Supplemental Results

Because Hmg2p degradation proceeds by the HRD quality control pathway, we wondered if NSG levels had any general effects on ER quality control. As a gauge of such actions we tested the effects of overexpression or removal of NSG proteins on the unfolded protein response (UPR), the conserved signaling pathway that monitors and responds to levels of misfolded ER proteins. We used an integrated GFP reporter of UPR activity (Patil and Walter, 2001), that allows the effects of drugs or genetic perturbations to be measured in vivo by flow cytometry (Bays et al., 2001b; Cronin et al., 2002). Overexpression of either NSG protein caused no change on the basal levels of the UPR, nor any alteration of the dose-response curve for DTT, an agent that stimulates the UPR (Fig. 1S, A). Similarly the presence of either single deletion or the double null mutation *nsg1Δnsg2Δ* had no discernable effects on the basal or stimulated UPR when compared to otherwise isogenic wild-type strains (control, Fig. 1S). Thus, despite its profound effects on Hmg2p stability, an absence or overabundance of NSG proteins had no effect on the tone or responsiveness of the UPR pathway.

Supplemental Materials and Methods

Reagents

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Berverly, MA). KOD HotStart DNA polymerase was obtained from Novagen Inc., Madison, WI. Anti-myc antibody was used as cell culture supernatant obtained by growing the 9E10 hybridoma cell line (CRL 1729 American Type Culture Collection) in RPMI1640 culture medium (GIBCO BRL, Grand Island, NY) with 10% fetal calf serum and supplements. Anti-HA.11 monoclonal antibodies were purchased as ascites fluid or affinity-matrix coupled from Covance Inc., Berkeley, CA. HRP-conjugated goat anti-mouse antibodies were purchased from Jackson Immuno Research. Chemiluminscence immunodetection reagents were obtained from Perkin Elmer. Serva Blue G was purchased from Serva Electrophoresis GmbH, Heidelberg, Germany. Digitonin was obtained from Fluka AG, Buchs, Switzerland, or Sigma. All other chemical reagents were obtained from Sigma, St. Louis, MO or Fisher Scientific, Fairlawn, NJ (chemicals) Hampton, NH (headquarters).

Plasmids and DNA

pRH1238 (*YIp/LEU2*, expressed *NSG2* from a *TDH3* promoter) was constructed by PCR amplification of NSG2 from genomic DNA and subcloning into expression vector pRH983 (YIp, *LEU2*). pRH1246 (*YIp/LEU2*, expressed *hemi-HRD1* from the *TDH3* promoter) was constructed by subcloning the region encoding the transmembrane domain of Hrd1p, referred to as hemi-Hrd1p (Gardner et al., 2000), into expression vector pRH981 (*YIp/LEU2*). pRH1574 (*YCp/ADE2/LEU2*, expressed *SEC61* from a *TDH3* promoter) was made by subcloning a BsaI-NarI fragment containing *ADE2* and *TDH3-SEC61* from pRH1348 (*YIp/ADE2*, expresses *SEC61* from a *TDH3* promoter) into pRS415 (*YIp/LEU)*. pRH1774, used for disruption of the *NSG1(YHR133c)* open reading frame, by PCR amplifying kanMX-deleted *nsg* allele from genomic DNA of the *YHR133c/yhr133cΔ* Research Genetics heterozygous diploid strain (strain 21961, Invitrogen, Carlsbad, CA). The resulting product, containing long flanking sequences upstream and downstream of the *nsg1::kanMX* coding region, was cloned into the SmaI

site of pRS406. The disrupting fragment was cut out of the plasmid and used for transformation to create the *nsg1Δ* null allele. pRH1775, used for disruption of the *NSG2(YNL156c)* open reading frame, was prepared in an identical manner, using the *YNL156c/ynl156cΔ* Research Genetics heterozygous diploid strain (strain 22056, Invitrogen, Carlsbad, CA). pRH1791 (*YIp/ADE2*, expressed *NSG1* from a *TDH3* promoter) was constructed by PCR amplification of the coding region from genomic DNA followed by subcloning into pRH1579 (*YIp/ADE2*, with *TDH3* promoter upstream of MCS). pRH1805 (*YIp/ADE2*, expressed *NSG1-3HA* from *TDH3* promoter) was constructed by addition of an in-frame 3HA coding region by SOEing and recloning into pRH1791 to create the tagged version of the coding region. pRH399, which is a promoterless version of 1myc-Hmg2p, was used to insert a single *myc* epitope tag into *HMG2* CDS expressed from its genomic locus by single site integration into the *hmg2::HIS3* null allele (Hampton and Rine, 1994). pDN431 (*YCp/URA3*, expresses *CPY*-HA*) was a gift from Davis Ng, Penn State. pDN431 (*YCp/URA3*, expresses *CPY*-HA*) was a gift from Davis Ng, Penn State.

In vitro ubiquitination of Hmg2p

In vitro ubiquitination of Hmg2p was evaluated in isolated microsomes by an assay characterized in detail in a separate publication (Garza et al., 2005). Briefly, microsomes from a *ubc7Δ* null yeast strain expressing Hmg2p-GFP and Hrd1p from the TDH3 promoter were prepared by glass bead lysis of 20 OD_{600} units of log-phase cells in 400 ml of MF buffer (20 mM Tris pH 7.5, 100 mM NaCl, 300 mM sorbitol, with the following protease inhibitors: 1mM PMSF, 260μM AEBSF, 100 μM leupeptin hemisulfate, 76 μM pepstatin A, 5mM ε-aminocaproic acid, 5mM benzamidine, and 142 μM TPCK) at 4°C, 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 2 x 400 ml rinses of the beads with MF to give 1.2 ml of crude microsomal lysate. The crude lysate was next briefly microfuged using full speed (14,000 x g) 5 second pulses to remove large cellular debris. Next the microsomes were pelleted by 30 minute, 21,000 g spin at 4 oC and then resuspended in B88 buffer (20 mM Hepes pH 6.8, 250 mM sorbitol

150 mM KOAc, 5mM MgOAc, 1mM DTT, and the same cocktail of protease inhibitors used in the lysis buffer), to a final volume of 125 microliters. At the same time, cytosol from a Ubc7p overexpressing, *hrd1Δ* null strain was prepared in the manner of Spang and Schekman (Spang and Schekman, 1998). Control cytosol was prepared in parallel from an otherwise identical *ubc7Δ* null strain. Briefly, 500 OD equivalents of cells were pelleted, rinsed once with water, once with B88 buffer, and resuspended in 500 ml of B88 buffer. The resulting suspension was poured into a liquid nitrogen-containing mortar, and the resulting fast-frozen pellet was ground with a pestle until a fine powder. The frozen powder was next transferred to a microfuge tube, raised to 1mM ATP with a 500 mM stock solution in H20 ph 7.5, and allowed to thaw on ice. The thawed cytosol lysate was centrifuged at 3000 x g for 5 min, to remove debris, and the resulting supernatent was removed and centrifuged at 20,000 x g for 15 minutes. Finally, the resulting 20,000 supernatent was removed and ultracentrifuged (100,000 x g) for 1 hr. The resulting supernatent was collected and measured for protein, and adjusted to 25 mg/ml protein for use in the ubiquitination assay.

The *in vitro* ubiquitination assay was initiated by addition of 20 µl of microsomes (with Hrd1p and Hmg2p), 12 µl of cytosol, and sufficient concentrated stock of ATP to yield 30 mM ATP (with Ubc7p, E1, ATP, and ubiquitin), followed incubation at 30 ^{o}C, typically for an hour. The assay is then terminated by solubilization with 200 ml of SUME with protease inhibitors and 5mM N-ethylmaleimide, followed by addition of 600 ml of IP buffer, and subsequent immunoprecipitation of Hmg2p and immunblotting for Hmg2p-GFP with anti-GFP monoclonal antibodies, or for ubiquitin using anti-ubiquitin (Zymed Laboratorie, South San Francisco) or anti-GFP monclonals as described (Bays et al., 2001b).

References for Supplemental Material

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Figure legend Supplemental

Figure 1S Overexpressed NSGs (A), or *nsgΔ* null mutations (B) did not cause or alter UPR signaling. A) A reporter strain expressing the UPREx4-GFP reporter, with either empty vector or plasmids overexpressing the indicated NSGs from the TDH3 promoter, were subjected to flow cytometry after a 4 hour incubation with 0, 0.5, or 2 mM DTT to examine the basal and stimulated UPR. Vertically placed histograms have identical fluorescence axes allowing visual comparison. B) The same experiment was performed on the reporter strain, with normal NSG genes or the indicated null mutations.

Table of yeast strains used in studies

Table 1. List of strain, and figures of usage

Strain	Genotype	Figure
RHY3119	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2 ura3-52::URA3::pTDH3-HMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Fig 2A left column
RHY3120	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2-pTDH3-NSG2 ura3-52::URA3::pTDH3-HMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Fig 2A left column
RHY3121	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2-pTDH3-hemiHRD1 ura3-52::URA3::pTDH3-HMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Fig 2A left column
RHY3127	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::pTDH3-HMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Fig 2A right column
RHY3128	*MATα ade2-101::ADE2::pTDH3-NSG1 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::pTDH3-HMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Fig 2A right column
RHY3213	*MATα ade2-101::ADE2::pTDH3-NSG1-3HA met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::pTDH3-HMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Fig 2A right column
RHY3219	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 leu2Δ ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2*	Fig 2B Fig 7A, B
RHY3220	*MATα ade2-101::ADE2::pTDH3-NSG1 met 2 lys2-801 his3Δ200 leu2Δ ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2*	Fig. 2B
RHY3221	*MATα ade2-101::ADE2::pTDH3-NSG1-3HA met 2 lys2-801 his3Δ200 leu2Δ ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 2B Fig 7A, B
RHY3472	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2 ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 2B
RHY3473	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2::pTDH3-NSG2 ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 2B
RHY3474	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2::pTDH3-hemiHRD1 ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 2B
RHY3254	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 leu2Δ ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2 [pDN431] (expressing CPY*-HA)*	Figure 3A
RHY3255	*MATα ade2-101::ADE2::pTDH3-NSG1 met 2 lys2-801 his3Δ200 leu2Δ ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2 [pDN431] (expressing CPY*-HA)*	Figure 3A
RHY3475	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2 ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2 [pDN431] (expressing CPY*-HA)*	Figure 3A
RHY3476	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2::pTDH3-NSG2 ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2 [pDN431] (expressing CPY*-HA)*	Figure 3A
RHY3477	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2::pTDH3-hemiHRD1 ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2 [pDN431] (expressing CPY*-HA)*	Figure 3A
RHY3322	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::pTDH3-6mycHMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 3B

RHY3323	*MATα ade2-101::ADE2::pTDH3-NSG1 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::pTDH3-6mycHMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 3B
RHY3324	*MATα ade2-101::ADE2::pTDH3-NSG1-3HA met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::pTDH3-6mycHMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 3B
RHY3488	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2 ura3-52::URA3::pTDH3-6mycHMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 3B
RHY3489	*MATα ade2-101 leu2Δ::LEU2::pTDH3-NSG2 ura3-52::URA3::pTDH3-6mycHMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 3B
RHY3490	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2::pTDH3-hemiHRD1 ura3-52::URA3::pTDH3-6mycHMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 3B
RHY3328	*MATa leu2,3-112::LEU2 ade2-1 ura3-52 pep4-3 sec61-2*	Figure 3C
RHY3329	*MATa leu2,3-112::LEU2::TDH3-NSG2 ade2-1 ura3-52 pep4-3 sec61-2*	Figure 3C
RHY3330	*MATa leu2,3-112::LEU2::TDH3-hemiHRD1 ade2-1 ura3-52 pep4-3 sec61-2*	Figure 3C
RHY3333	*MATa leu2,3-112 ade2-1 ura3-52 pep4-3 sec61-2 [pRH1574(ARS/CEN SEC61)]*	Figure 3C
RHY3168	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::4xUPRE::GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 4A
RHY3169	*MATα ade2-101::ADE2::pTDH2-NSG1 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::4xUPRE::GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 1SA
RHY3170	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2 ura3-52::URA3::4xUPRE::GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 1SA
RHY3171	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ::LEU2::pTDH3-NSG2 ura3-52::URA3::4xUPRE::GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 1SA
RHY1983	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::4xUPRE::GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 1SB
RHY2338	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::4xUPRE::GFP hmg2Δ::HIS3::pTDH3-1mycHMG2 nsg2Δ::kanMX*	Figure 1SB
RHY2344	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::4xUPRE::GFP hmg2Δ::HIS3::pTDH3-1mycHMG2 nsg1Δ::kanMX nsg2Δ::CaURA3MX*	Figure 1SB
RHY2345	*MATα ade2-101 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::4xUPRE::GFP hmg2Δ::HIS3::pTDH3-1mycHMG2 nsg1Δ::kanMX*	Figure 1SB
RHY3128	*MATα ade2-101::ADE2::pTDH3-NSG1 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::pTDH3-HMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 4
RHY3129	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::pTDH3-HMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2 cod1Δ::kanMX*	Figure 4
RHY3130	*MATα ade2-101::ADE2::TDH3-NSG1 met 2 lys2-801 his3Δ200 leu2Δ ura3-52::URA3::pTDH3-HMG2-GFP hmg2Δ::HIS3::pTDH3-1mycHMG2 cod1Δ::kanMX*	Figure 4
RHY3388	*MATa ade2-101 met 2 lys2-801 his3Δ200 trp1::hisG leu2Δ ura3-52 HMG1 hmg2Δ::pHMG2-1mycHMG2 nsg1Δ::kanMX*	Figure 5A
RHY3389[b]	*MATα ade2-101 met 2 lys2-801 his3Δ200 trp1::hisG leu2D ura3-52 HMG1 hmg2Δ::pHMG2-1mycHMG2 nsg2Δ::kanMX*	Figure 5A
RHY3391	*MATa ade2-101 met 2 lys2-801 his3Δ200 trp1::hisG leu2D ura3-52 HMG1 hmg2Δ::pHMG2-1mycHMG2 nsg1Δ::kanMX*	Figure 5A

	nsg2Δ::kanMX	
RHY3392	*MATa ade2-101 met 2 lys2-801 his3Δ200 trp1::hisG leu2D ura3-52 HMG1 hmg2Δ::pHMG2-1mycHMG2*	Fig 5As
RHY3487	*MATa ade2-101 met 2 lys2-801 his3Δ200 trp1::hisG leu2D ura3-52 HMG1 hmg2Δ::pHMG2-1mycHMG2 nsg1Δ::kanMX nsg2Δ::kanMX hrd1Δ::LEU2*	Fig 5B
RHY3353	*MATa ade2-101 met2 lys2-801 his3Δ200 trp1::hisG leu2Δ ura3-52 hmg1Δ::LYS2 hmg2Δ::pHMG2-1mycHMG2*	Fig 5C
RHY3355	*MATα ade2-101 met2 lys2-801 his3Δ200 trp1::hisG leu2Δ ura3-52 hmg1Δ::LYS2 hmg2Δ::pHMG2-1mycHMG2 nsg1Δ::kanMX nsg2Δ::kanMX*	Fig 5C
RHY1131	*MATa ade2-101 met2 lys2-801 his3D200 trp1::hisG ura3-52::URA3 HMG1 hmg2Δ::pHMG2-1mycHMG2*	Fig 6
RHY1215	*MATa ade2-101 met2 lys2-801 his3D200 trp1::hisG ura3-52::URA3::pTDH3-HMG2-GFP HMG1 hmg2Δ::pHMG2-1mycHMG2*	Fig 6
RHY3540	*MATa ade2-101::ADE2::pTDH3-NSG1-3HA met2 lys2-801 his3Δ200 trp1::hisG ura3-52::URA3::HMG2-GFP HMG1 hmg2Δ::pHMG2-1mycHMG2*	Fig 6
RHY3219	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 leu2Δ ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2*	Fig 2 Fig 7A, B
RHY3221	*MATα ade2-101::ADE2::pTDH3-NSG1-3HA met 2 lys2-801 his3Δ200 leu2Δ ura3-52 hmg2Δ::HIS3::pTDH3-1mycHMG2*	Figure 2 Fig 7A, B
RHY3445	*MATa ade2–101::ADE2::pTDH3-NSG1-3HA met 2 lys2-801 his3Δ200 ura3–52::HMG2cd hmg1::LYS2 hmg2::HIS3*	Fig 7B
RHY3485	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 ura3-52::URA3::pTDH3-HMGcd::pTDH2-myc$_L$-HMG2-GFP hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::kanMX*	Fig 8A
RHY3486	*MATα ade2-101::ADE2::pTDH3-NSG1 met 2 lys2-801 his3Δ200 ura3-52::URA3::pTDH3-HMGcd::pTDH2-myc$_L$-HMG2-GFP hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::kanMX*	Fig 8A
RHY4304	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 leu2Δ pep4::HIS3 ura3-52::URA3::pTDH3-HMG2-GFP HMG1 hmg2Δ::pHMG2-1mycHMG2 hrd1Δ::kanMX trp1::hisG::TRP1-pTDH3::HRD1-3HA ubc7::LEU2*	Fig 8C
RHY4305	*MATα ade2-101::ADE2::pTDH3-NSG1-3HA met 2 lys2-801 his3Δ200 leu2Δ pep4::HIS3 ura3-52::URA3::HMG2-GFP HMG1 hmg2Δ::pHMG2-1mycHMG2 hrd1Δ::kanMX trp1::hisG::TRP1-pTDH3::HRD1-3HA ubc7::LEU2*	Fig 8C
RHY4306	*MATα ade2-101::ADE2 met 2 lys2-801 his3Δ200 leu2Δ pep4::HIS3 ura3-52 HMG1 hmg2Δ::pHMG2-1mycHMG2 hrd1Δ::kanMX trp1::hisG::TRP1-pTDH3::HRD1-3HA ubc7::LEU2 ubc1::natR*	Fig 8C (cytosol donor no ubc7)
RHY4307	*MATα ade2-101::ADE2::pTDH3-UBC7-2HA met 2 lys2-801 his3Δ200 leu2Δ met2 pep4::HIS3 ura3-52 HMG1 hmg2Δ::pHMG2-1mycHMG2 hrd1Δ::kanMX trp1::hisG::TRP1-pTDH3::HRD1-3HA ubc7::LEU2 ubc1::natR*	Fig 8C (cytosol donor plus UBC7)

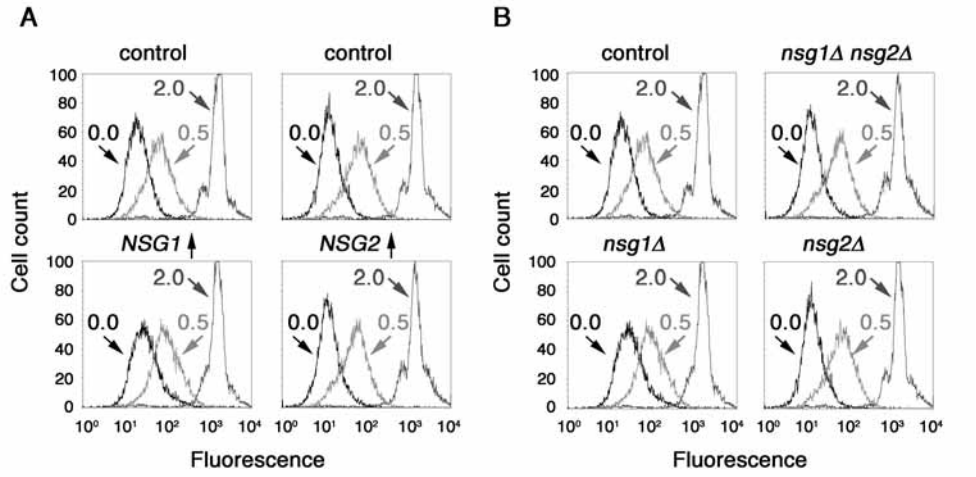
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Figure 1S