

Role of Cue1p in Ubiquitination and Degradation at the ER Surface

Thomas Biederer, Corinna Volkwein, Thomas Sommer*

Endoplasmic reticulum (ER) degradation of aberrant proteins is mediated by the ubiquitin-proteasome pathway. Here, a membrane-bound component of the ubiquitin system, Cue1p, was identified. It was shown to recruit the soluble ubiquitin-conjugating enzyme Ubc7p to the ER membrane. In the absence of Cue1p, unassembled and thus cytosolically mislocalized Ubc7p was unable to participate in ER degradation or in the turnover of soluble non-ER proteins. Moreover, ubiquitination by Cue1p-assembled Ubc7p and Ubc6p was a prerequisite for retrograde transport of luminal substrates out of the ER, which suggests that ubiquitination is mechanistically integrated into the ER degradation process.

Formation of ubiquitin conjugates requires three classes of enzymes: the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s or Ubc), and occasionally ubiquitin-protein ligases (E3s). E1 activates the highly conserved polypeptide ubiquitin under adenosine triphosphate (ATP) hydrolysis and transfers it to the Ubc. Both E1 and Ubc form a thioester bond between their active-site cysteine and ubiquitin. Ubc then attach ubiquitin to substrate proteins by an isopeptide bond that marks them for proteolysis by the 26S proteasome (1).

This proteolytic pathway is also involved in ER degradation, a process that helps to prevent malfunctions in the secretory pathway (2). The integral ER membrane protein Ubc6p (3) and the soluble Ubc7p are key components for ER degradation in yeast. Substrates of these enzymes include a mutant form of the multispanning ER membrane protein Sec61p (4), encoded by *sec61-2* (5), and a mutated carboxypeptidase Y (CPY*) of the ER lumen, encoded by *prc1-1* (6, 7). The proteolysis of CPY* occurs outside the ER and is preceded by retrograde transport from the ER lumen back into the cytosol (7). This transport is facilitated by components of the protein translocation machinery (8). We addressed the following issues: Are additional components of the ubiquitin system present at the ER membrane? How is Ubc7p involved in ER degradation? Are retrograde transport and ubiquitination functionally coupled processes?

Initially, we purified membrane-bound components of the ubiquitin system from yeast by their ability to form a thioester bond with ubiquitin in the presence of ATP (9). A wild-type cytosolic fraction II serving as source for cytosolic E1 and Ubc but devoid

of free ubiquitin was used to preload ubiquitin-Sepharose with these components. Two columns were prepared from this ubiquitin-Sepharose. Membrane proteins solubilized with the detergent CHAPS were applied to one column (10); the second column was loaded in parallel with the solubilization buffer and served as a control. Thioester-bonded proteins were eluted from both columns with dithiothreitol (DTT). A prominent protein of 23 kD was detected only in the eluate of the first column (Fig. 1A). This protein was purified to homogeneity by reversed-phase high-performance liquid chromatography (RP-HPLC), and its 29 NH₂-terminal amino acids were sequenced.

The cloned corresponding gene (11) codes for a protein of 203 amino acids with a calculated molecular weight of 22.8 kD. (Fig. 1B). We called this nonessential (11) gene *CUE1* (factor for coupling of ubiquitin conjugation to ER degradation). Cue1p was predicted to have a single NH₂-terminal membrane-spanning segment, and the COOH-terminus of Cue1p was oriented toward the cytosol (12). Cue1p could be extracted from crude microsomes by detergents, but not by urea, salt, or alkaline conditions (13), verifying that it was an integral membrane protein (Fig. 1C).

Sequence comparison revealed no similarity of Cue1p to previously identified components of the ubiquitin system. Its position within the ubiquitin system was determined by a ubiquitin affinity chromatography assay (14). A glutathione-S-transferase-ubiquitin fusion protein (GST-ubiquitin) was noncovalently immobilized on glutathione-Sepharose beads. After incubation of these beads with fraction II, enzymes of the ubiquitin system had formed thioester bonds with GST-ubiquitin. Elution of the bound proteins under nonreducing conditions with SDS preserved these thioester bonds. As a control, we used Ubc1p contained in fraction II. It bound efficiently to GST-ubiq-

uitin in the presence of ATP and could not be eluted with 3.0 M urea but could be eluted with SDS. The molecular weight of a population of Ubc1p was increased by 30 kD, which is the size of GST-ubiquitin. This modification was reversed by reducing conditions and thus represented a thioester-bonded form of Ubc1p (Fig. 2A). Therefore, detection of this molecular weight shift indicated covalent binding of the eluted proteins to GST-ubiquitin. The absence of this shift would indicate noncovalent binding. To analyze the behavior of membrane-bound enzymes of the ubiquitin system, we characterized Ubc6p in the following assay. Bead-bound GST-ubiquitin was preloaded with fraction II in the presence of ATP, and solubilized membrane proteins were subsequently added. Ubc6p behaved similarly to Ubc1p (Fig. 2B). Cue1p derived from wild-type membrane extracts also bound to immobilized GST-ubiquitin but was partially washed off with urea. No forms of Cue1p with an increased molecular weight were detected in the eluates. Binding of Cue1p was completely dependent on ATP (Fig. 2B). Thus, Cue1p neither formed a thioester bond with ubiquitin nor bound to ubiquitin noncovalently. Instead, it was likely that Cue1p was immobilized by noncovalent interaction with a thioester-bound enzyme of the ubiquitin system.

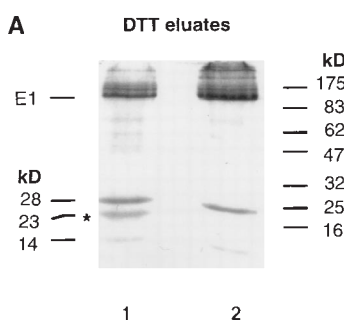
Using preparations from Ubc deletion strains, we identified this component. Purification of Cue1p by GST-ubiquitin affinity chromatography was not altered when we applied solubilized membrane proteins from Δ *ubc6* cells (Fig. 2B). Application of fraction II from Δ *ubc7* cells in the preloading step also had no effect on Cue1p binding (Fig. 2B). But when fraction II and membrane proteins were both derived from Δ *ubc7* cells, Cue1p did not bind to GST-ubiquitin any more (Fig. 2B). Thus, only membrane-bound Ubc7p could immobilize Cue1p. The association of Cue1p and Ubc7p was also demonstrated by immunofluorescence with antibodies specific for Cue1p (15). To this end, we constructed a myc-tagged version of Ubc7p (16). Applying solubilized membrane proteins from cells expressing Cue1p and Ubc7p^{myc} from multicopy vectors, both proteins could be coeluted from the anti-Cue1p immunofluorescence column. The antigenic peptide blocked their purification completely (Fig. 2C). These results confirmed that Cue1p and Ubc7p are assembled into a membrane-bound heterodimer or a larger protein complex. Whether both proteins bind each other directly, or whether additional components are also part of a putative complex, remains to be clarified.

The assembly of Cue1p with Ubc7p suggested that Cue1p determined the enzyme's

Max-Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13122 Berlin, Germany.

*To whom correspondence should be addressed. E-mail: tsommer@mdc-berlin.de

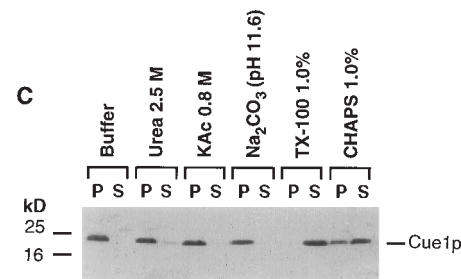
Fig. 1. Purification of Cue1p from crude yeast microsomes by ubiquitin affinity chromatography. (A) SDS-PAGE and Coomassie staining of the DTT eluates of ubiquitin affinity chromatographies corresponding to material purified from 4000 equivalents [defined in (10)] are shown (9). Lane 1 shows the eluate from a column



B

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1  MEDSRLILTL ILVFGVIFLK KFPQSNQHP S AQRLSATGVN AHGRPQGSTQ
51  NALRRTRGRVN GGHEVTTQMV ETVQNLA PNL HPEQIRYSLE NTGSVEETVE
101 RYLRCDEF SF PPGPEP SRAP MCANAAVDNN AAGGGEFNDP RKKNMICAEN
151 LLDKPHVDLN EDMNLSFKD LDIEERKRL L VWQARKNLET KLQSDKDLQS
201 LLT
    
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loaded sequentially with cytosolic fraction II and solubilized membrane proteins. Lane 2 shows the eluate of the control column loaded with fraction II and the solubilization buffer, in which only the cytosolic components of the ubiquitin system were contained. When both fraction II and solubilized membrane proteins had been applied, an additional band, corresponding to Cue1p, was coeluted, which is marked by an asterisk in lane 1. (B) Amino acid sequence of Cue1p (24). Underlined amino acids represent the putative membrane anchor; asterisks mark the sequenced amino acids. (C) Cue1p is an integral membrane protein. Membrane preparations were extracted with buffer containing 2.5 M urea or 800 mM potassium acetate, with Na₂CO₃ (pH 11.6), or with 1.0% detergent and 125 mM potassium acetate (13). P, pellet; S, supernatant.

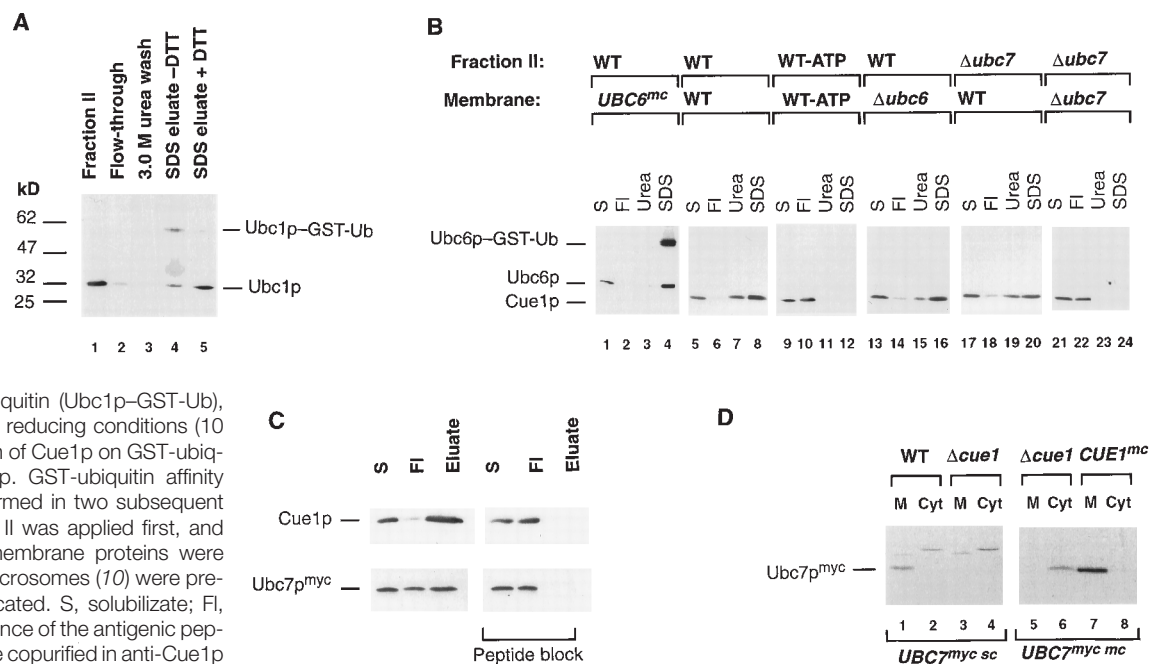
intracellular distribution (Fig. 2D). Ubc7p^{myc} expressed in $\Delta ubc7$ cells from a single-copy vector was detected solely in a crude microsomal fraction. In the absence of Cue1p, Ubc7p^{myc} was not detectable at all. In a $\Delta cue1$ strain, overexpressed Ubc7p^{myc} was found exclusively in the cytosol. Upon overexpression of both Ubc7p^{myc} and Cue1p from multicopy vectors, Ubc7p^{myc} sedimented exclusively with the membranes. Consistently, immunofluorescence microscopy indicated the Cue1p-

dependent localization of Ubc7p to the ER surface and possibly the nuclear membrane (16).

In order to ask if the interaction of Cue1p with Ubc7p at the ER membrane was functionally important, we analyzed the degradation of different substrates in pulse-chase experiments (17). First, the essential multispanning ER membrane protein Sec61p, which is a key component of the protein translocation apparatus (18), was investigated. Wild-type

and various *sec61-2* mutant cells were pulse-labeled at the allele's nonpermissive temperature. Under this condition, mutant Sec61p is short-lived in a wild-type background. After different chase periods, cells were lysed and Sec61p was immunoprecipitated (Fig. 3A). In the absence of Cue1p, proteolysis of Sec61-2p was abrogated as completely as in cells lacking Ubc7p. This observation might have been due to the drastically reduced level of Ubc7p in $\Delta cue1$ cells. To test this, we used cells

Fig. 2. Cue1p is assembled with Ubc7p. (A) Behavior of Ubc1p in GST-ubiquitin affinity chromatography (14). After application of fraction II to GST-ubiquitin Sepharose, a population of the eluted Ubc1p had a lowered mobility in SDS-PAGE as compared with Ubc1p contained in the starting material. This modification represents a thioester bond between Ubc1p and GST-ubiquitin (Ubc1p-GST-Ub), because it was reversed by reducing conditions (10 mM DTT). (B) Immobilization of Cue1p on GST-ubiquitin is mediated by Ubc7p. GST-ubiquitin affinity chromatography was performed in two subsequent rounds of binding. Fraction II was applied first, and thereafter the solubilized membrane proteins were added. Fraction II (9) and microsomes (10) were prepared from the strains indicated. S, solubilize; FI, flow-through. (C) In the absence of the antigenic peptide, Cue1p and Ubc7p were copurified in anti-Cue1p immunoaffinity chromatography (15). Membrane proteins were prepared from a $\Delta cue1 \Delta ubc7$ strain expressing Cue1p and Ubc7p^{myc} from multicopy vectors. (D) Partitioning of Ubc7p to crude microsomal preparations depends on Cue1p. Equal portions of membrane proteins and cytosol were loaded. Extracts were prepared (10) from a $\Delta ubc7$ strain expressing Ubc7p^{myc} from a single-copy vector (lanes 1 and 2) or from a $\Delta ubc7 \Delta cue1$ strain expressing Ubc7p^{myc} either from a single-copy (lanes 3 and 4) or a multicopy vector (lanes 5 and 6) or expressing both Ubc7p^{myc} and Cue1p from multicopy vectors (lanes 7 and 8). Cross-reactions with myc antibodies were detected at longer exposures (left panel). Proteins were expressed under control of their native promoters. Detection was performed after nonreducing [(A) lane 1 through 4 and (B)] or reducing [(A) lane 5, (C), and (D)] SDS-PAGE by immunoblotting. M, membrane proteins; Cyt, cytosol preparation; sc: single-copy ARS-CEN vector; mc, multicopy 2- μ m vector.



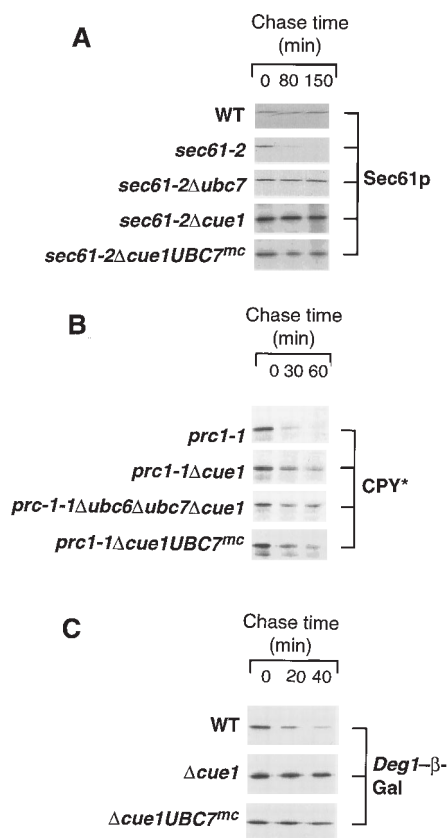


Fig. 3. Cue1p integrates Ubc7p into ER-bound ubiquitination and degradation. mc, multicopy 2- μ m vector. **(A)** Cue1p-assembled Ubc7p determines the half-life of mutant Sec61p. Pulse-chase analysis was performed as described (4). Equal amounts of cells of the indicated strains were shifted to the restrictive temperature of 38°C for 1 hour before the pulse-labeling with 35 S-methionine. At each time point of the chase, equal amounts of cells were lysed and microsomes were prepared. The proteins contained in the microsomes were solubilized and subjected to immunoprecipitation with Sec61p-specific antibodies. **(B)** Cue1p-assembled Ubc7p determines the half-life of CPY*. Pulse-chase analysis of CPY* was performed as described in (A), with the exception that the cells were always kept at 30°C and total lysates were prepared. **(C)** Cue1p-assembled Ubc7p determines the half-life of a fusion protein containing the *Deg1*-degradation signal. *Deg1*- β -galactosidase (β -Gal) was expressed from a multicopy vector, and pulse-chase analysis was done as described (19). All immunoprecipitates were analyzed by SDS-PAGE and fluorography.

lacking Cue1p but containing large amounts of overexpressed Ubc7p in the cytosol. This unassembled Ubc7p was unable to participate in ER degradation of Sec61-2p. In a similar pulse-chase approach, we determined the half-life of CPY* in different isogenic *prc1-1* mutant strains (Fig. 3B). In the absence of Cue1p, CPY* was significantly stabilized. The additional deletion of both *UBC6* and *UBC7* in the Δ *cue1* background had no further influence. Again, cytosolically mislocalized

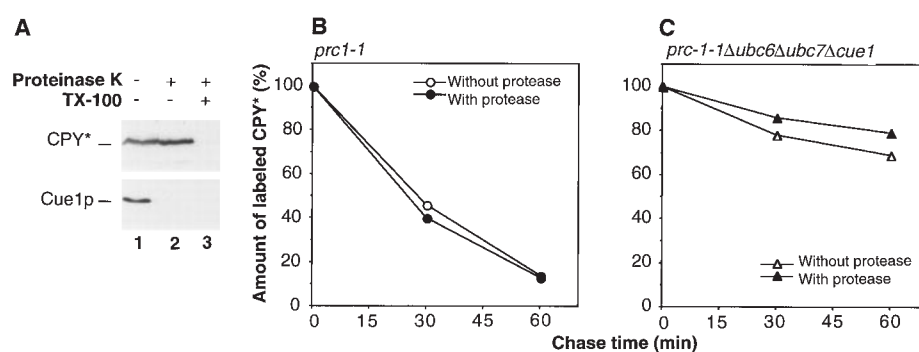


Fig. 4. In vivo analysis of retrograde transport of CPY* by a combined pulse-chase and protease-protection approach (21). **(A)** *prc1-1* cells were lysed with glass beads under conditions that allowed the formation of outside-out vesicles. The crude homogenates remained untreated or were treated with proteinase K in the absence or presence of the detergent Triton X-100. The protease accessibility of CPY* and Cue1p was detected by immunoblotting with specific antibodies. **(B)** No significant fraction of pulse-labeled CPY* was accessible to exogenously added proteinase K during ER degradation. *prc1-1* cells were pulse-labeled, and at each time point of the chase, cells were lysed. These homogenates were treated as described (21). Thereafter the homogenates were solubilized, precipitated with trichloroacetic acid, resuspended, and subjected to immunoprecipitation with CPY*-specific antibodies. Immunoprecipitates were quantitated after SDS-PAGE with a PhosphorImager. **(C)** Loss of ER-bound ubiquitination does not allow detection of accumulated CPY* outside the ER. *prc1-1* Δ *ubc6* Δ *ubc7* Δ *cue1* cells were analyzed as described in (B).

Ubc7p did not destabilize CPY*. Ubc6p and Ubc7p also recognize the *Deg1*-degradation signal of the short-lived soluble transcriptional repressor *mat α 2* (19). Unassembled Ubc7p was inactive in this pathway (Fig. 3C) and also in protection of cells against cadmium toxication (20). Thus, all identified Ubc7p-mediated pathways, including a pathway that originates outside the ER, absolutely required association with Cue1p at the ER.

Next, we analyzed the cellular distribution of CPY* in the presence and absence of the membrane-bound ubiquitination activity in vivo. If retrograde transport and ubiquitination on the ER surface are coupled processes, CPY* would be retained inside the ER in the absence of membrane-bound ubiquitination. If not, it would be transported into the cytosol and thus become sensitive to protease attack. To differentiate between these possibilities, we combined the pulse-chase approach with a protease protection assay (21). The integrity of the prepared microsomes as well as the activity of the protease and the accessibility of proteins on the cytosolic ER surface were controlled in parallel immunoblots (Fig. 4A). The pulse-labeled CPY* of *prc1-1* cells was insensitive to exogenously added proteinase K during its rapid degradation (Fig. 4B), indicating that cytosolic intermediates are rare species. In the absence of membrane-bound ubiquitination (*prc1-1* Δ *cue1* Δ *ubc6* Δ *ubc7*), CPY* was stabilized as expected, but no protease-accessible fraction of the accumulating protein was detectable (Fig. 4C). Thus, we propose that without the surface-bound ubiquitination activity, CPY* remained inside the microsomes, which in-

dicates a lack of retrograde transport.

Cue1p is the first factor known to localize a component of the ubiquitin system. The compartmental targeting of the soluble Ubc7p to the ER caused by assembly with Cue1p is a prerequisite for Ubc7p's function. This represents a new way to locally define substrate specificities of the ubiquitin system. Our observations strengthen the view of the ER surface as a cellular "ubiquitin conjugation platform." Even soluble non-ER proteins containing the *Deg1*-degradation signal are also targeted to this membrane-bound ubiquitination machinery before proteolysis. Because the components of the ubiquitin-proteasome pathway could be preassembled at the cytosolic surface of the ER, such a targeting step could increase the efficiency of ubiquitin-dependent proteolysis. In fact, previous results have indicated an ER localization of E1 and the 26S proteasome (22). Furthermore, retrograde transport of ER degradation substrates seems to be functionally coupled to ubiquitination on the ER surface. Such a coupling could prevent a substrate's escape into the cytosol before targeting it to the 26S proteasome, and the ongoing ubiquitination might contribute to the unidirectionality of the retrograde transport.

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9. Preparation of ubiquitin-Sepharose and cytosolic fraction II, and performance of ubiquitin affinity chromatography, were carried out essentially as described [S. Jentsch, J. P. McGrath, A. Varshavsky, *ibid.* **329**, 131 (1987)]. Ubiquitin-Sepharose was loaded with fraction II in the presence of 2.0 mM ATP; washed repeatedly with 25 mM tris (pH 7.4), 5 mM magnesium chloride, 2.0 mM ATP, and 0.2 mM DTT; and split into two halves. In parallel, microsomes were solubilized on ice with 25 mM tris (pH 7.5), 250 mM potassium acetate, 5 mM magnesium chloride, 0.2 mM DTT, 2.0 mM ATP, and 1.0% CHAPS. One-half of the ubiquitin-Sepharose was loaded with the solubilized membrane proteins, the other with the solubilization buffer. After being washed with 25 mM tris (pH 7.5), 1.0 M potassium chloride, and 1.0% CHAPS, the columns were washed repeatedly with solubilization buffer. Elution of bound material was performed with solubilization buffer without ATP but containing 20 mM DTT. These eluates were further fractionated by RP-HPLC on an Aquapore 300 C8 column (Applied Biosystems) with an acetonitrile gradient. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).
10. Microsome and cytosol preparations were performed essentially as described (4) [S. Panzner, L. Dreier, E. Hartmann, S. Kostka, T. A. Rapoport, *Cell* **81**, 561 (1995)].
11. *CUE1* was isolated from a genomic library (generated in the plasmid pSEY8) by hybridization with oligonucleotides. *CUE1* is located on chromosome XIII (YMR264W). A null allele of *CUE1* was constructed by amplification of 400 base pairs (bp) 5' from the start codon and 650 bp 3' from the stop codon by polymerase chain reaction (PCR) and insertion of the 2.2-kb fragment containing *LEU2*. A second disruption allele carries the *HIS3* gene inserted into the singular Eco RI site of *CUE1*. Null alleles were introduced into yeast cells by the one-step gene disruption method and monitored by Southern (DNA) hybridization [F. M. Ausubel *et al.*, *Short Protocols in Molecular Biology* (Greene Publishing Associates, New York, 1992)] Both disruptions showed no obvious growth defect (23).
12. Cue1p antibodies were raised against the peptide CKLQSDKDLQSLLT (24), corresponding to the COOH-terminus of the protein plus an additional NH₂-terminal cysteine. Affinity purification and immobilization of the antibodies were carried out as described (15). The antibodies detected a single protein of about 23 kD exclusively in crude microsomes of wild-type but not of *Δcue1* cells (23). Protease protection assays of intact microsomes revealed that the COOH-terminus of Cue1p was oriented toward the cytosol (23).
13. Microsomes (10) were extracted as in (3).
14. GST-ubiquitin was expressed and immobilized as described [M. Scheffner, J. M. Huibregtse, R. D. Vierstra, P. M. Howley, *Cell* **75**, 495 (1993)]. Binding of fraction II and CHAPS-solubilized membrane proteins was done on a small scale but essentially as in (9). The first elution was performed with 3.0 M urea and 1.0% CHAPS at room temperature, the second with nonreducing sample buffer containing 4.0% SDS. Bound GST-ubiquitin was not eluted with 3.0 M urea but with SDS (23). Binding of Cue1p to GST-ubiquitin was dependent on the preloading step with fraction II (23). Fractions were analyzed by immunoblotting.
15. Immunoaffinity chromatography was performed essentially as described [D. Görlich, S. Prehn, E. Hartmann, K. U. Kalies, T. A. Rapoport, *Cell* **71**, 489 (1992)]. Solubilization of microsomes and chromatography were performed in 25 mM tris (pH 7.5), 125 mM potassium acetate, 5 mM magnesium chloride, 1.0% Triton X-100 (Fluka, Buchs, Switzerland), 250 mM sucrose, 0.1 mM DTT, bovine serum albumin (1.0 mg/ml), 0.5 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), and chymostatin (5 μg/ml).
16. To tag the NH₂-terminus of Ubc7p with the myc epitope, a unique Sph I site was introduced into *UBC7* by mutagenesis [C. Papworth, J. C. Bauer, J. Braman, *Strategies* **9**, 3 (1996)]. A PCR fragment containing three successive myc epitopes [B. L. Schneider, W. Seufert, B. Steiner, Q. H. Yang, B. Futcher, *Yeast* **11**, 1265 (1996)] was introduced into this site. Ubc7p^{myc} was expressed under control of its native promoter from pRS416 (ARS/CEN) or pRS426 (2 μm). This version of Ubc7p was fully functional in degradation of mutant Sec61p (23). Using Ubc7p^{myc}, we also performed immunofluorescent microscopy. A perinuclear ring-shaped staining was visible with antibodies to myc, which was indistinguishable from that of Kar2p (the ER-luminal homolog of mammalian BiP). Cells that do not express the myc epitope showed virtually no staining (23).
17. Pulse-chase experiments and immunoprecipitation were done as described (4, 19). The wild-type strain used in this study was YWO2 (*mata*, *trp1-1(am)*, *his3-Δ200*, *ura3-52*, *lys2-801*, *leu2-3,-112*). RSY521 (*mata*, *leu2-3,-112*, *ura3-52*, *trp1-1*, *his4-401*, *HOL1-1*) and YFP338 (*mata*, *sec61-2*, *leu2-3,-112*, *ura3-52*, *ade2-3*, *pep4-3*) were kindly supplied by M. Rose and R. Schekman. YTX5 (*mata*, *Δubc6::HIS3*, *trp1-1(am)*, *his3-Δ200*, *ura3-52*, *lys2-801*, *leu2-3,-112*) and YTX93 (*mata*, *sec61-2*, *Δubc7::LEU2*, *leu2-3,-112*, *ura3-52*, *ade2-3*, *pep4-3*) were as described (4). Mutants used in this study [YTX105 (*mata*, *Δcue1::HIS3*, *trp1-1(am)*, *his3-Δ200*, *ura3-52*, *lys2-801*, *leu2-3,-112*), YTX106 (*mata*, *Δubc7::LEU2*, *trp1-1(am)*, *his3-Δ200*, *ura3-52*, *lys2-801*, *leu2-3,-112*), and YTX121 (*mata*, *sec61-2*, *Δcue1::LEU2*, *leu2-3,-112*, *ura3-52*, *ade2-3*, *pep4-3*)] were generated by direct transformation of null alleles (17). Null alleles of *UBC6* and *UBC7* have been described previously (3, 20). The *prc1-1* allele was introduced into haploid wild-type cells as described (7) to generate YTX140 (*mata*, *prc1-1*, *trp1-1(am)*, *his3-Δ200*, *ura3-52*, *lys2-801*, *leu2-3,-112*). Multiple mutants were generated by a second round of transformation [YTX141 (*mata*, *Δcue1::HIS3*, *prc1-1*, *trp1-1(am)*, *his3-Δ200*, *ura3-52*, *lys2-801*, *leu2-3,-112*), YTX142 (*mata*, *Δcue1::HIS3*, *Δubc7::LEU2*, *Δubc6::TRP1*, *prc1-1*, *trp1-1(am)*, *his3-Δ200*, *ura3-52*, *lys2-801*, *leu2-3,-112*)] or by crossing of single mutants and subsequent tetrad dissection [YTX133 (*mata*, *Δcue1::HIS3*, *Δubc7::LEU2*, *trp1-1(am)*, *his3-Δ200*, *ura3-52*, *lys2-801*, *leu2-3,-112*)].
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20. Growth of *Δcue1* and *Δcue1* cells overexpressing Ubc7p from a multicopy vector on minimal media containing cadmium (23) was tested as described in J. Jungmann, H. A. Reins, C. Schobert, S. Jentsch, *Nature* **361**, 369 (1993).
21. Pulse-chase (4) and protease protection assays (3) were done essentially as described. The cells were lysed in 50 mM tris (pH 7.5), 250 mM sucrose, and 10 mM EDTA with one volume of glass beads by four repeated cycles of mixing with a Vortex for 30 s at maximum speed, interrupted by 30-s incubations on ice. In every pulse-chase experiment, the extracts were untreated, treated with proteinase K (0.1 mg/ml), or treated with proteinase K (0.1 mg/ml) and 0.4% Triton X-100 on ice for 15 min. No immunoprecipitable CPY* was detected after treatment with proteinase K and detergent (23).
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24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Multistep Control of Pituitary Organogenesis

Hui Z. Sheng, Kenji Moriyama, Tsuyoshi Yamashita, Hung Li, S. Steven Potter, Kathleen A. Mahon, Heiner Westphal*

Lhx3 and *Lhx4* (*Gsh4*), two closely related LIM homeobox genes, determine formation of the pituitary gland in mice. Rathke's pouch is formed in two steps—first as a rudiment and later as a definitive pouch. *Lhx3* and *Lhx4* have redundant control over formation of the definitive pouch. *Lhx3* controls a subsequent step of pituitary fate commitment. Thereafter, *Lhx3* and *Lhx4* together regulate proliferation and differentiation of pituitary-specific cell lineages. Thus, *Lhx3* and *Lhx4* dictate pituitary organ identity by controlling developmental decisions at multiple stages of organogenesis.

Pituitary organogenesis is driven by a series of developmental decisions controlled by transcription regulators. *Pit-1*/GHF-1 (1–4) and *prophet-1* (5) direct establishment of certain pituitary cell lineages [for review, see (6)]. Targeted mutation of the *Lhx3* gene revealed its role in the specification of most pituitary lineages (7). This study focuses on earlier steps in pituitary organ formation. We analyze the effects of null mutations in *Lhx3* and *Lhx4* (8), a gene

closely related to *Lhx3* (8–11), and show that both genes direct formation of the pituitary gland in mice.

The anterior and intermediate lobes of the pituitary are derived from the oral ectoderm that invaginates to form Rathke's pouch (12). Rathke's pouch gives rise to at least six pituitary-specific cell lineages (6, 12).

Lhx3 and *Lhx4* are expressed throughout the invaginating pouch at day 9.5 of gesta-