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An In Vivo Assay to Quantify Stable Protein Phosphatase 2A (PP2A) Heterotrimeric Species

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Summary

Protein phosphatase 2A (PP2A) regulates a broad spectrum of cellular processes. The enzyme is, in fact, largely a collection of varied heterotrimeric species composed of a catalytic (C) subunit and regulatory (B-type) subunit bound together by a structural (A) subunit. One important feature of the C subunit is that its carboxy-terminus can be modified by phosphorylation and methylation. The mechanisms that trigger such posttranslational modifications, as well as their consequences, are still under investigation. However, data collected thus far indicate that these modifications alter the binding to B subunits for an AC dimer, thereby affecting the makeup of the PP2A species in the cell. In this chapter, we describe an in vivo assay for assessing stable PP2A heterotrimer formation that is based on specific subcellular localizations of PP2A heterotrimers. This assay can be used to study the impact of a wide variety of alterations (such as mutations and covalent modifications) on PP2A heterotrimer formation. We specifically describe the use of this assay to quantify the effects of methylation on the stable formation of $PP2A_{Rts1p}$ and $PP2A_{Cdc55p}$ heterotrimers.

Key Words: Protein phosphatase 2A; PP2A; GFP; methylation; phosphorylation

1. Introduction

Protein phosphatase 2A (PP2A), a major eukaryotic serine/threonine protein phosphatase, plays a critical role in a wide array of cellular processes, including DNA replication, RNA transcription, RNA splicing, and cell-cycle progression *(1–4)*. PP2A is able to participate in such a variety of processes, dephosphorylating multiple substrates, because of the enzyme's inherent heterogeneity. PP2A's heterogeneity is largely supplied via the B-type subunits. In mammals, five different classes of B-type subunits have been reported *(5)*. In addition, each class possesses several isoforms, generating the potential for more than 40 different PP2A heterotrimeric species to exist *(5)*.

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In the yeast *Saccharomyces cerevisiae*, the PP2A community is far simpler, encompassing only five genes. The A subunit is encoded by *TPD3* ***(6,7)***. The C subunits are encoded by two highly similar genes, *PPH21* and *PPH22* ***(8)***. *S. cerevisiae* has only two classes of B-type subunits and only one member in each class: *CDC55* encodes the B-class subunit ***(9)*** and *RTS1* encodes the B'-class subunit ***(10,11)***. Mutations in each PP2A subunit gene elicit complex pleiotropic phenotypes.

The complexity of PP2A in *S. cerevisiae* can be traced to the enzyme's community dynamics. These dynamics encompass three general areas.

1. Post-translational modifications of PP2A subunits (*see* **Fig. 1A**). The C subunits are methylated at their C-terminal leucine by Ppm1p and, by analogy with mammalian C subunits, can be phosphorylated on three residues by an unknown kinase(s) ***(12–15)***. Methylation and one phosphorylation event differentially affect stable formation of $PP2A_{Cdc55p}$ and $PP2A_{Rts1p}$ species ***(16–21)***. Likewise, Rts1p is phosphorylated in its N-terminus, but the function is unknown ***(11)***.
2. The stoichiometry of PP2A subunits (*see* **Fig. 1B**). The C subunits are the most abundant of the PP2A subunits ***(22)***. Rts1p (B-type subunit) is approx 12-fold more abundant than Cdc55p (B-type subunit), and Tpd3p (A subunit) serves as the limiting subunit for trimer formation ***(22)***. Thus, there is competition between B-type subunits for binding to the A-subunit.
3. The subcellular localization of PP2A (*see* **Fig. 1C**). One role of the B-type subunits is to target the heterotrimer to different cell-cycle–specific subcellular localizations via two different methods: Rts1p must be incorporated into a heterotrimer to achieve and/or maintain subcellular localization, whereas Cdc55p can be targeted to and accumulate at subcellular sites independent of heterotrimer assembly to a certain degree ***(22)***.

Cumulatively, all of the above factors endow just five proteins the flexibility needed to participate in multiple cellular events and pathways.

The idea for a novel quantitative assay to monitor stable heterotrimer formation arose from our recent studies defining these community dynamics ***(18,22–24)***. The crux of this assay is that $PP2A_{Rts1p}$ and $PP2A_{Cdc55p}$ heterotrimers exhibit distinct cell-cycle–specific subcellular localization patterns that can be easily visualized by fluorescence microscopy (*see* **Figs. 1C** and **2**) ***(22)***. $PP2A_{Rts1p}$ heterotrimers localize to the kinetochore of small/medium budded cells and to the bud neck of large budded, posttelophase cells (*see* **Figs. 1C** and **2**) ***(22)***. Conversely, $PP2A_{Cdc55p}$ heterotrimers localize to the bud tip of small/medium budded cells and to the bud neck of large budded, post-telophase cells (*see* **Figs. 1C** and **2**) ***(22)***. Moreover, GFP-Tpd3p and Rts1p-GFP subcellular localizations are entirely dependent on heterotrimer formation, whereas GFP-Cdc55p can maintain some subcellular localization independent of heterotrimer formation ***(22)***. Therefore, GFP-Tpd3p subcellular localization to the kineto-

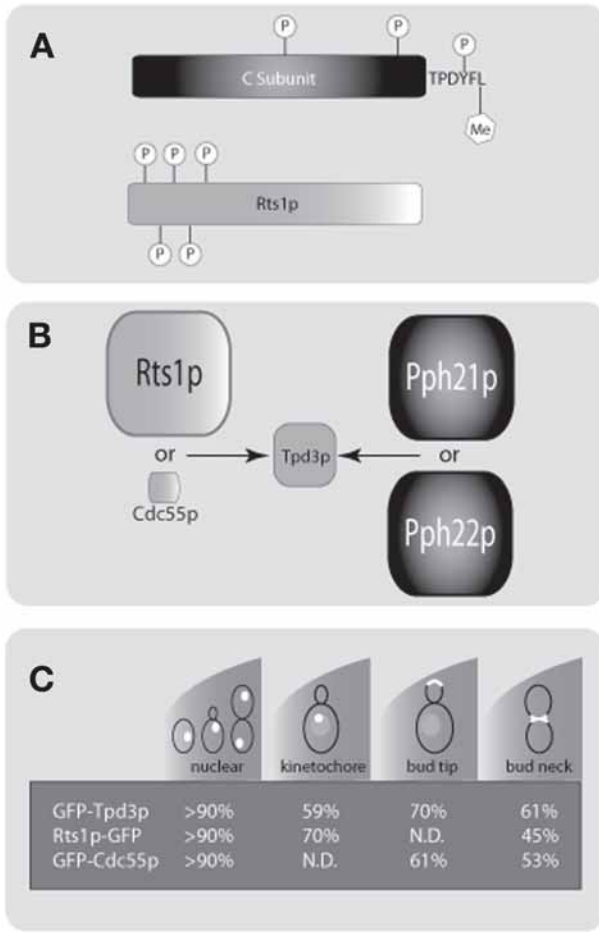

Fig. 1. The community dynamics of PP2A. **(A)** A schematic of PP2A subunit posttranslational modifications. The C-subunit of PP2A is modified with four post-translational modifications: methylation (Me) of the conserved C-terminal leucine, phosphorylation (P) of a conserved tyrosine two residues from the C-terminus, phosphorylation of an unidentified serine, and phosphorylation of an unidentified threonine. Rts1p is multiply phosphorylated in its first 216 amino acids. **(B)** A schematic of PP2A subunit stoichiometry. The size of the subunits reflect the relative abundances. In asynchronous *S. cerevisiae* cells, the ratio Rts1p : Cdc55p : Tpd3p : Pph21p : Pph22p is 4 : 0.25 : 1 : 4 : 4. **(C)** A summary of GFP-PP2A subunit localization patterns in asynchronous cells. Cells were divided into four classes: nonbudded, small/medium budded, large budded with one nucleus, and large budded with two nuclei. Cells in each class were scored as having or lacking the trademark localization in question. N.D. = not detected. (Part 1C was modified from **ref.** *22*, with permission from The American Society for Cell Biology.)

chore (via $PP2A_{Rts1p}$) and to the bud tip (via $PP2A_{Cdc55p}$) can be monitored and quantified as a measurement of stable $PP2A_{Rts1p}$ and $PP2A_{Cdc55p}$ heterotrimer formation, respectively. Quantification is done simply by visualizing cells expressing GFP-Tpd3p and scoring cells as having or lacking the trademark localization. Additionally, Rts1p-GFP and GFP-Cdc55p subcellular localizations can be used to verify the GFP-Tpd3p localization results.

Previously, quantitiation of PP2A heterotrimeric species was monitored solely via immunoprecipitation and Western analysis. These techniques, although very powerful, have intrinsic flaws that are avoided by our in vivo assay. For instance, our assay circumvents instability issues with Rts1p that occur upon cell lysis, it avoids artifactual in vitro association of subunits that might have been compartmentalized in vivo, it allows the measurement of effects on $PP2A_{Cdc55p}$ and $PP2A_{Rts1p}$ stable heterotrimer formation in the same cells, and it measures the functional localization of PP2A heterotrimers. It must be noted that our assay does not specifically measure heterotrimer assembly or heterotrimer formation. The assay directly measures localization, which we use as an indirect measurement of the formation of localization competent heterotrimers, hence the wording "stable heterotrimer formation."

2. Materials

1. *Saccharomyces cerevisiae* strain αW303 or other haploid or diploid strain with the appropriate genotype (e.g., appropriate mutant alleles of genes used as selectable markers for expression from plasmids) (*see* **Note 1**). The method described uses the strains and plasmid listed in **Note 2**.
2. Yeast extract, peptone, adenine, dextrose (YPAD; a variation of YPD/YEPD) medium to grow yeast under nonselection conditions (*see* **Note 3**). YPAD can be made via two methods:
   a. Dissolve 50 g YPD medium (BD Biosciences) and 80 mg adenine hemisulfate salt (Sigma) in 1 L of water. Be sure to fully dissolve the YPD medium, as the dextrose can burn upon autoclaving. Autoclave for 30 min to sterilize.
   b. Dissolve the following in order in 1 L of water: 20 g dextrose (Fisher Scientific), 80 mg adenine hemisulfate salt (Sigma), 10 g Bacto™ yeast extract (BD Biosciences), 20 g Bacto™ peptone (BD Biosciences). Be sure to fully dissolve the dextrose so that it does not burn upon autoclaving. Autoclave for 30 min to sterilize. Alternatively, dextrose can be made at a 20% (10X) solution that has been filter-sterilized to prevent darkening of the media and added to the solution after autoclaving.
3. Synthetic complete (SC or CM) medium to resuspend yeast for visualization of GFP-tagged proteins.
   a. Dissolve 26.7 g minimal SD base (BD Biosciences), 80 mg adenine hemisulfate salt (Sigma), and the appropriate amount of the appropriate dropout (DO) supplement (BD Biosciences) in 1 L of water. Autoclave for 30 min to sterilize.

   b. Dissolve the following in order in 1 L of water: 20 g dextrose (Fisher Scientific), 1.7 g yeast nitrogen base without amino acids and without ammonium sulfate (US Biological), 5 g ammonium sulfate, 80 mg adenine hemisulfate salt (Sigma), and the appropriate amount of the appropriate DO supplement (BD Biosciences) (*see* **Note 4**).
4. 1000X 4',6-diamidino-2-phenylindole (DAPI) solution: Prepare a 1 mg/mL DAPI stock solution by dissolving 1 mg DAPI (Sigma) in 1 mL of water. Store the stock solution at –20°C.
5. A BX60 epifluorescence microscope (Olympus) using a 10X UPlanFl objective (numerical aperture [N.A.] 0.30) (Olympus), a 40X UPlanFl objective (N.A. 0.75) (Olympus), a 100X UPlan Apo objective (N.A. 1.35) (Olympus), and a manual shutter. The microscope is equipped with the Endow GFP (EGFP) bandpass emission filter set #41017 (Chroma) and DAPI/Hoechst/AMCA filter set #31000v2 (Chroma), a MagnaFire CCD camera (Olympus, Model S99806; with an array of 1300 × 1030, 6.7-μm pixels) and a BH2-RFL-T3 100 W high-pressure mercury burner power supply unit (Olympus; 100–120 V, approx 2.8 A 50–60 Hz) or a comparable system (*see* **Note 5**).
6. Microscope slides, 3 × 1 × 1 mm (Fisher) and glass cover slips, 22 × 22 mm, No. 1 thickness (Sigma)

3. Methods

3.1. Growth of Cells

1. Inoculate a single colony of *GFP-TPD3 ppm1*Δ with YCp22 *PPM1*, *GFP-TPD3 PPM1* and *GFP-TPD3 ppm1*Δ from a fresh plate into 3–5 mL of the appropriate media (SC minus tryptophan [–Trp] for the first strain and SC or YPAD for the second and third) and grow shaking at 30°C overnight (*see* **Notes 6** and **7**). The next afternoon, inoculate 10 mL of the same media with 5 μL, 10 μL, and 50 μL of the overnight culture. This will ensure that at least one of the dilutions will be at an OD_{600} of 0.4–0.8 the following morning (*see* **Note 8**).
2. The next morning, choose cultures that are still growing in log phase (preferably OD_{600} of 0.4–0.8), add 10 μL of a 1-mg/mL stock of DAPI to each culture and continue shaking at 30°C for 1 h (*see* **Note 9**).

3.2. Preparation of Cells

1. Spin down one OD_{600} unit of each culture at 1000*g* (approx 3000 rpm in a microcentrifuge) for 3 min at room temperature.
2. If the cells were grown in YPAD (instead of SC), then wash the cells once in SC (*see* **Note 10**).
3. Gently resuspend each culture in 30 μL of SC by pipeting up and down (do not vortex the cells). Place 3.3 μL of each culture onto a microscope slide and place a cover slip over each. Two to three separate cultures can be placed side by side on one microscope slide.

4. Place the slide and cover slips inside of a folded Kimwipe® (Thomas Scientific) with the cover slip side down on a flat surface. Press down on the microscope slide and excess liquid will be wicked up by the Kimwipe. Look at the cells using a non-oil-immersion objective and transmitted light microscopy to see if the cells are floating in the suspension or if they are still. If they are floating, repeat the Kimwipe pressing technique until enough liquid is removed so that the cells are still.

3.3. Microscopic Analysis and Quantification

1. View each cell culture under the necessary magnification to view subcellular localization (we almost exclusively use 100× to photograph and score cells). There should be 40–80 cells in the field-of-view. Be sure that the cells are not too dense (e.g., not touching) so that cell morphology can be used to determine cell-cycle stages.
2. Capture a picture of the field using the EGFP filter set; and DAPI filter set, move to a new field and capture another set of pictures. Exposure times to capture the GFP and DAPI signals vary for each microscope and camera, but our system requires the following exposure times: DAPI, 0.05 s; GFP-Tpd3p, 3 s; Rts1p-GFP, 5 s; GFP-Cdc55p, 9 s.
3. Repeat this sequence until approx 400 cells (approx 10–20 fields) have been captured for each culture. Capture these fields as quickly as possible, preferably having the cells on the microscope slide for less than 20 min. If needed, make one slide at a time so that the cells are not sitting under the cover slip at room temperature any longer than necessary (*see* **Note 11**).
4. Using a lab counter (Fisher), score small/medium budded cells as having or lacking GFP-Tpd3p kinetochore and bud tip localization (*see* **Note 12**). These cells will all have one undivided nucleus (because they are all premitotic cells), thus there is no need to examine the DAPI pictures when scoring for these subcellular localizations. An example of the cells is shown in **Fig. 2**.
5. Score large budded, posttelophase cells as having or lacking GFP-Tpd3p bud neck localization. An example of GFP-Tpd3p localization is shown in **Fig. 2** and an example of DAPI-stained cells is shown in **Fig. 3**. Nuclear division must be monitored to determine which cells are post-telophase. This can be done via two methods (we usually use the first):
   a. Merge the DAPI and GFP captured files using Photoshop (Adobe) to assess nuclear positioning/cell-cycle phase and GFP-Tpd3p localization.
   b. Open each file and view them side by side to assess nuclear positioning/cell-cycle phase and GFP-Tpd3p localization.
6. Calculate the percentage of cells that display each trademark localization pattern (*see* **Note 13**). **Figure 1C** shows the percentage of wild-type cells that display the trademark PP2A localizations (*see* **Note 14**).
7. Divide the percentage obtained for each subcellular location by the corresponding percentage obtained at the same location for *GFP-TPD3 PPM1* cells and

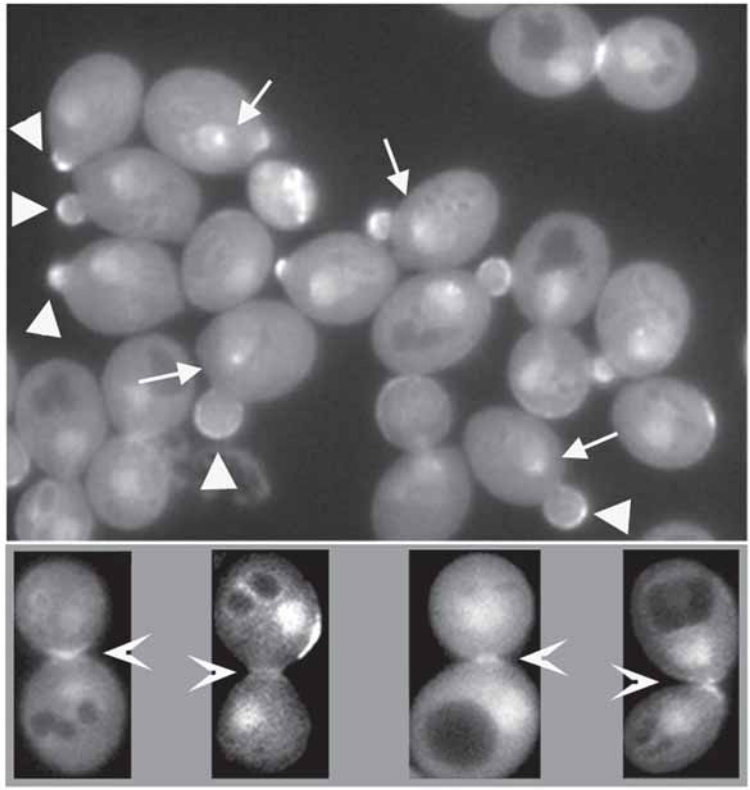

Fig. 2. GFP-Tpd3p localization. **(Top)** Asynchronous cells expressing GFP-Tpd3p were photographed using an EGFP filter set. Arrows denote kinetochore/spindle pole body localization; arrowheads denote bud tip localization. **(Bottom)** Single cells exhibit different phases of bud neck localization. Arrowheads denote bud neck localization. (Modified from **ref.** *22*, with permission from The American Society for Cell Biology.)

multiple by 100 to standardize all percentages to wild-type levels. An example of the results is shown in **Fig. 4**.

4. Notes

1. Although we used haploid cells for our studies, diploid cells are often better for microscopy. Diploid cells are larger and often yield a brighter GFP signal (because of the increased protein products). Diploid a/α W303 cells with two integrated copies of the GFP genes expressing GFP-Tpd3p, Rts1p-GFP, or GFP-Cdc55p yield a brighter GFP signal than the respective haploid strain. However, the signal from haploid cells is more than sufficient for these experiments.

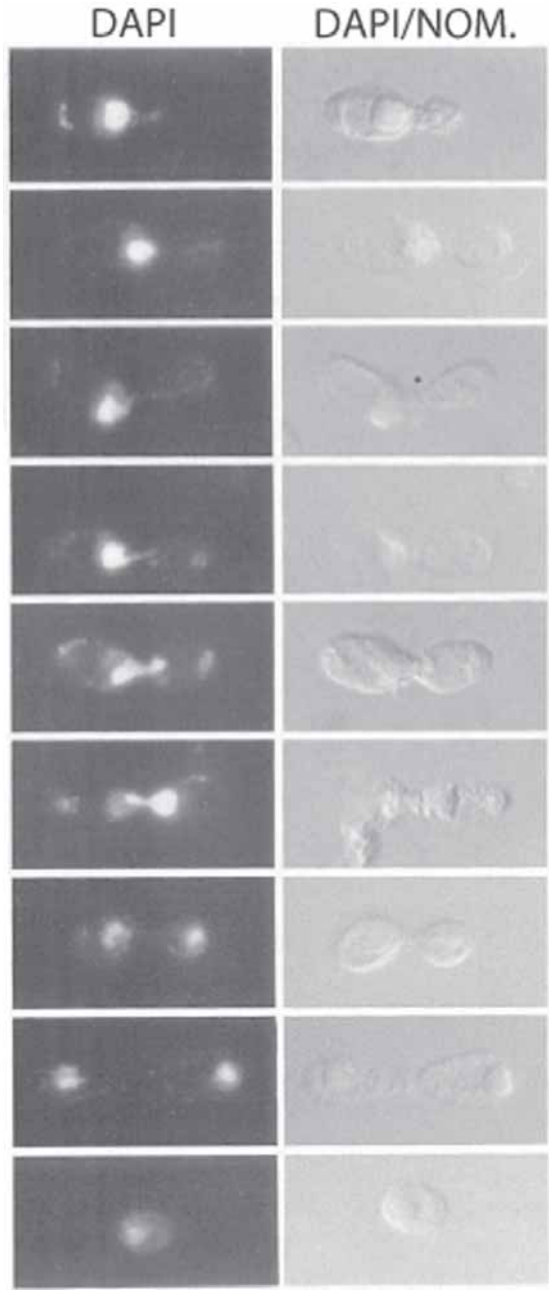

Fig. 3. DAPI-stained *S. cerevisiae* cells throughout the mitotic cell cycle. DAPI staining is shown in the left panels and DAPI with Nomarski is shown in the right panels. (Modified from **ref.** *33*, with permission from Elsevier.)

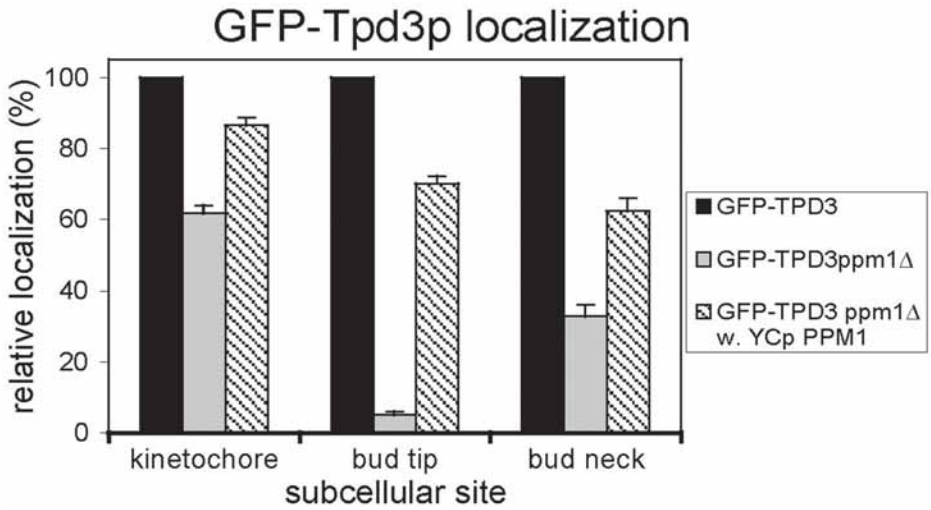

Fig. 4. Quantified GFP-Tpd3p kinetochore, bud tip and bud neck localization in *PPM1* and *ppm1*Δ cells. (Modified from **ref.** *17*, with permission from The American Society for Microbiology.)

2. We used two strains in this study: (MSG66) MATα *ade2–1 ura3–1 his3–11 trp1–1 leu2–3,112 GFP-TPD3* and (MSG261) MATα *ade2–1 ura3–1 his3–11 trp1–1 leu2–3,112 ppm1::KAN GFP-TPD3 (17)*. We used one plasmid in this study: (pMG528) YCp22 *PPM1*, *CEN* vector/*TRP1 (17)*.
3. Autofluoresence is a problem in *ade1* and *ade2* auxotrophic strains because of the accumulation of phosphoribosylaminoimidazole, a fluorescent metabolic precursor that accumulates in vacuoles *(25,26)*. Thus, it is preferable to use adenine prototrophs. However, *ADE* prototrophic strains sometimes lightly autofluoresce. High concentrations of adenine (80 μg/mL) will largely eliminate the autofluoresence of protoprophs and auxothrophs.
4. The DO supplement can be purchased as a premixed powder or can be made by adding the appropriate amino acids together *(27)*.
5. An excellent review on using GFP in yeast cells, hardware (e.g., microscopes, CCD cameras, and objectives), and long-term live cell imaging protocols was written by Tatchell and Robinson *(28)*.
6. We chose to write the methods using the *ppm1*Δ strain to show how lack of C-subunit methylation affects $PP2A_{Rts1p}$ and $PP2A_{Cdc55p}$ stable heterotrimer formation to different degrees. The assay can be used to test how other PP2A modifications (e.g., C-subunit phosphorylation) affect stable heterotrimer assembly as well *(17)*. Additionally, we feel that this assay could easily be modified to test the stable assembly of other multimeric complexes, as long as at least one of the subunit's localization depends on complex formation.

7. In our experience, the localization of a GFP-tagged protein can vary immensely depending on whether the gene expressing the GFP is integrated or expressed from a YCp series vector (e.g., YCp22, YCp33, andYCp111) *(29)* versus expressed from a pRS series vector (e.g., pRS313, pRS314, pRS315, and pRS316) *(30)*. The subcellular localizations of GFP-Tpd3p, GFP-Cdc55p, and Rts1p-GFP are nearly identical for integrated genes and those expressed from the YCp vector series. However, the localization changes dramatically when any of the three are expressed using the pRS vector series. This change in localization is likely the result of the copy number of the vectors. Although the YCp and pRS vector series both have a *CEN* sequence and are considered "single copy," the pRS series often accumulates to multiple copies in each cell *(31)*. Additionally, if we overexpress any of the PP2A subunits, the localization of the overexpressed subunit is disrupted and becomes ubiquitous throughout the cytoplasm. Furthermore, if we monitor the localization of a GFP-tagged subunit expressed from an integrated gene and overexpress a different subunit, the localization of the protein expressed from the integrated gene is disrupted. Thus, at least for PP2A, endogenous subunit stoichiometry is paramount for correct localization. For these reasons, we strongly recommend integrating the GFP-tagged gene and compare any protein levels from a vector-expressed gene to that of the integrated expression before doing any experiments from vector-expressed genes.
8. It is essential that the cultures all be at an OD_{600} between 0.4 and 1.0 at the time of the experiment. The subcellular localization of Rts1p-GFP, GFP-Tpd3p, and GFP-Cdc55p changes at higher OD units. The subunits no longer localize to specific regions of the cell, but are ubiquitous throughout the cytoplasm. This is likely because $PP2A_{Rts1p}$ and $PP2A_{Cdc55p}$ heterotrimers carry out cell-cycle-specific functions and these functions are not needed because cell densities increase and cells slow down and stop their division cycles (eventually entering G_O).
9. The DAPI can be used as a vital stain, but the background staining of cell bodies (largely mitochondria) is higher in live cells than in fixed cells. However, cells cannot be fixed for this assay because the GFP signal for GFP-Tpd3p, GFP-Cdc55p, and Rts1p-GFP does not survive fixation. We made *rhoo* strains via the protocol of Fox et al. *(32)* to decrease the background staining for publication-quality figures *(22)*, but this is not necessary just to determine pre-telophase vs telophase cells.
10. Cells grown in YPAD need to be washed in SC because the yeast extract and peptone are highly fluorescent. GFP-PP2A subunit localization is essentially the same in cells grown in YPAD and SC.
11. Microscope slides made in this manner start to dry out after 15–20 min. Large bubbles start appearing under the cover slip and the cells start to be displaced. Additionally, cells start to look "unhealthy" after sitting on the microscope slide for more than 20–30 min.
12. To be able to quickly score cells, first learn the cellular morphologies. The best way to do this is to DAPI stain a population of cells and study the placement of the nucleus with respect to the bud size (*see* **Fig. 3**) *(33)*. Once you can match the

bud size to the correct cell-cycle stage, use a lab counter to score "yes" or "no" for each subcellular localization.

13. To support the validity of our quantitation method, we used some of the same captured fields of cells and measured the area and intensity of the GFP signal at the bud tip and bud neck using NIH Image (National Institutes of Health, Bethesda, MD) and NIH ImageJ (National Institutes of Health, Bethesda, MD). The results with NIH Image and NIH ImageJ were very similar to our "yes"/"no" method, thus demonstrating the validity of our method. Using NIH Image to qauntitate signal intensities was much more tedious and time-intensive and yielded no more information than our simple scoring method.
14. The percentage of cells displaying any PP2A trademark localization never reaches 100%. The two reasons for this are: (1) PP2A heterotrimer localization is a dynamic, cell-cycle-specific process and (2) we photographed cells to score for localizations; in each photograph a certain percentage of cells were not in focus. When we focused on each cell the percentage did increase, but it never reached 100%. (3) The amount of PP2A localization in a population of cells likely represents a Gaussian distribution with a portion of cells having a localization level not detectable above background. To compensate for these issues, we divide each experimental percentage localization value obtained by the percentage localization value obtained for wild-type cells in that same category.

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