**YND1 INTERACTS WITH CDC55 AND IS A NOVEL MEDIATOR OF E4ORF4-INDUCED TOXICITY**

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Running title: YND1 is a novel mediator of E4orf4-induced toxicity

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Adenovirus E4orf4 protein induces protein phosphatase 2A (PP2A)-dependent non-classical apoptosis in mammalian cells and irreversible growth arrest in *S. cerevisiae*. Oncogenic transformation sensitizes cells to E4orf4-induced cell death. To uncover additional components of the E4orf4 network required for induction of its unique mode of apoptosis, we used yeast genetics to select gene deletions conferring resistance to E4orf4. Deletion of *YND1*, encoding a yeast Golgi apyrase, conferred partial resistance to E4orf4. However, Ynd1p apyrase activity was not required for E4orf4-induced toxicity. Ynd1p and Cdc55p, the yeast PP2A-B subunit, contribute additively to E4orf4-induced toxicity. Furthermore, concomitant overexpression of one and deletion of the other is detrimental to yeast growth, demonstrating a functional interaction between the two proteins. *YND1* and *CDC55* also interact genetically with *CDC20* and *CDH1/HCT1*, encoding activating subunits of the anaphase promoting complex / cyclosome (APC/C). In addition to their functional interaction, Ynd1p and Cdc55p interact physically and this interaction is disrupted by E4orf4, which remains associated with both proteins. The results suggest that Ynd1p and Cdc55p share a common downstream target whose balanced modulation by the two E4orf4 partners is crucial to viability. Disruption of this balance by E4orf4 may lead to cell death. NTPDase-4/Lapl70/UDPase, the closest mammalian homologue of Ynd1p, associates with E4orf4 in mammalian cells, suggesting that the results in yeast are relevant to the mammalian system.

**INTRODUCTION**

Adenovirus E4orf4 protein is a multifunctional viral regulator, which down-regulates expression of genes that have been activated by E1A and cAMP (1-4), induces hypophosphorylation of various viral and cellular proteins (1, 5), regulates alternative splicing of adenovirus mRNAs (5), and induces p53-independent apoptosis in transformed cells (6-8). Oncogenic transformation of primary cells sensitizes them to cell killing by E4orf4 (9), indicating that E4orf4 research may have exciting implications for cancer therapy. Induction of apoptosis by E4orf4 has been reported to be at least partially caspase-dependent in 293T cells, but it is caspase-independent in CHO and H1299 cells, suggesting that non-classical apoptotic pathways are involved (7, 10). In 293T cells, where E4orf4-induced apoptosis is caspase dependent, components of the death receptor pathway contribute to the process (10).

Protein phosphatase 2A (PP2A) is composed usually of three subunits, a catalytic C subunit, a scaffolding A subunit, and one of several regulatory B subunits (Brα-δ/B55, Brα-ε/B56, B"(PR72, PR130, PR59, PR48)), encoded by unrelated gene families. The B subunits dictate the cellular localization and substrate specificity of the phosphatase (reviewed in (11)). We have previously shown that E4orf4 interacts with PP2A through a direct association with the phosphatase Brα/B55 regulatory subunit (2). E4orf4 can also interact with PP2A molecules containing a different regulatory subunit, B"/B56 (12). The interaction of E4orf4 with an active PP2A enzyme is required for induction of apoptosis (9, 12, 13), however, only PP2A holoenzymes containing the Brα/B55 subunit mediate this process (12).
E4orf4 also associates with members of the Src kinase family leading to its Tyr-phosphorylation and to deregulation of Src signaling. E4orf4 Tyr-phosphorylation is correlated with a shift of E4orf4 accumulation from the nucleus to the cytoskeleton and to cell membranes and enhances E4orf4-induced apoptosis (14, 15).

To further dissect the E4orf4 apoptotic pathway, a genetic system was applied, using the yeast Saccharomyces cerevisiae. Although some components of the metazoan core machinery of cell death, such as Bcl-2 family members, are absent in yeast, it has been previously shown that this organism could serve as a powerful tool for apoptosis research (16). Since Src kinase family members and death receptor pathway components, known to play a part in E4orf4-induced apoptosis in mammalian cells, are absent in yeast, the yeast system was not expected to reveal the full spectrum of E4orf4-induced events in mammalian cells. However, E4orf4-induced apoptosis can occur in the absence of these effectors (10, 17), and yeast and mammalian PP2A subunits share an extensive homology. Thus the yeast system could reveal components of the apoptotic pathway regulated by the E4orf4-PP2A complex.

When expressed in the budding yeast S. cerevisiae, E4orf4 induces PP2A-dependent irreversible growth arrest (18, 19). Furthermore, Cdc55p, the yeast homologue of the PP2A-Bα/B55 subunit, is required for growth inhibition by E4orf4, whereas Rts1p, the yeast B'/B56 homologue, is dispensable for E4orf4-induced toxicity. To further demonstrate the relevance of the yeast system to E4orf4-induced apoptosis in mammalian cells, E4orf4 was randomly mutagenized in vitro by a chemical mutagen, and non-toxic E4orf4 mutants were selected in yeast (20). These mutants were shown to be non-apoptotic in mammalian cells, and demonstrated a reduced ability to associate with an active PP2A. These results underscore the high conservation of E4orf4 targets between yeast and mammalian cells. Using yeast, we demonstrated that E4orf4 functionally interacted with components of the yeast cell-cycle machinery. Thus, E4orf4 enhanced Cdc28 activity in an Mih1-dependent manner and inhibited the anaphase promoting complex / cyclosome (APC/C). Furthermore, E4orf4 physically associated with APC/C and targeted PP2A to this complex. E4orf4 was also shown to interact genetically with CDC20 and CDH1/HCT1, encoding two substrate-specific APC/C activating subunits. The end result of the interactions of E4orf4 with the cell cycle machinery was the induction of mitotic arrest (18).

We further showed that E4orf4 could cause G2/M arrest in mammalian cells as well, prior to induction of apoptosis (18). These results indicated that S. cerevisiae could be used as a tool to identify E4orf4 effectors, which are relevant to E4orf4-induced events in mammalian cells.

Nucleoside triphosphate diphosphohydrolases, also known as NTPDases, apyrases, or E-ATPases, hydrolyze a variety of nucleoside 5'-triphosphates and 5'-diphosphates with different nucleotide preferences. Most of the members of this family are integral membrane glycoproteins, and their catalytic sites face the extracellular medium or the lumen of intracellular organelles. Golgi- and ER-NTPDases may be important in hydrolysis of nucleotide diphosphates to the corresponding nucleoside monophosphates and consequently in the import of nucleotide sugars from the cytosol into the Golgi or the ER. They may thus have an impact on protein glycosylation. Members of the NTPDase family share five highly conserved sequence domains, called apyrase conserved domains (ACRs). Several residues involved in apyrase catalytic activity are found in the ACRs (reviewed in (21)).

In this study we undertook a classical genetics approach designed to identify yeast genes that are required for E4orf4-mediated toxicity in S. cerevisiae. The genetic analysis revealed that Ynd1p, a Golgi NTPDase, functionally interacts with Cdc55p to mediate E4orf4-induced toxicity. Biochemical studies demonstrated that Ynd1p associates with Cdc55p, and this interaction is disrupted in the presence of E4orf4, which remains associated with both proteins. However, Ynd1p enzymatic activity is not required for E4orf4-induced toxicity. Furthermore, NTPDase-4 (Lalp70/UDPase), a human Ynd1p homologue, associates with E4orf4 in mammalian cells, indicating its involvement in E4orf4-induced events.
EXPERIMENTAL PROCEDURES

Plasmids, yeast strains, mammalian cell lines, and media

Yeast were grown either in YPD (1% yeast extract, 2% Bacto peptone, 0.015% L-Tryptophan, 2% glucose) or in synthetic complete medium (22). For induction with galactose, cells were grown in synthetic medium with 2% raffinose overnight. They were then diluted to A600 0.3 and allowed to grow another 2 hrs before addition of galactose to 2%. Alternatively, cells were grown in 2% glucose to mid-log phase, washed once and resuspended in 2% galactose.

All yeast strains used in this study are listed in Table I, along with their sources. All the strains are isogenic with WT strain W303.

The mammalian cells utilized in this study were 293 cells (23).

The following plasmids used in this work, have been previously described: p414 GALL-E4orf4 and pDAD2-E4orf4 (18), YCp50-CDC55 (24), pDB20(HA-CDC55) (25), pGZ148 (expressing Myc-Ynd1p-HA from a yeast glycerol dehydrogenase promoter) (26), GW1-UDPase-Myc (expressing the fusion protein from the cytomegalovirus (CMV) promoter) (27), pLAL70-EGFP (28), pGAL-CA-GCN4 (a gift from D. Kornitzer). To generate the YND1 mutants E152Q and S189A, the pGZ148 plasmid was subjected to site-directed mutagenesis using the QuickChange XL kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions.

Generation of mutant yeast collections

Yeast strain W303, containing p414 GALL-E4orf4, was mutagenized with the transposon insertion library mTn-lacZ/LEU2 (29), as described in detail in http://ygac.med.yale.edu/mtn/insertion_libraries.st m. Briefly, yeast cells were transformed with NotI-digested library DNA, using the LiOAc method. After transformation, the cells were grown for 3 hrs in YPD medium, washed once in H2O and were then plated on galactose medium for selection of E4orf4-resistant yeast clones. Surviving colonies were picked and E4orf4 levels were assessed by Western blot analysis. Yeast clones expressing WT levels of E4orf4 were mated with the parent W303 strain and the resulting diploid cells were subjected to tetrad analysis to find those displaying Mendelian segregation of resistance to E4orf4.

Identification of transposon-disrupted genes

To determine the site of transposon insertion, genomic DNA immediately adjacent to the transposon was rescued in E. Coli, using a plasmid marked with URA3 (pRSQ2-URA3) to replace part of the transposon by recombination of lacZ sequences. (29). Yeast DNA was digested with EcoRI and circularized. Resulting plasmids containing a bacterial origin of replication, the beta-lactamase gene and a portion of the lacZ gene with adjacent yeast DNA, were recovered in bacteria. Plasmids were sequenced using a primer complementary to the 5’ end of the transposon. A detailed description of the process can be found in http://ygac.med.yale.edu/mtn/insertion_libraries.st m.

Knockout of YND1

A ynd1Δ strain in which the YND1 orf was replaced by a Kanamycine cassette was obtained from G. Guidotti (CTY182-8: Mat a, ura3-52, Ahis3, lys2, ynd1::KanR). To create a ynd1Δ strain with the W303 genetic background, the kanamycine cassette replacing the YND1 orf, flanked by 500 bp on each side, was PCR amplified from CTY182-8, using the 5’ oligonucleotide GTGAACGGACAAATTCTA and the 3’ oligonucleotide CTGTTGGTTTGGGCAAGC. The DNA product was transformed into W303 yeast cells and homologous recombination to the YND1 locus was verified by PCR.

Immunoblot analysis and co-immunoprecipitations of yeast and mammalian proteins

For immunobLOTS and co-immunoprecipitations, yeast extracts were prepared by bead beating cells for 4 min at 4°C in lysis buffer (250 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% Triton X-100, 50 mM NaF, 100 µM sodium vanadate, 100 nM okadaic acid (Calbiochem), 1 x complete protease inhibitor cocktail (Roche Molecular Biochemicals) and 1:250 Sigma protease inhibitor cocktail for use in yeast extracts, 2 µg/ml aprotinin (Sigma)). The lysates were spun to separate beads and debris from the clear lysate. The beads were washed twice more in the lysis buffer and immunoprecipitations were carried out in the same lysis buffer.
Mammalian cell extracts were prepared as described previously (9), using a lysis buffer containing 50 mM Hepes-KOH pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.1% NP40, 10% glycerol, 2 mM DTT, 1 x protease inhibitors cocktail (Sigma-Aldrich), 10 mM NaF, 50 mM β-glycerophosphate, 0.25 mM sodium vanadate, 5 mM okadaic acid. Antibodies to E4orf4 were covalently coupled to protein sepharose beads (9). Lysates, antibodies and protein A beads were rotated for 3 hrs at 4°C, and then washed three times in lysis buffer. For detection of Lalp70/UDPase, cell extracts were chromatographed on SDS PAGE after incubation at 37°C for 5 min, without boiling. Antibodies used in this work were: anti-HA (Covance, Berkeley, CA), anti-E4orf4 (6), anti-Cdc55p (30), anti-Tpd3p (from J.R. Broach), and anti-Myc monoclonal (9E10).

**NTPDase activity assays**

NTPDase enzymatic assays were performed using crude membrane preparations, as described by Zhong and Guidotti (26).

**Image acquisition and processing**

Yeast colonies were photographed with the Bio-Rad ChemiDoc™ documentation apparatus, or with a BI.S. Bio Imaging System (model 202D). Gels were scanned using a UMAX ASTRA 3450 scanner. Images were processed using Adobe Photoshop 5.0 or 7.0.

**RESULTS**

**Generation of a mutant yeast strain that is partially resistant to E4orf4 and identification of the mutated gene involved**

Yeast strain W303 containing p414 GALL-E4orf4, a URA3-marked, high copy episomal plasmid expressing E4orf4 from a galactose-inducible promoter, was transformed with a LEU2-marked, transposon-mutagenized yeast DNA library. Transformants were plated on leucine- and uracil-deficient semisolid medium containing galactose. An aliquot of the transformants was plated on medium containing glucose, to estimate the number of transformants obtained. Out of 121,000 transformants, sixty-one colonies grew on galactose. To exclude clones that might have survived due to loss of E4orf4 expression, Western blot analysis was performed on the viable colonies. Only five colonies expressed WT levels of E4orf4 upon induction on galactose. Following removal of the E4orf4-expressing plasmid from the resistant colonies by selection on 5-FOA, the colonies were mated with WT W303 cells, yielding 5 diploids. Tetrad analysis revealed that only one mutant exhibited a 2:2 Mendelian segregation of the E4orf4-resistant phenotype in a manner consistent with a single gene defect.

The gene whose disruption conferred partial resistance to E4orf4 was identified by plasmid rescue of genomic yeast sequences lying adjacent to the transposon as YND1 (YER005w), encoding a yeast Golgi apyrase (26, 31). Deletion of YND1 resulted in partial resistance of the yeast cells to E4orf4 (Fig. 1A), whereas it did not alter expression of the E4orf4 protein (Figs. 3B). Introduction of a WT YND1 gene into ynd1∆ cells reinstated E4orf4-induced toxicity (Fig. 1B). YND1 is specifically required for E4orf4-induced toxicity, since its deletion did not alter toxicity induced by overexpression of Candida Albicans GCN4 (Fig. 1C). These results indicate that YND1 is a novel component of the E4orf4 toxic pathway in yeast.

Ynd1p and Cdc55p contribute to separate E4orf4-induced pathways, which interact functionally

Since both Ynd1p and a PP2A heterotrimer containing the Cdc55p regulatory subunit contribute to E4orf4-induced growth arrest in yeast cells, we further investigated whether they were part of one or more E4orf4-induced pathways. A double mutant strain cdc55∆ynd1∆ was generated and galactose-induced E4orf4 toxicity was compared in WT cells, in the single mutant strains, cdc55∆ and ynd1∆, and in the double mutant (Fig. 2A,B). The cdc55∆ cells grew less well than the other strains on galactose, and produced smaller colonies. The colonies were slightly smaller when E4orf4 was expressed in the cdc55∆ cells, indicating that these cells were not fully resistant to E4orf4, as reported previously (19). The ynd1∆ cells were also partially resistant to E4orf4, generating smaller colonies in its presence. However, as demonstrated before, they were more resistant to E4orf4 than WT cells. The double mutant appeared to be fully resistant to
E4orf4, while still expressing WT E4orf4 protein levels (Fig. 2C). These results suggest that Cdc55p and Ynd1p have additive effects on E4orf4-induced events.

We have previously reported that overexpression of Cdc55p facilitates hypertoxicity of E4orf4 (18). To test whether Ynd1p was required for PP2A-mediated toxicity of E4orf4, Cdc55p was overexpressed in WT cells or in a Ynd1Δ genetic background, and its effect on E4orf4 toxicity was tested. For these experiments, E4orf4 was expressed from a weak GAL promoter and induced very little growth arrest by itself, and Cdc55p was expressed from a constitutive ADH promoter. As demonstrated in Fig. 3A, the presence of YND1 was not required for Cdc55p-mediated hypersensitization of yeast cells to E4orf4. Moreover, the ynd1Δ strain appeared to be supersensitive to the combination of E4orf4 and overexpressed Cdc55p compared with the WT yeast strain. In addition, ynd1Δ cells overexpressing Cdc55p grew slower than WT cells overexpressing this protein (compare colony sizes on glucose plates, Fig. 3A). The difference in sensitivity to E4orf4 did not result from altered levels of E4orf4, as seen in Fig. 3B. Thus, Ynd1p is not a downstream target of the E4orf4-PP2A complex, however it may functionally interact with Cdc55p.

To further test for a functional interaction between Cdc55p and Ynd1p, WT, cdc55Δ and cdc55Δynd1Δ cells were transformed with a plasmid expressing Ynd1p-HA from a glycerol dehydrogenase promoter. As seen in Fig. 4, although WT and cdc55Δynd1Δ cells were efficiently transformed with the pGDH-YND1-HA plasmid, no transformants were obtained in the cdc55Δ cells, suggesting that increased Ynd1p expression is lethal in this background. Furthermore, YND1-HA overexpression slowed down the growth of WT cells, and to a lesser extent that of cdc55Δynd1Δ cells (Fig. 4). These results suggest that too much Ynd1p is detrimental to cell growth even in the presence of Cdc55p, and that synthetic lethality in the cdc55Δ background occurs only when Ynd1p levels increase above a certain threshold.

To further examine the functional interaction between YND1 and CDC55, we tested whether deletion of YND1 suppressed phenotypes associated with inactivation of CDC55. cdc55 mutants demonstrate defective spindle checkpoint activity (32, 33), which could result from a diminished ability to promote dephosphorylation of Cdc28p (32), and from enhanced derepression of APC/C activity in the mutant cells, leading to a less effective inactivation of the APC/C by the spindle checkpoint pathway (18). In addition, Cdc55p antagonises the Tor signaling pathway, and cdc55Δ mutants exhibit rapamycin resistance (34). Accordingly, we tested whether deletion of YND1 suppressed these two phenotypes. To test whether the double mutant cdc55Δynd1Δ lost the checkpoint defect caused by loss of CDC55, we examined the benomyl sensitivity of this strain. Strains defective in the spindle checkpoint pathway are more sensitive to low levels of benomyl, a microtubule-depolymerizing agent, probably due to an increased proportion of cells that go through mitosis in the absence of an intact spindle (35, 36). Fig. 5 demonstrates that there is variability in the degree of benomyl sensitivity shown by several cdc55Δynd1Δ double mutants obtained from different tetrads. However, all of them grew better on benomyl than cdc55Δ. Thus, a ynd1Δ mutation can partially suppress the cdc55Δ spindle checkpoint defect.

The macrolide drug rapamycin inhibits yeast growth by inhibiting essential activities of Tor kinase that are mediated at least partially through Tap42. cdc55Δ strains are resistant to rapamycin, since Cdc55p is required to reverse Tor-mediated phosphorylation of Tap42, and in its absence even low levels of Tor kinase activity are sufficient for cell growth (34). When growth of cdc55Δynd1Δ double mutants on rapamycin was compared to growth of the single cdc55Δ mutant (Fig. 5), no consistent differences were found, indicating that the ynd1Δ mutation did not suppress the rapamycin-resistance of cdc55Δ.

Taken together, the results strongly support the conclusion that Ynd1p and Cdc55p control two separate pathways that interact functionally, possibly by sharing a common downstream target.

**Genetic interaction of YND1 with regulators of the APC/C**

Since deletion of YND1 can decrease the spindle checkpoint defect of cdc55Δ, YND1
possibly interacts genetically with genes encoding components of the spindle checkpoint or its downstream targets. Furthermore, we have previously shown that mutants defective in the substrate-specific activating subunits of APC/C, cdc20-1 and cdh1/hct1Δ, are supersensitive to E4orf4 and that CDC55 interacts genetically with CDC20, encoding a downstream target of the spindle checkpoint (18). Thus the pathway controlled by Cdc55p and Ynd1p, which is affected by E4orf4, could involve the substrate-specific APC/C activating subunits. To test this possibility, Ynd1p was overexpressed in yeast strains containing mutations in CDC20 and CDH1/HCT1, and growth of the transformed yeast was examined. As seen in Fig. 6A, overexpression of Ynd1p inhibited growth of the yeast strain containing a cdc20-1 mutation more efficiently than growth of the other strains on glucose medium. When yeast cells were grown on sublethal concentrations of benomyl (15µg/ml), overexpression of Ynd1p was highly toxic in the cdh1/hct1Δ mutant, and slightly toxic in the mad1Δ mutant, deficient in a component of the spindle checkpoint. Ynd1p overexpression inhibited WT yeast growth only at higher concentrations of benomyl (25µg/ml). The higher sensitivity of cdc20-1 and cdh1/hct1Δ yeast strains to Ynd1p expression was not caused by increased Ynd1p levels, as demonstrated in Fig. 6B. To test whether CDC55 affected growth of the cdh1/hct1Δ mutant cells similarly to YND1, WT and cdh1/hct1Δ yeast cells were transformed with plasmids expressing the two genes. Fig. 6C demonstrates that Cdc55p overexpression inhibited WT yeast growth only at higher concentrations of benomyl (25µg/ml). The higher sensitivity of cdc20-1 and cdh1/hct1Δ yeast strains to Ynd1p expression was not caused by increased Ynd1p levels, as demonstrated in Fig. 6B. To test whether CDC55 affected growth of the cdh1/hct1Δ mutant cells similarly to YND1, WT and cdh1/hct1Δ yeast cells were transformed with plasmids expressing the two genes. Fig. 6C demonstrates that Cdc55p overexpression is lethal in cdh1/hct1Δ cells, even in the absence of benomyl. We have previously shown that overexpression of Cdc55p was also supertoxic in cdc20-1 cells (18). Thus, overexpression of both Cdc55p and Ynd1p is synthetically lethal with mutations in the APC/C activating subunits, indicating a functional interaction between these proteins. No synthetic lethality was demonstrated between Ynd1p overexpression and mutations in CDC34, involved in G1/S transition, or in CDC15, a member of the mitotic exit network (results not shown). These mutations were also not supersensitive to E4orf4 expression (18).

E4orf4 physically interacts with Ynd1p and disrupts a Cdc55p-Ynd1p interaction

To test whether E4orf4 and Ynd1p physically interact, HA-tagged Ynd1p was expressed in ynd1Δ cells in the presence or absence of E4orf4, and cell extracts were prepared 3 hrs after induction with galactose. E4orf4 was immunoprecipitated from the various samples, and the presence of Ynd1p-HA in the immune complexes was detected by Western blot analysis. Fig. 7A demonstrates that E4orf4 coimmunoprecipitated with Ynd1p, as well as with the PP2A A subunit, Tpd3, previously reported to interact with E4orf4 (18). No Ynd1p-HA was immunoprecipitated in the absence of E4orf4. In a reciprocal experiment, Ynd1p-HA was immunoprecipitated from the same cell extracts, and as seen in Fig. 7B, the levels of E4orf4 found in immune complexes containing Ynd1p-HA were significantly higher than the levels of non-specific binding in the absence of Ynd1p-HA. These results indicate that E4orf4 specifically associates with Ynd1p in yeast cells. Next, we tested whether Cdc55p and Ynd1p, two E4orf4 binding partners, are present in a ternary complex. Ynd1p-HA was expressed in ynd1Δ cells together with E4orf4 or with an empty vector. Immunoprecipitation reactions were carried out with antibodies to Cdc55p, and the presence of Ynd1p-HA in the immune complexes was examined by Western blots. As seen in Fig. 7C, Ynd1p-HA coimmunoprecipitated with Cdc55p in the absence of E4orf4. However, when E4orf4 was present, no Ynd1p-HA was detected in the Cdc55p-containing immune complexes, even though Ynd1p-HA levels were similar in control and E4orf4-expressing cells. The level of another Cdc55p-interacting partner, Tpd3, in the immune complexes was not altered by the presence of E4orf4. These results indicate that in addition to the functional interactions between Cdc55p and Ynd1p, these proteins interact physically, and their interaction is disrupted in the presence of E4orf4.

Ynd1p NTPDase activity is not required for its contribution to E4orf4-induced toxicity

To further investigate the molecular mechanisms underlying the contribution of Ynd1p to E4orf4-induced toxicity, enzymatically-inactive Ynd1p variants were prepared. It has been previously reported that certain residues found in
ACR domains of apyrases are required for the catalytic activity of these enzymes. Alteration of residues E182 and S224 in NTPDase-3 caused total inactivation of this enzyme towards all nucleotide substrates (37), and equivalent mutations in NTPDase-1 dramatically reduced the activity of this enzyme towards both ATP and ADP (38). Since the ACR domains are highly conserved within apyrase family members, we mutated residues E152 and S189 in Ynd1p, which are equivalent to residues E182 and S224 in NTPDase-3 from mammalian cells. Plasmids expressing HA-tagged WT Ynd1p or the mutants were introduced into ynd1Δ cells, and the effect of mutations E152Q and S189A on Ynd1p enzymatic activity was assayed. The results shown in Fig. 8A,B demonstrate that although mutant S189A was expressed at the same levels as WT ynd1p, its GTPase and GDPase activities were decreased dramatically: more than 20-fold towards GTP and 3-fold towards GDP. It should be noted that endogenous GDPase activity levels in ynd1Δ cells are high since these cells contain another GDPase encoded by the GDA1 gene (26). The high background might explain the smaller net difference between WT and mutant GDPase activities. Mutant E152Q was expressed at lower levels, but its GTPase activity appeared to be reduced more than its expression. To determine whether enzymatic activity is required for the contribution of Ynd1p to E4orf4-induced toxicity, WT and mutant Ynd1 proteins were co-expressed with E4orf4 or with an empty vector, and cells were streaked on semi-solid glucose and galactose media. Growth of individual E4orf4-expressing colonies could only be detected when cells contained E4orf4 together with an empty vector, but not when cells were expressing E4orf4 together with any of the Ynd1 proteins (Fig. 8C). Thus, inactive Ynd1p variants can mediate E4orf4-induced toxicity. These results indicate that enzymatic activity is dispensable for the ability of Ynd1p to mediate E4orf4-induced effects.

Preliminary investigation of the relevance of the findings in yeast to the mammalian cell system

Since Ynd1p was discovered as a mediator of E4orf4-induced toxicity in yeast cells, we wanted to find whether human homologues of Ynd1p also interacted with E4orf4. The closest homologues of Ynd1p in mammalian cells are a Golgi UDPase and Lalp70p, encoded by two splice variants of the NTPDASE-4 gene. These proteins differ only by eight residues, which are inserted in-frame into the UDPase to generate Lalp70p (27, 28, 39). As seen in Fig. 9, E4orf4-specific antibodies immunoprecipitated Myc-tagged UDPase from 293 cell extracts expressing E4orf4 but not from extracts lacking the viral protein. These results indicate that E4orf4 and the Golgi UDPase associate in mammalian cells.

DISCUSSION

In this work we present genetic evidence that YND1, encoding a yeast Golgi apyrase, contributes to E4orf4-induced toxicity in yeast (Fig. 1). It is unclear why only one gene whose deletion conferred resistance to E4orf4 was obtained in the screen. At least two other genes, CDC55 and TPD3, encoding PP2A subunits that are required for E4orf4-induced toxicity (18), should have been obtained as well. However, it is possible that the amplified library used for the screen did not fully represent all non-essential yeast genes.

The genetic analysis presented here demonstrates that YND1 and CDC55 contribute in an additive manner to E4orf4-induced toxicity (Fig. 2). Since full resistance to E4orf4 is demonstrated in a ynd1Δcdc55Δ double mutant, it appears that Ynd1p and Cdc55p may be the only E4orf4 direct effectors, which mediate its toxic effect in yeast. Our results also demonstrate that YND1 interacts genetically with CDC55 (Figs. 3-5). Overexpression of Ynd1p and deletion of CDC55 are synthetically lethal (Fig. 4), and overexpression of Cdc55p inhibits growth of ynd1Δ cells and sensitizes them to E4orf4-mediated toxicity (Fig. 3). Thus Ynd1p and Cdc55p are not located downstream of each other in the same pathway but they interact functionally. The results are consistent with the proposal that Cdc55p and Ynd1p have opposite effects on a common downstream target, and that a balanced regulation of this target by Ynd1p and Cdc55p is crucial to yeast cell viability. Disruption of the balanced modulation of the unknown target by E4orf4 may underlie its toxic effect. The investigation of the physical associations between E4orf4 and its two effectors further support the genetic evidence. We found that Ynd1p and...
Cdc55p physically interact in the absence of E4orf4, however, when E4orf4 is expressed this interaction is disrupted (Fig. 7). These findings raise the possibility that the common target may be found in the Ynd1p-Cdc55p complex. It is unlikely, however, that removal of Ynd1p or Cdc55p from a complex containing their common target is the only effect that leads to E4orf4 toxicity, since E4orf4 expression is not equivalent to Ynd1p or Cdc55p deletion. Rather, overexpression of one protein and deletion of the other must both occur to cause toxicity in the yeast. Consistent with these observations, we found that, having disrupted the Ynd1p-Cdc55p interaction, E4orf4 remains associated with both its effectors (Fig. 7), possibly further affecting their activities. The results showing that YND1 deletion only partially suppresses cdc55∆ phenotypes (Fig. 5) suggest that Ynd1p does not affect all Cdc55p targets.

To date, Ynd1p has been described as a Golgi apyrase, whose enzymatic activity is required for regulation of nucleoside-sugar import into the lumen of the Golgi (21, 26). However, we show here that enzymatically-inactive variants of Ynd1p are as proficient in mediating E4orf4-induced activity as the WT protein (Fig. 8). Thus Ynd1p appears to have an additional function, which does not require its apyrase activity, and which contributes to the E4orf4-initiated pathway. It is not surprising, therefore, that we could not find an effect of E4orf4 on global protein glycosylation or on Ynd1p enzymatic activity (data not shown). There have been many reports in the literature demonstrating non-enzymatic regulatory roles of enzymes in cellular events (for example, refs. (40-42)). Thus, besides their catalytic activity, enzymes can provide a scaffold function, or can activate or inhibit other proteins by a physical interaction, unmasking or interfering with activating or inhibitory domains.

Our results further suggest a pathway in which a joint target for PP2A and Ynd1p may be functional. We have previously shown that mutants defective in the APC/C activating subunits Cdc20p and Cdh1/Hct1p are supersensitive to E4orf4-induced toxicity, and that cdc20-1 cells are supersensitive to Cdc55p overexpression (18). Here we show that cdh1/hct1Δ cells are more sensitive to Ynd1p and Cdc55p overexpression than WT cells, and that cdc20-1 cells are supersensitive to Ynd1p overexpression (Fig. 6). It appears therefore that the E4orf4 partners Ynd1p and Cdc55p, as well as E4orf4 itself, functionally interact with the two APC/C activating subunits. Moreover, E4orf4 was shown to associate with the APC/C in a complex that includes PP2A as well (18), further linking E4orf4 and its effectors to APC/C-controlled pathways. Cdc20p and Cdh1/Hct1p belong to two checkpoint pathways: the spindle checkpoint pathway in which APC/Ccdc20p is inactivated by an incomplete spindle through binding to Mad2, and the spindle position checkpoint in which APC/Ccdc1/hct1p is inactivated by incorrect spindle orientation through inhibition of the mitotic exit network (43). There is crosstalk between the two checkpoints, and Cdc20p is degraded in an APC/C-dependent manner during G1 and is thus likely to be a Cdh1/Hct1p substrate (44, 45). Furthermore, cdh1/hct1Δ mutant cells were reported to rely on the spindle assembly checkpoint pathway to prevent chromosome loss (46). These findings are consistent with the possibility that shared targets of Cdc20p and Cdh1/Hct1p exist, and such targets could be affected by Cdc55p, Ynd1p and E4orf4.

How could Ynd1p, an integral Golgi membrane protein (26, 31), impact mitotic regulation pathways, and functionally interact with APC/C activating subunits? Several precedents have been described for Golgi membrane proteins involved in mitotic control, although the molecular mechanisms underlying their mitotic involvement are not yet understood. These include GRASP65, shown to regulate spindle dynamics, perhaps through modulation of γ-tubulin recruitment to the centrosome (47); Pmr1, a Golgi membrane ATPase, which interacts genetically with the G2/M cyclin Clb3p, and may impact signaling to a cell cycle regulation pathway through N-linked glycosylation (48); and an N-linked, O-linked palmitoylated integral membrane protein, ADP/E3-11.6K, encoded by adenovirus. which interacts with the human cell cycle protein MAD2B through the ADP cytoplasmic or nucleoplasmic tail, an interaction that is biologically relevant to viral infection (49). Experiments designed to identify the joint Ynd1p-Cdc55p target, which are currently underway, are likely to provide more insights into the mechanisms involved in the functional interaction
between Ynd1p and the APC/C activating subunits.

Results shown in Fig. 9 indicate that Golgi UDPase, a human orthologue of Ynd1p (27), associates with E4orf4 in mammalian cells. A splice variant of the UDPase, Lalp70p, located in lysosomal/autophagic vacuoles (28), also associates with E4orf4 (results not shown). These results suggest that our findings in the yeast screen are relevant to E4orf4-induced events in mammalian cells, although further examination of the involvement of Ynd1p mammalian orthologues in E4orf4-induced apoptosis in human cells will be carried out in future experiments. At early stages of the apoptotic process E4orf4 is found in the nucleus, whereas it localizes to cell membranes at intermediate and late stages. The signals allowing a shift of E4orf4 from the nucleus to the membranes involve phosphorylation of E4orf4 by Src kinases (15), although it is not yet known why this translocation does not occur as an immediate early event in the E4orf4-mediated process. During its passage to cell membranes, E4orf4 could possibly interact with the human homologues of Ynd1p. In yeast, E4orf4 was also shown to localize to cellular membranes (results not shown).

In summary, the data presented here reveals a new component of an emerging E4orf4 signaling network assembled both in yeast and mammalian cells, which is involved in E4orf4-induced toxicity. These studies validate the advantage of utilizing non-mammalian model systems to the study of E4orf4-induced events. Future work will further clarify the contribution of NTPDase-4 to cancer cell-specific E4orf4-induced apoptosis.

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ABBREVIATIONS
E4orf4, early region 4 open reading frame 4; PP2A, protein phosphatase 2A, APC/C, anaphase promoting complex / cyclosome; wild type, WT.

FIGURE LEGENDS
Fig. 1 A ynd1∆ mutant is partially resistant to E4orf4-induced toxicity
A. WT and ynd1∆ cells were transformed with vector or pGALL-E4orf4 plasmid. Yeast cells were streaked on -URA galactose plates, on which E4orf4 expression is induced. Colonies were grown for three days at 30°C.
B. ynd1∆ cells were transformed with vector, pGALL-E4orf4, pGDH-YND1 or both E4orf4 and YND1, and were streaked on galactose plates, to induce E4orf4 expression. Colonies were grown for three days at 30°C.
C. WT and ynd1∆ cells were transformed with vector or pGALL-CaGCN4, encoding Candida Albicans GCN4, and were streaked on galactose plates, to induce Gcn4p expression. Colonies were grown for three days at 30°C.

Fig. 2 Cdc55p and Ynd1p have an additive effect on E4orf4-induced toxicity
A. WT and mutant yeast strains were transformed with vector (-) or with pGALL-E4orf4 (+). Transformed yeast cultures were serially diluted 5-fold, and plated on -URA glucose and galactose plates. Cells were grown for 3 days at 30°C.
B. A higher magnification of the last four dilutions plated on galactose is shown.
C. Proteins were prepared from an aliquot of the WT and cdc55∆ynd1∆ cells in A, and were subjected to Western blot analysis with E4orf4- and Tpd3-specific antibodies. Tpd3 (the PP2A A subunit) served as a loading control.

Fig. 3 Overexpression of Cdc55p in a ynd1∆ genetic background causes supersensitivity of the cells to E4orf4-induced toxicity
A. WT and ynd1∆ mutant cells were transformed with combinations of pDAD-E4orf4 (a weak expressor of E4orf4) and pADH-CDC55, as noted in the figure. Serial 5-fold dilutions of the various yeast samples were plated on -URA-LEU glucose or galactose plates and grown for three days at 30°C.
B. Proteins were prepared from an aliquot of the cells in A and were subjected to Western blot analysis with E4orf4- and Tpd3-specific antibodies. Tpd3 served as a loading control.

Fig. 4 Deletion of CDC55 and overexpression of YND1 are synthetically lethal
Wild type, cdc55∆, and cdc55∆ynd1∆ yeast cells were transformed with vector or pGDH-YND1, plated on -URA glucose plates, and grown for two days at 30°C.

Fig. 5 A YND1 deletion partially rescues the spindle checkpoint defect of cdc55∆
Wild type (WT), ynd1∆, cdc55∆ and several clones of cdc55∆ynd1∆ obtained from different tetrads were grown in liquid culture overnight (5A and 5B were obtained from two different sporulation events). Equal yeast concentrations were subjected to five-fold serial dilutions and were spotted on plates containing YPD medium alone, YPD containing 27.5 μg/ml benomyl, or YPD containing 100nM rapamycin. The cells were grown for two days (YPD alone) or three days (YPD with benomyl or rapamycin) at 30°C.

Fig. 6 cdc20-1 and cdh1/hct1∆ cells are supersensitive to overexpression of YND1 or CDC55
A. Wild type (WT), mad1∆, cdh1/hct1∆, and cdc20-1 cells transformed with an empty vector or with pGDH-YND1 were grown and subjected to serial dilutions, as described in the legend to Fig. 5. The cells were spotted onto -URA glucose plates containing: no drug, 15 μg/ml benomyl, or 25 μg/ml benomyl, and were grown for two days (no drug) or three days (benomyl) at 30°C.
B. Proteins were prepared from an aliquot of the cells in A and were subjected to Western blot analysis with HA- and Tpd3-specific antibodies. Tpd3 served as a loading control.

C. Wild type (WT) and cdh1/hct1 Δ cells were transformed with an empty vector, pGDH-YND1, or pADH-CDC55. The cells were grown on -URA glucose plates for three days at 30°C.

Fig. 7 E4orf4 physically interacts with Ynd1p and disrupts a Cdc55p-Ynd1p interaction
Ynd1Δ-derived yeast cells expressing Ynd1p-HA or E4orf4, as noted in the figure, were harvested and cell extracts were immunoprecipitated with E4orf4-specific antibodies (A), with anti-HA antibodies (B) or with Cdc55p-specific antibodies (C). Immune complexes (IP), as well as cell lysates (Lysate), were separated on SDS gels and subjected to Western blot analysis, using antibodies to HA, Tpd3p, Cdc55p or E4orf4, sequentially. The lysates shown represent 10% of extracts used for immunoprecipitation.

Fig. 8 Ynd1p enzymatic activity is not required for E4orf4-induced toxicity
WT YND1, an empty vector (Vec), or two YND1 mutants (S189A and E152Q) were introduced into Ynd1Δ yeast cells together with p414 GALL-E4orf4 or an empty vector. Crude membranes prepared from cells grown on glucose medium were used in apyrase assays with GTP or GDP substrates (A) and the amount of phosphate released was quantified. Endogenous levels of apyrase activity measured in vector-containing cells were subtracted from the results of each of the other samples and net activity of the exogenous constructs is shown. Similar amounts of the crude membranes were also chromatographed on SDS PAGE and subjected to Western blot analysis with antibodies recognizing the HA tag or Tpd3, which served as a loading control (B). In parallel, yeast cells containing the various plasmids were plated on glucose and galactose plates (C), and allowed to grow for 2 days (glucose) or 3 days (galactose) at 30°C.

Fig. 9 The human homologue of Ynd1p interacts with E4orf4
Various combinations of plasmids expressing UDPase-Myc and E4orf4 were cotransfected into 293 cells. Protein extracts were subjected to immunoprecipitation with E4orf4-specific antibodies and immune complexes (IP), as well as extracts before precipitation (Lysate), were chromatographed on SDS PAGE. Western blots were stained with Myc- and E4orf4-specific antibodies.

### TABLE I Yeast strains used in this study

All strains are isogenic with W303.

| Strain | Genotype | Reference or source |
|--------|----------|---------------------|
| W303-1A | a ura3-1 leu2-3,112 trp1-1 ade2-1 his3-11,15 GAL+ | R. Rothstein |
| KY520 | α ura3-1 leu2-3,112 trp1-1 GAL+ cdc55::LEU2 | (18) |
| KY679 | α ura3-1 leu2-3 tpd3::LEU2 | (18) |
| Ynd1Δ | a, α ura3-1 leu2-3,112 trp1-1 GAL+ ynd1::KAN | This work |
| Cdc55Δynd1Δ | α ura3-1 leu2-3,112 trp1-1 GAL+ cdc55::LEU2 ynd1::KAN | This work |
| KH123 | a ura3-52 leu2-3,112 trp1 can1 ade2 his3-11,15 mad1Δ::HIS3 | (50) |
| W321 | a ura3-1 leu2-3,112 trp1-1 ade2-1 his3-11,15 hct1Δ::LEU2 | (51) |
| A460 | a cdc20-1 ura3 leu2 trp1 his3 | A. Amon |
| MTY670 | a ura3-1 leu2-3,112 trp1-1 ade2-1 his3-11,15 cdc34-2 | M. Tyers |
| K1993 | a cdc15-2 ura3 trp1 leu2 | K. Nasmyth |
Fig. 1
Fig. 2
Fig. 3
Yeast strains:
Wild type

cdc55Δ

cdc55Δynd1Δ

Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 9
YND1 interacts with CDC55 and is a novel mediator of E4orf4-induced toxicity
Tsosnai Maoz, Roni Koren, Inbal Ben-Ari and Tamar Kleinberger

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