⌘ *Author's Choice*

Sequestration of Sup35 by Aggregates of huntingtin Fragments Causes Toxicity of [*PSI*$^+$] Yeast*

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Background: Yeast have been used to study hungtingtin toxicity.
Results: Both HttQ103 and HttQP103 are toxic in yeast with [*PSI*$^+$] prion. This toxicity is markedly rescued by a Sup35 fragment.
Conclusion: Sequestration of the essential protein, Sup35, contributes to Htt toxicity in yeast.
Significance: This research demonstrates the complex nature of Htt toxicity.

Expression of huntingtin fragments with 103 glutamines (HttQ103) is toxic in yeast containing either the [*PIN*$^+$] prion, which is the amyloid form of Rnq1, or [*PSI*$^+$] prion, which is the amyloid form of Sup35. We find that HttQP103, which has a polyproline region at the C-terminal end of the polyQ repeat region, is significantly more toxic in [*PSI*$^+$] yeast than in [*PIN*$^+$], even though HttQP103 formed multiple aggregates in both [*PSI*$^+$] and [*PIN*$^+$] yeast. This toxicity was only observed in the strong [*PSI*$^+$] variant, not the weak [*PSI*$^+$] variant, which has more soluble Sup35 present than the strong variant. Furthermore, expression of the MC domains of Sup35, which retains the C-terminal domain of Sup35, but lacks the N-terminal prion domain, almost completely rescued HttQP103 toxicity, but was less effective in rescuing HttQ103 toxicity. Therefore, the toxicity of HttQP103 in yeast containing the [*PSI*$^+$] prion is primarily due to sequestration of the essential protein, Sup35.

Yeast has been used extensively as a model system to study human neurodegenerative diseases including Huntington disease, Parkinson disease, Alzheimer disease, amyotrophic lateral sclerosis, and mad cow disease (1). In all of these diseases, protein misfolding leads to the generation of amyloid plaque formation. One of the best characterized misfolding diseases is Huntington disease, which is caused by the misfolding of a fragment of huntingtin protein when these fragments have more than 35–40 glutamines in their polyQ repeat region. Although aggregate formation has been associated with neurotoxicity, several recent studies in mammalian cells have shown that it is the diffuse intracellular huntingtin (Htt)[3] fragments that are the toxic species, whereas the inclusion, referred to as an aggresome (2), is in fact neuroprotective (3, 4). The aggresome, a large aggregate containing misfolded proteins as well as intermediate filaments, is formed by the misfolded proteins traveling along the microtubules to the centrosome. As to the nature of the diffuse Htt species causing toxicity in mammalian cells, this has been attributed to both misfolded monomers and soluble oligomers (4–8). The oligomers form prior to the fibrils (9), which then are organized into the amyloid plaque.

Studies extending the Huntington disease model to yeast showed that the formation of Htt aggregates is dependent on the length of the polyQ repeat region in the expressed fragments, just as was observed in mammalian cells (10). However, unlike mammalian cells, neither a long polyQ repeat region nor the presence of visible oligomers is sufficient to cause toxicity in yeast even though toxicity only occurs when Htt with expanded polyQ repeat regions forms visible aggregates (10, 11). Instead, the aggregation of Htt fragments is generally dependent on the yeast having one of its many prion proteins in the amyloid conformation (10). So far 10 prions have been identified in yeast, the most common of them are [*PSI*$^+$], [*URE3*], and [*PIN*$^+$] (12). These yeast prions contain an asparagine/glutamine-rich domain, which is prone to misfold into an amyloid conformation. The propagation of this amyloid conformation requires the molecular chaperone, Hsp104; inactivation of Hsp104 activity cures yeast of the prion phenotype. In these cured cells, regardless of the length of their polyQ repeat regions, the expressed Htt fragments are non-toxic (10, 11).

When Htt fragments with an expanded polyQ repeat region are expressed in yeast containing a misfolded prion, they form either multiple aggregates or one large aggregate, recently identified as an aggresome (13). Multiple aggregates of Htt fragments in the yeast cytosol can be toxic whereas no Htt toxicity has been associated with the Htt aggresome (13), suggesting that the aggresome is protective, just as has been proposed for mammalian cells. Aggresomes only occur when the Htt fragments with an expanded polyQ repeat region also have a polyproline region downstream of the polyQ repeat region. However, not all Htt polyQ fragments with a polyproline region form an aggresome, which shows the importance of amino acids flanking the Htt fragment (14). Furthermore, even when

Htt fragments with expanded polyQ repeat regions form multiple aggregates, whether the fragment is toxic depends on the amino acids flanking the polyQ repeat region (14). For example, the HttQ103-GFP fragment without an epitope tag is not toxic, but the same fragment with an N-terminal FLAG-epitope is toxic. This latter fragment has been used extensively in many yeast studies to better characterize the toxic nature of Htt fragments with expanded polyQ repeat regions (13, 15–19).

The toxic effect of multiple Htt aggregates appears to be due to the sequestration of proteins that have a polyQ repeat region by the Htt aggregates (20). Among the proteins that are sequestered in Htt aggregates are endocytic proteins such as Pan1 and Sla1 (17, 18). Along with the sequestration of these proteins, Htt aggregates have been shown to cause a defect in endocytosis both in [PIN$^+$] and [PSI$^+$] yeast (17, 19). However, the relationship between the endocytosis defect and toxicity has not been clearly defined since many of the proteins that affect endocytosis in yeast also affect the actin cytoskeleton. Therefore, which protein or proteins are sequestered and why this causes toxicity is unclear.

In the present study, we examined in detail the effect of expressing Htt fragments in yeast with different [PSI$^+$] prion variants in an effort to determine how sequestration causes toxicity. Our results establish that both HttQP103 and HttQ103 are toxic in yeast that contain the strong [PSI$^+$] prion variant, but only HttQ103 is toxic in the weak [PSI$^+$] prion variant. Expression of a C-terminal fragment of Sup35 essential for Sup35 function markedly rescued HttQP103 toxicity, but was less effective in rescuing HttQ103 toxicity. These results establish that in [PSI$^+$] yeast, the sequestration of the translation termination factor, Sup35, is a major factor contributing to the toxicity of HttQP103. This is the first defined protein of sequestration by Htt fragments that has been shown to cause toxicity in yeast.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The yeast strains in this study (gift from Dr. Susan Liebman, University of Illinois) are derivatives of 74-D694 (*MATa, ade1-14 leu2-3,112 his3-Δ200 trp1-289 ura3-52*). They had the following prion phenotype: [*psi*$^-$]/[*pin*$^-$], weak [*PSI*$^+$]/[*PIN*$^+$], strong [*PSI*$^+$]/[*PIN*$^+$], and [*psi*$^-$]/high [*PIN*$^+$] (21). To express huntingtin fragments, yeast were transformed with the different Htt fragments (gift from Dr. Michael Sherman (Boston University School of Medicine) under the control of the *GAL1* promoter cloned in the pYES2 vector as described previously (13, 18). The different Htt fragments, HttQ25, HttQP25, HttQ103, and HttQP103, were expressed with a FLAG tag at its N terminus and a GFP at its C terminus. The MC fragment of Sup35 was expressed from a *LEU2*-based centromeric plasmid pJ528-MC (22), which is under the control of *SUP35* promoter (500 bp) and fused to *SUP35* terminator (200 bp of 3′-UTR). Sup45 was expressed from the *SUP45* promoter from a *TRP1*-based centromeric plasmid (gift from S. Liebman, University of Illinois). *RNQ1* was deleted in weak and strong [*PSI*$^+$] strains by using homolog recombination of a PCR-amplified *Kan*MX disruption cassette from a yeast deletion strain (ATCC). The Δ*rnq1* strain was verified by PCR and Western analysis using an anti-Rnq1 polyclonal antibody from Dr. E. Craig (data not shown).

Observation of Yeast Growth and Induction—Yeast were grown at 30 °C on synthetic medium (SD; 0.7% yeast nitrogen base, 2% glucose) with complete supplement mixture (CSM) or the appropriate amino acid dropout supplement mixture. Galactose medium contains both 2% galactose and 2% raffinose in place of glucose. Strains having 2 micron *URA3*-based empty vector or plasmids containing the galactose-inducible Htt fragments were grown in SD-Ura medium to mid-log phase and then washed out twice in synthetic galactose medium without uracil (SGal-Ura) to remove residual glucose. To determine toxicity using the spot assay, cells in SGal-Ura medium (A_{600} = 0.5) were serially diluted 5-fold. The serial dilutions (5 μl) were plated onto a SGal-Ura plate with limited adenine. In analyzing the growth of yeast that expressed both the MC fragment of Sup35 and Htt fragments, cells with serial dilution were plated onto SGal-Ura-Leu plate with limited adenine. Images of the plates were taken after incubation of the plates for 6 days at 30 °C. To determine growth, the same number of cells calculated from optical density was plated on duplicate SD selection plates. The colonies that grew on the selection plates were counted after incubation at 30 °C for 6 days. All growth curves were from 3 independent experiments and for each time point, the average value and standard deviation are plotted.

Staining of Cells with PI—Yeast were suspended in PBS at A_{600} = 0.2. This yeast suspension (300 μl) was mixed with 4 μl of a stock 1 mg/ml PI solution (Sigma). Following incubation at room temperature for 30 min, the yeast were either imaged by confocal microscopy or analyzed for fluorescence by FACs.

Confocal Microscopy—Zeiss LSM 510 confocal microscope with a 100× objective was used to acquire fluorescence, PI staining, and DIC images simultaneously. GFP-Htt and PI were imaged using the 488-nm laser and the 543-nm laser, respectively. Cells were imaged in eight-well 25-mm^2 chambered coverslips (Lab-Tek, Rochester) using the identical settings for all images. Cells were imaged 23 h after induction of expression of the Htt fragments by growing cells in SGal medium.

Western Blot Analysis—Cell lysis and Western blotting were performed as described previously with minor modifications (23). The following antibodies were used: a polyclonal rabbit anti-Sup35 antibody made in our laboratory and anti-Pgk1 monoclonal antibody (Molecular Probes, Carlsbad, CA). Rnq1 was probed using an anti-Rnq1 polyclonal antibody (gift from E. Craig, Univ. of Wisconsin). Odyssey dye800-conjugated goat and dye800-conjugated donkey anti-mouse antibody were used as secondary antibody. Odyssey scanner and software have been used for detection of bands and quantitation of the intensity of bands on the blots represented in figures.

FACS Analysis—Yeast were precultured in glucose selection medium overnight before they were transferred into galactose selection medium to induce HttQ103 and HttQP103 expression. After 22 h in galactose medium, cells were collected by centrifugation, washed once with PBS, and then stained with propodium iodide. FACS analysis on the BD FACS Calibur was used to determine the percent of propodium iodide-positive cells in the yeast population.
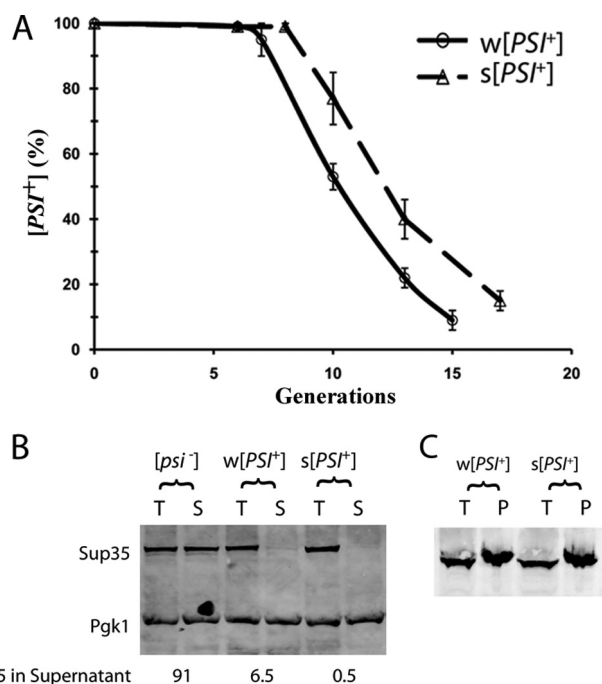
FIGURE 1. **Characterization of weak and strong [PSI⁺] variants.** *A*, curing of the weak (w) and strong (s) [PSI⁺] yeast variants by guanidine inactivation of Hsp104. Yeast were grown in SD with 5 mM guanidine and at the indicated generations, the extent of curing was determined by plating on 1/2YPD plates (0.5% yeast extract, 2% peptone, and 2% glucose, 2% agar). Colonies from each plate were counted after incubation at 30 °C for 4 days. [PSI⁺] cells yield white colonies on 1/2YPD plates, while cured cells ([psi⁻]) yield red colonies. The data are a compilation from three independent experiments. *B*, Western blot of the total and soluble Sup35 in yeast lysates. Yeast lysates were centrifuged at 100,000 rpm for 10 min in the Beckman TL-100 ultracentrifuge and the total lysates (*T*) and the supernatant fraction (*S*) were run on SDS gels. The prion phenotypes are as follows: *lanes 1 and 2*, [psi⁻]; *lanes 3 and 4*, weak [PSI⁺]; *lanes 5 and 6*, strong [PSI⁺]. Pgk1 was used as an internal loading control. The percent of the total Sup35 in the supernatant is given beneath the Western blot. *C*, Western blot of Sup35 showing the total Sup35 (*T*) and the resuspended pellet (*P*) following high speed centrifugation. The prion phenotypes are as follows *lanes 1 and 2*, weak [PSI⁺]; *lanes 3 and 4*, strong [PSI⁺].

RESULTS

Characterization of [PSI⁺] Variant—Prior to examining whether the weak and strong [PSI⁺] variants affect Htt toxicity, we first characterized the [PSI⁺] variants. It has been shown previously that the [PSI⁺] variants differ in their conformation so that there is greater severing of the prion seeds by Hsp104 in the strong [PSI⁺] variant than in the weak [PSI⁺] variant (24). This results in the strong [PSI⁺] variant having more seeds and less soluble Sup35 than the weak [PSI⁺] variant. In this study we initially examined the strong and weak [PSI⁺] variants that also contained the [PIN⁺] prion. First, to verify that the strong [PSI⁺] variant had a greater number of seeds than the weak variant, we measured the rate of curing of these variants when the yeast were treated with 5 mM guanidine to inactivate Hsp104. The curing curves in Fig. 1*A* shows that the strong [PSI⁺] variant took longer to cure than the weak [PSI⁺] variant. Specifically, there was a two generation difference between the variants when 40% of the cells were cured, and this difference in generation time became greater as the percentage of cured cells increased. Since the number of generations needed to cure [PSI⁺] is a measure of the number of prion seeds (25), these results confirm that there are more prion seeds present in the

strong than the weak [PSI⁺] variant, which in turn predicts that with fewer prion seeds, the weak [PSI⁺] variant should have more soluble Sup35 than the strong [PSI⁺] variant.
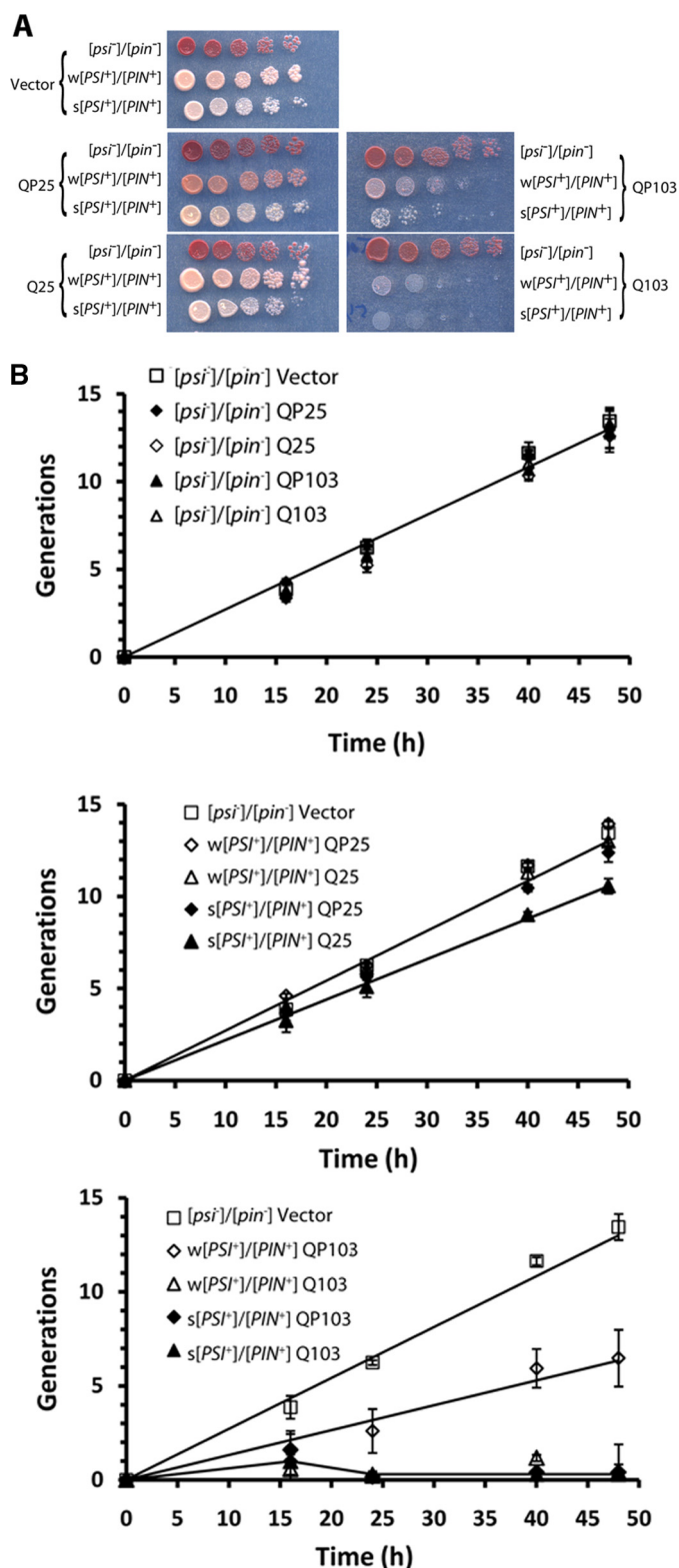
To test this prediction, we pelleted the misfolded Sup35 by high speed centrifugation of the yeast lysates. The soluble Sup35 present in the supernatants was then assayed using Western blot analysis. Fig. 1*B* shows that 5–7% of the total Sup35 remained in the supernatant of the weak [PSI⁺] yeast lysate, while <1% of the total Sup35 remained in the supernatant of the strong [PSI⁺] yeast lysate. Resuspension of the pellets showed that the Sup35 sedimented during the centrifugation (Fig. 1*C*). As expected, more than 90% of the total Sup35 remained in the supernatant of lysates from [psi⁻] yeast. Thus, the strong [PSI⁺] variant has more prion seeds and less soluble Sup35 than the weak [PSI⁺] variant.

Effect of Expressing Htt Fragments in the [PSI⁺]/[PIN⁺] Yeast— The effect of expressing Htt fragments on yeast growth was first examined in [PSI⁺]/[PIN⁺] yeast with either the weak or strong variant of the [PSI⁺] prion. HttQ25, HttQP25, HttQ103, and HttQP103 fragments were expressed under the control of the *GAL1* promoter. These fragments differ in the number of polyglutamines (25 *versus* 103) and the presence of a polyproline region at the C terminus. The effect of expressing these Htt fragments was examined using a spot assay in which the yeast were serially diluted on selection plates containing galactose to induce expression of the Htt fragments (Fig. 2*A*). The empty vector control shows that strong [PSI⁺]/[PIN⁺] yeast grew slightly slower than weak [PSI⁺]/[PIN⁺] yeast or yeast without prion. In agreement with previous studies, none of the Htt fragments caused toxicity in the absence of prion ([psi⁻]/[pin⁻]), nor did HttQP25 or HttQ25 cause toxicity in the presence of prion ([PSI⁺]/[PIN⁺]). In contrast, expression of HttQ103 and HttQP103 produced toxicity in both weak and strong [PSI⁺]/[PIN⁺] yeast. Comparison of the spot assays showed that HttQ103 was more toxic than HttQP103. Furthermore, while HttQ103 was highly toxic in both the weak and strong [PSI⁺] variants, HttQP103 was less toxic in the weak [PSI⁺] variant than in the strong [PSI⁺] variant.
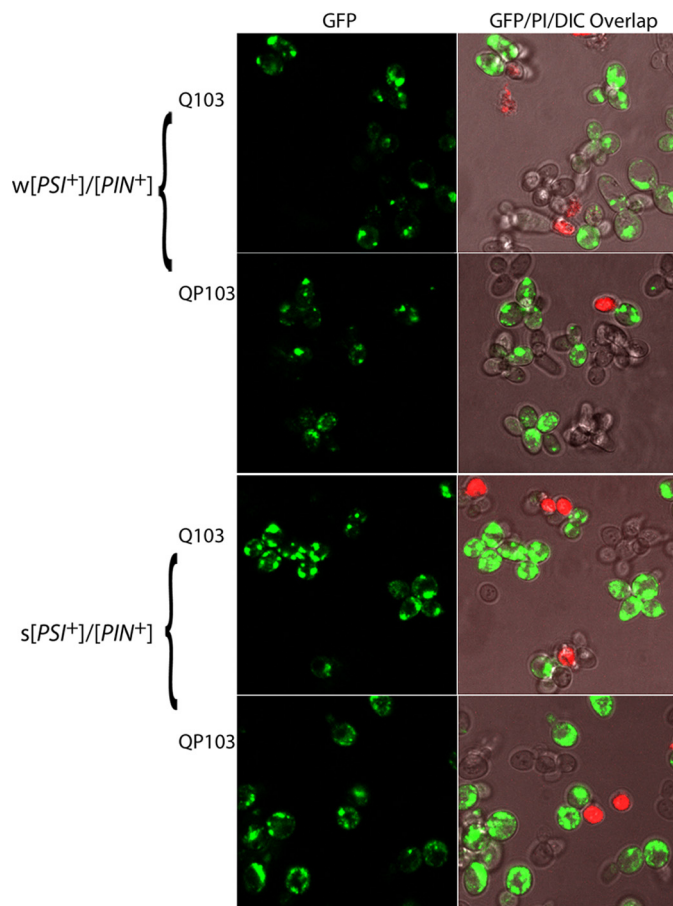
The dependence of Htt toxicity on the [PSI⁺] variant was confirmed by plating yeast grown in SGal-selection liquid medium, onto selection plates over a 48 h time period. Using colony count to measure growth, the data from the yeast expressing the different Htt fragments were plotted as generations *versus* time. Fig. 2*B* shows that the growth curves are in good agreement with the spot assays. None of the Htt fragments affected yeast growth in the absence of prion and expression of HttQP25 and HttQ25 had little, if any, effect on the growth of [PSI⁺]/[PIN⁺] yeast. Furthermore HttQP103 toxicity was much greater in yeast with the strong than the weak [PSI⁺] variant, while HttQ103 was highly toxic with both variants.

The growth data showed that the Htt toxicity was dependent on the [PSI⁺] variant and on the Htt fragment expressed. We therefore used confocal microscopy to examine the appearance of the GFP-labeled Htt fragments when expressed for 26 h in yeast with the different [PSI⁺] variants. As shown previously (18), all of the Htt fragments were diffusive in the prion free ([psi⁻]/[pin⁻]) cells (data not shown). In addition, HttQ25 and HttQP25 were diffusive in yeast regardless of the prion variant

FIGURE 3. **Confocal images of GFP-labeled Htt fragments expressed in either weak [PSI+]/[PIN+] or strong [PSI+]/[PIN+] yeast.** Images were obtained 26 h after induction of Htt expression. The *left panel* shows the GFP fluorescence, while the *right panel* shows an overlay of GFP fluorescence, PI staining, and DIC images of the yeast.

FIGURE 2. **Effect of Htt fragments expression on the growth of weak and strong [PSI+]/[PIN+] yeast.** Yeast without prion [psi−]/[pin−] were used as control. *A*, spot assay of yeast containing either empty vector plasmid or plasmid expressing the following Htt fragments: HttQ25, HttQP25, HttQ103, and HttQP103. Yeast were grown on SGal-Ura plate for 6 days at 30 °C. *B*, growth of yeast variants with empty vector or expressing different Htt fragments. Yeast grown in SGal-Ura liquid culture were plated on SD-Ura plates at indicated time points. The number of colonies on each plated was counted after 4 days at 30 °C.

(data not shown), in agreement with previous studies (11, 17). We next examined the expression of HttQ103 and HttQP103 in the weak and strong [PSI+]/[PIN+] yeast to determine whether their different toxicities were related to differences in aggregation. However, these fragments formed aggregates in all cells with similar appearance regardless of the prion variant present (Fig. 3). DIC staining showed that not all of the yeast cells were expressing GFP-labeled HttQ103 or HttQP103, which would explain the lack of growth of these cells when plated on selection plates. Evidently, the cells with the GFP-Htt aggregates are not proliferating, while the non-fluorescent cells are dying out with time in the selection medium. To detect dead cells in the yeast population, the yeast were stained with PI. In [PSI+]/[PIN+] yeast expressing either HttQ103 or HttQP103, around 10% of the yeast population was PI positive when measured by FACs analysis at 26 h. Therefore, there are relatively few dead cells in the yeast population after expressing the Htt fragments for 26 h even though the growth curves showed no proliferation of the yeast at this time. Since HttQ103 and HttQP103 are only toxic in yeast when aggregates are present, toxicity is evidently due to sequestration of essential protein(s) by the aggregates. However, differences in sequestration by HttQ103 and HttQP103 cannot be explained by differences in the appearance of the aggregates.
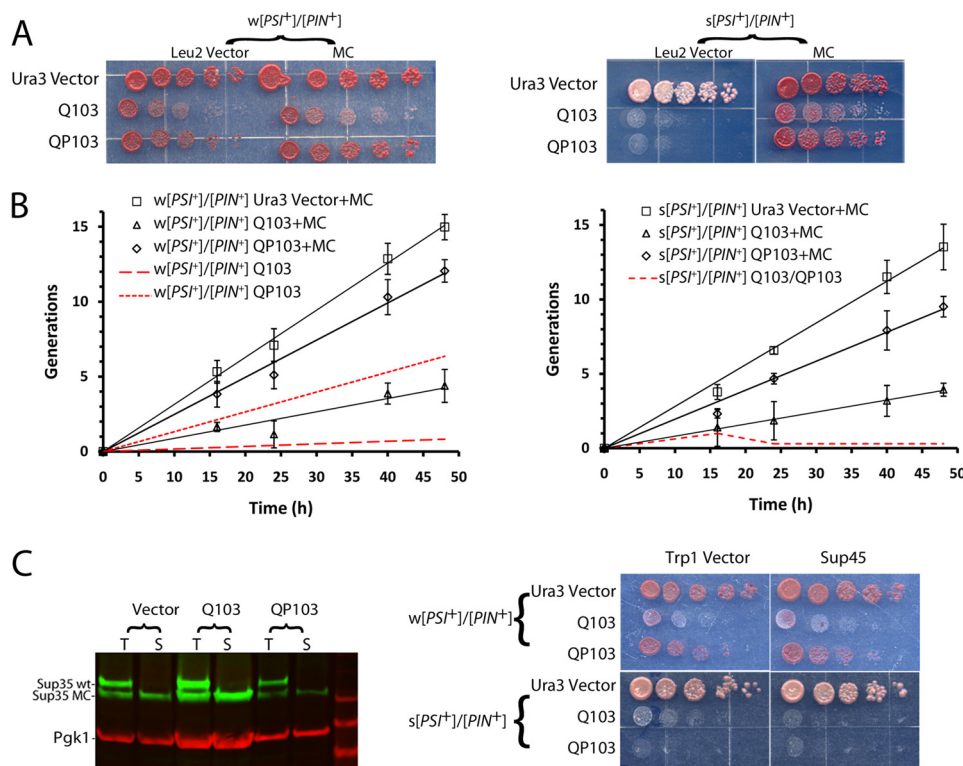
FIGURE 4. **Rescue of Htt toxicity by constitutive expression of the MC fragment of Sup35.** *A,* spot assay of weak and strong [*PSI*+]/[*PIN*+] yeast expressing Htt fragments and the MC fragment of Sup35. Yeast containing HttQ103, HttQP103, or empty Ura3 vector were transformed with the empty Leu2 vector or the MC vector. The yeast were spotted on SGal-Ura-Leu plates and grown for 6 days at 30 °C. The spot assays were done on the same SGal-Ura-Leu plate. *B,* effect of MC on the growth of weak and strong [*PSI*+]/[*PIN*+] expressing HttQ103 or HttQP103. Yeast containing empty vector or the Htt vectors were transformed with a plasmid that constitutively expresses MC. Yeast were grown in SGal-Ura-leu liquid culture and plated on SD-Ura-Leu plates at indicated time points. The number of colonies on each plated was counted after 4 days at 30 °C. The *red lines* are from the data in Fig. 2*B*. *C,* Western blot of MC level in yeast expressing Htt fragments. Lysates were prepared from strong [*PSI*+]/[*PIN*+] yeast expressing MC in addition to HttQ103, HttQP103, or empty Ura3 vector, which were grown in SGal-Ura-Leu medium for 24 h. Yeast total lysates and supernatant of high speed centrifugation were probed with an anti-Sup35 antibody detecting both MC fragment and wild type Sup35. Pgk1 protein level was used as a loading control. *D,* spot assay of weak and strong [*PSI*+]/[*PIN*+] yeast expressing Htt fragments and Sup45. Yeast containing HttQ103, HttQP103, or empty Ura3 vector were transformed with the empty Trp1 vector or Sup45 vector. The yeast were spotted on SGal-Ura-Trp plate and grown for 6 days at 30 °C. The spot assays comparing the growth of Sup45 expressing and empty vector yeast were done on the same SGal-Ura-Trp plate.

Rescue of Htt Toxicity in [PSI+]/[PIN+] Yeast—Since HttQP103 was much more toxic in yeast with the strong [*PSI*+]/[*PIN*+] variant than with the weak [*PSI*+]/[*PIN*+] variant, it seemed possible that Sup35 sequestration was contributing to HttQP103 toxicity since Sup35 is an essential protein and there is less soluble Sup35 present in the strong than the weak [*PSI*+] variant. To determine whether this is indeed the case, [*PSI*+]/[*PIN*+] yeast were transformed with a plasmid that constitutively expresses the MC fragment of Sup35 from the *SUP35* promoter to determine if this truncated fragment rescues Htt toxicity. Importantly, this MC fragment contains the C-terminal domain of Sup35, which is essential for translation termination, but lacks the N-terminal prion-forming domain. The effect of constitutive expression of the MC fragment on Htt toxicity was analyzed using the spot assay. As shown in Fig. 4*A*, MC rescued HttQP103 and HttQ103 toxicity in both weak and strong [*PSI*+]/[*PIN*+] yeast, but it was more effective in rescuing HttQP103 toxicity than HttQ103 toxicity. This suggests that more Sup35 is sequestered by the HttQP103 aggregates than by the HttQ103 aggregates. However, since MC does not completely rescue the toxicity of either HttQ103 or HttQP103, another essential protein, in addition to Sup35, appears to be sequestered by the Htt

aggregates. Importantly, the HttQ103 aggregates sequester more of this other essential protein than the HttQP103 aggregates, while the HttQP103 aggregates sequester more Sup35 than the HttQ103 aggregates.

To confirm the spot assays, we measured the growth of yeast with both weak and strong [*PSI*+]/[*PIN*+] prion variants expressing Htt fragments and MC as a function of time. Fig. 4*B* shows that the constitutive expression of MC partially rescued Htt toxicity in both weak and strong [*PSI*+]/[*PIN*+] yeast. Regardless of the [*PSI*+] variant, the growth rate of yeast expressing HttQ103 and MC was about 25% of the control rate, while the growth rate of yeast expressing HttQP103 and MC was about 75% of the control rate. These results confirm that MC rescues HttQP103 toxicity to a much greater extent than HttQ103 toxicity. To rule out that the difference in the extent of rescue is due to the expression level of MC, Western blot analysis was performed to measure the amount of MC using a polyclonal antibody that recognizes both MC and Sup35. Fig. 4*C* shows comparable levels of the MC fragment in control cells and cells expressing Htt fragments, after normalizing for protein levels using Pgk1 as an internal control. Furthermore, unlike full-length Sup35, the MC fragment remains in the supernatant after high speed centrifugation, which shows that a

limiting amount of MC does not account for the lack of complete rescue.

We also tested whether Htt toxicity is rescued by expressing Sup45 since Sup45 is an essential protein that forms a complex with Sup35 in translation termination (26). Furthermore, the sequestration of Sup45 has been shown to cause toxicity when Sup35 was highly overexpressed in $[PSI^+]$ yeast (21). To determine whether Sup45 rescues Htt toxicity, $[PSI^+]/[PIN^+]$ yeast were transformed with a plasmid that expresses Sup45 from its endogenous promoter. The spot assays show that Sup45 does not rescue Htt toxicity in yeast with either the weak or strong $[PSI^+]$ variants (Fig. 4D). Therefore, sequestration of Sup35, but not Sup45, by Htt aggregates is one factor that contributes to Htt toxicity in $[PSI^+]/[PIN^+]$ yeast.

Toxicity of Htt Fragments in $[PSI^+]/\Delta rnq1$ Yeast and Rescue of Toxicity by MC Fragment—To understand the nature of Htt toxicity in yeast, we next examined the effect of expressing the Htt fragments in yeast with only the $[PSI^+]$ prion, but not with the $[PIN^+]$ prion. To ensure the absence of the $[PIN^+]$ prion, $\Delta rnq1$ strains of yeast were constructed for both the weak and strong $[PSI^+]$ variants. Comparison of the spot assays of the $[PSI^+]/[PIN^+]$ and $[PSI^+]/\Delta rnq1$ yeast showed that the $[PIN^+]$ prion markedly increased the toxicity of the Htt fragments especially in yeast with the strong $[PSI^+]$ variant (Fig. 5A) The toxic effects of the Htt fragments also depended on the presence or absence of the polyproline region. HttQ103 was more toxic than HttQP103 regardless of the $[PSI^+]$ variant, as shown by the growth curves (Fig. 5B). Specifically, the growth rate was 60 and 80% of the control rate in the weak $[PSI^+]$ yeast expressing HttQ103 and HttQP103, respectively, while it was 25 and 50% of the control rate in strong $[PSI^+]$ yeast expressing HttQ103 and HttQP103, respectively. The fact that the Htt aggregates are more toxic in the presence of both $[PSI^+]$ and $[PIN^+]$ prions than with just the $[PSI^+]$ prion shows that $[PIN^+]$ increases the sequestration of essential proteins by the Htt aggregates.

Since the Htt fragments were more toxic in the presence of $[PIN^+]$, we examined the Htt distribution in yeast with only the $[PSI^+]$ prion to determine whether its aggregation state was altered in the absence of $[PIN^+]$ prion. After overnight induction of Htt with galactose medium, the $[PSI^+]/\Delta rnq1$ yeast were imaged by confocal microscopy. As shown in Fig. 5C, in addition to cells with multiple aggregates of HttQ103 and HttQP103, there were also cells with diffusive GFP fluorescence, especially in the weak $[PSI^+]/\Delta rnq1$ variant. From the confocal images, about 10% and 30% of the cells did not have aggregates in weak $[PSI^+]/\Delta rnq1$ yeast expressing HttQ103 ($n = 277$) and HttQP103 ($n = 593$), respectively. In contrast, aggregates were not detected in <5% and <15% of the strong $[PSI^+]/\Delta rnq1$ yeast expressing HttQ103 ($n = 250$) and HttQP103 ($n = 288$), respectively. Since the Htt toxicity is due to the presence of aggregates sequestering essential proteins, the diffusive cells are not contributing to the Htt toxicity in the $[PSI^+]/\Delta rnq1$ yeast, which would partly account for the decrease in toxicity of the Htt fragments in $[PSI^+]$ yeast in the absence of $[PIN^+]$.

Since these yeast contain only the $[PSI^+]$ prion, we examined whether sequestration of Sup35 is the sole cause of the toxicity produced by the Htt aggregates. This was tested by constitutively expressing the MC fragment of Sup35 in the weak and strong $[PSI^+]/\Delta rnq1$ strains. As shown by the spot assays (Fig. 6A), MC did not rescue the Htt toxicity in weak $[PSI^+]/\Delta rnq1$ yeast, which shows that sequestration of Sup35 is not contributing to Htt toxicity in this yeast strain. Consistent with this result, MC increased the growth rate of strong $[PSI^+]/\Delta rnq1$ yeast expressing HttQ103 and HttQP103 fragments to the rate that was obtained when weak $[PSI^+]/\Delta rnq1$ yeast expressed these fragments. With constitutive expression of MC, the growth rate of strong $[PSI^+]/\Delta rnq1$ yeast expressing HttQ103 was 55% of the control rate, while in yeast expressing HttQP103, the growth rate was 90% of the control rate (Fig. 6B). Since active Sup35 is not limiting, one or more other essential proteins is being sequestered by the Htt aggregates in $[PSI^+]/\Delta rnq1$ yeast and there is more sequestration of these proteins by the HttQ103 aggregates than by the HttQP103 aggregates. Therefore, taken together, our results establish that sequestration of other proteins aside from Sup35 is contributing to HttQ103 and HttQP103 toxicity in yeast with only the $[PSI^+]$ prion as well as in yeast with both the $[PSI^+]$ and $[PIN^+]$ prions.

Effect of Htt Fragments in $[psi^-]/[PIN^+]$ Yeast—Our results show that both HttQ103 and HttQP103 are much more toxic in the $[PSI^+]/[PIN^+]$ yeast than in the $[PSI^+]/[pin^-]$ yeast, which in turn, shows that $[PIN^+]$ is contributing to toxicity. To determine the effect of the $[PIN^+]$ prion alone on Htt toxicity, $[psi^-]/[PIN^+]$ yeast were transformed with the Htt plasmids and the toxicity of the Htt fragments was then analyzed using the spot assay. Fig. 7A shows the expression of HttQ103, but not HttQP103, caused a significant reduction in the growth of the $[PIN^+]$ yeast, in agreement with the results from the Sherman laboratory (18). These results were confirmed by the growth curves (Fig. 7B), which show that the growth rates of $[PIN^+]$ yeast were 25 and 80% of the control rate for yeast expressing HttQ103 and HttQP103, respectively. Since $[psi^-]$ yeast were used, the toxicity of the Htt fragments in $[PIN^+]$ yeast is caused by sequestration of one or more essential protein(s) other than Sup35, Evidently, there is greater sequestration of these proteins by the HttQ103 aggregates than by the HttQP103 aggregates.

Confocal images of the Htt fragments were taken in the $[psi^-]/[PIN^+]$ yeast to determine whether there was a difference in the appearance of the aggregates from those observed in the $[PSI^+]/[PIN^+]$ yeast (Fig. 3). Interestingly, even though the expression of HttQ103 and HttQP103 caused very different levels of toxicity in $[psi^-]/[PIN^+]$ yeast, the Htt aggregates had a similar appearance with multiple HttQ103 and HttQP103 aggregates present in the cytosol (Fig. 7C). The cells did not show one prominent aggregate characteristic of an aggresome (13) nor were there diffuse cells present in the population as occurred when yeast had only the $[PSI^+]$ prion. Therefore, gross differences in the appearance of the aggregates do not explain the differences in the observed toxicity. Instead, HttQ103 and HttQP103 amyloids must differ in their conformations to account for the differences in their sequestration of essential proteins and thus their toxicity.
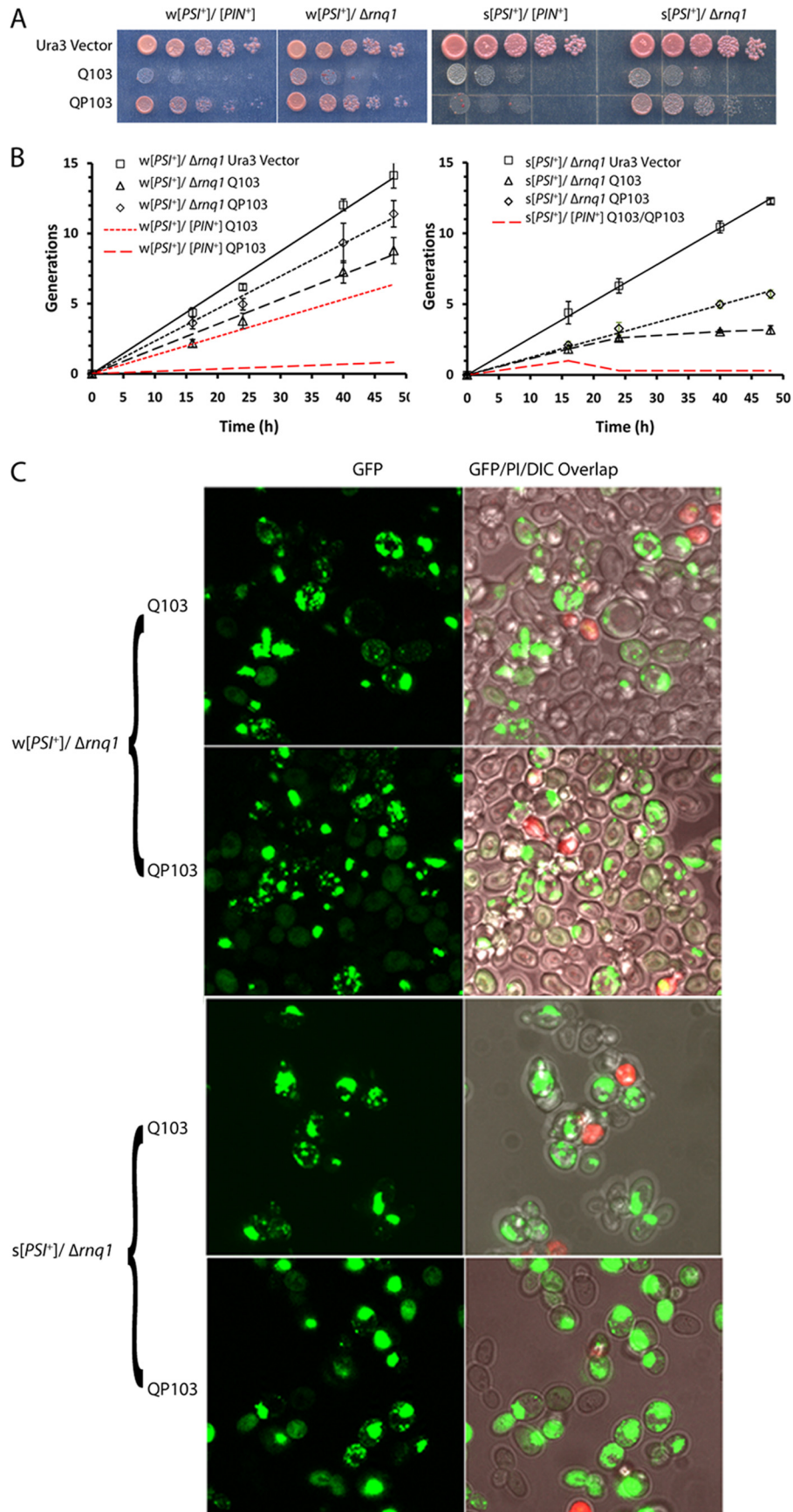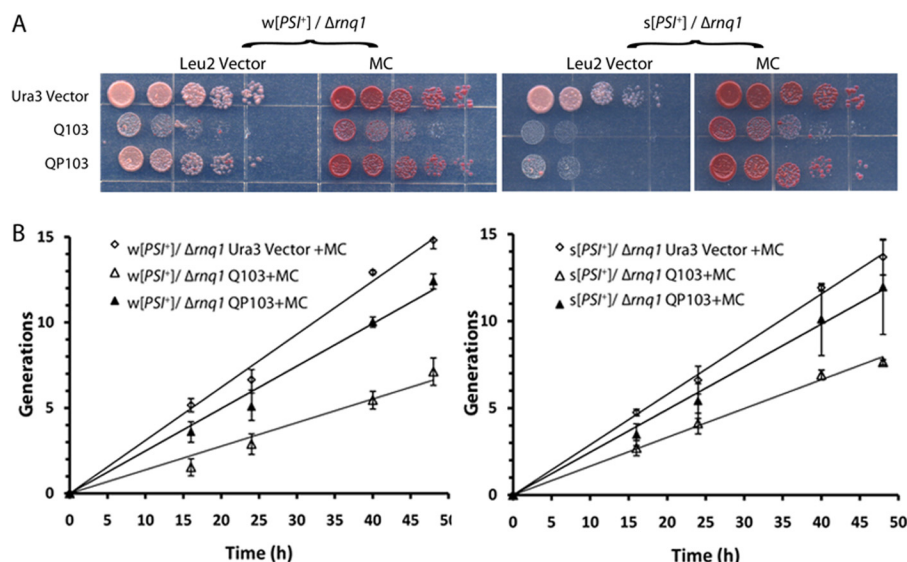
FIGURE 6. **Rescue of Htt toxicity by constitutive expression of MC in weak and strong [*PSI*⁺]/Δ*rnq1* yeast.** *A*, spot assays showing the effect of MC on Htt toxicity in weak and strong [*PSI*⁺]/Δ*rnq1* yeast. Yeast containing HttQ103, HttQP103, or empty Ura3 vector were transformed with the empty Leu2 vector or the MC vector. Yeast were spotted on SGal-Ura-Leu plate and grown for 6 days at 30 °C. The spot assays comparing the growth of yeast in the presence and absence of MC were done on the same SGal-selection plate. *B*, growth curves of weak and strong [*PSI*⁺]/Δ*rnq1* yeast constitutively expressing MC and either HttQ103 or HttQP103. The control is [*PSI*⁺]/Δ*rnq1* yeast constitutively expressing MC with the empty vector.

DISCUSSION

The expression of Htt fragments in yeast has been used to investigate the pathophysiology of Hungtinton disease. Surprisingly, the relationship between toxicity and the aggregation of the Htt fragments with pathologically long polyQ repeat regions (>40 polyQ) in the cytosol has proven to be more complex in yeast than in mammalian cells. In mammalian cells, the longer the polyQ repeat region, the greater the toxicity. Furthermore, recent data have suggested that, rather than the aggregate itself, it is the amount of diffuse intracellular Htt fragments (3, 27), that is both monomers and soluble oligomers of the Htt fragments, that apparently causes toxicity in mammalian cells (4, 5, 27).

On the other hand, many factors determine whether Htt fragments with extended polyQ repeats are toxic when expressed in yeast cells. Unlike in mammalian cells, an extended polyQ repeat region is not sufficient to cause toxicity in yeast. Rather, Htt fragments with extended polyQ repeat regions are only toxic in yeast that also have a misfolded prion protein, *e.g.* in [*PIN*⁺] or [*PSI*⁺] yeast. In yeast with misfolded prion proteins, the Htt fragments form visible aggregates, while in cells without a misfolded prion protein the Htt fragments are generally diffusive which suggests that the prion protein is promoting the formation of these Htt aggregates. In addition, the prion protein, itself, is affecting toxicity since expression of HttQ103 is toxic in both [*PIN*⁺] and [*PSI*⁺] yeast, whereas expression of HttQP103 causes pronounce toxicity only in [*PSI*⁺] yeast.

Interestingly, our results establish that the toxic effect of the Htt fragments is also dependent on the [*PSI*⁺] variant. In the absence of [*PIN*⁺] prion, both HttQ103 and HttQP103 were much more toxic in the strong [*PSI*⁺] variant than in the weak [*PSI*⁺] variant even though Htt aggregates were present in the majority of cells in both the weak and strong variants. To determine whether this toxicity was caused by the sequestration of Sup35, we tested whether the C-terminal fragment of Sup35 could rescue toxicity. The constitutive expression of the MC fragment of Sup35 markedly rescued HttQP103 toxicity, but was less effective in rescuing HttQ103 toxicity. Evidently, sequestration of other essential proteins is contributing more to the toxicity caused by HttQ103 than by HttQP103. As for HttQP103, its toxicity is mostly due to sequestration of Sup35, which indicates that the polyproline region of HttQP103 must be involved in Sup35 sequestration. Toxicity due to sequestration of Sup35 in [*PSI*⁺] yeast has also been observed when the Sup35 fragment, NMG (the N-terminal Gln/Asn-rich domain plus the middle domain of Sup35 fused to GFP), was overexpressed (21). Like the toxicity caused by HttQP103, toxicity due to NMG overexpression was rescued by expressing the C-terminal domain of Sup35. In addition, the Wickner laboratory has shown that an unusually strong [*PSI*⁺] phenotype causes lethality due to insufficient free Sup35 in the cytosol (28).

Our results show that the molecular basis of the toxicity caused by expression of Htt fragments in yeast is quite complex. First, this study showed that in addition to the HttQ103 fragment being toxic, in agreement with previous studies (11,

FIGURE 5. **Effect of Htt fragments expression on the growth of weak and strong [*PSI*⁺]/Δ*rnq1* yeast.** *A*, spot assays comparing Htt toxicity of [*PSI*⁺] yeast in the presence and absence of [*PIN*⁺]. The comparison between the growth of the [*PSI*⁺]/[*PIN*⁺] and the [*PSI*⁺]/Δ*rnq1* yeast were done on the same SGal-Ura selection plate. *B*, growth curves of weak and strong [*PSI*⁺]/Δ*rnq1* yeast with empty vector or expressing different Htt fragments. Yeast were grown in SGal-Ura liquid culture and plated on SD-Ura plates at indicated time points. The number of colonies on each plated was counted after 4 days at 30 °C. The *red lines* are from the data in Fig. 2*B*. *C*, confocal images of weak and strong [*PSI*⁺]/Δ*rnq1* yeast expressing HttQ103 or HttQP103. Yeast were incubated in galactose for 26 h to express Htt fragments. The *left panel* shows the GFP fluorescence, while the *right panel* shows an overlay of GFP fluorescence, PI staining, and DIC images of the yeast.
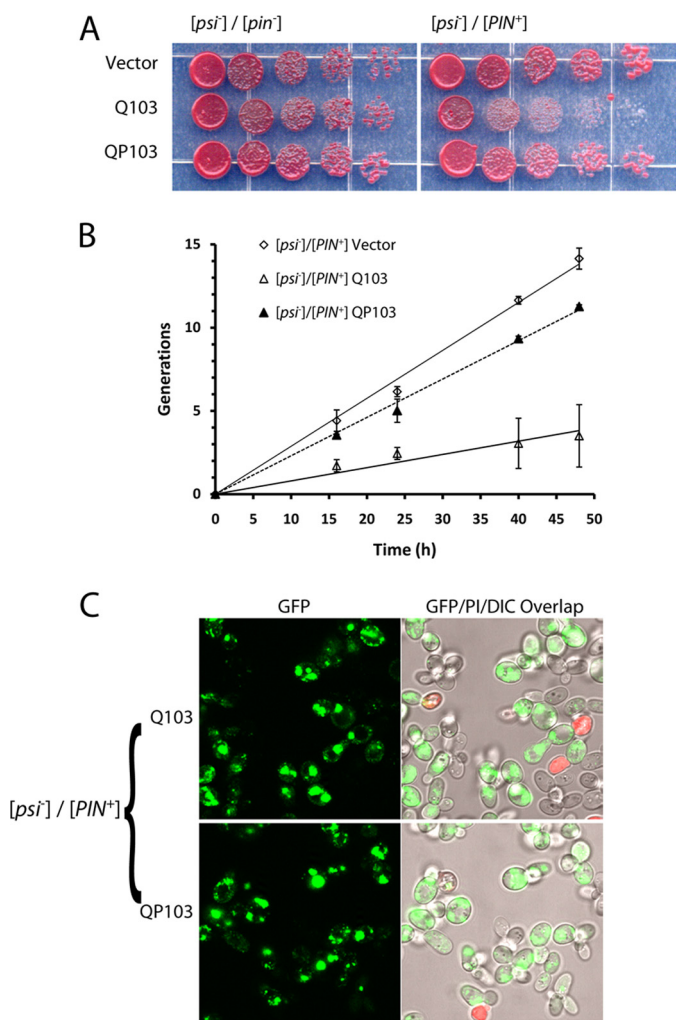
FIGURE 7. **Expression of HttQ103 or HttQP103 in [*psi⁻*]/[*PIN⁺*] yeast.** *A*, spot assay of [*psi⁻*]/[*pin⁻*] and [*psi⁻*]/[*PIN⁺*] yeast containing empty vector or expressing either HttQ103 or HttQP103. The spot assays comparing the growth of [*psi⁻*]/[*pin⁻*] and [*psi⁻*]/[*PIN⁺*] yeast were done on the same SGal-Ura plate. *B*, growth curves of [*psi⁻*]/[*PIN⁺*] yeast with empty vector or expressing either HttQ103 or HttQP103. Yeast were grown in SGal-Ura liquid culture and plated on SD-Ura plates at indicated time points. Colony count from each plate following incubation at 30 °C for 4 days was used to measure yeast growth. The number of colonies on each plated was counted after 4 days at 30 °C. *C*, confocal images of [*psi⁻*]/[*PIN⁺*] yeast expressing HttQ103 or HttQP103. Yeast were incubated in galactose for 26 h to express Htt fragments. The *left panel* shows the GFP fluorescence, while the *right panel* shows an overlay of GFP fluorescence, PI staining, and DIC images of the yeast.

16–18), we now find that the HttQP103 fragment is also toxic in yeast. Previous studies, which had expressed the HttQP103 construct in the W303 yeast strain, found that HttQP103 fragment was not toxic in this strain because it formed an aggresome rather than multiple aggregates. Disruption of the aggresome either by chemical or genetic tools resulted in HttQP103 becoming toxic to this strain of yeast. However, unlike in the W303 yeast strain, when HttQP103 was expressed in the 74-D694 yeast strain, which was used in this study, it formed multiple aggregates just like HttQ103. Second, we determined that different essential proteins are being sequestered by the HttQ103 and HttQP103 aggregates in strong [*PSI⁺*] yeast. Previous studies have clearly established that the HttQ103 aggregates sequester many proteins involved in endocytosis, which in turn leads to endocytic defects (17, 19). How-

ever, at this time, we cannot be sure if these endocytic defects are responsible for the toxicity caused by HttQ103 or the residual toxicity caused by the expression of HttQP103 that is not rescued by the C-terminal domain of Sup35 in strong [*PSI⁺*] yeast. In addition, it is not known whether the same essential proteins are sequestered by the HttQ103 aggregates in [*PIN⁺*] and [*PSI⁺*] yeast. Third, this study showed that the prion variant is a factor in determining the extent of the Htt toxicity. Since the prion conformation is different in [*PSI⁺*] variants (24), our results suggest that the prion conformation affects the amount and/or structure of the Htt aggregates that the prion variants induce. Finally, the toxic effect of expressing Htt is much more severe in yeast with both [*PSI⁺*] and [*PIN⁺*] prions than in yeast with only one of these prions. This indicates there is greater sequestration of essential proteins by the Htt aggregates in [*PSI⁺*]/[*PIN⁺*] yeast than in either [*psi⁻*]/[*PIN⁺*] or [*PSI⁺*]/[*pin⁻*] yeast; it is possible that the prion proteins act synergistically rather than additively.

The complex molecular basis of Huntington disease is also evident from studies on mammalian cells. Just as in yeast, Htt aggregates have been shown to sequester many different proteins including CBP, a coactivator of the transcription factor CREB, SP1, a transcriptional factor, and the Gln-Ala repeat transcriptional coactivator CA150 (29). Our yeast data suggest that the protein sequestered by the Htt aggregates may differ depending on whether the Htt fragments contain a polyproline region. In addition the toxicity caused by Htt may be dependent on the conformation of the misfolded protein. Not only Htt inclusions (30), but also misfolded monomer and soluble oligomers have been shown to be toxic to the cell (3–5). Even soluble Htt oligomers have been shown to exist in several conformations (6), which may lead to sequestration of different proteins in the cell. Importantly, Huntington disease belongs to a family of polyglutamine neurodegenerative diseases, which are all caused by an expanded polyQ repeat region. These different diseases affect different neurons, which may be due to the fact that different neurons require different levels of particular essential proteins. This complex molecular basis of Huntington disease may explain why it has been so difficult to understand the pathogenesis of polyglutamine disease in mammalian cells.

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