

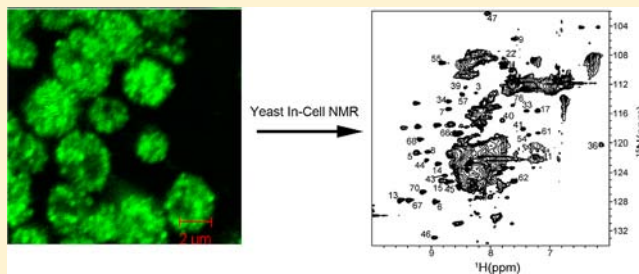
## Structure of Proteins in Eukaryotic Compartments

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### S Supporting Information

**ABSTRACT:** In-cell NMR in the yeast *Pichia pastoris* was used to study the influence of metabolic changes on protein structure and dynamics at atomic resolution. Induction of ubiquitin overexpression from the methanol induced AOX1 promoter results in the protein being localized in the cytosol and yields a well-resolved in-cell NMR spectrum. When *P. pastoris* is grown on a mixed carbon source containing both dextrose and methanol, ubiquitin is found in small storage vesicles distributed in the cytosol, and the resulting in-cell NMR spectrum is broadened. The sequestration of overexpressed proteins into storage vesicles, which are inaccessible to small molecules, was demonstrated for two unrelated proteins and two different strains of *P. pastoris*, suggesting its general nature.



### ■ INTRODUCTION

Elucidating the physicochemical properties of proteins inside living cells is the ultimate goal of biochemists. The dynamics of living cells are complex, and their reactions lie far from equilibrium. As a result, many *in vivo* processes are not adequately represented when the system is reconstituted *in vitro*.<sup>1</sup> In-cell NMR is one technique that allows us to observe selected (target) proteins in the crowded environment of the cytosol and to follow the changes in protein structure and dynamics during different stages of cellular physiology.<sup>2–4</sup>

In-cell NMR spectroscopy in bacterial cells is a well-established methodology<sup>4</sup> made possible by using powerful bacterial promoters that essentially shut down expression of endogenous proteins in favor of heterologous expression. By employing stable isotope labeling and NMR editing techniques, in-cell NMR results in signals from overexpressed proteins that are much stronger than background signals from endogenous proteins.<sup>5,6</sup> Protein overexpression does not work in higher eukaryotes because of the lack of strong promoters capable of producing adequate amounts of isotope-labeled proteins for detecting by in-cell NMR. Yeast, which can be used as a model system for eukaryotic cells,<sup>7,8</sup> does possess strong promoters, comparable to those found in bacterial cells,<sup>9</sup> that can be used for protein overexpression.<sup>10</sup>

Eukaryotic cells are much larger and more complex than bacterial cells. This complexity stems from the increased demand for specialization and stability in higher eukaryotes. Until now, in-cell NMR in eukaryotic cells has relied exclusively on exogenous sources for isotope-labeled protein. For example, protein microinjection is used to introduce isotope-labeled proteins into large *Xenopus laevis* oocytes.<sup>11,12</sup> More recently, phagocytosis of labeled proteins was used to deliver purified target protein into various human cells.<sup>2,13,14</sup> These techniques provide virtually background-free NMR spectra of proteins

inside different types of eukaryotic cells, but although powerful, they are not well-suited for studying the influence of intracellular biochemical processes on protein structure, since the target proteins do not undergo proper cellular processing.

Chemical reactions are impeded by the comparatively large volume of eukaryotic cells, which reduces the effective concentration of the reacting species. One natural solution to increase local concentrations is compartmentalization, which is not observed in prokaryotic cells.<sup>1</sup> For example, metabolic changes result in the sequestration of proteins to specialized compartments, where metabolites are processed and substrates are channeled from one enzymatic activity to another.<sup>15–18</sup> Recent reports suggest that proteins that are not involved in active metabolic pathways are targeted to protein storage bodies, possibly facilitating catabolite repression on the protein level.<sup>15,17,19</sup> Proteins may be closely packed in these compartments or in an aggregated state. The protein structure will necessarily be affected by close packing. Indeed, reports of hormonal proteins in an amyloid state suggest that proteins acquire different conformations under storage. The stored proteins are released and folded into functional form when conditions require.<sup>20–22</sup>

It is important to understand the protein conformations that can exist inside the cell.<sup>3,23</sup> For example, in drug design, drugs that target *in vitro* conformations may not interact with proteins confined to separate compartments or closely packed in protein storage bodies. Furthermore, proteins confined to cellular compartments may not be accessible to small effector molecules, thus thwarting cellular-based screening of otherwise effective drug candidates. In-cell NMR has the potential to reveal the hidden conformations that depend on various

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intracellular environments<sup>2,23</sup> and thereby help design experiments that are more effective at identifying potential drug candidates.

We developed a protocol for isotopic labeling and protein overexpression in the budding yeast *Pichia pastoris* that allows us to obtain high-resolution in-cell NMR spectra of yeast ubiquitin. By changing the metabolic state of the cells we are able to alter the intracellular location of overexpressed protein from cytosol to vesicles. We characterized changes in protein structure due to vesicular localization using immunomicroscopy, cellular fractionation, and in-cell NMR. The general nature of the observed phenomenon was demonstrated by overexpressing  $\beta$ -galactosidase, which was also packed into vesicles when the metabolic state was changed. The work suggests that in-cell NMR of proteins in yeast can be widely used for metabolic profiling experiments.<sup>24,25</sup>

## RESULTS

**Saccharomyces cerevisiae Ubiquitin is Overexpressed in *P. pastoris*.** We cloned *Saccharomyces cerevisiae* and human ubiquitins into plasmid pPIC6. These constructs integrate into the alcohol oxidase 1 (AOX1) locus of the *P. pastoris* strain X-33 genome<sup>9</sup> and places the heterologous gene under the control of the strong, methanol-induced AOX1 promoter (Figure 1A). To overexpress ubiquitin, the integrant was grown in buffered minimal dextrose (BMD). Dextrose is the preferred carbon source for yeast growth and is also a known suppressor of heterologous protein expression.<sup>26,27</sup> Protein overexpression was induced by switching the carbon source in the medium from dextrose to methanol by either adding methanol to BMD

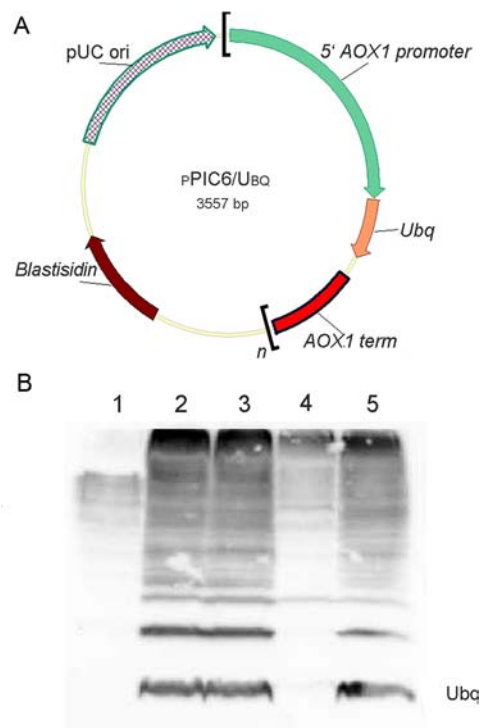
medium (BMDM) or washing the cells and resuspending them in buffered minimal methanol (BMM) medium. Protein overexpression proceeded for two days; additional methanol was added to the induced cultures after the first day of expression.

*S. cerevisiae* ubiquitin was strongly overexpressed in both BMM and BMDM media and was visible on Western blots (Figure 1B). Due to the large number of proteins in eukaryotic hosts, the ubiquitin band could not be easily identified by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We did not observe any improvement in protein overexpression by using multiple integrants of the construct. In spite of 96% sequence identity between *S. cerevisiae* and human ubiquitin, human ubiquitin was only weakly expressed and barely detectable on a Western blot. It is known that some heterologous sequences are not well-expressed in yeast.<sup>28</sup> Subsequently, all experiments were performed using *S. cerevisiae* ubiquitin.

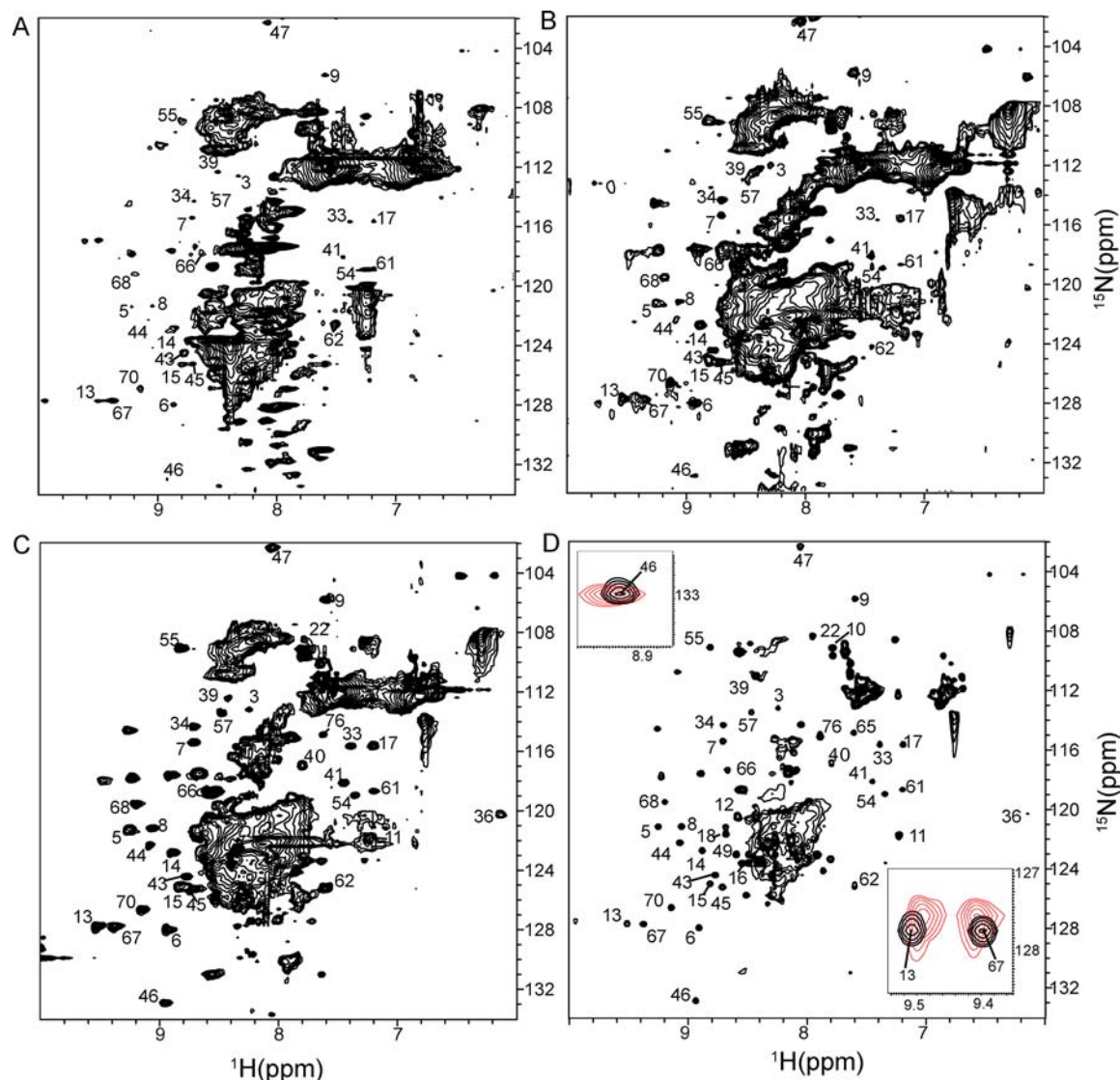
A long-term stability study of the cells overexpressing ubiquitin revealed that over a period of one month, the expression became much lower than that from fresh integrants. We attributed this loss of expression to recombination events in which the heterologous gene is spliced out of the genome. To restore expression levels, the yeast containing heterologous *S. cerevisiae* ubiquitin was reselected on blasticidin plates. Only the clones that produced maximum expression were chosen for further analysis.

**Ubiquitin Is Well Folded and Localized in the *P. pastoris* Cytosol.** The yeast cytosol occupies a majority of the intracellular space and has a relatively low viscosity; ubiquitin tumbling is not impaired by hydrodynamic drag or interactions with plasma cell membranes, thus allowing us to obtain high-quality in-cell NMR spectra. The in-cell <sup>1</sup>H{<sup>15</sup>N}-heteronuclear single quantum coherence (HSQC) NMR spectrum of ubiquitin is evident after 4 h of NMR data acquisition. The in-cell NMR spectrum acquired 24 h postinduction shows only a few well-resolved peaks (Figure 2A). Control experiments indicated that the signals arise exclusively from intracellular protein (Supplementary Figure 1, Supporting Information (SI)). The dispersion of the backbone amide protons from 6.5 to 10 ppm indicates that ubiquitin is well-folded inside *P. pastoris* cells. Background signals from small <sup>15</sup>N-labeled metabolites dominate the spectra in the region from 8 to 8.5 ppm and impede high-resolution analysis of ubiquitin peaks. Most of the peaks are broadened or missing, suggesting that ubiquitin interacts with large intracellular complexes. Indeed, eukaryotic host cells contain many proteins that contain ubiquitin binding domains. These proteins are often localized within large signal transduction complexes and can potentially provide binding sites for overexpressed ubiquitin. The in-cell NMR spectrum of ubiquitin acquired from *Xenopus laevis* cells was similarly broadened.<sup>2,12</sup>

The in-cell NMR spectrum of ubiquitin acquired 48 h postinduction (Figure 2B) contains most of the peaks expected for folded protein suggesting that a significant portion of ubiquitin is free to tumble inside the yeast. By overexpressing ubiquitin for two days most of the ubiquitin binding sites were saturated, allowing free ubiquitin to be observed. Nevertheless, residues, Leu8, Ile44, and Ala46, located in the hydrophobic patch involved in ubiquitin-protein interactions, were substantially broadened. An overlay of in-cell and lysate NMR spectra shows that in-cell ubiquitin amide protons exhibit small but characteristic chemical shift changes relative to those of the



**Figure 1.** In-cell NMR in yeast. (A) pPic6-Ubq plasmid used to integrate ubiquitin into *P. pastoris* strain X-33, and  $n = 1,2$  is the number of ubiquitin multimers. (B) Western blot showing ubiquitin expression in BMM and BMDM. Lane 1, uninduced; lane 2, 24 h induction in BMM; lane 3, 48 h induction in BMM; lane 4, 24 h induction in BMDM; and lane 5, 48 h induction in BMDM.



**Figure 2.** In-cell  $^1\text{H}\{^{15}\text{N}\}$ -HSQC NMR spectra of ubiquitin overexpressed in *P. pastoris*. (A) 24 h postmethanol induction without nitrogen starvation. (B) 48 h postmethanol induction without nitrogen starvation. (C) 48 h postmethanol induction with 6 h nitrogen starvation. (D) Lysate of 48 h sample with nitrogen starvation. Inserts are overlaps of selected peaks from C (red) and D (black).

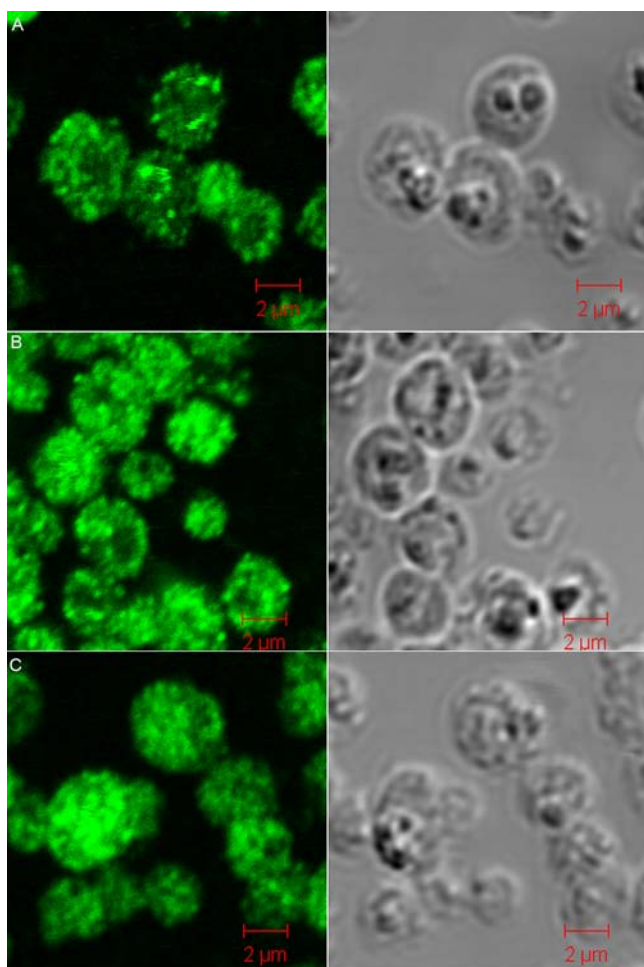
yeast lysate (Figure 2D, insets). The pH of the *P. pastoris* cytosol is 7.2,<sup>29</sup> which is slightly higher than that of the NMR buffer, 7.0. We assumed that the observed changes in chemical shifts are related to the difference between intra- and extracellular pH.

Because NMR peaks from small metabolites overlap ubiquitin peaks and hamper its structural analysis, we attempted to reduce metabolite peak intensities. We reasoned that NMR peaks from  $^{15}\text{N}$ -labeled background metabolites might be reduced by briefly starving the cells of nitrogen so that small metabolites are incorporated into large protein or nucleic acid structures and disappear from the  $^{15}\text{N}$ -edited NMR spectra. To achieve nitrogen starvation, the cells were washed after 24 or 48 h of methanol induction and resuspended into modified BMM medium, in which ammonium sulfate/chloride is replaced by sodium sulfate. After 6 h of nitrogen starvation, cells were prepared for in-cell NMR. The in-cell NMR spectrum of ubiquitin in nitrogen starved cells reveals a dramatically improved NMR spectrum (Figure 2C). The background signal is diminished, and ubiquitin peaks are clearly seen in between

8.5 and 8.0 ppm, a region previously dominated by small metabolite peaks. No changes in chemical shifts are observed between the cells with and without nitrogen starvation, suggesting that this step does not significantly change the interior of the yeast.

Immunofluorescence was used to determine the intracellular localization of overexpressed ubiquitin (Figure 3). The *P. pastoris* cell wall had to be completely removed to facilitate optimal antibody penetration during immunostaining. Due to its small size, ubiquitin tends to wash out of the spheroplasts during and after staining. To eliminate this problem, ubiquitin was immobilized inside the cells with formaldehyde before the cell wall was digested.

The yeast cells used for in-cell NMR are in the active growth stage since 40% of the imaged cells exhibit new buds. Ubiquitin is localized in the yeast cytosol as well as in brightly stained intracellular vesicles (Figure 3, left), which were identified as previously described protein storage bodies.<sup>15</sup> Yeast vacuoles, which are large, dark, and generally well-resolved in differential interference (DI) contrast images (Figure 3, right), play a role



**Figure 3.** Immunofluorescence and DI contrast microscopy of *P. pastoris* overexpressing ubiquitin. (A) 24 h postmethanol induction without nitrogen starvation. (B) 48 h postmethanol induction without nitrogen starvation. (C) 48 h postmixed dextrose/methanol induction. Immunofluorescent staining reveals a distribution of ubiquitin throughout the cytosol and in protein storage bodies. Vacuoles are evident as dark regions in DI contrast images.

in osmoregulation, homeostasis, and degradative processes. Overexpressed ubiquitin is excluded from vacuoles, suggesting that it does not function in protein degradation. There are no obvious differences in the localization of ubiquitin observed in cells grown in either methanol or dextrose/methanol. Control immunofluorescence experiments showed that nitrogen starvation did not affect the cellular localization of ubiquitin (Supplementary Figure 2, SI). We could not differentiate between the in-cell NMR spectra of cytosolic ubiquitin and that of ubiquitin trapped in cytosolic vesicles.

**Yeast Growth on Dextrose Packs Ubiquitin into Protein Storage Bodies.** Nutrients play a critical role in reorganizing the yeast cytosol. Different carbon sources influence protein expression by enhancing the production of metabolic enzymes necessary for optimal growth and suppressing proteins not involved in the metabolism of specific carbon sources. This phenomenon, described as catabolite inactivation, can influence protein expression on the transcriptional, translational, and post-translational level. We took advantage of catabolite inactivation to study ubiquitin compartmentalization in small protein storage bodies.<sup>15,16</sup>

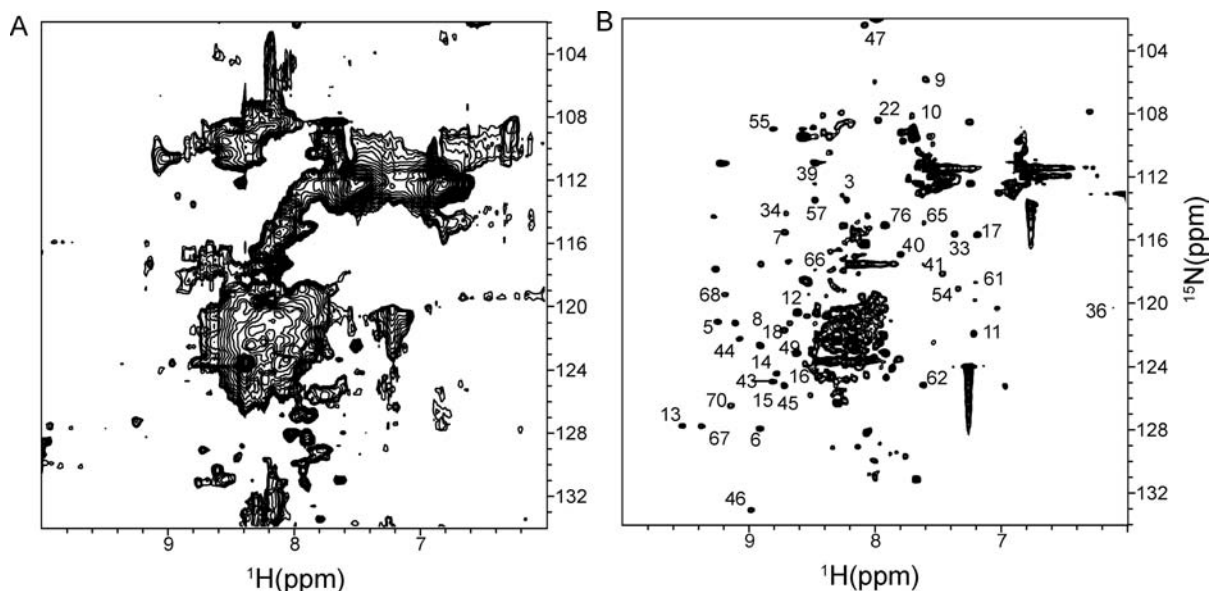
By growing *P. pastoris* on mixed dextrose/methanol carbon sources, the yeast are subject to the conditions of catabolite inactivation. At the same time, transcriptional repression of ubiquitin expression is overcome by methanol.<sup>26,30</sup> Similar experiments using mixed carbon sources were described by Egli et al.<sup>31</sup> We expected that ubiquitin produced under these conditions would be packed into intracellular vesicles, such as protein storage bodies,<sup>15</sup> rather than be released free in the cytosol where it may interfere with optimal metabolism of dextrose. Yeast were grown in <sup>15</sup>N-labeled minimal medium containing both dextrose and methanol. In-cell NMR samples were collected 24 and 48 h after induction with a 6 h nitrogen starvation at the end of the ubiquitin expression period. Western blots revealed that ubiquitin was overexpressed at the same level as when grown in methanol.

Only a few NMR peaks are visible in the in-cell NMR spectra of ubiquitin samples collected after 24 or 48 h inductions, suggesting that protein tumbling is restricted (Figure 4A). Overexpressed ubiquitin is packed into protein storage bodies in the fully or partially unfolded form, similar to proteins packed into bacterial inclusion bodies.<sup>20</sup> To investigate the structure of ubiquitin in the protein storage bodies, the cells, including their intracellular organelles, were lysed, and the NMR spectrum of the supernatant was collected. The supernatant contained sharp ubiquitin peaks (Figure 4B) that are similar to the peaks of yeast-expressed ubiquitin, suggesting that ubiquitin is packed into the vesicles in the folded form.

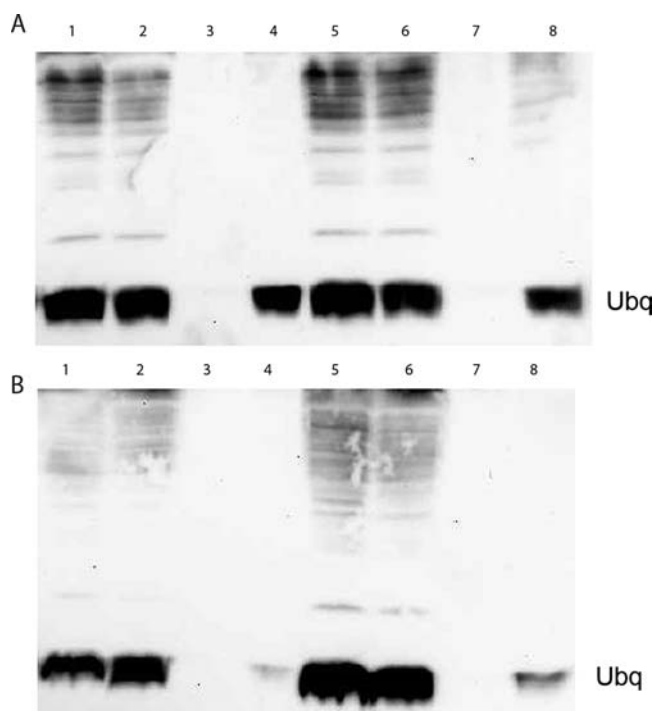
To further prove that ubiquitin is packed in storage bodies, we gently removed the cell walls, ruptured the plasma membranes, and fractionated the cells by using differential velocity centrifugation<sup>32</sup> (Figure 5). This procedure separates cytosolic proteins, the S fraction, from subcellular organelles, such as mitochondria, lysosomes, and microbodies, by sedimenting at 10 000g, to yield the membrane bound P1 fraction, and from smaller, lighter particles, such as ribosomes, endoplasmic reticulum fragments, cell membranes, and microsomes, by sedimenting at 100 000g, to yield the P2 fraction. Ubiquitin expressed by growing yeast in methanol is found in both membrane bound (P1) and the cytosolic (S) fractions, whereas ubiquitin expressed in yeast grown on mixed dextrose/methanol carbon sources is found only in the membrane bound fraction (P1) after 24 h, confirming its vesicular distribution. Control fractionation experiments showed that nitrogen starvation did not affect the cellular distribution of ubiquitin (Supplementary Figure 3, SI).

**Overexpressed  $\beta$ -Galactosidase Is Also Found in Storage Vesicles.** Packing protein into protein storage bodies is a novel feature of catabolite inactivation.<sup>30</sup> However, due to its function, ubiquitin is associated with many vesicular structures. To rule out the possibility that the observed phenomenon was specific to ubiquitin, we used a different strain of *P. pastoris* (GS115) that expresses the bacterial enzyme,  $\beta$ -galactosidase, and assessed its localization and activity in yeast grown using either methanol or mixed dextrose/methanol as carbon sources.

Immunofluorescence and fractionation studies showed that  $\beta$ -galactosidase is localized in protein storage bodies when yeast is grown in mixed dextrose/methanol medium (Supplementary Figures 4 and 5, SI).  $\beta$ -galactosidase expressed in yeast grown in methanol remains cytosolic. These observations confirm the general nature of the observed phenomenon of utilizing storage bodies during catabolite inactivation.



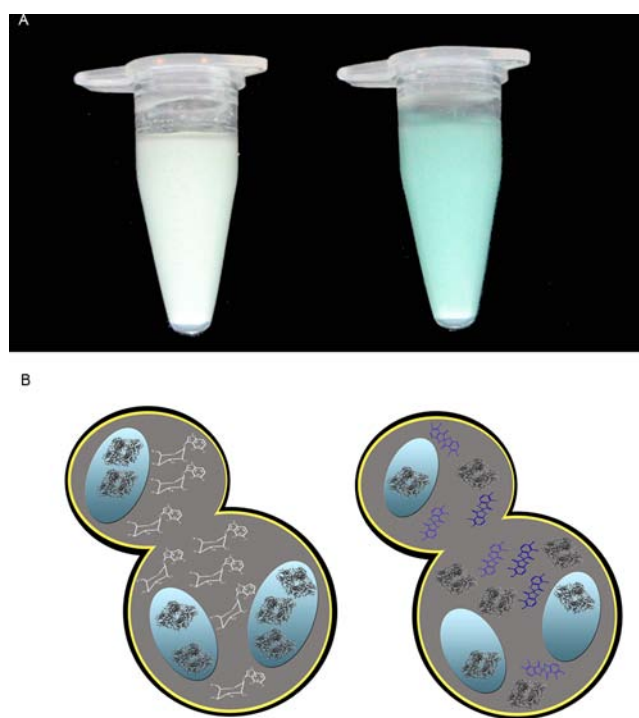
**Figure 4.**  $^1\text{H}\{^{15}\text{N}\}$ -HSQC NMR spectra of ubiquitin overexpressed on mixed dextrose/methanol medium. (A) In-cell 48 h postdextrose/methanol induction with nitrogen starvation. (B) Lysate of A.



**Figure 5.** Cellular localization of ubiquitin depends on growth conditions. (A) Ubiquitin is localized to the cytosol following methanol induction. Lane 1, 24 h total sample (TL); lane 2, 24 h low-speed precipitate (P1); lane 3, 24 h high-speed precipitate (P2); lane 4, 24 h supernatant (S); lane 5, 48 h TL; lane 6, 48 h P1; lane 7, 48 h P2; and lane 8, 48 h S. (B) Ubiquitin is localized to storage vesicles following mixed dextrose/methanol induction. Lane 1, 24 h TL; lane 2, 24 h P1; lane 3, 24 h P2; lane 4, 24 h S; lane 5, 48 h TL; lane 6, 48 h P1; lane 7, 48 h P2; and lane 8, 48 h S. Ubiquitin is localized in the P1 and c S fractions in methanol-induced cultures (lanes 4A and 8A) but is not present in the cytosol of mixed dextrose/methanol-induced cultures (lanes 4B and 8B). Anti-ubiquitin antibody was used to develop ubiquitin Western blots.

The enzymatic activity of  $\beta$ -galactosidase was assessed by adding its substrate, X-gal, directly to the yeast culture (Figure

6). Soluble X-gal diffuses across the plasma membrane into the yeast cytosol and is cleaved by  $\beta$ -galactosidase to yield the insoluble chromogenic product. Yeast containing  $\beta$ -galactosidase overexpressed in methanol exhibit a characteristic blue color after 4 h of incubation with X-gal. Yeast containing  $\beta$ -



**Figure 6.**  $\beta$ -galactosidase sequestered in the storage vesicles cannot cleave substrate. (A) *P. pastoris* with  $\beta$ -galactosidase overexpressed in methanol (right) or mixed dextrose/methanol (left) was exposed to X-gal for 4 h. (B)  $\beta$ -galactosidase inactivation. When overexpressed in methanol (right),  $\beta$ -galactosidase is largely cytosolic and reacts with X-gal, to generate the blue chromogenic reaction product. When overexpressed in mixed dextrose/methanol,  $\beta$ -galactosidase is sequestered into vesicles where it cannot react with X-gal.

galactosidase overexpressed in mixed dextrose/methanol do not exhibit any color change after 4 h of incubation, although after 6 h a slight blue color is evident. The results of these experiments show that  $\beta$ -galactosidase is active in the cytosol. Interestingly, the absence of  $\beta$ -galactosidase activity when localized to protein storage bodies may be due to the inability of the substrate to penetrate into these compartments since the enzyme is active when released from the vesicles (Supplementary Figure 6, SI).

## DISCUSSION

Yeast have been successfully used as a model organism to study basic biological processes inside eukaryotic cells.<sup>33</sup> The yeast two hybrid technique is widely used in proteomics to study protein–protein interactions.<sup>34</sup> These studies are limited since they are performed at the molecular level. In-cell NMR of proteins in yeast allows us to take the next step and visualize proteins and their interactions within the intracellular spaces inside living eukaryotic cells at atomic resolution.

In-cell NMR of eukaryotic cells has been demonstrated for proteins either injected or transported by active diffusion into a human or *Xenopus* cells.<sup>2,11,12</sup> While powerful, these techniques suffer from nonphysiological delivery of labeled proteins, thus limiting applications of in-cell NMR in these cells. We showed that in-cell NMR spectra of isotope-labeled proteins in yeast can be obtained by using the strong AOX induction/expression system in *P. pastoris*. Under our experimental conditions, in-cell NMR spectra can be collected within 6 h with a signal-to-noise ratio of 4:1. Since overexpressed proteins pass through the eukaryotic translational system, they can be properly targeted to physiologically relevant compartments and used to study changes in protein structure under different physiological conditions.

Yeast in-cell NMR has multiple advantages compared to in-cell NMR of bacterial and even other eukaryotic cells: The stability of the yeast cell wall essentially eliminates cell leakage problems that plague bacterial and eukaryotic in-cell NMR studies;<sup>35,36</sup> also, an array of genetic tools are available to control protein overexpression and the well understood genetics of yeast facilitate experimental designs to study protein structures under different physiological conditions.

A straightforward application of in-cell NMR in yeast revealed a high background level of signals that obscures a significant portion of the amide proton spectral range, from 7.5 to 8.5 ppm, a range essential to study protein structures. To resolve this problem we modified the standard sample preparation protocol by including a 6 h nitrogen starvation period following overexpression of the target protein. During this time, small metabolites that contain <sup>15</sup>N isotope are incorporated into either the target protein, thus enhancing the desired NMR signal, or into large cellular structures invisible to in-cell NMR. This dramatic improvement of the NMR signal allowed us to observe all but four NMR resonances of ubiquitin inside yeast cells.

Yeast is known to extensively reorganize its cytosol upon changes of environmental conditions, such as sources of nutrients.<sup>15</sup> Catabolic inactivation results in the inactivation of metabolic pathways of nonpreferred nutrients in favor of preferred ones.<sup>30</sup> We wanted to study changes in protein structure resulting from the metabolic changes of yeast grown in batch culture using different carbon sources. Growing yeast in mixed nutrients, dextrose and methanol, led to dramatic changes in the NMR spectrum of ubiquitin as compared to

those obtained when grown in methanol. When grown using mixed nutrients, ubiquitin NMR peaks largely disappear from the spectrum due to broadening. Our immunomicroscopy and fractionation analyses show that ubiquitin is sequestered into cytosolic vesicles, similar to the previously described protein storage depots.<sup>15,16</sup> Protein sequestration is nonspecific since the unrelated  $\beta$ -galactosidase protein is also sequestered in vesicles when yeast are grown in both dextrose and methanol. This process is unrelated to autophagy since vacuoles are largely devoid of the overexpressed protein.

The NMR spectrum of ubiquitin released from the vesicles by cell lysis is identical to that of folded ubiquitin, suggesting that proteins are maintained in their functional forms within the vesicles. This is different from hormonal proteins in neuronal cells or proteins in bacterial inclusion bodies that possess an amyloid-like structure.<sup>20,22</sup> What is impeding the tumbling of the proteins inside vesicles and broadening the NMR signal? NMR of proteins in small artificially made vesicles is well-studied and provides valuable data to test the effect of molecular confinement on protein structure.<sup>37</sup> The size of the storage vesicles, which was determined to be 20 nm,<sup>16</sup> provides enough space for free ubiquitin to tumble. We propose that the inner surface of the vesicles formed during metabolic changes has a nonspecific affinity for proteins that increases the concentration of the proteins inside the vesicles and slows protein tumbling.

The most important implication of our observation of proteins stored inside cytoplasmic vesicles relates to drug screening and therapy.<sup>38</sup> We showed that  $\beta$ -galactosidase inside the vesicles does not enzymatically cleave the colorimetric substrate (Figure 6 and Supplementary Figure 6, SI). There can be two explanations for this result: The enzyme inside the vesicle is in an inactive form or the substrate cannot penetrate through the vesicular wall. The former is unlikely since we concluded that proteins inside the vesicles maintain proper folding. Therefore the latter is likely to hold true. If the phenomenon of sequestration of cytosolic proteins into storage vesicles is of a general nature, then the specific nutritional conditions present during drug administration may cause drug targets to be unavailable for effective interference, the so-called food–drug interactions.<sup>39</sup> The ability to monitor the cytosolic distribution of protein targets by using in-cell NMR will help optimize conditions for drug–target interactions in drug screening. In-cell NMR in yeast, which allows us to study physiological biomolecular structures and interactions in eukaryotic cells, brings structural analyses one step closer to the coveted goal of understanding life processes at atomic resolution.<sup>40</sup>

## CONCLUSIONS

We described a new mechanism to deliver endogenous, physiologically processed target proteins into eukaryotic cells, which allowed us to visualize atomic resolution protein structures by using in-cell NMR. We used this technique to observe a novel process in which changes in the metabolic state of the cells resulted in target proteins being directed to subcellular compartments allowing changes in protein structure to be assessed as a function of cellular localization. Finally, a critical observation that small molecular substrates are unable to penetrate subcellular vesicles and interact with the target molecule has implications for drug screening.

## EXPERIMENTAL SECTION

**Chemicals and Reagents.** Restriction enzymes were from NEB. All other chemicals used were reagent grade or better.

**Plasmid Construction.** *UBI3* coding for yeast ubiquitin was amplified from *S. cerevisiae* strain BY4741 using the oligonucleotides 5'-TTTTTTCACGTGAAAATGCAGATTTTCGTCAAGACTTTGACC-3' and 5'-TTTTTTCGCGGTCAACCACCTCTTAGCCTTAGCACAAG-3'. The DNA was ligated into pPIC6A (Invitrogen) using the *PmlI* and *SacII* linker sites. pPIC confers blasticidin resistance and contains a pUC replication origin for selection using bacterial cells. Transformants were selected on low-salt LB plates [pH 8] containing 100  $\mu\text{g}/\text{mL}$  blasticidin. The resulting recombinant plasmid, pPIC-ScUbq, places ubiquitin transcription under the control of the alcohol oxidase (AOX1) promoter, which is induced by methanol.

To prepare a dimeric insert, DNA coding for ScUbq was digested from pPIC-ScUbq using the *BglII* and *BamHI* sites. The insert was simultaneously digested with *BglII* and *BamHI* and ligated using T4 ligase in the presence of 300 mM ATP for 2 h at 37 °C (Invitrogen protocol). Ligation between *BglII* and *BamHI* will result in the loss of both restriction sites, while ligation between *BglII* sites (tail-to-tail) and between *BamHI* sites (head-to-head) will maintain the restriction sites; as a result only head-to-tail multimers will remain intact. Dimerized insert was gel purified and ligated into the *BglII* and *BamHI* sites of pPIC (Invitrogen), which had been restriction digested and treated with antarctic phosphatase to prevent recircularization of the vector during ligation. Clones were selected on low-salt LB plates [pH 8] containing 100  $\mu\text{g}/\text{mL}$  blasticidin. The resulting recombinant plasmid, pPIC-2xUbq, places two expression cassettes for ubiquitin, each under the control of the alcohol oxidase (AOX1) promoter, which is induced by methanol.

**Preparation of Electroporation-Competent X-33 Cells.** *P. pastoris* X-33 yeast were grown overnight in YPD at 30 °C. 250 mL of YPD were inoculated with the overnight culture and grown overnight at 30 °C to an  $\text{OD}_{600}$  of  $\sim 1$ . The cells were centrifuged at 1500g for 10 min at room temperature (RT) resuspended in 10 mL of prewarmed (30 °C) YPD containing 2 mL of 1 M Hepes [pH 8.0] and 250  $\mu\text{L}$  of 1 M DTT. The cells were incubated for 30 min at 30 °C with no shaking. Forty mL of ice cold (sterile) water were added, and the cells were centrifuged at 1500g for 10 min at 4 °C. The cells were washed once with 25 mL of ice cold water and washed a second time with ice cold 1 M sorbitol. The cells were finally resuspended with 500  $\mu\text{L}$  of ice cold 1 M sorbitol containing 10% glycerol, aliquoted, and stored at  $-80$  °C or used immediately.

**Transformation of *P. pastoris*.** 0.5–1 mg of pPIC-ScUbq linearized by *SacI* digestion or 10–12 mg of nonlinearized pPIC-2xUbq was added to frozen competent cells and thawed on ice. The cells were transferred to a chilled (4 °C) electroporation cuvette and electroporated at 1500 V using a BTX Model ECM399 electroporator. One mL of ice-cold 1 M sorbitol was added immediately, and the cells were incubated at 30 °C with shaking at 200 rpm for 1 h. One mL of prewarmed (30 °C) YPD was added, and the incubation continued for 1 h, and 200  $\mu\text{L}$  aliquots were plated onto YPD/sorbitol (YPDS) agar supplemented with 300  $\mu\text{g}/\text{mL}$  of blasticidin. The cells were incubated for 2 more hours, after which 2 additional plates were spread with 200  $\mu\text{L}$  of cells. Plates were incubated at 30 °C for 3–5 days. When  $\leq 1$  mg of DNA was used in the transformation, a lawn of “dead” cells appeared, punctuated with large white colonies, which were further tested for the ability to express ScUbq. When larger amounts of DNA were used to transform the yeast, large white colonies were not clearly evident. In these cases, randomly sampled portions of the lawn were streaked onto YPD/blastidicin plates containing 300, 600, and 900  $\mu\text{g}/\text{mL}$  to isolate recombinant clones. *N.b.* plasmids containing two or more expression cassettes cannot be linearized with *SacI* since this enzyme will cut within each cassette, thereby yielding multiple linearized expression fragments.

**Isolating ubiquitin Overexpressing Recombinants.** Single colonies were grown in BMM (85 mM  $\text{KPO}_4$  [pH 6.0], 34 g/L yeast nitrogen base, 76 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1% methanol, 0.4  $\mu\text{g}/\text{mL}$  biotin)

medium with methanol as the sole carbon source. Biotin was added from a 0.2 mg/mL stock in 10 mM NaOH. Cell lysates were prepared, and Western blots performed (see SDS-PAGE Western analyses) to verify overexpression of ubiquitin. The resulting recombinant yeasts, pPIC-ScUbq/X-33 and pPIC-2xUbq/X-33, overexpress ubiquitin from the AOX1 promoter, which is induced by methanol.

**Protein Expression.** Cells were grown overnight at 30 °C in BMD (85 mM  $\text{KPO}_4$  [pH 6.0], 34 g/L yeast nitrogen base, 76 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.5% dextrose, 0.4  $\mu\text{g}/\text{mL}$  biotin). The cells were centrifuged at 2000g for 10 min at RT, washed with 85 mM  $\text{KPO}_4$  [pH 6.0], recentrifuged, and suspended in BMM or BMDM (85 mM  $\text{KPO}_4$  [pH 6.0], 34 g/L yeast nitrogen base, 76 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.5% dextrose, 1% methanol, 0.4  $\mu\text{g}/\text{mL}$  biotin) at an  $\text{OD}_{600}$  of 0.6. Methanol-induced protein overexpression proceeded for up to 48 h at 30 °C. Samples were taken at 24 and 48 h postinduction. Additional methanol was added to a concentration of 0.5% from a 10% stock solution after 24 h. For isotopic labeling, the ammonium sulfate in BMM or BMDM was replaced with 76 mM  $\text{Na}_2\text{SO}_4$  and 2 g/L  $^{15}\text{N}$ - $(\text{NH}_4)_2\text{Cl}$ . The  $\text{OD}_{600}$  of cell cultures was  $\sim 4$  and  $\sim 6.5$  after 24 and 48 h of protein overexpression, respectively, for cultures containing dextrose and  $\sim 2.5$  and  $\sim 4$  after 24 and 48 h, respectively, for cultures grown in methanol. Some samples were subjected to nitrogen starvation prior to harvesting: Cell samples were centrifuged at 2000g for 10 min at RT and resuspended in an equal volume of modified BMM or BMDM, in which ammonium sulfate/chloride was replaced with 76 mM sodium sulfate and incubated for an additional 6 h at 30 °C.

To overexpress  $\beta$ -galactosidase, *P. pastoris* strain GS115/pPICZ/*lacZ* (Invitrogen) was used. This strain expresses  $\beta$ -galactosidase from the methanol-induced AOX1 promoter. The overexpressed protein is fused at the C-terminus to a myc epitope and a polyhistidine tag. Cells were grown as above.

**$\beta$ -Galactosidase Activity Assay.** Cells were grown overnight at 30 °C in BMD. The cells were centrifuged at 2000g for 10 min at RT, washed with 85 mM  $\text{KPO}_4$  [pH 6.0], recentrifuged, and suspended in BMM or BMDM at an  $\text{OD}_{600}$  of 0.6. After 24 h of protein expression, X-gal and methanol were added to the cultures to a final concentration of 80  $\mu\text{g}/\text{mL}$  and 2%, respectively, and allowed to incubate for up to 6 h. Cell cultures in which  $\beta$ -galactosidase was overexpressed turned blue. Cell cultures in which  $\beta$ -galactosidase was not overexpressed remained white.

**In-Cell NMR Spectroscopy.**  $^{15}\text{N}$ -isotope-labeled ScUbq was prepared by growing X-33/ScUbq in BMM containing  $^{15}\text{N}$  ammonium chloride and methanol as the sole nitrogen and carbon sources, respectively. From 24 and 48 h cell cultures, 100 mL samples both with and without nitrogen starvation were centrifuged at 2000g for 10 min at RT and washed once with 85 mM  $\text{KPO}_4$  [pH 6.0] and once with 85 mM  $\text{KPO}_4$  [pH 6.0] containing 10% glycerol. The final sample was centrifuged, and the pellet was stored at  $-80$  °C and used within 2 days. To prepare cells for in-cell NMR spectroscopy, the pellet was washed with 10 mM  $\text{KPO}_4$  [pH 7.0] buffer and resuspended in 250  $\mu\text{L}$  of 10 mM  $\text{KPO}_4$  [pH 7.0] buffer, 100  $\mu\text{L}$  of  $\text{D}_2\text{O}$ , and 50  $\mu\text{L}$  of glycerol. The resultant slurry was transferred to an NMR tube, and the cells were packed down by centrifuging the NMR tube for 20 min at 21g.  $^1\text{H}\{^{15}\text{N}\}$ -HSQC NMR experiments were performed to provide superior sensitivity due to the favorable relaxation characteristics of  $^{15}\text{N}$  nuclei.<sup>41</sup> NMR data were collected at 303 K on a 700 MHz Avance II Bruker NMR spectrometer equipped with a cryoprobe. After NMR experiments, cell viability, checked by plating and colony counting, was estimated to be  $>90\%$ .

To prepare lysate samples, the cells were physically lysed: First, the cells were freeze–thawed five times by using a dry ice/ethanol bath. Second, the cells were sonicated three times for 1 min each using 0.3 s pulses from a Branson digital sonifier at 60% maximum power. The lysed cells were centrifuged at 12 100g for 20 min at RT, and  $\text{D}_2\text{O}$  was added to the supernatant to a final concentration of 20%. The  $^1\text{H}\{^{15}\text{N}\}$ -HSQC spectrum of each sample was acquired at 303 K using a Bruker 700 MHz spectrophotometer.

**SDS-PAGE Western Analyses.** Ubiquitin or  $\beta$ -galactosidase was expressed in *P. pastoris* yeast cells, as described in the Protein

Expression section. After 24 and 48 h of protein expression, 1 mL samples were obtained, and the cells were pelleted and stored at  $-20^{\circ}\text{C}$ . Cells were suspended in 200  $\mu\text{L}$  of yeast cracking buffer, 40 mM Tris-HCl [pH 6.8], 8 M urea, 5% SDS, 0.1 mM EDTA, 0.4 mg/mL bromophenol blue, containing 0.1%  $\beta$ -ME, and 1 tablet of protease inhibitor cocktail (Roche Molecular) per 50 mL and incubated at  $70^{\circ}\text{C}$  for 10 min. The solution was vortexed vigorously and then centrifuged at 12 000g for 1 min at RT. The samples were electrophoresed at 100 V for 1 h on a 16% (ubiquitin) or 10% ( $\beta$ -galactosidase) gel in Tris-glycine-SDS buffer using a Mini-PROTEAN 3 (Bio-Rad) apparatus. The gel was washed with Tris-glycine buffer containing 20% methanol and transferred to a 0.2  $\mu\text{m}$  nitrocellulose membrane (Bio-Rad) in Tris-glycine buffer containing 20% methanol using the Mini-PROTEAN 3 (Bio-Rad). The membrane was blocked for 2 h at RT with 1% bovine serum albumin (BSA) in Tris buffered saline containing 0.05% Tween 20 (TBS-T) and incubated overnight at  $4^{\circ}\text{C}$  with primary antibody. For ubiquitin, the primary antibody was rabbit polyclonal antiubiquitin IgG (Millipore). For  $\beta$ -galactosidase, the primary antibody was rabbit polyclonal anti-c-myc (QED Bioscience). The membrane was washed with TBS-T and incubated for 1 h at RT with goat antirabbit IgG (H+L) HRP conjugate secondary antibody (Millipore). The membrane was washed once with TBS-T and then with TBS. Western blots were developed by incubating the membrane in SuperSignal West pico chemiluminescent substrate (Thermo) for 5 min at RT, and the resulting chemiluminescence imaged by using a Bio-Rad ChemiDoc XRS equipped with Quantity One software.

**Cell Fractionation.** Ubiquitin or  $\beta$ -galactosidase was expressed in *P. pastoris* yeast cells, as described in the Protein Expression section. After 24 and 48 h of protein expression, 500 mL samples were obtained, the cells were washed once with 85 mM  $\text{KPO}_4$  [pH 6.0] and once with 85 mM  $\text{KPO}_4$  [pH 6.0] containing 10% glycerol. The cells were pelleted, stored at  $-80^{\circ}\text{C}$ , and used within 2 days. Thawed cells were sequentially washed (40 mL) with 85 mM  $\text{KPO}_4$  [pH 6.0], glass distilled water (GDW) and spheroplast buffer (SB), 40 mM  $\text{KPO}_4$  [pH 7.4], and 1.4 M sorbitol. The cells were spheroplasted in 20 mL of SB containing 1%  $\beta$ -ME and 20  $\mu\text{L}$  of zymolyase (MPBiomedicals), from a 250 U/g stock prepared in SB buffer at RT, for  $\sim 1$  h or until the  $\text{OD}_{600}$  of the solution decreased by 75%. The spheroplasts were centrifuged at 3270g for 5 min, washed 4 $\times$  with 20 mL of SB, and resuspended in 10 mL of HB buffer, 30 mM triethanolamine [pH 7.2], 1.2 M sorbitol, 1.3 mM EDTA, and 240 mg/L AEBSF containing 1 tablet of protease inhibitor cocktail (Roche Molecular) per 50 mL. The suspended cells were transferred to a dounce homogenizer, homogenized with 10 strokes, and recentrifuged. The pellet was resuspended in 10 mL of HB buffer and homogenized with 20 strokes and recombined with supernatant from the first douncing to produce 20 mL of total solution (TL). For 10 min at  $4^{\circ}\text{C}$ , 18 mL of TL were centrifuged at 19 520g to yield sample pellet P1. The resulting supernatant was centrifuged at 141 000g for 1 h at  $4^{\circ}\text{C}$  to yield two samples: pellet P2 and supernatant S. The P1 and P2 samples were each resuspended in 20 mL of lysis buffer, 30 mM triethanolamine [pH 7.2], 1.2 M sorbitol, and 1.3 mM EDTA.

Western blots were used to detect ubiquitin present in the TL, P1, P2, and S samples. The samples were mixed with an equal volume of Laemmli buffer,<sup>42</sup> and 5  $\mu\text{L}$  were loaded into each lane. The solutions were denatured at  $70^{\circ}\text{C}$  for 10 min prior to electrophoresis. Western blotting was performed as described in the SDS-PAGE Western section.

**Immunofluorescence Microscopy.** Ubiquitin or  $\beta$ -galactosidase was expressed in *P. pastoris* as described in the Protein Expression section. After 24 and 48 h of protein expression, 30 mL samples were mixed with 4 mL of 37% of formaldehyde for 1 h at  $28^{\circ}\text{C}$ . At 12 000g at RT for 1 min, 5 mL were centrifuged. The cells were washed with 1 mL of Tris-EDTA buffer (TE), 100 mM Tris-HCl [pH 8.0], and 100 mM EDTA. The cells were resuspended at an  $\text{OD}_{600}$  of  $\sim 3$  in TE containing 0.3%  $\beta$ -ME and incubated for 45 min at  $28^{\circ}\text{C}$ . The cells were centrifuged and resuspended in 1 mL of buffer S, 10 mM PIPES [pH 6.5], 1 M sorbitol, containing 0.3%  $\beta$ -ME. Twenty-five U of zymolyase was added and the cells were incubated for 60 min at  $28^{\circ}\text{C}$ .

The cells were washed 4 $\times$  in 1 mL of S buffer and suspended in 500  $\mu\text{L}$  of phosphate buffered saline (PBS), 2 mM  $\text{KH}_2\text{PO}_4$ /10 mM  $\text{Na}_2\text{HPO}_4$  [pH 7.4], 2.7 mM KCl, and 137 mM NaCl.

Each well of a 6 mm, 10 well slide was treated with 15  $\mu\text{L}$  of 1 mg/mL poly-D-lysine in 0.15 M sodium borate [pH 8.3] and incubated overnight at RT in a moist incubation chamber to prevent drying. The slides were rinsed with water and dried thoroughly before using. Into each well, 20  $\mu\text{L}$  of spheroplasted cells were spotted and allowed to settle for 10 min. The slides were rinsed with PBS and dried thoroughly. Each well was incubated with 20  $\mu\text{L}$  of 0.01% Triton X for 6 min, rinsed with PBS, incubated with 20  $\mu\text{L}$  of blocking solution, and 1% BSA in PBS for 2 h at RT and rinsed with PBS. The wells were incubated overnight at  $4^{\circ}\text{C}$  with primary antibody, diluted 1:500 in 0.1% BSA/PBS. The primary antibodies used were rabbit polyclonal IgG anti-ubiquitin (Millipore) and rabbit polyclonal antibody anti-c-myc (QED Bioscience). The slides were washed with PBS and incubated for 2 h at RT with the secondary antibody, goat antirabbit IgG (H+L) conjugated with Alexafluor 488, in 0.1% BSA/PBS.

The slides were rinsed with PBS and 5  $\mu\text{L}$  of SlowFade Gold antifade reagent (Invitrogen) were added to each well. The wells were covered and sealed. The finished slides were imaged using a Zeiss LSM 510 laser scanning confocal microscope with a 63 $\times$  oil-immersion objective. Fluorescence images were obtained by using an argon laser emitting at 488 nm. DI contrast images were obtained by using a mercury lamp.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Leakage test; immunofluorescence and DI contrast microscopy of *P. pastoris* overexpressing ubiquitin grown on methanol with nitrogen starvation; influence of nitrogen starvation on cellular distribution of ubiquitin overexpressed in methanol; immunofluorescence and differential interference contrast microscopy of *P. pastoris* overexpressing  $\beta$ -galactosidase; dependence of cellular distribution of  $\beta$ -galactosidase on growth conditions; and  $\beta$ -galactosidase is enzymatically active when released from the protein storage bodies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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