Substrate recognition in nuclear protein quality control degradation is governed by exposed hydrophobicity that correlates with aggregation and insolubility.

Eric K. Fredrickson, Pamela S. Gallagher, Sarah V. Clowes Candadai, and Richard G. Gardner

From the Department of Pharmacology, University of Washington, Seattle, WA, 98195, USA

*Running title: Hydrophobicity and insolubility in San1-mediated degradation

To whom correspondence should be addressed: Richard Gardner, Department of Pharmacology, University of Washington, Box 357280, 1959 NE Pacific Street, Seattle, WA, 98195, USA, Tel.: (206) 685-0506; Fax: (206) 685-3822; E-mail: gardnerr@uw.edu

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Background: San1 is a ubiquitin-protein ligase that recognizes exposed hydrophobicity in misfolded nuclear proteins.

Results: San1 prefers a window of exposed hydrophobicity that causes a particular level of protein insolubility.

Conclusion: The hydrophobicity/insolubility threshold suggests that San1 evolved to target highly aggregation-prone proteins.

Significance: Identifying the specific parameters of misfolding recognized by ubiquitin-protein ligases allows us to understand the nature of substrate targeting.

SUMMARY

Misfolded proteins present an escalating deleterious challenge to cells over the course of their lifetime. One mechanism the cell possesses to prevent misfolded protein accumulation is their destruction by protein quality control (PQC) degradation systems. In eukaryotes, PQC degradation typically proceeds via multiple ubiquitin-protein ligases that act throughout the cell to ubiquitinate misfolded proteins for proteasome degradation. What the exact feature of misfolding that each PQC ubiquitin-protein ligase recognizes in their substrates remains an open question. Our previous studies of the budding yeast nuclear ubiquitin-protein ligase San1 indicated that it recognizes exposed hydrophobicity within its substrates, with the threshold of hydrophobicity equivalent to that of 5 contiguous hydrophobic residues. Here, we uncover an additional parameter – the nature of the exposed hydrophobicity that confers San1-mediated degradation correlates with significant protein insolubility. San1 particularly targets exposed hydrophobicity that leads to insolubility and aggregation above a certain threshold. Our studies presented here provide additional insight into the details of misfolded nuclear protein recognition, and demonstrate that there is selectivity for the type of exposed hydrophobicity.

The continuous stochastic production of misfolded proteins is a problem faced by all cells. Proteins that have lost their native structures expose hydrophobicity that is normally buried within the core of their structures. The exposed hydrophobicity can lead to protein insolubility and the formation of intracellular aggregates (1). Aggregate formation and the long-term cellular persistence of aggregates is thought to be the underlying cause of many devastating human pathologies including Alzheimer’s, Parkinson’s, Huntington’s, and amyotrophic lateral sclerosis (2).

Due to the deleterious nature of misfolded proteins, cells have evolved several mechanisms to prevent their accumulation. One central means is the elimination of misfolded proteins via protein quality control (PQC) degradation. Eukaryotic PQC degradation is generally mediated by ubiquitin-protein ligases that ubiquitinate misfolded proteins for subsequent proteasome degradation (3). PQC ubiquitin-protein ligases have been identified in most cellular compartments including the endoplasmic reticulum (4-9), cytoplasm (10-13), and nucleus (14-18). Multiple PQC ligases often exist in the same compartment. For example, Hrd1 and Doa10 operate in the yeast endoplasmic reticulum.
(4,6,9,19,20), whereas San1 and Doa10 act in the yeast nucleus (15,21). Although a cellular compartment can contain multiple PQC ubiquitin-protein ligases, they generally do not target the same substrates (20,22-26), indicating that the compartment-specific PQC ligases are, for the most part, not functionally redundant.

The lack of functional redundancy suggests that each PQC ubiquitin-protein ligase might recognize a unique feature of misfolding in its substrates. This likely depends upon the specific cellular compartment and the spatial organization of the PQC ligases within that compartment. With this consideration in mind, it remains an open question as to which feature(s) of misfolding an individual PQC ubiquitin-protein ligase targets. If each PQC ligase recognizes exposed hydrophobicity, is the extent of exposed hydrophobicity, the types of hydrophobic residues, and/or the context in which the hydrophobicity is presented different for each PQC ligase? To answer these questions, we require detailed information as to what each individual PQC ubiquitin-protein ligase recognizes in its misfolded substrates.

We have been probing this by exploring the features of misfolding recognized by the yeast nuclear PQC ubiquitin-protein ligase San1. Recently, we found that San1 targets exposed hydrophobicity in its substrates, with a minimal window of 5 exposed hydrophobic residues necessary for substrate recognition by San1 (27). Here, we find that San1 prefers particular types of hydrophobic residues within that window, and this preference can be explained by the strength of the residues’ ability to cause insolubility and aggregation.

EXPERIMENTAL PROCEDURES

Yeast strains and plasmids—Yeast strains used in this study are BY4741 (met15Δ0 his3Δ1 trp1Δ0 leu2Δ0) (28), RGY506 (BY4741 san1Δ) RGY565 (BY4741 pdr5Δ), RGY1794 (BY4741 san1Δ pdr5Δ), and RGY1273 (his3Δ1 trp1Δ0 leu2Δ0 lys2Δ0 DBP5-αRED::HIS3 san1Δ::HygMX) (27). Standard yeast growth media and yeast genetic methods were employed (29). Plasmids used in this study are listed in Table 1. Standard cloning methods were used to construct each plasmid. The relevant portion of each plasmid was sequenced to verify the fusions. Oligonucleotide sequences and cloning details will be provided upon request.

Degradation Assays—Cycloheximide-chase assays were performed similar to previously described (15). Briefly, cells were grown at 30°C in liquid synthetic media with 3% raffinose to a culture density of ~1x10^7 cells/ml. Galactose was added (final concentration of 3%) and the cells were incubated for 2 hours at 30°C. Cycloheximide was added (final concentration of 50µg/ml) and the cultures were further incubated for 0-3 hours. In certain cases, MG132 (final concentration of 10µg/ml) was added 1 hour prior to cycloheximide addition. 2ml of cells were harvested and lysed at the appropriate time points after cycloheximide addition in 200µl SUMEB (8M urea, 1% SDS, 10mM MOPS pH6.8, 10mM EDTA, 0.01% bromophenol blue) by vortexing 5 minutes with 100µL of 0.5mm acid-washed glass beads. Lysates were incubated at 65°C for 10 minutes and then clarified for 5 minutes by centrifugation at 12,800x g. Proteins were resolved on SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with anti-GFP (Sigma) antibodies.

Solubility Assays—Solubility assays were adapted from a previously described protocol (30). Cells were grown in liquid synthetic media with 3% raffinose to a culture density of ~1x10^7 cells/ml. Galactose was added (final concentration of 3%) and cultures were incubated at 30°C for 2 hours. 4ml of cells were harvested and lysed in 200 µL of Lysis buffer (100 mM Tris pH7.5, 200mM NaCl, 1mM EDTA, 1mM DTT, 5% glycerol, and 0.1% Nonidet P40) + PMSF by vortexing 5 minutes at 4°C with 100µl of 0.5mm acid-washed glass beads. To remove unlysed cells, lysates were centrifuged at 700 x g for 1 minute at 4°C. 50µl lysate, representing the “total lysate”, was removed and added to 50µl SUMEB. Remaining lysate was centrifuged at 12,800x g for 15 minutes at 4°C. 100µl supernatant, representing the “soluble fraction”, was added to 100µl SUMEB. The pellet, representing the “insoluble fraction”, was resuspended in 100µl Lysis buffer plus 100µl SUMEB. All samples were incubated at 65°C for 10 minutes and then clarified for 5 minutes by centrifugation at 12,800x g. Proteins were resolved on SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with anti-GFP antibodies (Sigma).

Nondenaturing polyacrylamide gel electrophoresis—The soluble fractions generated from the solubility assays, with or without SDS (final concentration of 2%) added to the loading buffer, were loaded onto 8-16% Tris-Glycine PAGE gels (Lonza Rockland Inc). Running buffer was 25mM Tris and 192mM glycine. Gels were
run at 4°C. Proteins were transferred to nitrocellulose, and immunoblotted with anti-GFP antibodies (Sigma).

**Microscopy**—Cells (san1Δ DBP5-dsRed) that expressed each GFP<sup>NLS</sup>-peptide fusion protein were grown at 30°C in 3% raffinose media to ~ 0.5×10<sup>7</sup> cells/ml. Galactose was added (final concentration of 3%) and the cells were incubated for 8 hours at 30°C. Cells were harvested, fixed in 4% paraformaldehyde in 0.1 M sucrose for 15 minutes, washed in wash buffer (1.2 M sorbitol, 0.4 M KPO4), stained with DAPI for 10 minutes in wash buffer plus 2% Triton X-100, and washed two times in wash buffer. Cells were imaged on a Nikon Eclipse 90i microscope with a 100X objective, filters for GFP (excitation wavelength (450–490 nm), dichroic mirror (495 nm), and emission filter (500–550 nm)), DAPI-stained DNA (excitation wavelength (325–375 nm), dichroic mirror (400 nm), and emission filter (435–485 nm)), and dsRed (excitation wavelength (540–580 nm), dichroic mirror (585 nm), and emission filter (593–668 nm)), and a Photometrics Cool Snap HQ2 cooled CCD camera with NIS-Elements acquisition software.

**Image processing**—Western blots were scanned using an Epson Perfection V350 Photo scanner at 300 dpi. All images were processed with a Mac iMac or Pro computer (Apple) using Photoshop CS (Adobe). Decay curves for degradation assays and quantification for solubility assays were determined using ImageJ (http://rsb.info.nih.gov/ij/).

**RESULTS**

**San1 recognition of hydrophobicity correlates with substrate insolubility**—Previously, we found that San1 recognizes short hydrophobic peptides as degrons when fused to reporter proteins such as GFP possessing a nuclear localization sequence (GFP<sup>NLS</sup>) or the Gal4 activating domain used in 2-hybrid analyses (27,31). Although the peptide degrons are not misfolded proteins by themselves, they likely allow San1 recognition of the reporter proteins by mimicking the feature San1 recognizes in a misfolded protein – exposed hydrophobicity. As such, we can use these small peptides in a controlled way to determine the biochemical parameters of the exposed hydrophobicity that leads to San1 targeting. In our previous study exploring the minimal number of contiguous hydrophobic residues in the peptide degrons necessary for San1-mediated degradation, we found ≥5 contiguous hydrophobic residues were required in the peptide for recognition by San1 and ≤4 contiguous hydrophobic residues were insufficient (27 and Figure 1A). Intrigued by this sharp delineation, we wanted to understand the biochemical nature of this limit.

Because insolubility and aggregate formation are detrimental results of protein misfolding, we examined the in vivo solubility of GFP<sup>NLS</sup>-peptide fusions containing 3, 4, 5, 6, or 7 contiguous hydrophobic residues. We found that the threshold for San1-mediated degradation – ≥5 contiguous hydrophobic residues – correlated with the GFP<sup>NLS</sup>-peptide fusions becoming highly insoluble (Figure 1B). The GFP<sup>NLS</sup>-peptide fusions containing ≤4 contiguous hydrophobic residues were largely soluble (Figure 1B).

The GFP<sup>NLS</sup>-peptide fusion proteins present in the insoluble fraction are likely part of large inclusions as this fraction represents the cellular debris that pellets at 12,800 g. Therefore, we also examined if the GFP<sup>NLS</sup>-peptide fusions in the soluble fraction formed high molecular weight species typical of aggregates using nondenaturing polyacrylamide gel electrophoresis (32,33). Similar to the solubility assays, we found that the GFP<sup>NLS</sup>-peptide fusions with ≥5 contiguous hydrophobic residues formed mostly higher molecular weight species compared with the GFP<sup>NLS</sup>-peptide fusions with ≤4 contiguous hydrophobic residues that ran primarily as monomers (Figure 1C, left panel). These higher molecular weight species were sensitive to addition of SDS to the loading buffer (Figure 1C, right panel), indicating that they are likely not SDS-insensitive amyloids (34).

Lastly, we examined if the GFP<sup>NLS</sup>-peptide fusions formed visible cellular inclusions in vivo. Using fluorescence microscopy, we found that the GFP<sup>NLS</sup>-peptide fusions with ≥5 contiguous hydrophobic residues were predominantly nuclear localized and formed a single visible inclusion in the nucleus (Figure 1D), indicating that they do aggregate in vivo. By contrast, the GFP<sup>NLS</sup>-peptide fusions with ≤4 contiguous hydrophobic residues showed more uniform localization throughout the cell, though there was some enrichment in the nucleous (Figure 1D). Furthermore, there was no inclusion formation in cells expressing the ≤4 contiguous hydrophobic residue GFP<sup>NLS</sup>-peptide fusions (Figure 1D), which is consistent with their solubility and lack of aggregate formation (Figure 1B & C). The difference in overall cellular localization between the GFP<sup>NLS</sup>-peptide fusions with ≥5 contiguous hydrophobic residues, which
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primarily localize to the nucleus, and the GFP\textsuperscript{NLS} peptide fusions with ≤4 contiguous hydrophobic residues, which localize throughout the cytoplasm and nucleus, is likely due to the aggregated versus monomeric state of the GFP\textsuperscript{NLS}-peptide fusion in relation to the passive diffusion limit of the yeast nuclear pore. The GFP\textsuperscript{NLS}-peptide fusions are ~30 kDa in size and thus have a monomeric molecular weight below the passive <40kDa diffusion limit of the yeast nuclear pore (35,36). Although the GFP\textsuperscript{NLS}-peptide fusions with ≥5 contiguous residues share the same monomeric molecular weight as those with ≤4 contiguous residues, the formation of an aggregate with ≥2 molecules in the nucleus would render an aggregate above the size of the nuclear pore passive diffusion limit. Thus, all the GFP\textsuperscript{NLS}-peptide fusions can be trafficked into the nucleus via classic NLS-dependent import, but the GFP\textsuperscript{NLS}-peptide fusions with ≥5 contiguous hydrophobic residues cannot passively diffuse back into the cytoplasm as can the GFP\textsuperscript{NLS}-peptide fusions with ≤4 contiguous hydrophobic residues.

Altogether from the combined solubility, nondenaturing polyacrylamide gel electrophoresis, and microscopy results, it appears San1 recognizes exposed hydrophobicity that crosses a particular threshold of insolubility. This threshold coincides with aggregation and inclusion formation in the nucleus. This intriguing result prompted further experiments to dissect the parameters of the hydrophobicity nature of a San1 substrate.

Unexpected selectivity of San1 for the types of hydrophobic residues recognized in a 5-residue window—Hydrophobicity is a general term that encompasses multiple amino acids. Algorithms have been developed that score amino acid residues either on their hydrophobicity, such as the well-known Kyte-Doolittle hydropathy algorithm (37), or their propensity to occur in protein regions prone to aggregation, such as the AGGRESCAN algorithm (38). In Figure 2A and B, we have ranked the different hydrophobicity and aggregation propensity scores for each amino acid residue according to Kyte-Doolittle hydropathy or AGGRESCAN measures.

Because 5 contiguous hydrophobic residues is the minimal feature recognized by San1 (Figure 1), we wanted to examine the ability of homopentameric amino acid stretches to function as San1 degrons. Our initial prediction was that homopentameric stretches of the most hydrophobic residues – Ile, Val, Leu, and Phe – would allow San1-mediated degradation, and there might be variability with homopentameric stretches of lesser hydrophobic residues – Tyr, Trp, Met, and Ala. In actuality, the results differed unexpectedly from our predictions. When we examined the degradation of each homopentameric GFP\textsuperscript{NLS}-peptide fusion, we found three distinct groups. The first group consisted of 5Phe and 5Ile stretches, which were degraded primarily in a San1-dependent manner as we predicted (Figure 2C). The second group was composed of 5Leu, 5Val, and 5Ala stretches, which were degraded, but in a primarily San1-independent manner (Figure 2D). This was surprising as we had anticipated that the 5Leu and 5Val stretches, which are highly hydrophobic by both scoring measures (Figure 2A & B), would cause San1-mediated degradation similar to the 5Ile and 5Phe stretches. The final group encompassed 5Tyr, 5Met, and 5Trp stretches, which were stable (Figure 2E).

The correlation between San1-mediated degradation and the insolubility of the original GFP\textsuperscript{NLS}-peptide fusions (Figure 1B) prompted us to examine the insolubility of each homopentameric GFP\textsuperscript{NLS}-peptide fusion. As expected from their San1-mediated degradation, the 5Ile and 5Phe GFP\textsuperscript{NLS}-peptide fusions showed the highest levels of insolubility (Figure 2C). Also as expected from their complete stability, the 5Tyr, 5Met, and 5Trp GFP\textsuperscript{NLS}-peptide fusions were primarily soluble (Figure 2E). Interestingly, the 5Leu, 5Val, and 5Ala GFP\textsuperscript{NLS}-peptide fusions showed a range of solubilities from complete or near complete solubility (5Ala and 5Val) to insolubility close to that observed with 5Ile (5Leu) (Figure 2D).

Given the variability in the insolubility of the homopentameric GFP\textsuperscript{NLS}-peptide fusions, we further characterized the fusions using fluorescence microscopy because this parameter separated San1 substrates from nonsubstrates by the propensity for \textit{in vivo} inclusion formation. As expected from their San1-dependent degradation and large degree of insolubility, the 5Ile and 5Phe GFP\textsuperscript{NLS}-peptide fusions were predominantly nuclear and formed a single nuclear inclusion (Figure 3). In contrast, every other homopentameric GFP\textsuperscript{NLS}-peptide fusion localized throughout the nucleus and cytoplasm and did not form inclusions (Figure 3). Overall, these results again demonstrated that San1-dependent degradation generally correlates with the degree of insolubility and formation of inclusions \textit{in vivo}. However, the results were surprising if the
conventional hydrophobicity score of a residue was the sole initial consideration.

Degradation of the GFP<sup>NLS</sup>-5Val peptide does not occur via Ubr1 or Doa10–The San1-independent degradation of the 5Leu, 5Val, and 5Ala GFP<sup>NLS</sup>-peptide fusions indicated that there is another degradation pathway that likely recognizes a lower threshold of insolubility than San1. Because the 5Val GFP<sup>NLS</sup>-peptide fusion was degraded the most rapidly, we used it to explore some of the features of the San1-independent pathway(s). First, we examined if the San1-independent degradation of the 5Val GFP<sup>NLS</sup>-peptide fusion required the proteasome. Addition of the proteasome inhibitor MG132 significantly stabilized the 5Val GFP<sup>NLS</sup>-peptide fusion (Figure 4A), indicating that the bulk of its degradation was through the proteasome.

It was previously shown that some San1 substrates are also targeted by the ubiquitin-protein ligase Ubr1 (10,39). In addition, the ubiquitin-protein ligase Doa10 is localized to the nuclear membrane in part and functions in the degradation of nuclear proteins (21,40). Therefore, we tested if either of these ubiquitin-protein ligases were involved in the proteasome degradation of the 5Val GFP<sup>NLS</sup>-peptide fusion. Double deletion of both <i>UBR1</i> and <i>DOA10</i> had a very slight stabilizing effect on the degradation of the 5Val GFP<sup>NLS</sup>-peptide fusion (Figure 4B), indicating that they are not the major degradative pathways. Deletion of <i>SAN1</i> in <i>ubr1Δdoa10Δ</i> cells had an additional modest stabilizing effect (Figure 4B), revealing that San1 does recognize the 5Val GFP<sup>NLS</sup>-peptide fusion to a minor degree. Because the major known cytoplasmic and nuclear PQC degradation pathways do not appreciably target the 5Val GFP<sup>NLS</sup>-peptide fusion, it appears that another, as-yet-unknown, proteasome-dependent PQC degradation pathway exists and operates at a lower insolubility threshold than San1.

Substitution of a single Ile residue in the 5Val GFP<sup>NLS</sup>-peptide fusion causes San1-dependent degradation in a position-dependent manner–Because the 5Val GFP<sup>NLS</sup>-peptide fusion was very modestly stabilized after deletion of <i>SAN1</i> in <i>ubr1Δdoa10Δ</i> cells, we reasoned that, if we increased the hydrophobicity of the 5Val stretch, then we might be able to shift the degradation of the GFP<sup>NLS</sup>-peptide fusion towards increased San1 dependency. To do this, we replaced one Val within the homopentameric stretch with an Ile to create VVVIV, VVIVV, and VIVVV stretches fused to GFP<sup>NLS</sup>. As we anticipated, the VVVIV stretch resulted in the GFP<sup>NLS</sup>-peptide fusion being subject to primarily San1-dependent degradation (Figure 5A & C). Surprisingly, however, the closer the Ile substitution was to the hydrophilic portion of the peptide fusion, the less effect it had on converting the degradation of the GFP<sup>NLS</sup>-peptide fusion to San1 dependency (Figure 5A & C). The San1 dependency of degradation did correlate with the insolubility of the GFP<sup>NLS</sup>-peptide fusions containing the single Ile (Figure 5B), indicating the rules of hydrophobicity/insolubility still applied. From these results, we suspected that the hydrophilic linker sequence positioned near the hydrophobic residue stretch could influence the strength of the hydrophobic residues and mitigate the effect of hydrophobicity, which would alter San1’s ability to recognize its substrates. In support of this hypothesis, flanking sequences to aggregation-prone regions have been shown to influence protein aggregation (41-47).

To test further the idea that flanking hydrophobic residues can affect San1 recognition, we focused on the 5Ile and 5Phe GFP<sup>NLS</sup>-peptide fusions, which undergo predominantly San1-dependent degradation (Figure 2C). To increase the flanking hydrophilicity to its maximum potential, we substituted the charged residue Arg into the position immediately proximal to the 5Ile or 5Phe stretches in their respective GFP<sup>NLS</sup>-peptide fusions (Figure 6). We chose to add an Arg residue because it is the most hydrophilic by the Kyte-Doolittle hydrophobicity measure (Figure 2A), and thus should have the best capacity to reveal if increasing hydrophilicity alters San1-dependent degradation. Indeed, substitution of an Arg residue in the proximal position did increase the degradation rate of both the 5Ile and 5Phe GFP<sup>NLS</sup>-peptide fusions in <i>san1Δ</i> cells, and this was due to acquisition of partial San1-independent degradation observed in <i>san1Δ</i> cells (Figure 6). Altogether, we think the combined observations suggest that, not only must the insoluble nature of the exposed hydrophobicity be taken into account, but so too must the nature of flanking sequences.

Homoseptameric stretches show increased dependency on San1 for degradation–The results from substituting a single Ile in the 5Val stretch and addition of a single Arg before the 5Ile and 5Phe stretches suggested that the hydrophobicity of the homopentameric stretch is important but is likely also affected by adjacent residues. Accordingly, we surmised that increasing the homopentameric stretches to homoseptameric stretches – creating a greater hydrophobic stretch
adjacent to the hydrophilic sequence – might now lead to San1-dependent degradation of the 7Val and 7Leu GFP<sub>NLS</sub>-peptide fusions and possibly others. This was the case as we found that most homoseptameric stretches resulted in the acquisition of at least some San1-dependent degradation ranging from strong (7Tyr) to moderate (7Val, 7Leu) to weak (7Met) (Figure 7A). The exceptions were the 7Trp stretch, which remained stable, and the 7Ala stretch, which continued to be degraded in a San1-independent manner (Figure 7A). Comparison of the degradation rates between the homopentameric and the homoseptameric GFP<sub>NLS</sub>-peptide fusions in <em>SAN1</em> and <em>san1Δ</em> cells are plotted in Figure 7C. The change in San1 dependency in terms of degradation also prompted us to look at solubility of the homoseptameric GFP<sub>NLS</sub>-peptide fusions. We observed that the acquisition of non-dependent degradation also generally correlated with increased insolubility (Figure 7B). The observations suggest a hierarchy of hydrophobic residues in terms of their strength to cause insolubility and San1 recognition with Ile and Phe being the strongest, Tyr, Val, and Leu being the next strongest, and Met being the weakest.

**DISCUSSION**

PQC degradation systems preferentially target misfolded proteins over normally folded ones. That means they must recognize a feature of structural abnormality that differentiates the misfolded protein from its normal counterpart. Furthermore, because each cellular compartment typically possesses multiple PQC degradation systems that appear to be functionally distinct, each PQC system likely recognizes a unique feature of misfolding. Identifying what these features are for each PQC degradation system and how they differ is key to understanding their substrate selectivity.

In our initial examination of what San1 recognizes within its misfolded protein substrates, we discovered that San1 prefers a particular threshold of exposed hydrophobicity, which could be defined as the amount of hydrophobicity conferred by a window of ≥5 contiguous hydrophobic residues (27 and Figure 1A). Here, we have presented evidence that it is not simply exposed hydrophobicity, but that the hydrophobicity also correlates with a particular threshold of insolubility to allow San1 recognition. If we think about this in terms of substrate-binding affinity, San1’s substrate-binding mechanism likely requires a similar degree of hydrophobicity necessary for two hydrophobic sequences to associate strongly with each other to form an aggregate. Thus, the degree of San1 substrate insolubility serves as a proxy measure for how “strong” the exposed hydrophobicity must be in order to trigger San1 recognition. How San1 achieves substrate binding with the necessary selectivity and affinity for the appropriate hydrophobicity is not yet clear.

For simplicity, we can think of San1 operating within a particular spectrum of hydrophobicity that confers strong insolubility leading to aggregation (Figure 8). Protein aggregation is exceptionally deleterious in the nucleus (48), so San1’s selectivity makes sense from an evolutionary perspective of misfolded protein recognition. It would be physiologically advantageous for a robust nuclear PQC degradation system like San1 to target the types of misfolded proteins that are likely to be the most prone to insolubility and toxic aggregation. Supporting this, we found in an early study, in which we identified new San1 substrates using a 2-hybrid analysis, that 11 of 14 substrates destroyed predominantly in a San1-dependent manner were highly toxic in the absence of San1 (31).

It is important to note, we do not think San1 alone has the ability to target proteins once they aggregate. This thought is based on our previous studies examining San1’s ability to ubiquitinate denatured luciferase <em>in vitro</em> (31). We found that San1 was unable to ubiquitinate denatured luciferase if San1 was added after the denaturation step, but San1 was able to ubiquitinate denatured luciferase when San1 was added during the denaturation step. We interpreted this to mean that San1 can only recognize exposed hydrophobicity that is not buried within an aggregate. Based on this interpretation and our studies here, we don’t think San1 targets a certain type of aggregate but instead recognizes misfolded proteins that haven’t yet aggregated.

If San1 itself cannot recognize aggregated proteins, the question is how San1 mediates the degradation of highly insoluble proteins <em>in vivo</em> once they form aggregates? Previous studies have demonstrated that the Hsp70 chaperones Ssa1 and Ssa2 are required for the degradation of some San1 substrates <em>in vivo</em> (10,39,49), suggesting that the solubility and accessibility of San1 substrates could be maintained upstream of San1 by the action of protein chaperones. It is also important to understand how San1 substrates maintain their
solubility after ubiquitination by San1 because the proteasome has difficulty degrading aggregated proteins (50-52). We recently found that the maintenance of San1 substrate solubility upstream of the proteasome requires the AAA-ATPase Cdc48 (Gallagher P. and Gardner R., unpublished observations). Thus, disaggregation capabilities can be employed both upstream and downstream of San1 to maintain the solubility of highly aggregation-prone San1 substrates.

We do note that while hydrophobicity and insolubility above a particular threshold generally correlated with San1-mediated degradation, there were a few deviations. For example, the 5Leu GFPNLS-peptide fusion isn’t targeted by San1 but has insolubility similar to the 5Ile GFPNLS-peptide fusion (Figure 2). Also, the degradation of the 7Tyr GFPNLS-peptide fusion is primarily San1 dependent but it is more soluble than the 7Val and 7Leu GFPNLS-peptide fusions, which are subject to degradation that is less San1 dependent (Figure 7). The deviations from general insolubility could be due to the assay we used, which is a qualitative assay that cannot distinguish fine quantitative differences in solubility. In fact, we caution against comparing solubility/insolubility percentages between figures because of the day-to-day experimental variability in performing the assay. However, because the solubility assays for each figure were performed at the same time and run on the same gels, comparisons are valid within each figure. In addition to the qualitative nature of the solubility assay, there could be additional parameters to San1 recognition that we have yet to discover. Perhaps the shape of the residue side chains or the overall complexity of the residues in the hydrophobic window play a role in how San1 recognizes a substrate.

Our results also revealed that there is another potential nuclear PQC degradation pathway that appears to recognize a lower threshold of hydrophobicity and insolubility than San1. The pathway targets substrates for proteasome degradation, but it is not the previously described PQC degradation systems of Ubr1 or Doa10. That there might be two nuclear PQC degradation pathways operating in different ranges of exposed hydrophobicity/insolubility is intriguing in terms of how the cell might have evolved triage hierarchies for the repair and degradation of misfolded proteins (53,54). It could be that having separate PQC systems based on the degree of exposed hydrophobicity and insolubility allows the cell to repair misfolded proteins that haven’t exposed sufficient hydrophobicity to be in danger of rapid aggregation, yet quickly and robustly destroy those misfolded proteins that have. Identification and characterization of the additional nuclear PQC degradation pathway(s) will allow for in depth comparison of the targeting capabilities with San1.

REFERENCES

1. Hartl, F. U., Bracher, A., and Hayer-Hartl, M. (2011) Molecular chaperones in protein folding and proteostasis. Nature 475, 324-332
2. Wang, S. S., Wu, J. W., Yamamoto, S., and Liu, H. S. (2008) Diseases of protein aggregation and the hunt for potential pharmacological agents. Biotechnol. J. 3, 165-192
3. Fredrickson, E. K., and Gardner, R. G. (2012) Selective destruction of abnormal proteins by ubiquitin-mediated protein quality control degradation. Semin. Cell. Dev. Biol. 23, 530-537
4. Bordallo, J., Plemper, R. K., Finger, A., and Wolf, D. H. (1998) Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded luminal and integral membrane proteins. Mol. Biol. Cell. 9, 209-222
5. Fang, S., Ferrone, M., Yang, C., Jensen, J. P., Tiwari, S., and Weissman, A. M. (2001) The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. Proc. Natl. Acad. Sci. U.S.A. 98, 14422-14427
6. Hampton, R. Y., Gardner, R. G., and Rine, J. (1996) Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Mol. Biol. Cell 7, 2029-2044
7. Kaneko, M., Ishiguro, M., Niinuma, Y., Uesugi, M., and Nomura, Y. (2002) Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. FEBS Lett. 532, 147-152
8. Nadav, E., Shmueli, A., Barr, H., Gonen, H., Ciechanover, A., and Reiss, Y. (2003) A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. Biochem. Biophys. Res. Commun. 303, 91-97
9. Swanson, R., Locher, M., and Hochstrasser, M. (2001) A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes Dev. 15, 2660-2674
10. Heck, J. W., Cheung, S. K., and Hampton, R. Y. (2010) Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1. Proc. Natl. Acad. Sci. U.S.A. 107, 1106-1111
11. Jiang, J., Ballinger, C. A., Wu, Y., Dai, Q., Cyr, D. M., Hohfeld, J., and Patterson, C. (2001) CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. J. Biol. Chem. 276, 42938-42944
12. Murata, S., Minami, Y., Minami, M., Chiba, T., and Tanaka, K. (2001) CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep. 2, 1133-1138
13. Nillegoda, N. B., Theodoraki, M. A., Mandal, A. K., Mayo, K. J., Ren, H. Y., Sultana, R., Wu, K., Johnson, J., Cyr, D. M., and Caplan, A. J. (2010) Ubr1 and Ubr2 Function in a Quality Control Pathway for Degradation of Unfolded Cytosolic Proteins. Mol. Biol. Cell 21, 2102-2116
14. Fu, L., Gao, Y. S., Tousson, A., Shah, A., Chen, T. L., Vertel, B. M., and Sztul, E. (2005) Nuclear aggresomes form by fusion of PML-associated aggregates. Mol. Biol. Cell 16, 4905-4917
15. Gardner, R. G., Nelson, Z. W., and Gottschling, D. E. (2005) Degradation-mediated protein quality control in the nucleus. Cell 120, 803-815
16. Iwata, A., Nagashima, Y., Matsumoto, L., Suzuki, T., Yamanaka, T., Date, H., Deoka, K., Nukina, N., and Tsuji, S. (2009) Intra-nuclear degradation of polyglutamine aggregates by the ubiquitin proteasome system. J. Biol. Chem. 284, 9796-9803
17. Janer, A., Martin, E., Muriel, M. P., Latouche, M., Fujigasaki, H., Ruberg, M., Brice, A., Trottier, Y., and Sittler, A. (2006) PML clastosomes prevent nuclear accumulation of mutant ataxin-7 and other polyglutamine proteins. J. Cell Biol. 174, 65-76
18. Wang, Z., and Prelich, G. (2009) Quality control of a transcriptional regulator by SUMO-targeted degradation. Mol. Cell. Biol. 29, 1694-1706
19. Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A., and Hampton, R. Y. (2001) Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nat. Cell Biol. 3, 24-29
20. Huyer, G., Piluek, W. F., Fansler, Z., Kreft, S. G., Hochstrasser, M., Brodsky, J. L., and Michaelis, S. (2004) Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispansing membrane protein and a soluble luminal protein. J. Biol. Chem. 279, 38369-38378
21. Deng, M., and Hochstrasser, M. (2006) Spatially regulated ubiquitin ligation by an ER/nuclear membrane ligase. Nature 443, 827-831
22. Hill, K., and Cooper, A. A. (2000) Degradation of unassembled Vph1p reveals novel aspects of the yeast ER quality control system. Embo J. 19, 550-561
23. Wilhovsky, S., Gardner, R., and Hampton, R. (2000) HRD gene dependence of endoplasmic reticulum-associated degradation. Mol. Biol. Cell 11, 1697-1708
24. Taxis, C., Hitt, R., Park, S. H., Deak, P. M., Kostova, Z., and Wolf, D. H. (2003) Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. J. Biol. Chem. 278, 35903-35913
25. Carvalho, P., Gober, V., and Rapoport, T. A. (2006) Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. Cell 126, 361-373
26. Kanehara, K., Xie, W., and Ng, D. T. (2010) Modularity of the Hrd1 ERAD complex underlies its diverse client range. J. Cell Biol. 188, 707-716
27. Fredrickson, E. K., Rosenbaum, J. C., Locke, M. N., Milac, T. I., and Gardner, R. G. (2011) Exposed hydrophobicity is a key determinant of nuclear quality control degradation. Mol. Biol. Cell 22, 2384-2395
28. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115-132
29. Guthrie, C., and Fink, G. R. (1991) Guide to yeast genetics and molecular biology. Methods Enzymol. 194, 1-863
30. Theodoraki, M. A., Nillegoda, N. B., Saini, J., and Caplan, A. J. (2012) A network of ubiquitin ligases is important for the dynamics of misfolded protein aggregates in yeast. J. Biol. Chem. 287, 23911-23922
31. Rosenbaum, J. C., Fredrickson, E. K., Oeser, M. L., Garrett-Engele, C. M., Locke, M. N., Richardson, L. A., Nelson, Z. W., Hetrick, E. D., Milac, T. I., Gottschling, D. E., and Gardner, R. G. (2011) Disorder targets disorder in nuclear quality control degradation: a disordered ubiquitin ligase directly recognizes its misfolded substrates. *Mol. Cell* **41**, 93-106

32. Lefebvre, B. G., Comolli, N. K., Gage, M. J., and Robinson, A. S. (2004) Pressure dissociation studies provide insight into oligomerization competence of temperature-sensitive folding mutants of P22 tailspike. *Protein Sci.* **13**, 1538-1546

33. Jung, C. H., Na, Y. R., and Im, H. (2004) Retarded protein folding of deficient human alpha 1-antitrypsin D256V and L414 variants. *Protein Sci.* **13**, 694-702

34. Kryndushkin, D. S., Alexandrov, I. M., Ter-Avanesyan, M. D., and Kushnirov, V. V. (2003) Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. *J. Biol. Chem.* **278**, 49636-49643

35. Shulga, N., and Goldfarb, D. S. (2003) Binding dynamics of structural nucleoporins govern nuclear pore complex permeability and may mediate channel gating. *Mol. Cell. Biol.* **23**, 534-542

36. Shulga, N., Mosammaparast, N., Wozniak, R., and Goldfarb, D. S. (2000) Yeast nucleoporins involved in passive nuclear envelope permeability. *J. Cell Biol.* **149**, 1027-1038

37. Kyte, J., and Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132

38. Conchillo-Sole, O., de Groot, N. S., Aviles, F. X., Vendrell, J., Daura, X., and Ventura, S. (2007) AGGRESCAN: a server for the prediction and evaluation of "hot spots" of aggregation in polypeptides. *BMC Bioinformatics* **8**, 65

39. Prasad, R., Kawaguchi, S., and Ng, D. T. (2010) A nucleus-based quality control mechanism for cytosolic proteins. *Mol. Cell Biol.* **21**, 2117-2127

40. Furth, N., Gertman, O., Shiber, A., Alfassy, O. S., Cohen, I., Rosenberg, M. M., Doron, N. K., Friedler, A., and Ravid, T. (2011) Exposure of bipartite hydrophobic signal triggers nuclear quality control of Ndc10 at the endoplasmic reticulum/nuclear envelope. *Mol. Cell Biol.* **22**, 4726-4739

41. Angeli, S., Shao, J., and Diamond, M. I. (2010) F-actin binding regions on the androgen receptor and huntingtin increase aggregation and alter aggregate characteristics. *PLoS One* **5**, e9053

42. Beert, J., Jonckheere, W., Rudyak, S., Xu, J., Wilkinson, H., De Smet, F., Schymkowitz, J., and Rousseau, F. (2012) Aggregation gatekeepers modulate protein homeostasis of aggregating sequences and affect bacterial fitness. *Protein Eng. Des. Sel.* **25**, 357-366

43. Duennwald, M. L., Jagadish, S., Muchowski, P. J., and Lindquist, S. (2006) Flanking sequences profoundly alter polyglutamine toxicity in yeast. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 11045-11050

44. Ignatova, Z., Thakur, A. K., Wetzel, R., and Gierasch, L. M. (2007) In-cell aggregation of a polyglutamine-containing chimera is a multistep process initiated by the flanking sequence. *J. Biol. Chem.* **282**, 36736-36743

45. Lakhani, V. V., Ding, F., and Dokholyan, N. V. (2010) Polyglutamine induced misfolding of huntingtin exon1 is modulated by the flanking sequences. *PLoS Comput. Biol.* **6**, e1000772

46. Robertson, A. L., Horne, J., Ellisdon, A., and Bottomley, S. P. (2008) The structural impact of a polyglutamine tract is location-dependent. *Biophys. J.* **95**, 5922-5930

47. Siwach, P., Sengupta, S., Parihar, R., and Ganesh, S. (2011) Proline repeats, in cis- and trans-positions, confer protection against the toxicity of misfolded proteins in a mammalian cellular model. *Neurosci. Res.* **70**, 435-441

48. Woulfe, J. M. (2007) Abnormalities of the nucleus and nuclear inclusions in neurodegenerative disease: a work in progress. *Neuropathol. Appl. Neurobiol.* **33**, 2-42

49. Prasad, R., Kawaguchi, S., and Ng, D. T. (2012) Biosynthetic mode can determine the mechanism of protein quality control. *Biochem. Biophys. Res. Commun.* **425**, 689-695

50. Holmberg, C. I., Staniszewski, K. E., Mensah, K. N., Matouschek, A., and Morimoto, R. I. (2004) Inefficient degradation of truncated polyglutamine proteins by the proteasome. *Embo J.* **23**, 4307-4318

51. Snyder, H., Mensah, K., Theisler, C., Lee, J., Matouschek, A., and Wolozin, B. (2003) Aggregated and monomeric alpha-synuclein bind to the S6' proteasomal protein and inhibit proteasomal function. *J. Biol. Chem.* **278**, 11753-11759

52. Verhoef, L. G., Lindsten, K., Masucci, M. G., and Dantuma, N. P. (2002) Aggregate formation
inhibits proteasomal degradation of polyglutamine proteins. *Hum. Mol. Genet.* **11**, 2689-2700

53. McClellan, A. J., Tam, S., Kaganovich, D., and Frydman, J. (2005) Protein quality control: chaperones culling corrupt conformations. *Nat. Cell Biol.* **7**, 736-741

54. Wickner, S., Maurizi, M. R., and Gottesman, S. (1999) Posttranslational quality control: folding, refolding, and degrading proteins. *Science* **286**, 1888-1893

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FOOTNOTES

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To whom correspondence should be addressed: Richard Gardner, Department of Pharmacology, University of Washington, Box 357280, 1959 NE Pacific Street, Seattle, WA, 98195, USA, Tel.: (206) 685-0506; Fax: (206) 685-3822; E-mail: gardnerr@uw.edu

†The abbreviations used are: PQC, protein quality control; SDS, sodium dodecylsulfate; MOPS, 3-(N-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfone fluoride; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; DTT, dithiothreitol; NLS, nuclear localization signal; NIH, National Institute of Health; NIA, National Institute on Aging; NIGMS, National Institute of General Medical Sciences

FIGURE LEGENDS

**FIGURE 1.** *San1-mediated degradation correlates with substrate insolubility.* A, Cycloheximide-chase degradation assays were performed to assess the stability of GFP<sup>NLS</sup>-peptide fusions containing 3-7 contiguous hydrophobic residues in the peptide degron. Stability was examined in cells with (*San1*) or without (*san1*) the *San1* gene intact. Time after cycloheximide addition is indicated above the blots. GFP<sup>NLS</sup>-peptide fusions in all Western blots were detected using anti-GFP antibodies. Decay curves for degradation assays were determined using ImageJ. B, Solubility assays were performed to determine how the indicated GFP<sup>NLS</sup>-peptide fusion partitions between the insoluble pellet fraction (I) and the soluble supernatant fraction (S) from *San1* cells. Total lysate (T) indicates the total amount of GFP<sup>NLS</sup>-peptide fusion in cell lysates. Note that all samples from the solubility assays were run on the same SDS-PAGE gel so that direct comparisons could be made. Percentage of protein in the insoluble versus soluble fractions is listed below each appropriate lane. Relative levels of the insoluble and soluble fractions were determined using ImageJ. C, Nondenaturing polyacrylamide gel electrophoresis of GFP<sup>NLS</sup>-peptide fusions containing 3-7 contiguous hydrophobic residues in the peptide degron. Soluble fractions were isolated as in (B) and run on a nondenaturing 8-16% Tris-Glycine gel without or with SDS in the loading buffer. D, Microscopy of *san1Δ* cells expressing GFP<sup>NLS</sup>-peptide fusions containing 3-7 contiguous hydrophobic residues in the peptide degron.

**FIGURE 2.** *Homopentameric stretches of hydrophobic residues confer different San1 dependencies.* A-B, Kyte-Doolittle hydrophobicity and AGGRESCAN aggregation propensity scores for the indicated amino acid. C-E, Cycloheximide-chase degradation assays were performed to assess the stability of GFP<sup>NLS</sup>-peptide fusions containing homopentameric stretches of hydrophobic residues in the peptide degron. Stability was examined in cells with (*San1*) or without (*san1Δ*) the *San1* gene intact. Time after cycloheximide addition is indicated above the blots. GFP<sup>NLS</sup>-peptide fusions in the Western blots were detected using anti-GFP antibodies. Solubility assays were performed to determine how the indicated GFP<sup>NLS</sup>-peptide fusion partition between the insoluble pellet fraction (I) and the soluble supernatant fraction (S). Total lysate (T) indicates the total amount of GFP<sup>NLS</sup>-peptide fusion in cell lysates. Percentage of protein in the insoluble versus soluble fractions is listed below each appropriate lane. Relative levels of the insoluble and soluble fractions was determined using ImageJ.
A, appropriate lane. Relative levels of the insoluble and soluble fractions was determined using ImageJ. Another unknown PQC degradation pathway that recognizes the 5Val GFP gene intact. Time after cycloheximide addition is indicated above the blots. GFP

Decay curves for degradation assays were performed to assess the stability of GFP

Substitution of a single Arg residue immediately proximal to the 5Val GFP

Percentage of protein in the insoluble versus soluble fractions is listed below each appropriate lane. Relative levels of the insoluble and soluble fractions was determined using ImageJ. Decay curves for degradation assays were determined using ImageJ.

Substitution of a single Ile residue in the 5Val GFP

Decay curves for degradation data in (A) were determined using ImageJ.

Homoseptameric stretches show increased dependency on San1 for degradation. A, Cycloheximide-chase degradation assays were performed to assess the stability of GFP

Amount of protein remaining at each time point of the degradation assays in (A) and Figure 2 (B-D) was determined using ImageJ.

Model for San1 substrate recognition. The spectrum of protein solubility is represented with exposed hydrophobicity mapped to increasing insolubility. San1 appears to target misfolded proteins on the far end of the insolubility spectrum. Another unknown PQC degradation pathway that recognizes less exposed hydrophobicity and insolubility than San1 is noted.
TABLE 1
Plasmids used in this study
GFP, green fluorescent protein; NLS, nuclear localization signal.

| Plasmid       | Figure(s) | Plasmid description                                    |
|---------------|-----------|--------------------------------------------------------|
| pRG3789       | Figure 1  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTFVLYIII, LEU2, 2 micron} \) |
| pRG3790       | Figure 1  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSVLYIII, LEU2, 2 micron} \) |
| pRG3791       | Figure 1  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTVLYIII, LEU2, 2 micron} \) |
| pRG3792       | Figure 1  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTSIVIII, LEU2, 2 micron} \) |
| pRG3793       | Figure 1  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIII, LEU2, 2 micron} \) |
| pRG3784       | Figure 2  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTYVIII, LEU2, 2 micron} \) |
| pRG3785       | Figures 2 & 3 | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIII, LEU2, 2 micron} \) |
| pRG3786       | Figures 2 & 3 | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTVIVIII, LEU2, 2 micron} \) |
| pRG3787       | Figures 2 & 3 | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTVVIIV, LEU2, 2 micron} \) |
| pRG3788       | Figures 2 & 3 | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTVIIIV, LEU2, 2 micron} \) |
| pRG3436       | Figures 2, 3, 5, & 6 | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIII, LEU2, 2 micron} \) |
| pRG3437       | Figures 2 & 3 | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTWWWW, LEU2, 2 micron} \) |
| pRG3438       | Figures 2 & 3 | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTVVV, LEU2, 2 micron} \) |
| pRG3545       | Figures 2 & 3 | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIV, LEU2, 2 micron} \) |
| pRG3650       | Figure 5  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVVIV, LEU2, 2 micron} \) |
| pRG3785       | Figure 5  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVVIV, LEU2, 2 micron} \) |
| pRG3786       | Figure 5  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVVIV, LEU2, 2 micron} \) |
| pRG3991       | Figure 6  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIIV, LEU2, 2 micron} \) |
| pRG3993       | Figure 6  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIIIV, LEU2, 2 micron} \) |
| pRG3610       | Figure 7  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVVIVV, LEU2, 2 micron} \) |
| pRG3611       | Figure 7  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIIVV, LEU2, 2 micron} \) |
| pRG3613       | Figure 7  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIIVV, LEU2, 2 micron} \) |
| pRG3619       | Figure 7  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIIVV, LEU2, 2 micron} \) |
| pRG3620       | Figure 7  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIIVV, LEU2, 2 micron} \) |
| pRG3621       | Figure 7  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIIVV, LEU2, 2 micron} \) |
| pRG3622       | Figure 7  | \( \text{p}_{\text{GAL1}}^{\text{GFP}^{\text{NLS}}} \cdot \text{TSTSTTVIIVV, LEU2, 2 micron} \) |
Hydrophobicity and insolubility in San1-mediated degradation

Figure 1

A

Time (hrs):
TSTFVLYIII
"7mer"
SAN1 san1Δ
0 1 3 0 1 3

TSTSVLYIII
"6mer"

TSTSTLYIII
"5mer"

TSTSTSYYIII
"4mer"

TSTSTTIII
"3mer"

% remaining

0 1 3

3mer 4mer 5mer 6mer 7mer

B

% remaining

0 1 3

3mer 4mer 5mer 6mer 7mer

C

# of contiguous hydrophobic residues

7mer

no SDS

plus SDS

kDa

D

7mer DNA nuclear membrane overlay 6mer DNA
nuclear membrane overlay

5mer DNA nuclear membrane overlay

4mer DNA nuclear membrane overlay

3mer DNA nuclear membrane overlay
**Figure 2**

| A | Kyte-Doolittle hydrophobicity score | B | AGGREGSCAN aggregation propensity |
|---|-----------------------------------|---|----------------------------------|
| I | 4.5 | I | 1.822 |
| V | 4.2 | F | 1.754 |
| L | 3.8 | V | 1.594 |
| F | 2.8 | L | 1.38 |
| C | 2.5 | Y | 1.159 |
| M | 1.9 | W | 1.037 |
| A | 1.8 | M | 0.91 |
| G | -0.4 | C | 0.604 |
| T | -0.7 | A | -0.036 |
| S | -0.8 | T | -0.159 |
| W | -0.9 | S | -0.294 |
| Y | -1.3 | P | -0.334 |
| P | -1.6 | G | -0.535 |
| H | -3.2 | K | -0.931 |
| E | -3.5 | H | -1.033 |
| Q | -3.5 | Q | -1.231 |
| D | -3.5 | R | -1.240 |
| N | -3.5 | N | -1.302 |
| K | -3.9 | E | -1.412 |
| R | -4.5 | D | -1.836 |

**C**

| SAN1 | san1Δ |
|------|-------|
| T | I | S |
| 0 | 1 | 3 |
| TSTTYVILL | TSTTFHHFF | TSTTIIII |
| 45 | 55 | 1 |
| 43 | 57 | 3 |

**D**

| SAN1 | san1Δ |
|------|-------|
| T | I | S |
| 0 | 1 | 3 |
| TSTLLLLL | TSTVWWV | TSTAAAA |
| 34 | 66 | 0 |
| 10 | 90 | 0 |
| 100 | 0 | 0 |

**E**

| SAN1 | san1Δ |
|------|-------|
| T | I | S |
| 0 | 1 | 3 |
| TSTYYYYY | TSTMMMMM | TSTMMMM |
| 0 | 100 | 0 |
| 100 | 0 | 0 |
| 95 | 5 | 95 |
Figure 3
Figure 4
Figure 5

Panel A: Western blots showing the degradation of different peptides over time. The peptides are listed as follows: TSTSTVVVV, TSTSTIIIII, TSTSTVVVIV, TSTSTVVVV, TSTSTIVVV. The time points for each peptide are indicated as 0, 1, and 3 hours.

Panel B: Graph showing the percentage of degradation over time for the peptides in Panel A. The legend includes symbols for different peptides, such as VVVV, IIII, VIVV, and VVIV.

Panel C: Graph showing the degradation curve for the peptides in Panel A, with the x-axis representing time in hours and the y-axis representing the percentage of degradation.
Figure 6

[Image of a diagram showing the degradation of different proteins over time with bars representing the percentage remaining at different time points (0, 1, 3 hours) for both SAN1 and san1Δ strains.]
Figure 7
Figure 8

Hydrophobicity and insolubility in San1-mediated degradation
Substrate recognition in nuclear protein quality control degradation is governed by exposed hydrophobicity that correlates with aggregation and insolubility
Eric K. Fredrickson, Pamela S. Gallagher, Sarah V. Clowes Candadai and Richard G. Gardner

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