Protein phosphatase 2A (PP2A) is an essential eukaryotic serine/threonine phosphatase known to play important roles in cell cycle regulation. Association of different B-type targeting subunits with the heterodimeric core (A/C) enzyme is known to be an important mechanism of regulating PP2A activity, substrate specificity, and localization. However, how the binding of these targeting subunits to the A/C heterodimer might be regulated is unknown. We have used the budding yeast *Saccharomyces cerevisiae* as a model system to investigate the hypothesis that covalent modification of the C subunit (Pph21p/Pph22p) carboxyl terminus modulates PP2A complex formation. Two approaches were taken. First, *S. cerevisiae* cells were generated whose survival depended on the expression of different carboxyl-terminal Pph21p mutants. Second, the major *S. cerevisiae* methyltransferase (Ppm1p) that catalyzes the methylation of the PP2A C subunit carboxyl-terminal leucine was identified, and cells deleted for this methyltransferase were utilized for our studies. Our results demonstrate that binding of the yeast B subunit, Cdc55p, to Pph21p was disrupted by either acidic substitution of potential carboxyl-terminal phosphorylation sites on Pph21p or by deletion of the gene for Ppm1p. Loss of Cdc55p association was accompanied in each case by a large reduction in binding of the yeast A subunit, Tpd3p, to Pph21p. Moreover, decreased Cdc55p and Tpd3p binding invariably resulted in nocodazole sensitivity, a known phenotype of *cdc55* or *tpd3* deletion. Furthermore, loss of methylation also greatly reduced the association of another yeast B-type subunit, Rts1p. Thus, methylation of Pph21p is important for formation of PP2A trimeric and dimeric complexes, and consequently, for PP2A function. Taken together, our results indicate that methylation and phosphorylation may be mechanisms by which the cell dynamically regulates PP2A complex formation and function.

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Huijun Wei‡, Danita G. Ashby‡, Carlos S. Moreno‡, Egon Ogris§, Foong M. Yeong§, Anita H. Corbett‡, and David C. Pallas‡‡

From the ‡Department of Biochemistry and Winship Cancer Center, Emory University School of Medicine, Atlanta, Georgia 30322 and the §Institute of Molecular Biology, Vienna Biocenter, University of Vienna, A-1030 Vienna, Austria

The effects of reversible methylation of leucine 309 (18–22) on PP2A activity is unclear (20, 23, 24). C subunit methylation appears to occur in vivo in a cell cycle-regulated manner (25) and, thus, could be a mechanism for modulating PP2A function in a cell cycle-specific manner. Because PP2A methylation is reversible and the methyltransferase (23) and methyllesterase (26) enzymes have recently been cloned, PP2A methylation represents an attractive model system for the study of the effects of reversible protein methylation on protein-protein interactions.

The *Saccharomyces cerevisiae* genome contains two genes (PPH21 and PPH22) encoding PP2A catalytic subunits (27) and a single A subunit gene, *TPD3* (28). Deletion of either *PPH21* or *

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, Emory University School of Medicine, 1510 Clifton Rd., Atlanta, GA 30322. Tel.: 404-727-5620; Fax: 404-727-3954; E-mail: dpallas@emory.edu.

The abbreviations used are: PP2A, protein phosphatase 2A; C subunit, catalytic subunit; HA, hemagglutinin; PMT1, protein phosphatase methyltransferase-1 (mammalian); wt, wild type; Vpr, virion-associated accessory protein; YPD, yeast extract/peptone/dextrose.

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1570
PPH21 alone has no effect on yeast growth, although PP2A activity is reduced by 51 and 33%, respectively (27). Double deletion of both genes results in severe slow growth and a temperature-sensitive phenotype (29). Additional deletion of the PPH3 gene, which encodes a nonessential protein phosphatase that has residual overlapping function with PP2A, leads to cell death (29). S. cerevisiae has two B-type subunits, Cdc55p and Rts1p, that are, respectively, homologous to the B and B' mammalian B-type subunit families (30, 31). Cells lacking Cdc55p or Tpd3p are sensitive to drugs such as nocodazole and benomyl, which perturb microtubule stability, presumably because forms of PP2A containing these subunits are important for normal mitotic spindle checkpoint function (32, 33).

Although only one S. cerevisiae methyltransferase homolog (Ppm1p) exists that was found to be nonessential for growth under normal conditions (26), two putative PP2A methyltransferase homologs (Ppm1p and Ppm2p) exist (23) whose importance has not yet been determined. The first, Ppm1p, is highly homologous to the mammalian PP2A methyltransferase (PMT1) that was recently cloned, expressed, and shown to have activity by De Baere et al. (23). These authors also reported the existence of a second homologous protein in both mammalian cells (PMT2) and S. cerevisiae (Ppm2p) that has ~350 additional amino acids containing significant similarity to the kelch domain (23), which has been implicated in actin binding. To date, Ppm1p, Ppm2p, and the human Ppm2p homolog have not been shown to have PP2A methyltransferase activity, and therefore, the relative contributions of these enzymes toward methylating PP2A in vivo are not known.

We previously created a set of mutants targeting highly conserved residues in the carboxyl terminus of the mammalian PP2A catalytic (C) subunit. Several of these mutants showed decreased binding of B subunit and, in some cases, decreased C subunit methylation (10). Expression of these mutants in mammalian cells (Ppm2p) that has a temperature-sensitive phenotype (29). Additional deletion of both genes results in severe slow growth and a temperature-sensitive phenotype (29). Perhaps forms of PP2A containing these subunits are important for normal mitotic spindle checkpoint function (32, 33).

MATERIALS AND METHODS

Plasmids and PPH21 Mutant cDNAs—A 2.5-kilobase polymerase chain reaction product (forward primer, CCGGATCCGAGGACAAATCGTAAAGTCAGG; reverse primer, ACGCCTGGGCTCATTCTAACTGCAACAGTTATCGGGTGC) encoding the PPH21 gene was cut with BamHI and SacI and ligated into pBluescript SK+ vector (Stratagene). The two nucleotides preceding the ATG start codon were then mutated to CC by site-directed polymerase chain reaction mutagenesis to create an Ncol site (CCATGG) that includes the start codon. Subsequently, site-directed polymerase chain reaction mutagenesis was used to create the T364A (codon change: ACG to GCT), T364D (codon change: ACG to GAC), Y367E (codon change: TAC to GAA), Y367F (codon change: TAC to TTC), and L369A (deleted codon) mutations. WT and mutant PPH21 cDNAs were then subcloned into pRS316 (CEN6 URA3 amd) (35), pRS310 (CEN6 URA3 GAL1–10 amp) (36), pRS425 (2µ URA3 amp) (35), or pRS423 (2µ TRP1 amp) (35) vectors. The amino-terminal epitope-tagged versions of the mutants in pRS316 and pRS425 vectors were also made by inserting double-stranded oligonucleotides encoding the influenza hemagglutinin (HA) epitope (Tyr-Pro-Tyr-Asp-Pro-Asp-Tyr-Ala) followed by the thymine recognition site (Leu-Val-Pro-Arg-Gly-Ser) just before the start ATGs, making use of the NcoI site created by the use of an NcoI site (CCATGG) as described previously. Rts1p has a carboxy-terminal 6×His epitope tag (Rts1p-6×His) was obtained from R. Hallberg (37).

RESULTS

Carboxy-terminal Pph21p Mutants Are Functional—To investigate the possibility that PP2A might be regulated by reversible covalent modification of its carboxy terminus, we pre-
Table I

<table>
<thead>
<tr>
<th>Mammalian</th>
<th></th>
<th>S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>C subunit*</td>
<td>B subunit binding*</td>
<td>C subunit (Pph21p)</td>
</tr>
<tr>
<td>Vector</td>
<td>N/A</td>
<td>Vector</td>
</tr>
<tr>
<td>WD309aa†</td>
<td></td>
<td>WT (369aa)†</td>
</tr>
<tr>
<td>T304A</td>
<td>+</td>
<td>T364A</td>
</tr>
<tr>
<td>T304D</td>
<td>–</td>
<td>T364D</td>
</tr>
<tr>
<td>Y307E</td>
<td>–</td>
<td>Y367E</td>
</tr>
<tr>
<td>Y307F</td>
<td>–</td>
<td>Y367F</td>
</tr>
<tr>
<td>L309A</td>
<td>–</td>
<td>L369A</td>
</tr>
</tbody>
</table>

* Data for mammalian C subunit mutants are taken from Ogris et al. (10)‡; N/A, not applicable.
† Data from Figs. 3 and 4. Ts, temperature-sensitive for growth at 37 °C. For these assays: +, severe; +, intermediate; −, not consistently detectable; N/A, not applicable.
‡ Data from Fig. 2. N/A, not applicable; NT, not tested (because the HA-tagged version of this mutant could not be expressed).
§ WT yeast C subunit has 369 amino acids (aa), whereas WT mammalian C subunit has 309.

Previously created and analyzed a set of mutants targeting highly conserved residues in the carboxyl terminus of the mammalian PP2A catalytic (C) subunit (10) (see examples in Table I). Several of these mutants showed decreased binding to B subunit (10) and, in some cases, decreased C subunit methylation.2 The lack of a consistent phenotype in mammalian cells prompted us to move to a model system that was more amenable to genetic manipulation. Because the carboxyl terminus of PP2A is highly conserved from yeast to humans and PP2A methylation is conserved in yeast (40), we chose to construct cDNAs expressing S. cerevisiae Pph21p versions of a subset of these mutants (T364A, T364D, Y367E, Y367F, and L369A; see “Materials and Methods” and Table I) and to study these mutants in S. cerevisiae cells whose viability depends on their expression.

Ronne et al. (29) previously created an S. cerevisiae strain (H328) deleted for PPH21 and PPH3 that expresses Php22p under control of the GAL promoter. H328 is viable when grown in the presence of galactose but is inviable on glucose, indicating that these cells are dependent on production of Php22p from the galactose-inducible promoter for viability. To determine whether our Php21p mutants could support viability, H328 cells were transformed with plasmids expressing wt or mutant C subunits or vector only and then grown on galactose or glucose. Fig. 1 shows that although H328 cells transformed with empty vector are inviable on glucose, all the mutants or glucose. Fig. 1 shows that although H328 cells transformed with plasmids expressing wt or mutant C subunits or vector only and then grown on galactose but is inviable on glucose, indicating that the roles of these residues in B subunit binding are highly conserved.

When these same immunoprecipitates were probed with anti-T364D antibody (characterized in Fig. 2B), T364D and Y367E were found to bind greatly reduced levels of Tpd3p compared with wt Php21p, T364A, and Y367F (Fig. 2C). However, the effect of acidic substitution of Thr-364 and Tyr-367 on Tpd3p binding was less dramatic than on the association of Cdc55p. Decreased A subunit binding was also found previously for the corresponding mammalian C subunit tyrosine mutant (Y307E) but not for the corresponding mammalian threonine mutant (T304D) (10).

To determine whether loss of Cdc55p and Tpd3p binding affects the levels of these proteins in cells, lysates from cells expressing wt or mutant Php21p proteins were probed with antibodies to these proteins. Similar levels of these two proteins were found in all samples (Fig. 2D), indicating that decreased binding did not impact protein stability in vivo.

Mutations in Tyrosine 367 Cause a Temperature-sensitive Phenotype—Previously, a strain deleted for CDC55 was reported to be cold-sensitive (30) but not temperature-sensitive (28). Because the strain expressing our mutants, H328, has a different parental background (W303a) than the strain for which ΔCDC55 cold sensitivity was reported, we first analyzed the growth of a W303a CDC55 deletion strain (ADR496) at different temperatures. Although we could not detect a cold-sensitive phenotype (data not shown), this strain demonstrated a moderate temperature-sensitive phenotype (Fig. 3A).

To determine whether any of the C subunit mutants were temperature-sensitive or cold-sensitive for growth, H328 cells expressing untagged wt or mutant Php21p proteins were tested for growth at 14, 25, and 37 °C. Although none of the mutants displayed a cold-sensitive phenotype (data not shown), several

The Importance of C Subunit Carboxy-terminal Residues for Interaction with B Subunit Is Highly Conserved between Mammals and Yeast—We next analyzed amino-terminal HA-tagged versions of all the Php21p carboxyl-terminal point mutants except L369A for their ability to bind Cdc55p by immunoprecipitating them via their epitope tag and immunoblotting for coimmunoprecipitated Cdc55p. For unknown reasons, the addition of an amino-terminal HA tag to L369A resulted in undetectable expression of this mutant using several different constructs, and HA-tagged L369A was unable to support the growth of H328 cells. As described under “Materials and Methods,” we raised a polyclonal antibody to Cdc55p to use in this assay. Fig. 2A shows the characterization of this antibody and the validation of the coimmunoprecipitation assay. As expected, the antibody detected a strong Cdc55p band in lysates from wt cells (lane 1) that was missing in lysates from cells deleted for CDC55 (ΔCDC55; lane 2). Preimmune serum from the same rabbit did not detect the Cdc55p band (not shown). In addition, the antibody easily detected Cdc55p coimmunoprecipitated with HA-tagged wt C subunit (lane 4), whereas a parallel immunoprecipitate (lane 3) from cells expressing untagged wt Php21p had no detectable Cdc55p.

Fig. 2C shows the results of analyzing Cdc55p binding to all the Php21p mutants except L369Δ. Although the control immunoprecipitate from cells expressing untagged Php21p (lane 1) again contained no detectable Cdc55p, Cdc55p was specifically coimmunoprecipitated with wt HA-Php21p and with the HA-tagged T364A and Y367F mutants (lanes 2, 3, and 6, respectively). In contrast, Cdc55p could not be detected in immunoprecipitates of T364D (lane 4) or Y367E (lane 5) even on long exposures. These results parallel those obtained previously with the corresponding mammalian mutants (Table I), indicating that the roles of these residues in B subunit binding are highly conserved.

The antibody detected strongly reduced levels of Tpd3p compared with wt Php21p, T364A, and Y367F (Fig. 2C). However, the effect of acidic substitution of Thr-364 and Tyr-367 on Tpd3p binding was less dramatic than on the association of Cdc55p.
mutants were temperature-sensitive (Fig. 3B). Y367E showed the most severe temperature-sensitive phenotype, whereas Y367F and L369D showed intermediate sensitivity. wt Pph21p, T364A, and T364D did not demonstrate a temperature-sensitive phenotype. Thus, the temperature sensitivity of the mutants appears to be residue-specific. Furthermore, this temperature-sensitive phenotype does not correlate with their ability to bind Cdc55p in our assay (Table I).

Cells Expressing C subunit Mutants Defective in Cdc55p and Tpd3p Binding Are Nocodazole-sensitive—Several laboratories have shown that deletion of CDC55 or TPD3 results in sensitivity to the microtubule depolymerizing drug, nocodazole (32, 33). To determine whether decreased binding of Cdc55p and Tpd3p to C subunit results in the same phenotype, we tested whether H328 cells expressing Pph21p mutants unable to stably bind Cdc55p were more sensitive to nocodazole than wt Pph21p cells expressing T364A, Y367E, or L369Δ were very sensitive to nocodazole treatment, whereas cells expressing T364A showed the same sensitivity to the drug treatment as cells expressing wt Pph21p. T364D and Y367E do not bind Cdc55p, and from the mammalian data (Table I), L369Δ would be predicted not to bind. Decreased Cdc55p/Tpd3p binding therefore correlates well with nocodazole sensitivity. The only exception was that cells expressing Y367F had intermediate nocodazole sensitivity and yet bound similar amounts of Cdc55p and Tpd3p as T364A, which had wt sensitivity. This result suggests that Y367F has an additional defect.

PPM1 but Not PPM2 Encodes the Major PP2A Methyltransferase—We hypothesized that mutation of certain C subunit carboxyl-terminal residues may affect Cdc55p and Tpd3p binding indirectly by altering methylation of leucine 369. In particular, the fact that loss of leucine 369 causes sensitivity to nocodazole suggested that methylation of this residue might affect Cdc55p and Tpd3p function via regulation of PP2A complex formation. We therefore used a combined genetic and biochemical approach in S. cerevisiae to determine whether...
methylation of Pph21p is required for Cdc55p and/or Tpd3p association with C subunit and, consequently, for Cdc55p and/or Tpd3p function as assayed by resistance to nocodazole. First, the steady-state \textit{in vivo} C subunit methylation levels of \textit{wt} cells and of cells deleted for one or both of the putative \textit{S. cerevisiae} methyltransferase genes (\textit{DPPM1}, \textit{DPPM2}, and \textit{DPPM1DPPM2}) were compared using an assay employing a methylation-sensitive monoclonal antibody, 1d6, which is specific for unmethylated C subunit (see “Materials and Methods” and Fig. 5). Because base treatment demethylates C subunits, a lysate with methylated C subunits will have an increase in 1d6 signal intensity upon treatment with base, whereas a lysate containing only unmethylated C subunits will have no increase. Fig. 5B shows that deletion of \textit{PPM1} caused a great decrease in vivo C subunit methylation, whereas those expressing \textit{T364A} or \textit{T364D} are not. Cells were grown to saturation at 25 °C. 10-fold serial dilutions were then spotted onto YPD plates and incubated at 25 or 37 °C. For presentation, data from two different plates were photographically merged for each panel. In repeated experiments, \textit{Y367E} always showed a more severe temperature-sensitive phenotype than \textit{Y367F}, \textit{L369A}, and the \textit{CDC55} deletion strain. \textit{WT-CC} and all mutants have the two nucleotides just upstream of the ATG start codon changed to CC in order to create a NcoI site for additional constructs (see “Materials and Methods”).
To determine whether methylation is important for binding of the yeast B subunit, Rts1p, lysates from wt, ΔPPM1, ΔPPM2, and ΔPPM1ΔPPM2 cells expressing both HA-tagged Pph21p (or vector only) and carboxyl-terminal 6×His-tagged Rts1p (Rts1p-6×His) were immunoprecipitated with HA tag antibody. Probing of the immunoprecipitates for the presence of Rts1p-6×His using an anti-6×His tag antibody (Fig. 6C) showed that Rts1p-6×His coimmunoprecipitated specifically with HA-tagged C subunits from wt and ΔPPM2 cells but was not detectable in HA-tag immunoprecipitates from cells deleted for PPM1. Moreover, Tpd3p association was again found to be greatly reduced (Fig. 6C). Thus, methylation appears to be important for binding of Cdc55p, Tpd3p, and Rts1p to Pph21p.

To determine whether Rts1p-6×His was expressed at similar levels in these cells, lysates were probed with Tpd3p and anti-6×His tag antibodies. Relative to Tpd3p, Rts1p-6×His was expressed at a lower level in ΔPPM1 and ΔPPM1ΔPPM2 cells than in wt and ΔPPM2 cells (Fig. 6D). It is not possible to distinguish whether the reduced levels of Rts1p-6×His expression in ΔPPM1 and ΔPPM1ΔPPM2 cells are a cause or an effect of decreased association with C subunit. Although this lower level of expression is not sufficient to account for all the reduction in Rts1p-6×His association seen in Fig. 6C, it makes it difficult to determine the exact amount of reduction in Rts1p-6×His binding due to loss of C subunit methylation. When Rts1p-6×His was immunoprecipitated from lysates of these cells with an anti-6×His antibody, reduced but detectable levels of HA-Pph21p were found in ΔPPM1 and ΔPPM1ΔPPM2 cells compared with wt and ΔPPM2 cells (data not shown), indicating that Rts1p-6His binding is reduced but not abolished.

Deletion of PPM1 Leads to Nocodazole Sensitivity—Wt, ΔPPM1, ΔPPM2, and ΔPPM1ΔPPM2 cells were next analyzed for nocodazole sensitivity as a functional assay for loss of Cdc55p and Tpd3p association. ΔPPM1 and ΔPPM1ΔPPM2 cells were much more sensitive than wt cells to nocodazole treatment, indicating that deletion of PPM1 generates a phenotype consistent with loss of Cdc55p and Tpd3p association (Fig. 7) (32, 33). Although in the experiment shown, ΔPPM2 cells appeared to show some sensitivity to nocodazole at 4 h, they were wt in their sensitivity at 6 h, demonstrating that they did not have a substantially greater sensitivity to nocodazole than wt cells. Thus, loss of PPM1, but not of PPM2, leads to loss of C subunit methylation. This, in turn, appears to result in a large decrease in C subunit association with Cdc55p and Tpd3p, inducing sensitivity to nocodazole.

**DISCUSSION**

PP2A plays important roles in many cellular processes, and consequently, the cell controls its activity, localization, and substrate specificity through a complex set of covalent and noncovalent mechanisms. We have used the budding yeast *S. cerevisiae* as a model system to investigate the hypothesis that covalent modification of the C subunit (Pph21p) carboxyl terminus modulates PP2A complex formation. Our results demonstrate that Cdc55p binding to Pph21p was disrupted by either acidic substitution of potential carboxyl-terminal phosphorylation sites or by deletion of the gene for the yeast PP2A methyltransferase homolog, Ppm1p, which resulted in almost complete loss of Pph21p methylation. Loss of Cdc55p association was accompanied in each case by a large reduction in Tpd3p binding to C subunit. Moreover, decreased Cdc55p and...
Our findings provide evidence that this modification is capable of regulating protein-protein interactions in a manner similar to phosphorylation. The total amount of C subunit in mammalian cells is known to be tightly regulated and does not appear to vary over the course of the cell cycle (41). Because methylation of PP2A C subunit has been shown to fluctuate during the cell cycle in mammalian cells (25), methylation may be a key mechanism for regulating PP2A function in a cell cycle-dependent manner.

Our results show that Ppm1p is the major PP2A methyltransferase in *S. cerevisiae*. Although deletion of *PPM2* did not lead to significant change of the methylation level of Phf21p, we could not rule out that Ppm2p might have some PP2A methyltransferase activity. Instead, in repeated experiments, we found that the level of unmethylated Phf21p in ΔPPM1ΔPPM2 was slightly higher than in ΔPPM1, suggesting that Ppm2p may have low methyltransferase activity in *vivo*, perhaps on a specific subpopulation of PP2A or at a specific time in the cell cycle. Because Ppm2p has an ~350-amino acid extension that may be involved in actin binding, Ppm2p might localize to actin-containing structures (23) and be responsible for methylating a small fraction of C subunit localized to that part of the cytoskeleton.

Deletion of *PPM1* led not only to decreased Cdc55p, Tpd3p, and Rts1p binding but also to nocodazole sensitivity. The nocodazole sensitivity may be due to loss of Cdc55p and/or Tpd3p binding because previous studies have demonstrated that deletion of *CDC55* or *TPD3* leads to nocodazole sensitivity (32, 33). However, we cannot rule out an effect of Rts1p or another, unknown regulatory subunit whose binding may be affected by methylation. In any case, our results clearly show that changes in methylation level of C subunit can affect the function of PP2A in *vivo*.

The effects of mutation or methylation on the binding of one or more of the PP2A subunits could be indirect. Tpd3p has been shown to be required for Rts1p binding to C subunit (31), and Cdc55p presumably has a similar requirement based on mammalian *in vitro* data. However, the fact that mutation or loss of methylation leads to a more severe effect on Cdc55p binding to Tpd3p association suggests that loss of Cdc55p binding is not solely due to loss of Tpd3p association. Instead, decreased Cdc55p association may be directly affected by mutation or loss
of C subunit methylation and cause a destabilization of
A(Tpd3p)/C subunit heterodimeric complexes.

Deletion of the CDC55 gene has previously been shown to
result in a cold-sensitive phenotype (30). However, our
findings have shown that ADR496, a CDC55 deletion strain, displays
a temperature-sensitive phenotype, suggesting that Cdc55p may
be important in this strain for response to stress induced by
 Elevated temperature. RT51 or TPD3 deletion is also known to
cause a temperature-sensitive growth phenotype. H328 cells
expressing the T364D mutant, which does not interact stably with
Cdc55p, are not temperature-sensitive. Furthermore, both
\( \Delta PPM1 \) and \( \Delta PPM1 \Delta PPM2 \) strains displayed no tempera-
ture-sensitive phenotype even though Pph21p association with
D. It is possible that these modifications are transient and
اتion directly (16). Although we have not been able to detect
residues might regulate B subunit-directed PP2A functions by
 together, these results suggest that phosphorylation of these
residues abolishes Cdc55p (B subunit) binding, whereas
completely conserved in all organisms, suggesting that they have
been detected by our assay.

Prevention of the temperature-sensitive phenotype but cannot be
proved by Emory University in accordance with its conflict of interest
policies.

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