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Geranylgeranyl pyrophosphate (GGPP) is a potent regulator of HRD-dependent HMG-CoA reductase degradation in yeast

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HMG-CoA reductase (HMGR), the rate-limiting enzymes of sterol synthesis, undergoes feedback-regulated ER degradation in both mammals and yeast. The yeast Hmg2p isozyme is subject to ubiquitin-mediated ER degradation by the HRD pathway. We had previously shown that alterations in cellular levels of the 15-carbon sterol pathway intermediate farnesyl pyrophosphate (FPP) cause increased Hmg2p ubiquitination and degradation. We now present evidence that the FPP-derived, 20-carbon molecule geranylgeranyl pyrophosphate (GGPP) is a potent endogenous regulator of Hmg2p degradation. This work was launched by the unexpected observation GGPP addition directly to living yeast cultures caused high potency and specific stimulation of Hmg2p degradation. This effect of GGPP was not recapitulated by FPP, GGOH, or related isoprenoids. GGPP-caused Hmg2p degradation met all the criteria for the previously characterized endogenous signal. The action of added GGPP did not require production of endogenous sterol molecules, indicating it did not act by causing the buildup of an endogenous pathway signal. Manipulation of endogenous GGPP by several means showed that naturally made GGPP controls Hmg2p stability. Analysis of the action of GGPP indicated that the molecule works upstream of retrotranslocation, and can directly alter the structure of Hmg2p. We propose that GGPP is the FPP-derived regulator of Hmg2p ubiquitination. Intriguingly, the sterol-dependent degradation of mammalian HMGR is similarly stimulated by addition of GGOH to intact cells, implying that a dependence on 20-carbon geranylgeranyl signals may be a common conserved feature of HMGR regulation, that may lead to highly specific therapeutic approaches for modulation of HMGR.

The sterol or mevalonate pathway is highly conserved and essential in eukaryotes. Sterol synthesis entails stepwise construction of 5-carbon isoprenes from acetyl CoA, followed by condensations, cyclizations and modifications to form the wide array of sterol and non-sterol products produced by this versatile biosynthetic route ((1), and Fig. 1). The first committed step of the pathway is catalyzed by HMG-CoA reductase (HMGR), catalyzing mevalonic acid formation from HMG-CoA. HMGR undergoes multivalent regulation, including feedback regulated degradation that is conserved from yeast to mammals (2). When sterol pathway flux is high, HMGR degradation is fast, and steady state levels tend to be low. When sterol pathway flux is slowed, the degradation rate is slowed and HMGR levels tend to rise. In this way, the amount of HMGR is altered to meet changing cellular demand for sterol pathway products. The HMGR molecule consists of a globular C-terminal catalytic domain, connected to a multispanning ER anchor. The N-terminal multispanning transmembrane domain is responsible for regulated degradation, while the catalytic region can function autonomously and is not subject to regulation when freed from its ER anchor. Conversely, the N-terminal transmembrane region of HMGR is sufficient for feedback regulated degradation, allowing fusion of reporter genes such as lacZ or GFP to facilitate biochemical and genetic analysis of this process.

Feedback regulation of HMGR stability is observed in both mammals and yeast. As an avenue towards understanding the mechanistic details HMGR regulated degradation, we have

been studying this process in *S. cerevisiae*, using a combination of techniques made facile in this guide organism (2). Yeast expresses two isozymes of HMGR, Hmg1p and Hmg2p: Hmg1p is quite stable, while Hmg2p undergoes sterol-pathway regulated degradation. Hmg2p degradation depends on its multispanning N-terminal anchor, with a half-life that varies between ~10 minutes and many hours depending on the level of degradation signal from the sterol pathway. Hmg2p degradation proceeds by the HRD pathway (**H**mg CoA **R**eductase **D**egradation (2-4)), mediated by the Hrd1p ubiquitin ligase and a variety of other factors. The HRD pathway is also a principle pathway of ER-associated degradation (ERAD), responsible for the destruction of numerous misfolded and damaged proteins in the ER lumen or membrane. Thus, regulated Hmg2p degradation occurs by signals from the sterol pathway promoting Hmg2p entry into the HRD quality control pathway, possibly by selective misfolding of the normal Hmg2p molecule to a structure that is recognized as a misfolded protein by the HRD apparatus.

In our analyses of the sterol pathway signals that accelerate Hmg2p degradation, we discovered that altering cellular levels of 15-carbon farnesyl pyrophosphate (FPP) causes striking changes in Hmg2p stability (5). Increasing FPP by lowering the activity of the FPP-utilizing enzyme squalene synthase (SS), either by pharmaceutical or genetic means, causes increased ubiquitination and degradation of Hmg2p. Conversely, lowering cellular FPP levels either by upstream inhibition of its production, or by overexpression of SS, slows degradation of Hmg2p. Thus, we have long thought that the signal was FPP, or an FPP-derived isoprenoid. Direct studies on the structure of Hmg2p using limited proteolysis and thermal denaturation assays indicated that isoprenoids can directly alter the structure of Hmg2p, causing a reversible misfolding of the protein that might make it more susceptible to the HRD pathway (6,7). Since the most effective lipid in these assays was farnesol (FOH), derived from FPP, we speculated that either farnesol was the FPP-derived regulator, or that the FPP itself employed this feature of its structure to accelerate Hmg2p degradation.

Recently, in an attempt to directly stimulate Hmg2p degradation, as opposed to using drugs or mutants to alter the endogenous, FPP-derived signal, we tested the effect of directly adding various pathway molecules to intact cells of the highly permeable *pdr5Δ* null strain. The ability to directly stimulate Hmg2p degradation would allow more refined analysis of both the signal and the process of regulated HRD-dependent Hmg2p degradation. To our surprise, we found a highly specific potent effect of the extended 20-carbon geranylgeranyl pyrophosphate (GGPP), which is a natural derivative of FPP, but no effect of any other lipid tested including the primary candidate FPP. GGPP caused specific stimulation of Hmg2p ubiquitination and degradation. In fact, GGPP was equally effective in normal yeast without the *pdr5Δ* mutation, apparently crossing the intact yeast membrane and cell wall despite its large size and high charge. Moving forward from this serendipitous observation, we have conducted a detailed analysis of the root of this effect, and have concluded from the studies below that endogenously synthesized GGPP is in all likelihood the physiological, FPP-derived regulator of Hmg2p degradation. A clear but poorly understood role for geranylgeranyl lipids in regulated degradation of mammalian HMGR has been reported (8), thus providing an intriguing new connection between HMGR regulation in yeast and mammals. In addition, this non-sterol mechanism of stability regulation may direct us to new approaches to modulating HMGR for clinical benefit.

EXPERIMENTAL PROCEDURES

Reagents- Isoprenoids were purchased from Sigma-Aldrich (St. Louis, MO). Lovastatin and zaragozic acid were gifts from Merck (Rahway, NJ).

Strains and Media- Strains were derived from the S288C derivative genetic background except for strains obtained from other laboratories. Yeast were grown at 30°C with aeration as described previously (3), or at 25°C and 37°C where indicated. Cultures were logarithmically grown in minimal medium with 2% glucose and appropriate amino acid supplements to an OD_{600}<0.35. Standard yeast techniques were used to introduce plasmids, prepare gene deletions, and incorporate mutant alleles. Yeast strains are listed in Table S1 of *Supplementary Data*. RHY1015 was previously

described (5). The *bts1*Δ null strain was made by PCR-mediated homologous recombination of the nourseothricin gene (*NatR*) flanked by 50 base pairs of homology of the *BTS1* coding region. Null was confirmed by cold temperature sensitivity at 17˚C and PCR. All primer sequences are available upon request. The geranylgeranyl transferase type I subunit mutant allele *bet2-1* strain and its corresponding wildtype were provided by Susan Ferro-Novick (UCSD) (9) and the strain carrying the type II subunit mutant allele *cdc43-2* was provided by Jasper Rine (UC Berkeley)(10). The *cdc43-2* allele was complemented by the expression of *CDC43* on a CEN plasmid to serve as the control strain. This plasmid was constructed by subcloning *CDC43* from YEp(43)2 (also received from the laboratory of J. Rine) into pRS415. Complementation was confirmed by growth at the restrictive temperature of 37°C. The plasmid carrying the *GAL1* promoter-driven *BTS1* was a gift from Seichi Matsuda (Rice University, Houston, Texas). Plasmids YEpM6PN and YEpM6G53E, expressing *MRS6* and mutant *mrs6*^{G53E}, respectively, were provided by Antonella Ragnini-Wilson (University of Tor Vergata, Italy), and described previously (11,12).

Flow Cytometry- Flow cytometry was carried out as previously described (13,14). Yeast grown in minimal medium with 2% glucose and appropriate amino acids into log phase (OD_{600} < 0.2) were incubated with mevalonate pathway isoprenoid intermediates (typically 11 µM unless indicated differently), zaragozic acid (10 µg/mL), lovastatin (25 µg/mL), vehicle (for GGPP and other isoprenoids this consisted of addition of the same volume of the solution 3 parts 1 mM ammonia and 7 parts methanol), or cycloheximide (50 µg/mL) for times indicated. The BD Biosciences FACS Calibur flow cytometer measured the individual fluorescence of 10,000 cells. CellQuest software was used to analyze the data and plotted fluorescence vs. cell count histograms.

Whole Cell Lysates- Preparation of whole cell lysates was previously described (15). After each time indicated with GGPP (11 µM) or vehicle incubation, cells were pelleted (1 OD equivalent) and resuspended in 100 µL SUME (1% SDS, 8 M urea, 10 mM MOPS pH 6.8, 10 mM EDTA) with the following protease inhibitors (PIs): 1 mM phenylmethylsulphonyl fluoride (PMSF), 100 mM leupeptin hemisulfate, 76 mM pepstatin A, and 142 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). 100 µL of acid-washed glass beads were added to suspension and cells were broken by vortexing for 3 x 1-minute intervals with 1-minute intervals on ice between each vortexing. Following addition of 100 µL of 2x urea sample buffer (2xUSB: 8 M urea, 4% SDS, 10% ß-mercaptoethanol, 125 mM Tris, pH 6.8), the slurry was incubated at 50˚C for 10 minutes. Lysates were clarified by centrifugation for 5 minutes at 14,000 g. Proteins were resolved by 8% SDS-PAGE and transferred to nitrocellulose membrane blots. 5% nonfat-dried milk in Tris-buffered saline with tween (TBS-T: 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) was used to block blots and 2% non-fat dried milk in TBS-T was used in antibody incubations. Hmg2p-GFP was detected with anti-GFP (BD Biosciences, San Jose, CA) and 1myc-Hmg2-GFP with anti-myc 9E-10 antibody (hybridoma from ATCC, Manassas, VA). Goat anti-mouse conjugated with HRP (Jackson ImmunoResearch, West Grove, PA) recognized primary antibodies. Western Lightning chemiluminescence reagents (Perkin Elmer, Waltham, MA) were used for immunodetection.

In vivo ubiquitination- Yeast cells were grown in minimum media to an OD of 0.3 OD/mL. Cells were pelleted at 2500 rpm and resuspended with 4 mL fresh media. Either 4 µL DMSO or ZA was added or 20 µL of vehicle or GGPP (final concentration 11 µM) and incubated for 10 minutes before lysis. Cells were pelleted at 3500 rpm and resuspended in 200 µL of SUME with the following PIs: 1 mM PMSF, 260 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 100 mM leupeptin hemisulfate, 76 mM pepstatin A, 5 mM -aminocaproic acid, 5 mM benzamidine, and 142 mM TPCK and 5 mM n ethylmaleimide (NEM). Glass lysis beads were added to meniscus. Tubes were vortexed for 6 x 1-minute intervals with incubations on ice for 1 minute in between. Liquid was removed from beads and placed in fresh tube. Beads were washed with 600 µL IPB (15 mM Na_2HPO_4, 150 mM NaCl, 2% Triton X-100, 0.1% SDS, 0.5% DOC, 10 mM EDTA, pH 7.5) with PIs and NEM, and combined with initial lysate. The lysis suspension was cleared of unbroken cells and large debris by centrifugation at 14,000 g for 5 min. The supernatant was transferred to fresh tube,

to which 15 µL anti-GFP was added. The IP was incubated for 5 min on ice, and then subjected to centrifugation for 5 min at 14,000 g. The supernatant was transferred to a fresh tube and the IP was incubated overnight at 4˚C. Protein A sepharose was added and incubated for an additional two hours. The protein-bound beads were washed once with IPB and once with IPW (50 mM NaCl, 10 mM Tris, pH 7.5). The beads were aspirated to dryness and proteins were eluted with 55 µL 2xUSB. Proteins were separated by SDS PAGE (8%) and transferred to nitrocellulose membranes. After membrane autoclaving (16), ubiquitinated Hmg2p-GFP were detected with monoclonal anti-ubiquitin (Zymed Laboratories, South San Francisco, CA). Levels of Hmg2p-GFP were detected with monoclonal anti-GFP.

Protease Protection Assay- Microsomes were prepared from the strain expressing the myc_L-Hmg2p-GFP for trypsin digestion as previously described (7)). The microsomes were resuspended in XL buffer (1.2 M sorbitol, 5 mM EDTA, 0.1 M KH_2PO_4/K_2HPO_4, final pH 7.5). Vehicles, farnesol (200 µM), or GGPP (20 µM) was added prior to incubation of 150 µg/mL trypsin (Sigma-Aldrich) for 0, 2, and 10 minutes. An equal volume of 2xUSB was added to stop the reactions. The samples were then separated by 14% SDS-PAGE and immunoblots were detected with anti-myc 9E-10 antibody.

In vitro ubiquitination- Assay was carried out as previously described (17) with the exception that microsome donor strains do not express *HRD1* from the *TDH3* promoter. Briefly, microsomes from a *ubc7*Δ null yeast strain expressing 3HA-Hrd1p from the *HRD1* promoter and Hmg2p-GFP (WT or NR1) from the *TDH3* promoter were prepared by glass bead lysis. 25 OD_{600} units of log-phase cells were lysed with 500 µL of membrane fractionation buffer (MF: 20 mM Tris pH 7.5, 100 mM NaCl, 300 mM sorbitol) with same PIs as in *In vivo ubiquitination*. At 4 °C, cells were lysed using hand vortexing for 6 x 1-minute intervals with 1-minute intervals on ice between each vortexing. The lysate was collected and pooled with two bead rinses with MF to give the crude microsomal lysate and cleared of cellular debris. The microsomes were pelleted at 14,000 g for 45 minutes at 4 °C and then resuspended in B88 buffer (20 mM Hepes pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM MgOAc, 1 mM DTT) with PIs, to a final concentration of 0.3 OD equivalent units/µL.

Cytosol was prepared from a *hrd1*Δ *ubc7*Δ double null control strain and its overexpressing Ubc7p counterpart in a in a similar manner as the Schekman lab (18). 500 OD equivalents of cells were pelleted, rinsed twice with water, once with B88 buffer, and resuspended in 500 µL of B88 buffer with PIs. The resuspended cells were transferred to a chilled mortar containing liquid nitrogen and ground with a pestle to form a the frozen powder. 1 mM ATP from a 500 mM stock solution in H_20 pH 7 was added to thawed cytosol. This crude cytosol lysate was centrifuged at 3,000 g for 5 min to remove large debris, and then the resulting supernatant was centrifuged at 20,000 g for 15 minutes. The lysate was further clarified by ultracentrifugaton at 100,000 g for one hour. Cytosol concentrations were determined by Bradford reagent and adjusted with B88 to 20 mg/mL. MG-132 (Sigma Aldrich) was added to a final concentration of 300 µM to microsome and cytosol preparations.

One in vitro ubiquitination reaction consisted of 10 µL microsomes and 12 µL cytosol. ATP (500 mM stock) was added to each reaction to a final concentration of 30 mM. The reactions were incubated for one hour at 30°C. The assay was stopped by solubilization with 200 µl of SUME with PIs and 5 mM NEM. 600 µL IPB with PIs and NEM was added and followed by addition of 15 µL rabbit polyclonal anti-GFP. Immunoprecipitation, separation by SDS-PAGE, and detection of unmodified and ubiquitinated Hmg2p-GFP were carried out as described above in *In vivo ubiquitination*.

RESULTS

Direct addition of GGPP to yeast cell cultures enhances Hmg2p degradation - Our earlier studies indicated that a molecule derived from FPP is the principle signal for HRD-dependent degradation of Hmg2p (5). Those studies depended on the indirect production of the FPP-derived signal by genetic or pharmacological means. For example, addition of the squalene synthase inhibitor zaragozic acid (ZA) causes buildup of the FPP substrate of this enzyme, leading to strongly increased ubiqutination and degradation of Hmg2p. We explored if addition of various

isoprenoids to yeast cell cultures could directly stimulate Hmg2p.

We tested a variety of isoprene molecules formed along or from the sterol pathway including our original candidate molecule FPP, the 10-carbon precursor geranyl pyrophosphate (GPP), the two 5-carbon precursors dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), and the 20-carbon geranylgeranyl pyrophosphate (GGPP), which is derived from FPP (Fig. 1). To facilitate our tests, we employed the normally regulated, non-catalytic reporter Hmg2p-GFP (5,20), consisting of the eight-spanning N-terminal transmembrane anchor followed by GFP. Hmg2p-GFP undergoes normally regulated degradation that can be monitored biochemically or by in vivo flow cytometry of whole-cell GFP. Log-phase cells expressing Hmg2p-GFP were treated by direct addition of various test molecules to the culture medium for 1 hour, followed by flow cytometry to evaluate changes in steady-state levels due to enhanced degradation.

Of the molecules tested, GGPP alone had an effect on Hmg2p-GFP levels and this effect was quite strong (Fig. 2A). In contrast, even very high concentrations of FPP did not affect Hmg2p-GFP levels (data not shown), despite its clear role as a precursor of the in vivo signal. Because GGPP is formed in vivo from FPP and IPP (Fig. 1), we also tested adding this pair in combination, but they similarly had no effect, and squalene, which lies downstream of FPP along the sterol pathway (Fig. 1) (5), was also without effect, as expected (data not shown).

This unique action of 20-carbon GGPP was reminiscent of stimulated degradation of mammalian HMGR caused by direct addition of non-phosphorylated geranylgeraniol (GGOH) to cultured cells (8). Nevertheless, a range of GGOH concentrations added to yeast cultures up to 50 times that of the lowest maximally effective GGPP concentration did not alter levels of Hmg2p-GFP (Fig. 2B). Although the 15-carbon alcohol farnesol (FOH) derived from FPP had strong effects in our in vitro structural assays (7), FOH is highly toxic to yeast cells and thus could not be tested over the time scales normally used. However, short term studies of FOH in yeast indicate that this molecule was similarly without effect (data not shown). Thus, only GGPP had a direct effect on Hmg2p, and a striking one, prompting further investigation of this action.

The concentration dependence of GGPP was determined (Figs. 2C & 2D). Logarithmically growing cells were incubated with various concentrations of GGPP for one hour, followed by flow cytometry of the cultures or immunoblotting of lysates prepared as described in *Experimental Procedures*. In both assays, the approximate EC_{50} was 2 μM, and the lowest maximally effective concentration being approximately 11 μM.

GGPP enhances Hmg2p ubiquitination - Regulated degradation of Hmg2p proceeds by the HRD ERAD pathway, which employs the Hrd1p ubiquitin ligase to ubiquitinate Hmg2p (4). The effect of GGPP on Hmg2p-GFP similarly required an intact HRD pathway, and was absent in a *hrd1*Δ null (Fig. 3A; compare *HRD1* to *hrd1Δ*).

Increasing the signal for Hmg2p degradation with agents such as ZA causes increased ubiquitination. The effect of GGPP on Hmg2p-GFP ubiquitination was evaluated by immuno-precipitation (IP) followed by immunoblotting (IB) for Hmg2p-GFP or ubiquitin. Identical cultures of cells were treated with GGPP, ZA or their respective vehicles for just 10 minutes, followed by lysis and IP/IB analysis for ubiquitination. Like ZA, GGPP added directly to the yeast cells causes sudden and profound ubiquitination of Hmg2p-GFP (Fig. 3B).

Effect of GGPP meets hallmarks of Hmg2p regulated degradation - An extensive mutagenic analysis showed that lysines 6 and 357 of Hmg2p are each required for Hmg2p regulated degradation (21). Loss of either lysine rendered GGPP ineffective in stimulating Hmg2p degradation (Fig. 4A). Similarly, the highly stable Hmg1p was not affected by GGPP (Fig. 4B).

A number of distinct Hmg2p mutants undergo HRD-dependent degradation but are unregulated by the FPP-derived signal. That is, their HRD-dependent degradation is the same at all levels of the FPP-derived signal. We examined two of these mutants, NR1 and 6myc-Hmg2p, to further test the specificity of the GGPP effect. NR1-Hmg2p-GFP contains a five-amino acid change in the sterol sensing domain within the transmembrane domain of Hmg2p, and 6myc-Hmg2p-GFP has a segment of the lumenal transmembrane domain replaced with 6 myc epitopes (7). Each of these proteins undergoes HRD dependent degradation unaffected

by changing the level of the FPP-derived signal. GGPP incubation did not influence NR1-Hmg2p-GFP or 6myc-Hmg2p-GFP levels in conditions where the wild-type Hmg2p-GFP underwent the expected degradation (Figs. 5A & 5B). Taken together, these *in cis* studies indicated that the effect of GGPP shows the same high specificity as the physiological signal derived from FPP.

GGPP action is not through squalene synthase inhibition - The squalene synthase inhibitor zaragozic acid (ZA) induces Hmg2p degradation by causing FPP levels to build-up, and acts very rapidly (5). GGPP addition had a similar rapid effect on stability (Fig. 2E) and ubiquitination (Fig. 3B). Thus, it was important to test whether GGPP was also a potent inhibitor of squalene synthase, or alternatively, operated in a manner distinct from inhibition of this enzyme. Since ZA has a structure that is reminiscent of isoprenoids, this is a formal possibility. We tested this idea by several criteria that convincingly demonstrate GGPP acts directly on Hmg2p regulation rather than through squalene synthase inhibition.

The effect of ZA is dependent on its inhibition of squalene synthase, encoded by the yeast *ERG9* gene ((22), Fig. 1). Not surprisingly, overexpression of squalene synthase shifts the dose-response curve for ZA induced stimulation of Hmg2p degradation strongly to the right. Specifically, we have shown that stimulation of Hmg2p degradation requires significantly higher concentrations of ZA when the squalene synthase gene is overexpressed by using the strong *TDH3* promoter to drive the *ERG9* gene (5). This provided a useful test for assessing if GGPP was also an inhibitor of squalene synthase.

We directly compared the effects of squalene synthase overexpression on ZA or GGPP stimulation of Hmg2p-GFP degradation. The indicated concentrations of GGPP or ZA were incubated with otherwise identical strains expressing normal genomic *ERG9*, or *ERG9* driven by the strong *TDH3* promoter. While the ZA showed the expected decrease in potency as a stimulant of Hmg2p-GFP degradation in the strains with its overexpressed target enzyme, GGPP had identical potency in each strain, indicating that its effects were not mediated by inhibition of squalene synthase (Fig. 6A).

Mevalonate pathway inhibition does not block effect of GGPP - The action of ZA depends on flux through the mevalonate pathway, since stimulation of Hmg2p degradation by ZA is caused by a build-up of the pathway intermediate FPP (Fig. 1). Thus, the action of ZA is inhibited by the upstream inhibitor lovastatin, which at sufficient doses blocks the production of FPP needed for stimulation of Hmg2p degradation (5,23). We wondered if the action of GGPP was independent of sterol pathway flux; this would be further evidence that GGPP does not operate by inhibition of squalene synthase, or any other downstream enzyme. We tested the effect of lovastatin on GGPP-mediated lowering of Hmg2p-GFP. Cells were pretreated with lovastatin for one hour and then incubated with either GGPP or ZA for an additional hour. As expected, ZA did not stimulate Hmg2p degradation in these conditions (Fig. 6B), or even when added at the same time as lovastatin (data not shown). In contrast, the GGPP effect was identical in the presence or absence of lovastatin in these conditions. Taken together, these results indicate that GGPP acts directly on the degradation process of Hmg2p-GFP, rather than by altering the production or levels of a sterol-pathway derived signal.

Intracellular levels of GGPP influence Hmg2p stability - We turned our attention to the possibility that endogenously made GGPP was involved in regulated degradation of Hmg2p. GGPP is synthesized from FPP by the geranylgeranyl pyrophosphate synthase encoded by *BTS1* ((9) and Fig. 1A). We first tested if overexpression of *BTS1* would increase the degradation of Hmg2p, using a plasmid expressing *BTS1* under the control of the strong galactose promoter. In galactose, Hmg2p-GFP levels were significantly lower in strains carrying the *BTS1* plasmid. When these *BTS1*-expressing cells were treated with lovastatin, Hmg2p-GFP levels rose (Fig. 7). As expected, overexpression of *BTS1* had no effect on the unregulated NR1-Hmg2p-GFP or 6myc-Hmg2p-GFP versions of Hmg2p.

The *BTS1* overexpression test indicated that endogenously produced GGPP can promote Hmg2p degradation. We next addressed if naturally-made GGPP participated in Hmg2p regulation, by examining Hmg2p degradation in a *bts1Δ* null mutant, which is viable but cold sensitive (9). The *bts1Δ* null was confirmed by direct PCR analysis and the reported cold sensitive growth phenotype: *bts1*Δ null colonies showed

slowed growth at 30 °C that was exacerbated at 17˚C (*Supplemental Data,* Fig. S1).

We compared the rate of Hmg2p-GFP degradation in *BTS1* and *bts1*Δ null strains by addition of cycloheximide followed by flow cytometry at the indicated times. An untreated culture aliquot was also included in the run to indicate Hmg2p-GFP starting levels. Hmg2p-GFP degradation was reproducibly slower in the *bts1Δ*, showing a 1.6 fold slowing of degradation (Figs. 8A & 8B). This altered rate of degradation was not observed when the identical experiment was done with unregulated NR1-Hmg2p-GFP, indicating the difference was due to altered degradation signal. We were somewhat surprised by this small effect, since lovastatin strongly inhibits Hmg2p degradation, and this action is preserved in the *bts1Δ* strain (data not shown). However, it has been suggested that GGPP can be synthesized by both *BTS1* and by FPP synthase (25), indicating that the *bts1Δ* null may still produce GGPP by this alternate route.

The *bts1Δ* null shows that decreasing the efficiency of GGPP production stabilizes Hmg2p. Because it appears that GGPP might be made by either Bts1p or FPP synthase (25), we used the Mrs6 protein as an independent means to manipulate intracellular GGPP levels (12). This protein mediates binding between substrate, GGPP and the geranylgeranylation enzymes that add the 20-carbon isoprene unit to the C-termini of various proteins. Strong expression of Mrs6p results in a higher ratio of ungeranylgeranylated substrates, which has been attributed by a variety of tests to lowering of free intracellular GGPP by overexpressed Mrs6p. Using the plasmids from those studies, *MRS6* overexpression was tested for effects on Hmg2p degradation, along with a mutant version, *mrs6*^{G53E}, that does not lower free GGPP.

Overexpression of Mrs6p caused elevated levels of Hmg2p-GFP, but not unregulated 6myc-Hmg2p-GFP tested in parallel (Fig. 8C). Conversely, cells expressing the mutant Mrs6p^{G53E} actually showed decreased Hmg2p-GFP levels, and again there was no effect on unregulated 6myc-Hmg2p-GFP. *mrs6*^{G53E} has been reported to cause a decrease in cellular geranylgeranyl transferase activity (12), possibly increasing free GGPP levels that would enhance regulated degradation of Hmg2p-GFP. These results with *MRS6* are again consistent with a role of endogenous GGPP in regulation of Hmg2p stability.

A possible interpretation of both the GGPP manipulation and the Mrs6p studies was that a critical regulatory protein was geranylgeranylated to an active form that caused Hmg2p degradation. However, many of our studies have cast doubt on this model. Protein prenylation is thought to occur soon after translation, without build-up of unprenylated precursors. Yet, Hmg2p regulation occurs in the absence of proteins synthesis, even when protein synthesis has been blocked for significant time before the FPP-derived signal is introduced.

Prenylation was not implicated in Hmg2p degradation - We confirmed the independence of GGPP's effects from protein synthesis. Cells expressing Hmg2p-GFP were treated with cycloheximide for one hour, after which GGPP, ZA, or vehicle were added, followed by continued incubation and flow cytometry at various times. Despite this long period of protein synthesis inhibition, both GGPP and ZA caused increased rate of Hmg2p degradation, showing that their regulatory effects work long after protein synthesis has ceased (Fig. 9a). Although this strongly implies that GGPP operates independently of prenylation, we also directly tested the effect of mutations in the geranylgeranyl transferase (GGTase) enzymes that transfer the isoprene group from GGPP to target proteins.

Yeast express two GGTases: Type I and Type II. Type I GGTase consists of two subunits, Cdc43p and Ram2p, and Type II of Bet2p, Bet4p, and Mrs6p (reviewed in (26)). We employed temperature sensitive alleles of Type II GGTase subunit *bet2-1*, or Type I GGTase subunit, *cdc43-2* to examine if Hmg2p degradation was affected by deficiencies in either type of geranylgeranylation. We assayed Hmg2p-GFP degradation after stopping protein synthesis with cycloheximide at both permissive and restrictive temperatures, using both normally regulated Hmg2p-GFP and unregulated NR1-Hmg2p-GFP. Neither mutation, even after prolonged incubation at the non-permissive temperatures, showed any defect in degradation specific for the regulated Hmg2p-GFP protein (Figs. 9B & 9C).

Furthermore, all the prenylation mutants remained responsive to addition of GGPP (data not shown).

GGPP affected Hmg2p in vitro proteolysis - These studies indicate that the regulation of Hmg2p may involve a direct interaction of a sterol-pathway derived molecule with Hmg2p. Consistent with with this idea, we have shown that a number of isoprene-containing molecules, principally farnesol, will reversibly alter the structure of Hmg2p to a more unfolded state, that may be better recognized by the HRD quality control pathway (7). These studies were done using myc_L-Hmg2p-GFP, a normally regulated version of Hmg2p-GFP with an added lumenal myc tag, to allow limited proteolysis of the microsome-bound protein, to assess changes in structure caused by the added isoprene. We confirmed that, like farnesol, GGPP directly altered the structure of myc_L-Hmg2p-GFP to a similar extent when added directly to the microsomes (Fig. 10A), and that it did not have these effects on the NR1 variant of myc_L-Hmg2p-GFP protein (data not shown), showing that by this structural transition assay that meets a number of criteria appropriate for relevance in Hmg2p regulation, GGPP would be considered a positive regulator.

GGPP was without effect on Hmg2p in vitro ubiquitination - We had previously developed an in vitro ubiquitination assay to better understand Hmg2p degradation. In vitro ubiquitination of Hmg2p is HRD dependent, requires the correct E2 Ubc7p, is specific for the appropriate lysines, shows Cdc48p-dependent retrotranslocation, and is sensitive to chemical chaperones ((27,28), and unpublished observations). However, we have so far not observed any effect of addition of FPP to this reconstituted system. We tested the effects of GGPP on in vitro ubiquitination both in conditions where Hrd1p is overexpressed (not shown), and when Hrd1p is at native levels. In neither case did GGPP addition to the in vitro reaction cause changes in Hrd1p-mediated ubiquitination of Hmg2p (Fig. 10B), despite its conformity to many criteria of physiologically appropriate ubiquitination. Interestingly, addition of GGOH, or GGPP to ubiquitination assays for mammalian HMGR similarly had no effect, despite its ability to accelerate HMGR degradation in the mammal as well (8).

Intact retrotranslocation machinery was not required for GGPP-induced Hmg2p ubiquitination - It has been proposed that the dependence of mammalian HMGR degradation on geranylgeranyl isoprenes may be due to stimulation of HMGR retrotranslocation (8). To test if GGPP-stimulation of Hmg2p-GFP ubiquitination was coupled to retrotranslocation, we evaluated the ubiquitination state of Hmg2p in a retrotranslocation-deficient *cdc48-3* strain exposed to GGPP. We introduced the mutant *cdc48-3* allele into our strain background and confirmed that wildtype Hmg2p-GFP and mutant NR1-Hmg2p-GFP were stable in this background by flow cytometry (*Supplemental Data,* Fig. S2). We also confirmed that this mutant strongly inhibits in vitro retrotranslocation of Hmg2p-GFP (17). The presence of the *cdc48-3* allele had no effect on GGPP-stimulated ubiquitination of Hmg2p (Fig. 10C), despite it having a strong effect on Hmg2p-GFP stability in vivo (Fig. S2) and Hmg2p-GFP retrotranslocation in vitro. The NR1-Hmg2p-GFP was unaffected by GGPP in this pair of strains, confirming maintained specificity of GGPP's action in the presence of the Cdc48p deficiency. Consistent with this, addition of GGPP directly to an in vitro retrotranslocation assay for Hmg2p had no effect on the process observed in this manner either (data not shown).

GGPP enhanced degradation of full-length Hmg2p - The Hmg2p-GFP reporter protein is regulated identically to catalytically active Hmg2p in all ways tested. Therefore, we expected and confirmed that the full-length, catalytically active Hmg2p was similarly responsive to GGPP. Strains expressing full-length Hmg2p tagged with a single myc epitope or its lysine 6 mutant K6R counterpart were exposed to GGPP for 0, 1, or 3 hours. Wildtype Hmg2p degradation was accelerated and the stable lysine mutant was resistant, precisely as determined with alterations of cellular GGPP (21) (Fig. 10D).

DISCUSSION

In these studies we demonstrate that the mevalonate-derived molecule GGPP can function as a positive regulator of Hmg2p stability, and is, in all likelihood, the FPP-derived molecule that controls Hmg2p stability in response to changes in the mevalonate pathway. This work was initiated by the still-surprising observation that GGPP added directly to living yeast cells caused a high-

potency (low micromolar EC_{50}), immediate change in Hmg2p stability, resulting in enhanced ubiquitination and rapid degradation, similar to the response to the squalene synthase inhibitor zaragozic acid. The effect of GGPP was highly specific for regulated Hmg2p. Non-regulated and stable variants of Hmg2p, or normally stable Hmg1p were all entirely unaffected by GGPP. Intracellular elevation of GGPP by overexpression of the *BTS1* gene, encoding GGPP synthase, similarly caused a drastic and specific hastening of Hmg2p degradation, again consistent with its role as an intracellular regulator of Hmg2p.

The striking similarities between addition of GGPP and addition of squalene synthase (SS) inhibitor zaragozic acid (ZA) led us to examine the idea that GGPP was in fact a potent inhibitor of this key sterol pathway enzyme. However, the potency of GGPP was unaffected by elevation of SS to levels that drastically affected that of ZA, indicating it does not work through inhibition of this enzyme. Furthermore, exogenous GGPP still stimulated Hmg2p degradation in the presence of lovastatin concentrations that fully blocked the effects of ZA, again consistent with GGPP operating directly as opposed to acting through inhibition of squalene synthase, or any sterol pathway enzyme downstream of HMGR for that matter.

Since GGPP is naturally derived from FPP, it seems reasonable that this molecule is generated from FPP to control Hmg2p stability. In fact, naturally generated GGPP similarly altered stability of Hmg2p, since the *bts1Δ* null allele showed reproducible increased stability of Hmg2p specific for the normally regulated version of the protein. Furthermore, alteration of GGPP pools by overexpression of wild-type or mutant versions of the prenylation cofactor Mrs6p also showed the expected changes in Hmg2p stability consistent with endogenous GGPP playing a role in Hmg2p regulation. We were initially surprised that the effects of the *bts1Δ* null were not stronger. If this were the only source of GGPP, and if GGPP were the key regulator, then we would have expected the *bts1Δ* null to cause complete stabilization of Hmg2p, similar to the effects of lovastatin treatment, or mutations in Hmg2p that render it refractory to regulatory signals (21). A recent study of prenyl synthetases in both *S. pombe* and *S. cerevisiae* offers a possible explanation. It has recently been reported that sufficient expression of yeast FPP synthase (Erg20p), which is along the mevalonate pathway, can suppress specific phenotypes of the *bts1Δ*, indicating that this enzyme can function as a GGPP synthase as well (25). Considering the great similarity in the two reactions, this is perhaps not terribly surprising. Thus, it is likely that the GGPP generated in the cell is a product of the actions of both the dedicated Bts1p enzyme and the bifunctional Erg20p. That redundancy may also explain why the viable *bts1Δ* null mutant has never been isolated in the several screens performed in our laboratory for mutants that stabilized normally regulated Hmg2p. Although these observations fit a model that normally made GGPP is the key FPP-derived regulator of Hmg2p, they will require future direct analysis to confirm this important idea.

Perhaps the most natural idea for the action of a regulatory isoprenoid like GGPP would be as part of a prenylation reaction that renders a regulatory protein functional. However, it seems unlikely that GGPP acts in this capacity in control of Hmg2p stability. First, even when protein synthesis is blocked for an hour, the response to added GGPP is still intact and observable (Fig. 9A), even though it is thought that prenylation occurs soon after translation in most situations. Furthermore, temperature sensitive mutants of either GGPP-employing prenylation mutant had no effect on the stability of regulated Hmg2p. Both of these observations are consistent with prenylation not playing a role in Hmg2p regulation by GGPP.

The alternative model would be that GGPP, or something derived from it, directly affects Hmg2p entry into the HRD pathway. In biochemical assays of Hmg2p structure, we showed that some isoprenoids could cause reversible misfolding of the Hmg2p transmembrane domain, which could render it more susceptible to detection by the HRD quality control pathway. The effect is highly specific for normally regulated Hmg2p, and the same structural transition can be observed when the in vivo signal is elevated prior to isolating the assay microsomes (7). The most effective molecule in this in vitro assay was farnesol, although direct addition of farnesol (or FPP) to cells has no effect on Hmg2p stability, or on the in vitro Hmg2p ubiquitination assays we have so far devised. Similarly, GGPP also caused this in vitro

structural effect, and so at least in this readout of Hmg2p regulation can have direct effects, although how these translate to more effective entry of Hmg2p into the HRD pathway is not clear.

The action of GGPP in vivo appears to be fairly upstream, in that we see increased ubiquitination of Hmg2p immediately after addition of the molecule to cells, and in conditions where the proteasome or Cdc48p-dependent retro-translocation are inhibited. Thus, there is a dichotomy between our in vivo results that indicate ubiquitination is rapidly and primarily elevated, and our in vitro results that indicate ubiquitination is not affected. There is an interesting parallel to the regulation of mammalian HMGR as studied in the Debose-Boyd laboratory, which unlike Hmg2p is primarily keyed to levels of lanosterol in the cell; when lanosterol levels are high, the mammalian gp78 homologue of Hrd1p is recruited to HMGR and ubiquitination is accelerated (28). Along with this distinct role for sterols that separates mammalian HMGR regulation from that of Hmg2p, there is a common theme in the involvement of 20-carbon geranylgeranyl isoprenoids. Addition of the 20-carbon alcohol GGOH (GGPP without its two phosphates) to mammalian cells causes an increase in HMGR degradation above that caused by sterols alone (8). The authors posit that the added GGOH is converted into GGPP, which then has still-unknown effects on HMGR degradation. Whether this is the same process in both species, or the same isoprene being involved in different processes, will be an important question to address in the future.

There are several surprising features to our current work that warrant further study. The ease and potency with which direct addition of GGPP to living yeast cultures affects Hmg2p degradation was quite unexpected. Our normal experience has been that addition of anionic organic molecules, such as mevalonate, is at best a poor route for delivery to the cytoplasm (3). In fact, GGPP was used as a negative control in our first experiments, due to our presumption that it could not get into the cells. Thus, there is either a high affinity transport system for GGPP, or the intact yeast has a surprising hole in its permeability barrier for this molecule. Although we have not addressed this issue yet, we did attempt to test if there was a transporter with an inhibition experiment. Because FPP added directly to cells had no effect, we tested if very high concentrations of FPP would antagonize the effects of added GGPP. However, no concentration of FPP had any effect on added GGPP, even when in great excess to the active compound. Because FPP has no effect on its own, this observation does not inform us about the nature of GGPP influx, which, despite its inherent interest, remains a mystery demanding future study.

Our earlier work has strongly implicated endogenous FPP as a source for the principle regulator Hmg2p in vivo. Elevation of FPP by a number of means increases Hmg2p ubiquitination, and lowering FPP decreases ubiquitination (5). Those studies include both genetic and pharmacological means, and form a highly consistent picture of a positive regulator derived from FPP. Thus, we were surprised that addition of FPP to intact cells has no effect on Hmg2p. It could be that the transport of 15-carbon FPP does not occur, while the very similar 20-carbon GGPP structure is transported. While this seems unlikely, the question requires further investigation. If the FPP is indeed transported, then why is it ineffective at stimulating Hmg2p degradation, while endogenously produced FPP appears so central? We wondered if added FPP could not be converted into GGPP due to a lack of the other precursor molecule IPP, but simultaneous addition had no effect either. One explanation may be that the synthesis of GGPP is tightly coupled to FPP production, perhaps by channeling between the enzymes that produce FPP and those that use if for GGPP synthesis, including both Bts1p and Erg20p (FPP synthase) itself. This would explain the striking difference between adding the product and the substrate of the GGPP reaction, and warrants further study.

Tight coupling of FPP to GGPP production would mean that significant levels of GGPP should be expected during the normal operation of the sterol pathway, and especially when FPP utilization is halted with ZA or by genetic down-modulation of squalene synthase. There are two lines of evidence that indicate that this is the case. In our own earlier work (20), we tested the effects of various manipulations of the mevalonate pathway on the labeling pattern of Carbon-14 acetate as measured by thin layer chromatography.

Although our primary interest in those studies was the detection of oxysterols produced by the "alternate pathway" that arises from a buildup of dioxidosqualene, the entire pattern of acetate products was delivered by this approach. Upon revisiting these TLC autoradiograms, we discovered that the most prominent sterol-pathway product produced by inhibition of SS with ZA or by promoter shutoff had an identical Rf to GGOH, to the complete exclusion of FOH, for which standards were run in those assay. We confirmed this by directly comparing standards of GOH, FOH, and GGOH in identical TLC systems, which showed that the prominent band had the expected mobility of GGOH. In Fig. S3, for the convenience of the reader, we have reproduced the picture of the TLC of one of these experiments from (20), with an arrow indicating the prominent band with the mobility of GGOH. Clearly, this 20-carbon skeleton is abundantly produced in conditions where FPP utilization by SS is lessened. Similarly, in bulk lipid studies recently performed to evaluate the isoprenes produced when HMGR activity is elevated in yeast (29), the proportion of geranylgeranyl isoprenes produced is approximately the same as that of FPP, again indicating efficient conversion of FPP into GGPP when flux, and thus the degradation signal, is high.

Taken together, these results present a strong case for the FPP-derived signal that controls Hmg2p stability either being, or having as a direct precursor, GGPP. Since we are fairly confident that the GGPP is not employed in a prenylation reaction to make its regulatory hand felt, a product derived from GGPP would most likely be a more rare isoprenoid. It will be interesting to directly test some of the structural analogues of GGPP that cannot be hydrolyzed to the free alcohol to evaluate the effectiveness of the GGPP structure itself in this assay (30). Unraveling the nature of this striking effect of GGPP on Hmg2p regulation will allow us to understand the details of regulated HRD pathway degradation of Hmg2p, and perhaps the common facets of mechanism that connect the regulation of HMGR and Hmg2p, separated by over a billion years of evolution, but joined by a common selective force to regulate the sterol pathway in highly divergent walks of eukaryotic life.

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FOOTNOTES

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-The abbreviations used are: DMAPP, dimethylallyl pyrophosphate; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; FPP, farnesyl pyrophosphate; GFP, green fluorescent protein; GGOH, geranylgeraniol; GGPP, geranylgeranyl pyrophosphate; Hmg-CoA, 3-hydroxy-3-methylglutaryl CoA; HMGR, HMG-CoA reductase; IPP, isopentenyl pyrophosphate; SS, squalene synthase; ZA, zaragozic acid

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FIGURE LEGENDS

Figure 1. Key isoprenoids of the mevalonate pathway.

Figure 2. Direct addition of GGPP to yeast cell culture medium decreased Hmg2p levels. (A) GGPP lowers Hmg2p steady state levels. Cells expressing Hmg2p-GFP were incubated for one hour with the vehicle control or 11 μM of the following mevalonate pathway isoprenoids: IPP, DMAPP, GPP, FPP, GGPP. For each treatment, the GFP fluorescence of 10,000 cells was measured by flow cytometry. Histograms of number of cells vs. GFP fluorescence are shown in this and all subsequent figures. (B) Geranylgeraniol did not lower Hmg2p steady state levels like GGPP. Concentrations ranging from 0 to 100 μM of GGOH were incubated with cells expressing Hmg2p-GFP, followed by flow cytometry to measure GFP levels. (C) GGPP dose response by immunoblotting of Hmg2p-GFP. Whole cell lysates were prepared from cells that were incubated with indicated concentrations of GGPP for one hour. Hmg2p-GFP levels were measured by immunoblotting with anti-GFP. (D) GGPP dose response of Hmg2p-GFP levels by flow cytometry. Each data point represents mean fluorescence of 10,000 cells incubated with indicated concentration of GGPP for one hour. (E) GGPP effected Hmg2p levels on similar time scale as squalene synthase inhibitor zaragozic acid. Whole cell lysates were prepared from cells incubated with 10 μg/mL (13 μM) ZA, 11 μM GGPP, or the vehicle for indicated time lengths. Hmg2p-GFP levels were detected by immunoblotting with anti-GFP.

Figure 3. GGPP-mediated decline in Hmg2p levels was dependent on the Hrd1p E3 ligase. (A) Absence of Hrd1p blocked GGPP effect on Hmg2p-GFP levels. GGPP (11 μM) or its vehicle control was incubated with WT or *hrd1*Δ null cells for one hour. Flow cytometry was carried out to evaluate Hmg2p-GFP levels. (B) In vivo Hmg2p ubiquitination. Cells were incubated with either ZA (13 μM), GGPP (11 μM), or their respective vehicle controls for 10 minutes. Lysates were prepared, immunoprecipitated with anti-GFP, and immunoblotted for ubiquitin and GFP.

Figure 4. GGPP effects on Hmg2p-GFP mutants and Hmg1p. (A) Stabilizing lysine mutants K6R and K357R of Hmg2-GFP were unaffected by GGPP. Flow cytometry was carried out to evaluate Hmg2p-GFP levels after cells were incubated with 11 μM GGPP or vehicle for 1.5 hrs. (B) Levels of stable Hmg1p did not change in response to GGPP. Strains expressing Hmg1p-GFP or Hmg2p-GFP were subjected to incubation with GGPP and GFP fluorescence was measured by flow cytometry.

Figure 5. Unregulated mutants of Hmg2p-GFP were unresponsive to GGPP. (A,B) Hmg2p-GFP mutants NR1-Hmg2p-GFP (A) and 6myc-Hmg2p-GFP (B) were tested for an effect by GGPP. Strains expressing each mutant or WT Hmg2p-GFP were compared for effects of 1 hour incubation with 11 μM GGPP or vehicle.

Figure 6. GGPP action was distinct from zaragozic acid. (A) Overexpression of squalene synthase (*ERG9*) did not alter Hmg2p responsiveness to GGPP. Strains expressing WT or high levels of squalene synthase (from *TDH3* promoter) were incubated with indicated concentrations of ZA or GGPP. Following one hour incubation, Hmg2p-GFP fluorescence was measured by flow cytometry. (B) Lovastatin preincubation did not block the GGPP effect. Cells were either untreated or preincubated with lovastatin (25 μg/mL) for one hour before addition of either ZA (13 μM) or GGPP (11 μM) for another additional hour of incubation, followed by GFP flow cytometry.

Figure 7. Increasing endogenous production of GGPP synthase lowers Hmg2p levels. (A) WT Hmg2p-GFP steady state levels are lower in cells expressing galactose-driven *BTS1* compared to control whereas lysine mutant K6R-Hmg2p-GFP remain unchanged. Lovastatin treatment for four hours reversed *BTS1* overexpression on Hmg2p-GFP levels. (B) Like *A*, unregulated NR1-Hmg2p-GFP levels were not

influenced by high expression of Bts1p or inhibiting the pathway with lovastatin like its corresponding WT expressing strain.

Figure 8. Hmg2p stability increased with lowered GGPP levels. (A) Hmg2p degradation was slowed in *bts1*Δ. Hmg2p-GFP degradation was evaluated in WT and *bts1*Δ by cycloheximide chase. GFP fluorescence was measured by flow cytometry after adding cycloheximide for the indicated time lengths. (B) Graphical representation of Hmg2p-GFP degradation rates measured in *A*. (C) Overexpression of Mrs6p increased Hmg2p-GFP levels. Strains expressing Hmg2p-GFP or the unregulated 6myc-Hmg2p-GFP in cells with empty vector (e.v.) and cells overexpressing *MRS6* or mutant *mrs6*^{G53E} cells were subjected to GFP flow cytometry.

Figure 9. Protein synthesis was not required for GGPP-mediated Hmg2p degradation. (A) GGPP-stimulated Hmg2p-GFP degradation occurred independently of protein synthesis. Cells expressing Hmg2p-GFP were untreated (no CX) or preincubated with protein synthesis inhibitor cycloheximide (CX, 50 μg/mL) for one hour before addition of vehicle, 11 μM GGPP, or 13 μM ZA for incubation of an additional hour. (B) Normal geranylgeranylation was not required for GGPP-mediated Hmg2p-GFP degradation. Graphic representation of Hmg2p degradation evaluated in WT and GGtase mutants *bet2-1* (*top*) and *cdc43-2* (*bottom*) at permissive (25˚C or 30˚C) and restrictive temperatures (37˚C) by cycloheximide chase. Cells were shifted to restrictive temperature one hour before two hour cycloheximide exposure. Graph displays percent of Hmg2p-GFP remaining in cells treated with cycloheximide with respect to cells treated in the same exact manner but without cycloheximide.

Figure 10. In vitro and in vivo analysis of GGPP effects. (A) GGPP increases rate of proteolytic digestion of 1myc-Hmg2p-GFP. Microsomes prepared from strain expressing lumenally tagged myc$_L$-Hmg2-GFP were incubated with either vehicle controls, 200 μM farnesol or 20 μM GGPP in the presence of trypsin for indicted times, and immunoblotted with anti-myc. (B) GGPP did not induce in vitro WT Hmg2p-GFP ubiquitination. In vitro reactions consisted of mixing microsomes expressing WT or NR1 Hmg2p-GFP with either *ubc7*Δ (Δ) or *UBC7* cytosol. Prior to adding 30 μM ATP to initiate reactions the following were added to the mixtures: no drug (ND), vehicle (V), 10 μM (10) or 80 μM (80) GGPP (G). GFP antisera was used to immunoprecipitate WT and NR1 Hmg2p-GFP. Ubiquitinated Hmg2p-GFP was detected with anti-ubiquitin and levels of Hmg2p-GFP were detected by immunoblotting with anti-GFP. Note that the unregulated NR1 *UBC7*-dependent ubiquitination occurred regardless of GGPP presence. (C) Intact retrotranslocation was not required for GGPP-induced Hmg2p-GFP ubiquitination. WT and NR1 Hmg2p-GFP was immunoprecipitated from *CDC48* and *cdc48-3* strains exposed to either vehicle (V) or 11 μM GGPP (G) for 10 minutes. Ubiquitinated Hmg2p-GFP and its levels were detected as in *B*. (D) GGPP stimulated degradation of catalytically active, full-length Hmg2p. Strains expressing catalytically active WT or mutant K6R Hmg2p with a single myc epitope were incubated with 11 μM GGPP. Whole cell lysates were prepared after indicated incubations. Full-length Hmg2p was detected with anti-myc.

Figure 1

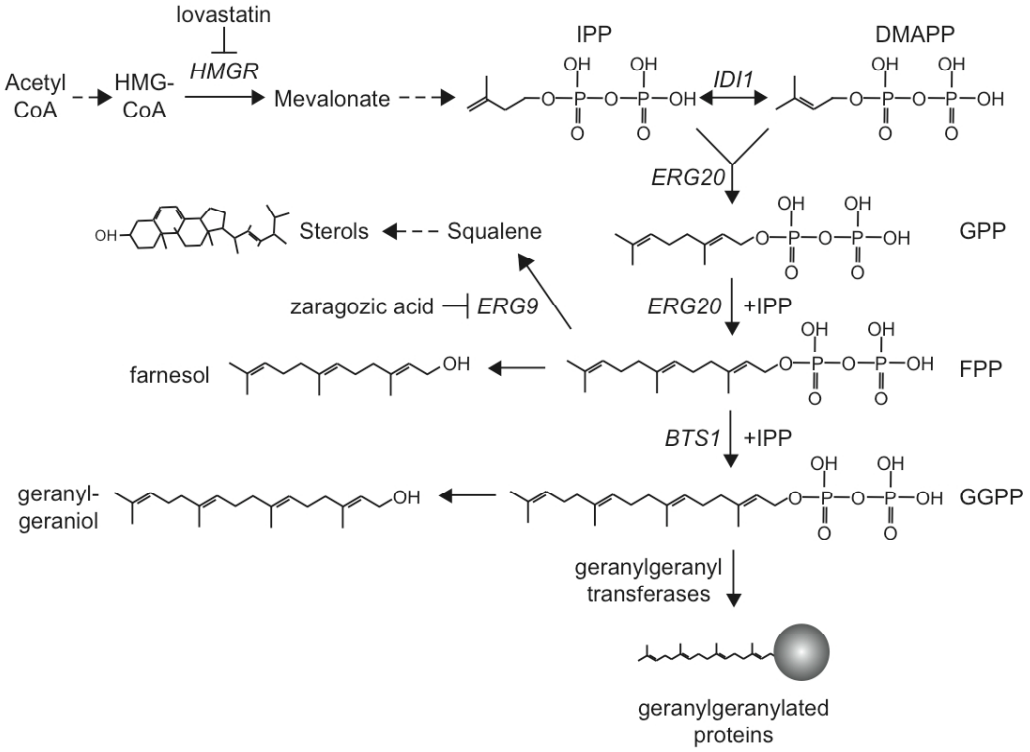

Figure 2

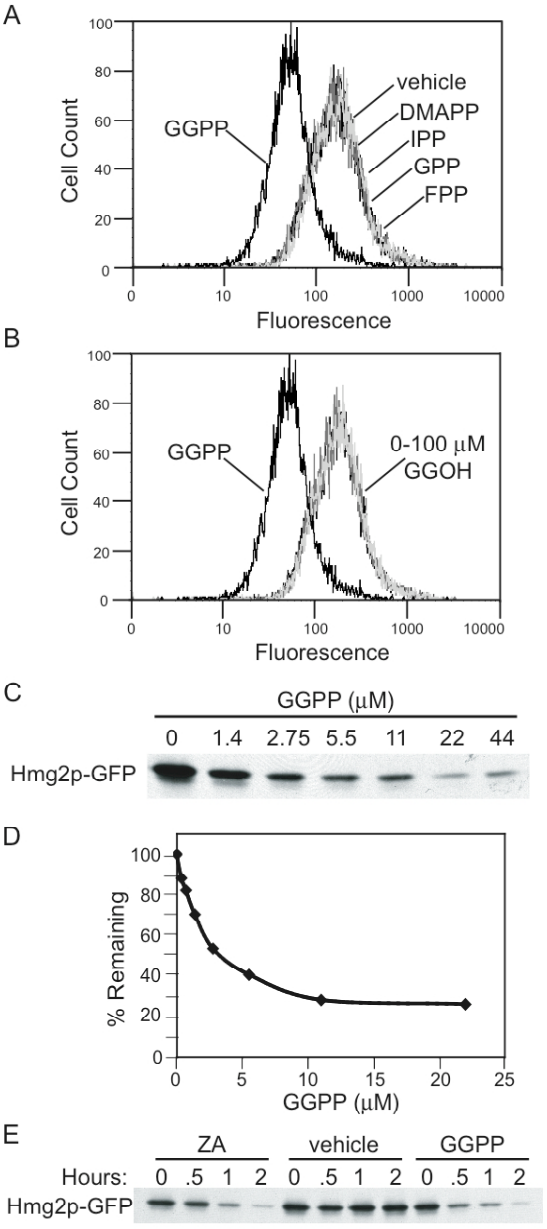

Figure 3

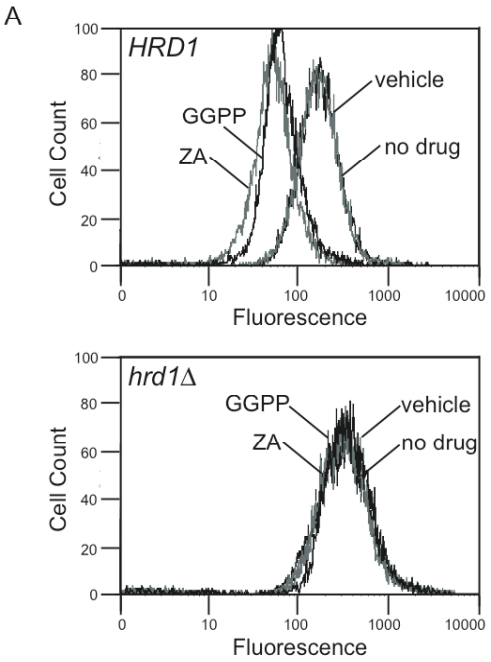

Figure 4

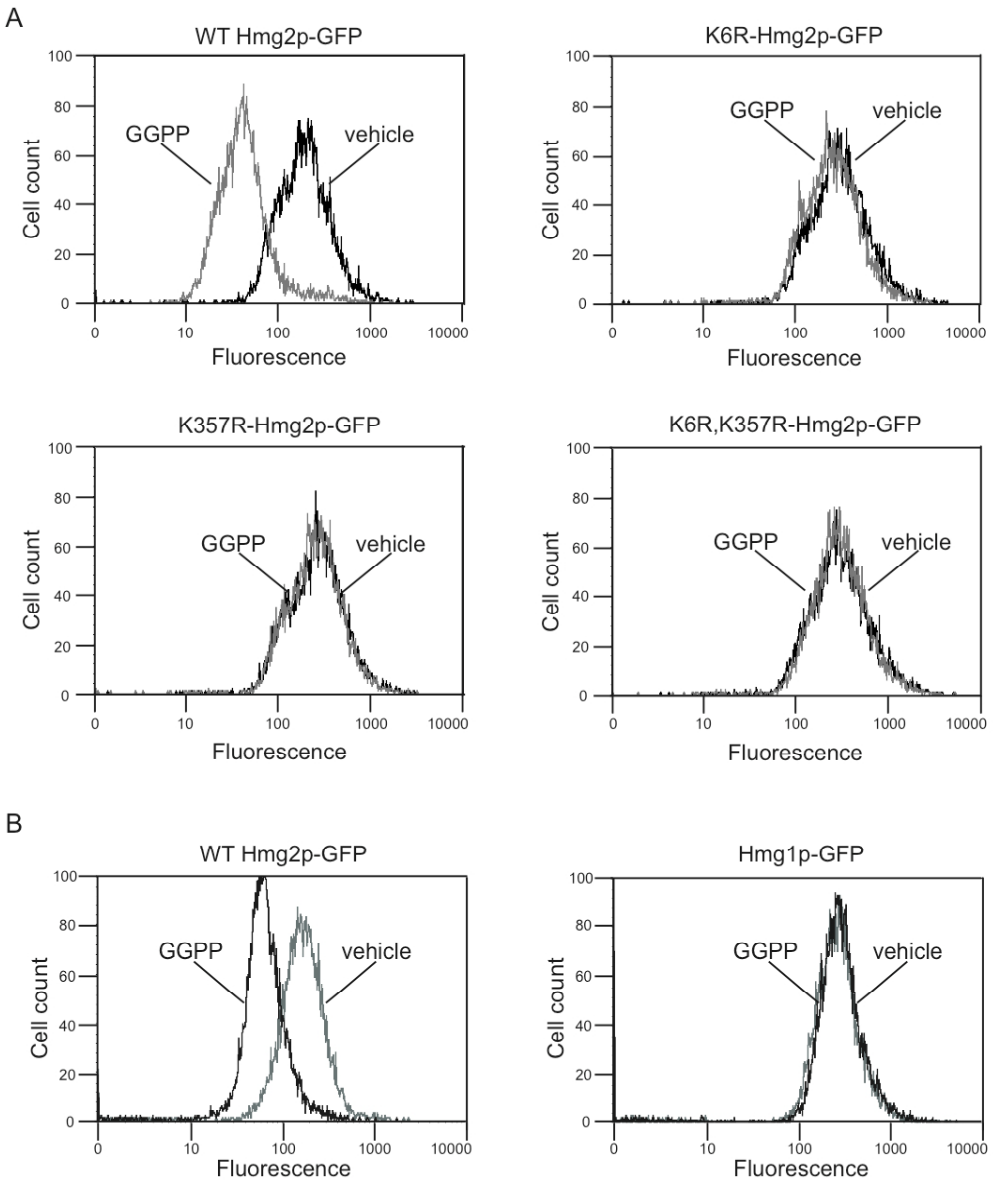

Figure 5

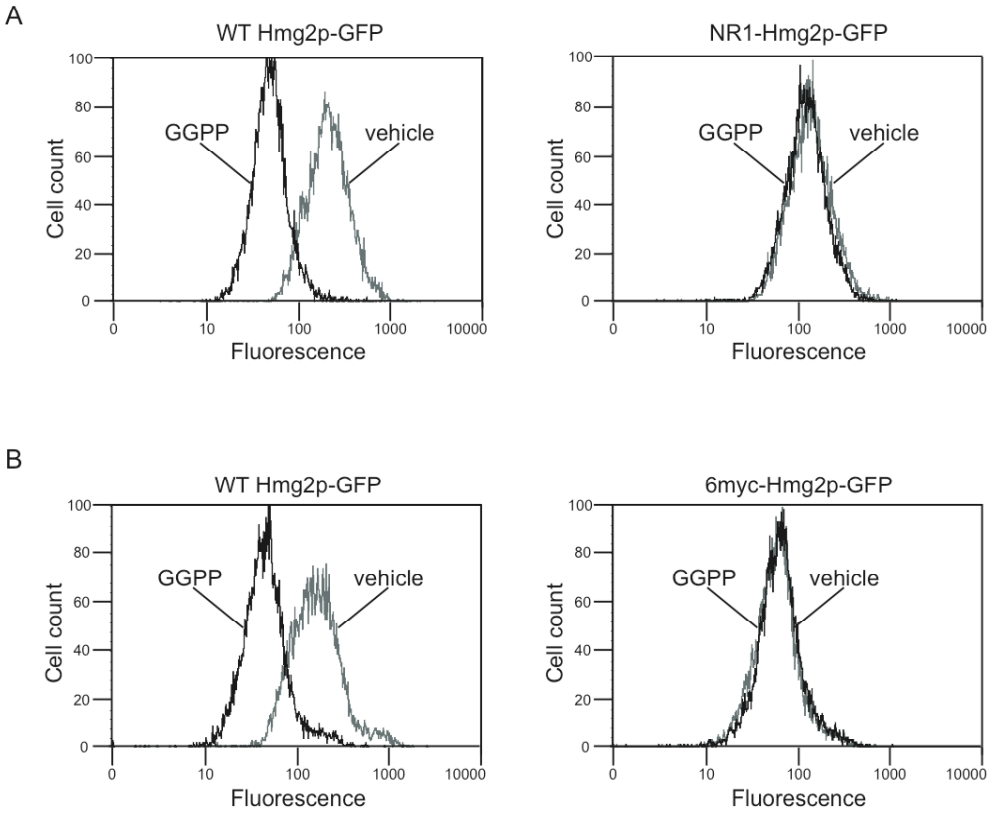

Figure 6

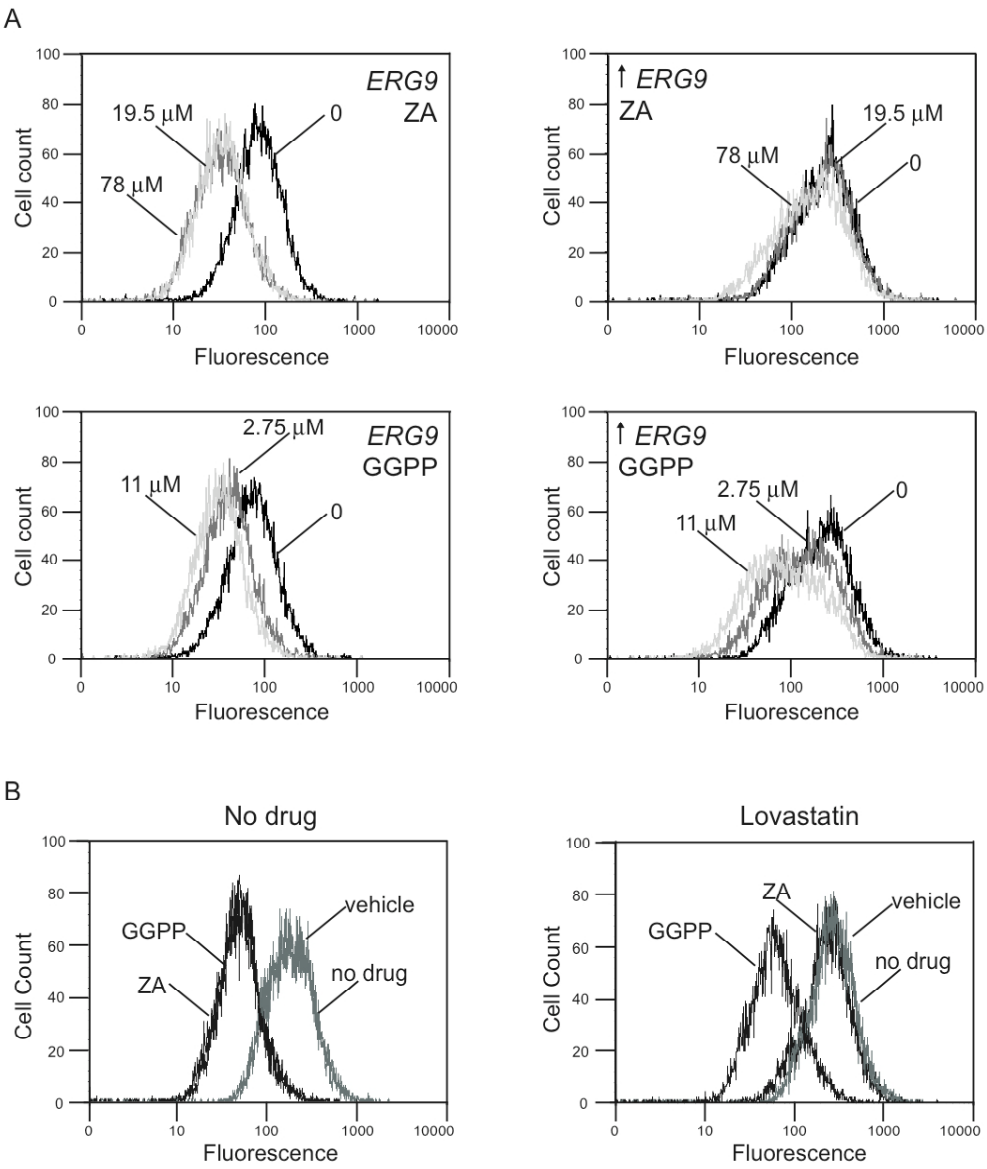

Figure 7

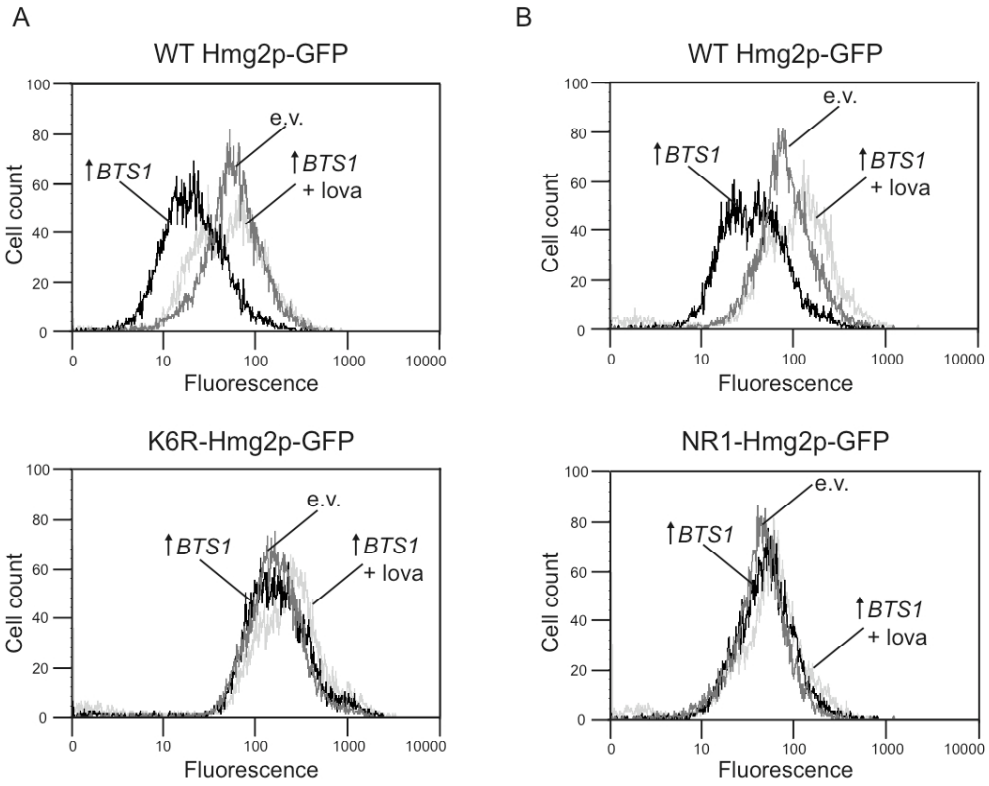

Figure 8

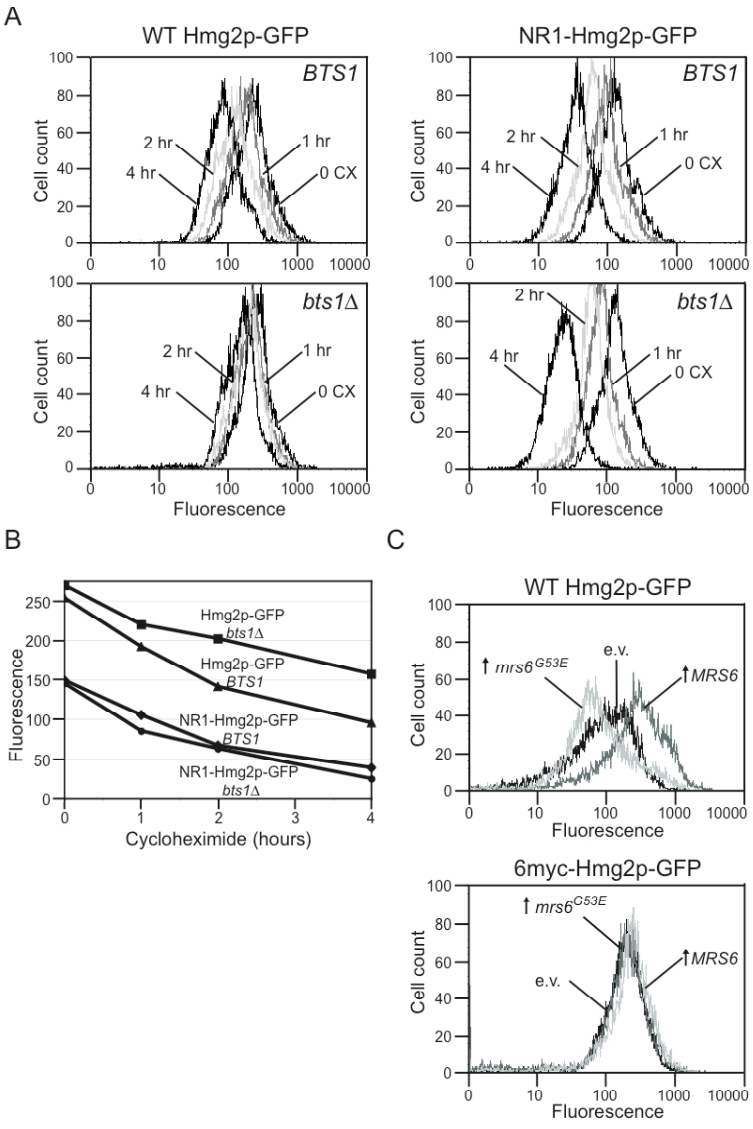

Figure 9

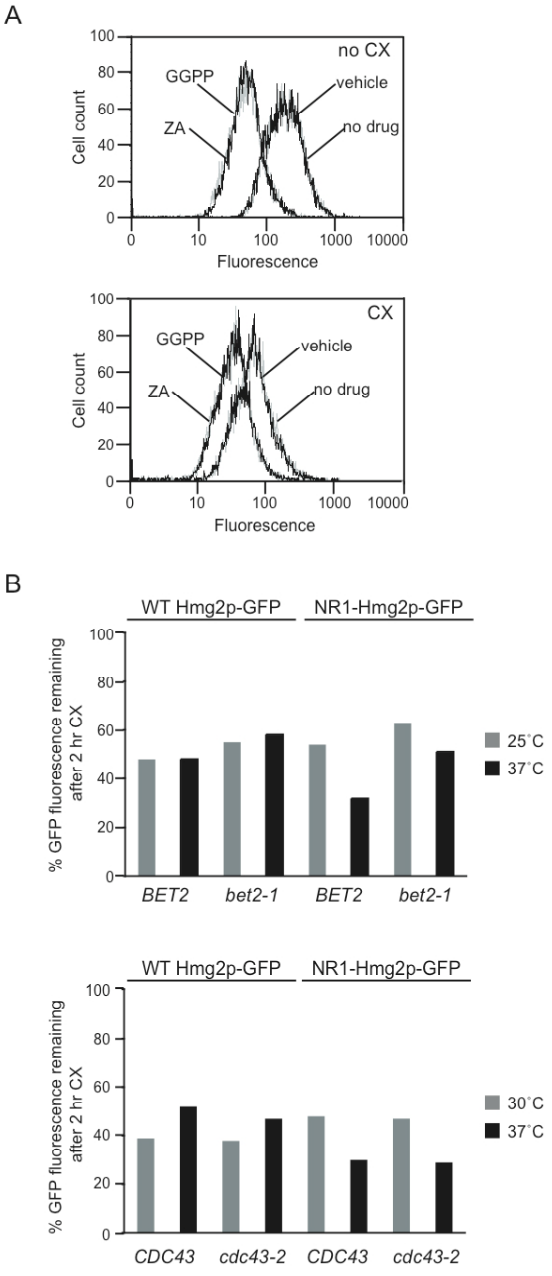

Figure 10