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Supplemental Information

Tumor suppressor protein p53 expressed in yeast can remain diffuse, form a prion, or form unstable liquid-like droplets

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Supplemental Figures

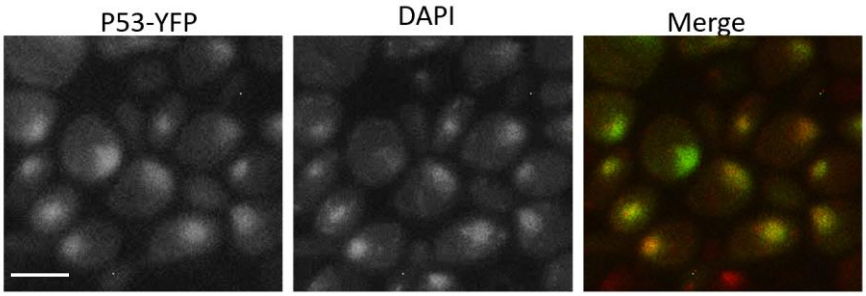

Figure S1 (related to Figure 1). In non-prion cells, p53-YFP is diffuse in the nucleus. P53 is expressed constitutively from pGPD-p53-EYFP in strain yIG397. Cells were grown overnight on plasmid selective glucose media. Nuclei were stained with DAPI. Size bar indicates 5µM.

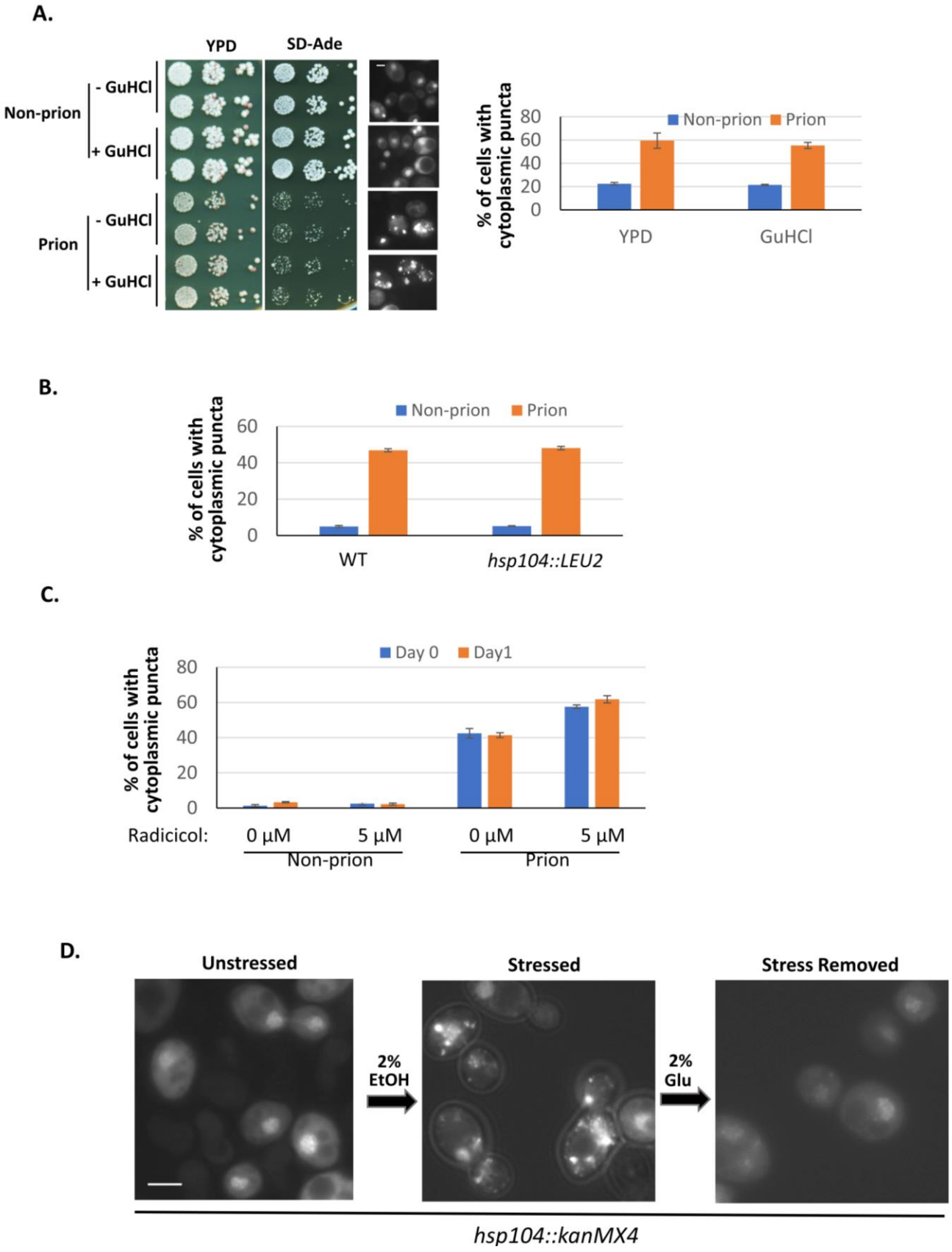

Figure S2. Chaperones tested have no effect on p53 prion retention or stress granule formation (related to Figures 1 and 5). *A. Growth in guanidine did not cause loss of the p53 prion.* Strain L3672 containing the p53 prion and its isogenic non-prion control (L3671) were streaked sequentially for 4 passages on YPD and YPD + 5mM GuHCl plates. Cells were then

normalized to OD_{600} =3, $1/10^{th}$ serially diluted in water and spotted on complex glucose (YPD) and glucose -Ade (SD-Ade). Also shown are photographs of p53-EYFP fluorescence in cells and the percent of cells with cytoplasmic foci based on 6 replicates of 150-500 cells each with standard error of the mean. There was no statistical difference whether or not cells were grown on guanidine. B. Disruption of *HSP104 did not cause loss of the p53 prion. HSP104* was disrupted in cytoductants of L3663 transformed with *pGPD-p53-EYFP* containing the p53 prion as well as in isogenic controls lacking the p53 prions. The fraction of cells with cytoplasmic puncta was determined and plotted. Standard error of the mean bars shown were based on 3 replicates where 200-400 cells were scored for each replica. There was no statistical different whether or not *HSP104* was disrupted. *C. HSP90 is not required for p53 prion maintenance.* To test whether Hsp90 effects p53 prion propagation, prion (L3672) and isogenic non-prion control (L3671) cells were grown in complete glucose liquid media without or with 5 µM radicicol, a potent Hsp90 inhibitor. The percent of cells with nuclear diffuse and cytoplasmic p53-EYFP puncta are shown. The standard error of the mean bars are based on 3 replicates with 200-600 cells for each replica. *D. Stress droplets form even in the absence of HSP104.* Exponentially growing BY4741 *hsp104Δ* cells transformed with pGPD-p53-EYFP grown in plasmid selective glucose medium (unstressed) were resuspended in 2% ethanol medium for 1 hr (stressed) and then resuspended in glucose medium for 30 min (stress removed). Size bar indicates 5µM.

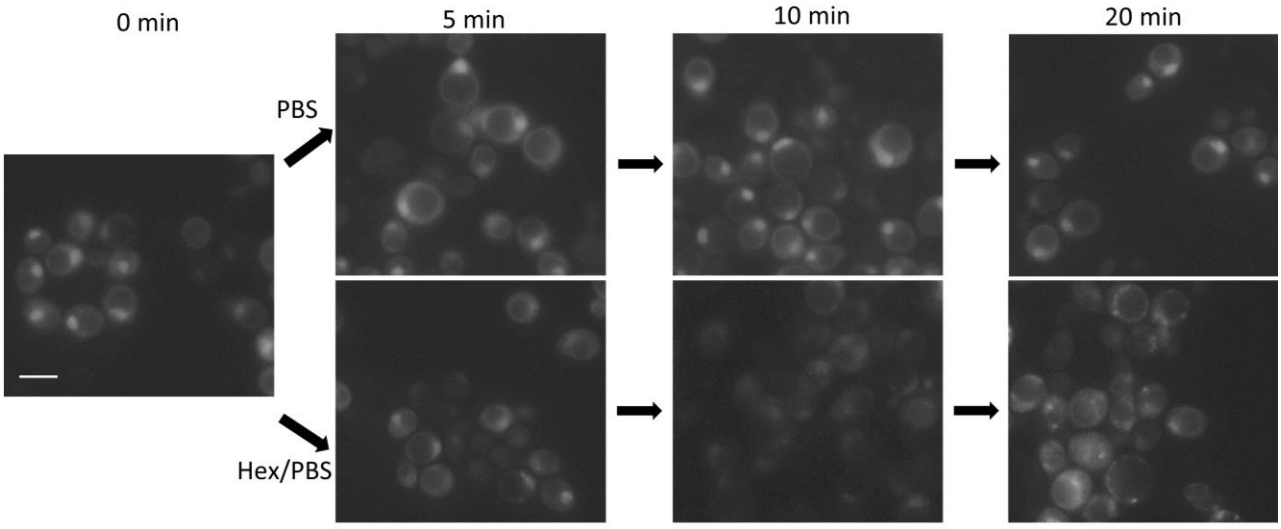

Figure. S3. 1,6-Hexanediol causes the appearance of wild type p53-EYFP cytoplasmic dots (related to Figure 5). yIG397 cells transformed with pGPD-p53-EYFP grown on glucose plasmid selective media were treated with 10% 1,6-hexanediol. Fluorescence was assayed at 5, 10 and 20 min. 1,6-Hexanediol induced p53 foci started to appear at 15 min and were plentiful by 20 min. Foci were not seen at either 5 or 10 min. Size bar indicates 5µM.

Transparent Methods

Yeast Strains, Plasmids, and Cultivation.

Media, cultivation and transformation procedures were standard (Sherman, 2002). Yeast with *ade2* mutations were maintained on complex glucose medium (YPD) supplemented with adenine (200 μg/ml) to prevent the appearance of *ade2* suppressors. Synthetic glucose media (SD) was supplemented with required amino acids. Strains and plasmids used are listed in Tables S1 and S2, respectively. The yeast strain used to assay for p53 activity and in which we selected the p53 prion was yIG397 (*MATα ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100, ura3-1 URA3 3xRGC::pCYC1::ADE2*) (kindly supplied by A. Inga, U of Trento, Italy) (Inga et al., 1997) (Flaman et al., 1995; Ishioka et al., 1993), with the integrated plasmid pLS210 (Flaman et al., 1995) that contains an *ADE2* reporter gene under three copies of the p53 consensus binding sequence from the ribosomal gene cluster (RGC) immediately upstream of minimal promoter CYC1 with a *URA3* selectable marker. Expression of *ADE2* was used to assay for p53 function in yIG397, L3671, L3672 and L3628 by analyzing growth and color of yeast by spotting 10X serially diluted suspensions of cells (initially normalized to OD_{600} = 3) on plasmid selective media with limiting adenine (5 μg/ml). When yIG397 was transformed with plasmids expressing wild type p53, it was prototrophic for adenine, yielding white colonies on plates containing a limited amount of adenine or YPD. Without functional p53, yIG397 is red and with limited p53 function it is pink. The non-prion control strain, L3671, was yIG397 co-transformed with pADH1-p53 and pGAL1-p53-EYFP. The prion strain induced in L3671 is L3672.

Strains L3719 and L3628 were used as recipients for transfection. L3719 and L3628 are a diploid strains we made by respectively selecting for *MAT***a**/*MAT***a** or *MATα*/*MATα* resulting from mitotic crossovers following low level UV irradiation of the *MAT***a**/*MATα* strain SGY6001 (*MAT***a**/*MATα lys-801/ lys-801 ade2-101/ ade2-101 trp1-Δ63/ trp1-Δ63 his3-Δ200/ his3-Δ200 leu2-Δ1/ leu2-Δ1 URA3::3xRGC::p-cyc1::ADE2::ura3-52/ ura3-52*) kindly provided by Samir K Maji, IIT, India (Sengupta et al., 2017, 2020). Strain L3663 (*MAT***a** *kar1 ura2 his- leu2* [PIN^+] [rho^-]) was used as a recipient for cytoduction. It was made by inducing 3385, kindly supplied by R.B. Wickner, N.I.H. (Wickner, 1994), to become [rho^-] by streaking on medium with ethidium bromide.

HSP104 was disrupted in cytoductants of L3663 transformed with *pGPD-p53-EYFP* containing the p53 prion as well as in isogenic controls lacking the p53 prions. As described previously (Zhou et al., 1999), the disruptions were obtained with the one-step gene replacement method (Rothstein, 1983) by transforming the prion and non-prion cytoductants with a purified *Pvu*I and *Hind*III fragment containing *HSP104::LEU2* isolated from plasmid pYABL5 (Chernoff et al., 1995)**.** The disruptions were verified by PCR as described previously (Zhou et al., 1999).

Plasmid pADH1-p53 (pLS76=pRS415, p*ADH1-p53, CEN LEU2*), which expresses human p53 cDNA under control of constitutive *ADH1* promoter, was kindly provided by Dr. Richard Iggo, Bergonie Cancer Institute, France (Inga et al., 1997). This plasmid was used to screen for p53 prion cells. Gateway cloning was used to construct plasmids with wild-type or R175H mutant p53 expression controlled by *GAL1 or GPD1.* Human p53 without a stop codon was PCR amplified from pLS76 or pLS40 (*pGAL-p53 R175H, CEN, TRP1*) (Inga et al., 1997) and cloned into pDONR221 using a BP reaction to build p2506 or p2516 (respectively, pDONR221-p53 and pDONR221-p53mt R175H without stop codons). The p53 fragments in the entry clones were then transferred to p2258 (pAG414 *GAL1-ccdB-EYFP, TRP1, CEN*) and p2474 (pAG413 *GPD-ccdB-EYFP, HIS3, CEN*) (Alberti et al., 2007) by an LR reaction to respectively build pGAL1-p53-EYFP (p2489=pAG414 *GAL1-p53-EYFP, TRP1, CEN*) and pGPD-p53-EYFP (p2517=pAG413, *GPD-p53-EYFP, HIS3, CEN*) or pGAL-p53-R175H-EYFP (p2518=pAG414 *GAL1-p53-R175H-EYFP, TRP1, CEN*). The p53 sequences in the destination clones were confirmed (Genomic Center, University of Nevada, Reno) and strain yIG397 transformed with pADH1-p53 (pLS76=pRS415, p*ADH1-p53, CEN, LEU2*) and pGAL1-p53-EYFP (p2489=pAG414 *GAL1-p53-EYFP, TRP1, CEN*) (L3671) was confirmed to be white on adenine limiting (5 μg/ml), plasmid selection media.

TABLE S1. Yeast strains used, related to Figures 1-6

Strains	Description	Reference
yIG397	*MATα ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 URA3::3xRGC::p-cyc1::ADE2::ura3-1*	(Inga et al., 1997)
L3671	*yIG397* transformed with *pADH1-p53* (*LEU2*) and *pGAL1-p53-EYFP* (*TRP1*)	This study
L3672	L3671 with p53 prion	This study
3385	*MAT***a** *kar1 ura2 leu2 his3* [PIN^+]	(Wickner, 1994)
L3663	[rho^-] version of 3385	This study
L3719	*MAT***a**/*MAT***a** *lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 URA3::3xRGC::p-cyc1::ADE2::ura3-52/ ura3-52*	This study
L3628	*MATα/MATα lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 URA3::3xRGC::p-cyc1::ADE2::ura3-52/ ura3-52*	This Study
BY4741 *hsp104Δ*	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hsp104Δ*::*KanMX4 deletion strain in BY4741 background*	(Winzeler et al., 1999)

TABLE S2. Plasmids used, related to Figures 1-6

Short Name, and SWL Laboratory Plasmid #	Original Plasmid Name/Description	Reference
pADH1-p53, pLS76	pADH1-p53 (*CEN*, *LEU2*)	(Inga et al., 1997)
p2258	pAG414-GAL-ccdB-EYFP (*CEN*, *TRP1*)	Addgene plasmid #14215
p2474	pAG413-*GPD-ccdB-EYFP* (*CEN*, *HIS3*)	Addgene plasmid #14214
pGAL-p53-EYFP, p2489	pGAL1-p53-EYFP (*CEN*, *TRP1*)	This study
pGPD-p53-EYFP, p2517	pGPD1-p53-EYFP (*CEN, HIS3*)	This study
pGPD-p53-R175H-EYFP, p2518	pGPD1-p53-R175H-EYFP (*CEN, HIS3*)	This study
pEdc3-mCH, p2137	pRP1575 Edc3-mCh (*CEN, TRP1*)	(Buchan et al., 2008)
pDcp2-RFP, p2136	pRP1155 Dcp2-RFP (*CEN, LEU2)*	(Teixeira et al., 2005)
p812	pYABL5, *HSP104::LEU2*	(Chernoff et al., 1995)

Selection for p53 Prion

L3671 was patched all over plasmid selective adenine limiting glucose plates. After robust growth these were velveteen replica-plated to each of 4 galactose and 4 glucose plasmid selective adenine limiting plates, where respectively p53-EYFP was, or was not, expressed. After 5 days incubation at 30°C each of these plates were replica-plated to glucose plasmid selective adenine limiting media to end the transient p53-EYFP overexpression caused by previous growth on galactose. Plates were grown for 3 days at 30°C. They were then transferred to room temperature for 3 weeks for further growth and to allow the development of the red/pink color diagnostic for cells with reduced *ADE2* expression expected of strains with p53 prion.

Induction and Removal of Stress
Cells transformed with pGPD-p53-EYFP (p2517) or pGAL-p53-R175H-EYFP (p2518) were grown to exponential phase in plasmid selective media. For ethanol stress, cells were washed twice with deionized water, resuspended and incubated with shaking for 30 min in plasmid selective 2% ethanol media and resuspended in plasmid selective glucose medium for stress removal. For heat stress, cells grown in plasmid selective glucose medium were incubated for 10 min in a 46˚C water bath before being returned to 30˚C. For glucose starvation, cells were washed twice with deionized water, resuspended in synthetic medium without any carbon source and incubated for 20 min with shaking. To remove glucose starvation stress, cells were harvested, resuspend in plasmid selective glucose medium and incubated for 20 min with shaking.

Sedimentation Analysis and Immuno-blotting
L3671 (non-prion control) and L3672 (prion) cells grown on dextrose or galactose plasmid selective media were harvested and lysed as described previously (Park et al., 2019). To compare levels of proteins in supernatant vs. pellet, normalized cleared cell lysates (150 µl of 100 µg of protein/ml) were centrifuged at 80,000 rpm for 30 min at 4°C. After the supernatant was removed and saved, pellets were washed with lysate buffer containing a protease inhibitor cocktail and PMSF protease inhibitor, recentrifuged at 80,000 rpm for 10 min and resuspended in 150 µl of lysate buffer with protease inhibitors. Boiled proteins in equal volumes total (T), supernatant (S) and pellet (P) fractions were resolved by PAGE that was immunoblotted with anti-p53 (DO-1, Santa Cruz Biotechnology, Santa Cruz CA). The blots were then stripped and reprobed with loading control anti-PGK (Novex, Thermo Fisher Scientific, Waltham, MA).

Fluorescence Microscopy, Thioflavin T Staining, DAPI Staining and 1,6-Hexanediol Treatment
To see EYFP labeled p53 aggregates in p53 prion subclones, cytoductants or transfectants, we examined cells taken from plates after 5 days growth at 30^{o}C on plasmid selective galactose medium (for prion subclones with GAL1-p53-EYFP) or 3 days growth on plasmid selective glucose medium (for transfectants and cytoductants with GPD1-p53-EYFP). P53 cytoplasmic foci were also stained with ThT in fixed cells as in (Johnson et al., 2008). Visualization of EYFP labeled or ThT stained cytoplasmic p53 was done with a Nikon Eclipse E600 fluorescent microscope (100X oil immersion) equipped with FITC, YFP, mCH and CFP filter cubes. P53-EYFP was visualized in the YFP channel, and ThT was viewed in the CFP channel. Cells expressing EYFP that were fixed for ThT staining exhibited reduced EYFP fluorescence. Both mCH and RFP were viewed with an mCH filter. To visualize nuclei, cells were stained with 1 µ g/ml 40,60-diamidino-2- phenylindole (DAPI) in 1XPBS for 10 min after fixation for 1 hr with 4% paraformaldehyde and permeabilized for 30 min with 60% EtOH. To determine if p53 foci in cells had the property of being dissolved by the aliphatic alcohol, 1,6-hexanediol shown to dissolve liquid-like over solid-like assemblies (Kroschwald et al., 2017), prion cells or cells under stress conditions were suspended in 10% 1,6-hexanediol and 10 µg/ml digitonin for 5 min before examining them for foci.

Cytoduction
[*RHO*$^{+}$] p53 prion strain L3672 or [*RHO*$^{+}$] non-prion isogenic control L3671, respectively containing or not containing p53 prion aggregates, were mated with recipient, L3663 (*MAT***a** *kar1 ura2 his3 leu2* [*PIN*$^{+}$] [*rho*$^{-}$]) transformed with pGPD-p53-EYFP (*CEN, HIS3*) or pGPD-p53-R175H-EYFP (*CEN, HIS3*) for about 8 hours until zygotes were visible. The mating mixtures were then spread on synthetic glucose medium lacking histidine (SD-His) to select for single colonies, that were then patched on a master plate (SD-His) and replicated onto YPGly (complete medium with 2% glycerol as the sole carbon source) media where recipients that were cytoduced (Conde and Fink, 1976) to become [*RHO*$^{+}$], by gaining donor cytoplasm, can grow. Colonies picked from plasmid selective glucose media and confirmed to be *MAT***a** Ura^{-} Leu^{-} His^{+} were scored as cytoductants and examined under a fluorescent microscope to score for the presence or absence of EYFP foci.

In vitro Polymerization of P8 Peptide

Aggregation-prone p53-derived peptide, p8 (NH2-PILTIITL-COOH, purchased from GeneScript USA Inc. Piscataway, NJ) was dissolved in 0.5 ml of 5.0 % D-mannitol and 0.01% sodium azide (pH 5.5) at a concentration of 1 mM in 1.5 ml Eppendorf tubes and placed in a water bath sonicator at its maximum output for 3 cycles of 20 sec resulting in a clear solution (Ghosh et al., 2014). The peptide was polymerized at room temperature with mild shaking for 8 hours and polymerization was confirmed with a ThT binding assay: 2 µl of 1 mM of P8 was mixed with 2 µl of the 1mM ThT in Tris-HCl (pH 8.0) and the volume was adjusted with 5% D-mannitol and 0.01% sodium azide (pH5.5) to give a final peptide concentration of 50 µM before measuring the fluorescence with a spectrofluorometer (SpectraMax M5, Molecular Devices, San Jose, CA), with excitation 450 nm and an emission 460-500 nm (Naiki et al., 1989).

Preparation of Yeast Spheroplasts for Transfection with Cell Lysates or P8 Polymer

Transfection was as previously described (Tanaka et al. 2004). Crude cell lysate either from prion (L3672) or non-prion (L3671) p53 cells, or *in vitro* made P8 polymer, were co-transformed along with pGPD-p53-EYFP (*HIS3, CEN*), into a recipient lacking p53 (L3628). Prion and non-prion p53 cells were grown on plasmid selective glucose media (SD-Leu-Trp) for 2 days at 30^{o}C. Harvested cells were then resuspended in STC buffer (1M sorbitol, 10 mM CaCl2, 10 mM Tris-HCl pH7.5) including 10 mM PMSF, and an anti-protease cocktail for yeast (Sigma, St. Louis, MO) and lysed by vortexing with glass beads. Cell debris was removed by centrifugation 2X at 4°C for 5 min at 8,000 g. The clear cell-free lysates or polymer of P8 were sonicated (output 4) for 30 seconds 3X using a micro tip (Misonix XL-2000 Misonix Inc. Farmingdale, NY). Fresh spheroplasts of recipient strain L3628 were obtained using lyticase and maintained in STC buffer at 4°C and were mixed with sonicated lysates or polymer of P8 along with pGPD-p53-EYFP (*HIS3, CEN*) (20 µg/ml) and salmon sperm DNA (100 µg/ml). After heat shock in the presence of 25% PEG 3350 the spheroplasts were incubated in SOS (1M sorbitol, 0.3 YPD, 10 mM $CaCl_2$) at 30^{o}C and added to SD-His with limiting adenine agar medium supplemented with 1M sorbitol. This was then overlaid on SD-His plates and incubated at 30^{o}C to select for transfectants. Transfectants confirmed to be His^{+}, Lys^{-}, Leu^{-}, and Trp^{-}, that therefore contained the pGPD-p53 -EYFP without plasmids from the donor, were assayed for the presence of p53-EYFP foci.

Supplemental References

Alberti, S., Gitler, A.D., and Lindquist, S. (2007). A suite of Gateway cloning vectors for high-throughput genetic analysis in Saccharomyces cerevisiae. Yeast *24*, 913-919.

Buchan, J.R., Muhlrad, D., and Parker, R. (2008). P bodies promote stress granule assembly in Saccharomyces cerevisiae. J Cell Biol *183*, 441-455.

Flaman, J.M., Frebourg, T., Moreau, V., Charbonnier, F., Martin, C., Chappuis, P., Sappino, A.P., Limacher, I.M., Bron, L., Benhattar, J.*, et al.* (1995). A simple p53 functional assay for screening cell lines, blood, and tumors. Proceedings of the National Academy of Sciences of the United States of America *92*, 3963-3967.

Ishioka, C., Frebourg, T., Yan, Y.X., Vidal, M., Friend, S.H., Schmidt, S., and Iggo, R. (1993). Screening patients for heterozygous p53 mutations using a functional assay in yeast. Nature genetics *5*, 124-129.

Johnson, B.S., McCaffery, J.M., Lindquist, S., and Gitler, A.D. (2008). A yeast TDP-43 proteinopathy model: Exploring the molecular determinants of TDP-43 aggregation and cellular toxicity. Proc Natl Acad Sci U S A *105*, 6439-6444.

Naiki, H., Higuchi, K., Hosokawa, M., and Takeda, T. (1989). Fluorometric determination of amyloid fibrils in vitro using the fluorescent dye, thioflavin T1. Anal Biochem *177*, 244-249.

Rothstein, R.J. (1983). One-step gene disruption in yeast. Methods Enzymol *101*, 202-211.

Sengupta, S., Maji, S.K., and Ghosh, S.K. (2017). Evidence of a Prion-Like Transmission of p53 Amyloid in Saccharomyces cerevisiae. Molecular and cellular biology *37*.

Sengupta, S., Maji, S.K., and Ghosh, S.K. (2020). Retraction for Sengupta et al., "Evidence of a Prion-Like Transmission of p53 Amyloid in Saccharomyces cerevisiae". Molecular and cellular biology *40*.

Sherman, F. (2002). Getting started with yeast. Methods Enzymol *350*, 3-41.

Teixeira, D., Sheth, U., Valencia-Sanchez, M.A., Brengues, M., and Parker, R. (2005). Processing bodies require RNA for assembly and contain nontranslating mRNAs. RNA *11*, 371-382.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J.D., Bussey, H*., et al.* (1999). Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science *285*, 901-906.

Zhou, P., Derkatch, I.L., Uptain, S.M., Patino, M.M., Lindquist, S., and Liebman, S.W. (1999). The yeast non-Mendelian factor [ETA+] is a variant of [PSI+], a prion-like form of release factor eRF3. EMBO J *18*, 1182-1191.