a-Synuclein Causes Non-Specific Toxicity in vps34 Yeast

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α-Synuclein Causes Non-Specific Toxicity in vps34 Yeast

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Summary

α-Synuclein is implicated in Parkinson’s Disease, a neurodegenerative disease that destroys midbrain neurons. The misfolding and subsequent aggregation of this protein is likely to cause cell death. A major hypothesis in the field is that increasing α-synuclein’s rate of degradation may prevent its aggregation and toxicity. The prevalent model for α-synuclein degradation is via the proteasome, and malfunctions in this pathway have been shown to increase α-synuclein accumulation and toxicity. However, increasing evidence suggests that the Multivesicular Body (MVB) sorting pathway is involved in protein degradation via the lysosome. To test the role of the MVB sorting pathway for the degradation of wild-type and mutant α-synucleins, we asked if α-synuclein would accumulate and increase toxicity in yeast that lacked one of the MVB proteins. Previously, Price and Shresetta showed that the absence of vps28, an MVB protein, caused toxicity in yeast expressing α-synuclein (Eukaryon). We tested another protein, vps34, a PI 3-kinase acting upstream in the processes involved in the MVB pathway. The absence of vps34 was toxic to yeast and this toxicity was severely exacerbated in the presence of any foreign protein, including α-synuclein. Future research will examine several other essential lysosomal pathway factors in mediating α-synuclein toxicity.

Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disease affecting about 1 million people in North America (Greenamyre and Hastings, 2004). In PD, dopaminergic neurons in the substantia nigra pars compacta (SNpc), in the midbrain die. These basal ganglia neurons secrete dopamine which is necessary for smooth and coordinated muscle movement (Gliasson and Lee, 2003). Loss of dopamine results in most of the clinical symptoms of PD which include resting tremors, slowness of movement, rigidity, postural instability, and depression (Wolters and Braak, 2006). Round eosinophilic inclusions comprised of a halo of radiating fibrils, known as Lewy bodies, found within SNpc neurons and dystonecous neurites (Lewy neurites) are the pathological hallmarks of PD (Gliasson and Lee, 2003; Dawson and Dawson, 2003). Misfolded and aggregated α-synuclein is the primary filamentous component of Lewy bodies (Spillantini, 1997; Spillantini et al., 1998).

α-Synuclein is a 140 amino acid protein found in pre-synaptic nerve terminals in neurons (Spillantini, 1998; McLean et al., 2000; Choi et al., 2004). Although the precise function of α-synuclein is unclear, it is known to play a role in synaptic plasticity and remodeling (Kahle et al., 2000). Recently, α-synuclein was shown to protect nerve terminals against injury in conjunction with other synaptic proteins (Chandra et al., 2005). Over the past 10 years, three missense point mutations in α-synuclein have been discovered in families with PD: A30P, A53T, and E46K (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004). These familial mutations on chromosome 4 are associated with early-onset PD and may cause the misfolding and subsequent aggregation of α-synuclein in Lewy bodies (Zabrocki et al., 2005). The duplication or triplication of the α-synuclein gene is also known to cause PD (Ibanez et al., 2004). In 2001, McNaught et al., found that protein degradation is impaired by 33-42% in PD patients, providing evidence that PD is caused by impaired degradation of α-synuclein leading to the formation of protein-rich Lewy bodies containing misfolded α-synuclein.

A possible way of treating PD is by degrading misfolded and aggregated α-synuclein. There is genetic and chemical evidence to show that α-synuclein is degraded by the ubiquitin-proteasome system (UPS; Thrower et al., 2000; Holtz and O’Malley, 2003; McNaught 2002; Webb et al., 2003). Familial mutations in parkin, an E3 ubiquitin ligase, and mutations in ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) inhibit the UPS in PD patients (Kifata et al., 1998; Leroy et al., 1998). Furthermore, McNaught et al. (2002), provided strong evidence that defects in the UPS underlie PD pathology and toxicity.

Studies have shown that the UPS is not the only organelle involved in α-synuclein degradation (Webb et al., 2003). In fact, monomeric and aggregated α-synuclein has been shown to inhibit the UPS, rather than defects in the UPS causing α-synuclein aggregation (Snyder et al., 2003). Further, pharmacological inhibition of the UPS did lead to an increase in cellular levels of α-synuclein (Rideout and Stefanis, 2002; Biasini et al., 2004). This suggests that another pathway of protein degradation—the endosome/lysosome pathway—may be involved.

The yeast vacuole acts as the lysosome, which degrades extracellular molecules, membrane and endo-membrane proteins, and organelles. It also degrades nuclear and cytoplasmic proteins, making it a possible target for α-synuclein degradation. Webb et al., provided evidence that in addition to the proteasome, the lysosome also degrades α-synuclein. Moreover, inhibition of the lysosome leads to an increase in the intracellular levels of α-synuclein (Webb et al., 2003; Lee et al., 2004; Cuervo et al., 2004).

Proteins are sent to the lysosome via endocytic pathways. The multivesicular body (MVB) sorting pathway to degradation by the lysosome/vacuole sorts proteins that are to be degraded by the lysosome by targeting them into the lumen of endosomes (Katzmann et al., 2001). Proteins that are to be recycled or sent elsewhere are kept at the limiting membrane of the endosome (Katzmann et al., 2002). The fusion of the endosome with the lysosome delivers the contents of the MVBs to the lysosome for degradation, while proteins on the limiting membrane of the MVBs remain on the limiting membrane of the lysosome (Katzmann et al., 2001).

The MVB sorting pathway is composed of more than 15 vacuolar protein sorting (vps) proteins which work alone or in complex. Monoubiquitination
serves as a recognition signal for proteins to be degraded through the MVB sorting pathway (Katzmann et al., 2001). The ESCRT-I complex, a 350kDa protein complex composed of vps23, vps28, and vps37 functions downstream of vps27, while vps15 and vps34 which form a complex are upstream of vps27 (Katzmann et al., 2003). Vps34 is a PI 3-kinase. Vps27 is then recruited to the enriched phospholipid layer of the endosome membrane, where it can bind ubiquitinated MVB cargo and recruit and activate ESCRT-I (Katzmann et al., 2003). ESCRT-II and ESCRT-III are composed of other vps proteins and act downstream of ESCRT-I. Since α-synuclein is known to bind membranes (Choi et al., 2004), it may be degraded through the MVB sorting pathway to the lysosome/vacuole.

Previously, our lab showed that knocking out vps28, a component of ESCRT-I lead to modest toxicity (Price and Shrestha, 2005). We hypothesize that knocking out vps34 and expressing wildtype and mutant forms of α-synuclein, will cause toxicity, since vps34 is required for the proper functioning of the MVB sorting pathway to protein degradation by the lysosome.

An S. cerevisiae model developed in our lab (Sharma et al., 2006) was used to assess the lack of vps34 with expression of α-synuclein, as yeast have already been shown to be useful model systems for the study of study neurodegenerative diseases, including Huntington’s disease and PD (Outeiro et al., 2003). The toxicity of wildtype and mutant (A30P, E46K and A53T) α-synucleins was assessed in vps34 mutants using growth curve analysis through optical density data and dilution serial spotting. Western blotting was done to examine the levels of wildtype and mutant α-synucleins in vps34 strains. These data shed light on the role of vps34 in the degradation of wildtype and mutant α-synucleins.

Results

Wildtype α-synuclein is toxic to vps34 yeast
To assess the effects of knocking out vps34 and expressing α-synuclein in the cells, an optical density analysis at 600 nm was done. Untagged α-synuclein was expressed in vps34 yeast by growing cells in galactose media. As seen in figure 1, cells lacking vps34 showed moderate toxicity. At 24 hours post induction, yeast lacking vps34 had barely grown, while those with vps34 had an absorbance value of 1.5. Strains not expressing any α-synuclein are shown at the top graph and serve as controls. These strains were grown in SC-Ura glucose (Refer to methods).

Wildtype α-synuclein tagged to GFP is also toxic to vps34 yeast
In order to assess the localization of α-synuclein in vps34 yeast, a GFP tag was ligated to the C-terminal of the α-synuclein gene (Refer to methods). As seen in figure 2, α-synuclein-GFP is highly toxic to vps34 yeast. This strain did not grow at all, until about 48 hours, however, the parent strain, expressing vps34 and α-synuclein-GFP had already reached saturation point by 24 hours. A sharp increase in growth was observed after 48 hours in vps34 yeast expressing α-synuclein-GFP.

GFP alone is extremely toxic to vps34 yeast
Since the toxicity associated with α-synuclein-GFP was greater than that associated with α-synuclein, an optical...
density analysis was done in a vps34 strain expressing GFP alone. Surprisingly, GFP was extremely toxic to vps34 yeast. There was virtually no growth even after 72 hours. However, the parent strain expressing vps34 reached saturation density before 24 hours (figure 3).

Another Foreign Protein, LacZ, is also toxic to vps34 yeast
Green fluorescent protein (GFP) is a reporter gene which is used extensively by scientists to study protein localization in model systems. The toxicity associated with GFP in vps34 yeast led to the analysis of another reporter gene; LacZ. As seen in figure 4, the vps34 strain expressing LacZ, is highly toxic. There is barely any growth in this strain up to 36 hours post induction; however, the parent strain reaches saturation in only 36 hours. In vps34 yeast, toxicity due to expression of LacZ is less than the toxicity associated with GFP.

Spotting analysis of vps34 yeast shows high toxicity
To further analyze the toxicity associated with the vps34 strain, a dilution series spotting assay was performed. The three familial α-synuclein mutants, A30P-α-synuclein, E46K-α-synuclein and A53T-α-synuclein were transformed in wild type parent strain and vps34 yeast. The empty pYES2 plasmid and GFP were used as controls. Cells were plated on inducing and non-inducing media (figure 5). vps34 transformants can be seen to have grown less than parent strain transformants on non-inducing media. In inducing media, parent strain transformants grow well. However, vps34 transformants do not grow at all, with the exception of some growth in the empty pYES2 transformant. Growth curve data showing toxicity in any vps34 transformant expressing foreign proteins supported the dilution series spotting data.

Vps34 affects α-synuclein expression
Western analysis was done on parent strain and the vps34 transformants to confirm α-synuclein expression. Cell lysates were prepared after 24 hours of protein induction. Lysates were run on two gels, one for the blotting membrane (figure 6A) and one for the Coomassie control (figure 6B). As expected, no protein expression was seen in cells transformed with the pYES2 vector (lane 1). Lane 2 showed the expected size of the green fluorescent protein at about 36 kDa. For wildtype and mutant forms of α-synuclein (lanes 3-6), bands were seen at 60 kDa, which is about 8 kDa greater than the standard size of monomeric α-synuclein. In our S. cerevisiae model, α-synuclein was previously shown to consistently migrate 6-8 kDa higher than expected (Sharma et al., 2006). Band intensities of wildtype and mutant α-synucleins in the parent strain (4741) are comparable. In 4741 transformants, GFP, wildtype and mutant α-synucleins showed multiple bands, as seen in lanes 2 through 6. This indicates that proteins are degraded either during cell lysis or in vivo (Sharma et al., 2006).

In the empty pYES2 vps34 transformant, no protein was seen, as expected (lane 7). Importantly, the lack of vps34 completely suppressed α-synuclein expression (lanes 8-12).

Galactose media is toxic to vps34 yeast
A growth curve analysis was done with the parent strain and vps34 strain grown in SC-Ura glucose and SC-Ura galactose (figure 7). Both strains reach saturation Density by the 24 hour time point when grown in non-
Figure 5. Dilution series spotting.
4741 and vps34 transformants were grown on non-inducing (glucose) and inducing (galactose) media. Growth of vps34 yeast was much less than parent strain on non-inducing media. When expressing GFP and α-synuclein, vps34 yeast did not grow at all. Slight growth was seen in the pYES2 control.

Figure 6. α-Synuclein expression in vps34
(A) Western Blotting. α-synuclein was probed with an Anti–V5 AP 1° antibody. As expected, the empty pYES2 transformant showed no protein. A band was seen at 36 kDa corresponding to expected size of GFP. Bands were seen for wildtype and mutant α-synucleins at about 60 kDa. Multiple bands are seen in lanes 2-6. No protein was seen for any of the vps34 transformants.
(B) Coomassie Staining. For parent strain 4741, band intensities are comparable, corresponding to equal amounts of protein being loaded. However, for vps34 transformants, bands 7, 8 and 10 are darker, indicating that more protein was loaded in these lanes.
Density of about 1.5 at the same time point. Figure 7. Growth curve analysis of saturation density by 24 hours. In galactose, and galactose.

The toxicity associated with untagged \( \alpha \)-synuclein is a potential therapy. Genetic and chemical evidence points to the role of the ubiquitin system in degrading \( \alpha \)-synuclein. However, studies have shown that the lysosome also degrades \( \alpha \)-synuclein (Webb et al., 2003). Knocking out \( \text{vps28} \), a component of the MVB sorting pathway in mediating \( \alpha \)-synuclein toxicity. Absence of \( \text{vps28} \), a component of the MVB sorting pathway to degradation via the lysosome has been shown to increase \( \alpha \)-synuclein toxicity (Willingham et al., 2003; Price and Shrestha, 2005). In this paper, we examined the role of \( \text{vps34} \) a PI 3-kinase, in the MVB pathway, in mediating \( \alpha \)-synuclein toxicity.

**Foreign proteins including \( \alpha \)-synuclein are toxic to \( \text{vps34} \) yeast**

\( \text{vps34} \) is a PI 3-kinase which phosphorylates PI on endosomes to PI(3)P. Endosomal membranes containing PI(3)P, then target \( \text{vps27} \) which binds to ubiquitinated cargo on endosomes and recruits and activates the ESCRT-I complex (Katzmann et al., 2003). The kinase activity of \( \text{vps34} \) is probably not restricted to endosomal membranes and is essential for survival when foreign proteins, like \( \alpha \)-synuclein, GFP and LacZ are expressed.

The toxicity associated with untagged wildtype \( \alpha \)-synuclein in \( \text{vps34} \) yeast was exacerbated when GFP was tagged to the C-terminal of the \( \alpha \)-synuclein gene. This increase in toxicity was shown to be caused by the addition of GFP, as \( \alpha \)-synuclein alone was less toxic to \( \text{vps34} \) yeast. This may have occurred due to some adverse effect of GFP on metabolism in \( \text{vps34} \) yeast. It can also be possible that \( \alpha \)-synuclein has a protective function, as \( \alpha \)-synuclein-GFP was less toxic than GFP alone. Research will have to be conducted to study the interactions of GFP in \( \text{vps34} \) yeast.

Studies using \( \text{vps34} \) yeast have previously been done (Katzmann et al., 2003); however, parent strain 4741 was not used. The extreme toxicity observed when expressing wildtype or mutant \( \alpha \)-synuclein, GFP or LacZ is most likely a strain specific sensitivity of the 4741 parent strain. Different strains of yeast are known to be sensitive to different proteins. Unpublished results from our lab show that the E46K-\( \alpha \)-synuclein mutant is moderately toxic in the 4741 parent strain, but not in other isogenic strains like 5-1.

**\( \alpha \)-Synuclein expression in \( \text{vps34} \) yeast**

The absence of \( \alpha \)-synuclein in the western blot analysis, suggests that the cells decreased \( \alpha \)-synuclein expression in order to survive. Unpublished data from our lab, show that in yeast knocked out for \( \alpha \)-ketoglutarate dehydrogenase, a mitochondrial enzyme, \( \alpha \)-synuclein expression is also greatly reduced. Thus, reduction in \( \alpha \)-synuclein expression may be used by the cell to survive.

**Toxicity associated with galactose media**

SC-Ura galactose caused toxicity in untransformed \( \text{vps34} \) yeast. Yeast prefer glucose, and are known to grow slower in galactose media. However, SC-Ura galactose caused more than usual toxicity in the untransformed \( \text{vps34} \) strain. It is possible that \( \text{vps34} \) plays a role in galactose metabolism. This toxicity may also be strain specific. The effect of galactose could be studied in \( \text{vps34} \) knockouts in other yeast strains to determine if galactose toxicity is a general phenomenon.

The MVB sorting pathway to the lysosome has been implicated in \( \alpha \)-synuclein degradation (Willingham et al., 2003). Knocking out \( \text{vps28} \), a component of the ESCRT-I complex of the MVB pathway was shown to cause toxicity in cells expressing wildtype and mutant forms of \( \alpha \)-synuclein (Price and Shrestha, 2005). In this study, we have shown that \( \alpha \)-synuclein causes non-specific toxicity in \( \text{vps34} \) yeast. Future research will examine several other \( \text{vps} \) proteins, like \( \text{vps22} \) and \( \text{vps27} \) in mediating \( \alpha \)-synuclein toxicity.

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**Methods**

**Strains and Transformation:**

A30P and E46K mutant \( \alpha \)-synuclein were created using site directed mutagenesis from human wild type \( \alpha \)-synuclein (Invitrogen). A53T mutant alpha-synuclein
and human wild type α-synuclein cDNAs were gifts from Christopher Ross (John Hopkins University). In order to tag the synuclein cDNAs with GFP, the synuclein cDNAs were subcloned into the mammalian pcDNA3.1/C-terminal GFP expression vector (Invitrogen). After amplification, the synucleins were subcloned into pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). α-Synuclein expression plasmids were transformed into competent E. coli grown on LB ampicillin media for selection. Plasmids were then isolated and transformed as described (Burke, 2000) into URA-3 deficient S. cerevisiae 4741, and vps34 strains. Yeast cells were grown on synthetic-complete media lacking uracil (SC-Ura) for selection and grown on LB ampicillin media for selection. Plasmids were transformed into competent competent cells with 10M of Antibody Wash for 5 minutes each time. For PKG controls, the membranes were incubated in 10M of Secondary Antibody Solution for 30 minutes, and washed four times with 20 mL of Antibody Wash for 5 minutes each time. Membranes were then rinsed with 20M of water for 2 minutes, thrice. 5M of Chromogenic Substrate was used to incubate the membranes until bands of desired intensity could be seen. Membranes were washed again with 20 mL of water three times after which they were dried on a clean piece of filter paper under infrared light.

Coomasie Blue Staining:
After protein samples were run on the 10-20% Tris-Glycine SDS gel, the gel was stained with Coomassie Blue [Coomassie Staining Solution:50% (v/v) methanol,0.05% (v/v) Coomassie brilliant blue R-250 (Bio-Raseur Purge), 1% (v/v) acetic acid, 40% H2O]. The solution was prepared in deionised water, by dissolving the Coomassie brilliant blue R-250 in methanol before adding acetic acid and water. Staining was carried on for two hours. The gel was then destained with Destaining Solution [7% (v/v) acetic acid, 5% (v/v) methanol, 88% H2O], overnight on a rotary shaker. The gel was then washed three times, for two minutes each time in deionised water (50mL). 35mL of Gel-Dry Drying Solution was added to the gel-tray and shaken for 5minutes in the StainEase gel Staining Tray. A sheet of cellophane was immersed in the Gel-Dry Drying Solution for 20 seconds, after which it was placed on one side of the DryEase Gel Drying Frame. Another wetter cellophane was put on top of the gel and air bubbles and wrinkles were removed. The frame was aligned and the plastic clamps were fastened onto the four edges of the frames. The gel dryer assembly was allowed to sit upright on a bench top for 48 hours.

Toxicity Analysis:
Growth Curve: For the OD600 analysis, transformed knockouts and the 4741 parent strain were grown overnight in 10mL of SC-URA glucose at 30°C in a shaking incubator at 200 rpm. Cells were harvested at 1500 x g for 5 min at 4°C, and were washed twice with 5 mL H2O. Cells were resuspended in 10 mL H2O and were counted. Flasks with 35mL SC-URA galactose and 35mL SC-URA glucose (for controls) were inoculated to a 2.0 x 10^6 cells/mL density. Absorbance readings were taken at 0,3,6,12,18,24,36 and 48 hours at 600nm using a Hitachi-U-2000 Spectrophotometer. Absorbance readings were plotted against time points to produce a growth curve.

Spotting:
Transformed knockouts and 4741 parent strain were

Cell Lysates:
BY 4741, and vps34 knockout strains with wild-type α-synuclein-GFP, A30P-GFP, E46K-GFP, A53T-GFP, GFP and parent plasmid were grown overnight at 30°C in a shaking incubator at 200rpm in 10mL SC-URA glucose. Cells were washed three times with 10mL H2O and resuspended in 2mL H2O. To induce protein expression, 1mL of cell suspension was used to inoculate 5mL of SC-URA galactose. Cultures were then incubated for 24 hours at 30°C, in a shaking incubator at 200rpm. Cells in each culture were counted to determine the cell density. 2.5 x 10^7 cells were taken from each culture and washed with 1mL 50mM Tris (pH 7.5) and 10mM NaCl [ 100mL: 95mL of H2O; 5mL of Tris 1M pH 7.5; 0.065g/l NaCl]. Cells were resuspended in 30mL Electrophoresis Sample Buffer [(EB) 2% SDS, 80mM Tris pH 6.8], 10% glycerol, 1.5% DTT, 1 mg/mL bromophenol blue], and various protease inhibitors and solubilizing agents [1% Triton-X 100, 1mM phenylmethlysulfonyl fluoride (PMSF), 1mM benzamide, 1mM sodium orthovanadate, 0.7µg/mL pepstaton A, 0.5µg/mL leupeptin, 10µg/mL E64, 2µg/mL aprotinin and 2µg/mL chymostatin]. The cell mixtures were then vortexed and heated at 100°C for 3 minutes. 0.3 grams of 0.5mm glass beads were added to the cell mixtures and vortexed for 2 minutes. 70 µL ESB was added to each tube and samples were heated again at 100°C for 1 minute.

Western Analysis:
20µL cell lysates were loaded into 10-20% Tris-Glycine SDS gels (Invitrogen) and electrophoresed in 1x Tris-Glycine SDS running buffer ([diluted to 1x from 10x]: 29.0g Tris Base, 144.0g Glycine, 10.0g SDS, 1.0L Di H2O, pH 8.3], at 130 volts.10µL of SEEBLUE protein ladder was used. Gels were then transferred onto polyvinylidene difluoride (PVDF) membranes, in 1x transfer buffer ([diluted to 1x from 25x]: 18.2g Tris base, 90.0g Glycine, to 500mL DiH2O, pH 8.3]. PVDF membranes were presoaked in methanol, H2O and 1x transfer buffer. The PVDF membrane was placed on a foam pad immersed in 1x transfer buffer. The gel was placed on the PVDF membrane and another foam pad soaked in transfer buffer was placed onto the gel. The protein was transferred for 1.5 hours at 30 volts. Western Breeze® Chromogenic Immunodetection protocol was used to probe for the proteins of interest. Membranes were placed in 10mL of blocking solution (Western Breeze) and incubated for 30 minutes on a rotary shaker set at 1 revolution/sec. Membranes were rinsed twice with 20 mL of H2O for 5 minutes each time. Primary antibody solution was prepared by diluting the Anti–V5 AP 1 ^i in 7mL of H2O, 2mL Blocker/Diluent (Part A) and 1mL Blocker/Diluent (Part B). The dilution was 1:2000 (5 µL in 10mL of Primary Antibody Diluent). Membranes were incubated with 10M of Primary antibody diluent for 1 hour. Antibody was removed and saved, and then membranes were washed four times with 20 mL of Antibody Wash for 5 minutes each time. For PGK controls, the membranes were incubated in 10mL of Secondary Antibody Solution for 30 minutes, and washed four times with 20 mL of Antibody Wash for 5 minutes each time. Membranes were then rinsed with 20mL of water for 2 minutes, thrice, 5mL of Chromogenic Substrate was used to incubate the membranes until bands of desired intensity could be seen. Membranes were washed again with 20 mL of water three times after which they were dried on a clean piece of filter paper under infrared light.
grown in 10mL SC-URA glucose overnight at 30°C in a shaking incubator at 200rpm. Cells were harvested at 1500 x g for 5 min at 4°C, and were washed twice with 5 mL H2O. Cells were resuspended in 10 mL DI H2O and counted. 2.0 x 10^7 cells/mL were removed from the cultures and resuspended in 1 mL H2O. 100 µl of this culture was added to the first lane of a microtiter plate. The next 5 lanes contained 80 µl H2O. 20 µL of the 100 µl of culture was removed from the first lane and added to the second. After mixing, 20 µL from the second lane was pipetted and added to the third lane and so on, until there were 5 lanes with five-fold serial dilutions for each cell culture. These cells were plated by inserting a frogger into the microtiter plate and plating cells onto SC-URA glucose and SC-URA galactose media plates. Plates were grown at 30°C for 3 days and pictures were taken.

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