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Cdc55p-Mediated E4orf4 Growth Inhibition in *Saccharomyces cerevisiae* Is Mediated Only in Part via the Catalytic Subunit of Protein Phosphatase 2A[▽]

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The adenovirus early region 4 open reading frame 4 (E4orf4) protein specifically induces p53-independent cell death of transformed but not normal human cells, suggesting that elucidation of its mechanism may provide important new avenues for cancer therapy. Wild-type E4orf4 and mutants that retain cancer cell toxicity also induce growth inhibition in *Saccharomyces cerevisiae*, which provides a genetically tractable system for studying E4orf4 function. Interaction with the protein phosphatase 2A (PP2A) B regulatory subunit is required for E4orf4's effects, suggesting that E4orf4 may function by regulating B subunit-containing heterotrimeric PP2A holoenzymes ($PP2A_{BAC}$), which consist of a B subunit complexed with the PP2A structural (A) and catalytic (C) subunits. However, it is not known whether E4orf4-induced growth inhibition requires interaction with the PP2A C subunit or whether E4orf4 might have PP2A B subunit-dependent effects that are independent of $PP2A_{BAC}$ holoenzyme formation. To test these possibilities in *S. cerevisiae*, we disrupted the stable formation of $PP2A_{BAC}$ heterotrimers and thus E4orf4/C subunit association by PP2A C subunit point mutations or by deletion of the gene for the PP2A methyltransferase, Ppm1p, and assayed for effects on E4orf4-induced growth inhibition. Our results support a model in which E4orf4 mediates growth inhibition and cell killing both through $PP2A_{BAC}$ heterotrimers and through a B regulatory subunit-dependent pathway(s) that is independent of stable complex formation with the PP2A C subunit. They also indicate that Ppm1p has a function other than regulating the assembly of PP2A heterotrimers and suggest that selective PP2A trimer inhibitors and PP6 inhibitors may be useful as adjuvant anticancer therapies.

The adenovirus protein E4orf4 is a multifunctional protein that plays an important role in controlling the alternative splicing pattern of adenovirus L1 mRNA by inhibiting the splicing repressor activity of certain host SR proteins (9, 22). E4orf4 also moderates transcriptional activation mediated by the adenovirus E1A protein, including AP-1 (c-*fos*/jun-B heterodimer) transcription factor activity and transactivation of the adenovirus E2 and E4 promoters (6, 24, 30, 36). E4orf4 mediates these effects at least in part by inducing the dephosphorylation of viral and host proteins, including E1A, c-*fos*, and SR proteins, and by regulating the expression of jun-B (24, 36). Expression of E4orf4 alone in mammalian cells induces G_2/M arrest (25) followed by p53-independent cell death in cancer cells, but not in normal human cells (32, 42), thus making E4orf4 a potentially useful tool for cancer therapy. Moreover, investigating the mechanisms underlying E4orf4 function may help identify important drug targets for cancer treatment.

E4orf4 induction of cell death in transformed cells requires an interaction with the serine/threonine protein phosphatase PP2A (24, 43). PP2A is a highly conserved, ubiquitous eukaryotic protein phosphatase that has broad biological roles in important cellular functions, such as metabolism, initiation of DNA replication, cell cycle control, and apoptosis, and has been implicated in the development of human cancers (20, 21, 37). The core enzyme of PP2A is a heterodimer consisting of a catalytic C subunit and a structural A subunit. This core enzyme can associate with one of a number of distinct B-type subunits to form a wide variety of heterotrimeric holoenzymes (21). In mammalian cells, there are three B-type subunit families (B/B55, B′/B56, and B″/PR72/130) and a putative fourth family (striatin family), each of which has multiple isoforms. In *S. cerevisiae*, PP2A C subunits are encoded by two genes, *PPH21* and *PPH22*, whereas the PP2A A subunit is encoded by a single gene, *TPD3* (54). Moreover, two B-type families are represented: a single PP2A B/B55 subunit encoded by *CDC55* (18, 57) and a single B′/B56 subunit encoded by *RTS1* (45).

Of the different PP2A heterotrimers, only $PP2A_{B\alpha AC}$ heterotrimers have been shown to be critical for E4orf4-induced growth inhibition and cell killing of transformed cells. E4orf4 interacts with the $PP2A_{B\alpha AC}$ holoenzyme complex through direct interaction with the Bα subunit (24). Expression of PP2A Bα antisense inhibits the induction of cell death by E4orf4 (43), and overexpression of Bα increases cell death (44). In addition, E4orf4 mutants with defects in complex formation with PP2A Bα cannot induce cell death (31). Thus, the PP2A Bα interaction with E4orf4 is clearly required for E4orf4-induced cell death.

Expression of E4orf4 also inhibits the growth of *S. cerevisiae*, resulting in irreversible arrest of cells in G_2/M and elongated

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cell morphology (41). Multiple observations suggest that the mechanism of E4orf4-induced growth inhibition in *S. cerevisiae* is very similar to the mechanism of E4orf4-induced cell death in mammalian cells. First, E4orf4 arrests both mammalian and yeast cells in G_2/M (25, 41). Second, E4orf4 interacts with the PP2A Bα subunit in mammalian cells and the PP2A B subunit, Cdc55p, in *S. cerevisiae*, and this interaction is required for E4orf4 function in both cell types (24, 41, 44). Third, E4orf4 mutants that cannot bind to the PP2A B subunit and cannot induce cell death in mammalian cells are also unable to induce growth inhibition in yeast and vice versa (2, 41). Fourth, E4orf4 induces accumulation of reactive oxygen species both in mammalian cells and in yeast cells (25, 27). Therefore, analysis of the mechanism of E4orf4-induced growth inhibition in yeast is likely to provide insight into the mechanism of E4orf4-induced cell death in mammalian cells.

The carboxy terminus of the PP2A C subunit is highly conserved from yeast to humans and is modified by both methylation and phosphorylation. In both mammalian and yeast cells, addition and removal of a single methyl group on the carboxy-terminal leucine of the PP2A C subunits differentially regulates the formation of certain PP2A heterotrimers (16, 50, 58–60). In yeast, deletion of the gene for the PP2A methyltransferase Ppm1p almost abolishes ($\sim$20-fold reduction) stable formation of PP2A heterotrimers containing the Cdc55p subunit, while PP2A heterotrimers containing Rts1p are reduced less than 2-fold ($\sim$40%) (16, 58, 59). Mutation of the catalytic subunit carboxy terminus to mimic phosphorylation or to remove the site of methylation has also been shown in yeast to disrupt $PP2A_{BAC}$ heterotrimers and, in some cases, $PP2A_{B'AC}$ heterotrimers, providing useful tools for investigating PP2A function in E4orf4-induced growth inhibition and cell killing (10, 16, 58, 59).

Although it has generally been assumed that E4orf4 functions through the $PP2A_{BAC}$ heterotrimeric complex because of the importance of the PP2A B subunit for E4orf4-induced growth inhibition/cell killing in both mammalian and yeast cells, little is known about the involvement of the A and C subunits. Previous results from yeast using a nonquantitative assay indicated that Tpd3p is required for full E4orf4-induced growth inhibition (25), but the relative importance of Tpd3p and Cdc55p has not been quantitatively compared. Moreover, a requirement for the PP2A C subunit in E4orf4-induced growth inhibition and cell killing has not been demonstrated. Thus, it is possible that the B subunit (Cdc55p) mediates the growth inhibition function of E4orf4 in part through a pathway that is independent of the A subunit (Tpd3p), C subunit (Pph21p/Pph22p), or both.

One possibility for a PP2A C subunit-independent pathway could be that the B subunit (Cdc55p) provides a function to E4orf4 through association with another PP2A family catalytic subunit. In mammalian cells, PP2A A and B subunits have been reported to form trimeric complexes with PP5, the mammalian homolog of the *S. cerevisiae* phosphatase Ppt1p (28). In *S. cerevisiae*, the PP2A A subunit (Tpd3p) and B subunit (Cdc55p) have also been proposed to interact with Sit4p, the mammalian homolog of protein phosphatase 6 (PP6) (4), to form a heterotrimeric complex (38). Thus, the possibility exists that the B subunit may mediate E4orf4-induced growth inhi-

bition and cell killing through multiple PP2A family C subunits and perhaps even independently of PP2A C subunits.

In the present study we have quantitatively compared the effects of PP2A A subunit (*TPD3*) deletion and PP2A B subunit (*CDC55*) deletion on E4orf4 growth inhibition in *S. cerevisiae* in order to determine whether E4orf4 mediates some of its effects via Cdc55p independently of Tpd3p. We have also used PP2A C subunit mutants and deletion of the PP2A methyltransferase gene *PPM1* to disrupt $PP2A_{Cdc55p/Tpd3p/Pph21(22)p}$ heterotrimer formation (and thus the association of E4orf4 with Pph21p/Pph22p) to determine the importance of Pph21p/Pph22p association with E4orf4 for E4orf4-mediated growth inhibition. Moreover, we have assayed whether E4orf4 associates with Sit4p in *S. cerevisiae* and have analyzed the effects of deletion of genes for various PP2A family phosphatase catalytic subunits, including Sit4p on E4orf4-induced growth inhibition, to determine whether E4orf4 might function through, or genetically interact with, any of these phosphatases. Our observations support a new model in which E4orf4 mediates growth inhibition and cell killing both through $PP2A_{BAC}$ heterotrimers and through a B regulatory subunit-dependent pathway that is independent of the PP2A C subunit. Our analysis also indicates that Ppm1p has a function other than regulating the assembly of PP2A heterotrimers. Finally, our results suggest that selective PP2A trimer inhibitors and PP6 inhibitors may be useful as adjuvant anticancer therapies.

MATERIALS AND METHODS

Yeast strains. All yeast strains that were used in this study are described in Table 1. BY4741 wild-type and deletion strains from the *S. cerevisiae* knockout collection were a kind gift from the laboratory of Anita Corbett or were obtained from Research Genetics. W303a and its isogenic derivative, ADR496, were obtained from A. Murray. $\Delta TPD3$ cells were obtained from the laboratory of Richard Hallberg. DEY3 cells were originally created by Evans and Stark (11). These cells are deleted for *PPH21*, *PPH22*, and *PPH3* and contain a *URA3* maintenance plasmid that expresses Pph22p from its own promoter (11). $\Delta CDC55$ $\Delta TPD3$ cells were constructed by crossing ADR496 and $\Delta TPD3$ cells and screening for colonies able to grow on medium lacking histidine or leucine; then, double deletions of the *CDC55* and *TPD3* genes were confirmed by PCR. PCR-based gene deletion (5) utilizing a set of previously described vectors (46) was used to make additional knockout strains in *S. cerevisiae*; all primers used are listed in Table 2, and the markers used are indicated in Table 1. The primers in Table 2 used for deletion of the *CDC55* gene in DEY3 cells were previously published by Roopchand and colleagues (41). Strains deleted for *RTS1* demonstrated temperature sensitivity at 37°C, consistent with previous data (45). The double deletion strain $\Delta SIT4$ $\Delta CDC55$ was created by deletion of *CDC55* in the $\Delta SIT4$ strain obtained from Research Genetics. Each deletion strain was confirmed by PCR, at a minimum with a sense primer upstream of the deleted gene and an antisense primer internal to the drug resistance marker inserted. In addition, deletion of *CDC55* in the $\Delta SIT4$ $\Delta CDC55$ strain was confirmed by Western blotting for Cdc55p.

Yeast media. Yeast were grown in YPD (1% yeast extract, 2% Bacto peptone, 2% glucose), synthetic complete (SC), or synthetic dropout media. Growth media were either prepared according to standard recipes (1) or obtained from Bio101 (Carlsbad, CA). To induce expression of E4orf4 from the *GAL1-10* promoter, 2% galactose was used instead of glucose. The 5-fluoroorotic acid (5-FOA) medium consisted of 2% glucose, 0.67% yeast nitrogen base without amino acids, the required auxotrophic supplements, 50 µg/ml uracil, and 0.5 mg/ml 5-FOA.

Plasmids. pRS424 (2µm *TRP1 A*) (8) and pRS314 (CEN6 *TRP1 A*) (46) were gifts from the laboratory of Anita Corbett. To provide appropriate selection markers for PCR-based gene disruption of yeast with limited selection markers, two plasmids were used. pFA-KanMX (56) was used for PCR of *KAN* cassettes, and pAG32 (hphMX4) (17) was used for PCR of hygromycin B (hph) cassettes. pRS314-*PPH21* wild-type and T364A, T364D, Y367E, Y367F, and L369Δ plasmids were subcloned from corresponding pRS316 plasmids that were described

TABLE 1. Yeast strains used in the study

Yeast strain	Genotype	Reference or source
W303-1A	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*	35
DEY3[a]	*MATa pph21Δ::LEU2 pph22Δ1::HIS3 pph3Δ1::LYS2 lys2-951 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 ssd1-d1 {YCpDE8 PPH22} W303*	11
ADR496[a]	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc55Δ::HIS3*	33
ΔTPD3[a]	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 tpd3Δ::LEU*	54
BY4741[b]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
ΔCDC55[b]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cdc55Δ::HIS3*	This study
ΔPPH3[b]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pph3Δ::KAN*	Research Genetics
ΔPPT1[b]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ppt1Δ::KAN*	Research Genetics
ΔPPZ1[b]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ppzΔ::KAN*	Research Genetics
ΔSIT4[b]	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sit4Δ::KAN*	Research Genetics
YDP001[b] (ΔSIT4ΔCDC55)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sit4Δ::KAN cdc55Δ::HIS3*	This study
YDP002[a] (ΔCDC55ΔTPD3)	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc55::HIS3 tpd3::LEU W303*	This study
YDP003[a] (ΔRTS1)	*MATa pph21Δ::LEU2 pph22Δ1::HIS3 pph3Δ1::LYS2 lys2-951 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 ssd1-d1 rts1::KAN {YCpDE8 PPH22} W303*	This study
YDP004[a] (ΔCDC55)	*MATa pph21Δ::LEU2 pph22Δ1::HIS3 pph3Δ1::LYS2 lys2-951 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 ssd1-d1 cdc55::KAN {YCpDE8 PPH22} W303*	This study
YDP005[a] (ΔPPM1)	*MATa pph21Δ::LEU2 pph22Δ1::HIS3 pph3Δ1::LYS2 lys2-951 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 ssd1-d11 ppm1::KAN {YCpDE8 PPH22} W303*	This study
YDP006[a] (ΔPPM1ΔCDC55)	*MATa pph21Δ::LEU2 pph22Δ1::HIS3 pph3Δ1::LYS2 lys2-951 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 ssd1-d1 ppm1::KAN cdc55::HYG {YCpDE8 PPH22} W303*	This study

[a] Isogenic with W303-1A.
[b] Isogenic with BY4741.

previously (58). The Pph21p mutants are referred to by the position mutated preceded by the wild-type amino acid (single letter code), followed by the introduced residue. pYES2 and pYES2-GAL-HA-E4orf4 have been previously published (41). Yeast transformations were performed as previously described (7).

Plasmid shuffle method. DEY3 cells, which contain a Pph22p-expressing plasmid with a *URA3* selectable marker, were transformed with *TRP* marker-containing pRS314 plasmids expressing wild-type Pph21p or one of the Pph21p mutants, T364A, T364D, Y367E, Y367F, or L369Δ, and then grown on SC-Trp plates. Colonies were picked and streaked on 5-FOA plates to select for cells without the *URA3*-containing plasmid. Cells grown on 5-FOA plates were then streaked on SC-Ura and SC-Trp plates to confirm the loss of the *URA3* plasmid and the presence of the *TRP1* plasmid.

Serial dilution spotting assay for measuring E4orf4-induced growth inhibition of *S. cerevisiae* cells. Growth inhibition by E4orf4 was quantitatively assessed using serial dilution cell growth assays. Cells were grown at 30°C in 2% glucose medium for 2 days or to saturation. Cells were then diluted with sterile H$_2$O to the concentration of ~2 × 10^6 cells/ml. Four 10-fold serial dilutions were then made with sterile H$_2$O, and 5 μl of each dilution was spotted on agar plates containing 2% glucose or 2% galactose. If not indicated in the figure legends, photographs were taken 2 to 3 days after spotting. In some cases, cells were grown on 2% raffinose medium for 2 h before dilution and spotting.

Antibodies. Anti-hemagglutinin (HA) tag antibodies 16B12 and F-7 were obtained from Berkeley Antibody Company and Santa Cruz Biotechnology, Inc. PP6 antibody directed against a C-terminal peptide was obtained from Stratagene. Sit4p antibody was a kind gift from Yu Jiang (38). An affinity-purified rabbit polyclonal antibody specific for Pph21p and Pph22p was used to visualize Pph21p proteins in cell lysates (16). Ppm1p rabbit polyclonal antiserum (RK3332) was raised to two Ppm1p peptides (SKEDTAKSPFLIDQGRYKC and QSNLKESRNLEMPTLMTYNSKEKYASRC), corresponding to Ppm1p residues 153 to 170 and 248 to 274, respectively, with an additional carboxy-terminal cysteine for coupling to keyhole limpet hemocyanin using the Pierce Inject kit. The specificity of this antibody was evaluated using affinity purification and wild-type and ΔPPM1 cells (not shown). Cdc55p antibody, a gift from the laboratory of Egon Ogris, has been previously described (58).

Preparation of cellular extracts, immunoprecipitation, and Western blotting.

TABLE 2. Primers used for yeast gene disruption

| Gene	Primers
CDC55::KAN	5'-CCTCATAAAATCTAGCCAACATATCGAGGTCAAACTGGAGAGGATATCAAGCTTGCCTCG-3' 5'-GAATTCAAGTTCAATTTAAATTTCAATTAAAACAGTAGTAGTATGTGGGGAAGATATGGGGTCGACACTGGATGGCGGC-3'
CDC55::HIS3	5'-GGAGTTTGCACAAGACAAG-3' 5'-GAAGTGATGAAAGAAGTCC-3'
CDC55::HYG	5'-TCCTTGTGACTCCGCATAAACTAGATGATAAAGAGTACAAACAAGTCGCCCAGCTGAAGCTTCGTACGC-3' 5'-AACGGTAAGCATATTAAGATCAAATTAGTTGAGGCTGTAAATAAAAAGCATAGGCCACTAGTGGATCTG-3'
RTS1::KAN	5'-CGGCCTACTTGATGCTCTACC-3' 5'-CTAAACTTCCTCACTTCTTCG-3'
PPM1::KAN	5'-CTTATGCCATAAGAAGCAG-3' 5'-TGGAACAATGCCCTGCATC-3'
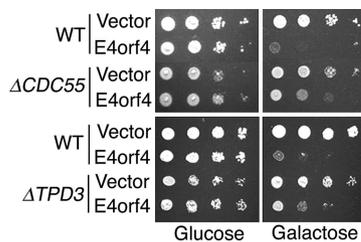
FIG. 1. Deletion of either *CDC55* or *TPD3* partially rescues *S. cerevisiae* cells from E4orf4-induced growth inhibition to a similar extent. A serial dilution cell growth assay (10-fold dilutions from left to right) was carried out as described in Materials and Methods with W303 wild-type (WT), Δ*CDC55*, and Δ*TPD3* cells expressing either control empty vector (vector) or vector containing galactose-inducible E4orf4 (E4orf4). The growth of these cells on glucose- and galactose-containing plates is shown. The lower panels were grown longer than the upper panels because loss of Tpd3p causes slower growth. However, it is clear that, relative to vector controls, loss of *CDC55* or *TPD3* provides a similar relative extent of rescue from E4orf4-induced growth inhibition. The results shown are representative of results obtained from three independent experiments.

Yeast cells were harvested and lysed in lysis buffer (100 mM Tris-Cl pH 7.5, 200 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol) containing 1 mM phenylmethylsulfonyl fluoride and yeast cocktail inhibitor (Sigma) as previously described using glass beads and vortexing (49, 58) or a mini-beadbeater (BIOSPEC). The lysate protein concentration was determined using Bio-Rad DC protein assay reagent, and then a one-half volume of radioimmunoprecipitation assay buffer (50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) was added. Immunoprecipitation of E4orf4-PP2A complexes via HA-tagged E4orf4 was then performed using anti-HA tag monoclonal antibody (16B12 or F-7) plus protein A-Sepharose beads and incubation at 4°C for 1 h 30 min. After immunoprecipitation, the samples were boiled in gel loading buffer and analyzed by 10% or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were transferred to nitrocellulose membranes and immunoblotted. Bands were visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. Bands were quantitated using a Fluor S-Max chemilumimager (Bio-Rad, Richmond, CA) that directly measures band intensities without the use of film via a supercooled charge-coupled-device camera that provides linear data over 4.8 orders of magnitude. This method yielded highly reproducible results that did not vary with image capture times.

Cell culture and creation of stable lines that express vector control or PP6-directed shRNAs. Human 293T cells for generating lentiviral stocks and H1299 human lung cancer cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 10% CO_2 at 37°C. To create H1299 lines that stably express shRNA vector (vector control) or PP6-directed shRNA, cells were infected with shRNA lentiviruses. These lentiviruses were generated using a three-plasmid-based lentivirus system (34) (available from The RNAi Consortium [TRC] at the Broad Institute). The targeted sequence in the PP6 shRNA lentivirus we used was 5'-CCAGAACGACAACGCCATATT-3' (TRC ID TRCN0000002765). After infection with the appropriate lentivirus, infected cells were selected with puromycin (2 μg/ml) until a control dish of uninfected H1299 cells was completely dead. After incubating at least one additional day to allow the cells to recover from puromycin treatment, immunoblotting was used to confirm the knockdown of PP6. Cells were then used for experiments, and aliquots of cells were frozen for later use. Although PP6 knockdown was quite stable (data not shown), cells were used within 10 days after selection and thawing.

Adenovirus infection and WST-1 cell viability assay. A 96-well plate was seeded with 100 μl of H1299 cells per well at a concentration of 2.5×10^4 cells/ml. The next day, cells were infected with control adenovirus (CMVrtTA) at a multiplicity of infection (MOI) of 10 or with E4orf4-expressing adenovirus (CMVrtHA-E4orf4) and CMVrtTA adenovirus, each at an MOI of 5, and then E4orf4 expression was induced by the addition of 1 μg/ml doxycycline. Four days after infection, cell viability was assayed in a water-soluble tetrazolium salt (WST-1) killing assay (Roche).

RESULTS

Disruption of PP2A$_{BAC}$ complexes by deletion of *TPD3* rescues E4orf4-induced slow growth in *S. cerevisiae* to the same extent as deletion of *CDC55*. We first wanted to determine whether the B subunit (Cdc55p) might provide a function to E4orf4 that is independent of the A subunit (Tpd3p). We reasoned that if this were the case, then *CDC55* deletion should rescue *S. cerevisiae* cells from E4orf4 growth inhibition more effectively than *TPD3* deletion.

To test this possibility, serial dilution cell growth assays were carried out to compare the effects of deletions of *TPD3* and *CDC55* on E4orf4-induced growth inhibition of W303 *S. cerevisiae* cells. Wild-type, Δ*TPD3*, and Δ*CDC55* cells were transformed with a galactose-inducible E4orf4 plasmid, and cell growth was compared on glucose and galactose media. Figure 1 shows that when E4orf4 was induced on galactose plates, the growth of the wild-type cells was strongly inhibited. Deletion of *CDC55* could partially rescue E4orf4-induced slow growth, confirming the previous finding that E4orf4 functions through Cdc55p-dependent and Cdc55p-independent pathways (41). Deletion of *TPD3* partially rescued E4orf4-induced slow growth to a similar extent as deletion of *CDC55*, suggesting that Cdc55p does not provide a function to E4orf4-induced growth inhibition that is independent of the A subunit.

Because Δ*TPD3* cells grow more slowly under normal growth conditions than Δ*CDC55* cells (54), we used a second approach to compare the importance of Tpd3p and Cdc55p for E4orf4-induced growth inhibition. We reasoned that if Cdc55p provided a Tpd3p-independent function to E4orf4, then double deletion of *CDC55* and *TPD3* should rescue E4orf4-mediated growth inhibition more effectively than deletion of *TPD3* alone. Δ*CDC5* Δ*TPD3* cells and Δ*TPD3* cells grow at a similar rate and thus should provide a fair comparison that is not affected by the general health of the cells. Therefore, we compared the growth rates in liquid medium of wild-type, Δ*TPD3*, and Δ*CDC55* Δ*TPD3* cells expressing E4orf4 or containing an empty vector (control). The average doubling time for cells with or without E4orf4 was calculated from three independent experiments, and the ratios of doubling times of cells with E4orf4 to cells without E4orf4 were compared. As indicated in Table 3, E4orf4 caused an ~58% increase in the doubling time of wild-type cells. Consistent with the results from serial dilu-

TABLE 3. Double deletion of *CDC55* and *TPD3* does not rescue cells from E4orf4-induced growth inhibition more effectively than deletion of *TPD3* alone[a]

Yeast strain	t_d ratio
Wild type (W303)	1.58 ± 0.08
Δ*tpd3*	1.11 ± 0.12
Δ*cdc55* Δ*tpd3*	1.13 ± 0.09

[a] Cells were grown in 2% glucose medium to log phase. Cells were then washed and grown in 2% raffinose medium for 2 h. Cells were then counted and diluted to equal number of cells or were diluted to an optical density at 600 nm (OD_{600}) of 0.05. The growth of the cells was monitored by counting cells using a hemacytometer or by measuring the OD_{600}. To accurately measure the OD, cells were diluted at some point so that the OD_{600} reading would be below 1.0. The doubling times (t_d) of the cells were calculated using the cell numbers and times from the exponential phase for regression analysis. The ratios of the t_d for cells expressing Eforf4 over the t_d for cells with vector only are reported as mean ± standard deviation.

tion cell growth assays, indicating that deletion of *TPD3* can partially rescue E4orf4-induced slow growth (Fig. 1), E4orf4 caused only an ~11% increase in the doubling time of Δ*tpd3* cells. Double deletion of *TPD3* and *CDC55* did not further diminish the growth inhibition effect of E4orf4 compared to deletion of *TPD3* alone (Table 3), indicating that Cdc55p does not mediate an E4orf4-induced growth inhibition function in these cells through a pathway independent of Tpd3p.

Disruption of Cdc55p association with the C subunit by mutating the PP2A C subunit does not have the same effect on E4orf4-induced growth inhibition as loss of Cdc55p expression. Although our data indicate that Cdc55p and Tpd3p both appear to be equally involved in E4orf4-mediated growth inhibition, the importance of the PP2A catalytic C subunits (Pph21p/Pph22p) has not been directly tested. It is difficult to assess the contribution of the PP2A C subunits by deletion, because double deletion of *PPH21* and *PPH22*, the redundant genes that encode the yeast C subunit, severely impairs yeast cell growth. For example, Δ*PPH21* Δ*PPH22* cells in a W303 background grow very poorly on glucose-containing medium and are inviable on galactose-containing medium, even in the absence of E4orf4 expression (reference 40 and unpublished data). To circumvent this problem, we used alternative approaches to disrupt Cdc55p (and thus E4orf4) association with the PP2A catalytic subunit that did not involve deletion of the genes for both catalytic subunits. As a first approach, we tested whether Pph21p mutants defective in $PP2A_{Cdc55p/Tpd3p/Pph21(22)p}$ complex formation could rescue E4orf4-induced growth inhibition. We reasoned that if E4orf4 mediated its Cdc55p-dependent growth inhibitory effects solely via this trimeric complex, then disruption of Cdc55p (and thus E4orf4) binding to the PP2A catalytic subunit with these mutants would rescue E4orf4-mediated growth inhibition to a similar extent as deletion of *CDC55*.

We previously demonstrated that certain mutations in the C terminus of Pph21p abolish yeast $PP2A_{BAC}$ heterotrimeric complex formation (15, 16, 58). Specifically, Pph21p mutants with acidic substitutions of threonine 364 or tyrosine 367, termed T364D and Y367E, do not associate stably with Cdc55p, while mutants with conserved neutral substitutions at these same positions, T364A and Y367F, associate with Cdc55p at near-wild-type levels. In addition, a Pph21p mutant lacking the carboxy-terminal leucine, L369Δ, did not form PP2A heterotrimers containing Cdc55p (15, 16). In this study, we directly tested for the ability of these mutants to associate with E4orf4 by examining whether they could be coimmunoprecipitated with epitope-tagged E4orf4. The results shown in Fig. 2 indicate that while wild-type Pph21p and the neutral substitution mutant controls T364A and Y367F were easily detected in E4orf4 immunoprecipitates, T364D, Y367E, and L369Δ did not specifically associate with E4orf4.

Next, to assess the importance of E4orf4 association with the PP2A C subunit for E4orf4-induced growth inhibition, we assayed the ability of E4orf4 to inhibit the growth of cells expressing only exogenous wild-type or mutant PP2A catalytic subunits. The DEY3 strain was used for these experiments because it has the chromosomal *PPH21* and *PPH22* PP2A catalytic subunit genes deleted and a *URA*-selectable maintenance plasmid that expresses Pph22p from its own promoter (Table 1). We used a plasmid shuffle technique described in Materials and Methods to create cells expressing only exoge-
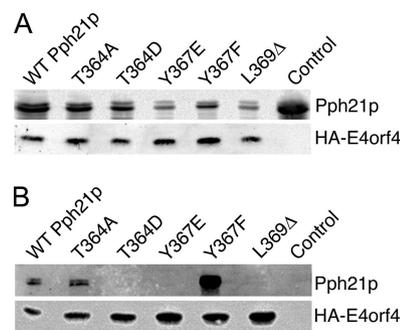

FIG. 2. The Pph21p mutants T364D, Y367E, and L369Δ do not associate stably with E4orf4. DEY3 yeast cells containing a galactose-inducible pYES2 vector encoding HA-E4orf4 and a noninducible pRS314 vector encoding either wild-type (WT) Pph21p or one of the indicated mutant Pph21p proteins were induced with galactose and then lysed as described in Materials and Methods. As a control, DEY3 cells containing empty pYES2 vector and pRS314 encoding wild-type Pph21p were also grown on galactose-containing medium and lysed (control). Because E4orf4 expression varies between the cell lines, cell lysates were analyzed by Western blotting for E4orf4 (not shown), and lysate amounts used for immunoprecipitation were normalized to contain equal amounts of E4orf4. Aliquots of these normalized lysates were analyzed by SDS-polyacrylamide electrophoresis, transferred to nitrocellulose, and immunoblotted with HA tag antibody and antibody recognizing Pph21p. E4orf4 was immunoprecipitated via its HA tag from the remainder of the normalized lysates, and immunoprecipitates were washed, analyzed by SDS-polyacrylamide electrophoresis, transferred to nitrocellulose, and then immunoblotted with HA tag antibody and antibody recognizing Pph21p. (A) Western blots showing the relative amounts of E4orf4 and Pph21p in cell lysates used for immunoprecipitation after normalization for E4orf4 expression. (B) Western blots of HA tag immunoprecipitates from the lysates shown in panel A, demonstrating that E4orf4 does not associate stably with T364D, Y367E, and L369Δ. Results shown are representative of three separate experiments, except that E4orf4 bound Y367F at a level similar to wild-type Pph21p in other experiments.

nous wild-type or mutant Pph21p proteins from the Pph21p promoter (12) and then introduced a plasmid expressing galactose-inducible E4orf4 so that we could determine the sensitivity of these cells to growth inhibition by E4orf4. For the growth inhibition assay, serial dilution cell growth spotting assays were performed in which we compared the growth of these cells on glucose and galactose (Fig. 3A). As a control, the same cells with an empty vector (no E4orf4) were also compared. As expected, E4orf4 inhibited to a similar extent the growth of cells expressing wild-type Pph21p or the control Pph21p mutants containing neutral substitutions, T364A and Y367F, which still form stable $PP2A_{Cdc55p/Tpd3p/Pph21p}$ heterotrimers (58) and associate with E4orf4 (Fig. 2B). Surprisingly, E4orf4 also strongly inhibited the growth of cells expressing Y367E and L369Δ to the same level as wild-type cells, indicating that the formation of stable $PP2A_{Cdc55p}$ heterotrimers may not be required for Cdc55p-mediated E4orf4-induced growth inhibition in yeast. Moreover, although in repeated experiments cells expressing T364D were more resistant to E4orf4-induced slow growth than wild-type cells (see Fig. 4, below, for another example), they were not nearly as resistant to E4orf4-induced growth inhibition as cells deleted for *CDC55* (Fig. 3A). Thus, three separate PP2A catalytic subunit mutants that prevent stable association with E4orf4 are unable to provide
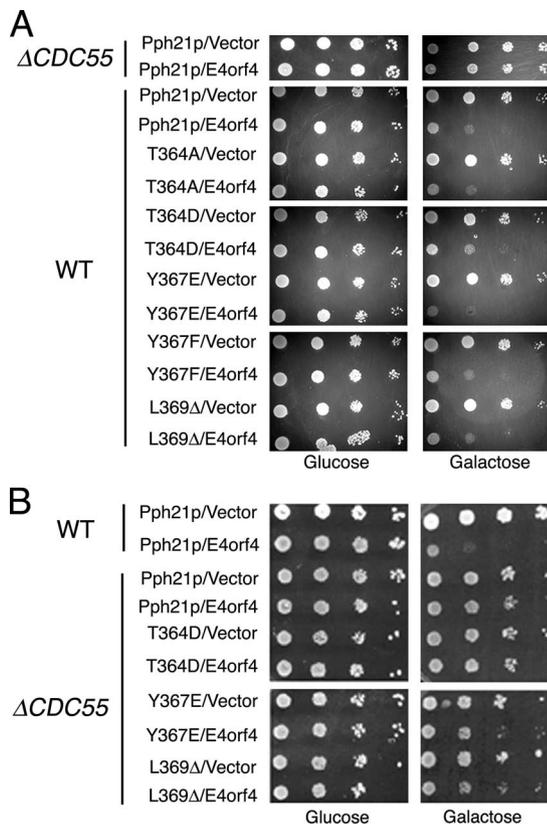
FIG. 3. Analysis of *S. cerevisiae* strains expressing PP2A catalytic subunit mutants unable to form complexes with Cdc55p and E4orf4. (A) Disruption of the PP2A$_{B(Cdc55p)AC}$ complex subunit by PP2A catalytic subunit mutations does not rescue E4orf4-induced growth inhibition to the same extent as deletion of *CDC55*. Wild-type (WT) DEY3 cells expressing only exogenous wild-type Pph21p or one of the indicated mutant Pph21p proteins were transformed with empty vector (vector) or with a plasmid expressing galactose-inducible E4orf4 (E4orf4). A serial dilution cell growth assay (10-fold dilutions from left to right) of these cells was carried out on glucose and galactose plates as described in Materials and Methods. A small amount of rescue from E4orf4-induced growth inhibition was consistently seen with the T364D mutant-expressing DEY3 cells (also see Fig. 4, below), but this rescue was not seen with the other Pph21p mutant-expressing DEY3 cells. Also shown for comparison is the almost complete rescue obtained when *CDC55* was deleted in DEY3 cells (Δ*CDC55*). The results shown are representative of at least four separate experiments for both the mutant analysis and for the Δ*CDC55* rescue. (B) DEY3 cells expressing T364D, Y367E, or L369Δ can all be rescued substantially from E4orf4-induced growth inhibition by deletion of *CDC55*. WT and Δ*CDC55* DEY3 cells expressing exogenous wild-type Pph21p (Pph21p) and Δ*CDC55* DEY3 cells expressing the indicated mutant Pph21p proteins were transformed with empty vector (vector) or with vector expressing galactose-inducible E4orf4 (E4orf4). A serial dilution cell growth assay (10-fold dilutions from left to right) of these cells was carried out on glucose and galactose plates as described in Materials and Methods. DEY3 cells expressing T364D Pph21p were rescued completely, while DEY3 cells expressing T367E or L369Δ were rescued substantially but not completely. The results shown are representative of results obtained from two independent experiments.

the same rescue from E4orf4-induced growth inhibition that deletion of *CDC55* provides.

These results are consistent with the hypothesis that E4orf4-induced growth inhibition involves one or more Cdc55p-dependent effects that are independent of PP2A$_{BAC}$ heterotrimer

formation. However, an alternative possibility is that these mutations not only disrupt PP2A$_{BAC}$ heterotrimer formation but also have an additional effect that enhances E4orf4-induced growth inhibition, thus preventing full rescue. If this were the case, cells expressing Pph21p mutants should show increased sensitivity to E4orf4 in a Δ*CDC55* background compared to cells expressing wild-type Pph21p (i.e., expression of these mutants should also prevent deletion of *CDC55* from providing full rescue). We therefore compared E4orf4-induced growth inhibition of Δ*CDC55* cells expressing wild-type, T364D, Y367E, or L369Δ Pph21p. The results in Fig. 3B show that deletion of *CDC55* rescued cells expressing wild-type or T364D Pph21p to the same extent, indicating that T364D has no Cdc55p-indpendent effects that enhance E4orf4-induced growth inhibition. On the other hand, cells expressing Y367E or L369Δ Pph21p consistently showed a slightly reduced rescue upon deletion of *CDC55* (Fig. 3B and data not shown), indicating that these mutants have a Cdc55p-independent effect that enhances E4orf4-induced growth inhibition. Together, the results in Fig. 3A and B indicate that disruption of stable formation of PP2A$_{BAC}$ heterotrimers by PP2A catalytic subunit mutation partially rescues E4orf4-induced growth inhibition but, in the case of Y367E and L369Δ, this effect is not seen because they have an additional, unknown effect that enhances E4orf4-induced growth inhibition. Nevertheless, in all cases deletion of *CDC55* substantially rescued E4orf4-induced growth inhibition of DEY3 cells expressing these mutants (Fig. 3B), strongly supporting the existence of a Cdc55p-dependent, PP2A$_{B(Cdc55p)AC}$ heterotrimer-independent effect(s) important for E4orf4-induced growth inhibition.

Downregulation of Rts1p function enhances E4orf4-induced cytotoxicity. What might the Cdc55p-independent effect of the Y367E and L369Δ Pph21p mutants (but not T364D) be that enhances E4orf4-induced growth inhibition even in the absence of *CDC55* (Fig. 3)? Previous analyses showed that T364D forms complexes with Rts1p (60% of wild-type level) but is unable to associate with Cdc55p, while Y367E and L369Δ are unable to form complexes with either Rts1p or Cdc55p (16, 58). We therefore hypothesized that greatly reduced complex formation of Pph21p with Rts1p (and thus reduced Rts1p function) in DEY3 cells expressing Y367E and L369Δ enhanced E4orf4-induced growth inhibition. To test this hypothesis we deleted *RTS1* in DEY3 cells and compared the E4orf4-induced growth inhibition of these cells with that of the parental DEY3 cells to see if loss of Rts1p indeed made cells more sensitive to E4orf4-induced growth inhibition. Both cells expressing wild-type Pph21p and cells expressing the T364D mutant were analyzed. Consistent with our results shown in Fig. 3, wild-type DEY3 cells expressing the T364D mutant showed a partial rescue from E4orf4-induced growth inhibition relative to wild-type DEY3 cells expressing wild-type Pph21p, while deletion of *CDC55* gave full rescue (Fig. 4A). However, *RTS1* deletion made both wild-type and T364D Pph21p-expressing cells more sensitive to E4orf4-induced growth inhibition (Fig. 4A), effectively masking any rescue by T364D. This result indicates that loss of Rts1p function does indeed enhance E4orf4-induced cytotoxicity. Moreover, we found in separate experiments that in the absence of Rts1p, cells expressing T364D are inhibited to a similar extent by E4orf4 as cells expressing the two mutants lacking Rts1p bind-
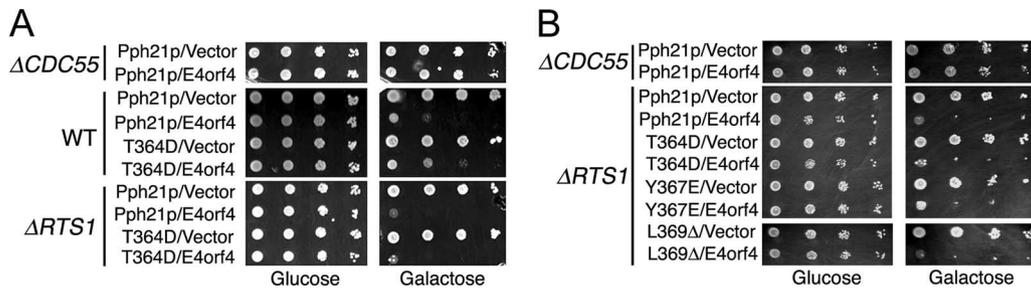
FIG. 4. Loss of Rts1p function enhances E4orf4-induced cytotoxicity. (A) $\Delta CDC55$, wild-type (WT), and $\Delta RTS1$ DEY3 cells expressing galactose-inducible E4orf4 or empty vector and either the wild-type (Pph21p) or T364D (T364D) Pph21p were analyzed for E4orf4-induced growth inhibition by a serial dilution cell growth assay (10-fold dilutions from left to right). While deletion of $CDC55$ completely rescued cells from E4orf4-induced growth inhibition, deletion of $RTS1$ enhanced E4orf4-induced growth inhibition in cells expressing wild-type or T364D Pph21p. The results shown are representative of results obtained from three independent experiments. (B) In a separate experiment, $\Delta RTS1$ DEY3 cells expressing wild-type Pph21p (Pph21p) or T364D, Y367E, or L369Δ mutant Pph21p were analyzed for E4orf4-induced growth inhibition. In the absence of Rts1p, cells expressing wild-type Pph21p or T364D, Y367E, or L369Δ mutant Pph21p have a similar sensitivity to E4orf4-induced growth inhibition. $\Delta CDC55$ cells were included in the assay as a control. In the $\Delta RTS1$ cells analyzed on a galactose plate in this figure, the single dots in the Pph21p/E4orf4, T364D/E4orf4, Y367E/E4orf4, and L369Δ/E4orf4 rows are not colonies but rather accidental marks made with the pipette tips used in the assay. The results shown here are representative of results obtained from two independent experiments.

ing, Y367E and L369Δ (Fig. 4B). Together, these results support our hypothesis that differences in Rts1p association may explain why the T364D Pph21p mutant, but not the Y367E and L369Δ mutants, provides a partial rescue from E4orf4-induced growth inhibition in wild-type DEY3 cells.

Disruption of PP2A_BAC complexes by deletion of the PP2A methyltransferase gene, *PPM1*, does not rescue E4orf4-induced growth inhibition. Our results with PP2A catalytic subunit mutants unable to form stable PP2A_BAC heterotrimers suggest that there is a Cdc55p-dependent function(s) in E4orf4-induced growth inhibition that does not require stable Cdc55p association with Pph21p/Pph22p. To test this idea by an independent approach, we deleted the PP2A methyltransferase gene, *PPM1*, to disrupt E4orf4 association with PP2A catalytic subunits. Loss of PP2A methylation due to deletion of *PPM1* nearly abolishes the stable formation of PP2A_Cdc55p heterotrimers when assayed in a coimmunoprecipitation assay (58, 59) or an in vivo association assay (16). To determine whether Pph21p can specifically associate with E4orf4 in $\Delta PPM1$ cells, HA-tagged E4orf4 was expressed in wild-type and $\Delta PPM1$ cells, the HA-tagged E4orf4 was immunoprecipitated, and the immunoprecipitates were analyzed for the presence of Pph21p. While Pph21p could be readily detected in the E4orf4 immunoprecipitate in wild-type cells, it was greatly reduced in the E4orf4 immunoprecipitate from $\Delta PPM1$ cells (Fig. 5A). Quantitation indicated that the levels of immunoprecipitated Pph21p from $\Delta PPM1$ cells are only about 5% of the amount from wild-type cells.

Given that deletion of *PPM1* was an effective way to disrupt E4orf4 association with Pph21p, we transformed wild-type and $\Delta PPM1$ cells with a plasmid expressing galactose-inducible E4orf4 and tested the effect of the deletion of *PPM1* on E4orf4-induced growth inhibition. When E4orf4 expression was induced, it inhibited growth of wild-type and $\Delta PPM1$ cells to the same extent (Fig. 5B), further supporting the idea that substantial interaction of E4orf4 with the PP2A catalytic subunits, Pph21p/Pph22p, is not required for E4orf4 to inhibit cell growth in *S. cerevisiae*.

Cells deleted for *PPM1* cannot be rescued from E4orf4-induced growth inhibition by deletion of *CDC55*. We were somewhat surprised that deletion of *PPM1* did not provide any rescue from E4orf4-induced growth inhibition in our experiments described above (Fig. 5B), given that it dramatically reduces the formation of PP2A_Cdc55p heterotrimers. One possible explanation was that the very small amount of PP2A_Cdc55p heterotrimers present in the $\Delta PPM1$ cells is sufficient to mediate E4orf4-induced growth inhibition. If this scenario were true, then additional deletion of *CDC55* in the $\Delta PPM1$ cells should rescue these cells from E4orf4-induced growth inhibition. To test this hypothesis, we constructed a $\Delta PPM1$ $\Delta CDC55$ double mutant and compared the E4orf4-induced growth inhibition of these cells with wild-type and $\Delta PPM1$ cells (Fig. 5C). Consistent with our results in Fig. 5B, E4orf4 efficiently inhibited the growth of both wild-type and $\Delta PPM1$ cells. Surprisingly, however, the $\Delta PPM1$ $\Delta CDC55$ cells were also inhibited to a similar extent as wild-type and $\Delta PPM1$ cells, indicating that cells that do not express Ppm1p cannot be rescued by deletion of the gene encoding Cdc55p. Thus, Ppm1p must have another function in addition to regulating PP2A_Cdc55p heterotrimer formation, and this function must intersect with E4orf4 signaling.

Loss of Sit4p enhances E4orf4-induced growth inhibition. One possibility for an additional function of Ppm1p that may intersect with E4orf4 signaling is that Ppm1p might regulate another phosphatase in addition to PP2A. There are two mammalian phosphatases, PP4 and PP6, that share approximately 60% amino acid identity with PP2A and have identical carboxy-terminal amino acids, including a carboxy-terminal leucine, the known site of carboxyl methylation for both PP2A and PP4 (19). PP6 has a functional homolog in *S. cerevisiae* termed Sit4p (4) that shares these similarities with Pph21p/Pph22p. While it is not yet known whether PP4 and Sit4p are indeed methylated, it seems likely that they would be based on the critical similarities between their sequences and those of PP2A and PP4 C subunits. Thus, Sit4p is a potential substrate
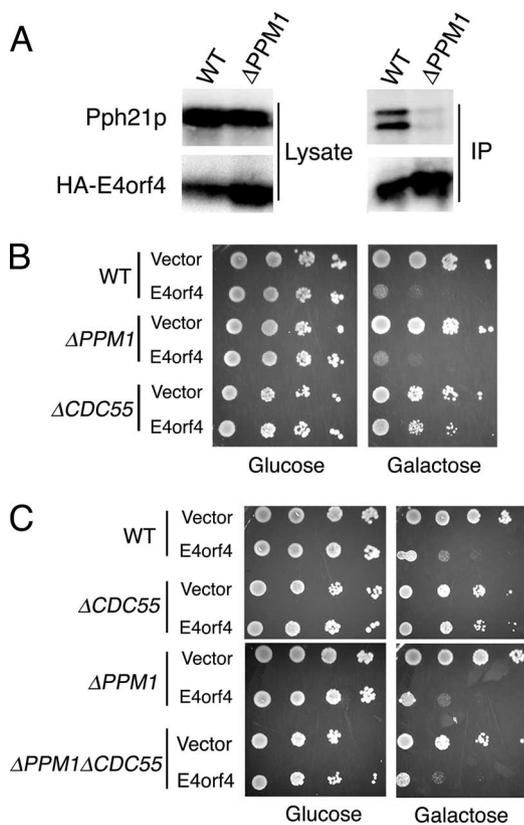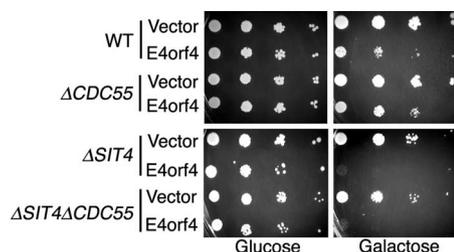
FIG. 6. Loss of Sit4p enhances E4orf4-induced growth inhibition of *S. cerevisiae* in both the presence and absence of Cdc55p. Serial dilution cell growth assays (10-fold dilutions from left to right) for wild type (WT), Δ*CDC55*, Δ*SIT4*, and Δ*SIT4* Δ*CDC55* BY4741 cells transformed with empty vector or with vector expressing galactose-inducible HA-E4orf4 were carried out on glucose and galactose plates as described in Materials and Methods. The results shown are representative of results obtained from at least four independent experiments.

FIG. 5. The PP2A methyltransferase, Ppm1p, regulates a *CDC55*-independent function in E4orf4-induced growth inhibition. (A) Deletion of *PPM1* greatly diminishes the interaction between E4orf4 and Pph21p. Wild-type DEY3 cells (WT) expressing wild-type Pph21p and HA-E4orf4 and Δ*PPM1* DEY3 cells expressing wild-type Pph21p and HA-E4orf4 were lysed, and E4orf4 complexes were immunoprecipitated using an anti-HA tag antibody. The immunoprecipitates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted for HA-E4orf4 and Pph21p. Deletion of *PPM1* disrupts the vast majority of the E4orf4-Pph21p association. The results shown are representative of results obtained from two independent experiments. (B) Deletion of *PPM1* does not rescue E4orf4-induced growth inhibition. WT, Δ*PPM1*, and Δ*CDC55* (control) DEY3 cells were analyzed for sensitivity to E4orf4-induced growth inhibition by a serial dilution cell growth assay (10-fold dilutions from left to right). The results shown are representative of results obtained from three independent experiments. (C) Deletion of *CDC55* cannot rescue Δ*PPM1* cells from E4orf4 growth inhibition. WT, Δ*CDC55*, Δ*PPM1*, and Δ*PPM1* Δ*CDC55* DEY3 cells were analyzed for sensitivity to E4orf4-induced growth inhibition by a serial dilution growth assay (10-fold dilutions from left to right). The results shown are representative of results obtained from three independent experiments.

of Ppm1p. We therefore tested whether deletion of *SIT4* would mimic *PPM1* deletion with regard to E4orf4 growth inhibition.

To test this possibility, we exploited the existence of Δ*SIT4* cells in the *S. cerevisiae* knockout collection (BY4741 strain background) and compared wild-type, Δ*CDC55*, Δ*SIT4*, and Δ*SIT4* Δ*CDC55* cells for growth inhibition by E4orf4. The results of a typical experiment are shown in Fig. 6. Comparison of the growth inhibition of wild-type and Δ*SIT4* cells showed that loss of Sit4p greatly sensitizes cells to E4orf4-induced growth inhibition (Fig. 6). Moreover, while deletion of *CDC55* partially rescued wild-type cells in this strain background,

CDC55 deletion was unable to rescue the E4orf4-induced growth inhibition of the Δ*SIT4* cells (Fig. 6). Thus, deletion of *SIT4* has a more severe effect than deletion of *PPM1*, but it is similar to deletion of *PPM1* in that deletion of *CDC55* shows no rescue of Δ*SIT4* or Δ*PPM1* cells in a serial dilution growth assay (Fig. 5C and 6).

There are at least four other PP2A-related serine/threonine phosphatases in yeast, Ppg1p, Pph3p, Ppt1p, and Ppz1p. It has been shown that Ppg1p and Pph3p are not required for E4orf4 function (25, 44). To determine whether other PP2A family serine/threonine phosphatase catalytic subunits might also interact genetically with E4orf4-induced growth inhibition, we next assayed by serial dilution cell growth assays whether individual deletion of *PPT1* or *PPZ1* would rescue or enhance E4orf4-induced growth inhibition upon E4orf4 expression. No effect on E4orf4-induced growth inhibition was observed upon deletion of either of these phosphatases (data not shown), indicating that the effects seen with Sit4p deletion are unique compared with other PP2A-related phosphatases.

Knockdown of PP6, the mammalian Sit4p homolog, by shRNA greatly enhances E4orf4 killing of H1299 human non-small cell lung carcinoma cells. Regardless of whether Sit4p is a direct target of Ppm1p, our results show that deletion of Sit4p greatly enhances E4orf4-induced growth inhibition in *S. cerevisiae*. This result in yeast suggested that inhibition of PP6 in mammalian cells might have synergistic effects with E4orf4 for killing cancer cells. We therefore used lentivirus-mediated shRNAs to knock down mammalian PP6 in H1299 human lung cancer cells to determine if this would affect E4orf4-induced cell killing. As seen in Fig. 7, endogenous PP6 was almost undetectable in the PP6 shRNA H1299 cells (2.5% of control levels when quantitated). Interestingly, no increase in cell death was detected over vector control (pLKO) cells, even with this level of knockdown (Fig. 7; compare the first two columns), suggesting that PP6 may not be an essential protein in these cells. However, when wild-type E4orf4 was expressed in the PP6 knockdown and vector control cells via an adenovirus vector, PP6 knockdown clearly enhanced E4orf4 killing (Fig. 7; compare the third and fourth columns). When a mutant (R69-75A) E4orf4 defective for binding PP2A and for killing (31) was expressed in these cells, little to no cell killing was detected

A

PP6   Vector

PP6c

PP2Ac

B

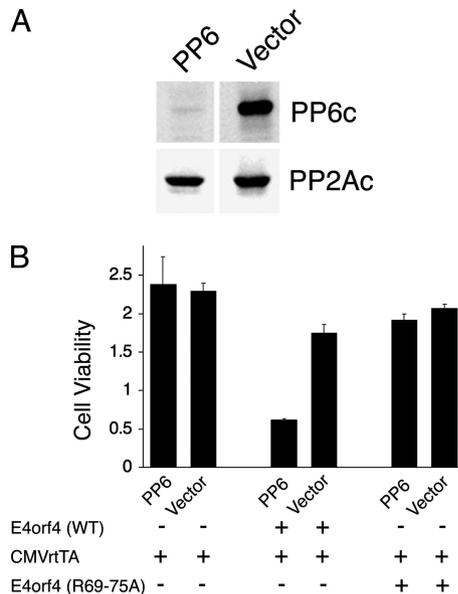| E4orf4 (WT) | - | - | + | + | - | - |
| CMVrtTA | + | + | + | + | + | + |
| E4orf4 (R69-75A) | - | - | - | - | + | + |

FIG. 7. Knockdown of PP6 in H1299 cells enhances killing by E4orf4. (A) Knockdown of PP6 by shRNA. Lysates from H1299 cells expressing shRNA directed against the catalytic subunit of PP6 (PP6) and vector control (vector) H1299 cells were analyzed by SDS-PAGE and immunoblotting with anti-PP6 catalytic subunit (PP6c) and anti-PP2A catalytic subunit (PP2Ac; loading control) antibodies. PP6c protein levels were quantitated using a Bio-Rad Fluor-S Max chemilumimager and normalized to the amount of PP2Ac. Results from two separate experiments indicated that PP6 was reduced >98% in the PP6 knockdown cells. (B) Knockdown of PP6 enhances E4orf4 killing of H1299 cells. PP6 catalytic subunit shRNA (PP6) and vector control (vector) cells were infected as indicated (+ or −) at an MOI of 5 with adenovirus expressing wild-type (WT) E4orf4 or R69-75A mutant E4orf4 and at an MOI of 5 with CMVrtTA helper adenovirus. When no wild-type or mutant E4orf4-expressing virus was used, the MOI of CMVrtTA virus was increased to 10 to control for nonspecific effects of adenovirus infection. After 48 h, cell viability was assayed by a water-soluble tetrazolium salt (WST-1) killing assay. Shown are the results from one experiment performed in triplicate. Error bars indicate standard deviations. Similar results were obtained in three independent experiments, except that expression of the R69-75A E4orf4 mutant usually showed no killing over control.

in the presence or absence of PP6 knockdown (Fig. 7B, fifth and sixth columns, and data not shown). Taken together, these results indicate that PP6 is important for cell viability in the presence of E4orf4 protein and that inhibitors of PP6 may be useful in E4orf4 cancer therapy or drug therapy based on the E4orf4 cancer cell toxicity mechanism.

DISCUSSION

Expression of adenovirus E4orf4 protein specifically induces p53-independent cell death of transformed human cells but not normal human cells (32, 42), suggesting that elucidation of the E4orf4-induced growth inhibition pathway(s) could provide important new avenues for cancer therapy. The requirement for the PP2A B subunit in E4orf4 function has led to the hypothesis that E4orf4 functions in part through changing the activity, substrate specificity, or localization of PP2A$_{BAC}$ heterotrimers. However, the results of our current study support a new model in which E4orf4 mediates growth inhibition and cell

killing both through PP2A$_{BAC}$ heterotrimers and through a B regulatory subunit-dependent pathway that is independent of stable complex formation with the PP2A C subunit.

The clearest support for a PP2A$_{BAC}$ heterotrimer-independent, B subunit-dependent pathway in E4orf4-induced growth inhibition comes from our results with DEY3 cells expressing the PP2A catalytic subunit mutant T364D. Expression of this mutant in place of the wild-type PP2A catalytic subunit consistently gave only a partial rescue from E4orf4 growth inhibition, while deletion of the gene encoding Cdc55p gave full rescue in the same cells. The partial rescue afforded by T364D is not likely to be due to a small amount of residual heterotrimer formation with Cdc55p (and Tpd3p) because of the following: (i) no heterotrimer formation with Cdc55p was detected for this mutant in either an immunoprecipitation or an in vivo association assay (16, 58); (ii) cells expressing this mutant in place of the wild-type PP2A catalytic subunit are defective in the spindle checkpoint, a known Cdc55p function (58); (iii) we could not detect a complex between T364D and E4orf4 in this study (Fig. 2). In addition, the fact that deletion of CDC55 could fully rescue DEY3 cells expressing T364D from E4orf4-induced growth inhibition argues against the possibility that the T364D mutation has a Cdc55p-independent growth inhibitory effect that reduces its ability to rescue cells from E4orf4-induced growth inhibition. Further support for a C subunit-independent, B/A subunit-dependent pathway in E4orf4-induced growth inhibition comes from the finding that deletion of CDC55 provided substantial rescue to DEY3 cells expressing Y367E or L369Δ mutant PP2A catalytic subunits (Fig. 3 and 4 and data not shown). The inability of these mutants to form complexes with Cdc55p and E4orf4 is also well documented (16, 58) (Fig. 2). Thus, our data show directly for the first time that the PP2A catalytic subunit is indeed a target for E4orf4, but that E4orf4 mediates substantial growth inhibition in a Cdc55p/Tpd3p-dependent manner that does not require stable complex formation between E4orf4 and the catalytic subunit of PP2A.

Our results also indicate that Rts1p, the S. cerevisiae PP2A B′ subunit, has some function that opposes E4orf4-induced growth inhibition. The correlation between the ability of PP2A catalytic subunit mutants to partially rescue E4orf4-induced growth inhibition and their ability to bind Rts1p indicates that at least part of Rts1p's opposing function is carried out via PP2A$_{Rts1p}$ heterotrimers. Shtrichman and colleagues previously reported that mammalian B′ subunits associate with E4orf4 (44). It is tempting to speculate that E4orf4 might bind Rts1p to block its inhibitory function toward E4orf4 growth arrest. However, the association of E4orf4 with mammalian B′ subunits has been challenged, and data from a study with S. cerevisiae suggest that E4orf4 may not bind PP2A$_{Rts1p}$ heterotrimers (41). Whether or not it is a direct target of E4orf4, Rts1p may oppose E4orf4-induced growth inhibition at G$_2$/M. Rts1p associates with the catalytic subunit of the cell cycle regulatory kinase Cdc28p and with Clb2p, a B-type cyclin that regulates Cdc28p in G$_2$/M (53). E4orf4 has been reported to associate with the anaphase-promoting complex (APC) (25), which regulates progression through mitosis by modulating the stability of several proteins, including Pds1p (yeast securin) and Clb2p (reference 55 and references therein). Like deletion of RTS1, mutation of CDC28 has been reported to make yeast

cells hypersensitive to E4orf4 (25), leading us to hypothesize that Rts1p might regulate Cdc28/Clb2p to oppose E4orf4. Regardless of the mechanism, our results indicate that Cdc55p and Rts1p have opposite effects on E4orf4-induced growth inhibition. Furthermore, they are consistent with reports that B′ subunits have a prosurvival role in *Drosophila melanogaster* (26, 47) and mammalian cells (48).

An important implication of our results is that the methyltransferase Ppm1p has a function independent of regulating PP2A$_{Cdc55p}$ and PP2A$_{Rts1p}$ heterotrimer formation. Especially striking is the observation that in the absence of Ppm1p, E4orf4-induced growth inhibition became Cdc55p-independent, as assayed by serial dilution cell growth assays. We considered that the inability of *CDC55* deletion to rescue Δ*PPM1* cells from E4orf4-induced growth inhibition might be partially explained by the small (<2-fold) reduction in PP2A$_{Rts1p}$ heterotrimers caused by *PPM1* deletion (16). However, we have previously shown that expression of T364D Pph21p in place of wild-type Pph21p also causes the same reduction in PP2A$_{Rts1p}$ heterotrimers as *PPM1* deletion (16), yet the growth inhibition of T364D-expressing DEY3 cells by E4orf4 can be fully rescued by deletion of *CDC55* (Fig. 4). Thus, it appears that Ppm1p can function independently of Cdc55p and Rts1p. It is possible that Ppm1p may have another substrate(s) in addition to the PP2A catalytic subunits and that this substrate regulates a target relevant to E4orf4-induced growth inhibition. One candidate for such an additional substrate is Sit4p, the yeast homolog of the catalytic subunit of the mammalian phosphatase PP6, which shares ~60% identity with PP2A, including a carboxy-terminal leucine, the site of PP2A (and PP4) methylation. Consistent with the possibility that Sit4p might be a target of Ppm1p, the enhanced E4orf4-induced growth inhibition caused by deletion of the gene encoding Sit4p could not be rescued by deletion of *CDC55*. Although deletion of *SIT4* enhanced E4orf4-induced growth inhibition while deletion of *PPM1* did not, this difference may be due partly to the fact that Ppm1p also regulates PP2A$_{Cdc55p}$ heterotrimer formation, which would be expected to reduce E4orf4-induced growth inhibition. Consistent with this idea, even though Δ*PPM1* cells showed no rescue in the serial dilution growth assay upon *CDC55* deletion, deletion of *PPM1* does result in an ability to tolerate higher E4orf4 expression (Y. Li and D. C. Pallas, unpublished results), a phenotype previously described for *CDC55* deletion cells (41). Based on a previous report suggesting that Cdc55p may form a heterotrimeric complex with Tpd3p and Sit4p, it was also possible that E4orf4 might target Sit4p directly through Cdc55p. However, we have been unable to detect a physical association between Sit4p and E4orf4 (Li and Pallas, unpublished).

Given the recent report that E4orf4 hijacks Rho GTPase-dependent actin dynamics to kill cancer cells (39), it is of interest that Sit4p has been clearly implicated in the regulation of actin dynamics in yeast (3, 23, 52). *SIT4* deletion increases the time necessary for repolarization of the actin cytoskeleton after cellular stress (3), and Sit4p interacts with Bem2p, a protein with GTPase-activating protein (GAP) homology that regulates actin dynamics and cellular morphogenesis. Moreover, *SIT4* deletion is synthetically lethal with *BEM2* deletion, and both *SIT4* deletion and *BEM2* mutant phenotypes can be suppressed by a common suppressor, *SSD1-vl* (13, 14, 23, 51).

Consistent with our observation that *CDC55* deletion and *SIT4* deletion have opposite effects on E4orf4-induced growth inhibition, genetic data from *S. cerevisiae* indicate that Sit4p and Bem2p function in opposition to Cdc55p (18, 23). However, although the links to actin are intriguing, other possible mechanisms of enhancement of E4orf4-induced growth inhibition by *SIT4* deletion include Sit4p effects on regulation of G$_1$/S progression or mitosis or a combination of these pathways.

Regardless of the mechanism of enhancement of E4orf4-induced growth inhibition by deletion of *SIT4*, our results with shRNA-mediated downregulation of the mammalian Sit4p homolog, PP6, in H1299 human lung cancer cells indicate that downregulation of this phosphatase has similar effects in mammalian cells—enhancing E4orf4 cancer cell toxicity. It was especially striking that PP6 downregulation had no noticeable effects on cell viability in the absence of E4orf4 expression. PP6 downregulation failed to enhance the killing of H1299 cells when an E4orf4 mutant defective in binding to PP2A$_{BAC}$ heterotrimers was used, indicating that PP6 knockdown synergizes with the PP2A-dependent E4orf4 cell killing pathway. After more details of this pathway are elucidated for the benefit of therapeutic mimicry of the E4orf4 mechanism, our results suggest that PP6 inhibition should be considered as part of that cancer cell-killing strategy. It is also possible that PP6 downregulation may have the potential for enhancing other cancer therapeutics. Consistent with this possibility, PP6 was recently identified in an siRNA screen of human kinases and phosphatases as having a cell survival role in HeLa cells (29).

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