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Conditional Depletion of Nuclear Proteins by the Anchor Away System (*ms# CP-10-0125*)

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Abstract

Nuclear proteins play key roles in the regulation of many important cellular processes. In *Saccharomyces cerevisiae*, many genes encoding nuclear proteins are essential. Here we describe a method termed Anchor Away that can be used to conditionally and rapidly deplete nuclear proteins of interest. It involves conditional export of the protein of interest out of the nucleus and its subsequent sequestration in the cytoplasm. This method can be used to simultaneously deplete multiple proteins from nucleus.

Nuclear-localized proteins play key roles in the regulation of a wide variety of important cellular processes such as transcription, DNA replication, DNA repair and many others. In *Saccharomyces cerevisiae*, deletions of many genes encoding nuclear-localized proteins render the resulting strains inviable. While this underscores the critical roles that nuclear proteins play in cellular pathways, the inviability of strains harboring null mutants also makes them difficult to study.

One solution is to use conditional alleles such as temperature sensitive (ts) mutants. Unfortunately, the procedure to isolate temperature sensitive mutants is tedious and labor-intensive. Furthermore, it is often unclear how such mutations affect the function of the protein of interest. In practical terms, inactivating temperature sensitive alleles requires shifting cells to non-permissive temperatures that are outside the range of normal growth conditions, thereby triggering cellular stress responses. This makes temperature sensitive alleles ill-suited for the study of nuclear proteins that affect the stress response, as the temperature shifts required for target protein inactivation may have effects that cannot be easily separated from the actual pathway under investigation.

Alternatively, conditional alleles can be generated by the so called "double-shutoff" method, which can then be used to study essential nuclear proteins (Bartel et al. 1990; Moqtaderi et al. 1996). In this method, the gene of interest is placed under the control of a copper-regulated promoter and the amino-terminus of protein sequence is modified by the addition of a polypeptide that targets the protein product to the ubiquitin degradation pathway. In an appropriate strain, the addition of copper results in the transcriptional repression of the target mRNA as well as the conditional destruction of existing target protein molecules. However, this procedure also activates cellular stress responses. An added drawback to the double shut-off system is that it is kinetically slow, with inactivation taking place over a period of hours (Moqtaderi et al. 1996). This slow kinetic profile makes it more difficult to separate

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Internet Resources

http://www.sequence.stanford.edu/group/yeast_deletion_project/Deletion_primers_PCR_sizes.txt.

<http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/laemmli.html>

the primary effects directly caused by the targeted protein inactivation from the indirect (secondary and later) effects that are the result of changes in downstream protein products.

Recently, a new strategy has been developed to conditionally and rapidly deplete nuclear proteins of interest (Haruki et al. 2008). This method, termed Anchor Away, involves conditional export of the protein of interest out of the nucleus and its subsequent sequestration in the cytoplasm. Anchor Away takes advantage of the fact that the human proteins FKBP12 and FRAP are able to strongly interact only in the presence of Rapamycin, a small molecule that is taken up very rapidly by yeast cells growing in liquid culture (Brown et al. 1994; Chen et al. 1995). In a typical Anchor Away experiment, FKBP12 is fused to a highly abundant anchor protein, in this case the ribosomal RPL13A, while the FKBP12-Rapamycin-binding domain of FRAP (FRB) is fused to a target protein of interest. As part of the process of ribosomal maturation, ribosomal proteins (including the RPL13A-FKBP12) are transiently imported into the nucleus, where they are assembled into ribosome particles in concert with ribosomal RNA, and are subsequently exported to the cytoplasm for function. In the absence of Rapamycin, RPL13A-FKBP12 and the target protein-FRB fusion proteins do not interact, and the target protein functions normally. On the other hand, in the presence of Rapamycin, the anchor protein (RPL13A-FKBP12) will form a stable ternary complex with the target protein-FRB chimera while transiting through the nucleus. Ribosomes incorporating the RPL13A-FKBP12 anchor are then exported to the cytoplasm, dragging along the target protein-FRB fusion protein that is linked to the ribosome via a Rapamycin bridge (Fig. 1). Cytoplasmic sequestration of the target protein is rapid and nearly complete in less than an hour after Rapamycin treatment for most proteins studied so far (Haruki et al. 2008 and Fig.2).

This UNIT describes the step-by-step method of conditionally and rapidly depleting a nuclear-localized target protein of interest by Anchor Away. Included are instructions on how to construct yeast strains for Anchor Away and how to deplete target proteins from the nucleus. To make this system work, a yeast strain needs to be constructed with the following four components: (1) the FRB-tagged protein of interest, (2) the FKBP12-tagged anchor protein (RPL13A), (3) an *fpr1* deletion and (4) a *Tor1-1* mutation. Some yeast strains (e.g. HHY168 in the W303-1A background; Haruki et al. 2008) already have the latter three components engineered in, and only require the introduction of the FRB-domain to the protein of interest prior to use.

PCR-Mediated One-Step Protein Tagging

To construct an Anchor Away strain, the target nuclear protein needs to be tagged with FRB. The nucleotide sequences corresponding to the FRB tag can be amplified by PCR, and can then be inserted to the gene of interest in the yeast genome by homologous recombination (based on the procedure described in UNITS 3.17 and 13.7).

Materials

Template DNA (1 to 10ng of plasmid of pFA6a-FRB-KanMX6, Haruki et al. 2008, containing sequence encoding FRB tag and KanMX6 selective marker)

Oligonucleotide primers (for tagging (Step 1) and colony PCR confirmation (Step 7))

QIAGEN PCR purification kit or an equivalent

Yeast strains for tagging (HHY168, Haruki et al. 2008, Mat α *tor1-1 fpr1::NatMX4* RPL13A- FKBP12::TRP1)

YPD+Kan plate (UNIT 13.1, YPD plate containing 200 μ g/ml Geneticin from Sigma)

YPD+Rapa plate (UNIT 13.1, YPD plate containing 1µg/ml Rapamycin from Tecoland Corp.)

Plasmid and yeast strain are available from European *Saccharomyces cerevisiae* Archive for Functional Analysis, <http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/laemmli.html> (see also Haruki et al. 2008). Additional reagents and equipment for amplification of DNA by PCR (UNIT 15.1), agarose gel electrophoresis (UNIT 2.5A), DNA extraction and precipitation (UNIT 2.1A), purification of DNA (UNIT 2.6) and yeast transformation (UNIT 13.7).

Amplify tagging sequence

1. Design PCR primers for the tagging cassette as follows (Longtine et al. 1998, see Fig.1). Pick 40 to 50 nucleotides of sequence from the sense strand at the 3' end of the target gene immediately upstream of the stop codon (do not include the stop codon) and add this sequence to the 5' end of the forward primer whose sequence is shown below. Similarly, pick 40 to 50 nucleotides of sequence from the antisense strand at the 3' end of the target gene immediately downstream of the stop codon (do not include the stop codon) and add to the 5' end of the reverse primer.

Forward primer: 5'- 40-50 nt of GENE-SPECIFIC SENSE SEQUENCES
cggatccccgggtaattaa -3'

Reverse primer: 5'- 40-50 nt of GENE-SPECIFIC ANTI-SENSE
SEQUENCES **gaattcgagctcgtttaaac** -3'

This primer pair is used to amplify the FRB tag and a selective marker from the plasmid template.

2. Amplify tagging cassette by PCR with high fidelity thermo-stable DNA polymerase (see UNIT 15.1)

Purify PCR product (See UNIT 2.1A, 2.5A and 2.6)

3. Analyze a fraction (5–10µl) of the reaction mix by agarose gel electrophoresis to verify the specificity and yield of the expected product. The expected size of the correct PCR product is approximately 1.8 kb.
4. Purify amplified DNA from the PCR reaction mix with QIAgen PCR purification kit or an equivalent product.
5. Alternatively, extract amplification product with buffered phenol/chloroform and then precipitate DNA with 100% ethanol (UNIT 2.1A).

Transform yeast cells with PCR product (See UNIT 13.7)

Select transformants on corresponding selective plates. If yeast strain HHY168 and the plasmid pFA6a-FRB- KanMX6 are used, select transformants on YPD+Kan plates.

Identification of transformants with successful FRB-tagging

6. Re-streak transformants and the parental HHY168 strain on a YPD plate as well as a YPD+Rapa plate. Incubate for 1–2 days at 30°C.

If deletion of the target gene has a growth phenotype, the correct transformants will replicate that growth phenotype when grown on YPD+Rapa plates.

Whereas HHY168 will grow well overnight, transformants with successful tagging of an essential gene will exhibit very little or no growth on YPD+Rapa

plate. If loss-of-function of the gene of interest has no phenotype, correct transformants need to be identified by colony PCR.

7. Perform colony PCR to confirm or identify correct FRB tagging at the gene of interest (See UNIT 15.1). It is important to include the parental strain (HHY168) as a negative control.
8. Check tagging by Western with antibody against FRB tag from Alexis Biochemicals.

Protocol for conditionally deplete the target nuclear protein by addition of Rapamycin

To conditionally deplete the target nuclear protein, yeast cells should be grown to log phase, typically OD_{600nm} of 0.3 to 0.6. Rapamycin should then be added directly to the medium for a final concentration of 1 µg/ml. After a desired duration (typically <60 min), cells can be analyzed or processed as needed. The following example illustrates a typical time-course experiment for ChIP analysis of Rpo21-FRB, the largest subunit of RNA polymerase II (Pol II).

Materials

YPD medium (UNIT 13.1)

Anchor away strain (Rpo21- FRB) and control strain HHY168 (HHY168 is available from European *Saccharomyces cerevisiae* Archive for Functional Analysis, <http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/laemmli.html>.)

Rapamycin (from Tecoland Corp., 1000x stock – 1 mg/ml in ethanol)

Rapamycin dissolved in ethanol is stable for several weeks if stored at –20C. Since poor Rapamycin activity is one of the major causes of inefficient Anchor-Away, old stocks of Rapamycin should be used with caution or avoided altogether.

Formaldehyde (37% solution)

Glycine 2.5M

Antibody against the C-terminal domain of the largest subunit of Pol II such as 8WG16 (Covance)

Experimental procedures for performing Anchor Away

1. In the morning of the day before the anchor away experiment, inoculate yeast anchor away strain Rpo21-FRB and control strain HHY168 into 5 ml YPD medium. In the afternoon, measure OD_{600nm} of cultures and dilute yeast cells into 500ml of fresh YPD medium, so that OD_{600nm} of cultures will reach 0.4 by the next morning.
2. In the morning of the Anchor Away experiment, measure OD_{600nm} every 30 min to plot growth curves. When OD_{600nm} reaches 0.4, remove 40 ml yeast cells from the flask and fix the 40ml of cells by adding formaldehyde to a final concentration of 1% (formaldehyde will crosslink proteins to DNA).
3. Add Rapamycin to the rest of cells to a final concentration of 1 µg/ml.
4. In addition to the 0 min time point in step (2) above, remove and fix 40 ml of cells 20, 40, 60, 80, 100, 120 and 150 min after the addition of Rapamycin.

5. Add 10 ml of 2.5M Glycine to each sample after 20 min of crosslinking in order to terminate the crosslinking reaction.
6. Process samples and prepare chromatin as described (see UNIT21.3) and (Fan et al. 2008).
7. Perform ChIP assay with antibody against the carboxyl terminal domain of the largest subunit of Pol II (from Covance) as described (see UNIT21.3). Determine the extent and kinetics of depletion of Pol II at different gene loci by quantitative PCR.

COMMENTARY

Background Information

The main advantages of the Anchor Away system over temperature sensitive mutants include: 1. It is easier to generate Anchor Away strains than to isolate temperature sensitive mutants, especially in the cases where multiple proteins are to be analyzed; 2. Under normal growth conditions, Anchor Away strains are more likely to exhibit normal growth and are in general healthier than most corresponding temperature sensitive mutant strains; 3. The working mechanism for Anchor Away has been established, while the mechanism(s) underlying how temperature sensitive mutants mediate their effects are often unknown and can vary a great deal even among different ts mutations in the same gene. The advantage of the Anchor Away system over both temperature sensitive and double-shutoff strains is that the relatively mild condition required for target protein inactivation in Anchor Away strains generally do not activate the stress response pathways, thereby allowing for easier interpretation of results that are not complicated with unintended stress response-specific data. An additional advantage of the Anchor Away system is that multiple FRB-tagged proteins can be simultaneously depleted from nucleus. This is particularly useful to study proteins with redundant or overlapping functions, where nuclear depletion of a single target protein has minimal or no phenotypic effect.

Since Rapamycin is toxic to the wild type yeast cells, mutations need to be introduced into yeast cells in order to alleviate this toxicity. In yeast, the Rapamycin- Fpr1p complex binds to Tor1p and Tor2p and causes cell cycle arrest in the G1 phase (Heitman et al. 1991; Lorenz and Heitman 1995). Therefore, the *FPR1* gene has to be deleted and the wild-type genomic *TOR1* allele has to be replaced by its Rapamycin-resistant *tor1-1* counterpart in order for strains to be suitable for Anchor Away (Cafferkey et al. 1993). Deletion of *FPR1* also increases the interaction between FRB and FKBP12, making Anchor Away-based depletion more efficient in the process.

In principle, any abundant protein that shuttles between different cellular compartments has the potential to serve as an anchor protein. The anchor protein has to sequester a target protein to biologically irrelevant sites with respect to the target's function, thereby preventing the target protein from functioning properly. In another example, the proton pump PMA1 was used as an anchor protein to sequester target proteins that normally shuttle between the nucleus and cytoplasm to plasma membrane (Haruki et al. 2008).

Critical Parameters

In general, DNA amplification and purification guidelines outlined in UNIT3.16 should be applied to this protocol to ensure sufficient high quality DNA to be used in the transformation. Competent cells preparation and transformation guidelines outlined in UNIT13.7 should be closely followed in order to ensure high transformation efficiency of yeast. However, the following points deserve special attention.

Screening transformants—For essential genes or genes whose deletion results in obvious growth defects, transformants can be quickly screened based on their impaired growth on Rapamycin-containing plates as compared to regular YPD plates lacking Rapamycin. Correct transformants will not grow or grow slowly on Rapamycin-containing plates. To make just a few Rapamycin plates, appropriate amount of Rapamycin can be spread on the surface of regular YPD plates. Transformants can then be further confirmed by colony PCR with gene-specific and cassette-specific primer pairs and Western with an antibody against the FRB tag from Alexis Biochemicals.

If a protein of interest binds to DNA, the extent of its depletion in nucleus can be monitored by ChIP analysis using an antibody against it or against the FRB portion. If the protein is difficult to ChIP, GFP-FRB tag instead of FRB tag can be used, as the addition of GFP tag allows for the monitoring of the depletion status of target protein by fluorescence microscopy.

Freezing Stocks of Anchor Away yeast strains—Some Anchor Away strains die faster at 4°C on a plate than others, so it is important to prepare yeast strain stocks from a fresh plate (personal communications with Dr. U.K. Laemmli at University of Geneva, Switzerland).

Troubleshooting

No or few transformants—To increase transformation efficiency, after heat shock, yeast cells can be spun down, resuspended in 2 ml YPD and transferred to a test tube at 30°C to recover for 4 hr or overnight before being applied on selective plates.

Insufficient depletion of Target Proteins—Different target proteins may have different depletion kinetics, so it is important to perform pilot experiments in order to determine the optimal rapamycin treatment time for each target protein. If the protein of interest binds to DNA, the depletion kinetics in nucleus can be monitored by ChIP analysis using an antibody against it or against the FRB portion. Extensive sonication usually results in smaller chromatin fragment and improved sensitivity in ChIP assay (Fan et al. 2008).

Anticipated Results

Amplification of tagging cassette usually results in a 1.8 kb DNA product. It is recommended to purify the desired DNA product by electrophoresis and gel purification. A successful transformation usually produces 20–100 transformants. Generally, half of the transformants contain correctly tagged target proteins. Depletion of target protein from the nucleus is usually very quick, as shown in Fig.2, Pol II association with genes tested decreases dramatically after 20 min of treatment with Rapamycin, and signals decrease to background level after 1 hr treatment.

Time Consideration

Preparation of tagging cassette by PCR requires about 2 hr. Purification of DNA requires 30 min to 2 hr, depending on methods used. After yeast cells have reached the desired growth phase, the transformation protocol requires about 3 hr. It usually takes 2 to 4 days for transformants to appear. Colony PCR confirmation requires about 3 hr and the growth assay on Rapamycin containing plate requires 1 to 2 days. The Anchor Away experiment takes 1 to 3 hr, depending on the target protein and assay.

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Figure 1.

Scheme of the Anchor Away System: ligand-dependent depletion of nuclear proteins.

- A.** In the absence of Rapamycin, anchor protein RPL13A-FKBP12 and target protein YFG-FRB (a transcription factor in this case) do not interact. YFG-FRB functions normally on chromatin. The rectangle represents the cell and the circle represents the nucleus.
- B.** In the presence of Rapamycin, anchor protein RPL13A-FKBP12 and target protein YFG-FRB form ternary complex via Rapamycin. The maturation of the ribosome takes the target protein YFG-FRB to the cytoplasm and away from nucleus, thereby inhibiting the transcription function of YFG-FRB.

Figure 2. Rapid nuclear depletion of Rpo21-FRB after Rapamycin addition. Relative Pol II occupancy at CDC19 and PMA1 coding regions was determined at different time points after Rapamycin addition. Pol II occupancy before Rapamycin addition was set to 100.