannot replace Ubc1, *i.e.* Ubc4 does not build polyubiquitin chains, unlike its highly homologous human relative, UbcH5.

In summary, we found that the essential function performed by Ubc4 is HECT E3-dependent and that nonessential but critical functions of Ubc4 with RING E3s, such as the APC, do not require Ubc4 to elongate poly-Ub chains. Thus, although yeast Ubc4 has 80% identity to its human ortholog UbcH5c, the data presented here suggest that these homologous E2s play different roles in the process of Ub transfer. Although UbcH5 can both transfer the first Ub and builds poly-Ub chains, the roles of Ubc4 in yeast do not require the ability to extend poly-Ub chains with RING E3s.

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