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G Protein Mono-ubiquitination by the Rsp5 Ubiquitin Ligase*[S]

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Emerging evidence suggests that ubiquitination serves as a protein trafficking signal in addition to its well characterized role in promoting protein degradation. The yeast G protein α subunit Gpa1 represents a rare example of a protein that undergoes both mono- and poly-ubiquitination. Whereas mono-ubiquitinated Gpa1 is targeted to the vacuole, poly-ubiquitinated Gpa1 is directed instead to the proteasome. Here we investigate the structural requirements for mono- and poly-ubiquitination of Gpa1. We find that variants of Gpa1 engineered to be unstable are more likely to be poly-ubiquitinated and less likely to be mono-ubiquitinated. In addition, mutants that cannot be myristoylated are no longer mono-ubiquitinated but are still poly-ubiquitinated. Finally, we show that the ubiquitin ligase Rsp5 is necessary for Gpa1 mono-ubiquitination *in vivo* and that the purified enzyme is sufficient to catalyze Gpa1 mono-ubiquitination *in vitro*. Taken together, these data indicate that mono- and poly-ubiquitination have distinct enzyme and substrate recognition requirements; whereas poly-ubiquitination targets misfolded protein for degradation, a distinct ubiquitination apparatus targets the fully mature, fully myristoylated G protein for mono-ubiquitination and delivery to the vacuole.

Cell surface receptors transmit external sensory and chemical stimuli to the intracellular space via G proteins. In yeast, G protein-coupled receptors initiate a signaling cascade that leads to new gene transcription, cell cycle arrest, morphological changes, and eventually mating. Mating is the process by which **a** and α cells fuse to form an **a**/α diploid cell. This mating process is initiated by cell type-specific peptide pheromones; haploid **a**-type cells secrete **a**-factor pheromone, which binds to receptors on the surface of α-type cells, whereas α-type cells secrete α-factor that acts exclusively on **a**-type cells (1).

Transmission of the pheromone signal requires a receptor, a heterotrimeric G protein complex including α, β, and γ subunits, and a series of protein kinases as well as a transcription factor. As with other G protein systems, pheromone binding to its receptor stimulates an exchange of GDP for GTP on the Gα subunit (Gpa1), which induces a conformational change and dissociation from the Gβγ (Ste4/18) subunit complex (1, 2). The dissociated Gβγ in turn transmits and amplifies the signal to effector proteins that produce an intracellular response. Signaling persists until GTP is hydrolyzed to GDP by the Gα subunit, which promotes its re-association with Gβγ. Thus, the Gα subunit serves primarily to regulate the levels of free Gβγ. Indeed, cells lacking the Gα subunit gene cannot sequester Gβγ, resulting in permanent activation of the mating response and cell cycle arrest (3, 4). Recent evidence suggests that the GTP-activated Gα subunit can also modulate signaling through a direct interaction with phosphatidylinositol 3-kinase in yeast (5).

Given their function as mediators between cell surface receptors and intracellular effectors, G proteins are well positioned to serve as targets of regulation. Indeed, Gα proteins undergo a variety of modifications including (i) phosphorylation, (ii) myristoylation, (iii) palmitoylation, and (iv) ubiquitination (6–8). Of these modifications, the least characterized is phosphorylation. Gpa1 is phosphorylated at serine 200 (9), and phosphorylation in this case appears to preclude myristoylation.[3] However, the responsible kinase and the upstream stimulus have not been identified. Another major modification is *N*-myristoylation, which is an irreversible process that occurs co-translationally on most Gα proteins including yeast Gpa1. Myristoylation of Gpa1 is required to promote proper plasma membrane localization and heterotrimeric G protein complex formation (10). Yeast mutations in the *N*-myristoyltransferase (*NMT1*) gene or mutations that replace the myristoylated glycine-2 residue of Gpa1 result in constitutive activation of the pheromone response pathway (11). A third modification is palmitoylation. Nearly all Gα proteins including Gpa1 are palmitoylated (7, 12, 13). Palmitoylation of Gα subunits is post-translational, reversible, and largely dependent on prior myristoylation and delivery to the plasma membrane (14, 15). Finally, ubiquitination is the process by which a protein is covalently modified by addition of a single (mono) or polymeric (poly) form of ubiquitin (16). Generally speaking, mono-ubiquitinated substrates are recognized by a cellular trafficking machinery that transports the target protein to the vacuole (the yeast counterpart to the lysosome), where it is degraded by resident vacuolar proteases (17). Poly-ubiquitinated substrates, on the other hand, are recognized and degraded by the proteasome protease complex (18).

Emerging evidence suggests that ubiquitination acts largely as a trafficking signal in addition to its well documented role as a degradation signal (17, 19). Restated, a protein that undergoes mono- or poly-ubiquitination must first translocate to either the vacuole or the proteasome before it can be degraded. This

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.
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trafficking function is well documented for mono-ubiquitinated plasma membrane proteins such as Ste2 and Ste3 (17, 20, 21). Poly-ubiquitination has likewise been demonstrated to act as a trafficking signal for some plasma membrane proteins. For example, the mammalian V2 vasopressin receptor and the β_2 adrenergic receptor are internalized and degraded in a poly-ubiquitin-dependent manner (22, 23). In some instances such as the insulin-like growth factor I receptor, both lysosomal and proteasomal pathways are employed, further suggesting that either mono- or poly-ubiquitination can serve as a trafficking signal for membrane-bound proteins (24).

Ubiquitination plays a particularly important role in regulation of the yeast mating response. Of the pheromone signaling components, either mono- or poly-ubiquitination has been reported for the cell surface receptors Ste2 and Ste3 (20, 21), the G protein α subunit Gpa1 (25, 26), the GTPase accelerating protein Sst2 (27), and the downstream protein kinase Ste7 (28, 29). Mono-ubiquitination of Ste2 and poly-ubiquitination of Ste7 and Sst2 are induced by pheromone, and these modifications are thought to represent feedback loops leading to pheromone desensitization (in the case of Ste2 and Ste7) and resensitization (in the case of Sst2).

Whereas a large number of proteins undergo ubiquitination, only a few are known to undergo both mono- and poly-ubiquitination. Prominent examples include the human tumor suppressor p53 (30), proliferating cell nuclear antigen (31), and the general amino acid permease Gap1 (32). Such examples have the potential to reveal the regulatory mechanisms and functional consequences for each type of ubiquitin modification, each acting on the same protein substrate.

Gpa1 has been shown to undergo both mono- and poly-ubiquitination, and available evidence suggests that these two processes are distinct (25, 26). In yeast lacking Pep4, an aspartyl protease required for activation of vacuolar proteases (33), Gpa1 accumulates in the mono-ubiquitinated form and becomes visibly concentrated in vacuoles (26). In contrast, yeast harboring a temperature-sensitive mutation in Cim3, a subunit required for proteasomal protease activity (34), exhibit elevated Gpa1 poly-ubiquitination and accumulation in the cytoplasm (26). Here we show that Gpa1 mono- and poly-ubiquitination are catalyzed by different ubiquitin ligases, each having distinct substrate requirements, and with different functional consequences; whereas poly-ubiquitination is a trafficking signal used for turnover of misfolded or structurally unstable protein, mono-ubiquitination serves as a trafficking signal for the turnover of fully myristoylated, fully mature protein. More generally, these findings reveal how a single protein is targeted for two different forms of ubiquitination, each having profoundly distinct consequences for trafficking and degradation of the substrate.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Standard methods for growth, maintenance, and transformation of yeast and bacteria for the manipulation of DNA were used throughout. Yeast *Saccharomyces cerevisiae* strains used in this study were BY4741 (*MAT***a** *leu2*Δ *met15*Δ *his3*Δ *ura3*Δ), BY4741-derived mutants lacking *PEP4* (*pep4*Δ::*KanMX*, Research Genetics, Hunstville, AL), LHY488 (*MAT***a** *his3-*Δ*200 leu2*Δ*1 ura3-52 lys2-801 trp1*Δ*63 ade2-101*), LHY489 (LHY488 *cim3-1*) (provided by Linda Hicke, Northwestern University), YGS5 (*MAT***a** *ura3-*52 *lys2 ade2*^{oc} *trp1 leu2-1 gpa1*::hisG *ste11*$_{ts}$) (10), and *ste7*Δ *gpa1*Δ (BY4741, *ste7*Δ::*KanMX, gpa1*::*hisG*) in which the *GPA1* open reading frame was disrupted by integration of a HisG-*URA3*-HisG cassette followed by counter-selection with 5-fluoroorotic acid as described previously (10). Doxycycline-sensitive yeast strains (*MAT***a** *URA3*::*CMV-tTA his3-1 leu2-0 met15-0*) were purchased as part of the yeast Tet-promoter Hughes Collection (yTHC, Open Biosystems) described in Mnaimneh *et al.* (35).

Yeast shuttle plasmids used here were pAD4M (2 μm amp^R *LEU2 ADH1* promoter/terminator), pAD4M-GPA1, pRS406-GPA1-GFP (26), pRS316-GPA1, which contains *GPA1* under the control of its native promoter (10), pRS423-FUS1-lacZ (36), and pRS405-GPA1-GFP-*pep4*. pRS405-GPA1-GFP-*pep4* was constructed by first cloning the *GPA1* open reading frame plus 600 base pairs of upstream promoter sequence into the XbaI/ClaI sites of pUG35 vector (provided by Johannes Hegemann, Heinrich-Heine-Universität), which places *GPA1* in-frame with green fluorescent protein (GFP).[4] The resulting *GFP* fusion was subcloned from pUG35 (including the pUG35 terminator sequence) into the XbaI/EagI sites of pRS405 (Stratagene). The DNA fragment corresponding to base pairs 239–782 of the *PEP4* gene was cloned into the XhoI site of the resulting pRS405-GPA1-GFP vector. The resulting plasmid was linearized with BbvCI (New England Biolabs) and integrated at the *PEP4* locus, simultaneously disrupting the *PEP4* gene and introducing *GPA1-GFP*.

All point mutations were constructed using the QuikChange site-directed mutagenesis kit (Stratagene). Oligonucleotide sequences were as follows (5′-3′; reverse compliment not shown): *F406A*, CATGCCCATAAGAAAGTACGCTCCTGATTACCAGGGAC; *V340A*, GTTTCGAAGGAATTACAGCAGCTTTATTTGTTTTAGCAATGAG; *L385A*, GGTTCAAAGATACACCGTTTATTGCGTTTTTAAATAAAATTGATTTG; *F369A*, GCATGAATCAATAATGCTAGCTGACACGTTATTGAACTC; *F383A*, GTGGTTCAAAGATACACCGGCTATTTTGTTTTTAAATAAAATTG; *G2A*, CTAGTTCTAGAATGGCGTGTACAGTGAGTAC; *S200A*, CTGATAGAAACAACAGTGCTAGAATTAACCTACAGG; *G2A/4K*, ATCCCATGATGGCGTCTACAGTGAGTAAGAA.

Computational Protein Stability Estimation—Identification of destabilizing mutations in yeast Gpa1 was accomplished using *Medusa* protein modeling software (37). *Medusa* models a protein in atomic resolution and features a physical force field as well as a rapid amino acid side-chain packing algorithm to accurately recapitulate changes in protein fold stability upon amino acid substitution (38, 39). Because the structure of yeast Gpa1 is unknown, we used its mammalian ortholog, Gα_i, as the modeling system. Gα_i exhibits 67% sequence similarity with Gpa1, and the Gα_i structure has been solved by high resolution x-ray crystallography (PDB code 1agr) (40). Using *Medusa*, we systematically evaluated the predicted

[4] The abbreviations used are: GFP, green fluorescent protein; DTT, dithiothreitol; DIC, differential interference contrast; 4K, four lysine; Ub, ubiquitin.

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stability change upon substitution of each native amino acid with alanine along the entire $G\alpha_i$ sequence. The stability of each mutant was computed with the relaxed structure, and the mutation-induced stability change ($\Delta\Delta G$) was obtained by subtracting the wild type stability (39). Finally, the destabilizing mutations were mapped onto the yeast Gpa1 sequence, and only conserved residues were considered further. To minimize the likelihood of disrupting G protein function, we avoided mutating residues associated with nucleotide catalysis, conformational changes (switch regions), or other known protein binding regions (2).

Growth, Transcription, and Protein Turnover Assays—Pheromone-dependent growth inhibition (halo) and reporter-transcription assays were conducted as described previously (36). Doxycycline repressible strains ($TetO_7$-ORF) were grown to early log phase, then diluted with selectable media containing doxycycline hyclate (Sigma-Aldrich) at 10 μg/ml final concentration, grown to early log phase ($A_{600\ nm} \sim 1.0$), and harvested. To evaluate Gpa1 stability in the absence of protein translation, early-log phase cultures were treated with cycloheximide at 10 μg/ml final concentration, and aliquots were harvested at the indicated time points. Unless otherwise noted, all cells were harvested by the addition of 1 M NaN_3 (10 mM final concentration), centrifugation, washing with ice-cold 10 mM NaN_3, and stored at −80 °C. Protein extracts were generated by glass bead lysis in trichloroacetic acid as described previously (41), resolved by 10% SDS-PAGE, and transferred to nitrocellulose membranes. The resulting membranes were probed with Gpa1 antibodies at 1:1000 (42). Immunoreactive species were visualized by chemiluminescent detection (PerkinElmer Life Sciences LAS) of horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad).

Microscopy—Cells were visualized by differential interference contrast (DIC) and fluorescence microscopy using an Olympus Fluoview 1000 confocal microscope, with the following laser lines: 488 nm (blue argon, for GFP) and 633 nm (red helium neon for DIC). Images (12-bit Tiff) were analyzed using ImageJ (NIH).

Escherichia coli Expression of Glutathione S-Transferase- and His_6 Fusion Proteins—pLIC-HIS-GPA1 plasmids were generated by ligation-independent cloning as described previously (43). *GPA1* was PCR-amplified from genomic DNA (forward primer, 5′-TACTTCCAATCCAATGCGATGGGGTGTACAGTGAGTAC-3′; reverse primer, 5′-TTATCCACTTCCAATGCGCTATATAATACCAATTTTTTTAAGGTTTTGC-3′), annealed to the gapped His_6 vector pMCSG7 (from Jason Snyder and John Sondek, University of North Carolina), and transformed into BL21-CodonPlus (DE3)-RIPL *E. coli* (Stratagene). An overnight culture from a single colony grown at 37 °C in 2× YT (yeast extract and Tryptone) with 100 μg/ml carbenicillin was diluted 1:100 in fresh medium and grown to $A_{600\ nm} \sim 0.6$ followed by induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside and incubation at room temperature for 5 h with shaking. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris, pH 8.0, 400 mM NaCl, 2 mM $MgCl_2$, 25 mM Imidazole, 1 mM DTT, 5% glycerol) supplemented with 20 μM GDP and proteinase inhibitor tablets (Roche Applied Science). The cell suspension was homogenized with an Emulsiflex-C5 homogenizer (Avestin), and the resulting lysate was cleared by two centrifugations, one at 2,000 × *g* for 15 min and a second at 45,900 × *g* for 30 min. The supernatant was incubated with lysis buffer-equilibrated Ni-Sepharose™ 6 Fast Flow resin (General Electric) for 2 h rotating at 4 °C. The resin was harvested by centrifugation and washed 4 times with lysis buffer followed by elution with lysis buffer containing 250 mM Imidazole. The elution was simultaneously mixed with His-tagged tobacco etch virus protease (to remove the N-terminal 6HIS from Gpa1) and dialyzed in 1 liter of dialysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM DTT, 5% glycerol, 20 μM GDP) overnight followed by incubation with Ni-Sepharose resin for 1 h to remove tobacco etch virus protease and cleavage products. Flow-through from the Ni-Sepharose was concentrated using Vivaspin concentrators (Vivascience AG).

Rsp5, Ubc5, and protein kinase A (PKA)-ubiquitin (ubiquitin containing a cAMP-dependent protein kinase phosphorylation site suitable for radioactive phosphorylation) were expressed from the pET21b expression vector (Novagen) in BL21p *E. coli* (Stratagene). Glutathione *S*-transferase-Rsp5 expression was induced by the addition of 0.33 mM isopropyl β-D-1-thiogalactopyranoside at 16 °C overnight. Lysates were generated by sonication in lysis buffer A (1× phosphate-buffered saline, 1 mM DTT) followed by ultracentrifugation at 15,000 × *g*. The cleared supernatant was passed over a GSTrap column (Amersham Biosciences), washed, and then eluted by thrombin (Sigma-Aldrich) cleavage overnight at 4 °C in elution buffer (50 mM Tris pH 8.0, 1 mM DTT). The cleaved protein was passed over GSTrap again and the flow-through was collected and further purified by anion exchange chromatography using HiTrap Q (Amersham Biosciences). Ubc5 and protein kinase A-ubiquitin expression was induced by the addition of 0.33 mM isopropyl β-D-1-thiogalactopyranoside at 25 °C overnight. Lysates were generated as above but in lysis buffer B (50 mM $NaPO_4$ pH 7.6, 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol) and then passed over HiTrap His (Amersham Biosciences) followed by elution in lysis buffer B containing 500 mM imidazole and dialyzed into 50 mM Tris, pH 8.0, 1 mM DTT. The dialyzed protein was further purified by anion exchange (HiTrap Q) and size exclusion chromatography (S75 resin) (Amersham Biosciences).

In Vitro Ubiquitination Assay—1 μM human E1 activating enzyme Uba1 (Boston Biochem), 1 μM yeast E2 conjugating enzyme Ubc5, and 0.2 μM yeast E3 ligase Rsp5 (or the catalytically inactive mutant $Rsp5^{C777A}$) were incubated with 0.2 μM Gpa1, 5 μM protein kinase A-ubiquitin, and 4.8 mM ATP (Sigma-Aldrich) in 1× ubiquitination buffer (25 mM Tris, pH 7.5, 50 mM NaCl, 4 mM $MgCl_2$) for 30 min at room temperature. Reactions were quenched by adding SDS-PAGE loading buffer containing 100 mM DTT followed by SDS-PAGE and immunoblotting with anti-Gpa1 antibody as described above.

RESULTS

Myristoylation Is Required for Mono-ubiquitination of Gpa1—It is well established that Gpa1 is myristoylated, palmitoylated, phosphorylated, and either mono-ubiquitinated or poly-ubiq-

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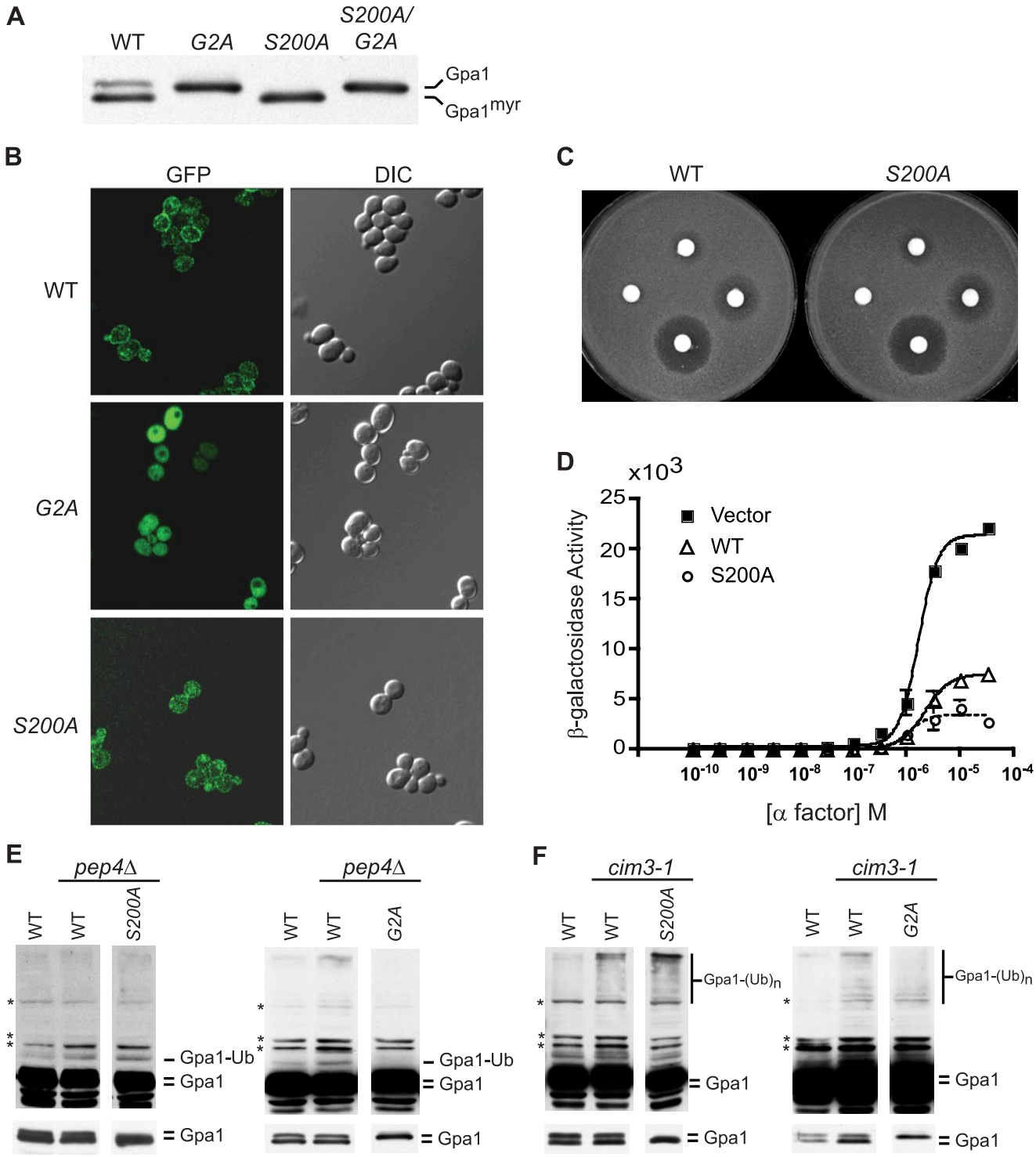

FIGURE 1. **Myristoylation is required for mono-ubiquitination of Gpa1.** *A*, anti-Gpa1 immunoblot of strain BY4741 transformed with an overexpression plasmid (pAD4M) containing either wild type *GPA1* (*WT*), *gpa1*^{G2A} (*G2A*), *gpa1*^{S200A} (*S200A*), or the double mutant as indicated. *B*, DIC and fluorescence microscopy images of BY4741 transformed with an integrating plasmid (pRS406) containing the native *GPA1* promoter and either wild type *GPA1* or the indicated mutant fused to the yeast-enhanced GFP (*left*). *C*, pheromone-induced growth inhibition assay of strain (*ste7*Δ *gpa1*Δ) transformed with single copy plasmids pRS315-*STE7* and either pRS316-GPA1 or pRS316-GPA1^{S200A}, with each gene under the control of its native promoter. Transformed cells were plated onto solid medium and exposed to paper discs containing α-factor pheromone (*clockwise from left*: 1.5, 4.5, 15, and 45 μg). *D*, cells expressing *FUS1-lacZ* reporter and overexpressing (pAD4M) Gpa1 or Gpa1^{S200A} treated with the indicated concentrations of mating pheromone for 90 min. Results are the mean ± S.E. for three individual experiments each performed in triplicate. *E* and *F*, a proteasomal protease-defective mutant (*cim3-1*), a vacuolar protease-deficient mutant (*pep4*Δ), or the corresponding isogenic wild type strains transformed with plasmid pAD4M-GPA1, pAD4M-gpa1^{S200A}, or pAD4M-gpa1^{G2A} and analyzed by immunoblotting with anti-Gpa1 antibodies. Note that all lanes shown within a single panel are from a single gel and are treated identically through all stages of image acquisition. Poly-ubiquitinated Gpa1 (*Gpa1-(Ub)$_n$*), mono-ubiquitinated Gpa1 (*Gpa1-Ub*), myristoylated, and non-myristoylated Gpa1 (*Gpa1*) and common nonspecific bands (*) are indicated. *Top* and *bottom panels* are identical except for exposure time.

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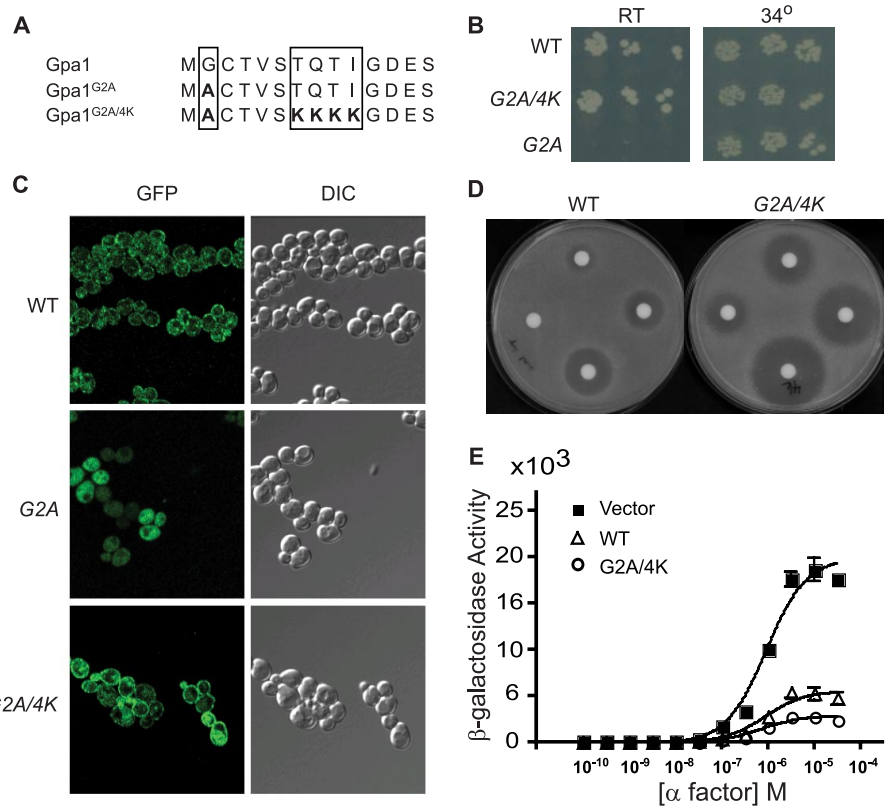

FIGURE 2. **An N-terminal polybasic stretch can substitute for Gpa1 myristoylation.** *A*, table of Gpa1 N-terminal mutants and their corresponding sequences. *B*, strain YGS5 expressing single copy (pRS316) Gpa1 (*WT*), Gpa1^{G2A} (*G2A*), or Gpa1$^{G2A/4K}$ (*G2A/4K*) were grown at 34 °C to saturation, spotted in serial 2-fold dilutions, and incubated either at 34 °C or room temperature (*RT*). *C*, DIC and GFP fluorescence of strain BY4741 expressing integrated (pRS406) Gpa1-GFP, Gpa1^{G2A}-GFP, or Gpa1$^{G2A/4K}$-GFP. *D*, strain (*ste7*Δ *gpa1*Δ) transformed with single copy plasmids pRS315-STE7 and either pRS316-GPA1 or pRS316-GPA1$^{G2A/4K}$ analyzed with the pheromone-induced growth inhibition assay as described above. *E*, BY4741 cells expressing *FUS1-lacZ* reporter and overexpressing (pAD4M) Gpa1 or Gpa1$^{G2A/4K}$ treated with the indicated concentrations of mating pheromone for 90 min. Results are the mean ± S.E. for four individual experiments each performed in triplicate.

uitinated. Our objective here was to determine the structural determinants leading to mono- *versus* poly-ubiquitination of Gpa1. The data available suggest that myristoylation and mono-ubiquitination are coordinately regulated, as they have opposing effects on plasma membrane localization. Whereas myristoylation is required for plasma-membrane targeting of Gpa1, mono-ubiquitination directs the protein from the plasma membrane to the vacuole where it is degraded (26). Given that Gpa1 normally exists in both myristoylated and non-myristoylated forms within the cell (11, 42), we first considered whether the two types of ubiquitination are dictated by the myristoylation status of the protein.

Initially we characterized variants of Gpa1 that are either non-myristoylated or fully myristoylated. Substitution of the N-terminal Gly residue of Gpa1 yields a protein that cannot be myristoylated (Gpa1^{G2A}) and, as a consequence, is redirected from the plasma membrane to endomembranes and cytoplasm (Fig. 1, *A* and *B*) (10). Gpa1 phosphorylated at serine 200 is also incapable of being myristoylated,[3] and so conversely a non-phosphorylated mutant (Gpa1^{S200A}) is fully myristoylated (Fig. 1*A*). We used both of these variants to investigate the requirement of myristoylation for ubiquitination of Gpa1.

Because this is the first analysis of a fully myristoylated form of Gpa1, we documented the functionality of the Gpa1^{S200A} mutant *in vivo*. We found that substitution of serine 200 does not alter Gpa1 localization as determined by fluorescence microscopy of a GFP-tagged protein (Fig. 1*B*). Gpa1^{S200A} also exhibits normal pheromone-induced cell division arrest, as determined by the growth arrest (halo) assay (Fig. 1*C*). As a third measure of signaling competence, we tested the effect of Gpa1 overexpression on pheromone-induced gene transcription. Overexpression of wild type Gpa1 dampens pheromone-induced pathway activation by providing excess Gα to sequester free Gβγ at the plasma membrane. Using a transcription reporter assay (*FUS1* promoter, *lacZ* reporter), we found that overexpression of Gpa1^{S200A} also dampens pheromone-induced gene transcription, comparable with that observed with overexpression of wild type Gpa1 (Fig. 1*D*). We conclude that Gpa1^{S200A} is fully myristoylated and fully competent to transmit the pheromone signal.

We then considered whether myristoylation is required for mono-ubiquitination of Gpa1. To address this question, we tracked mono-ubiquitination of protein that is partially myristoylated (Gpa1), non-myristoylated (Gpa1^{G2A}), or fully myristoylated (Gpa1^{S200A}). To best detect the mono-ubiquitinated species, we overexpressed Gpa1 in cells lacking the master vacuolar protease Pep4 (*pep4*Δ). In these cells Gpa1 mono-ubiquitination is evident as a single band that migrates ~8–10 kDa higher than the native protein (~63 kDa *versus* ~54–56 kDa) as detected with anti-Gpa1 antibodies (supplemental Fig. 1) (26). We found that wild type and fully myristoylated Gpa1^{S200A} exhibited similar levels of mono-ubiquitination (Fig. 1*E*, *left*). In contrast, non-myristoylated Gpa1^{G2A} lacked any detectable mono-ubiquitination (Fig. 1*E*, *right*). Thus, myristoylation appears to be a prerequisite for mono-ubiquitination of Gpa1.

Next we evaluated the contribution of myristoylation to poly-ubiquitination. In this case we expressed Gpa1 in cells harboring a temperature-sensitive allele of *CIM3* (*cim3-1*), encoding a component of the 26 S proteasome complex (34). In the *cim3-1* strain Gpa1 poly-ubiquitination is evident as a ladder of high molecular weight bands. Laddering was not observed in extracts from cells transformed with empty vector, indicating that the high molecular weight immunoblot signal was specific to Gpa1 (supplemental Fig. 1). Wild type Gpa1 and fully myristoylated Gpa1^{S200A} exhibited nearly equivalent levels of poly-ubiquitination (Fig. 1*F*, *left*). Likewise, poly-ubiquitination of Gpa1^{G2A} was clearly visible, albeit diminished (Fig. 1*F*,

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right). Taken together these data reveal that myristoylation is required for mono-ubiquitination but not for poly-ubiquitination of Gpa1.

An N-terminal Polybasic Stretch Can Substitute for Gpa1 Myristoylation—Myristoylation is required for mono-ubiquitination as well as for plasma membrane targeting of Gpa1, as noted above. Therefore, it is possible that proper membrane localization, rather than myristoylation, is required for Gpa1 mono-ubiquitination. To test this possibility we created a mutant form of Gpa1 that is not myristoylated but is nevertheless properly localized at the plasma membrane. Our approach was similar to one employed by Deschenes and co-workers (44), who showed that a stretch of basic amino acids can substitute for lipid acylation and thereby restore membrane localization to Ras. Accordingly, we introduced a polybasic stretch consisting of four lysine residues close to the N terminus of Gpa1, where myristoylation and palmitoylation normally occur. We refer to this mutant as Gpa1$^{G2A/4K}$, indicating that it contains four lysine substitutions but lacks the ability to be myristoylated (G2A) (Fig. 2*A*).

We then established the functionality of Gpa1$^{G2A/4K}$. Cells that exclusively express non-myristoylated Gpa1^{G2A} are inviable, presumably because Gα and G$\beta\gamma$ no longer co-localize, allowing the G$\beta\gamma$ complex to signal constitutively, resulting in cell cycle arrest (10). To determine whether Gpa1$^{G2A/4K}$ can overcome the loss of myristoylation, we expressed the mutant in cells that lack endogenous *GPA1* (strain YGS5). The absence of Gpa1 ordinarily leads to constitutive signaling and cell cycle arrest; however, YGS5 is viable at 34 °C because of a temperature-sensitive mutation that blocks the signal downstream of the G protein (*ste11*^{ts}). At the permissive temperature for growth where signaling is restricted, cells expressing either Gpa1^{G2A} or Gpa1$^{G2A/4K}$ grew well. At the restrictive temperature for growth, where signaling proceeds normally, expression of Gpa1$^{G2A/4K}$ allowed normal growth, whereas Gpa1^{G2A} caused growth arrest (Fig. 2*B*). Gpa1$^{G2A/4K}$ is also properly localized at the plasma membrane as determined by fluorescence microscopy, whereas Gpa1^{G2A} localizes diffusely throughout the cytoplasm (Fig. 2*C*). Finally, Gpa1$^{G2A/4K}$ is signaling-competent as determined by the halo and transcription-reporter assays described above, although the cells were unusually sensitive to pheromone-induced growth arrest (Fig. 2, *D* and *E*). Taken together, these data confirm that Gpa1$^{G2A/4K}$ is properly localized and functional despite the absence of myristoylation.

We then used Gpa1$^{G2A/4K}$ to determine whether plasma membrane localization (in the absence of myristoylation) is sufficient for mono-ubiquitination of Gpa1. As shown in Fig. 3, mono-ubiquitinated Gpa1 was readily detected in both wild type and *pep4*Δ cells. In contrast, we failed to observe any modification of Gpa1$^{G2A/4K}$ in either cell type (Fig. 3). Gpa1^{4K}, which can be myristoylated, was still mono-ubiquitinated, suggesting that the polybasic stretch itself does not affect Gpa1 mono-ubiquitination. In contrast, poly-ubiquitination of Gpa1$^{G2A/4K}$ was comparable with that of wild type Gpa1 (Fig. 4), whereas poly-ubiquitination of Gpa1^{4K} was enhanced slightly. We conclude that Gpa1 must be myristoylated to undergo mono-ubiquitination *in vivo*. In contrast, neither myristoylation nor plasma membrane targeting is required for Gpa1 poly-ubiquitination.

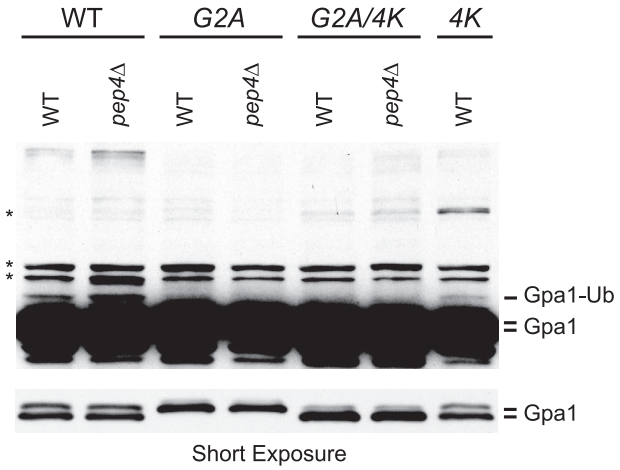

FIGURE 3. **Plasma membrane localization is not sufficient for Gpa1 mono-ubiquitination.** pAD4M-GPA1 (*WT*) or the indicated point mutant expressed in *pep4*Δ or the corresponding isogenic wild type strain and analyzed by immunoblotting with anti-Gpa1 antibodies, as described above. *Top* and *bottom panels* are identical except for exposure time. The slower mobility of G2A/4K relative to G2A is presumed to occur because of the effect of substituting four lysine residues on SDS-PAGE mobility of the protein.

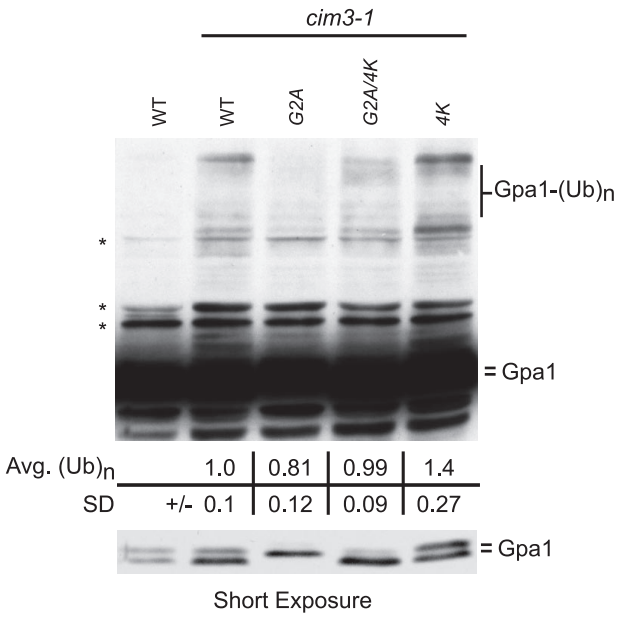

FIGURE 4. **Plasma membrane localization is sufficient for Gpa1 poly-ubiquitination.** pAD4M-GPA1 (*WT*) or the indicated point mutant expressed in *cim3-1* or the corresponding isogenic wild type strain and analyzed by immunoblotting with anti-Gpa1 antibodies as described above. Quantitation of the poly-ubiquitinated form of Gpa1 (laddering) relative to native Gpa1 is the average ± S.D. for three independent experiments. *Top* and *bottom panels* are identical except for exposure time.

Gpa1 Protein Folding Mutants Are Targeted for Poly-ubiquitination but Not Mono-ubiquitination—The data presented above indicate that mono-ubiquitination requires that Gpa1 is properly myristoylated. In contrast, poly-ubiquitination is unaffected by the loss of myristoylation. Thus, we next considered if poly-ubiquitination instead targets Gpa1 that is misfolded. To test this hypothesis, we engineered Gpa1 variants that are structurally unstable and then compared mono- and poly-ubiquitination of each variant. Accordingly, we employed

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a protein modeling suite, *Medusa* (37), to identify Gpa1 destabilizing mutations based on the three-dimensional structure of the protein. The structure of Gpa1 has not been determined; however, the structure of human $G\alpha_i$, which shares 67% sequence similarity with yeast Gpa1, has been solved to high resolution by x-ray crystallography (PDB code 1agr) (40). Benchmarked on a large set of experimentally characterized mutations, *Medusa* has been used to accurately recapitulate changes in the structural stability of proteins upon amino acid substitution (37–39). Using this method, we identified five residues within the hydrophobic core of the protein that are conserved between yeast and human, substitution of which (to alanine) is predicted to destabilize Gpa1 (Table 1). Mutations that interfere with the catalytic or known protein binding regions of $G\alpha$ were avoided.

We then examined all five Gpa1 mutants in the proteasome-deficient *cim3-1* cells and compared the levels of poly-ubiquitination by immunoblotting. For every Gpa1 mutant, poly-ubiquitination was elevated relative to the wild type protein (Fig. 5*A*). In contrast, Gpa1 mono-ubiquitination was unaffected or reduced in every case (Fig. 5*B*). These data indicate that misfolded Gpa1 is targeted for poly-ubiquitination rather than mono-ubiquitination.

TABLE 1

Relative free energies of Gpa1 protein fold-destabilizing mutations

Medusa protein modeling software was used in combination with the high resolution crystal structure of human $G\alpha_i$ (PDB code 1agr) to identify a set of five point mutations that are conserved in the yeast $G\alpha$ protein Gpa1 and predicted to create a variety of protein fold stabilities relative to the wild type protein (see "Experimental Procedures"). The structural stability of the mutant protein is computed using the relaxed structure, and the mutation-induced stability change ($\Delta\Delta G$) is obtained by subtracting the wild type stability. Mutations with higher $\Delta\Delta G$ values are predicted to have the greatest negative effect on $G\alpha_i$ protein fold stability.

$G\alpha_i$ mutation	Corresponding Gpa1 mutation	Predicted $\Delta\Delta G$[a]
Y287A	F406A	10.23
I221A	V340A	10.20
L266A	L385A	9.10
F250A	F369A	7.29
I264A	F383A	7.28

[a] Relative to wild type.

Rsp5 Is Necessary and Sufficient for Mono-ubiquitination of Gpa1—The data presented above indicate that mono- and poly-ubiquitination are distinct processes with distinct substrate specificity requirements and unique functional consequences for Gpa1. Whereas the fully mature and fully myristoylated G protein is mono-ubiquitinated and delivered to the vacuole, misfolded Gpa1 is instead poly-ubiquitinated and degraded by the proteasome. As a further test of this model, we sought to determine whether the two types of ubiquitination employ different ubiquitinating enzymes. Two lines of evidence suggest that Rsp5 might be involved in Gpa1 ubiquitination. First, Rsp5 is a HECT-type E3 ubiquitin ligase involved in mono-ubiquitination and endocytosis of multiple membrane-localized proteins including the pheromone receptor Ste2. Second, Rsp5 is membrane-bound *in vivo* (45, 46). To test the hypothesis that Rsp5 is required for mono-ubiquitination of Gpa1, we overexpressed Gpa1 in a strain where *RSP5* gene expression is repressed upon the addition of doxycycline ($TetO_7$-RSP5). The data were compared with a wild type strain in which the promoter element is present but not attached to any open reading frame ($TetO_7$-WT) (35). As shown in Fig. 6, we observed a loss of Gpa1 mono-ubiquitination after *RSP5* repression but no change in untreated cells or in the control $TetO_7$-WT cells with or without doxycycline treatment (Fig. 6*A*). Conversely, we observed a significant increase in Gpa1 poly-ubiquitination after *RSP5* repression. Thus, Gpa1 mono-ubiquitination and poly-ubiquitination are reciprocally regulated in the absence of Rsp5. Because mono-ubiquitination serves as an endocytosis and vacuolar sorting signal, we tested the ability of Gpa1 to undergo vacuolar translocation in the presence or absence of Rsp5. Whereas Gpa1-GFP is normally detected in the vacuolar compartment, vacuolar Gpa1 was barely detectable after Rsp5 repression (Fig. 6*B*). Thus, Rsp5 is required for mono-ubiquitination and proper translocation of Gpa1 to the vacuole.

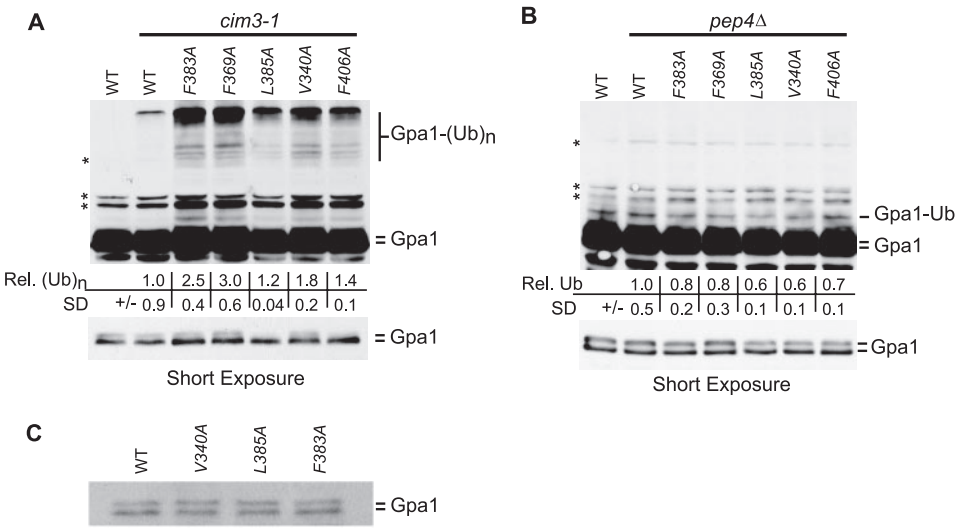

FIGURE 5. **Gpa1 poly-ubiquitination, but not mono-ubiquitination, is elevated for protein folding mutants.** *A*, immunoblot of mutant *cim3-1* and the isogenic wild type strain transformed with plasmid pAD4M-GPA1 (*WT*) or the indicated point mutant grown at the restrictive temperature of 37 °C for 4 h. Whole cell extracts were resolved by 10% SDS-PAGE and immunoblotting with anti-Gpa1 antibodies. *B*, mutant *pep4*Δ and the isogenic wild type strain transformed with plasmid pAD4M-GPA1 or the indicated point mutant and grown to early-log phase at 30 °C. The difference in exposures between *cim3-1* and *pep4*Δ experiments is required to ensure visualization of either the mono-ubiquitin band (which is in close proximity to the Gpa1 bands) or poly-ubiquitin laddering. Quantitation of the poly-ubiquitinated (*cim3-1* strain) or mono-ubiquitinated (*pep4*Δ strain) Gpa1 relative to native Gpa1 is the average ± S.D. for at least three independent experiments. *C*, *ste7*Δ *gpa1*Δ cells expressing pRS315-STE7 and pRS316-GPA1 or the indicated mutant were grown to early log phase and analyzed by immunoblotting with anti-Gpa1 antibodies.

To further characterize the structural determinants necessary for Gpa1 mono-ubiquitination, we examined the protein for potential Rsp5 docking sites. Rsp5 contains WW protein interaction domains that bind and recruit substrates or substrate adaptor proteins containing one or more short PY motifs (PP*X*Y) within their primary structure (47, 48). However, the P*X*Y portion of the motif is the minimal

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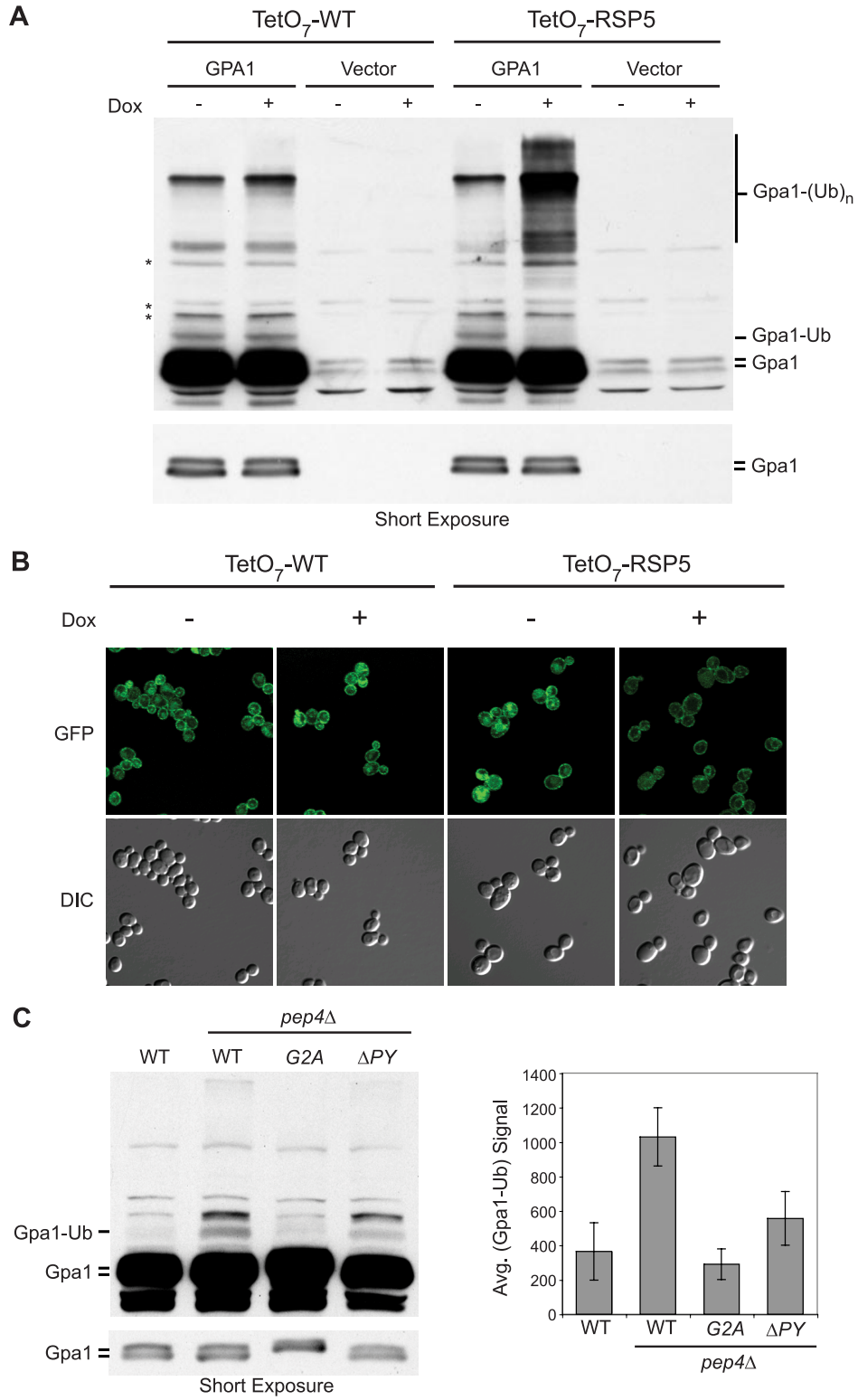

FIGURE 6. **The ubiquitin ligase Rsp5 is required for Gpa1 mono-ubiquitination *in vivo*.** *A*, cells harboring a doxycycline-repressible promoter attached to the *RSP5* open reading frame (*TetO$_7$-RSP5*) or to a non-expressible genetic element (*TetO$_7$-WT*) were transformed with the overexpression plasmid pAD4M-GPA1 or empty vector. Transformants were grown to early log phase and then split, and half were treated with doxycycline (*Dox*) at 10 μg/ml for 16 h, diluted in doxycycline-containing or free medium, and grown to early log phase. Cells were harvested, lysed, and analyzed by immunoblotting with anti-Gpa1 antibodies. *B*, DIC and GFP fluorescence of strain TetO$_7$-WT or TetO$_7$-RSP5 harboring pRS405-GPA1-GFP-*pep4* integrated into the *PEP4* locus, thereby disrupting expression of the vacuolar protease Pep4. *C*, mono-ubiquitination of overexpressed wild type (WT) and non-myristoylated (*G2A*) Gpa1 was compared by Gpa1 immunoblotting with that of a Gpa1 double mutant P407A/Y409A (*ΔPY*) lacking the essential residues of a putative Rsp5 binding motif (PY motif) (*left*). Quantitation of the resulting immunoblots was performed in triplicate using cells from three different transformants.

requirement for establishing an interaction with Rsp5, whereas nonpolar hydrophobic residues can be tolerated at the first position of the motif (*e.g.* LP*X*Y or AP*X*Y) (47, 48). Gpa1 contains a single non-canonical PY motif (FPDY) that contains a nonpolar hydrophobic residue in position 1. To determine whether the FPDY segment contributes to Rsp5-mediated ubiquitination, we replaced the conserved proline and tyrosine with alanine (FADA = ΔPY). These substitutions resulted in a significant but incomplete reduction in Gpa1 mono-ubiquitination *in vivo* (Fig. 6*C*). Taken together these data reveal that Rsp5 expression as well as an Rsp5 binding motif is necessary for the proper mono-ubiquitination of Gpa1.

To directly determine whether Rsp5 ubiquitinates Gpa1, we conducted an *in vitro* ubiquitination assay using purified proteins. To this end, we combined purified recombinant Rsp5 or catalytically-inactive Rsp5-(C777A) with Uba1 activating enzyme, Ubc5 conjugating enzyme, ubiquitin, Gpa1, and ATP. We allowed the *in vitro* reaction to proceed for 30 min after which we analyzed Gpa1 ubiquitination by immunoblotting. When incubated with Rsp5, Gpa1 mono-ubiquitination was detectable as a distinct band supershifted relative to native Gpa1 (Fig. 7). In contrast, Gpa1 incubated with the catalytically inactive Rsp5 mutant (Rsp5-(C/A)) or without E1 activating enzyme showed no evidence of ubiquitination (Fig. 7). Although less obvious, some Rsp5-dependent poly-ubiquitination of Gpa1 was also observed in the reaction. Taken together these data reveal that Rsp5 is both necessary and sufficient for the mono-ubiquitination of Gpa1. Furthermore, it appears that a distinct enzyme is needed for Gpa1 poly-ubiquitination and that such an enzyme acts preferentially on misfolded protein.

DISCUSSION

The yeast G protein α subunit, Gpa1, undergoes both mono- and

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poly-ubiquitination. Either type of ubiquitination results in alternate Gpa1 trafficking outcomes; whereas mono-ubiquitination promotes translocation to the vacuole, poly-ubiquitination results in translocation to the proteasome. We have used Gpa1 as a model protein to investigate the factors underlying the two types of ubiquitination. We find that mutants that are not myristoylated are no longer mono-ubiquitinated but still are poly-ubiquitinated *in vivo*. Conversely, we find that variants of Gpa1 engineered to be unstable are less likely to be mono-ubiquitinated and more likely poly-ubiquitinated. Finally, we show that the ubiquitin ligase Rsp5 is necessary for Gpa1 mono-ubiquitination *in vivo* and is sufficient for this process to occur *in vitro*. We conclude that Gpa1 mono- and poly-ubiquitination are functionally distinct processes controlled by different E3 ligases, each with distinct substrate requirements; whereas Rsp5-catalyzed mono-ubiquitination targets myristoylated G protein to the vacuole, poly-ubiquitination by a different E3 ligase targets misfolded protein to the proteasome (Fig. 8).

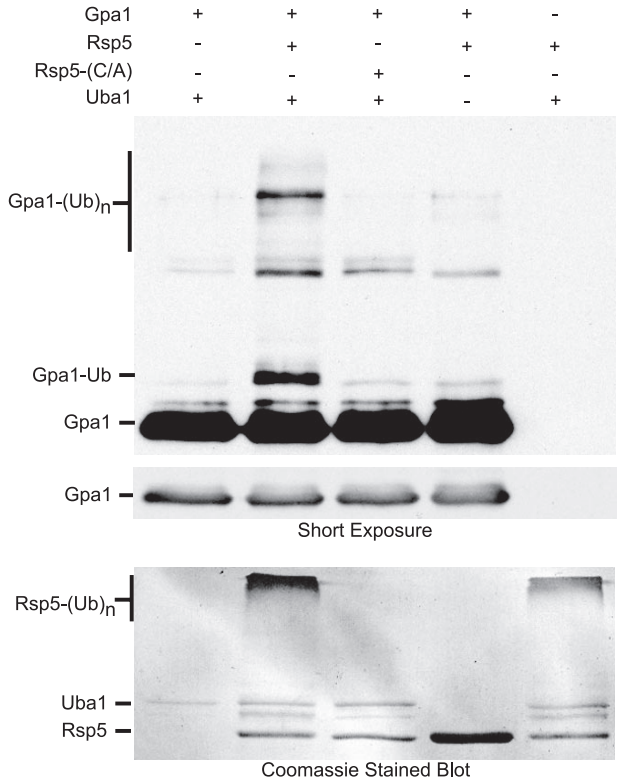

FIGURE 7. **Rsp5 ubiquitinates Gpa1 *in vitro*.** Purified recombinant wild type or catalytically-inactive Rsp5 (*Rsp5-(C/A)*), Gpa1, and Uba1 (E1) were incubated as indicated in the presence of Ubc5, ATP, and ubiquitin for 30 min at room temperature followed by quenching with SDS-PAGE loading buffer containing DTT. Samples were resolved by 10% SDS-PAGE and immunoblotting with anti-Gpa1 antibody (*top panels*). The nitrocellulose blot was stained with Coomassie Blue to detect Rsp5 and Uba1.

We have identified Rsp5 as the ligase responsible for mono-ubiquitination and vacuolar translocation of Gpa1. Rsp5 is a membrane-bound E3 ligase responsible for ubiquitination of many substrates including the G protein-coupled receptor Ste2. Whereas Ste2 mono-ubiquitination occurs in a pheromone-stimulated manner (45, 46), Gpa1 mono-ubiquitination appears to be a constitutive process. Mono-ubiquitination in either case contributes to clearing of fully mature and fully functional protein from the plasma membrane. Just as Ste2 must be present at the cell surface and competent to bind pheromone, we propose that Gpa1 must be myristoylated and present at the plasma membrane to be mono-ubiquitinated. The possibility exists that mono-ubiquitination of Gpa1 also occurs at endomembranes.

The precise role of myristoylation during Gpa1 mono-ubiquitination is not clear. We have reconstituted Rsp5-catalyzed mono-ubiquitination *in vitro* using Gpa1 purified from *E. coli*, which lacks the N-myristoyltransferase Nmt1 and, therefore, produces non-myristoylated G protein. Because Rsp5 can ubiquitinate Gpa1 in the absence of myristoylation *in vitro*, it is possible that the absence of Gpa1$^{G2A/4K}$ mono-ubiquitination may be due to the orientation of the G protein relative to the membrane; we consider this plausible because the interaction of plasma membrane with myristate and the N-terminal polybasic stretch are likely to be different. Alternatively, myristoylation may be necessary to enhance an otherwise weak interaction between Gpa1 and Rsp5.

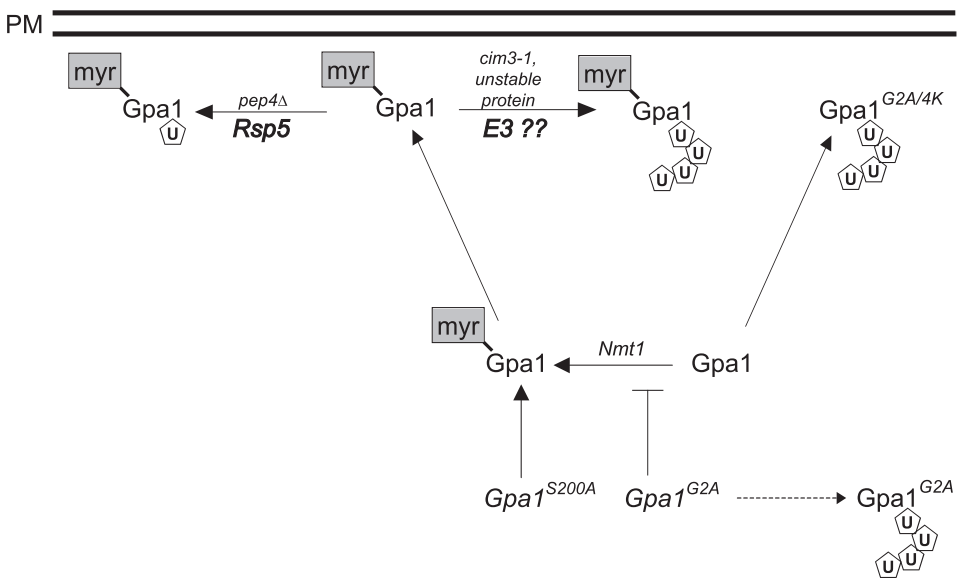

FIGURE 8. **Determinants of Gpa1 mono- and poly-ubiquitination.** The fully myristoylated and fully mature form of Gpa1 (*e.g.* Gpa1^{S200A}) is mono-ubiquitinated and translocated to the vacuole for degradation (enriched in *pep4*Δ cells). Non-myristoylated (*e.g.* Gpa1^{G2A} or Gpa1$^{G2A/4K}$) or misfolded Gpa1 is poly-ubiquitinated and degraded by the proteasome. Rsp5 is necessary and sufficient for Gpa1 mono-ubiquitination. The enzyme responsible for Gpa1 poly-ubiquitination is unknown. *PM*, plasma membrane.

We have shown further that mutation of an Rsp5 binding or PY motif in Gpa1 reduces mono-ubiquitination. In contrast, recent evidence indicates that many Rsp5 substrates lack a clear PY motif, and binding to Rsp5 is instead provided by an adaptor protein that mediates functional interaction between the substrate and the ligase (49).

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Although our data indicate that an adaptor is not necessary for Gpa1 mono-ubiquitination, the possibility remains that an adaptor protein may enhance ubiquitination by Rsp5 *in vivo*.

Interestingly, cells lacking Rsp5 exhibit a dramatic increase in Gpa1 poly-ubiquitination. In the absence of Rsp5, poly-ubiquitination may be allowed to occur predominantly and, thereby, compensate for the loss of vacuolar degradation. Alternatively, Rsp5 may be involved in down-regulating Gpa1 poly-ubiquitination through direct targeting of another ubiquitin ligase. In any case, an additional E3 ligase other than Rsp5 is likely responsible for poly-ubiquitination of Gpa1 *in vivo*. We have demonstrated that Gpa1 misfolding mutants may be more susceptible to poly-ubiquitination by such a ligase. To this end we used the *Medusa* protein-modeling suite, which has been previously shown to accurately predict mutation-induced differences in protein folding (38, 39). The success of this approach relied in part on the high resolution crystal structure of $G\alpha_i$, a protein with a high degree of similarity to Gpa1. Therefore, the mutations we have described here may be useful in studying $G\alpha_i$ as a potential ubiquitination substrate. Notably, $G\alpha_i$ has been shown to be down-regulated by the overexpression of GIPN (GAIP interacting protein N terminus), a putative E3 ubiquitin ligase that is localized to the plasma membrane (50). The *Medusa* protein-modeling suite could also be used to study the effects of protein misfolding on other signaling proteins that share high similarity between organisms. Candidates include numerous ubiquitinated signaling components in yeast and mammals (8, 51, 52), including G protein-coupled receptors (20–23, 53–59) and G proteins (25, 53, 60–63).

Historically, investigation of G protein modification has focused on fatty acylation. Many cellular proteins are poly-ubiquitinated and subsequently degraded by the proteasome. More rarely, proteins are mono-ubiquitinated and targeted for endocytosis. However, it has remained unclear how proteins are selected for one or the other form of ubiquitination. Here we have identified functional determinants leading to mono- *versus* poly-ubiquitination of a single protein substrate, the G protein α subunit Gpa1. We have shown that the fully mature, fully myristoylated form of the protein undergoes mono-ubiquitination, whereas misfolded protein is instead poly-ubiquitinated. We have shown further that Rsp5 is both necessary and sufficient for the mono-ubiquitination and vacuolar translocation of Gpa1. Finally we have demonstrated how a protein fold prediction algorithm can be used to improve understanding of Gα protein ubiquitination and its relationship to protein fold stability. Given the ubiquity of G proteins as signal transducers and of ubiquitination as a mechanism of cell regulation, the integration of genetic, biochemical, and computational modeling approaches employed here should prove useful in the study of other G proteins as substrates for ubiquitination.

REFERENCES

1. Dohlman, H. G., and Thorner, J. W. (2001) *Annu. Rev. Biochem.* **70,** 703–754
2. Sprang, S. R. (1997) *Annu. Rev. Biochem.* **66,** 639–678
3. Dietzel, C., and Kurjan, J. (1987) *Cell* **50,** 1001–1010
4. Miyajima, I., Nakafuku, M., Nakayama, N., Brenner, C., Miyajima, A., Kaibuchi, K., Arai, K., Kaziro, Y., and Matsumoto, K. (1987) *Cell* **50,** 1011–1019
5. Slessareva, J. E., Routt, S. M., Temple, B., Bankaitis, V. A., and Dohlman, H. G. (2006) *Cell* **126,** 191–203
6. Chen, C. A., and Manning, D. R. (2001) *Oncogene* **20,** 1643–1652
7. Escriba, P. V., Wedegaertner, P. B., Goni, F. M., and Vogler, O. (2007) *Biochim. Biophys. Acta* **1768,** 836–852
8. Wang, Y., and Dohlman, H. G. (2006) *Circ. Res.* **99,** 1305–1314
9. Li, X., Gerber, S. A., Rudner, A. D., Beausoleil, S. A., Haas, W., Villen, J., Elias, J. E., and Gygi, S. P. (2007) *J. Proteome Res.* **6,** 1190–1197
10. Song, J., Hirschman, J., Gunn, K., and Dohlman, H. G. (1996) *J. Biol. Chem.* **271,** 20273–20283
11. Stone, D. E., Cole, G. M., de Barros Lopes, M., Goebl, M., and Reed, S. I. (1991) *Genes Dev.* **5,** 1969–1981
12. Song, J., and Dohlman, H. G. (1996) *Biochemistry* **35,** 14806–14817
13. Manahan, C. L., Patnana, M., Blumer, K. J., and Linder, M. E. (2000) *Mol. Biol. Cell* **11,** 957–968
14. Wedegaertner, P. B., and Bourne, H. R. (1994) *Cell* **77,** 1063–1070
15. Degtyarev, M. Y., Spiegel, A. M., and Jones, T. L. (1994) *J. Biol. Chem.* **269,** 30898–30903
16. Pickart, C. M. (2001) *Annu. Rev. Biochem.* **70,** 503–533
17. d'Azzo, A., Bongiovanni, A., and Nastasi, T. (2005) *Traffic* **6,** 429–441
18. Voges, D., Zwickl, P., and Baumeister, W. (1999) *Annu. Rev. Biochem.* **68,** 1015–1068
19. Hicke, L. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 195–201
20. Hicke, L., and Riezman, H. (1996) *Cell* **84,** 277–287
21. Roth, A. F., and Davis, N. G. (1996) *J. Cell Biol.* **134,** 661–674
22. Martin, N. P., Lefkowitz, R. J., and Shenoy, S. K. (2003) *J. Biol. Chem.* **278,** 45954–45959
23. Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) *Science* **294,** 1307–1313
24. Vecchione, A., Marchese, A., Henry, P., Rotin, D., and Morrione, A. (2003) *Mol. Cell. Biol.* **23,** 3363–3372
25. Madura, K., and Varshavsky, A. (1994) *Science* **265,** 1454–1458
26. Wang, Y., Marotti, L. A., Jr., Lee, M. J., and Dohlman, H. G. (2005) *J. Biol. Chem.* **280,** 284–291
27. Hao, N., Yildirim, N., Wang, Y., Elston, T. C., and Dohlman, H. G. (2003) *J. Biol. Chem.* **278,** 46506–46515
28. Wang, Y., and Dohlman, H. G. (2002) *J. Biol. Chem.* **277,** 15766–15772
29. Wang, Y., Ge, Q., Houston, D., Thorner, J., Errede, B., and Dohlman, H. G. (2003) *J. Biol. Chem.* **278,** 22284–22289
30. Li, M., Brooks, C. L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003) *Science* **302,** 1972–1975
31. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) *Nature* **419,** 135–141
32. Risinger, A. L., and Kaiser, C. A. (2008) *Mol. Biol. Cell* **19,** 2962–2972
33. Rothman, J. H., Howald, I., and Stevens, T. H. (1989) *EMBO J.* **8,** 2057–2065
34. Finley, D., Tanaka, K., Mann, C., Feldmann, H., Hochstrasser, M., Vierstra, R., Johnston, S., Hampton, R., Haber, J., McCusker, J., Silver, P., Frontali, L., Thorsness, P., Varshavsky, A., Byers, B., Madura, K., Reed, S. I., Wolf, D., Jentsch, S., Sommer, T., Baumeister, W., Goldberg, A., Fried, V., Rubin, D. M., Glickman, M. H., and Toh-e, A. (1998) *Trends Biochem. Sci.* **23,** 244–245
35. Mnaimneh, S., Davierwala, A. P., Haynes, J., Moffat, J., Peng, W. T., Zhang, W., Yang, X., Pootoolal, J., Chua, G., Lopez, A., Trochesset, M., Morse, D., Krogan, N. J., Hiley, S. L., Li, Z., Morris, Q., Grigull, J., Mitsakakis, N., Roberts, C. J., Greenblatt, J. F., Boone, C., Kaiser, C. A., Andrews, B. J., and Hughes, T. R. (2004) *Cell* **118,** 31–44
36. Hoffman, G., Garrison, T. R., and Dohlman, H. G. (2002) *Methods Enzymol.* **344,** 617–631
37. Ding, F., and Dokholyan, N. V. (2006) *PLoS Comput. Biol.* **2,** e85
38. Yin, S., Ding, F., and Dokholyan, N. V. (2007) *Nat. Methods* **4,** 466–467
39. Yin, S., Ding, F., and Dokholyan, N. V. (2007) *Structure* **15,** 1567–1576
40. Tesmer, J. J., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) *Cell* **89,** 251–261
41. Lee, M. J., and Dohlman, H. G. (2008) *Curr. Biol.* **18,** 211–215
42. Dohlman, H. G., Goldsmith, P., Spiegel, A. M., and Thorner, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9688–9692

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43. Stols, L., Gu, M., Dieckman, L., Raffen, R., Collart, F. R., and Donnelly, M. I. (2002) *Protein Expression Purif.* **25,** 8–15
44. Mitchell, D. A., Farh, L., Marshall, T. K., and Deschenes, R. J. (1994) *J. Biol. Chem.* **269,** 21540–21546
45. Dunn, R., and Hicke, L. (2001) *J. Biol. Chem.* **276,** 25974–25981
46. Dunn, R., Klos, D. A., Adler, A. S., and Hicke, L. (2004) *J. Cell Biol.* **165,** 135–144
47. Sullivan, J. A., Lewis, M. J., Nikko, E., and Pelham, H. R. (2007) *Mol. Biol. Cell* **18,** 2429–2440
48. Gupta, R., Kus, B., Fladd, C., Wasmuth, J., Tonikian, R., Sidhu, S., Krogan, N. J., Parkinson, J., and Rotin, D. (2007) *Mol. Syst. Biol.* **3,** 116
49. Belgareh-Touze, N., Leon, S., Erpapazoglou, Z., Stawiecka-Mirota, M., Urban-Grimal, D., and Haguenauer-Tsapis, R. (2008) *Biochem. Soc. Trans.* **36,** 791–796
50. Fischer, T., De Vries, L., Meerloo, T., and Farquhar, M. G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8270–8275
51. Marchese, A., Paing, M. M., Temple, B. R., and Trejo, J. (2008) *Annu. Rev. Pharmacol. Toxicol.* **48,** 601–629
52. Shenoy, S. K. (2007) *Circ. Res.* **100,** 1142–1154
53. Obin, M. S., Jahngen-Hodge, J., Nowell, T., and Taylor, A. (1996) *J. Biol. Chem.* **271,** 14473–14484
54. Marchese, A., and Benovic, J. L. (2001) *J. Biol. Chem.* **276,** 45509–45512
55. Chaturvedi, K., Bandari, P., Chinen, N., and Howells, R. D. (2001) *J. Biol. Chem.* **276,** 12345–12355
56. Cohen, B. D., Bariteau, J. T., Magenis, L. M., and Dias, J. A. (2003) *Endocrinology* **144,** 4393–4402
57. Wolfe, B. L., Marchese, A., and Trejo, J. (2007) *J. Cell Biol.* **177,** 905–916
58. Jacob, C., Cottrell, G. S., Gehringer, D., Schmidlin, F., Grady, E. F., and Bunnett, N. W. (2005) *J. Biol. Chem.* **280,** 16076–16087
59. Cook, L. B., Zhu, C. C., and Hinkle, P. M. (2003) *Mol. Endocrinol.* **17,** 1777–1791
60. Naviglio, S., Pagano, M., Romano, M., Sorrentino, A., Fusco, A., Illiano, F., Chiosi, E., Spina, A., and Illiano, G. (2004) *Cell. Signal.* **16,** 1229–1237
61. Obin, M., Lee, B. Y., Meinke, G., Bohm, A., Lee, R. H., Gaudet, R., Hopp, J. A., Arshavsky, V. Y., Willardson, B. M., and Taylor, A. (2002) *J. Biol. Chem.* **277,** 44566–44575
62. Hamilton, M. H., Cook, L. A., McRackan, T. R., Schey, K. L., and Hildebrandt, J. D. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 5081–5086
63. Mouledous, L., Neasta, J., Uttenweiler-Joseph, S., Stella, A., Matondo, M., Corbani, M., Monsarrat, B., and Meunier, J. C. (2005) *Mol. Pharmacol.* **68,** 467–476

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