Using fluorescence microscopy to determine whether Gal80p subcellular redistribution is a key mechanistic element of the GAL gene switch.

A Senior Honors Thesis

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One of the most important mechanisms driving cellular functions is transcriptional regulation of gene expression. Changes in gene expression are responsible for driving adaptations to environmental changes, as well as driving development and cellular differentiation. Transcriptional regulation is one of the important ways by which cells control their protein levels, so understanding the many mechanisms that cells use to control gene expression at the transcriptional level is paramount to figuring out how a cell works. Transcription involves the recruitment of RNA polymerase to the promoter of a gene to facilitate the creation of an mRNA copy of the gene. Regulation of this process is necessary to stop unneeded genes from being transcribed and to make sure that required genes continue to be transcribed.

Our lab works on a transcriptional regulator switch in the eukaryotic budding yeast, *Saccharomyces cerevisiae*. The yeast have both asexual and sexual modes of reproduction, resulting in haploid and diploid life cycles, respectively. The facile genetics offered by haploid and diploid yeast provide powerful analytical approaches. Moreover, powerful molecular genetic tools such as targeted gene replacement make budding yeast an excellent model organism for studying transcriptional regulation. Research in this area is relevant to medicine since the mechanisms of transcription are mainly conserved from yeast to humans.

One of the most genetically well-studied transcriptional switches is the galactose (GAL) gene switch in *S. cerevisiae* (1, 2, 3, 4). This gene switch controls the expression of twelve genes involved in galactose metabolism, with most encoding for enzymes. All GAL genes are transcribed at very low levels in the absence of galactose, and are rapidly induced (within 4 minutes) in the presence of galactose only if glucose is absent.

Glucose inhibits activation of the GAL genes, as yeast, as well as many other organisms, have evolved to utilize the preferred carbon source first (Fig 1).

The GAL gene switch is composed of three proteins: Gal4p, Gal80p, and Gal3p. Gal4p specifically binds to a 17 base-pair sequence in the upstream activator sequence of the galactose gene promoter (UAS_{gal}), and also contains an activation domain (AD) required for recruitment of transcription machinery to the promoter. The Gal4AD has been shown to bind to SAGA, TBP, TFIIB, and other mediator proteins of the transcriptional machinery (5, 6, 7). SAGA and other transcription factors assemble at the promoter to form a pre-initiation complex (PIC) (6). The PIC is required for RNA polymerase II recruitment (6). Gal80p is bound to Gal4p in the absence of galactose and blocks the AD of Gal4p, inhibiting the binding events required for recruitment of RNA polymerase. GAL genes are activated by a series of events involving the three regulator proteins, beginning with the binding of a galactose molecule and ATP to Gal3p. Galactose-and-ATP-bound Gal3p relieves Gal80p's inhibition of Gal4p (8, 9, 10). The GAL switch controls the expression levels of Gal80p and Gal3p, which are upregulated in the presence of galactose (2).

How Gal3p relieves Gal80p's inhibition of Gal4p is controversial. Since the first evidence from Leuther and Johnston in 1992 suggesting non-dissociation, a non-dissociation model has been widely accepted, based on the idea that Gal3p can enter the nucleus and bind to the Gal4p-bound Gal80p (Fig 2) (11). According to the non-dissociation model, the binding of Gal3p changes Gal80p's conformation and Gal80p shifts to an alternate binding site on Gal4p, relieving inhibition of Gal4p's activation domain (11). This model predicts an activated three-protein complex, in which Gal4p

would be actively recruiting RNA polymerase and promoting transcription of the GAL genes. A Gal3p-Gal80p-Gal4p tripartite complex was demonstrated *in vitro* by Platt and Reece in 1998 using an electrophoretic mobility shift assay (EMSA) with 30-fold excess of a Gal3 mutant protein (12). This non-dissociation model has been widely featured in review articles and textbooks (13).

Beginning in 2000, published work from our lab has suggested a different model for the GAL gene switch mechanism. Gang Peng, a former graduate student in Dr. Hopper's lab, showed through indirect immuno-fluorescence experiments and by cell fractionation analysis that Gal3p is detectable only in the cytoplasm and that Gal80p is in both the cytoplasm and the nucleus (14). Gang Peng also demonstrated that induction was not impaired by the anchoring of Gal3 protein to the inner plasma membrane and intracellular vesicle membranes with a N-myristoylation sequence (Myr-Gal3p), or by anchoring Gal3p to the outer mitochondrial membranes using a signal anchor sequence (Mom-Gal3p) (15). By tagging Gal80p with green fluorescent protein (GFP) and a nuclear localization sequence (NLS), he also showed by creating heterokaryons (diploids with two, non-fused haploid nuclei) that Gal80p could shuttle between the nucleus and cytoplasm (14). The heterokaryon data suggests that Gal80 contains a nuclear export signal (NES) in addition to an NLS, since Gal80 was able to cross the nuclear membrane in both directions. Vepkhia Pilauri, another member of Dr. Hopper's Lab, also demonstrated using fluorescence recovery after photobleaching (FRAP), that Gal80p shuttling between the nucleus and cytoplasm is rapid (Pilauri and Hopper, unpublished data).

The data from Gang Peng's work (14, 15) argue against the current textbook non-dissociation model, as Gal3p could not directly bind to the Gal80-Gal4 protein complex *in vivo* if Gal3p was present only in the cytoplasm. Gang Peng also showed by making Gal80p more nuclear by addition of an SV40-NLS, that induction was impaired (14). Moreover, he showed by chromatin immunoprecipitation (ChIP) that the occupancy of Gal80p on Gal4p at the promoter decreases substantially in response to galactose (15).

The results of Gang Peng's experiments, cited above, support a dissociation model, in that binding of galactose-induced Gal3p to free-floating Gal80p in the cytoplasm shifts the equilibrium of Gal80p to make it more cytoplasmic and less nuclear. Such a decrease in the steady-state concentration of Gal80p in the nucleus would make Gal80p less likely to be associated with DNA-bound Gal4p, relieving Gal80p's inhibition of Gal4p's transcription activation domain. This dissociation model was published in PNAS in 2002 (15) (Fig 3).

My project was aimed at testing the idea that the concentrations of Gal80p in the cytoplasm and nucleus change when the cells are exposed to galactose. My approach has been to use the intensity of light emitted by double-mCitrine-tagged Gal80p in fluorescence microscopy to measure nuclear and cytoplasmic concentrations of Gal80p before and after induction. By defining the nucleus and cytoplasm using a nuclear marker, H2B-mCherry, I aim to assess whether Gal80p does indeed redistribute to become more cytoplasmic and less nuclear upon induction, as predicted by our model.

Materials and Methods

The yeast strains used in this project were derived from lab strain Sc723 (*MATa ade1 ile leu2-3,112 ura3-52 trp-HIII his3-Δ1 MEL1 LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3*) as

previously described by Blank *et al.* 1997 (8) including *gal3Δ* strain Sc724 (*MATa ade1 ile leu2-3,112 ura3-52 trp-HIII his3-Δ1 MEL1 LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3 gal3-Δ3::LEU2*) and *GAL80s-2* mutant strain Sc729 (*MATa ade1 ile leu2-3,112 ura3-52 trp-HIII his3-Δ1 MEL1 LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3 GAL80^{s-2}*). Sc723, Sc724, and Sc729 were transformed by Dr. Fenglei Jiang with a ~3.6kb SmaI-SnaBI cassette from pFJ46KAN containing two copies of mCitrine (YFP) along with a kanamycin resistance marker flanked by regions of homology for the endogenous *GAL80* locus. Sc723-46K named as Sc856, Sc724-46K named as Sc857, and Sc729-46K named as Sc868. Sc856, Sc857, and Sc868 were the strains used in my project.

I transformed the yeast strains with a plasmid carrying a gene for mCherry-tagged DNA histone H2B and a tryptophan selectable marker (gift from Dr. Fenglei Jiang). For imaging, yeast cells were grown to mid-log phase at 30°C in synthetic tryptophan dropout media with 3% v/v glycerol, 2% w/v lactic acid, extra adenine (.001% w/v), 1% w/v raffinose, and 0.05% w/v glucose (16).

PCR-based confirmation of the strains used in this study was performed using yeast colony PCR based on a protocol from the Amberg Lab at SUNY Upstate Medical University. Primers used were specific for the 3` end of the *GAL80* locus (G80seq1) and the 5` end of the fluorescent tag (FP-int). The G80seq1 primer sequence was GTTCCAGTGTCATGCAGT and the FP-int primer sequence was CTTCACCTTCACCGGAGACAG. PCR conditions were as follows: one yeast colony, 200 µl/ml Zymolyase 100T, 625 µM of each primer, 200 µM each dNTP, and 100 µg/ml BSA in a 50µl Taq reaction mixture. The PCR cycles were as follows: 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min

at 72°C, and then an extra 10 min at 72°C.

Semi-quantitative yeast colony growth assays were conducted using yeast bearing the *HIS3*-reporter under the control of the *GAL1* promoter. Growth was assayed on synthetic histidine dropout plates at 30°C with 10 mM 3-AT, 2% w/v lactic acid, 3% v/v glycerol, 0.05% w/v glucose (16). The +gal plates also contained 2% w/v galactose.

For fluorescence microscopy, cells were incubated with 25 μg/ml cycloheximide for 10 min prior to addition of galactose for induced samples. Induced samples were incubated in 2% w/v galactose for 15 min. Non-induced samples were incubated for 15 minutes without the addition of galactose after 10 minute incubation in cycloheximide.

After incubation, induced and non-induced cells were spun down to increase cell density and then wet mounted onto a thin pad of 2% w/v agarose to reduce random cell motion during imaging. Images were acquired using a Zeiss Axioplan 2 microscope using a digital-imaging system designed by 3I Imaging of Colorado. Images were captured and analyzed using Slidebook 4.2 software for PC. Images were captured between 15 and 25 minutes of induction to minimize temporal variation of Gal80p expression.

When using the Slidebook 4.2's image-analysis capability to obtain data, it was necessary to consistently and accurately define the nucleus and cytoplasm in each analyzed cell using two separate masks, one for each defined area. A mask is a virtual overlay of an image that is used to isolate specific areas of the image without modifying the original image. I defined the nuclear mask as the area of the cell expressing mCherry above a minimum threshold. The cytoplasmic mask was defined as the area of the cell below the mCherry threshold but with expression of YFP above a minimum threshold

intensity. This minimum threshold of YFP for the cytoplasm is needed to exclude the cells' environment from the measurements. In other words, I used mCherry expression to define the nucleus, while using the YFP minimum threshold intensity to exclude the extracellular space and define the cytosol. Because of the variation between slides and cultures, minimum thresholds were based on the intensity of the fluorescence in each image.

Results

My first attempts to measure Gal80p levels in the nucleus and cytoplasm relied on Gal80p tagged with mCitrine (a variant of yellow fluorescent protein or YFP) expressed from a plasmid. To clarify, I will refer to mCitrine as YFP for the remainder of this paper. Expression from a plasmid yielded inconsistent results due to variations in plasmid copy number per cell. To obtain more consistent expression levels of double-YFP-tagged GAL80p, I elected to use a yeast strain with a *2YFP GAL80* fusion gene cassette integrated into the yeast chromosome via homologous recombination onto the 3` end of the endogenous copy of *GAL80* (gift from Dr. Fenglei Jiang).

Parallel experiments performed independently by myself and Dr. Fenglei Jiang have determined that double-YFP gives a brighter signal than a single copy of YFP in microscopy experiments. Incorporating the gene into the genome negates the need for the cells to carry a plasmid carrying the *GAL80-2YFP*. This makes Gal80-2YFP protein concentrations among cells more consistent, since gene expression from a plasmid can be quite variable. Genomic integration of the fluorescent tag was confirmed by PCR analysis and fluorescence microscopy. Also, I performed galactose-induction *HIS3*-reporter spotting growth assays to assess whether the relatively large fluorescent tag

interferes with Gal80p's function. Fortunately, tagging Gal80p with two copies of YFP does not affect the function of the GAL switch as a whole.

It was important for my measurements that I could determine the location of the nucleus in each cell. Because I was only concerned with measuring the intensity of YFP from Gal80p, it was not necessary to integrate the mCherry into the H2B locus to improve consistency among cells. Successful identification of the nucleus with mCherry allowed me to begin collecting data.

Variations in plasmid copy number of H2B-mCherry between cells made it necessary to create three to five separate masks defining the nuclei. This made the nuclei more sharply defined overall, and made it easier to include more cells within the summed nuclear mask, created by combining the three to five separate masks.

Because Gal80p expression is controlled by the GAL switch, nacent Gal80p protein being produced on the ribosomes in the cytoplasm between the time of induction and image acquisition could artificially increase the Gal80p intensity in the cytoplasm. Cycloheximide was used to inhibit all protein synthesis in the yeast cells. Margery Evans, a member of Dr. Hopper's Lab, has demonstrated by western blots that cycloheximide does indeed halt production of Gal80p, as well as other proteins (Evans and Hopper, unpublished data).

The *GAL80-2YFP* (*GAL3* WT) test strain exhibited a nuclear to cytoplasmic YFP intensity ratio that was 15-22% lower in induced cells than in non-induced cells (Figs. 4 and 5) ($p < 10^{-14}$, Student's t-test). Though modest, the difference in nuclear intensity between induced and uninduced cells is detectable by eye (Fig 6). This change in the intensity ratio was similar in cells treated with cycloheximide ($p < 10^{-6}$, Student's t-test),

suggesting that during 15-25 minutes of induction, in non-treated cells, newly synthesized Gal80-2YFPp does not have a noticable effect on measured intensity. I also tested negative control strains including a *gal3Δ* deletion strain and *GAL80s-2* super-repressor mutant, which are both defective for the Gal3p-Gal80p interaction (Fig 4 and 5).

The *gal3Δ* deletion strain (8, 14) contains *GAL3* with a *CaURA* insertion that makes the protein non-functional. Yeast with a *gal3Δ* deletion demonstrates a non-inducible phenotype, as the non-functional Gal3p is unable to bind Gal80p in the presence of galactose. The fluorescent *gal3Δ* strain demonstrated a negligible intensity difference between induced and non-induced cells. The *GAL80s-2* mutant is a dominant, non-inducible mutant that is able to bind to Gal4p but not to Gal3p (17, 18, 19, 20). Like the *gal3Δ* deletion strain, *GAL80s-2* did not demonstrate an appreciable redistribution of Gal80p. Importantly, this observed lack of a redistribution in the two control strains establishes that the redistribution of Gal80p is dependent on the Gal3p-Gal80p interaction.

Discussion

The data presented here leads to two important conclusions: Gal80p redistributes to become less nuclear upon induction with galactose, and the Gal80p redistribution is dependent upon the Gal3p-Gal80p interaction. Also, observation of a redistribution of Gal80p upon galactose induction in the presence of cycloheximide suggests an increase in Gal3p, Gap80p, and Gal4p expression is not required for the GAL switch to remain active 15-25 minutes after induction, though as the proteins degrade over time, GAL

switch function may become highly variable, depending on which proteins in the switch degrade more quickly.

To express the differences in intensity, I have elected to report the data as a nuclear to cytoplasmic ratio. It has been shown that the size of the nucleus of an average *S. cerevisiae* cell tends to be consistent at 7% of total cell volume (21). This suggests that a decrease in the fluorescence intensity of the nucleus would result in a relatively small increase in the cytoplasmic intensity. In 2006, Ramsey *et al.* reported that there is a 7-fold excess of Gal3p over total cellular Gal80p (22). Using a GFP-reporter, Gang Peng demonstrated with western blots using antibody against GFP that there was an approximate 8- to 12-fold excess of Gal3p over Gal80p (Peng and Hopper, unpublished data). Dr. Xiaorong Tao, another member in the Hopper Laboratory, also did the same experiment using a 6HIS tag. He also found an approximate 8-fold excess of Gal3p (Tao and Hopper, unpublished data).

Data from Ramsey *et al.* also indicates that the steady-state distribution of Gal80p is 50% nuclear and 50% cytoplasmic (22). Assuming Gal3p is only cytoplasmic and that Gal3p binds Gal80p in a 1:1 monomer-to-monomer ratio (23), data from Ramsey *et al.* suggests that there is roughly a 14-fold excess of Gal3p over Gal80p in the cytoplasm. My results suggest that roughly 22% of nuclear Gal80p becomes cytoplasmic upon induction, so overall distribution of Gal80p in the cell would become 39% nuclear and 61% cytoplasmic. Correcting for the volume difference between the cytoplasmic space and the nucleus—assuming the cytoplasm is 93% of the cell volume—a 22% drop in intensity in the nucleus after induction translates to only a 1.56% increase in intensity in the cytoplasm. Also, because the cytoplasmic space is so much larger than the nucleus,

images of a single cross-section of the yeast cell reveal a higher percentage of the total nuclear size than of the total cytoplasmic size, which would make the intensity decrease detected in the cytoplasm even less than the estimated 1.56%.

However, this estimate assumes that 93% of the cell is cytoplasm. Because of the cell volume taken up by other organelles and membranes, namely the vacuole, the cytoplasmic volume would be only a fraction of the non-nuclear volume. Measurements of the vacuole put it at about 25% of total cell volume (24). This would shift the estimated cytoplasmic volume to about 68% of the cell volume. This means the increase in cytoplasmic intensity would be closer to 2.27%. So in reporting my data, I should be able to use the relatively constant cytoplasmic intensity to correct for differences in total Gal80p intensity between cells and between images. Importantly, the fact that there is only an estimated 2.27% increase in cytoplasmic intensity indicates that the intensity increase in the cytoplasm predicted by our model cannot be detected using this method.

It is critical to determine whether the redistribution of Gal80p observed in my experiments would be significant enough to allow DNA-bound Gal4p to become activated. However there is a wide range of estimates of the concentrations of Gal4p and Gal80p in the nucleus, as well as different measurements of the K_D for Gal80p's binding to Gal4p. For example, using the estimates from Ramsey *et al.*, I calculated a Gal4p nuclear concentration, both DNA-bound and free-floating, of about 205 nM (22). In contrast, Anders *et al.* estimate the Gal4p concentration in the nucleus to be about 80 nM (25). According to titration experiments performed by Melcher and Xu, the K_D of Gal80p, when allowed to bind to 0.09nM DNA-bound Gal4p, was 0.3 nM (26). However, Lohr *et al.* note the K_D of Gal80p for DNA-bound Gal4p to be 5 nM (2).

It is also important to point out that it is unknown whether the dimer, monomer, or both forms of Gal80p are able to shuttle, and that it is also unknown what form of Gal80p is able to bind to Gal4p. Without knowing these details, it is not feasible to calculate the affect of a 22% drop in nuclear Gal80p concentration on the GAL switch, due to the lack of measurements made under similar conditions for all the necessary data, namely the K_D of Gal80p for Gal4p, concentrations of Gal80p and Gal4p, and the number of Gal4p associated with the DNA at any one time.

Future Work and Acknowledgements

Future work could include extensions of the experiments, taking advantage of additional genetic controls. For example, deletion of *GAL4* should render the gene switch inactive, but would be expected to result in an observable redistribution after galactose induction, allowing us to view the redistribution without an increase in protein levels due to GAL gene transcription, and without using cycloheximide. One could also take advantage of Gal3p anchored to membranes within the cytoplasm using the N-Myr and Mom tags discussed earlier on the redistribution of Gal80p. The prediction is that Gal80p redistribution would still occur with anchored Gal3p. Additional negative controls could include mutants of Gal3p that fail to bind to Gal80p (*Gal3*^{80NB}) as an alternative to deletion of *GAL3*, and testing of additional *GAL80s* super-repressor mutants using cycloheximide.

Another major experiment would be to capture the redistribution of Gal80p in real-time using confocal microscopy looking at early induction. This could be done using the strains similar to the one used in my experiments. However, this would have to be done with a more stable fluorophore than mCitrine, as mCitrine becomes photobleached

easily (27). Using a more photostable fluorescent tag would allow many pictures of the same cell without a major loss in fluorescence intensity due to photobleaching.

A very informative experiment to test a key prediction of our model would be to use fluorescence resonance energy transfer (FRET) to observe the Gal3p-Gal80p interaction before, during, and after induction. According to our dissociation model, the FRET signal should initially occur only in the cytoplasm. The possible ability of Gal80p to pull Gal3p into the nucleus when they are bound after galactose induction has not been tested. If Gal80p could in fact pull Gal3p into the nucleus, the FRET signal would also be visible in the nucleus after a time.

It would be very informative to measure the concentrations of free-floating Gal4p in the nucleus, DNA-bound Gal4p, as well as Gal80 and Gal3 concentrations in vivo, to allow for a calculation of the affect a redistribution of Gal80p on the GAL switch.

This project was conducted in the lab of my advisor, Dr. James E. Hopper. My experiments are to be contributed as part of a broader-content manuscript that will be submitted for publication by Dr. Fenglei Jiang, who is doing her post-doctoral work in Dr. Hopper's lab. I wish to thank Dr. Fenglei Jiang for her invaluable guidance during my time in the lab and also for her gifts of strains and plasmids used in my research. I would also like to thank Dr. James E. Hopper for allowing me to work in his lab, for providing the funds for my research through NIH, and for critical evaluation of the manuscript. Finally, I wish to thank my colleagues Margery Evans, Onur Egriboz, and Dr. Xiaorong Tao for stimulating discussions and encouragement, and the College of Arts and Sciences Honors Committee at The Ohio State University for the ASC Research Scholarship given in recognition of my research.

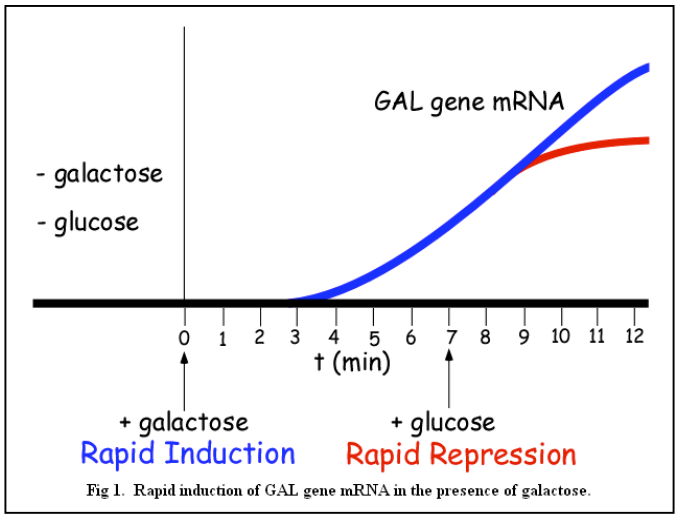

Fig 1. Rapid induction of GAL gene mRNA in the presence of galactose.

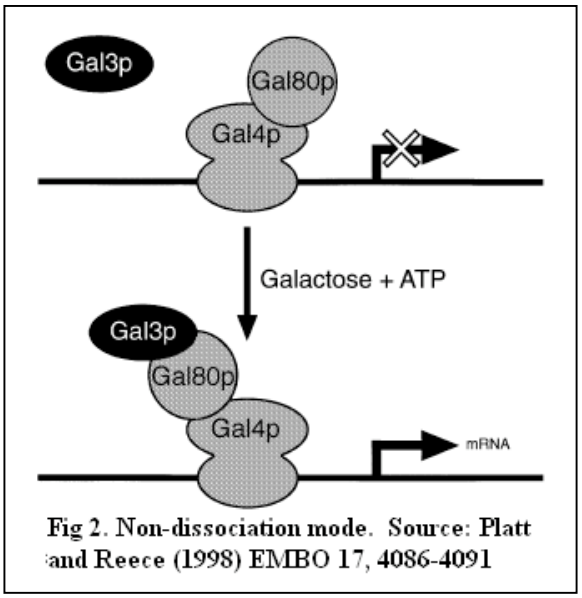

Fig 2. Non-dissociation mode. Source: Platt and Reece (1998) EMBO 17, 4086-4091

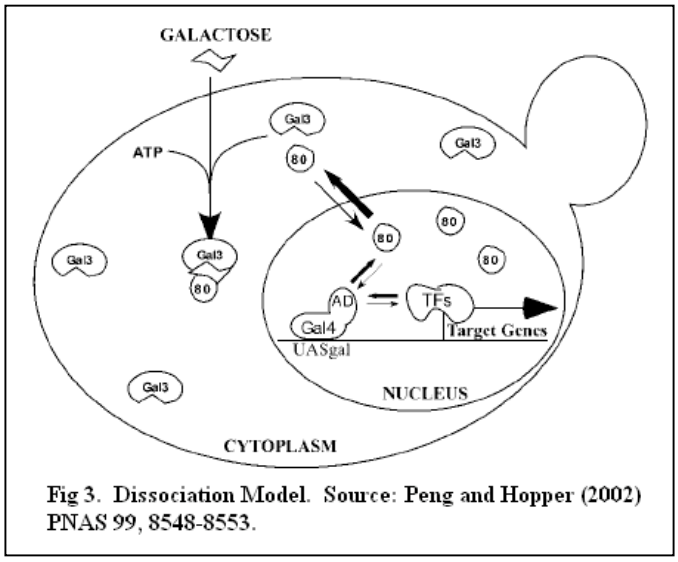

Fig 3. Dissociation Model. Source: Peng and Hopper (2002) PNAS 99, 8548-8553.

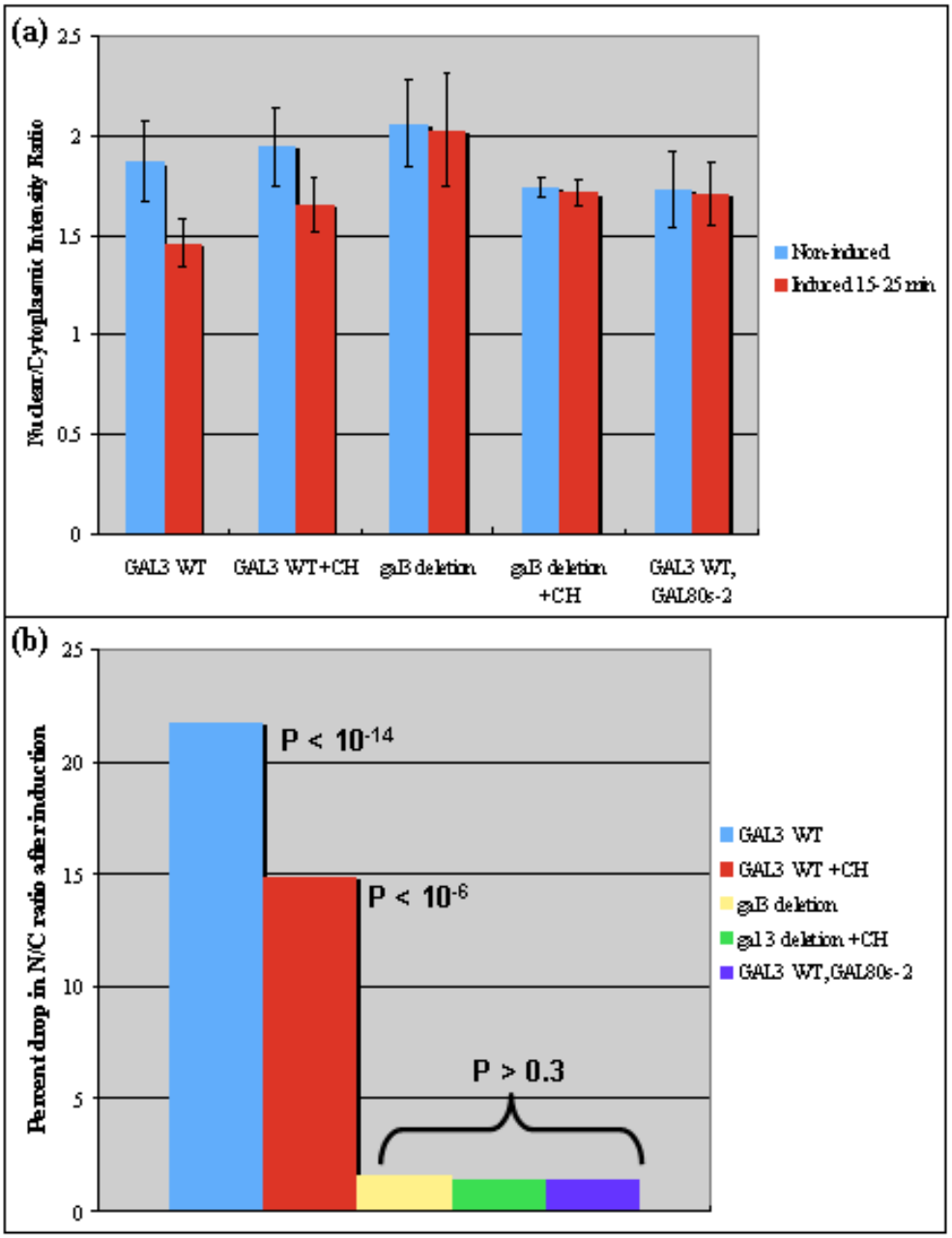

Fig 4. Graphical intensity data gathered from images of Gal80-2YFP protein in *GAL3* wildtype, *gal3Δ* strains and *GAL80s-2*. No cycloheximide data for *GAL80s-2* mutant. (a) Comparison of nuclear/cytoplasmic intensity ratio of induced to non-induced in all test strains. (b) Percent drop of nuclear/cytoplasmic ratio in all test strains.

		Non-induced	Induced	Comparison**	
GAL3 WT	average intensity	**1.862**	**1.457**	% difference	21.7508
	standard deviation	0.2005	0.1227	difference	0.405
	# of images	70	35	p-value†	9.4E-14
	# of cells (est*)	457	131		
GAL3 WT +CH	average intensity	**1.942**	**1.652**	% difference	14.9331
	standard deviation	0.1992	0.1323	difference	0.29
	# of images	42	13	p-value†	5.1E-06
	# of cells (est*)	789	362		
gal3 deletion	average intensity	**2.061**	**2.028**	% difference	1.60116
	standard deviation	0.2179	0.2863	difference	0.033
	# of images	30	14	p-value†	0.3198
	# of cells (est*)	101	71		
gal3 deletion +CH	average intensity	**1.737**	**1.712**	% difference	1.43926
	standard deviation	0.055	0.0685	difference	0.025
	# of images	12	6	p-value†	0.67831
	# of cells (est*)	135	75		
GAL3 WT, *GAL80s-2*	average intensity	**1.728**	**1.703**	% difference	1.44676
	standard deviation	0.1925	0.1571	difference	0.025
	# of images	65	37	p-value†	0.49935
	# of cells (est*)	189	106		

Fig 5. Intensity data for *GAL3* WT strains and control strains *gal3* deletion and *GAL80s-2* mutant. *Total cell counts are estimates based on the measured volume of cytoplasmic and nuclear masks. **Comparison made between non-induced and induced cells. †p-value determined using two-tailed Student's t-test.

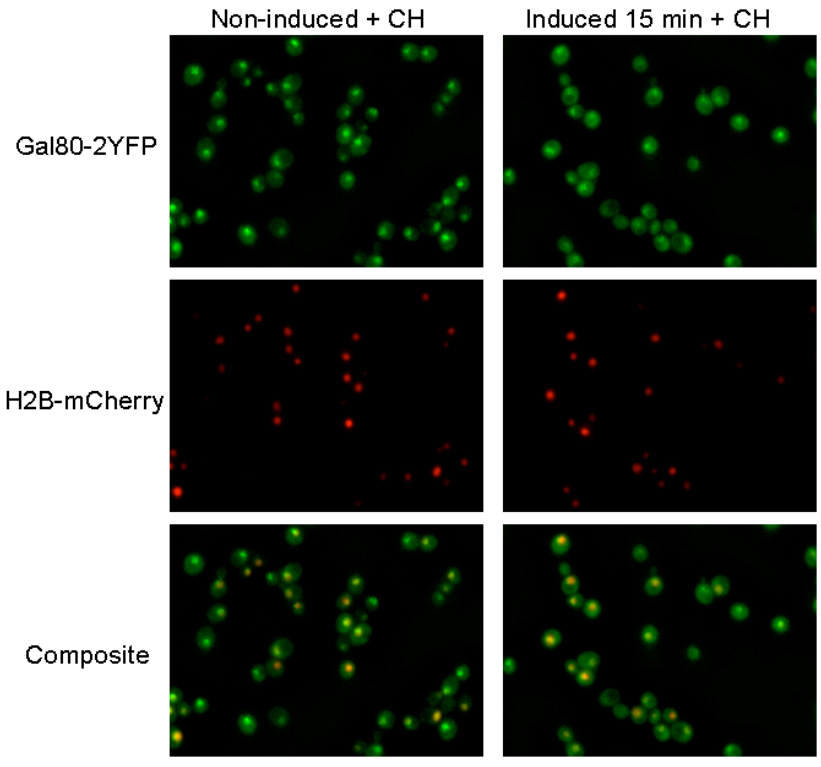

Fig 6. *GAL80* tagged with two-copies of YFP transformed with a vector containing *H2B* tagged with mCherry (nuclear marker) in a *GAL3* WT background.

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