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Degradation-Mediated Protein Quality Control in the Nucleus

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Summary

Protein quality control degradation systems rid the cell of aberrant proteins, preventing detrimental effects on normal cellular function. Although such systems have been identified in most subcellular compartments, none have been found in the nucleus. Here, we report the discovery of such a system in *Saccharomyces cerevisiae*. It is defined by San1p, a ubiquitin-protein ligase that, in conjunction with the ubiquitin-conjugating enzymes Cdc34p and Ubc1p, targets four distinct mutant nuclear proteins for ubiquitination and destruction by the proteasome. San1p has exquisite specificity for aberrant proteins and does not target the wild-type versions of its mutant substrates. San1p is nuclear localized and requires nuclear localization for function. Loss of *SAN1* results in a chronic stress response, underscoring its role of protein quality control in the cell. We propose that San1p-mediated degradation acts as the last line of proteolytic defense against the deleterious accumulation of aberrant proteins in the nucleus and that analogous systems exist in other eukaryotes.

Introduction

Aberrant proteins can be produced in the cell by mutation, transcriptional or translational errors, incorrect folding, imbalanced subunit synthesis, improper trafficking, or damage caused by environmental conditions or metabolic byproducts (Goldberg, 2003). Accumulation or persistence of aberrant proteins within the cell can often have deleterious consequences. For example, aberrant proteins may lose regulation, may form inactive complexes that compete with functional complexes, may assemble into aggregates that eliminate protein function or cause toxicity, or may introduce harmful activities if mislocalized. In humans, accumulation of aberrant proteins may underlie the pathology of diseases such as Alzheimer's, Huntington's, Parkinson's, and prion pathologies like Creutzfeld-Jakob's (Shastry, 2003).

To minimize harmful effects from aberrant proteins, the cell possesses a set of protein quality control (PQC) systems that operate in distinct ways. Some PQC systems function in repair, such as protein chaperones that refold or sequester aberrantly folded proteins (Stirling et al., 2003). Protein chaperones reside in every major cellular compartment and function either by refolding misfolded proteins (e.g., Hsp70 and Hsp90), binding to misfolded proteins and preventing aggregation (e.g., Hsp40), or disrupting protein aggregates (e.g., Hsp104). Not all aberrant proteins can be repaired, however. For these lost causes, the cell has PQC systems that function in disposal, such as proteolytic systems that destroy proteins that are mutant, damaged, or misfolded and recalcitrant to refolding (Goldberg, 2003). PQC degradation systems have been identified in the cytoplasm, the secretory pathway, and mitochondria. In the cytoplasm and endoplasmic reticulum (ER), PQC degradation is primarily brought about by protein-ubiquitination complexes that mark proteins for proteasomal degradation (Hampton, 2002; McDonough and Patterson, 2003; Trombetta and Parodi, 2003). PQC degradation in the cytoplasm and ER can also occur via transport to the lysosome/vacuole (Trombetta and Parodi, 2003). In the mitochondria, localized proteases function in PQC degradation (Arnold and Langer, 2002). Repair and degradation systems may not be mutually exclusive, as various protein chaperones appear to interface with PQC degradation systems (McDonough and Patterson, 2003; Trombetta and Parodi, 2003).

How the cell targets and destroys aberrant proteins depends upon how and when aberrant proteins are produced. Aberrant proteins may be evident immediately upon production due to errors in synthesis or folding. It is estimated that nearly a third of all newly synthesized proteins are defective and destroyed during or shortly after synthesis (Schubert et al., 2000), indicating that PQC degradation systems may be intimately linked with the production process. Properly synthesized proteins can become aberrant even after they are performing their normal functions. Postproduction damage can be caused by physical or chemical means including heat, irradiation, free radicals, and changes in hydration or osmolarity (Goldberg, 2003). It is not clear if particular PQC degradation systems are dedicated exclusively to production-level defects or postproduction damage or if they target both classes of aberrant proteins.

In contrast to the cytoplasm, ER, and mitochondria, very little is known about PQC in the nucleus. A number of chaperones have been implicated in protein refolding and disaggregation in the nucleus (Parsell et al., 1994; Rossi and Lindquist, 1989), but no nuclear PQC degradation systems have been identified. Because there is no functional protein synthesis in the nucleus, the cell would not have to contend with production defects in the nucleus unless they eluded cytoplasmic PQC systems. Nuclear proteins can be damaged by the same stresses that damage proteins in other cellular compartments; thus, the cell must contend with aberrant proteins that arise within the nucleus. It is not clear if PQC degradation systems exist in the nucleus and, if so, whether they would be similar to those in other cellular compartments where protein synthesis occurs. Yet, similar to the protein synthetic compartments, accumulation of aberrant proteins in the nucleus can have deleterious effects. For example, aberrant protein accumulation in the nucleus likely underlies the pathology

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of Huntington's disease (HD) and oculopharyngeal muscular dystrophy (OPMD) (Brais, 2003; Jana and Nukina, 2003). With a significant amount of regulated degradation occurring in the nucleus via ubiquitination and nuclear-localized proteasomes (Wojcik and DeMartino, 2003), the nucleus certainly has the potential machinery for PQC degradation. In fact, nuclear aggregates of mutant polyglutamine- and polyalanine-expanded proteins in HD and OPMD, respectively, are ubiquitinated and associated with proteasome subunits (Abu-Baker et al., 2003; Calado et al., 2000; Davies et al., 1997; Waelter et al., 2001), providing some evidence that PQC degradation systems may be operating in the nucleus.

Given the potential importance of PQC degradation in the nucleus, we asked if such a system existed in the budding yeast, *Saccharomyces cerevisiae*.

Results

Mutant Nuclear Proteins Are Degraded in a *SAN1*-Dependent Manner

We searched for nuclear PQC degradation pathways in yeast working under the assumption that temperature-sensitive mutant nuclear proteins might be substrates for such a pathway. This idea was based on the observation that some temperature-sensitive proteins in other cellular compartments are recognized as aberrant and destroyed by PQC machinery, despite the fact that the mutant proteins often possess normal or near-normal activity (Betting and Seufert, 1996; Bordallo et al., 1998). In these cases, the temperature-sensitive phenotype results from reduced steady-state levels of the protein and can be suppressed by inhibiting the protein's degradation, thus allowing the mutant protein to accumulate to functional levels (Bordallo et al., 1998).

We speculated that if a general nuclear-localized PQC degradation system exists, then the temperature-sensitive phenotypes of different mutant nuclear proteins with disparate normal functions would be suppressed by a common extragenic mutation. We focused on temperature-sensitive alleles of nuclear protein genes that had been previously identified, particularly those for which suppressor analyses had been reported. From our search, we found two independently conducted genetic analyses: one targeting the *sir4-9* allele (Schnell et al., 1989) and the other targeting the *cdc68-1* allele (Xu et al., 1993). Both analyses identified mutations in the gene *SAN1* that suppressed the respective temperature-sensitive phenotypes. *SAN1* made an excellent candidate gene for a nuclear PQC degradation factor given the different nuclear functions of Sir4p, which is a structural component of silent chromatin at the silent mating-type loci and telomeres (Aparicio et al., 1991; Rine and Herskowitz, 1987), and Cdc68p, which facilitates DNA replication, transcriptional initiation, and elongation (Belotserkovskaya et al., 2003; Brewster et al., 2001; Formosa et al., 2001). In fact, the mutant Cdc68-1 protein was previously shown to be unstable compared to wild-type Cdc68p (Xu et al., 1995), and *SAN1* was required for the mutant protein's degradation (Evans et al., 1998).

To assess San1p's role in the QC degradation of mutant nuclear proteins, we first confirmed the *SAN1* requirement for Cdc68-1p degradation (Figure 1A). Next, we examined the stability of the mutant Sir4-9 protein and found that it too was degraded in a *SAN1*-dependent manner, whereas the wild-type version of Sir4p was stable in the presence of *SAN1* (Figure 1B). Thus, mutations in both the Sir4 and Cdc68 proteins caused them to be rapidly degraded in the presence of *SAN1,* while their wild-type counterparts were stable.

To facilitate additional genetic and biochemical assays, single Myc-tagged versions of wild-type Sir4p and the mutant Sir4-9p were created. While we found that N- or C-terminal addition of epitope sequences to Sir4p abrogated its silencing function and, in the case of N-terminal tags, resulted in aberrant degradation (data not shown), we found that conversion of the internal native sequence of Sir4p, ^{372}EQKMKEDADL, to the highly similar c-Myc epitope sequence, EQKLIS EEDL, had no effect on *SIR4* silencing function (data not shown). Furthermore, overexpression of each internal Myc-tagged protein from the constitutive *TDH3* promoter did not alter the stability of wild-type Sir4p or the *SAN1*-dependent degradation of mutant Sir4-9p (Figure 1C).

We next determined if San1p's degradative function was limited to the mutant Sir4-9 and Cdc68-1 proteins or if San1p acted more generally in aberrant nuclear protein degradation. To do so, we examined the stability of two other temperature-sensitive mutant nuclear proteins, Cdc13-1p and Sir3-8p. Detection of Cdc13 proteins was facilitated by the addition of a 9× Myc epitope sequence to the C terminus, which does not alter wild-type Cdc13p function (Qi and Zakian, 2000). Detection of Sir3 proteins was facilitated by replacement of the wild-type Sir3p sequence, ^{426}ETDNEMNG NGK, with the VSV epitope sequence, ^{426}YTDIEMNR LGK, which had no effect on *SIR3* silencing function (data not shown). Similar to the mutant Cdc68-1 and Sir4-9 proteins, the mutant Cdc13-1 and Sir3-8 proteins were rapidly degraded, and each was stabilized in the absence of *SAN1* (Figures 1D and 1E, respectively). Cdc13-1p stabilization in *san1*Δ cells was complete, whereas Sir3-8p stabilization was partial. Both wild-type Cdc13p and Sir3p were stable in the presence of *SAN1* (Figures 1D and 1E, respectively).

Although the absence of *SAN1* stabilized both Cdc13-1p and Sir3-8p, neither the *cdc13-1* nor the *sir3-8* temperature-sensitive phenotype was suppressed by the *san1*Δ allele (data not shown; Schnell et al., 1989). This lack of phenotypic suppression contrasted with the *san1*Δ effect on the *sir4-9* and *cdc68-1* phenotypes (Schnell et al., 1989; Xu et al., 1993), indicating that the stabilized Cdc13-1 and Sir3-8 proteins are likely to be nonfunctional at the restrictive temperatures. Nevertheless, the fact that all four mutant proteins were aberrantly degraded in a *SAN1*-dependent manner is consistent with San1p functioning in nuclear PQC degradation.

San1p Is a RING-Domain Ubiquitin-Protein Ligase

Analysis of its sequence revealed that San1p contained a variant version of the canonical RING domain (from residues 165–279; Figure 2A), which is common among

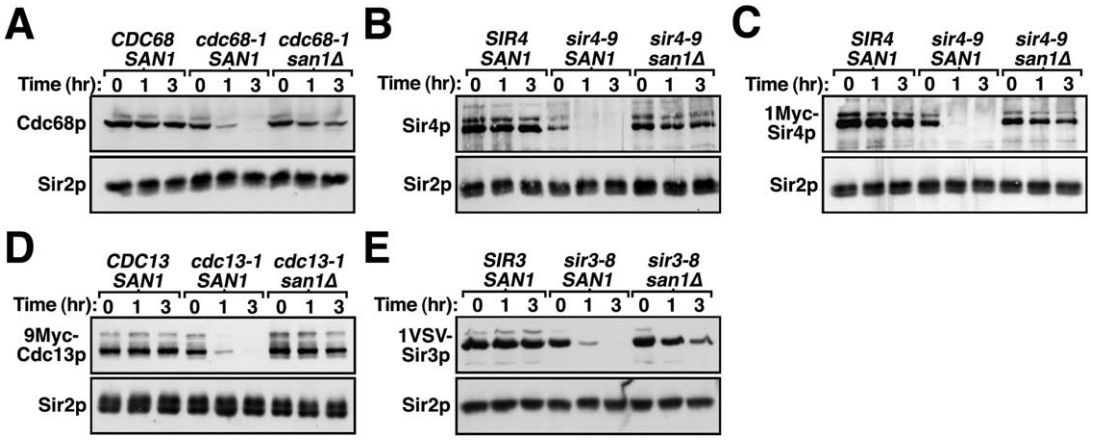

Figure 1. Degradation of Mutant Nuclear Proteins Requires *SAN1*

Cycloheximide-chase assays of cells expressing the indicated wild-type or mutant protein were performed to assess stability in the presence or absence of *SAN1*. Time after addition of cycloheximide is indicated above each lane. Images are of the same blot, first probed with either anti-Cdc68p, anti-Sir4p, anti-Myc, or anti-VSV antibodies to detect the appropriate protein and subsequently probed with anti-Sir2p antibodies to assess Sir2p levels as a loading control.

(A) Cdc68-1p degradation, (B) Sir4-9p degradation, (C) 1Myc-Sir4-9p degradation, with expression from the *TDH3* promoter, (D) 9Myc-Cdc13-1p degradation, and (E) 1VSV-Sir3-8p degradation, with expression from the *TDH3* promoter.

a subset of ubiquitin-protein ligases (Jackson et al., 2000). In San1p's RING domain, the fifth canonical zinc binding His/Cys residue is replaced by Gly (Figure 2A). Because mutation of any of the conserved zinc binding Cys or His residues results in loss of activity in other ubiquitin-protein ligases tested (Bays et al., 2001; Joazeiro et al., 1999; Lorick et al., 1999), the presence of a Gly residue in this position might be expected to disrupt RING domain structure and impair or prevent potential ubiquitin-protein ligase activity. To test San1p for ubiquitin-protein ligase activity, we used a standard in vitro autoubiquitination assay (Joazeiro et al., 1999; Lorick et al., 1999). The addition of a purified GST fusion of San1p to purified ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, ubiquitin, and ATP resulted in autoubiquitination of GST-San1p (Figure 2B). This effect was not observed with GST alone or when any critical component of the reaction was excluded (Figure 2B). The activity was also RING domain dependent, as replacement of either Cys257 or Cys279 with Ser abolished the ubiquitin-protein ligase activity of the GST-San1p fusion (Figure 2C). Thus, San1p possessed

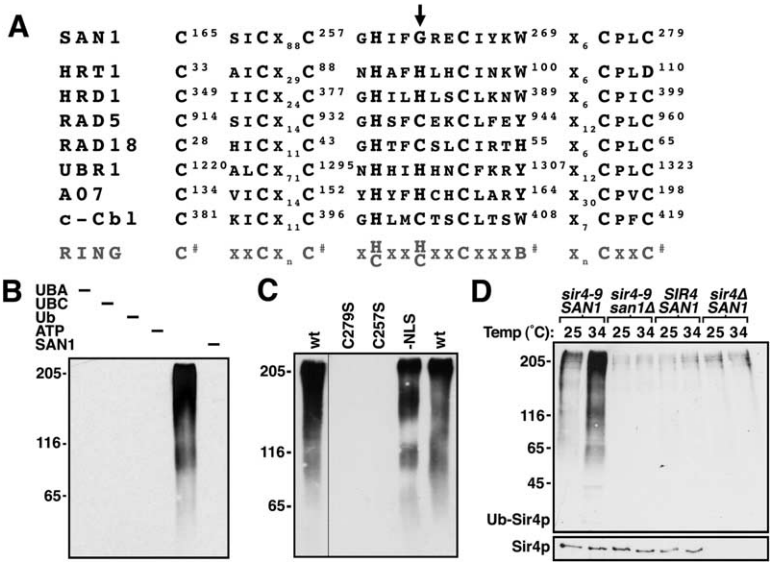

Figure 2. San1p Is a Ubiquitin-Protein Ligase

(A) The RING domain of San1p is compared to the RING domain of five yeast and two mammalian ubiquitin-protein ligases. Consensus RING domain is in gray on the bottom. Zinc binding cysteine and histidine residues are in large typeface. The "B" represents a bulky, hydrophobic residue common among ubiquitin-protein ligases. The arrow points to the noncanonical glycine in San1p's RING domain.

(B) Purified GST-San1p was added to a reaction containing ubiquitin-activating enzyme (UBA), ubiquitin-conjugating enzyme (UBC), ubiquitin (Ub), and ATP. Dashes above gel lanes indicate which reagent was excluded from final reaction mixture. Blot was probed with a monoclonal anti-ubiquitin antibody. Numbers on left indicate positions of protein molecular weight markers.

(C) San1p ubiquitin-protein ligase activity requires the RING domain. Purified GST-San1p containing the indicated mutations was used in an in vitro ubiquitin-ligase assay described in (B).

(D) San1p is required for Sir4-9p ubiquitination in vivo. Sir4 protein was immunoprecipitated from lysates of cells expressing either 1Myc-Sir4-9p, 1Myc-Sir4p, or no Sir4p, and the indicated *SAN1* allele. Blots of the immunoprecipitates were probed with anti-HA antibodies to detect ubiquitin (top panel) or anti-Myc antibodies to assess Sir4p levels (bottom panel).

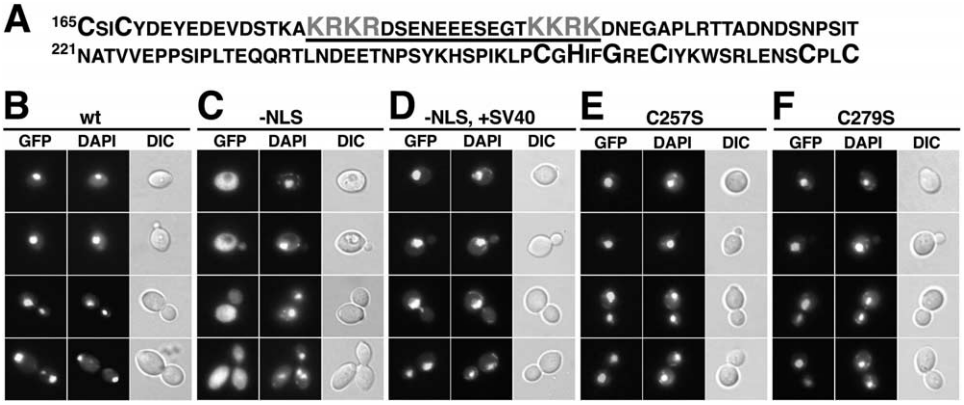

Figure 3. San1p Is Nuclear Localized

(A) The San1p RING domain. Zinc binding cysteine and histidine residues are in large black typeface. Residues comprising the bipartite nuclear localization sequence are in large gray typeface, with the entire sequence underlined.

(B–F) Cells expressing the indicated San1p-3HSV-GFP fusion were fixed with 4% paraformaldehyde, stained with DAPI, and examined by fluorescence microscopy as previously described (Biggins et al., 1999). Single cell images were taken showing different stages of the cell cycle. Left panels show GFP fluorescence, middle panels show DAPI-stained DNA, and right panels show bright field images. Cells expressing (B) *SAN1-3HSV-GFP*, (C) *SAN1(-NLS)-3HSV-GFP*, (D) *SAN1(-NLS)-3HSV-SV40NLS-GFP*, (E) *SAN1(C257S)-3HSV-GFP*, or (F) *SAN1(C279S)-3HSV-GFP* are shown.

in vitro RING-dependent ubiquitin-protein ligase activity, which is in agreement with recent findings (Dasgupta et al., 2004).

Next, we assessed San1p activity in vivo by determining if one of its cellular substrates, Sir4-9p, was ubiquitinated in a San1p-dependent manner. Immunoprecipitates of Sir4-9p contained high molecular weight ubiquitin conjugates that increased dramatically after incubation at the nonpermissive temperature (Figure 2D), consistent with the temperature-sensitive degradation of Sir4-9p. Importantly, the ubiquitination of Sir4-9p required the presence of *SAN1*. Furthermore, *SAN1*-dependent ubiquitination was specific for only the mutant Sir4-9p, as the stable wild-type Sir4p showed no ubiquitination. Together, our in vivo and in vitro observations indicate that San1p is a ubiquitin-protein ligase that acts in the ubiquitination and degradation of aberrant nuclear proteins.

Nuclear Localization of San1p Is Required for Its Function

It was evident that San1p was involved in the QC degradation of mutant nuclear proteins, but it was not clear if San1p carried out its function in the nucleus per se. Indeed, San1p resides in the nucleus, as evidenced by colocalization of GFP-tagged San1p with DAPI-stained nuclear DNA (Figure 3B). By analyzing San1p's sequence with PROSITE (http://au.expasy.org/prosite/), we found that San1p has a putative bipartite nuclear-localization sequence (NLS) from residues 182–199 (Figure 3A). In fact, the NLS was bona fide, as mutagenesis of the San1p NLS (Lys182Cys, Arg183Ser, Lys184Ala, Arg185Thr, Lys197Ser, Lys198Gln, Arg199Ser) resulted in predominantly cytoplasmic localization (Figure 3C). Although the NLS resides within a loop of the RING domain, RING domain structure was not critical for nuclear localization, as substitution of Cys257 or Cys279 with Ser had no effect on San1p-GFP nuclear localization (Figures 3E and 3F).

Importantly, mislocalization of San1p by the NLS mutations abrogated the degradation of Sir4-9p and Cdc68-1p and suppressed the temperature sensitivity of *cdc68-1* cells in a manner similar to that observed when *SAN1* was deleted or the RING domain was mutated (Figure 4). However, the NLS-deficient San1p still retained normal ubiquitin-protein ligase activity in vitro (Figure 2C), indicating that mutation of the NLS did not adversely affect RING-domain activity. Curiously, the NLS mutations resulted in a slower mobility of San1p in SDS-PAGE gels (Figures 4A and 4B). It is not clear why this change occurred; perhaps the NLS-deficient San1 protein was subject to additional or new modifications that also interfered with San1p function. To rule out this possibility, we added the SV40 NLS onto the C terminus of the NLS-deficient San1p. This addition restored nuclear localization (Figure 3D) and in vivo degradation function (Figure 4), indicating that disruption of San1p nuclear localization by mutations to the endogenous NLS simply prevented San1p from gaining access to its nuclear substrates and did not alter another function of San1p.

Ubc1p and Ubc3p/Cdc34p Function in San1p-Mediated Degradation

In addition to a ubiquitin-protein ligase (E3), the covalent attachment of ubiquitin to substrates requires the action of two other enzymes: a ubiquitin-activating enzyme (UBA or E1), which forms a high-energy, thiol-ester bond with ubiquitin in a reaction that requires ATP, and a ubiquitin-conjugating enzyme (UBC or E2), which accepts the charged ubiquitin from the activating enzyme by forming an identical, high-energy, thiol-ester

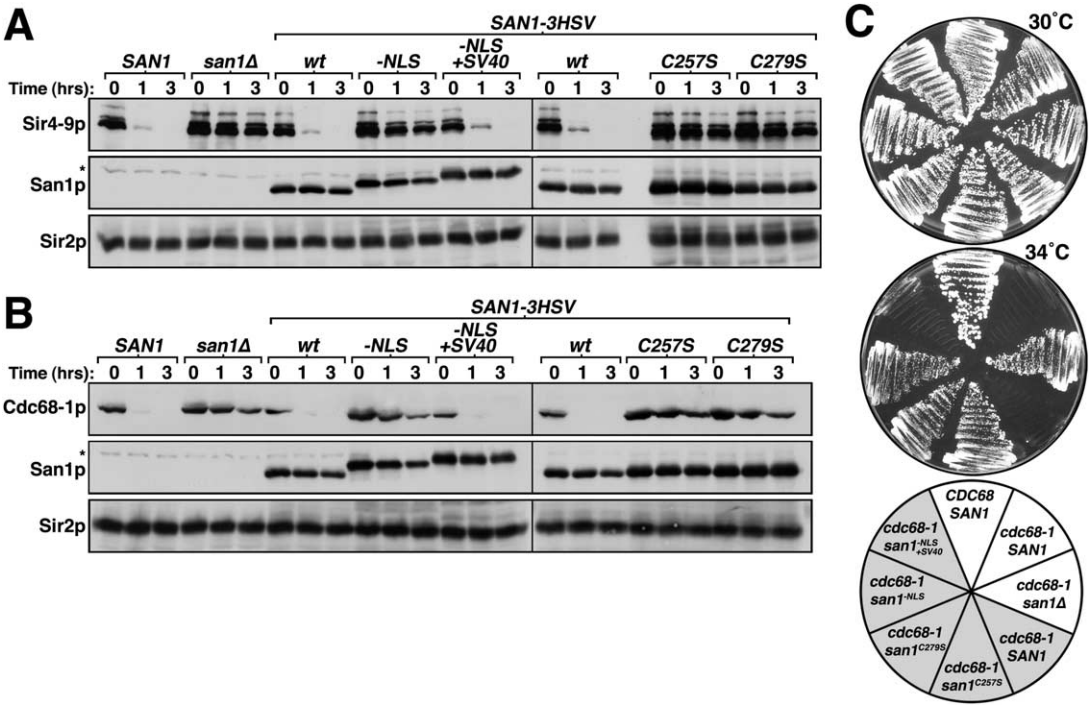

Figure 4. Nuclear Localization of San1p Is Required for Function

(A and B) Cycloheximide-chase assays of cells expressing the indicated San1p substrates were performed to assess stability in the presence of various *san1* alleles. Images are of the same blot, probed sequentially with anti-Myc or anti-Cdc68p antibodies, anti-HSV antibodies, and anti-Sir2p antibodies. The degradation of (A) 1Myc-Sir4-9p and (B) Cdc68-1p was analyzed.

(C) Strains containing the indicated *SAN1* and *CDC68* alleles were streaked onto YC Trp plates and incubated at the indicated temperatures for 3 days. Gray triangles in the plate map indicate strains in which the indicated *SAN1-3HSV* allele covered the *san1Δ* allele.

bond with ubiquitin (Jackson et al., 2000). *S. cerevisiae* has a single essential UBA, Uba1p, and eleven UBCs, ten of which are not essential for viability (Jackson et al., 2000).

To identify the UBC(s) participating in *SAN1*-mediated degradation, Sir4-9p degradation was examined in the absence of each nonessential *UBC* gene. *ubc1Δ* was the only single null allele that had a detectable effect on Sir4-9p degradation (Figure 5A). However, loss of *UBC1* resulted in partial stabilization of Sir4-9p, unlike the *san1Δ* allele, indicating that Ubc1p likely acts in concert with another UBC and San1p to ubiquitinate target proteins. Accumulation of Sir4-9p proteolytic fragments in *ubc1Δ* cells (Figure 5A, arrows) might indicate that Ubc1p activity is important in a processive aspect of *SAN1*-mediated degradation.

The remaining untested UBC was *CDC34/UBC3,* which is a member of the SCF complex and is essential for yeast cells to progress through the cell cycle (Deshaies, 1999). To assess a possible role of *CDC34* in San1p-mediated degradation, we analyzed Sir4-9p degradation in a strain carrying the *cdc34-2* allele (Liu et al., 1995). When compared to a wild-type *CDC34* strain, Sir4-9p was stable in the *cdc34-2* strain (Figure 5B). Degradation was restored when the *cdc34-2* allele was complemented by introduction of the wild-type *CDC34* gene (Figure 5B), indicating that the *cdc34-2* allele itself was responsible for Sir4-9p stability.

We further tested the involvement of *UBC1* and *CDC34* in *SAN1*-mediated degradation by examining the effect of overexpressing catalytically inactive versions of each UBC on Sir4-9p degradation. Mutation of the active-site Cys residue in UBCs destroys their function, and overexpression of the mutant can dominantly interfere with the function of the wild-type cognate (Banerjee et al., 1995; Madura et al., 1993). We created catalytically inactive mutants by substituting a Ser at Cys88 for Ubc1p and at Cys95 for Cdc34p. Overexpression of either mutant *UBC* from the galactose-inducible *GAL1,10* promoter significantly stabilized Sir4-9p (Figure 5C).

Given the nuclear-restricted activity of *SAN1* and nuclear localization of Sir4p (Palladino et al., 1993), it was possible that overexpression of any nuclear-localized catalytically inactive UBC might affect Sir4-9p degradation. To address this, we made a catalytically inactive variant of Rad6/Ubc2p, which is located in the nucleus (Ulrich and Jentsch, 2000). In contrast to $CDC34^{C95S}$ or $UBC1^{C88S}$, $RAD6^{C88S}$ overexpression had little effect on Sir4-9p degradation (Figure 5C).

Ubc1p also functions with Ubc7p in degradation of proteins at the ER (Bays et al., 2001; Friedlander et al., 2000). However, loss of *UBC7* or overexpression of the $UBC7^{C89S}$ allele did not stabilize Sir4-9p (Figures 5A and 5C), indicating that Ubc1p function in Sir4-9p degradation is distinct from its ER function. The lack of

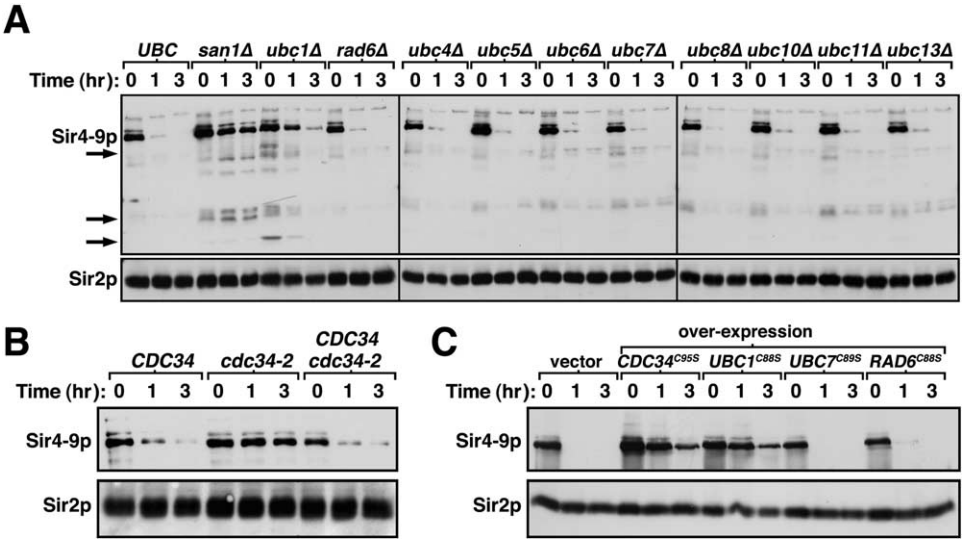

Figure 5. Ubc1p and Cdc34p Are Required for Optimal Degradation of San1p Substrates

(A) Cycloheximide-chase assays of cells expressing 1Myc-Sir4-9p were performed to assess stability in the absence of the individual *UBC* genes. Images are of the same blot, probed sequentially with anti-Sir4p antibodies and anti-Sir2p antibodies.

(B) Cycloheximide-chase assays of cells expressing 1Myc-Sir4-9p were performed to assess stability in the presence of the *cdc34-2* allele. Images are of the same blot, sequentially probed with anti-Myc antibodies and anti-Sir2p antibodies.

(C) Cycloheximide-chase assays of cells expressing 1Myc-Sir4-9p were performed to assess stability in the presence of the individual catalytically inactive *CDC34*^{C95S}, *UBC1*^{C88S}, *UBC7*^{C89S}, and *RAD6*^{C88S} alleles. Images are of the same blot, which was probed as in (B).

effect seen with the *UBC7*^{C89S} and *RAD6*^{C88S} alleles indicates that Sir4-9p stabilization due to overexpression of the *CDC34*^{C95S} or *UBC1*^{C88S} alleles is not a general feature of overexpressing dominant-negative versions of UBCs. Similar stabilizing effects on Cdc68-1p degradation were observed after overexpression of the *UBC1*^{C88S} or *CDC34*^{C95S} alleles but not the *UBC7*^{C89S} or *RAD6*^{C88S} alleles (data not shown).

The Proteasome Is the Primary Protease Involved in San1p-Mediated Degradation

Because *SAN1*-mediated degradation is ubiquitin dependent, the proteasome is the likely protease involved. In fact, the previously described *cdc68-1* suppressor analysis identified mutations in the proteasomal 19S regulatory particle subunit *RPT6/SUG1/CIM3* that suppressed *cdc68-1* temperature sensitivity (Xu et al., 1993; Xu et al., 1995). To test this idea more thoroughly, we examined the degradation of the Sir4-9 protein in a strain containing the *cim3-1* allele and found that Sir4-9p was stable (Figure 6A). Sir4-9p was also very stable in the presence of *cim5-1*, a mutant allele of another proteasomal 19S subunit (Figure 6A). Finally, to test whether the proteasome's 20S catalytic activity was required for *SAN1*-mediated degradation, we added the proteasome inhibitor MG-132 to cells and assayed for protein stability. Addition of MG-132 stabilized Sir4-9p, Cdc13-1p, and Cdc68-1p (Figure 6B). Thus, *SAN1*-dependent QC degradation requires a functional 26S proteasome.

San1p Does Not Regulate Wild-Type Versions of Its Mutant Substrates

Our observations support a model in which San1p is part of a nuclear-localized PQC degradation machinery. However, San1p could also be a regulator of the wild-type proteins whose mutant versions were its degradation substrates. To see if this were the case, we determined if loss of *SAN1* affected any functions normally associated with the wild-type proteins. Cdc13p is a single-stranded DNA binding protein that regulates telomere length (Smogorzewska and de Lange, 2004). When we examined telomere length in *san1Δ* cells, we saw no significant difference in global telomere length compared to wild-type *SAN1* cells (see Figure S1A in the Supplemental Data available with this article online). Sir4p and Sir3p function together in silent chromatin formation at the silent mating-type loci and telomeres (Aparicio et al., 1991; Rine and Herskowitz, 1987). When we assayed effects on silencing using telomeric reporter genes (Gottschling et al., 1990), we found telomeric silencing was unaffected by loss of *SAN1* (Figure S1B). Cdc68p functions as part of the FACT complex in transcriptional initiation and elongation (Belotserkovskaya et al., 2003; Brewster et al., 2001; Formosa et al., 2001). When comparing gene expression profiles from wild-type or *san1Δ* cells grown in complete synthetic media (YC) or rich media (YEPD; Table 1), we found no difference in expression for genes affected by mutations in *CDC68/SPT16* (Kaplan et al., 2003; Rowley et al., 1991). We also found no significant difference in expression for genes regulated by *SIR4*-mediated silent chromatin at telomeres, the silent mating loci, or for genes regulated by the mating loci (Wyrick et al., 1999). Altogether, we found no evidence that *SAN1* normally regulates any of the processes in which the wild-type Cdc13, Sir4, Sir3, and Cdc68 proteins normally function.

In contrast to our results, it was recently claimed that wild-type Sir4p is degraded in a *SAN1*-dependent man-

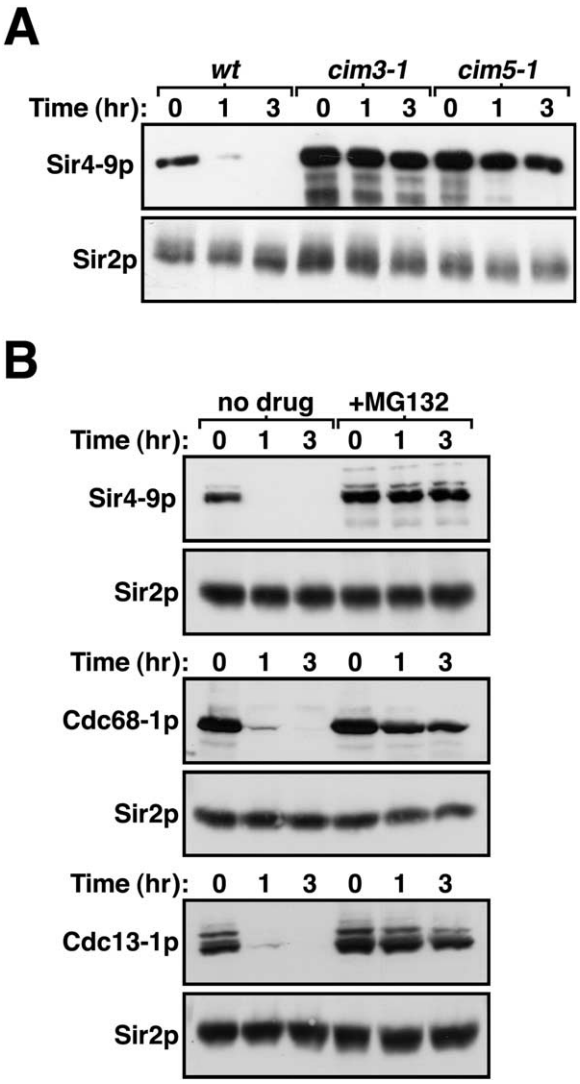

Figure 6. The Proteasome Is Required for Degradation of Mutant Nuclear Proteins

(A) Cycloheximide-chase assays of cells expressing 1Myc-Sir4-9p were performed to assess stability in the presence of either the *cim3-1* or *cim5-1* alleles. Images are of the same blot, probed sequentially with anti-Myc antibodies and anti-Sir2p antibodies.

(B) Cycloheximide-chase assays of cells expressing either wild-type or mutant protein were performed to assess stability in the presence or absence of the proteasome inhibitor MG-132. Assays were performed in *pdr5*Δ cells to increase sensitivity of cells to MG-132 (Chernova et al., 2003). Images are of the same blot, sequentially probed with anti-Myc or anti-Cdc68p antibodies and anti-Sir2p antibodies.

ner (Dasgupta et al., 2004). To resolve this difference, we examined the degradation of wild-type Sir4p in five different strain backgrounds, including the ones reported in the recent study. We found that wild-type Sir4p was stable in every strain background tested. In our hands, mutant Sir4-9p was the only version of Sir4p that was degraded (Figures 1 and S1C). Although we cannot explain these differences, we are confident from our reproducible observations that San1p acts specifically upon mutant versions of Sir4p, Sir3p, Cdc68p, and Cdc13p and not as a regulator of the wild-type proteins.

A Cellular Stress Response Is Induced in the Absence of *SAN1*

Given San1p's role in degrading aberrant nuclear proteins, we speculated that its activity would be important for cellular viability under growth conditions that create aberrant proteins. Consequently, *san1*⊿ and wild-type cells were compared for their ability to grow at elevated temperatures or in the presence of $CdCl_2$, in the presence of canavanine, or after exposure to UV, MMS, EMS, or hydrogen peroxide. In no instance did we observe a growth difference between *SAN1* and *san1*⊿ cells (data not shown). In fact, *san1* mutant alleles have never been identified in any screen looking for mutations that cause sensitivity to cell stress. A possible explanation for the lack of a cell-stress phenotype is the compensatory upregulation or activation of other pathways in the absence of *SAN1*.

To explore this possibility further, we carefully compared the transcript-microarray profiles of wild-type and *san1*⊿ cells. When we placed the genes affected differentially in *san1*⊿ cells in order of increased expression, the top thirty upregulated genes for cells grown in YC media were highly represented by those that are induced under cellular stress (Causton et al., 2001; Jelinsky et al., 2000). Using the *Saccharomyces* Genome Database Gene Ontology (GO) Term Finder tool (http://db.yeastgenome.org/cgi-bin/GO/goTermFinder), 12 of the 30 genes belonged to the class of proteins involved in cellular stress ($p = 1.2 \times 10^{-8}$), including seven protein chaperones: *HSP26*, *HSP12*, *HSP42*, *HSP104*, *HSP82*, *HSC82*, and *SSA4* (Table 1). Furthermore, transcript-microarray profiles from the literature revealed that 29 of the 30 genes are induced 2-fold or greater under defined cellular stress conditions (Causton et al., 2001; Jelinsky et al., 2000) and 18 contain a stress response element (STRE) in their promoters (Burchett et al., 2002; Cameroni et al., 2004; DeRisi et al., 1997; Grant et al., 2000; Lagorce et al., 2003; Moskvina et al., 1998) or are regulated by *MSN2/MSN4* (Causton et al., 2001). Although the induction of these genes in our transcript-microarray analysis was modest, ranging from 1.3- to 2.1-fold increases, independent verification by quantitative Northern analyses indicated that some had increased expression by as much as 6-fold (data not shown).

In contrast, when cells were grown in YEPD media, there was no similar transcriptional signature for *san1*⊿ cells. Fewer genes were upregulated in *san1*⊿ cells under these conditions, and only three of the top thirty genes upregulated during growth in synthetic medium were also upregulated during growth in YEPD (Table 1). The numerous differences between these growth media make it difficult to assess the source of the cell stress resulting from growth in synthetic media. Nevertheless, our data indicate that *san1*⊿ cells grown in synthetic media are under a cellular stress that induces the expression of numerous protein chaperones that operate in the nucleus.

Table 1. Genes Upregulated in *san1*Δ Cells

YC Media					
ORF	Gene	Fold Change	p Value	STRE	Stress Induced
YFL014W	HSP12	2.1	3.1×10^{-8}	Yes[1,3,8]	Yes[3,6]
YBR072W	HSP26	1.9	5.0×10^{-8}	Yes[1,3,8]	Yes[3,6]
YNL111C	CYB5	1.7	5.8×10^{-7}		Yes[3,6]
YGR234W	YHB1	1.6	5.7×10^{-8}		Yes[6]
YJR048W	CYC1	1.6	5.1×10^{-8}		Yes[6]
YHR087W		1.5	1.6×10^{-4}	Yes[1,3]	Yes[3,6]
YML128C	MSC1	1.5	5.3×10^{-6}	Yes[1,3]	Yes[3,6]
YER103W	SSA4	1.4	1.1×10^{-6}	Yes[1,3,8]	Yes[3,6]
YER044C	ERG28	1.4	4.6×10^{-6}		Yes[6]
YGR248W	SOL4	1.4	1.9×10^{-3}	Yes[3,4]	Yes[3, 6]
YGR008C	STF2	1.4	1.0×10^{-5}	Yes[1,3]	Yes[3,6]
YLL026W	HSP104	1.4	4.4×10^{-4}	Yes[8]	Yes[3,6]
YDL130W-A	STF1	1.4	7.1×10^{-5}		
YDR070C		1.4	1.0×10^{-4}	Yes[1,3]	Yes[3,6]
YIL111W	COX5B	1.4	2.7×10^{-5}	Yes[3,8]	Yes[3,6]
YMR251W-A	HOR7	1.4	2.9×10^{-4}		Yes[6]
YAR015W	ADE1	1.3	3.7×10^{-6}		Yes[6]
YPR160W	GPH1	1.3	2.6×10^{-3}	Yes[1,3,8]	Yes[3,6]
YMR105C	PGM2	1.3	2.5×10^{-3}	Yes[1,3,8]	Yes[3,6]
YKR080W	MTD1	1.3	4.4×10^{-5}		Yes[6]
YMR186W	HSC82	1.3	2.5×10^{-5}		Yes[6]
YNL015W	PBI2	1.3	1.2×10^{-5}	Yes[3,7]	Yes[3,6]
YMR196W		1.3	1.0×10^{-5}	MSN2/MSN4[3]	Yes[3]
YLR205C	HMX1	1.3	4.4×10^{-5}		Yes[3,6]
YLR217W		1.3	3.6×10^{-5}		Yes[3]
YPL240C	HSP82	1.3	7.3×10^{-5}	Yes[6]	
YDR171W	HSP42	1.3	1.7×10^{-3}	Yes[3,8]	Yes[3,6]
YMR250W	GAD1	1.3	1.5×10^{-3}	Yes[2,3]	Yes[3,6]
YCL035C	GRX1	1.3	1.4×10^{-5}	Yes[3,5]	Yes[3,6]
YFR053C	HXK1	1.3	2.4×10^{-3}	Yes[1,3,8]	Yes[3,6]
YEPD Media					
ORF	Gene	Fold Change	p Value	STRE	Stress Induced
YOL152W	FRE7	2.0	3.3×10^{-10}		
YPR123C		1.6	4.4×10^{-9}		
YPR124W	CTR1	1.5	3.3×10^{-9}		Yes[6]
YGR164W		1.5	2.1×10^{-8}		
YNL111C	CYB5	1.4	1.8×10^{-8}		Yes[3,6]
YLR214W	FRE1	1.4	2.6×10^{-8}		
YJR048W	CYC1	1.4	1.8×10^{-5}		Yes[6]
YER044C	ERG28	1.4	7.3×10^{-8}		Yes[6]
YML075C	HMG1	1.3	4.8×10^{-7}		
YGR234W	YHB1	1.3	5.5×10^{-7}		Yes[6]
YPR200C	ARR2	1.3	1.8×10^{-5}		
YLR158C	ASP3-3	1.3	1.6×10^{-7}		Yes[6]
YOL106W		1.3	9.1×10^{-5}		

Genes whose expression is increased by greater than 1.3-fold in *san1*Δ cells compared to wild-type *SAN1* cells are listed above. For complete data set, including genes that are downregulated, see Table S3. Genes that possess a STRE in their promoter are listed. Genes that are regulated by various stress conditions are annotated with the appropriate references below.

[1] Burchett et al., 2002
[2] Cameroni et al., 2004
[3] Causton et al., 2001
[4] DeRisi et al., 1997
[5] Grant et al., 2000
[6] Jelinsky et al., 2000
[7] Lagorce et al., 2003
[8] Moskvina et al., 1998

Discussion

SAN1 Defines a Nuclear PQC Degradation System within the Nucleus

In this study, we provide evidence for a PQC system that resides within the nucleus and degrades aberrant proteins. The defining member of this degradation system is San1p, a ubiquitin-protein ligase that requires nuclear localization for its proper function. San1p mediates the ubiquitination of aberrant nuclear proteins, which ultimately leads to their degradation by the proteasome. The critical evidence that San1p is part of a PQC degradation system comes from its specific destruction of mutant nuclear proteins, while it leaves the

wild-type counterparts of these proteins unscathed. Yet San1p is not a global degradation factor; loss of *SAN1* does not stabilize PQC substrates of the endoplasmic reticulum (Dasgupta et al., 2004).

Because San1p is a ubiquitin-protein ligase, it must act in concert with ubiquitin-conjugating enzymes. Of the eleven UBCs in *S. cerevisiae*, only mutant alleles of *CDC34* and *UBC1* stabilized San1p substrates. Formally, it is possible that other UBCs are involved in San1p-mediated degradation, given that some UBCs appear to be redundant in other degradation pathways (Betting and Seufert, 1996; Chen et al., 1993; Seufert and Jentsch, 1990). Nevertheless, both Ubc1p and Cdc34p are found in the nucleus (Blondel et al., 2000; Huh et al., 2003), which is consistent with their role in the degradation of San1p substrates. Previously, the only function assigned to Cdc34p was as part of the SCF complex, where it mediates the regulated ubiquitination of numerous substrates, including those required for cell-cycle progression (Deshaies, 1999). Thus, Cdc34p involvement in nuclear PQC degradation defines a new role for the UBC. Ubc1p has already been shown to function in PQC degradation at the ER as part of the HRD complex (Bays et al., 2001; Friedlander et al., 2000), so involvement in nuclear PQC degradation is an extension of Ubc1p function.

The involvement of Cdc34p and Ubc1p in the degradation of San1p substrates and the nature of San1p itself opens up intriguing parallels with PQC degradation in the ER, mediated by the RING-domain ubiquitin-protein ligase Hrd1p (Bays et al., 2001; Deak and Wolf, 2001). The closest homolog to San1p in the *S. cerevisiae* genome is Hrd1p, with 24% identity and 40% similarity shared between them in a 174 residue stretch comprising the RING domain and an adjacent C-terminal region (https://proteome.incyte.com/proteome/YPD/SAN1.html). Ubc1p is required for both the normal degradation of Hrd1p-dependent substrates (Bays et al., 2001; Friedlander et al., 2000) and San1p-dependent substrates (Figure 5). Cdc34p shares the greatest homology with Ubc7p (Ptak et al., 2001), which interacts with Hrd1p and is the primary UBC required for Hrd1p-mediated degradation (Bays et al., 2001; Deak and Wolf, 2001). Given the homology between Hrd1p and San1p and the involvement of identical and highly similar UBCs, we speculate that the two PQC degradation systems may have evolved from a common pathway, with Hrd1p anchored to the ER and San1p targeted to the nucleus.

Although we have identified four mutant protein substrates of the *SAN1* system, we cannot yet explain what hallmarks of the mutant proteins are recognized as "aberrant" by this system (e.g., exposed hydrophobic patches, atypical posttranslational modifications, aggregation, etc.). Clearly, whatever is recognized as aberrant does not necessarily coincide with complete loss of protein activity. Otherwise, the *sir4-9* and *cdc68-1* phenotypes would not have been suppressed by deleting *SAN1*.

We also do not yet know what properties of San1p allow it to recognize aberrant proteins. Is aberrant protein targeting an autonomous feature of San1p, or does San1p engage ancillary factors, such as protein chaperones, to target aberrant proteins for ubiquitination? It also is not clear if San1p targets aberrant proteins from all regions of the nucleus or if San1p action is restricted to aberrant chromatin-associated proteins such as the ones examined in this study.

SAN1 Reduces Cellular Stress

In support of San1p's role in nuclear PQC, *san1⊿* cells grown in synthetic media had a transcript-microarray signature indicative of a cellular stress response. Of the 30 upregulated genes, 29 are induced under cellular stress conditions, 18 have STRE in their promoters, and 7 are protein chaperones. Of the chaperones, *HSP26* and *HSP104* are implicated in nuclear protein homeostasis (Parsell et al., 1994; Rossi and Lindquist, 1989); overexpression of *HSP82* or *HSC82* partially suppresses the temperature sensitivity of the *cdc13-1* allele (Grandin and Charbonneau, 2001), which encodes one of the San1p substrates we identified, and Hsp12p, Hsp26p, Hsp104p, and Ssa4p are localized to the nucleus (Chughtai et al., 2001; Huh et al., 2003; Kawai et al., 1999; Rossi and Lindquist, 1989). The increased expression of these genes in *san1*Δ cells is consistent with the idea that loss of *SAN1* results in the accumulation of aberrant proteins in the nucleus, which in turn triggers a cellular response that attempts to refold or otherwise sequester the aberrant nuclear proteins. By extension, we propose that San1p normally rids the nucleus of aberrant proteins, and its presence alleviates the need for such a response.

The increased expression of protein chaperones may buffer the cell from dramatic phenotypic effects when *SAN1* is absent. In fact, the low level of chronic stress that *san1*Δ cells are under may precondition them for additional challenges, similar to what occurs in thermotolerance (Kregel, 2002). This may explain why a significant growth difference between *san1*Δ and wild-type cells was not observed under standard lab conditions or after exposure to physical and chemical challenges. The use of alternate mechanisms to handle aberrant proteins when degradation is alleviated appears to be typical for PQC, as evidenced by what occurs in ER PQC (Friedlander et al., 2000; Travers et al., 2000).

The induction of the stress response in *san1*Δ cells might also be explained if *SAN1* facilitates the rapid and continuous degradation of a positive regulator of stress response genes. In the absence of *SAN1*, the positive regulator would rise above a critical threshold required for activation of the stress response. However, we find this explanation unlikely, since a similar stress response was not detected when cells were grown in YEPD media. Instead, the differential response implies that the metabolism of cells grown in synthetic media generated more aberrant nuclear proteins than when cells were grown in rich media.

San1p as a Last Line of Proteolytic Defense against Aberrant Nuclear Proteins

One implication of our findings is that the *SAN1* system functions downstream of protein production, recognizing determinants in aberrant proteins that were not detected by earlier cytoplasmic PQC checkpoints, which in principle should act on aberrant proteins before they become nuclear localized. If San1p substrates were de-

stroyed by cytoplasmic PQC, the degradation and, consequently, the temperature-sensitive phenotypes of both the Sir4-9 and Cdc68-1 proteins would not be suppressed by loss of *SAN1* function (Schnell et al., 1989; Xu et al., 1993). Why are cytoplasmic PQC degradation pathways unable to target the mutant San1p substrates for destruction? Perhaps there are environmental differences between the cytoplasm and nucleus, such that the determinants of aberrancy are only revealed upon import into the nucleus. Alternatively, such determinants may be hidden from cytoplasmic PQC by proteins that function as escorts prior to nuclear import, or the determinants may be uncovered during nuclear import itself. Given the failure of earlier PQC checkpoints to recognize these aberrant proteins, it may be that the cell uses San1p as a last line of proteolytic defense against accumulation of aberrant proteins in the nucleus, initiating degradation when other PQC pathways upstream in the hierarchy of PQC fail.

As noted above, a lack of *SAN1*-mediated nuclear PQC degradation manifests a stress response in *S. cerevisiae*. This may be indicative of a cellular pathology that can occur in any eukaryotic cell, including those associated with some neurodegenerative diseases in which there is accumulation and aggregation of mutant proteins in the nucleus. From our sequence-based searches, we have identified homologs of San1p in a number of fungi (data not shown). Using those in subsequent iterative searches, we found proteins with regions of similarity, including the noncanonical RING domain, in metazoa and plants, indicating that an analogous pathway may exist in other species. In metazoans, nuclear PQC may be most critical in cell types that remain in a nondividing state for long periods of time, such as neural and muscle cells. We speculate that the severity in onset of nuclear protein aggregation diseases may be influenced by the activity of this or a similar pathway.

Experimental Procedures

Materials and Reagents

Reagents were from New England Biolabs (Beverley, Massachusetts), Sigma (St. Louis, Missouri), and Fisher (Pittsburgh, Pennsylvania). Nitrocellulose (Protran, pore size 0.2 μM) was from Schleicher and Schuell (Keene, New Hampshire). Mouse anti-Myc, anti-VSV, and anti-HA antibodies were from Sigma (St. Louis, Missouri) and used at 1:10,000 dilutions. Mouse anti-HSV antibody was from Novagen (Madison, Wisconsin) and used at a 1:10,000 dilution. Goat anti-Sir2p polyclonal antibodies and donkey anti-goat HRP-conjugated antisera were from Santa Cruz Biotechnology (Santa Cruz, California) and used at a 1:2000 dilutions. Rabbit anti-Cdc68p antisera was kindly provided by Tim Formosa (University of Utah) and used at a 1:1000 dilution. Affinity-purified rabbit anti-Sir4p antibodies were kindly provided by Danesh Moazed (Harvard University) and used at a 1:2000 dilution. Rabbit anti-Sir4p antisera were from Covance (Princeton, New Jersey), after providing them with GST-Sir4p (C1) purified as previously described (Moazed et al., 1997), and used at a 1:500 dilution. ECL chemiluminescence reagents, sheep anti-mouse, and donkey anti-rabbit HRP-conjugated antisera were from Amersham Biosciences (Piscataway, New Jersey) and used at 1:2000 dilutions. FastFlow rProtein G-agarose was from Repligen (Waltham, Massachusetts). Purified yeast E1 enzyme, human UbcH5a, ubiquitin, Mg-ATP, and MG-132 were from Boston Biochem (Boston, Massachusetts). GST-Bind resin was from Novagen. Talon resin was from BD Biosciences Clontech (Palo Alto, California).

Plasmids and Yeast Strains

Standard molecular biology techniques were used. All plasmids used in this study and brief descriptions of their construction are listed in Table S1.

Standard yeast methods were used. Recipes for YEPD media and YC media can be found at http://www.fhcrc.org/labs/gottschling. All yeast strains used in this study are listed in Table S2.

Degradation Assays

Protein degradation was assessed by cycloheximide-chase assays similar to previously described (Gardner and Hampton, 1999), with some changes. Cells were usually grown in YEPD media to an approximate cell density of 2×10^7 cells/ml at the permissive temperature of 20°C or 25°C before addition of cycloheximide (50 μg/ml final concentration), and further incubated at the restrictive temperatures of 30°C, 34°C, or 37°C for 0–3 hr after addition of cycloheximide. In some cases, cells were grown first in YC + 3% raffinose, with galactose added to a final concentration of 3% and cultures incubated for 4 hr prior to addition of cycloheximide. In other cases, the proteasome inhibitor MG-132 was added to a final concentration of 25 μg/ml, and cultures were further incubated at 25°C for 30 min prior to addition of cycloheximide. Cells were lysed in 200 μl SUMEB (8 M urea, 1% SDS, 10 mM MOPS [pH6.8], 10 mM EDTA, 0.01% bromophenol blue) + 10 mM PMSF. Fifteen to thirty microliter samples of the cellular lysates were resolved on 8% SDS-PAGE gels, the proteins transferred to nitrocellulose, and immunoblotted with the appropriate antibody.

Ubiquitination Assays

GST fusions of San1p were purified per manufacturer's instructions (Novagen GST-Bind kit) from 100 ml LB + amp cultures, which were initially grown at 37°C to an optical density (OD_{600}) of 0.3. IPTG was added to a final concentration of 0.8 mM and the cultures were incubated for 6 hr at 37°C. Cells were harvested by centrifugation and then frozen at –80°C overnight. Frozen cells were lysed in 20 ml lysis buffer (20 mM Tris-HCl [pH8.0], 200 mM NaCl, 1 mM EDTA, 1 mM EGTA) + 10 mM PMSF + 10 mM benzamide + 200 μg/ml lysozyme. Clarified lysates were loaded onto 1.5 ml glutathione columns prewashed with 5 ml lysis buffer. Columns were washed with 20 ml lysis buffer then 20 ml wash buffer (20 mM HEPES-KOH [pH7.6], 350 mM NaCl, 1 mM DTT). Bound proteins were eluted with 10 ml wash buffer + 10 mM glutathione. Samples containing purified GST-San1 protein were combined and concentrated using a Centricon10 (Millipore, Bedford, Massachusetts).

In vitro ubiquitination assays were performed similar to previously described (Lorick et al., 1999), with some changes. Fifteen microliter reactions containing 0.08 μg ubiquitin activating enzyme (yeast Uba1p), 0.1 μg ubiquitin conjugating enzyme (human UbcH5a), 1.75 μg purified GST-San1p (or its mutant variants or GST alone), 2.5 μg ubiquitin, 2 mM Mg-ATP, 50 mM Tris-HCl (pH7.5), 2.5 mM $MgCl_2$, and 0.5 mM DTT were incubated at 25°C for 90 min. Fifteen microliters SUMEB was added to each sample and incubated at 65°C for 10 min. The entire reaction was resolved on 8% SDS-PAGE gels, the proteins transferred to nitrocellulose, and immunoblotted with anti-ubiquitin monoclonal antibody (Kahana and Gottschling, 1999).

In vivo ubiquitination assays were performed similar to previously described (Gardner and Hampton, 1999), with modifications. Strains expressing either 1Myc-Sir4p or 1Myc-Sir4-9p and a triple HA-epitope-tagged ubiquitin expressed from the constitutive *TDH3* promoter were grown in 20 ml YC Ura^- media to an approximate cell density of 2×10^7 cells/ml. Cultures were divided into 2–5 ml aliquots, with one aliquot incubated at 25°C and the other incubated at 34°C for 20 min. Cellular lysates were prepared in 200 μl SUME + 10 mM PMSF + 5 mM N-ethylmaleimide (NEM). 1Myc-Sir4 proteins were immunoprecipitated in 1 ml IP buffer (15 mM Na_2PO_4, 150 mM NaCl, 2% Triton-X100, 0.1% SDS, 0.5% deoxycholate, 10 mM EDTA [pH7.5]) + 10 mM PMSF + 5 mM NEM + 20 μl anti-Sir4p antibodies. 100 μl FastFlow rProtein G-agarose was used to capture the immunocomplexes. Proteins were eluted from beads using 60 μl SUMEB + 5% β-mercaptoethanol and incubation at 65°C for 10 min. Ten microliter or forty microliter samples were resolved on 8%

SDS-PAGE gels, proteins transferred to nitrocellulose, and immunoblotted with either an anti-Myc antibody or an anti-HA antibody.

Transcript DNA Microarrays

Transcript DNA microarrays were performed similar to previously described (Fazzio et al., 2001). Three independent *san1⊿* cultures and three independent wild-type cultures were grown to midlog phase at 30°C in 50 ml YEPD or YC media. Dye-reversal experiments were performed to identify sequence-specific dye biases. Statistical analysis of microarray data was performed as previously described (Cullen et al., 2004). The entire normalized data set is in Table S3.

Supplemental Data

Supplemental Data include one figure, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/120/6/803/DC1/.

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Accession Numbers

The GEO accession number for the series of transcript array datasets is GSE2159.