Insight into Parkinson's Disease and α-Synuclein Degradation via the Lysosome: α-Synuclein Localization Changes in Vps28Δ

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Summary

The neurodegeneration pathology in Parkinson's disease patients predominantly targets dopaminergic neurons in the midbrain. These neurons accumulate aggregated alpha-synuclein, which may be linked to cell death. The misfolding and buildup of α-synuclein is thought to trigger its accumulation and aggregation. An attractive hypothesis states that excess amounts of α-synuclein are due to dysfunctional degradation of the protein. Until recently, the proteasome was considered the major site for degrading alpha-synuclein, but recent studies suggest that the lysosome may also be involved. To test this latter hypothesis, we employed a budding yeast model for α-synuclein aggregation and toxicity to genetically evaluate the role of the multivesicular body (MVB) pathway, which is a major route used by proteins to target the yeast vacuole for degradation. ESCRT-1 is a major protein complex in the MVB pathway. We asked whether alpha-synuclein would accumulate and increase toxicity in yeast that lacked important ESRCT-1 components, in this case vps28 or mvb12. We demonstrate that the absence of vps28 altered wildtype, A53T, and E46K α-synuclein localization. Specifically, α-synuclein shifted from being localized primarily on the plasma membrane to being diffuse and aggregated within the cytoplasm. In contrast, the mvb12Δ strain retained plasma membrane α-synuclein localization. Our preliminary data indicates that the MVB pathway is involved in α-synuclein degradation, but not all proteins within ESRCT-1 participate. Complete analysis of the remaining ESCRT-I proteins and other ESCRT complexes is needed to fully understand the role of sorting proteins and MVBs in the α-synuclein lysosome degradation pathway.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, second only to Alzheimer's disease. PD affects over 1 million people in the U.S. (Greenamyre and Hastings, 2004). Symptomatically, patients develop movement deficits such as muscular rigidity, tremors, altered posture, and bradykinesia (Giasson and Lee, 2003). These symptoms are caused by the characteristic death of dopaminergic neurons in the substantia nigra of the midbrain, which is linked to the formation of α- synuclein aggregates in the dying neurons (Spillantini et al. 1998).

α-Synuclein is a 140 amino acid protein, which aggregates and is known to interact with phospholipids. Despite this knowledge, its precise function remains unknown. α-synuclein colocalizes with synaptic vesicles and is thought to be involved in vesicular transport (Cabin et al. 2002). α-Synuclein misfolding correlates with both sporadic and familial Parkinson's disease. Although other mutants are linked to PD, in all cases α-synuclein aggregates. In sporadic PD, α-synuclein misfolds for unknown reasons; in familial PD, ,mutations in α-synuclein cause misfolding. There are three α-synuclein mutations: A30P, A53T, and E46K, (Polymeropoulos et al., 1997; Kruger et al. 1997, Zarranz et al. 2004). In all PD, however, insoluble α-synuclein aggregates are unable to be degraded.

Death of specific dopaminergic neurons is linked to α-synuclein misfolding, aggregation, and to impaired degradation and oxidative damage. If α-synuclein aggregates have a toxic effect on cells, then maybe degrading them could reduce cell death. α- Synuclein is a key player in PD; thus, it is thought that if α- synuclein degradation is better understood, to the possible treatment development could be possible.

Cytoplasmic proteins are degraded by the proteasome, and lipid-binding proteins are generally degraded in the lysosome. For the past ten to fifteen years, pharmacological and genetic evidence shows that α-synuclein is degraded via the ubiquitin-proteasome system (UPS), (Cummings *et al.,* 1999; Walter *et al.*, 2001). Mutations in proteasome enzymes accelerate neuropathology in mice (Rideout, 2004), while inhibition of proteasomal function accelerates α-synuclein aggregation and consequently increases cell death (McNaught et el. 2002). Recently, however, pharmacological evidence demonstrates that when lysosomal function is impaired, α-synuclein accumulates, resulting in an increase in toxicity and cell death (Lee et al. 2004). Although promising, this evidence lacks genetic support suggesting that the lysosome is α-synuclein's degradation site.

Membrane bound proteins reach the lysosome via the multivesicular body (MVB) pathway. Monoubiquitinated proteins are recruited by a late endosome, MVB, and are sorted into the MVB lumen. Once the proteins are sorted into MVB vesicles, the MVB fuses with the lysosome and releases the captured proteins for degradation (Katzmann et al. 2001; Klinodky and Kim 2000). Interestingly, α- synuclein aggregates in PD patients are ubiquitinated, suggesting that misfolded, accumulated α-synuclein is being tagged for degradation but not reaching the degradation site. Dysfunctional degradation and aggregate resistance to degradation are possible causes of α-synuclein aggregation and improper degradation.

In the MVB/lysosome pathway, proteins are recruited by the endosome's membrane. This multipart membrane consists of many proteins and three protein complexes required for transport, called endosomal sorting complexes (ESCRT): ESCRT-I, -II, and -III (Katzmann et al. 2001; Katzmann et al. 2003). ESCRT-I is made up of four proteins. In our study, the role of two such proteins, vps28 and mvb12, are analyzed in order to further understand the route for lysosomal degradation (Katzmann et al. 2001; Katzmann et al. 2003).

Budding yeasts are very practical organisms to study the MVB/lysosome pathway in PD because their vacuole is analogous to the lysosome (Outerio and Lindquist, 2003; Klionsky and Kim 2000). Additionally, the complete knockout library for yeast is available, providing means to study the genetic role of proteins in the MVB/lysosome pathway. Our yeast model was used to

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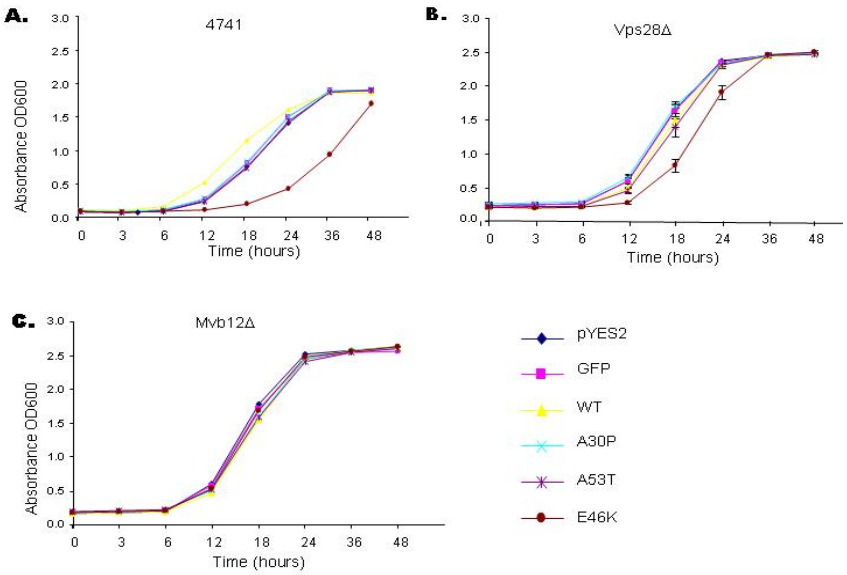

Figure 1. α-Synuclein Toxicity Analysis in BY4741 Parent Strain, Vps28D and Mvb12D. Averaged absorbance readings at 0, 3, 6, 12, 18, 24, 36, and 48 hours were plotted against time to produce growth curves. pYES2, GFP, A30P, A53T, and E46K cells were analyzed for BY4741, vps28D, and mvb12D. a) At 18, 24, and 36 hours, post induction, growth was reduced in E4K in 4741. Toxicity was not observed in other plasmids in 4741. b) Growth was reduced in E46K in Vps28D at 18, 24, and 36 hours post induction. Reduced growth was not observed in the other plasmids. c) In mvb12D, no toxicity was observed in E46K. Growth was not reduced in all other plasmids, cells were healthy.

provide evidence for α-synuclein degradation via the lysosome, using the MVB/endosomal pathway to gain access to the lysosome. Vps28 and mvb12 knockout strains expressing α-synuclein were used to evaluate any affect on α-synuclein localization, expression, and toxicity to the cell, an indication of perturbed degradation. Protein deletions showed no increase in toxicity or expression; however, vps28Δ did increase α-synuclein cytoplasmic accumulation.

Results

α-Synuclein has No Effect on Vps28Δ and Mvb12Δ Cell Growth

To test if α-synuclein is toxic to MVB knockout yeast, optical density growth analysis was performed at 600 nm. Absorbance readings were taken at 0, 3, 6, 12, 18, 24, 36, and 48 hours post-induction. Averaged absorbance readings were plotted against time to produce a growth curve. Growth patterns were observed in vps28Δ and mvb12Δ expressing WT, A30P, A53T, and E46K, and were compared to, parent strain, BY4741 growth curves done by Michael White. In both vps28Δ and mvb12Δ all constructs, with the exception of E46K, grew similarly to cells in the parent strain (Fig. 1). Vps28Δ cells expressing E46K showed exhibited decreased growth 18 and 24 hours. This growth pattern was similar to that observed in the parent strain (Fig. 1A and 1B). At 18 hours, E46K had less absorbance than all other cell types. Finally, a growth curve analysis was done with E46K cells in BY4741 and vps28Δ strains (data not shown). Absorbance readings were similar at all times, and no additional toxicity was observed. In mvb12Δ E46K toxicity was not observed; The E46K cells grew equal to cells in all other vectors (Fig. 1C).

α-Synuclein Localization Changes in Vps28Δ

α-Synuclein-GFP localization was evaluated in GFP fluorescence images acquired at 24 and 48 hours post-induction. Empty vector and GFP-pYES2 were used as controls in our GFP fluorescence microscopy. Parent strain (BY4741) microscopy pictures are courtesy of Michael Zorniak. Parent strain images were used as a template to which vps28Δ images were compared. In 4741, WT and A53T α-synuclein was membrane bound, and A30P was cytoplasmically diffuse after 24 and 48 hours (Fig. 2A and 2D), consistent with previous studies (Sharma, 2007). However, at 24 hours, E46K α-synuclein was both membrane localized and cytoplasmically diffuse. By 48 hours E46K was predominantly cytoplasmically diffuse. Similar to 4741, both GFP and A30P in vps28Δ are cytoplasmically diffuse at 24 and 48 hours. However, WT and A53T, in vps28Δ become predominantly diffuse (Fig. 2B and 2E). E46K was both membrane localized and diffuse at 24 and 48 hours (Fig. 2B and 2E).

No Change in α-Synuclein Localization in Mvb12Δ

GFP fluorescence microscopy was used to analyze α-synuclein localization in mvb12Δ at 24 and 48 hours post-induction. Compared to BY4741, mvb12Δ showed no change in α-synuclein localization. A30P remained diffuse, while WT, A53T, and E46K α-synuclein were membrane localized. (Fig. 2C and 2F).

No Change in α-Synuclein Expression in BY4741 Parent Strain and Vps28Δ

α-Synuclein expression was evaluated in BY4741 and vps28Δ at 48 hours post-induction α-Synuclein-V5 expression was measured using a V5 antibody. α-Synuclein expression was equal in all constructs in 4741 and Vps28Δ. A PGK antibody was used as a loading control confirming equal loading amounts. pYES2, empty vector, was used as a negative control. In both the parent strain and vps28Δ wildtype and mutant α-synuclein were equally expressed.

Discussion

Although the formation of α-synuclein aggregates is linked to impaired degradation, we are still unclear on whether aggregates resist degradation or if dysfunctional degradation exists. In order to answer these questions, we first need to better understand α-synuclein degradation. For the past four years, an increasing number of studies

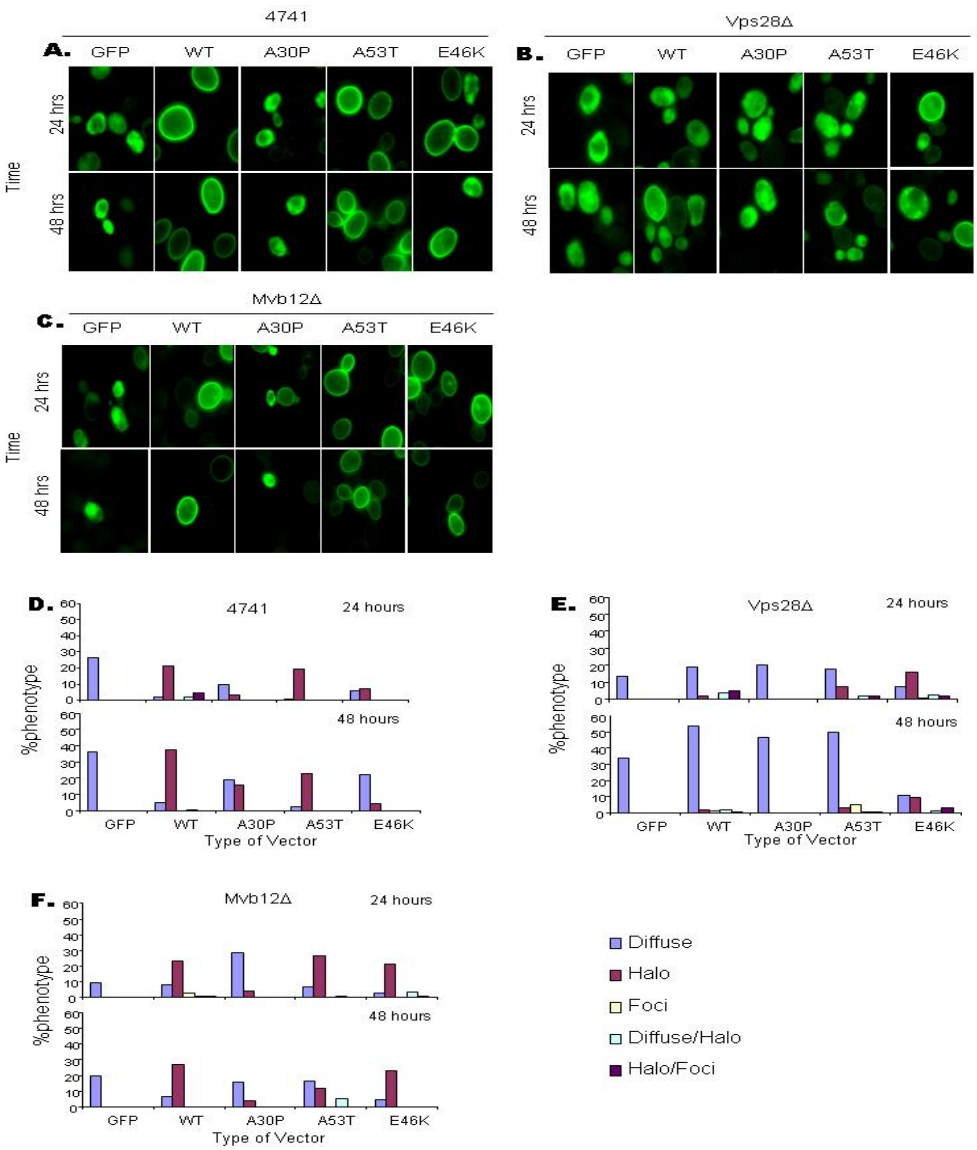

Figure 2. Change in a-Synuclein Localization in Vps28D. WT, A30P, A53T, and E46K a-synuclein with C-terminus GFP tags localization was visualized in BY4741, vps28D, and mvb12D. Images were taken at 24 and 48 hours post-induction. Approximately 750 cells were counted for each construct and percent phenotypes were plotted against time. A and D) WT and A53T a-synuclein in BY4741 was membrane localized (halo) at 24 and 48 hours. E46K was memebrane localized at 24 hours and was cytoplasmically diffuse (diffuse) at 48 hours. A30P was cytoplasmically diffuse at both time points. B and E) In vps28D WT, A30P and A53T a-synuclein was predominantly cytoplasmically diffuse. C and F) In mvb12D a-synuclein localization was the same as in 4741.

have provided evidence suggesting that α-synuclein is degraded by the lysosome (Lee et al., 2004; Cuervo et al., 2004). However, genetic evidence is needed to further support this hypothesis. Our study provides some insight into α-synuclein degradation via the MVB/lysosome pathway.

Compromising the MVB/lysosome Pathway Alters α-Synuclein's Membrane Localization

Our preliminary data implicates the MVB/endosome pathway in the regulation of α-synuclein localization, suggesting that the lysosome is a site for α-synuclein degradation. In our study, an ESCTR-1 sorting protein known as vps28 was knocked out. Subsequently, α-synuclein shifted from being primarily localizing on the membrane to being largely diffuse throughout the cytoplasm. This suggests that vps28 has a role in α-synuclein's phospholipid interaction. Now in the cytoplasm, α-Synuclein is susceptible to the formation of intracellular inclusions and will no longer be degraded by the lysosome.

However, the vps28 knockout had no major effects on toxicity patterns or on α-synuclein expression. E46K cells were the only cells that showed toxicity in vps28Δ E46K toxic growth pattern in vps28Δ was similar to the E46K toxic effect in BY4741 parent strain. When comparing parent strain and vps28Δ E46K growing curves, both were very similar, indicating that the toxic effect observed in the vps28Δ strain was simply due to the E46K mutation and not to the knocked out vps28 protein. Overall, with the exception of E46K in mvb12Δ, cells had no change in growth patterns, suggesting that cells lacking these MVB proteins remain as healthy as parent strain cells.

We also know that not all proteins in this degradation pathway have an effect on α-synuclein. When mvb12, another ESCRT I protein, was knocked out, there

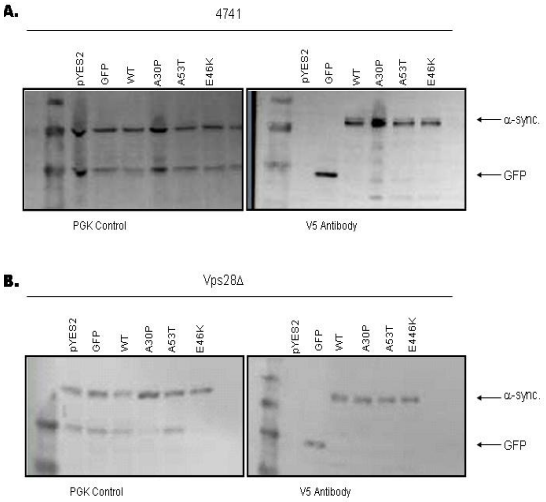

Figure 3. a-Synuclein Expression Analysis in BY4741 Parent Strain and Vps28D. Westem blots analysis was used to evaluate a-synuclein expression in BY4741 and vps28D 24 hours post induction. PGK antibody was used as a loading control. GFP and pYES2 were also controls. A) WT, A30P, A53T, and E46K α-synuclein have equal expression in 4741; B) WT, A30P, A53T, and E46K a-synuclein also show equal expression in vps28D.

was no change in α-synuclein localization, toxicity, or expression. While ESCRT-1 is involved, not all of its protein components may be critical for the regulation of α-synuclein degradation. This also suggests that other proteins or components in this pathway may be essential for the degradation pathway, but are not specific to the regulation of α-synuclein degradation, and that only some are necessary.

Absence of α-Synuclein Toxicity

The first question to answer relates to the seeming paradox evident in cells with compromised degradation pathways: why do such cells grow healthily and show no signs of toxicity? One reason for this is the fact that membrane localization is needed for toxicity. In vps28Δ cells, which show different membrane localization, α-synuclein is no longer found on the membrane. Instead, it is often located in the cytoplasm. However, we also realize that cells with membrane localization, including the parent and mvb12 strain, are also healthy. This is because in order for α-synuclein to have a toxic effect, membrane localization is necessary. A second reason is that, because the lysosome is being compromised, the proteasome may be activated in order to compensate for lysosomal loss. Since α-synuclein is found in the cytoplasm of vps28Δ cells, the proteasome would be the likely degradation site, which is also known to play a role in α-synuclein degradation (Cummings *et al.,* 1999; Walter *et al.*, 2001; Ma *et al.* 2002). Finally, protective factors or responses may be induced when there is lysosomal dysfunction in the cell.

Our data, showing that a compromised MVB/lysosome degradation pathway alters α-synuclein localization, provides preliminary evidence that α-synuclein is degraded by the lysosome and uses the MVB pathway to get to the lysosome. Although we observed no toxicity or change in α-synuclein expression, we did observe a change in α-synuclein localization with the elimination of one MVB protein. In this study we have shown that although not all proteins in the MVB/endosome pathway play a role in α-synuclein localization, ESCRT-1's vps28 protein does indeed play a role, implicating the pathway for α-synuclein degradation.

Future research will complete analysis on remaining ESCRT I proteins and other ESCRT complexes for their ability to regulate α-synuclein misfolding, localization, and toxicity. In order to further understand the role of the MVB/lysosome pathway in α-synuclein degradation, we need to first understand which proteins are most important for degradation. Also, we would like to test synergistic effects by combining MVB dysfunction with either pharmacologically compromised proteasomes or induced oxidative stress. Since we suspect that the proteasome is compensating for lysosomal loss, we would expect to see toxicity in cells that have compromised proteasomes and lysosomes. Anther option is to run a western blot for proteasomal enzymes in order to see if there is increased expression of these proteins in vps28Δ, which we would expect. And finally, we must evaluate induction of apoptosis, autophagy, and vacuolar integrity as cellular responses to reduced MVB regulation of α-synuclein degradation.

Methods

α-Synuclein Constructs

Human wild-type and A53T mutant α-synuclein cDNAs were a gift from Christopher Ross (Johns Hopkins University). A30P and E46K were created from wild-type and α-synuclein using site-directed mutagenesis (Invitrogen) and mutations were confirmed by sequencing (University of Chicago). Wild-type and mutant α-synuclein cDNAs were subcloned into the pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). All α-synuclein forms were tagged with GFP using a two-step cloning strategy, with GFP either tagged at the N- or C-terminus. α-Synuclein cDNAs were first subcloned into mammalian expression vectors, pcDNA3.1/C-terminal GFP (Invitrogen) in order to be fused with GFP at the C-terminus. GFP tagged α-synuclein genes were then PCR-amplified and subcloned into pYES2.1/V5-His-TOPO yeast expression vector (Invitrogen). Chemically competent *E. coli* cells were transformed with the α-synuclein, α-synuclein-GFP, and GFP-– α-synuclein pYES2.1/V5-His-TOPO vectors. The parent pYES2.1 vector (Invitrogen) and GFP in pYES2.1/V5-His-TOPO vector were used as controls.

Yeast Strains

BY4741 (mat a), vps28Δ (BY4741 parent), and mvb12Δ (BY4741 parent) were purchased from Open Biosystems.

Yeast Expression

α-Synuclein expression plasmid vectors were transformed into budding yeast strains as described in Burke et al. (2000). For selection, yeast cells were grown on synthetic-complete media lacking uracil (SC-Ura). Presence of α-synuclein constructs was confirmed by PCR. The pYES2.1 vector, containing a galactose inducible promoter (GAL1), allowed for regulated α-synuclein expression. Yeast cells were first grown overnight in SC-Ura glucose (2%) or SC-Ura raffinose (2%) media at 30°C and 200 rpm. Cells were washed with water and diluted to log-phase (5 x 10^6 cells/ml) in SC-URA galactose (2%) media to induce expression and grown to the time points desired for various measurements.

Western Analysis

Yeast cells ($2.5x10^7$ cells/mL) were washed in 50 mM Tris (pH 7.5), 10 mM NaN3 and solubilized in Electrophoresis Sample Buffer (ESB; Burke, 2000) containing 2% sodium dodecyl sulfate (SDS), 80 mM Tris (Ph 6.8), 10% glycerol, 1.5% dithiothreitol, 1 mg/ml bromophenol blue, and a cocktail of protease inhibitors and solubilizing agents (1% Triton-X 100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.7 mg/ml pepstatin A, 0.5 mg/ml leupeptin, 10 mg/ml E64, 2mg/ml aprotinin, and 2 mg/ml chymostatin). Samples were run on pre-cast 10-20% Tris-Glycine gels (Invitrogen) using SDS containing running buffer. SeeBlue (Invitrogen) was used as the molecular standard. Gels were transferred to PVDF membranes and Western blot was performed with different monoclonal antibodies using standard protocols and detected for alkaline phosphatase activity: anti-V5 (Invitrogen) and anti-phosphoglycerokinase (PGK; Molecular Probes) for most expression experiments, as a measure of loading control in duplicate blots.

Growth Curve Analysis
Cells were grown in 10 ml SC-URA glucose overnight at 30°C, 200 rpm. Cells were harvested at 1500 x g for 5 min at 4°C, and were washed twice in 5 ml H20. Cells were re-suspended in 5 mL H_20 and were counted. Flasks with 25 ml SC-URA galactose were inoculated to $2.0x10^6$ cells/ml density. At 0, 3, 6, 12, 18, 24, 36 and 48 hours, and in duplicate measurements, 1 ml of cell culture was removed and placed in a cuvet to measure absorbance using a Hitachi U-2000 Spectrophotometer. Averaged absorbance readings were plotted against time to produce a growth curve.

GFP Microscopy
Cells were grown overnight in 10 ml SC-URA glucose at 30°C at 200 rpm. Protein expression was induced with SC-URA galactose media. After a time specific induction, cells were harvested at 1500xg at 4°C for 5 minutes and were washed twice with 5 ml H_20. Cells were re-suspended in 100-1000ml SC-URA glucose, and 10 ml cell suspension was pipeted onto a slide. Cells were visualized under a Nikon TE2000-U fluorescent microscope and images were acquired and analyzed using Metamorph 4.0 imaging software.

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