Video Article

Analysis of the Development of a Morphological Phenotype as a Function of Protein Concentration in Budding Yeast

Debarati Mukherjee[1], Arpita Sen[1], R. Claudio Aguilar[1]

[1]Department of Biological Sciences and Purdue Center for Cancer Research, Purdue University

Correspondence to: R. Claudio Aguilar at claudio@purdue.edu

URL: http://www.jove.com/video/1863
DOI: doi:10.3791/1863

Keywords: Microbiology, Issue 37, Morphological phenotype, protein concentration, live cell imaging, cell division

Date Published: 3/24/2010

Citation: Mukherjee, D., Sen, A., Aguilar, R.C. Analysis of the Development of a Morphological Phenotype as a Function of Protein Concentration in Budding Yeast. *J. Vis. Exp.* (37), e1863, doi:10.3791/1863 (2010).

Abstract

Gene deletion and protein overexpression are common methods for studying functions of proteins. In this article, we describe a protocol for analysis of phenotype development as a function of protein concentration at population and single-cell levels in *Saccharomyces cerevisiae*. Although this protocol is based on the overexpression of a protein, it can easily be adapted for morphological phenotypes dependent on suppression of protein expression. Our lab is interested in studying the signaling properties of the endocytic adaptor protein epsin. To that purpose we used a dominant negative approach in which we over-expressed the conserved Epsin N-Terminal Homology (ENTH) domain in order to interfere with the functions of endogenous epsin-2 (Ent2 or YLR206W). We observed that overexpression of the ENTH domain of Ent2 (ENTH2) in wild type cells led to a cell division defect that is dependent on the mislocalization of a family of scaffolding proteins, septins.

Video Link

The video component of this article can be found at http://www.jove.com/video/1863/

Protocol

A. Cell Culture and Protein Induction

This section describes cell culture conditions, cell-cycle synchronization with Nocodazole, followed by induction of protein expression from a regulatable promoter in yeast cells.

1. The wild type yeast strain W303 (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) is transformed with high copy (2μ) plasmid DNA that contains our gene of interest (ENTH2) under the control of a methionine-repressible promoter *(MET25*). 2mM methionine is sufficient to suppress promoter activity, while media lacking methionine allows maximal expression. Hence, a range of methionine concentrations can be used to express the protein at the desired level. 1 Transformation of plasmid DNA in W303 cells is done using the LiAc-TE method according to standard procedures.[2]

   Note: This experiment should be performed by growing the cells overnight in the presence of 2mM methionine to ensure protein expression only at background levels. Next morning, protein expression should be induced by resuspending the cells in media lacking methionine immediately following cell-cycle synchronization (approximately 4h). However, we have found that the ENTH2-dependent cell division phenotype is induced only after a considerable concentration of ENTH2 has built up in the cells (usually after 4-6h of protein induction[3]), making the time course for analysis of all manifestations of the phenotype very lengthy (at least 16h). We have found that growing the cells overnight in the presence of 0.2mM methionine represses the morphological phenotype but allows subcritical protein concentration that can be rapidly increased to phenotype-inducing levels when transferred to media lacking methionine. This permits detailed analysis of severe manifestations of the cell division defect in a relatively shorter period of time. We hence recommend that the experimenter determine the methionine concentration that is sub-threshold for phenotype induction on a case-by-case basis.
2. Pick six colonies from a plate containing recently transformed cells and inoculate in 50 ml of selective media with 0.2mM methionine in a sterilized conical flask using standard sterile techniques. Incubate overnight at 30°C in with shaking at 200 rpm. *(For more information on standard yeast culture conditions, see ref. 4)*
3. Next morning, estimate cell density by measuring the optical density of the culture at 600nm. Transfer the equivalent to 20 OD_{600nm} of cells into a sterile centrifuge tube and harvest by spinning at 2200 rpm for 5min at room temperature.
4. Resuspend cells in YPD at a concentration of 0.4 OD_{600nm}/ml and add Nocodazole to a final concentration of 15μg/ml of from a stock of 1mg/ml in DMSO. Incubate for 4h at 30°C with shaking at 200rpm.

   Note: Alternative arrest methods including arrest at the G0 phase using alpha-factor or at G1-S phase using hydroxyurea can be used. We have found that arrest at mitosis using Nocodazole is simple and effective enough for our purposes.

5. After 4h, check percentage of cells arrested in mitosis by counting the number of cells with equal sized buds by viewing under the microscope. If the arrest is greater than 90%, proceed to the following step. Otherwise, maintain cells in Nocodazole until the desired percentage of mitotic arrest is achieved.
6. Harvest cells by centrifugation at 2200 rpm for 5min and wash with ice cold water three times. After the final wash, resuspend the pellet in selective media lacking methionine at a cell density of ~ 0.5 OD_{600nm}/ml. Transfer half the volume of cells to a new sterile tube and add methionine to a final 0.2mM concentration. This will serve as a control for effects due to basal levels of protein expression. This marks the first time-point of the experiment (time = 0h). Analyze cells every hour for 6h.

B. Analysis of Phenotype Development

Note: All the analysis methods described below do not need to be performed simultaneously.

Determination of protein expression levels by western Blotting

1. At every time-point, measure the OD_{600nm} of the culture and harvest 2 OD_{600nm} equivalents of cells by centrifugation at 2200 rpm for 5 mins.
2. Resuspend the pellet in 1ml sterile water and transfer the suspension to a 1.5ml eppendorf tube. Repeat the centrifugation to obtain a pellet. Aspirate the supernatant.
3. Resuspend the pellet in 50µl SDS-PAGE laemmli sample buffer (50mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and 0.006% bromophenol blue). Add 50µl of acid-washed glass beads (0.2-0.4 microns diameter) and vortex for 1 min.
4. Boil tubes on a 95°C heat block for 2 mins, vortex again for 30 secs and resume boiling for 5 mins. Freeze the protein sample at -20°C.
5. Run protein samples on a 12% SDS-PAGE gel, transfer onto a nitrocellulose membrane and perform Western blotting with appropriate antibodies to detect the protein of interest. In this example, we probe the presence of haemagglutinin (HA) tagged ENTH domains by incubating with mouse anti-HA antibody (Covance, Princeton, NJ) at 1:2000 dilution in Blotto-Tween (1XPBS, 5% non-fat dry milk, 0.1% Tween). HRP tagged goat anti-mouse secondary antibody (Pierce) at a dilution of 1:2500, also in Blotto-Tween, is allowed to bind the primary antibody on the membrane for 1h at room temperature. Supersignal West Pico Chemiluminescent Substrate is used to detect HRP tagged anti mouse antibody.
6. Quantify protein expression levels by densitometry.

This section describes two methods of tracking phenotype development as a function of increasing protein concentration. The first method is cell-population based, while the second studies it at the level of a single cell.

Quantification of cell-division phenotype and cell death

1. At each time-point, transfer 1ml cell suspension to a sterile eppendorf tube using sterile techniques and harvest cells by centrifugation at 2200 rpm for 5 mins.
2. Aspirate the supernatant and resuspend the pellet in 70µl media, followed by 30µl Methylene blue from a stock of 1mg/ml in sterile water. Methylene blue is a blue vital dye that becomes colorless in the reducing environment of the cytosol hence dead cells can be identified by their blue color.
3. Pipette ~6µl of this suspension on a clean glass slide and place a coverslip over the drop. Press the coverslip gently to form a thin, uniform layer of cells between the glass interfaces.
4. Divide the coverslip into 9 quadrants and take 3 images per quadrant as indicated in Fig. 2. The number of cells/field should be no larger than 30 cells to allow accurate quantification. Each coverslip can be imaged for approximately 15 mins before air bubbles damage the preparation.
5. Count the number of cells showing the phenotype under study. The cell division defect induced upon ENTH2 overexpression leads to cell death and hence, we also count the number of dead cells identified by their blue color.
6. Calculate the percentage of phenotypic cells.
7. Plot the data as shown in Fig. 3.

Visualization of phenotype-specific defects using fluorescence microscopy

Cell-division defective cells, as seen upon ENTH2 overexpression, are often characterized by cell wall defects and septin mislocalization3 (visualized as GFP-fusion proteins). The cell wall of budding yeast can easily be visualized by staining with Calcofluor White (CfW) which binds irreversibly to chitin in yeast cell walls.

1. At every time-point, aliquot 1ml of culture in a sterile eppendorf tube and harvest cells by centrifugation at 2200 rpm for 5 mins at room temperature.
2. Resuspend the cell pellet in 100µl of 1mg/ml CfW solution in sterile water and incubate for 5 mins at room temperature in the dark.
3. Harvest cells by centrifugation and wash 3 times with 1X PBS.
4. Resuspend the pellet in 100µl 1X PBS and image under the microscope using UV optics (cell wall) and FITC filter (GFP-septin) at 100X magnification.
5. Quantify percentage of cells with cell wall defect and septin mislocalization by following steps 4-6 in section B.2.1

B.3. Determination of phenotype development at the single cell level: Time-lapse video microscopy.

1. Clean a glass slide with a concave depression with 70% alcohol and allow to air dry.
2. Boil 0.06g agarose in 5ml selective media lacking methionine in a sterile glass tube in a microwave until the agarose dissolves completely.
3. Quickly pipette 200µl of the agarose containing media in the slide depression and invert another clean glass slide over it, making sure that no air bubbles are trapped underneath.
4. When the gel solidifies, move the slide on top smoothly away from the depression slide, keeping their surfaces parallel at all times. This ensures that the surface of the agarose bed is flush with the surface of the depression slide. Clean the slide region surrounding the agarose bed with a delicate tissue. The slide is now ready for use.

5. At time = 4 hours, transfer 1ml of cells from the culture to a sterile eppendorf tube and harvest by centrifugation at 2200 rpm for 5 mins at room temperature.

   Note: It is recommended a later time-point be chosen to assure high levels of protein expression so that phenotype development is guaranteed. Pilot experiments are required to determine induction times on a case-by-case basis.
6. Aspirate the supernatant and resuspend cells in 100µl of fresh selective media lacking methionine.
7. Apply petroleum jelly to the edges of the coverslip. Then place a drop of cell suspension at the center of the agarose bed and spread uniformly by gently pressing a coverslip over them.
8. Seal the edges of the coverslip with petroleum jelly and fix its position by lining the edges with nail polish. Wait until the nail polish dries.
9. Place the slide on a heated stage of a microscope and choose a field that contains no more than 10 cells spaced adequately. As cells divide, the field will get crowded over time.
10. Take an image of the field every 5 minutes for ~5 hours. Phenotype progression can be studied by assembling the images into a movie using ImageJ software (*Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2005*)

Note: We use Zeiss Axiovert 200M microscope, Zeiss Axiocam MRm monochrome digital camera and Carl Zeiss Axiovision image acquisition software (version 4.4) for live cell imaging. Temperature control is achieved by placing slides on a heated stage maintained at 30°C by the PeCon Tempcontrol 37 device.

In order to avoid re-adjusting the focus repeatedly, it is convenient to program the image acquisition software to automatically acquire a few z-stack images at every time-point and choose the image with the best focus later when assembling the movie. Some image acquisition softwares have the option of autofocus for time course experiments. It is convenient to use that option in when available.

Wild type yeast cells grow best at 30°C hence temperature control is critical. In order to ensure adequate heat, it is helpful to provide an external source of heat in addition to the heated stage. For example, we leave the transmitted white light of the microscope on for the entire duration of imaging.

It is also critical to avoid air-bubbles when preparing the agarose bed and cell sandwich. The air entrapped within the bubbles expands when heated over time and tend to push cells away from the field being imaged.

It is also important to ensure that individual cell positions within the field do not change appreciably over the duration of the imaging process, especially during re-focusing attempts. Image stabilization plugins are available for ImageJ to rectify small shifts in cell position after the movie has been assembled. (http://www.cs.cmu.edu/~kangli/code/Image_Stabilizer.html).

Discussion

This protocol describes how to monitor the development of a morphological phenotype (yeast cell unable to undergo proper cell division) upon protein overexpression. When doing this procedure it's important to remember to harvest yeast cells by pelleting at the recommended centrifugation speed as faster speeds may damage cells and obscure results. Methylene blue and Calcofluor white should be added to live cells just prior to imaging as they are toxic. This procedure can also be easily adapted for phenotypes observed under protein repression conditions, provided the target is expressed from a controllable promoter.

Acknowledgements

We thank Brian G. Coon and Claudia B. Hanna for helpful discussions and support. This project was supported by start-up funds from the Dep. of Biological Sciences, Purdue University to R. Claudio Aguilar and an American Cancer Society Institutional Research Grant to R. Claudio Aguilar through the Purdue Cancer Center.

References

1. Rönicke, V., Graulich, W., Mumberg, D., Müller, R. and Funk, M. Use of conditional promoters for expression of heterologous proteins in Saccharomyces cerevisiae. Methods Enzymol.283, 313-322 (1997).
2. Gietz, R. D., and Woods, R. A. High efficiency transformation with lithium acetate in Molecular Genetics of Yeast: A Practical Approach. Oxford University Press Inc., New York.1994. pp. 121-134.
3. Mukherjee, D., Coon, B.G., Edwards, D.F., Hanna, C. B., Longhi, S. A., McCaffery, J. M., Wendland, B., Retegui, L. A., Bi, E., and Aguilar, R. C. The yeast endocytic protein Epsin 2 functions in a cell-division signaling pathway. J Cell Sci 122, 2453-2463 (2009).
4. Sherman, F. Getting started with yeast in Guide to Yeats genetics and Molecular Biology. Academic Press Inc., San Diego. 1991. pp. 3-21.

