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Differential Roles of Transcriptional Mediator Subunits in Regulation of Multidrug Resistance Gene Expression in *Saccharomyces cerevisiae*

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The multiprotein transcriptional Mediator complex provides a key link between RNA polymerase II and upstream transcriptional activator proteins. Previous work has established that the multidrug resistance transcription factors Pdr1 and Pdr3 interact with the Mediator component Med15/Gal11 to drive normal levels of expression of the ATP-binding cassette transporter-encoding gene *PDR5* in *Saccharomyces cerevisiae*. *PDR5* transcription is induced upon loss of the mitochondrial genome (ρ^0 cells) and here we provide evidence that this ρ^0 induction is Med15 independent. A search through other known Mediator components determined that Med12/Srb8, a member of the CDK8 Mediator submodule, is required for ρ^0 activation of *PDR5* transcription. The CDK8 submodule contains the cyclin C homologue (CycC/Srb11), cyclin-dependent kinase Cdk8/Srb10, and the large Med13/Srb9 protein. Loss of these other proteins did not lead to the same block in *PDR5* induction. Chromatin immunoprecipitation analyses demonstrated that Med15 is associated with the *PDR5* promoter in both ρ^+ and ρ^0, whereas Med12 recruitment to this target promoter is highly responsive to loss of the mitochondrial genome. Coimmunoprecipitation experiments revealed that association of Pdr3 with Med12 can only be detected in ρ^0 cells. These experiments uncover the unique importance of Med12 in activated transcription of *PDR5* seen in ρ^0 cells.

INTRODUCTION

The transcriptional Mediator complex is a group of more than 20 polypeptide chains that serves as a link between upstream activator proteins and the RNA polymerase II machinery (reviewed in Casamassimi and Napoli, 2007). The Mediator complex can be isolated in at least two different forms. The core Mediator (C-Mediator) lacks a four-protein module that is present in the larger L-Mediator complex. L-Mediator consists of C-Mediator and the so-called CDK module, which consists of the large proteins Med12/Srb8 and Med13/Srb9, the cyclin-dependent kinase Cdk8/Srb10, and the cyclin C (CycC)/Srb11 (Borggrefe *et al.*, 2002; Samuelsen *et al.*, 2003). Genetic and biochemical analyses of C- and L-Mediator argued that the C-Mediator acted as a positive regulator of transcription, whereas the L-Mediator served a repressive activity (Holstege *et al.*, 1998; Elmlund *et al.*, 2006).

More recent work suggests that this binary view of the function of C- and L-Mediator is insufficient to explain the roles of these complexes. For example, transcription of the *Drosophila Distal-less* gene requires Med12 but not Cdk8 or CycC (Carrera *et al.*, 2008). Additionally, CDK8 is required for β-catenin–driven expression of several target genes important in colon cancer (Firestein *et al.*, 2008) and certain p53-regulated genes (Donner *et al.*, 2007). Coupled with earlier observations that loss of CDK module components in yeast led to induction of a large number of genes (Holstege *et al.*, 1998), both negative and positive roles for the L-Mediator seem likely.

We have previously described the role of a Mediator component called Med15/Gal11 (Suzuki *et al.*, 1988) in wild-type expression of a gene from *Saccharomyces cerevisiae* involved in multiple or pleiotropic drug resistance called *PDR5*. Expression of the ABC transporter protein-encoding *PDR5* gene requires the presence of at least one of the Zn_2Cys_6 zinc cluster–containing transcription factors Pdr1 and Pdr3 (Delaveau *et al.*, 1994; Katzmann *et al.*, 1994). Analysis of the interaction between Pdr1 and Med15 was driven by the observation that these two proteins are in direct contact (Thakur *et al.*, 2008). Hyperactive mutant forms of both Pdr1 and Pdr3 required Med15 to drive elevated levels of transcription as measured by use of a *PDR5-lacZ* reporter gene. However, closer examination of these data indicated that, although *med15*Δ strains exhibited lowered levels of *PDR5-lacZ* expression, introduction of hyperactive alleles of either *PDR1* or *PDR3* still led to a nearly 10-fold increase in β-galactosidase activity. We interpret these data to argue that both Pdr1 and Pdr3 still activate gene expression even in the absence of Med15, suggesting the presence of additional Mediator component targets. Here, we confirm this possibility for Pdr3-dependent activation of gene expression.

Pdr3 control of *PDR5* transcription occurs via the same Pdr1/Pdr3 response elements (PDREs) present in the *PDR5* promoter (Katzmann *et al.*, 1996), but Pdr3 is differentially regulated from Pdr1. Loss of the mitochondrial genome (ρ^0 cells) strongly induces *PDR5* transcription in a Pdr3-depen-

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Table 1. Strains used in this study

Strain	Genotype	Reference
SEY6210	*MATα leu2-3, -112ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻*	Scott Emr
SEY6210 ρ^0	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Mel⁻* ρ^0	Hallstrom and Moye-Rowley (2000)
PSY21	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ MED15-TAP::HIS3MX6*	This study
PSY42	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ med12Δ::kanMX4*	This study
PSY43	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 med12Δ::kanMX4 Mel⁻* ρ^0	This study
PSY44	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ med31Δ::kanMX4*	This study
PSY45	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 med31Δ::kanMX4 Mel⁻* ρ^0	This study
PSY46	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ med3Δ::TRP1*	This study
PSY47	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 med3Δ::TRP1 Mel⁻* ρ^0	This study
PSY48	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ med20Δ::kanMX4*	This study
PSY49	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 med20Δ::kanMX4 Mel⁻* ρ^0	This study
PSY50	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ cycCΔ::kanMX4*	This study
PSY51	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 cycCΔ::kanMX2 Mel⁻* ρ^0	This study
PSY52	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ med12Δ::hphMX2*	This study
PSY53	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 med12Δ::hphMX2 med15Δ::kanMX2 Mel⁻*	This study
PSY54	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ med12Δ::hphMX2* ρ^0	This study
PSY55	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 med12Δ::hphMX2 med15Δ::kanMX2 Mel⁻* ρ^0	This study
PSY56	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ med13Δ::kanMX4*	This study
PSY57	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 med13Δ::kanMX2 Mel⁻* ρ^0	This study
PSY59	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ MED12-TAP::HIS3MX6*	This study
PSY60	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ MED12-TAP::HIS3MX6 Mel⁻* ρ^0	This study
PSY79	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ cdk8Δ::TRP1*	This study
PSY80	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 cdk8Δ::TRP1 fzo1Δ::kanMX2 Mel⁻* ρ^0	This study
PSY81	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ MED12-TAP::HIS3MX6 med15Δ::kanMX4*	This study
PSY82	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 MED12-TAP::HIS3MX6 med15Δ::kanMX4 Mel⁻* ρ^0	This study
PSY83	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 med15Δ::kanMX4 Mel⁻ fzo1::natMX*	This study
KGS42	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 med15Δ::kanMX4 Mel⁻*	This study
KGS61	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pdr1-2::hisG, pdr3-1::hisG MED15-TAP::HIS3MX6*	This study
KGS63	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ pdr1-2::hisG, pdr3-1::hisG fzo1::kanMX MED15-TAP::HIS3MX6*	This study
KGS86	*MATα leu2-3, -112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 Mel⁻ MED15-TAP::HIS3MX6*	This study

dent but a Pdr1-independent manner (Hallstrom and Moye-Rowley, 2000). Additionally, changes in the levels of an enzyme involved in mitochondrial biosynthesis of phosphatidylethanolamine also acts via Pdr3 to induce *PDR5* transcription (Gulshan *et al.*, 2008). Because loss of Med15 reduced but did not eliminate the mitochondria-to-nucleus or retrograde induction of *PDR5*, we examined other nonessential Mediator components for their participation in this Pdr3 regulatory pathway. Loss of the Mediator component Med12/Srb8 prevented the retrograde activation of *PDR5* seen in ρ^0 cells. Med12 only plays an important role in *PDR5* expression in ρ^0 cells and is consistent with the view that this Mediator component may be intimately associated with retrograde regulation of multidrug resistance in this yeast.

MATERIALS AND METHODS

Yeast Media

Yeast cells were grown in YPD (2% yeast extract, 1% peptone, 2% glucose) under nonselective conditions or appropriate synthetic complete (SC) media under selective conditions (Sherman *et al.*, 1979) at 30°C with shaking. Drug resistance was measured by the spot test assay on plates with or without drug as indicated. Midlog phase cells were serially diluted in water (1:10), and dilutions were spotted on plates that were then incubated at 30°C for 2–3 d and photographed. Briefly, gradient plates were produced by pouring 25 ml of medium containing the final desired drug concentration into a square 100-mm Petri dish held at a constant angle (roughly 20° degrees from the horizontal; Katzmann *et al.*, 1995). The medium was allowed to solidify and additional 25 ml of medium was overlaid. Once this second layer of medium had solidified, the plates were used within 24 h. Cycloheximide, 0.2 mg/ml, was used for all assays except for Figure 6 in which 0.25 μg/ml cycloheximide was present in the medium. Transformation was performed using the LiOAc technique (Ito *et al.*, 1983). Assays for β-galactosidase activity were carried out on permeabilized cells using *o*-nitrophenyl-β-D-galactopyranoside as substrate as described (Guarente, 1983).

Strain construction

Yeast strains used in this study were derived from SEY6210 or BY4742, and their genotypes are listed in Table 1. Deletion strains of open reading frames (ORFs) of *MED15/GAL11*, *MED3/PGD1*, *MED31/SOH1*, *MED20/SRB2*, *MED12/SRB8*, *MED13/SRB9*, and *CycC/SRB11* containing the *kanMX4* cassette were obtained from Open Biosystems (Huntsville, AL) in the BY4742 background. PCR primers specific for nucleotide sequences 200 base pairs upstream and 200 base pairs downstream of the *kanMX4* gene replacement were used to PCR-amplify the specific locus (primer list is available on request). Disruption cassettes were then transformed into SEY6210 ρ^+ and ρ^0 genetic backgrounds to get the corresponding disruptions. Deletion in the *CDK8/SRB10* gene was made using plasmid pFA6a-TRP1 by PCR-based disruption as described (Longtine *et al.*, 1998). All disruption alleles were confirmed by PCR. Strains containing tandem affinity purification (TAP) tag fusions of *MED15/GAL11*, *MED12/SRB8*, and *MED3/PGD1*in SEY6210 ρ^+ and ρ^0 were constructed by transforming these strains with a TAP-*HIS3MX6* cassette amplified from the Open Biosystems TAP tag strain collection (Ghaemmaghami *et al.*, 2003). The enhanced green fluorescent protein (eGFP) cassette flanked by targeting sequences was amplified from plasmid pYM29 and pYM30 (Janke *et al.*, 2004) with S2-primer, the *reverse complement* of 45–55 bases downstream of the STOP-codon including STOP) of *MED12*, followed by 5-ATCGATGAATTCGAGCTCG-3 and S3-primer, 45–55 bases before the STOP-codon (excluding the stop codon) of *MED12*, followed by 5-CGTACGCTGCAGGTCGAC. This PCR fragment was transformed into cells, resulting in eGFP tagging of *MED12* in the SEY6210 ρ^+ and ρ^0 genetic backgrounds.

Plasmids

Ace1-*PDR3*, *PDR5*-, *SNQ2*-, *YOR1-lacZ*, pRS315-*PDR3/PDR3-11* plasmids have been described previously (Katzmann *et al.*, 1994; Decottignies *et al.*, 1995; Katzmann *et al.*, 1995; Hallstrom and Moye-Rowley, 2000; Zhang *et al.*,

2005). The Myc-Pdr1-expressing plasmid (pPS1) was constructed by transferring a Myc-tagged wild-type *PDR1* (Mamnun *et al.*, 2002) allele into pRS315.

Quantitative Reverse Transcriptase-PCR mRNA Measurements

Cells were grown to midlog phase in the absence of any drugs. Total RNA was prepared, subjected to reverse transcription, and analyzed as described previously (Shahi *et al.*, 2007).

Fluorescence Microscopy

The strains carrying different GFP-tagged versions of *MED12* were grown to saturation. These cultures were then reinoculated at a starting optical density at 600 nm (OD_{600}) of 0.1. Cells were allowed to grow for 2 h after which 1 μg/ml 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) was added to stain DNA, and cultures were further grown for 2 h to an approximate OD_{600} of 0.4–0.6, after which 5 μl of each was placed on a glass slide, overlaid with a coverslip, and examined under the 100× oil objective of an Olympus BX60 microscope (Melville, NY). Images were captured on a Hamamatsu digital camera (C4742–95; Bridgewater, NJ).

Western Analysis

Cell lysates were prepared as described in Mamnun *et al.* (2002). Cells were grown overnight in rich or selective medium. Cells equivalent to 5 OD_{600} were lysed with 250 μl of YEX lysis buffer (1.85 M NaOH, 7.5% β-mercaptoethanol), incubated for 10 min on ice, and precipitated with 250 μl of 50% (wt/wt) trichloracetic acid for 10 min on ice. The protein precipitate was dissolved in 100 μl of sample buffer (40 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 0.1 mM EDTA, 0.1 g/l bromophenol blue, supplemented with 1% β-mercaptoethanol and 10% 1 M Tris-base). Aliquots of 10 or 40 μl of cell-free extracts, corresponding to 0.5 (TAP-tagged Mediator subunits) or 2 OD_{600} (Pdr3) were separated by SDS-PAGE in 6% gels and blotted to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in phosphate buffered-saline. Polyclonal antiserum against TAP, 1:5000 dilution (Open Biosystems) or a commercially available monoclonal anti-hemagglutinin (HA) antibody 1:1000 dilution (Covance, Madison, WI) was used for immunodetection. Proteins on immunoblots were visualized using the ECL chemiluminescence detection system (Thermo Scientific, Waltham, MA).

Chromatin Immunoprecipitation

Fifty milliliters of the desired media was inoculated with saturated overnight culture and grown until the desired cell density was reached (usually ~A_{600} 1.0). Formaldehyde at a final concentration of 2% final was added to the cultures and incubated at room temperature 10–20 min with occasional swirling. Formaldehyde cross-linking was quenched by addition of 2.5 M glycine to a final concentration of 250 mM. Cells were then lysed in chromatin immunoprecipitation (ChIP) lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, and protease inhibitors), and the chromatin was sheared by sonication (Model 550 sonic dismembrator, Fisher Scientific, Pittsburgh, PA). An aliquot of the samples was saved to be used as input. The sheared chromatin was then immunoprecipitated using anti-tandem affinity purification (TAP) antibody (Open Biosystems) and protein A-agarose beads (Santa Cruz Biotechnology,). Agarose beads were then washed with ChIP wash buffer (10 mM Tris (pH 8.0), 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 1 mM EDTA), and precipitates were eluted in ChIP elution buffer (50 mM Tris pH 8.01% SDS10 mM EDTA). The eluted precipitates and input samples were incubated overnight at 65°C to reverse cross-links. DNA was then precipitated and subjected to quantitative PCR (qPCR) analysis. Target DNA in the immunoprecipitated sample was quantified by generating a standard curve with a 10-fold dilution series of the nonimmunoprecipitated sample (input DNA) for each DNA. The ratio of immunoprecipitated to input signals was calculated for the gene of interest and divided by the corresponding ratio for the control (no antibody) to input.

Coimmunoprecipitation Assay

Cells were grown to log phase, washed with spheroplast solution I (1 M sorbitol, 10 mM $MgCl_2$, 30 mM dithiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, 50 mM K_2HPO_4), and resuspended in spheroplast solution II (1 M sorbitol, 10 mM $MgCl_2$, 30 mM dithiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, 50 mM K_2HPO_4, 25 mM sodium succinate, pH 5.5) containing oxylyticase followed by incubation at 30°C for 30 min. Spheroplasts were pelleted by centrifuging at 5000 rpm for 20 min at 4°C in a Beckman JA-20 rotor (Fullerton, CA). These spheroplasts were either stored at −80°C or lysed immediately using glass beads and Nonidet P-40 lysis buffer (1% Nonidet P-40/Triton X-100, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.2) with 2 mM EDTA, 200 μM sodium vanadate, and 50 mM sodium fluoride. Lysis was performed by shaking cell suspensions on a Tomy shaker at 4°C. Protein extracts were clarified by centrifuging lysates at 14,000 rpm for 5 min at 4°C. The lysates were incubated with anti-TAP antibody for 2 h at 4°C on rotator. After which, protein A agarose beads, washed and resuspended in the same buffer, were added to it and incubated for 4 h for immunoprecipitation. Finally, the beads were washed, and immunoprecipitated proteins were recovered by adding 3× Laemmli dye (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 2% bromphenol blue dye). Immunoprecipitated proteins along with input proteins were then loaded on a 6% polyacrylamide gel and analyzed by Western blotting with anti-HA, anti-Myc or anti-TAP antibodies.

RESULTS

Med15/Gal11 Is Not the Sole Target of Pdr3-regulated Transcriptional Activation

Gain-of-function alleles of *PDR3* (*PDR3-11*) are capable of inducing expression of *PDR5-lacZ* by 10-fold when compared with wild-type Pdr3 (Kean *et al.*, 1997; Nourani *et al.*, 1997). As described earlier (Thakur *et al.*, 2008), although loss of Med15 reduced *PDR5* expression to 10% of that seen in *MED15* background, introduction of the *PDR3-11* allele into *med15*Δ cells still induced this lower basal expression level by a factor of 10. To confirm that this effect on reporter gene expression was seen on the natural *PDR5* locus, we introduced a low-copy-number plasmid containing or lacking *PDR3-11* into isogenic wild-type and *med15*Δ cells. These transformants were then grown to midlog phase and placed on media containing cycloheximide, a drug known to be detoxified by the action of Pdr5 (Leppert *et al.*, 1990). Plates were incubated at 30° and then photographed (Figure 1A).

Even in the absence of Med15, cells containing *PDR3-11* were much more resistant to cycloheximide than cells with the wild-type *PDR3* gene. This behavior supports the view that the increased transcriptional activation of Pdr3-11 compared with Pdr3 is retained even in the absence of Med15. To ensure that the increased drug resistance seen here was due to increased expression of the authentic *PDR5* locus, expression of the Pdr5 protein was assessed using a polyclonal antibody directed against this ABC transporter protein (Egner *et al.*, 1995). The transformants described above were grown to midlog phase, whole cell protein extracts prepared and analyzed by Western blotting with anti-Pdr5 antibody.

Consistent with the high-level drug resistance seen in the presence of Pdr3-11, the highest levels of Pdr5 expression were present in *MED15* cells expressing this hyperactive regulatory protein compared with the same strain bearing the empty vector plasmid (Figure 1B). Importantly, loss of Med15 lowered the expression in the presence of either plasmid but the presence of the *PDR3-11* allele still induced Pdr5 expression. These findings suggest that the transcriptional stimulation of *PDR5* elicited by the hyperactive Pdr3-11 protein was still present even in the absence of Med15.

On the basis of the previous study of interaction of hyperactive Pdr1 protein with Med15 (Thakur *et al.*, 2008), we anticipated that Pdr3-11 will interact more effectively with Med15 than its wild-type counterpart. This increased interaction would recruit more Mediator complex to the *PDR5* promoter and lead to the enhanced transcriptional activation and Pdr5-dependent phenotypes demonstrated above. To test this expectation, a coimmunoprecipitation assay was carried out to directly compare the association of wild-type and hyperactive Pdr3 with Med15. One complication of this comparison comes from the autoregulatory nature of *PDR3* gene expression (Delahodde *et al.*, 1995). Because levels of Pdr3 are controlled by the activity of Pdr3 itself, Pdr3-11 accumulates to a higher level than does wild-type Pdr3.

To avoid this complication, we used a modified *PDR3* promoter to drive expression of both the wild-type and hyperactive forms of Pdr3. This promoter was described previously (Zhang *et al.*, 2005) and, in brief, contains copper response elements (CuRE; Pena *et al.*, 1998) in place of the

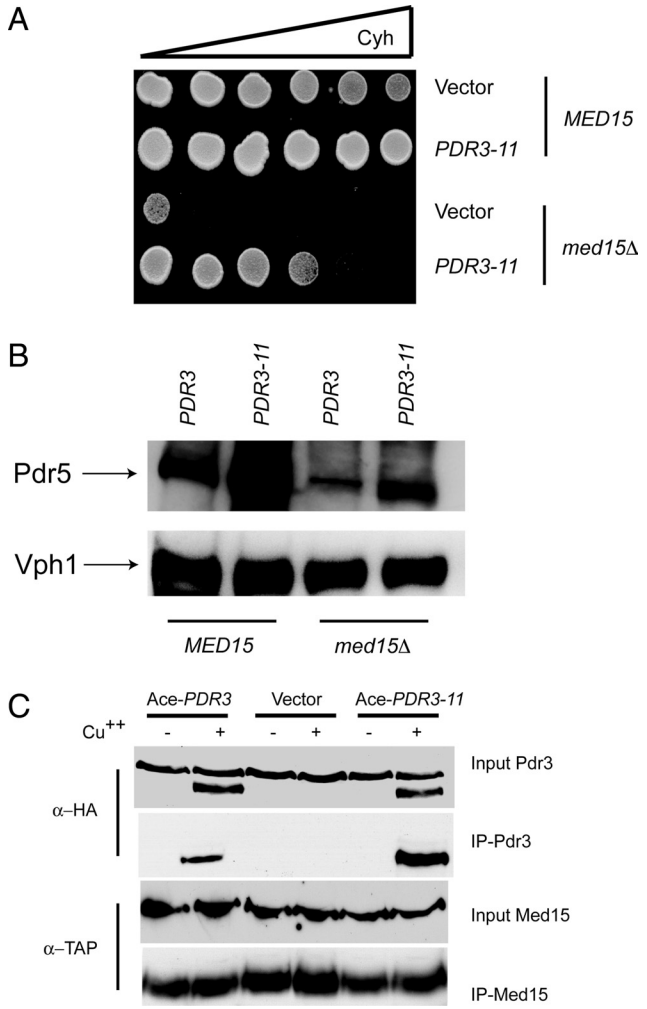

Figure 1. Pdr3-11 elevates drug resistance and Pdr5 expression in the absence of Med15. (A) Isogenic strains containing or lacking *MED15* were transformed with the empty vector plasmid pRS315 (Vector) or the same plasmid containing the hyperactive *PDR3-11* allele (*PDR3-11*). Transformants were grown to midlog phase and then 1000 cells/spot were placed on a YPD plate containing a gradient of cycloheximide (Cyh; increasing concentration indicated by the bar of increasing width). Except where noted, cycloheximide was present at 0.2 μg/ml. (B) Whole cell protein extracts were prepared from the transformants above. Equal amounts of protein were resolved on SDS-PAGE and analyzed by Western blotting using an antibody directed against Pdr5 or the vacuolar ATPase subunit Vph1. (C) A strain lacking both Pdr1 and Pdr3 (*pdr1*Δ *pdr3*Δ) and expressing a Med15-TAP fusion protein was transformed with a low-copy-number plasmid (Vector) or the same plasmid expressing wild-type Pdr3 (Ace-*PDR3*) or the hyperactive form of Pdr3 (Ace-*PDR3-11*) under control of the copper responsive Ace1 transcription factor. Transformants were grown to early log phase and split into subcultures. Subcultures were untreated (−) or induced with copper (+) to control expression of the different forms of Pdr3. After this treatment, whole cell protein extracts were prepared under native conditions. Aliquots of this total protein extract were reserved as controls for input protein with the remainder subjected to immunoprecipitation using an anti-TAP antibody. Immunoprecipitates were washed and then denatured in Laemmli sample buffer. Both immunoprecipitated and input samples were electrophoresed on SDS-PAGE and analyzed by Western blotting for Pdr3 (anti-HA antibody) and Med15 (anti-TAP antibody).

native PDREs normally found in the *PDR3* promoter. These CuREs are bound by the copper-inducible Ace1 transcription factor and eliminate autoregulation of *PDR3*, allowing both the wild-type and hyperactive forms of Pdr3 to accumulate to the same levels. These modified *PDR3* genes carried on low-copy-number plasmids are referred to as Ace1-*PDR3* and Ace1-*PDR3-11*, respectively. These plasmids were introduced into a strain containing a TAP-tagged form of Med15. Transformants were grown to early log phase and induced with copper to produce the two different forms of Pdr3, and whole cell protein lysates were prepared. Med15-containing protein complexes were recovered by anti-TAP immunoprecipitation and then analyzed by Western blotting with anti-HA (detects Pdr3) and anti-TAP antibodies (Figure 1C).

This coimmunoprecipitation analysis demonstrated that Pdr3-11 was more effective at association with Med15 compared with the wild-type Pdr3. Together, these data allow two important conclusions to be reached. First, Pdr3-11 exhibits increased interaction with Med15, stimulating elevated Mediator recruitment that, in turn, is likely to explain the increased expression of *PDR5* in this background. Second, although Med15 is an important target of Pdr3-mediated transcriptional activation of *PDR5*, other routes exist for Pdr3 to activate expression of this key target gene. These experiments all rely on a genetically hyperactive allele of *PDR3* to induce *PDR5* expression. Our previous work has demonstrated that loss of the mitochondrial genome (ρ^0 cells) strongly induced the activity of wild-type Pdr3, which in turn stimulated *PDR5* transcription (Hallstrom and Moye-Rowley, 2000; Zhang and Moye-Rowley, 2001). To determine if ρ^0 signaling exhibited a Med15 dependence similar to that seen for genetically activated Pdr3, the effect of removing Med15 from ρ^0 cells was evaluated directly.

Med15 Lowers But Does Not Eliminate ρ^0 Induction of PDR5

An isogenic series of ρ^+ and ρ^0 cells containing or lacking *MED15* was constructed by standard techniques. These strains were transformed with the *PDR5-lacZ* reporter plasmid and transformants assayed for two features of *PDR5* expression (Figure 2A). First, appropriate transformants were analyzed for Pdr5-dependent cycloheximide resistance by placing transformants on medium containing an inhibitory concentration of this drug. Second, levels of *PDR5*-dependent β-galactosidase activity were determined from these same transformants.

Strains containing a normal mitochondrial genome dosage but lacking Med15 were extremely sensitive to cycloheximide as shown earlier (Thakur *et al.*, 2008). Strikingly, cycloheximide resistance was still induced in ρ^0 derivatives. Although ρ^0 still enhances the cycloheximide resistance of a *med15*Δ strain, Med15 was clearly required for normal drug resistance in both ρ^+ and ρ^0 genetic backgrounds.

Expression of the *PDR5-lacZ* reporter gene correlated well with the observed effects on cycloheximide resistance. *PDR5*-dependent β-galactosidase activity increased by sevenfold upon loss of the mitochondrial genome, irrespective of the presence of Med15 (Figure 2B). Although this fold induction was maintained, loss of Med15 reduced *PDR5-lacZ* expression under both conditions by ~2.5-fold. These data indicate that Med15 is required for normal *PDR5* expression in ρ^+ and ρ^0 cells but is not necessary for ρ^0-dependent induction of expression. A *SNQ2-lacZ* gene was also assayed in these backgrounds as this gene is known to be responsive to Pdr3 regulation but not induced by mitochondrial signals (Zhang and Moye-Rowley, 2001). Expres-

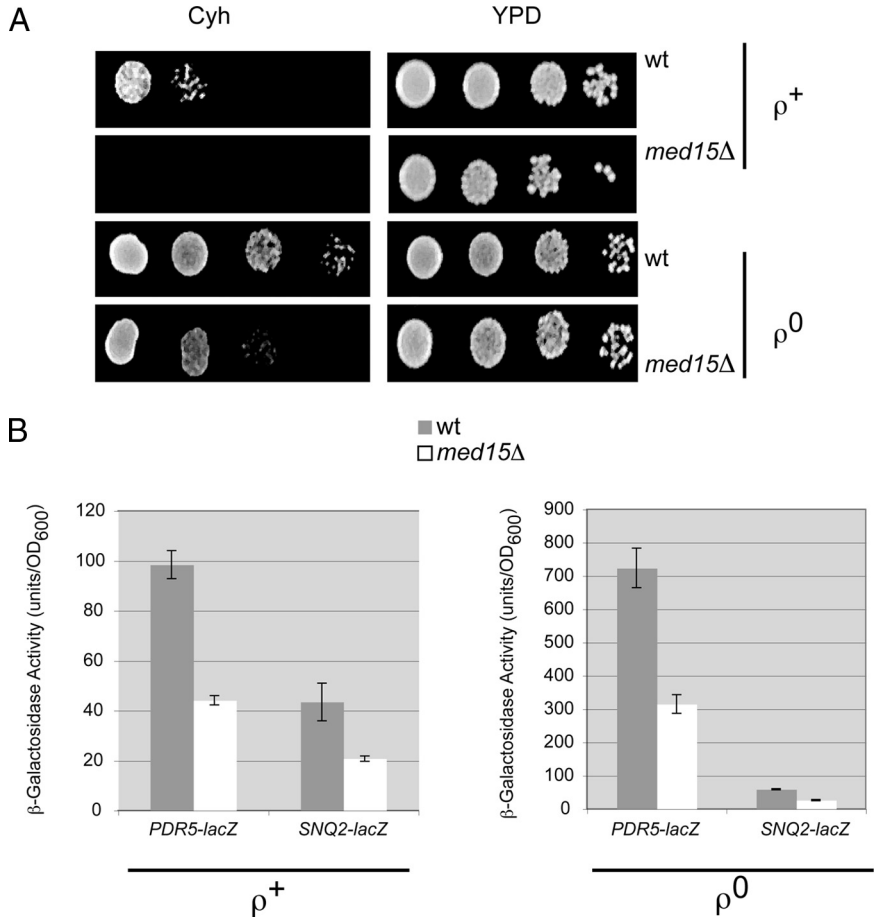

Figure 2. Mitochondrial signals still induce *PDR5* in *med15*Δ cells. (A) Isogenic wild-type and *med15*Δ cells containing (ρ^+) or lacking (ρ^0) their mitochondrial genomes were tested for cycloheximide resistance by spotting 10-fold serial dilution of midlog phase cells on rich medium (YPD) or YPD medium containing cycloheximide (Cyh). (B) Strains from above were transformed with low-copy-number plasmids containing gene fusions between *PDR5*- or *SNQ2*- and *Escherichia coli lacZ*. Transformants were assayed for β-galactosidase activity as described (Guarente, 1983).

sion of *SNQ2* serves as a control for the specificity of ρ^0 signaling to Pdr-regulated genes.

Med12 Component of Mediator Complex Is a Key Contributor to Induced Pdr3 Transcriptional Control

The Mediator complex consists of more than 20 proteins and can be subdivided into multiple domains: head, middle, tail, and the CDK8 module (see Casamassimi and Napoli, 2007; for a review; Figure 3A). Biochemical isolation of Mediator from yeast extracts demonstrated that Mediator could be obtained as core Mediator (C-Mediator: head, middle, and tail) as well as L-Mediator (core Mediator + CDK8 module; Borggrefe *et al.*, 2002; Samuelsen *et al.*, 2003). Early experiments argued that C-Mediator was involved in positive regulation of transcription, whereas L-Mediator was thought to be a negative transcriptional regulator (reviewed in Bjorklund and Gustafsson, 2005). More recent studies (Larschan and Winston, 2005; Donner *et al.*, 2007; Firestein *et al.*, 2008) have called into question this clear-cut division of action and evidence is accumulating that L-Mediator is also involved in positive control of gene expression.

To probe the role of other Mediator components in Pdr3-mediated control of *PDR5* transcription, disruption mutations in various nonessential Mediator subunits were generated in isogenic wild-type and ρ^0 strains. Representative Mediator subunits were selected to disrupt function of the tail (Med3/15), middle (Med31), head (Med20), or the CDK8 module (Med12). Appropriate strains were then tested for their ability to tolerate cycloheximide as resistance to this translation inhibitor is mediated primarily by *PDR5* (Figure 3B). Growth on rich medium containing a nonfermentable carbon source (YPGE) was also used to identify petite strains that can induce *PDR5* expression in some cases (Zhang and Moye-Rowley, 2001).

We used this resistance assay as a first approximation for Pdr3-dependent transcriptional activation. We have already demonstrated the importance of Med15 in both drug resistance and expression of *PDR5* in ρ^+ cells (Thakur *et al.*, 2008). Loss of Med15 or Med20 caused striking cycloheximide sensitivity but also caused cells to grow more slowly on rich medium, indicating the fundamentally important nature of these Mediator subunits in global gene expression. Mutants lacking Med3 were also quite sensitive to cycloheximide but grew normally in the absence of drug. Med12, the Mediator component we shall focus our studies on later, could be eliminated from ρ^+ cells with no major consequences to the phenotypes analyzed here. Loss of Med31 produced similar phenotypes to the *med20*Δ strain with the exception that *med31*Δ mutants were unable to grow on YPGE medium.

The ability of an isogenic series of ρ^0 strains lacking these same Mediator components was also tested for the response to these toxic compound challenges. Previous work has demonstrated that ρ^0 activation of Pdr3 function leads to a large increase in resistance to cycloheximide (Hallstrom and Moye-Rowley, 2000). Loss of either Med12 or Med20 prevented this increased resistance to both these Pdr3-regulated compounds, whereas none of the other Mediator subunit disruptions exhibited this pattern of tolerance.

As the focus of our study was to identify Mediator subunits, in addition to Med15, that play a role in Pdr3 activa-

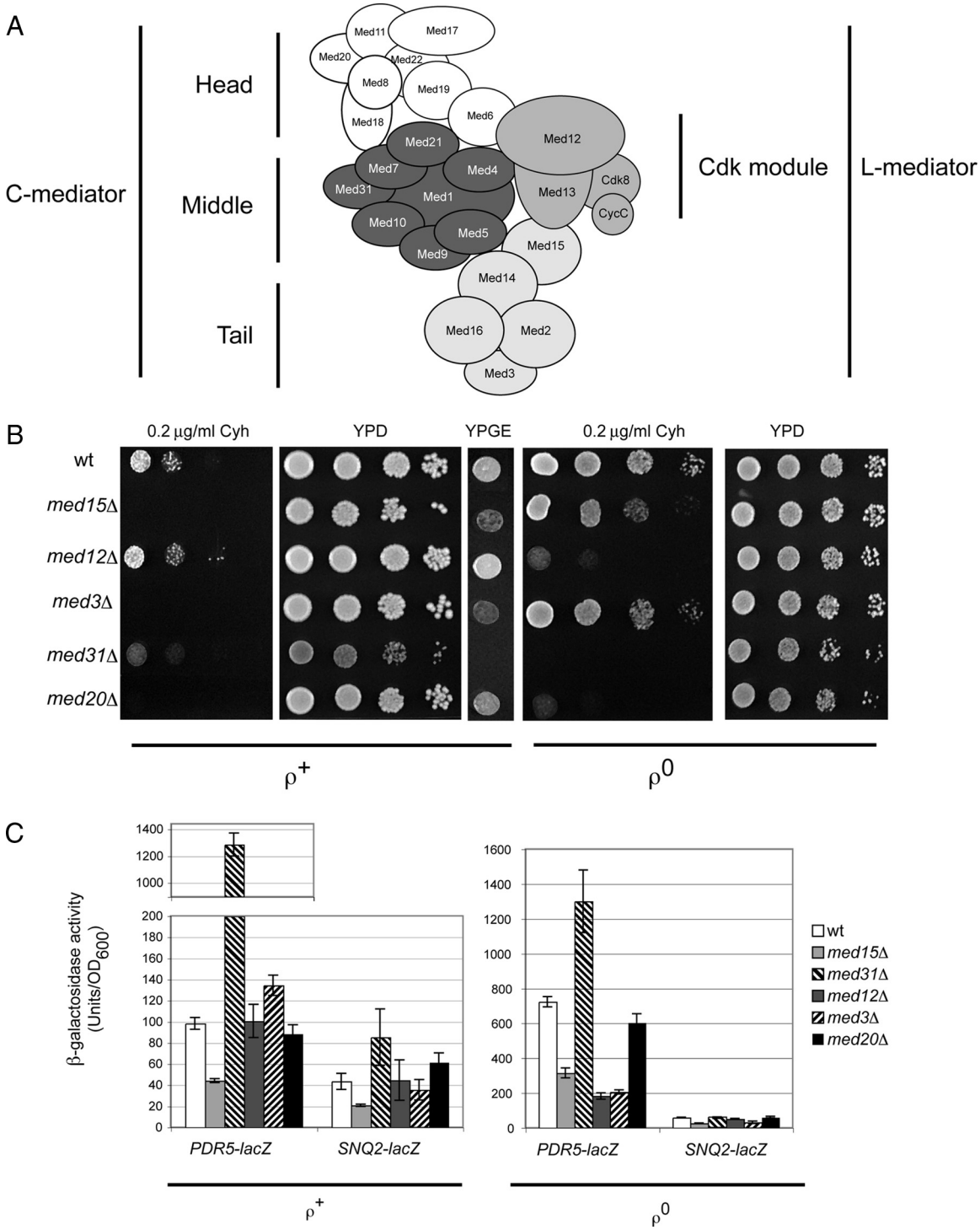

Figure 3. Mediator components influence resistance phenotypes in nonidentical ways. (A) The various subdomains of the Mediator complex are illustrated at the top of the figure and discussed in the text. This model is adapted from an earlier publication (Casamassimi and Napoli, 2007). (B) Isogenic ρ^+ and ρ^0 cells containing the disruption mutations indicated at the top of the figure were grown to midlog phase, and 10-fold serial dilutions were placed on media indicated. Strains containing all Mediator components are denoted as wild-type (wt) in both ρ^+ and ρ^0 backgrounds. The two media lacking all drugs are YPD and YPGE. Cycloheximide (Cyh) plates are based on YPD medium. (C) Isogenic ρ^+ and ρ^0 cells containing the indicated Mediator disruption mutations listed at the right of the figure were transformed with plasmids containing the *PDR5*- or *SNQ2-lacZ* reporter genes and grown to midlog phase. The levels of β-galactosidase activity were measured as described above.

tion of *PDR5*, we wanted to confirm that the phenotypic changes described above were correlated with changes in *PDR5* expression. Because any Mediator subunit is likely to affect transcription of hundreds of genes (for example, see

van de Peppel *et al.,* 2005), these alterations in drug resistance could be a composite phenotype resulting from alterations in global gene expression. To directly examine the effect of these Mediator subunit mutants on *PDR5* expression, the mutant strains used above were transformed with a low-copy-number plasmid containing a *PDR5-lacZ* reporter gene. Transformants were assayed for *PDR5*-dependent β-galactosidase activity as described (Guarente, 1983). Additionally, *SNQ2-lacZ* also served both as a Pdr specificity control as well as a control for any effects on expression of the *lacZ* reporter gene as was reported for mutants lacking Med5 or Med10 (Tabtiang and Herskowitz, 1998).

The *PDR5-lacZ* reporter gene was induced by eightfold in ρ^0 cells compared with isogenic ρ^+ cells as seen before (Hallstrom and Moye-Rowley, 2000; Figure 3C). Loss of Med31 dramatically elevated *PDR5-lacZ* expression in ρ^+ cells, but this reflects the likelihood that the *med31*Δ strain, although originally constructed in a ρ^+ background, has converted to ρ^0 in the absence of this Mediator subunit. This suggestion is supported by the failure of the *med31*Δ mutant constructed in the initially ρ^+ strain to grow on YPGE medium (Figure 3B). Although loss of Med3 prevented *PDR5* induction in ρ^0 cells, the usual ρ^0-mediated increase in cycloheximide resistance was still observed in this background. Loss of Med15 reduced expression of both reporter genes in ρ^+ and ρ^0 cells. Strikingly, loss of Med12 had no effect on *PDR5-lacZ* expression in ρ^+ cells but strongly reduced the ρ^0-dependent induction of this reporter gene. The *med12*Δ strains did not significantly alter *SNQ2-lacZ* expression. The other Mediator disruption mutants failed to exhibit *PDR5* expression that correlated with their cycloheximide resistance profile and were not studied further.

Three important conclusions can be drawn from this analysis. First, loss of Med12 caused a selective defect in ρ^0-activated gene expression of *PDR5*. Second, although *med15*Δ lowered *PDR5* expression in ρ^+ cells as reported previously (Thakur *et al.,* 2008), *PDR5* was still induced in a *med15*Δ ρ^0 cell by about eightfold, equivalent to the fold change seen in wild-type cells. Finally, interpretation of the results of a Mediator mutant in terms of a consistent effect on both drug resistance and *PDR5* expression is complicated by the multiple roles of these proteins in transcription of many genes. Only in the case of Med12 and Med15 was a clear link seen between the drug resistance phenotype and *PDR5-lacZ* expression. Together, these data suggest that Med15 is required for Pdr3-mediated activation of *PDR5* expression under all conditions, whereas Med12 is only required for ρ^0-induced expression.

Nonidentical Roles of CDK8 Mediator Subunits in PDR5 *Regulation*

Med12 is a large subunit (167 kDa) of the CDK8 Mediator subcomplex that also contains the proteins Med13, Cdk8, and CycC (reviewed in Carlson, 1997). Previous studies have argued that these four proteins define a common central function (Balciunas and Ronne, 1995; Kuchin *et al.,* 1995; Liao *et al.,* 1995), but recently this view has been challenged (Carrera *et al.,* 2008). To determine if the other three CDK8 subcomplex components played roles in *PDR5* expression similar to that of Med12, disruption mutant strains were constructed lacking each of these other genes in isogenic ρ^+ and ρ^0 cells. These mutants were tested for their ability to tolerate cycloheximide and activate expression of a *PDR5-lacZ* reporter gene as described above.

Loss of Med12 or CycC prevented the large increase in cycloheximide resistance seen in ρ^0 cells (Figure 4A). Disruption mutants lacking *CDK8* or *MED13* did not affect the increases in cycloheximide resistance normally seen in a ρ^0 background. Further differences between CDK8 subunit members were seen when disruptions were generated in an initially ρ^+ strain. Mutants lacking Med13 or CycC were petite, whereas both *cdk8*Δ and *med12*Δ strains retained the ability to grown on nonfermentable carbon sources. The *med13*Δ mutant also exhibited elevated cycloheximide resistance when compared with all other strains. Loss of Med13 is likely to cause cells to become ρ^0 (see below).

These same strains were then transformed with low-copy-number plasmids containing gene fusions between *PDR5* or *SNQ2* to *lacZ* and β-galactosidase activities determined for all transformants (Figure 4B). Loss of Med13 in an initially ρ^+ background caused an increase in *PDR5-lacZ* expression equivalent to that seen in a wild-type ρ^0 cell. This result coupled with the fact that *med13*Δ cells fail to grow on YPGE medium supports the view that Med13 is required to prevent cells from becoming ρ^0. Interestingly, loss of CycC modestly elevated *PDR5-lacZ,* although not to the extent seen for *med13*Δ mutants. Strains lacking CycC were unable to grow on YPGE, which indicates that these cells are petite. When these same disruption mutations are generated in a ρ^0 background, *PDR5-lacZ* expression was found to be unaltered from wild-type except for *med12*Δ and *cycC*Δ ρ^0 strains. Loss of Med12 prevented ρ^0 induction of *PDR5,* whereas loss of CycC reduced *PDR5* induction less than half that of wild-type cells. We believe the expression of *PDR5* in ρ^+ and ρ^0 is equivalent because loss of CycC from the wild-type cell caused these cells to convert to ρ^0. As seen before, *SNQ2* expression was not significantly affected in these backgrounds (Zhang and Moye-Rowley, 2001).

These data are consistent with the view that Med12 is required for wild-type induction of the *PDR5* promoter in ρ^0 cells. Previous work has demonstrated that ρ^0 induction of *PDR5* proceeds through activation of the Pdr3 transcription factor (Hallstrom and Moye-Rowley, 2000). Pdr3 expression is also induced in ρ^0 cells via engagement of an autoregulatory circuit involving Pdr3 activation of its own transcription (Delahodde *et al.,* 1995). To determine if the effect of Med12 in ρ^0 cells is restricted to the *PDR5* promoter, expression of Pdr3 was evaluated. A low-copy-number plasmid containing an epitope-tagged version of *PDR3* was introduced into an isogenic series of ρ^+ and ρ^0 cells containing or lacking *MED12* or *MED15*. Transformants were analyzed for their level of Pdr3 expression by Western analysis (Figure 5A).

Expression of Pdr3 was induced in response to loss of the mitochondrial genome as seen before (Hallstrom and Moye-Rowley, 2000). However, loss of either Med12 or Med15 elicited distinct responses in Pdr3 expression. A *med15*Δ ρ^+ strain produced Pdr3 levels that were well below those of either wild-type or *med12*Δ cells. The absence of Med15 still supported ρ^0 induction of Pdr3 expression, although the induced level achieved was less than in a *MED15* ρ^0 cell. Strikingly, no difference was seen in Pdr3 expression when comparing ρ^+ and ρ^0 *med12*Δ cells. These data indicate that Med15 is required for Pdr3-mediated activation of gene expression under all conditions, whereas Med12 is selectively important in ρ^0 activation. Additionally, the effects of Med12 and Med15 are exerted at both the *PDR3* and *PDR5* promoters.

To ensure that the differences observed by Western blotting were caused by changes in transcription, we analyzed the levels of *PDR3* and *PDR5* mRNA in isogenic ρ^+ and ρ^0 cells containing or lacking the *MED12* gene. Transcript levels of *ACT1* were also assessed as control for a gene insensitive to these genetic manipulations. Total RNA samples

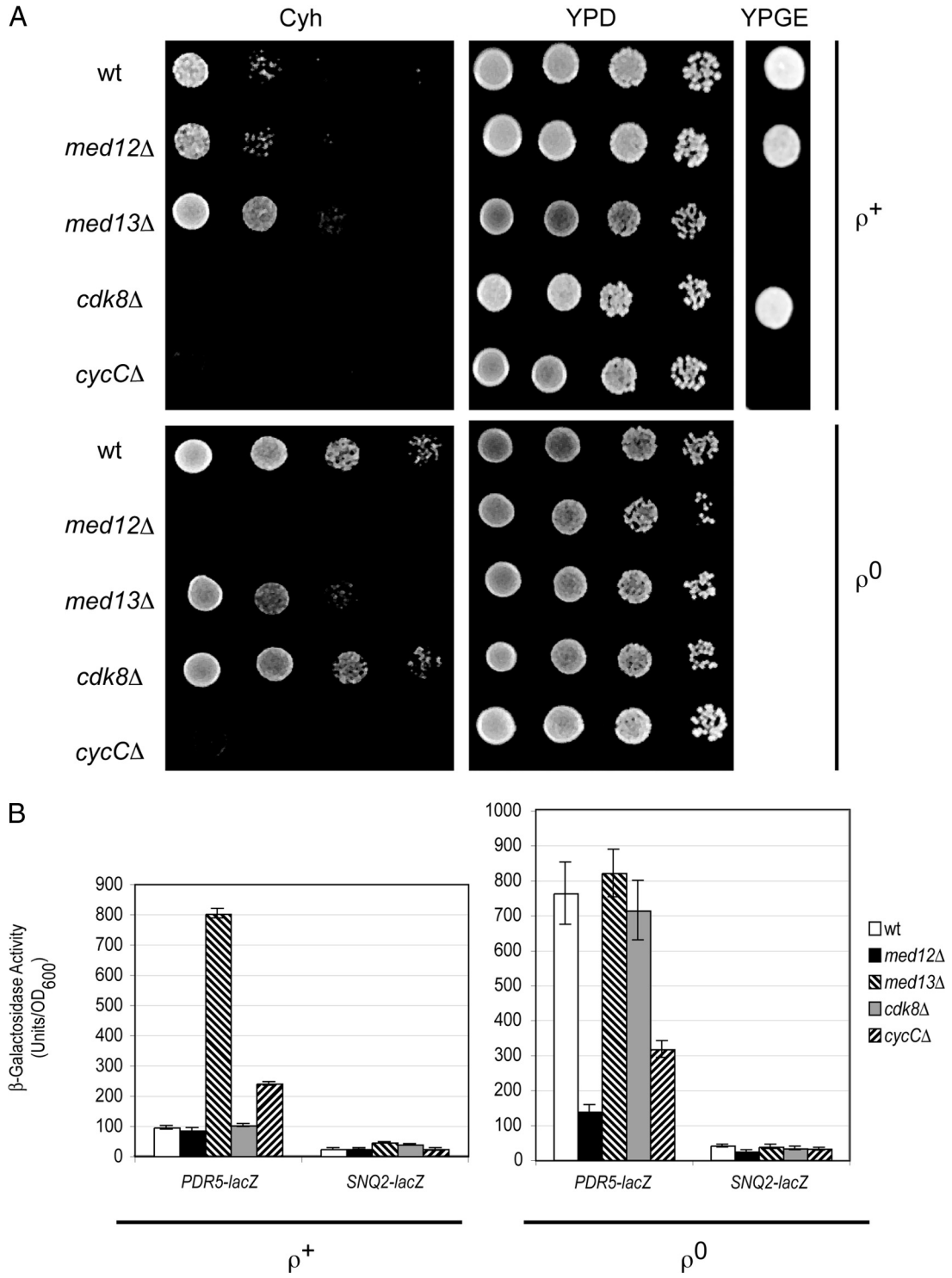

Figure 4. Nonidentical roles of Cdk module subunits of Mediator in *PDR5* activation. (A) Isogenic ρ^+ and ρ^0 cells containing the indicated disruption mutations in the four subunits of the Cdk module were grown to midlog phase, serially diluted as above and tested for their ability to grow on rich medium (YPD), YPD containing cycloheximide (Cyh), or rich medium with glycerol/ethanol as the carbon source (YPGE). (B) The strains described above were transformed with the indicated reporter plasmids and β-galactosidase activities determined.

were reverse-transcribed and analyzed by real-time PCR measurements (Shahi *et al.*, 2007; Figure 5B).

The changes in *PDR3* and *PDR5* mRNA levels correlated very well with the previously determined levels of epitope-tagged Pdr3. *PDR3* mRNA levels were nearly 10-fold elevated in ρ^0 cells, whereas no detectable difference could be seen between the relative transcript levels produced in *med12*Δ and *med12*Δ ρ^0 cells. This same behavior was observed for *PDR5* mRNA production. The simplest interpretation of these data are that Med12 is required to permit

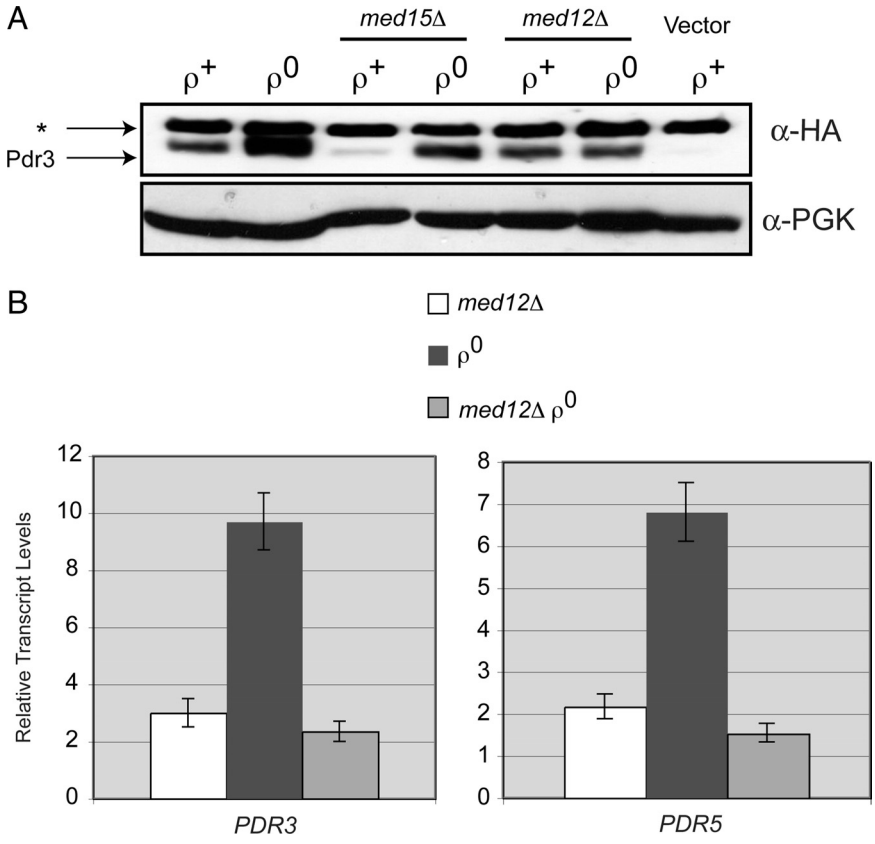

Figure 5. Differential roles of Med12 and Med15 in autoregulation of *PDR3* expression. (A) Isogenic ρ^+ and ρ^0 cells with the indicated relevant genotypes were transformed with a low-copy-number plasmid containing an epitope-tagged allele of *PDR3* or the empty vector plasmid (Vector). Transformants were grown to midlog phase, and protein extracts prepared and analyzed by Western blotting using an anti-HA antibody. The position of epitope-tagged Pdr3 is indicated at the left, and the asterisk (*) denotes the position of a nonspecific signal that cross reacts with the anti-HA antibody. Levels of phosphoglycerate kinase were assessed as a loading control. (B) Quantitative reverse transcription-PCR analysis of *PDR3* and *PDR5* mRNA levels in ρ^+ and ρ^0 cells containing or lacking *MED12* was carried out as described (Shahi *et al.*, 2007). Total RNA was extracted from these strains, and specific primers were used to detect *PDR3* and *PDR5* mRNA. Transcript levels are relative to the level seen in ρ^+ cells, and *ACT1* mRNA was analyzed in every sample as a control transcript.

Pdr3 to positively regulate transcription at both the *PDR3* and *PDR5* promoters in response to ρ^0 signaling.

Retrograde Signaling Contribution of Med12

Retrograde signaling refers to control of nuclear gene expression in response to mitochondrial dysfunction (reviewed in Butow and Avadhani, 2004) and was initially described for the ρ^0 induction of the citrate synthase-encoding gene *CIT2* by the Rtg1/Rtg3 transcription factors (Parikh *et al.*, 1987). The data above provided evidence that loss of Med12 blocked, whereas loss of Med15 only reduced ρ^0 induction of *PDR5* expression. Other work has demonstrated that elevated expression of the mitochondrially localized phosphatidylserine decarboxylase enzyme (Psd1) also leads to *PDR5* induction, even in ρ^+ cells (Gulshan *et al.*, 2008). To determine if these Mediator components were also involved in Psd1 signaling, a strain that overproduced this enzyme was constructed and analyzed for the degree of cycloheximide resistance produced in the presence or absence of Med12 or Med15 (Figure 6A).

Increased expression of Psd1 led to an elevation in cycloheximide resistance as we have documented previously (Gulshan *et al.*, 2008). Loss of Med15 prevented any Psd1-dependent increase in cycloheximide tolerance, whereas removal of Med12 had no effect on this regulatory circuit. Psd1 signaling, unlike ρ^0 activation of *PDR5* expression, is Med12 independent.

To support the conclusion that the action of Med15 in Psd1-induced cycloheximide resistance is likely to come about through the direct action of this Mediator component on the Pdr pathway, ChIP experiments were carried out. A strain containing a *MED15*-TAP fusion protein was transformed with a high-copy-number plasmid overproducing Psd1 or the empty vector alone. Chromatin was prepared and subjected to ChIP as described (Gulshan *et al.*, 2005). The presence of Med15-TAP at the *PDR3* and *PDR5* promoters was evaluated by qPCR with primers capable of detecting these two transcriptional control regions (Figure 6B).

Increased levels of Med15 were detected at both the *PDR3* and *PDR5* promoters when Psd1 was overproduced. These data support and extend previous work demonstrating that Pdr3 requires Med15 to normally positively regulate gene expression (Thakur *et al.*, 2008). Taken in whole, our findings implicate Med15 as a key contributor to Psd1 control of Pdr3-mediated gene regulation in ρ^+ cells, whereas Med12 is dispensable under these same conditions.

Previous studies on retrograde regulation defined a suite of genes that were induced in ρ^0 cells but did not require Pdr3 for this induction (Zhang *et al.*, 2005). The finding that Med12 was critical in Pdr3-mediated retrograde regulation prompted us to determine if Med12 influenced ρ^0 induction of other retrograde regulated genes. The D-lactate dehyrogenase– and aconitase-encoding genes *DLD1* and *ACO1* were selected as both of these transcripts showed robust induction in ρ^0 cells and were insensitive to the presence of Pdr3 (Zhang *et al.*, 2005). RNA prepared from isogenic ρ^+ and ρ^0 cells containing or lacking Med12 was used to make total cDNA. Levels of *DLD1*, *ACO1*, and *ACT1* transcripts were measured by qPCR using appropriate primers (Figure 6C).

Loss of Med12 reduced transcription of *DLD1* and *ACO1* in both ρ^+ and ρ^0 cells. This Med12 dependence is different from that seen for *PDR5* since expression of this ABC transporter-encoding gene was only reduced by loss of Med12 from a ρ^0 strain, not ρ^+ cells. This analysis indicates that Med12 controls expression of *DLD1* and *ACO1* irrespective

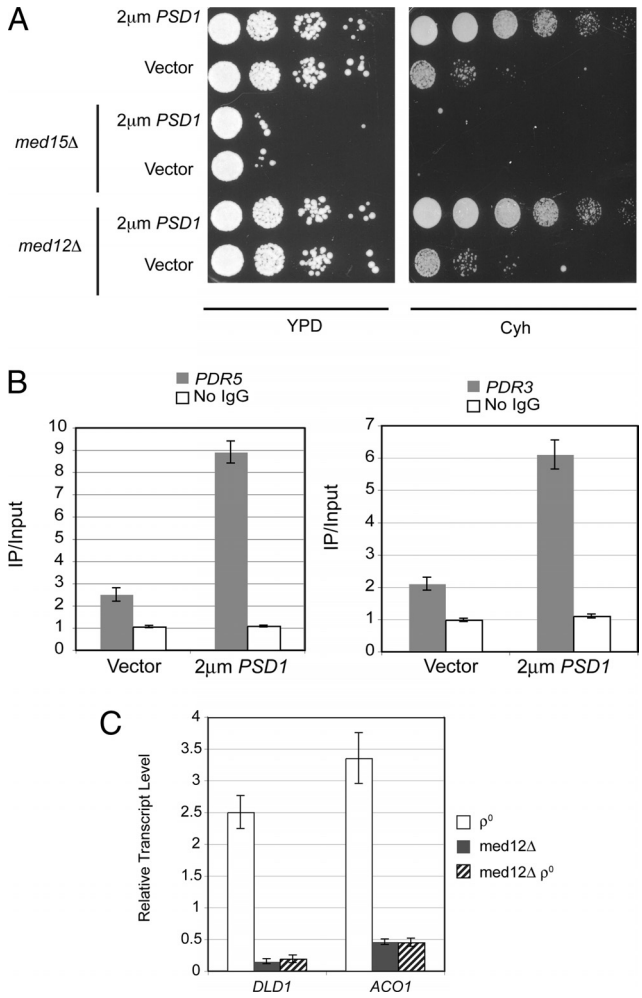

Figure 6. Med12 is not required for Psd1 signaling to *PDR5* in ρ^+ cells. (A) Wild-type cells or isogenic *med15*Δ and *med12*Δ derivatives were transformed with high-copy-number plasmids containing (2 μm *PSD1*) or lacking (Vector) the *PSD1* gene. Transformants were grown to midlog phase and spotted as 10-fold serial dilutions on YPD plates with or without cycloheximide (Cyh). (B) Cells expressing a Med15-TAP fusion protein and containing the wild-type gene dosage of *PSD1* (wt) or the high-copy-number plasmid bearing *PSD1* (2 μm *PSD1*) were processed for ChIP as described (Gulshan *et al.*, 2005). Aliquots of total chromatin (Input) or chromatin that was immunoprecipitated with anti-TAP antibody (Med15-associated chromatin) were analyzed by qPCR by using primers specific for the *PDR5* promoter (left) or the *PDR3* promoter (right). (C) Relative transcript level analysis by reverse transcriptase qPCR analysis of *DLD1* and *ACO1* mRNA levels in ρ^+ and ρ^0 cells containing or lacking *MED12* was carried out as described above. Quantitation of qPCR was carried out as described above.

of mitochondrial status, whereas its role in Pdr3-responsive retrograde regulation is restricted to ρ^0 cells.

Med12 Is a Critical Determinant of the Mediator Complex Required for ρ^0 Activation of PDR5 *Transcription*

Med15 has been associated with the tail subdomain of the Mediator complex, whereas Med12 is a component of the CDK8 subdomain (Davis *et al.*, 2002). Interestingly, loss of Med15 has been found to cause a reduction in recruitment of the CDK8 subdomain to Gal4-responsive promoters (Larschan and Winston, 2005). As shown above, both Med12 and Med15 contribute to ρ^0 activation of *PDR5* transcription. To explore the mechanism through which these Mediator components contribute to retrograde regulation, isogenic ρ^+ and ρ^0 cells were constructed containing or lacking *MED12*, *MED15* or both of these genes. These strains were tested for their ability to tolerate cycloheximide as above (Figure 7A).

Loss of Med15 caused a loss of cycloheximide resistance in ρ^+ cells. Interestingly, although *med12*Δ cells had no detectable cycloheximide sensitivity in ρ^+ cells, removal of Med12 from *med15*Δ mutants suppressed the cycloheximide phenotype seen in single *med15*Δ strains. A ρ^0 *med12*Δ mutant failed to grow normally at the concentration of cycloheximide used, whereas a ρ^0 *med15*Δ grew slower than isogenic ρ^0 cells but better than the ρ^0 *med12*Δ mutant. A ρ^0 *med12*Δ *med15*Δ mutant was indistinguishable from a ρ^0 *med12*Δ mutant strain. These data indicated that although Med15 was important under both ρ^+ and ρ^0 conditions, Med12 was indispensable in a ρ^0 background.

To determine if these phenotypic effects could be explained by their effects on *PDR5* expression, these same strains were transformed with the *PDR5*- and *SNQ2-lacZ* reporter plasmids described above. Transformants were grown to midlog phase, and levels of β-galactosidase activity were measured (Figure 7B).

Comparison of *PDR5*-directed β-galactosidase activity present in isogenic ρ^+ and ρ^0 *med15*Δ cells indicated that normal fold induction of *PDR5-lacZ* was retained, although the absolute level of *PDR5* expression was lower than in the presence of Med15. These results indicated that Med15 exerted roughly equivalent effects in both ρ^+ and ρ^0 cells. The presence of a *med12*Δ allele, either alone or in combination with *med15*Δ, had no influence on *PDR5* expression in ρ^+ cells. Conversely, loss of Med12 prevented significant induction of *PDR5-lacZ* in ρ^0 cells. *SNQ2-lacZ* levels were not significantly influenced by these changes in genetic background, arguing for the specific influence of these Mediator components on activation of *PDR5* transcription.

The data above suggested that Med12 played a uniquely important role in mediating Pdr3-dependent induction of *PDR5* expression seen in ρ^0 cells, whereas Med15 was involved in expression irrespective of mitochondrial genome status. Because Mediator subunits affect the transcription of many genes (Holstege *et al.*, 1998), the association of both Med15 and Med12 with the *PDR5* promoter in ρ^+ and ρ^0 cells was evaluated by ChIP. Isogenic ρ^+ and ρ^0 cells that contained either a *MED12*-TAP or *MED15*-TAP fusion gene were grown to midlog phase, total chromatin prepared and analyzed by ChIP using anti-TAP antibodies. Samples of total chromatin were reserved as input controls. Both total and immunoprecipitated DNA were analyzed by PCR using a primer pair that directed amplification of the *PDR5* promoter. PCR amplified DNA was qPCR (Figure 8A).

Recruitment of both Med12 and Med15 to the *PDR5* promoter was increased in ρ^0 cells but to different degrees. Med15 was present at higher levels at the *PDR5* promoter in ρ^+ cells than Med12, consistent with the greater dependence of *PDR5-lacZ* expression in ρ^+ cells on Med15. These data are consistent with the view that Med12 regulation of *PDR5* transcription is tightly linked to mitochondrial genome status, whereas Med15 plays an important role in both ρ^+ and ρ^0 cells. To determine if association of Pdr3 and Med12 was responsive to mitochondrial genome status, we carried out a coimmunoprecipitation analysis.

Expression of *PDR3* is positively autoregulated in ρ^0 cells (Hallstrom and Moye-Rowley, 2000). To avoid complications caused by unequal Pdr3 levels in ρ^+ and ρ^0 cells, we used the plasmid containing the copper-regulated form of

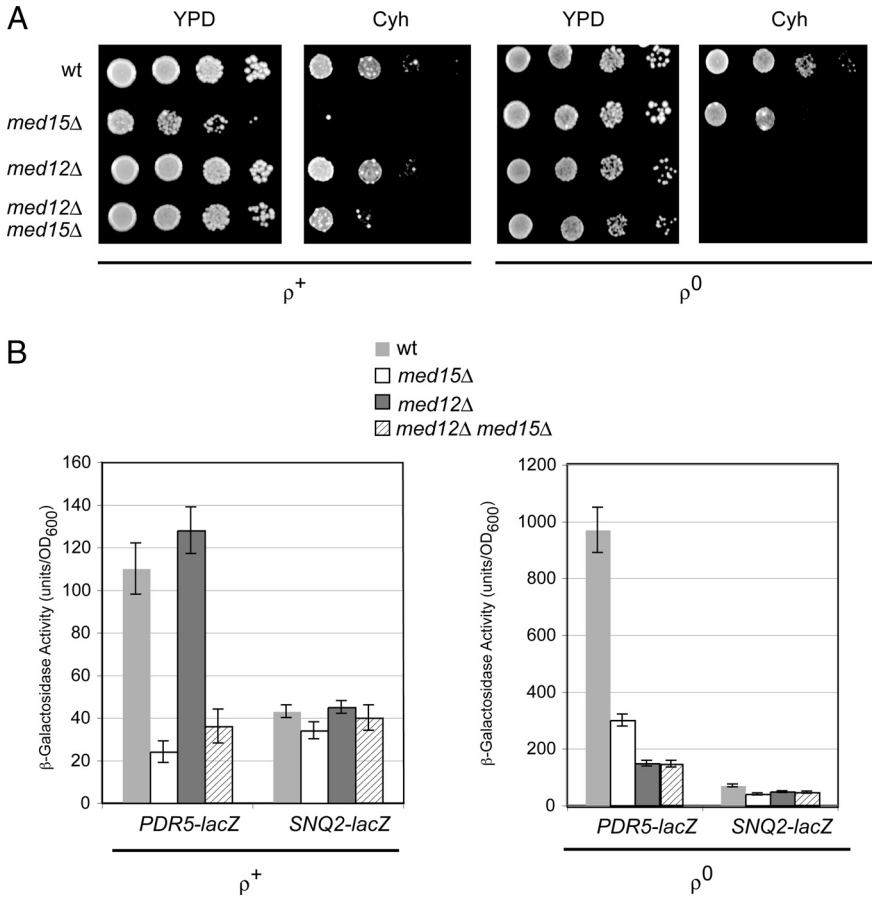

Figure 7. Comparison of Med12 and Med15 roles in control of *PDR5* expression. (A) Isogenic ρ^+ and ρ^0 strains containing disruption mutations of the either *MED15* and/or *MED12* were grown to midlog phase, and serial dilutions were tested for their ability to grow on YPD or YPD-containing cycloheximide (Cyh) media. (B) The strains described above were transformed with the indicated reporter genes and assayed for the levels of β-galactosidase produced in appropriate transformants.

the *PDR3* gene described above. This construct was introduced into an isogenic series of ρ^+ and ρ^0 cells. Additionally, we constructed a ρ^0 *med15*Δ strain to evaluate any requirement for the presence of this Mediator component in recruitment of Med12. Finally, all these strains were engineered to contain the *MED12*-TAP fusion gene. Appropriate transformants were grown with or without copper in the medium (to induce Pdr3 production), lysates were prepared, and immunoprecipitation was carried out using anti-TAP antibody. These immunopreciptates were analyzed by Western blotting with anti-TAP (to ensure equal recovery of Med12-TAP) and anti-HA (to detect epitope-tagged Pdr3).

Pdr3 did not associate with Med12 in ρ^+ cells but was easily detectable in a ρ^0 background (Figure 8B). Loss of Med15 had no significant effect on Pdr3–Med12 association, arguing that Pdr3–Med12 association in ρ^0 cells occurs independently of Med15. Taken as a whole, these data are consistent with the view that Med12 is an essential cofactor in ρ^0 stimulation of *PDR5* transcription by Pdr3.

A possible explanation for the dramatic difference seen in Med12:Pdr3 interaction in comparison of ρ^+ and ρ^0 cells could be provided by exclusion of Med12 from the nucleus in ρ^+ cells as Pdr3 has been shown to be nuclear in these cells (Mamnun *et al.*, 2002). A *MED12*-eGFP fusion gene was constructed in ρ^+ and ρ^0 cells and subcellular distribution evaluated by fluorescence microscopy (Supplemental Figure 1). Med12-eGFP was found in the nucleus irrespective of mitochondrial genome status. We interpret these data to indicate that posttranslational modification(s) of Pdr3 and/or Med12 are responsible for the observed difference in association of these two proteins.

Pdr1 encodes a protein that is 33% identical to Pdr3 (Delaveau *et al.*, 1994; Katzmann *et al.*, 1994). These proteins exhibit extensive functional overlap and have previously been demonstrate to interact with Med15 in ρ^+ cells (Thakur *et al.*, 2008). We wondered if Med12 could associate with Pdr1 as this Mediator subunit does with Pdr3. To address this question, isogenic ρ^+ and ρ^0 *pdr1*Δ *pdr3*Δ strains expressing either a Med12-TAP or Med15-TAP fusion protein were transformed with a plasmid producing a Myc-tagged form of Pdr1. Protein extracts were made and processed for immunoprecipitation using an anti-TAP antibody. Samples of the input and anti-TAP precipitated proteins were analyzed by Western blotting using anti-Myc and anti-TAP antibodies (Figure 8C).

Med12 did not associate with Pdr1 in either ρ^+ or ρ^0 cells. The Myc-Pdr1 protein was able to associate with Med15 as expected, confirming that this epitope-tagged protein functions normally. These data support the view that the specific association of Pdr3 with Med12 in ρ^0 cells is a determinative feature allowing this factor to induce a unique program of target gene expression in response to loss of the mitochondrial genome.

DISCUSSION

The Mediator complex is a critical link between the action of transcription factors and RNA polymerase II–dependent gene transcription. Biochemical experiments have provided evidence that two different forms of Mediator can be isolated from cells: C-Mediator, containing ~20 proteins, and C-Mediator containing an additional the four protein CDK8

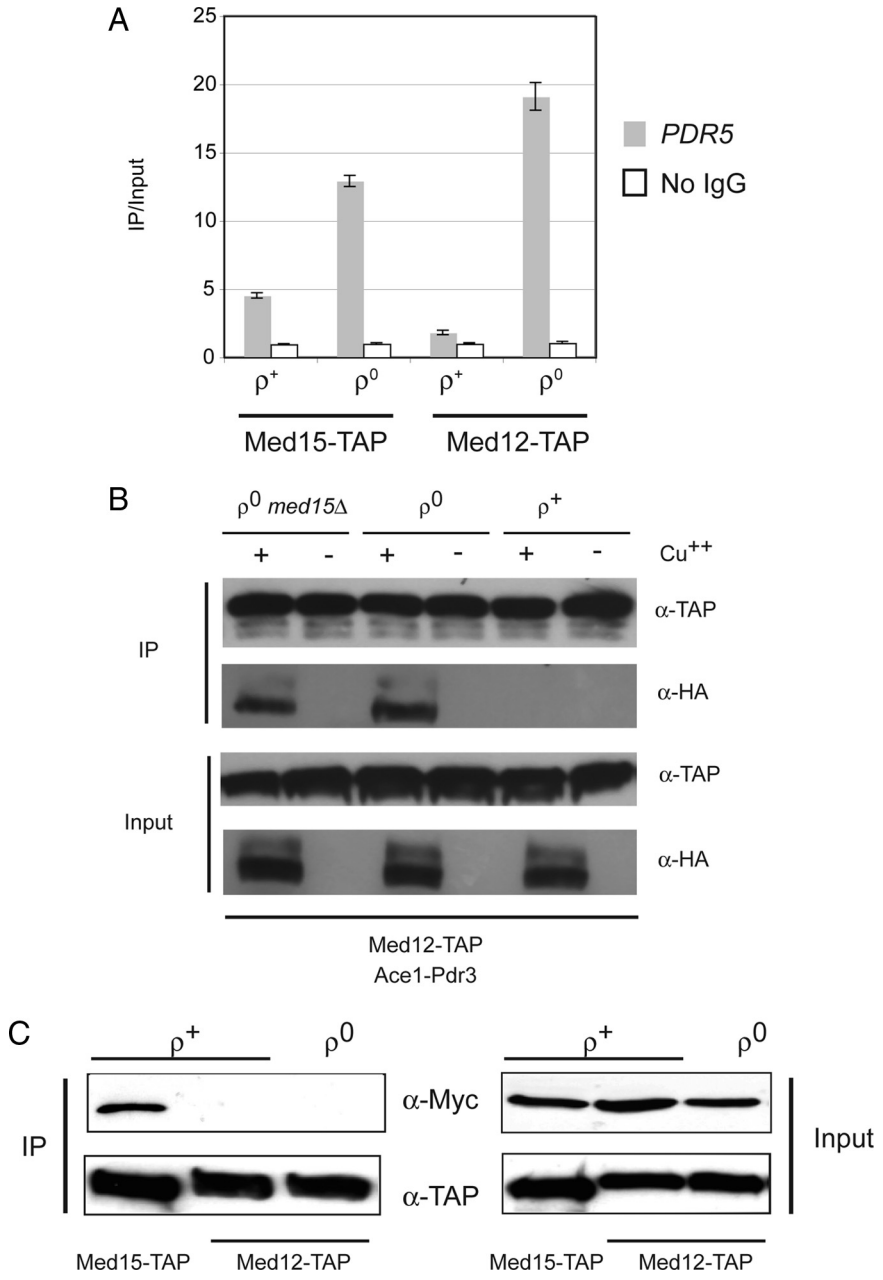

Figure 8. Med12 recruitment to the *PDR5* promoter and its association with Pdr3 is highly inducible in ρ^0 cells. (A) Isogenic ρ^+ and ρ^0 cells expressing the indicated Mediator subunit-TAP fusion protein were processed for ChIP as described above. Immunoprecipitated (IP) DNA was analyzed by qPCR using primers designed to amplify the *PDR5* promoter. Antibody independent DNA recovery was estimated by performing identical immunoprecipitations but without the addition of any primary antibody (No IgG) The ratio of IP to input signals was calculated for the signal from the *PDR5* promoter primer pairs divided by the corresponding value for the control (No IgG). The mean and SEs of the resulting normalized IP/Input ratios from replicate cultures, calculated with three PCR measurements, are plotted. (B) Strains with the indicated relevant genotypes (top of panel) expressing both Med12-TAP and epitope-tagged Pdr3 (under copper control: Ace1-Pdr3) were processed for coimmunoprecipitation using anti-TAP antibody as described in Figure 1. Immunoprecipitated samples were analyzed by Western blotting with anti-TAP (detects Med12-TAP) and anti-HA (detects epitope-tagged Pdr3). (C) Isogenic ρ^+ and ρ^0 *pdr1*Δ *pdr3*Δ strains expressing the indicated Mediator subunit-TAP fusion protein were transformed with a low-copy-plasmid expressing Myc-Pdr1 and processed for coimmunoprecipitation using anti-TAP antibody. Input and anti-TAP immunoprecipitated (IP) samples were electrophoresed on SDS-PAGE and subjected to Western blotting using anti-TAP (detects mediator subunit) and anti-Myc (detects epitope-tagged Pdr1) antibodies.

subcomplex (L-Mediator; Casamassimi and Napoli, 2007). Early studies with these two different forms of Mediator suggested that the core complex was involved in positive transcriptional control, whereas addition of the CDK8 subcomplex was associated with repression of target gene expression (Bjorklund and Gustafsson, 2005). More recent experiments, including the present work, indicate that this simple view of the regulatory roles of the different forms of Mediator is inadequate to explain the activities of this transcriptional regulatory complex.

Med12 was first identified as a negative regulator of invertase gene expression (Carlson *et al.*, 1984) and was later found to interact with C-terminal mutant forms of RNA polymerase II (Hengartner *et al.*, 1995). Global microarray analyses of various Mediator components demonstrated that loss of the CDK8 constituents typically led to increased transcript levels (van de Peppel *et al.*, 2005). Biochemical experiments also provide evidence that the isolated CDK8 module is capable of inhibiting Mediator-dependent transcription when added to an in vitro system (Knuesel *et al.*, 2009). However, these observations are balanced by demonstrations in yeast (Larschan and Winston, 2005), *Drosophila* (Loncle *et al.*, 2007), and mammalian cells (Donner *et al.*, 2007) that normal levels of gene expression requires the presence of functional CDK8 subcomplex. Together, these findings are most consistent with the view that the CDK8 subcomplex influences transcriptional regulation in a context-dependent manner and is capable of inhibiting or stimulating gene expression.

Med15 has already been shown to be an important participant in the positive transcriptional regulation of *PDR5* by both Pdr1 and Pdr3 (Thakur *et al.*, 2008). The gain-of-function form of Pdr3 exhibits enhanced association with Med15, consistent with increased recruitment of this Mediator com-

ponent during transcriptional activation by this hyperactive transcription factor. This interpretation is supported by previous data demonstrating that *PDR5* activation by the Pdr3-11 factor is compromised in the absence of Med15 (Thakur *et al.*, 2008). In opposition to the dependence of Pdr3-11 on Med15, our preliminary evidence suggests that loss of Med12 has no significant influence on Pdr3-11-mediated gene activation, at least when this measurement is done in ρ^+ cells (data not shown). We interpret these findings as further support for the strictly inducible nature of Pdr3 interaction with Med12 and that the gain-of-function form of Pdr3 enhances interaction with Med15 rather than Med12.

Comparison of the phenotypes of different CDK8 subcomplex members indicates that disruption mutant alleles of these genes cause very different phenotypic effects, inconsistent with a common role for all these subcomplex proteins. Earlier work using global transcriptional profiling as a measure of the in vivo roles of the CDK8 subcomplex members demonstrated that loss of individual components led to common transcriptional defects (van de Peppel *et al.*, 2005). Our experiments reveal additional complexity in the activities of the CDK8 subcomplex through focus on *PDR5* transcription. Med12 has a unique importance in *PDR5* expression in ρ^0 cells but is dispensable in a ρ^+ background. This finding illustrates the differential requirements for Mediator components when different transcriptional demands are placed on a gene. Med12 is critical to maintain high-level *PDR5* transcription in ρ^0 cells but unimportant in when less *PDR5* expression is required, such as in ρ^+ cells. Evaluation of the importance of a given Mediator subunit in expression of any gene should be conducted under conditions of varying transcription rates to avoid missing important contributions.

Another striking feature of the behavior of CDK8 disruption mutants was the generation of a petite phenotype in *CYCC* and *MED13* mutants but not in either *CDK8* or *MED12* disruptants. We believe that the *cycc*Δ and *med13*Δ mutants are ρ^0 for two reasons. First, ethidium bromide staining indicated that mitochondrial nucleoids are absent from both mutants. Second, both mutants exhibit elevated expression of *PDR5*, a diagnostic indicative of ρ^0 status (Hallstrom and Moye-Rowley, 2000). An interesting feature of the elevated *PDR5-lacZ* levels in the *cycc*Δ strain is the reduced expression compared with that seen in the *med13*Δ background. We suggest that CycC is required for full induction of *PDR5* in response to loss of the mitochondrial genome. This is provocative in light of the lack of an effect on *PDR5-lacZ* expression seen in the corresponding ρ^0 *cdk8*Δ strain. Because Cdk8:CycC form a kinase–cyclin pair (Liao *et al.*, 1995), these data support the view that CycC may have Cdk8 independent roles.

Finally, the singular importance of Med12 in ρ^0 activation of *PDR5* transcription provides an alternative possibility to the induction of Pdr3 activity seen in the absence of the mitochondrial genome. We speculate that changes in the ability of Pdr3 to associate with Med12 could explain both the autoregulation of *PDR3* and activation of *PDR5* in ρ^0 cells. Because we have demonstrated that Pdr3–Med12 association is greatly enhanced in ρ^0 cells, it is possible that Pdr3 may not be directly modified in this genetic background but rather that Med12 is the key target. Previously, others have demonstrated that protein kinase A influences gene expression through control of Med13 phosphorylation (Chang *et al.*, 2004). Given the global rewiring of gene expression seen when comparing transcriptional profiles of ρ^+ and ρ^0 cells (Epstein *et al.*, 2001; Traven *et al.*, 2001; Devaux *et al.*, 2002), targeting a global transcriptional regulator like Med12 would provide a parsimonious means of both specifically inducing Pdr3-dependent transcriptional events as well as to trigger the range of responses necessary to ensure viability in response to loss of the mitochondrial genome.

Induced Pdr3–Med12 interaction would also explain one of the poorly understood features of ρ^0 induction of *PDR5* in wild-type cells. A variety of experiments including *lacZ* fusion genes and Western blot measurements (Hallstrom and Moye-Rowley, 2000; Mamnun *et al.*, 2002) have indicated that in ρ^+ cells, levels of Pdr3 only approach 1% of the levels of Pdr1. Because both Pdr1 and Pdr3 bind to the same DNA elements (Katzmann *et al.*, 1996), the ability to induce Pdr3 in the presence of an excess of Pdr1 has been difficult to explain. If Med12 is specifically recruited to Pdr3 to enhance the transactivation capability of this factor, then this would allow Pdr3 activity to be elevated even in the presence of a large excess of Pdr1. Once the autoregulatory induction of *PDR3* is complete, levels of Pdr3 rise by a factor of 10 (Delahodde *et al.*, 1995), increasing the levels to a dose more comparable to that of Pdr1.

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