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The Groucho-associated Phosphatase PPM1B Displaces Pax Transactivation Domain Interacting Protein (PTIP) to Switch the Transcription Factor Pax2 from a Transcriptional Activator to a Repressor*

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Background: The Groucho co-repressor proteins inhibit transcription by a variety of described mechanisms.
Results: PPM1B interacts with Groucho4 and is localized to DNA in a Groucho-dependent manner, and phosphatase activity is required for transcriptional silencing.
Conclusion: PPM1B plays a vital role in Groucho-mediated transcriptional silencing of Pax-regulated genes.
Significance: We describe a novel mechanism of Groucho-mediated transcriptional repression.

Pax genes encode developmental regulatory proteins that specify cell lineages and tissues in metazoans. Upon binding to DNA through the conserved paired domain, Pax proteins can recruit both activating and repressing complexes that imprint distinct patterns of histone methylation associated with either gene activation or silencing. How the switch from Pax-mediated activation to repression is regulated remains poorly understood. In this report, we identify the phosphatase PPM1B as an essential component of the Groucho4 repressor complex that is recruited by Pax2 to chromatin. PPM1B can dephosphorylate the Pax2 activation domain and displace the adaptor protein PTIP, thus inhibiting H3K4 methylation and gene activation. Loss of PPM1B prevents Groucho-mediated gene repression. Thus, PPM1B helps switch Pax2 from a transcriptional activator to a repressor protein. This can have profound implications for developmental regulation by Pax proteins and suggests a model for imprinting specific epigenetic marks depending on the availability of co-factors.

Embryonic development requires cell signaling molecules to specify positional information and nuclear effectors that translate such signals into tissue specific outputs. Genetic screens in flies and mutational analyses in mice identified families of DNA-binding proteins that are essential for embryonic development and cell lineage specification. Among these, the vertebrate Pax genes encode proteins required for development of the eye, the central nervous system, the kidney, the vertebral column, thyroid, and the B cell lineage (1, 2). Although the necessity for Pax proteins in development and their associations with human disease is clear, the molecular mechanisms of transcriptional regulation by Pax proteins remain obscure.

Pax proteins are divided into families based on sequence homology of the DNA binding paired domain, the presence or absence of an octapeptide repressor sequence, and the presence of a carboxyl-terminal homeodomain (3). The Pax2/5/8 (paired box 2/5/8) subfamily proteins have nearly identical DNA-binding domains and share the octapeptide sequence (YSINGILG) that binds to the co-repressor Groucho4 (Grg4/Tle4) (4). Pax2 is also serine/threonine-phosphorylated within the carboxyl-terminal activation domain that is necessary for mediating transcriptional activation (5). Phosphorylation of the Pax2 carboxyl-terminal domain can be mediated by WNT signaling or by the activation of JNK (6). Pax2 is critical for specifying the renal epithelial lineage in the developing kidney (7, 8), a process that depends on both WNT signals and JNK activity (9).

At Pax2 DNA binding sites, the Pax2 protein recruits the adaptor PTIP, encoded by the *Paxip1* gene, to link Pax2 to an Mll3/4 (mixed lineage leukemia 3/4), also known as KMT2C/D (lysine methyltransferase 2C/D) histone methyltransferase complex that marks Pax2 binding sites with high levels of H3K4 mono-, di-, and trimethylation (10). Such epigenetic marks are known to impart active or accessible chromatin signatures to enable gene expression (11). During differentiation of embryonic stem cells, these epigenetic marks are established in a lineage specific manner and correlate with the loss of pluripotency (12). PTIP is essential for postgastrulation development and, together with the H3K4 methyltransferases, is widely expressed in most all cell types (13, 14). It is likely that Pax proteins provide some of the locus and tissue specificity for histone methylation complexes during development and cell lineage decision making. However, genetic and molecular analyses indicate that Pax proteins can also mediate gene suppression through the interactions with co-repressors such as Grg4/Tle4 (4, 6, 15).

The Grg/TLE proteins have nuclear localization signals and multiple WD40 repeat domains but no intrinsic DNA binding activity. Rather, Grg proteins complex with specific DNA-binding proteins (16) and promote gene repression and chromatin condensation (17). In addition, Grg proteins can interact with histone deacetylases to repress transcription (18). Grg4 binds

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directly to Pax2/5/8 through the conserved octapeptide domain (4). Groucho may directly interact with the core machinery to inhibit some step in the transcription cycle such as preinitiation complex assembly, promoter release, or elongation (19, 20). In developing B cells, Grg4 interacts with Pax5 and PU.1 to repress subsets of B cell-specific genes (21). In the spinal cord and the developing kidney (6), Grg4 expression overlaps with Pax2, which specifies renal epithelial cells from the intermediate mesoderm (8) and subsets of neurons in the CNS (22–24). In cell culture, Grg4 suppresses Pax2-mediated transcriptional activation and inhibits phosphorylation of the Pax2 activation domain by WNT signaling and JNK (6). The Grg4/Pax2 interaction displaces the adaptor PTIP,[2] prevents H3K4me, and recruits the arginine methyltransferase PRMT5 (protein arginine methyltranserase 5) and the Polycomb repressor 2 complex to impart H4R3 and H3K27 methylation (15), two marks associated with gene silencing.

In this report, we show that the phosphatase PPM1B is an essential component of the Grg4 silencing complex. PPM1B associates with Grg4 and is recruited to Pax2 DNA binding sites in a Grg4-dependent manner. Furthermore, PPM1B can dephosphorylate the Pax2 activation domain and displace PTIP, a protein that contains multiple BRCT domains capable of binding phosphoserine residues. The data suggest a mechanism in which Grg4-dependent recruitment of a phosphatase is essential for removing the adaptor PTIP, thereby switching Pax2 from an activator to a repressor.

TABLE 1
Primers used in the experiments

Cloning Mus musculus PPM1B into Tag 3b and pRSETA vectors			
Top:	5'-GTTCCGAGATCTATGGGTGCATTTTTGGATAAACC-3'		
Bot:	5'-GCCTAGGAATTCTCATATATTTTCAGCACTCCTCT-3'		
Mutation of amino acid 179 from arginine to glycine in the mouse sequence:			
Top:	5'-CCAGTGGAGAAGGAGGGCATTCAAAACGCAGGAGGC-3'		
Bot:	5'-GCCTCCTGCGTTTTGAATGCCCTCCTTCTCCACTGG-3'		
Mutation of nucleotide sequence in mouse PPM1B to be resistant to sh2:			
Top:	5'-CAGAAGTCTGGGGAGGAGGGTATGCCTGATCTTGCC-3'		
Bot:	5'-GGCAAGATCAGGCATACCCTCCTCCCCAGACTTCTG-3'		
Primers for PPM1B knockdown in HEK 293 cells		Location On cDNA	Mismatches between Human and Mouse PPM1B
Sh 1:	5'-GCAGTGGGAGTTATGATTTCA-3'	400-420	5 nucleotides
Sh 2:	5'-GGAAGGAATGCCTGATCTTGC-3'	987-1007	0 nucleotides
Sh 3:	5'-GCTGACTAGTTACAGGCTAG-3'	1242-1262	0 nucleotides
Sh 4:	5'-ACACAAGGGAAGTCGAGATAA-3'	840-860	1 nucleotide
Sh 5:	5'-AACTTCTGGAGGAGATGCTGA-3'	1226-1246	2 nucleotides
Sh 6:	5'-AATGCAGGAAAGCCATACTGA-3'	1344-1364	4 nucleotides
PCR primers for ChIP:			
PRS4 Top:	5'-GCTACCGGACTCAGA TCTCG-3'		
PRS4 Bot:	5'-TGCGAAGTGGACCTCGGACC-3'		
RAP1A Top:	5'-CTTTAAGCGGACTCCGGAAC-3'		
RAP1A Bot:	5'-CTCCTCCTCCTCCCT CCTCT-3'		
PCR primers for RT-PCR			
β-actin Top:	5'-GGCCACGGCTGCTTC-3'		
β-actin Bot:	5 -GTTGGCGTACAGGTCTTTGC-3'		
RAP1A Top:	5'-TGGATACTGCAGGGACAGAGCAAT-3'		
RAP1A Bot:	5'-ACATCTTCCGTGTCCTTAACCCGT-3'		

EXPERIMENTAL PROCEDURES

Plasmids—CMV-Pax2HA and FLAG-Grg4 plasmids have been described (15). Hexahistidine Pax2, containing amino acids 197–415 of Pax2, has been previously described (6). Myc-PPM1B was made by inserting a PCR-amplified full-length cDNA of murine PPM1B (Open Biosystems) into the BamHI-EcoRI site of pCMVTag3B (Agilent Technologies). Primers used to amplify DNA sequences are listed in Table 1. The phosphatase-defective PPM1B mutant (ΔPPM1B) was constructed by substitution of the arginine residue at position 179 with glycine (25) using oligonucleotide-directed mutagenesis with a QuikChange kit (Stratagene). Because the human and mouse cDNA sequences that are targeted by PPM1B short hairpin (sh) 2 were identical, nucleotide 990 was mutated from A to G, and nucleotide 993 was mutated from A to T using the QuikChange kit. The amino acid sequence is preserved, but the cloned DNA is resistant to the short hairpin. Hexahistidine PPM1B or the enzymatic mutant was made by inserting the PCR fragment into the BamHI-EcoRI sites of pRSETA (Invitrogen). All expression plasmids were sequenced for verification. Constitutively active, recombinant JNK was purchased from Upstate Biotechnology (Lake Placid, NY).

Antibodies—Rabbit antibodies to Pax2 and PTIP are described (10). Rabbit IgG (011-000-003; IP and ChIP) and goat IgG (005-000-003; IP and ChIP) were from Jackson Immunoresearch Laboratories. Anti-FLAG (F-3165) and anti-β-actin (A-1978) were from Sigma-Aldrich. Anti-PPM1B (A300–886A) was from Bethyl Labs. Anti-H3K4me3 (ab8580) and anti-polymerase II CTD (ab12073) were purchased from Abcam. Mouse anti-GFP (sc9996) and goat anti-Grg4/TLE4 (sc13377) were from Santa Cruz Biotechnology.

Protein Purification and Mass Spectometry—As described previously (15), 40 mg of nuclear extract prepared from HEK 293 cells transfected with FLAG-Grg4 was fractionated on Phosphocellulose (P11, Whatman) and DEAE-Sephacel (Pharmacia) and eluted stepwise with increasing concentrations of KCl. The peak Grg4 fractions were incubated overnight with anti-M2 FLAG agarose at 4 °C. After stringent washing, proteins were eluted at 4 °C with 150 ng/ml 3X-FLAG peptide (SIGMA). The elutions were concentrated on Microcon columns (Millipore) and resolved on a 12% SDS-PAGE gel. Gels were stained with colloidal Coomassie (Invitrogen), and excised bands were sequenced by the Harvard Microchemistry Facility. For purification with anti-Grg4 antibodies, 800 mg of nuclear extract from FLAG Grg4-transfected HEK293 cells was fractionated on P11 column, DEAEsephacel, CM-Sepharose (Pharmacia), SP-Sepharose (Pharmacia), and Q-Sepharose with increasing concentrations of KCl. Peak fractions containing Grg4 were pooled and loaded onto the next matrix. Proteins eluted from the Q-Sepharose matrix were immunoprecipitated overnight with either 20 μg of goat IgG or 20 μg of goat anti-Grg4 that had been coupled to protein G beads. After extensive washing, proteins bound to beads were eluted in 0.9 ml of 0.1 M glycine, pH 3.0, neutralized with 0.1 ml of 1 M Tris, pH 8.0, and concentrated with Strataclean beads (Agilent Technologies, Santa Clara, CA); 85% of the sample was separated by SDS-

[2] The abbreviations used are: PTIP, Pax transactivation domain-interacting protein; PPM1B, protein phosphatase magnesium-dependent isoform β; IP, immunoprecipitation; sh, short hairpin; PRS, Pax response sequence.

PAGE on a 10% gel and stained with colloidal Coomassie; and the excised bands were sequenced by MS/MS.

Cell Culture, Transfection, and Cell Lysate Preparation—HEK293 cells were cultured in DMEM (450 mg/dl glucose) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin in 5% CO_2, 95% air at 37 °C. 10-cm plates were transfected with 1–2 μg of DNA and 12 μl of FuGENE 6 as described (Roche Molecular Biochemicals). After 40 h of transfection, cells were harvested and nuclear extracts or whole cell lysates were prepared as described (26). HEK293 cells with an integrated PRS-EGFP were transfected with 1 μg of empty vector, 1 μg of CMV-PAX2, 1 μg of CMV-Pax2, and 2 μg of FLAG-Grg4 or FLAG-Grg4 (2 μg) as described (10).

Coimmunoprecipitation and Immunoblotting—For IPs, 1 mg of HEK293 nuclear extract of cells transfected with Flag-Grg4 was cleared with protein A-agarose or protein G-Sepharose (Amersham Biosciences). Antibody (2–4 μg) was added, and protein complexes were immobilized on agarose beads. After washing with IP buffer, the beads were boiled in 2× Laemmli sample buffer and separated by SDS-PAGE with 2% input. Immunoblotting was performed as previously described (26). All co-IPs were also conducted in the presence of 200 μg/ml ethidium bromide (27).

Dephosphorylation Assay—50 milliunits of recombinant JNK were used to phosphorylate 2 μg of hexahistidine-Pax2-(197–415) in 50 μl of kinase buffer (25 mm HEPES, pH 7.4, 20 mm $MgCl_2$, 0.5 mm EGTA, 12.5 mm β-glycerophosphate, 0.1 mm orthovanadate, 0.5 mm NaF) containing 20 μm ATP for 30 min at 30 °C as described (5). Nuclear extracts were prepared in Z buffer without Mg from 10 plates of HEK 293 cells that had the stable universal short hairpin negative control or cells that had PPM1B sh4. 2 ng of phosphorylated Pax2 was incubated with either 20 μg of nuclear extract, 10 or 30 ng of recombinant hexahistidine-PPM1B or mutant PPM1B in the presence or absence of various phosphatase inhibitors for 30 min at 30 °C in 20 μl of dephosphorylation buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mg/ml ovalbumin, 0.1% Tween 20, 1× protease inhibitors) containing no Mg^{2+} or Ca^{2+} or with 5 mM Mg^{2+} or Ca^{2+} as previously described (28). The following phosphatase inhibitors were added to the incubations before the addition of PPM1B: 5 μM microcystin-LR, 5 mM tetramisole, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 6 mM EDTA, 20 mM NaF, and Sigma phosphatase inhibitors Set 1 and 2 (Sigma) as described (28). The reactions were terminated by the addition of 6 μl of 6× SDS loading buffer and were subjected to SDS-PAGE followed by transfer to polyvinylidene difluoride membranes and Western blotting with Pax2 antibody and PPM1B antibody.

RNA Interference and Rescue of PPM1B in HEK293 Cells—Six oligonucleotides (Table 1) targeting the coding sequence of human PPM1B were designed using the BLOCK-IT RNAi designer tool (Life Technologies). Short hairpin constructs were made as described (Clontech). Briefly, hairpin constructs against human PPM1B mRNA were cloned into the pSiren-RetroQ (Clontech) vector system and transduced into GFP reporter cell lines. Control cell lines were transduced with the Clontech Universal short hairpin negative control oligonucleotide. The cells were selected with 2 μg/ml puromycin for 2 weeks. Knockdowns were confirmed by Western blotting. Stable GFP reporter cell lines containing the short hairpin negative control or PPM1B short hairpin were transfected with 1 μg of empty CMV vector, 1 μg of CMV-PAX2, 1 μg of CMV-Pax2 and 2 μg of FLAG-Grg4, 1 μg of CMV-Pax2, 2 μg of FLAG-Grg4, and 0.2 μg of Myc-PPM1B or with 1 μg of CMV-Pax2, 2 μg of FLAG-Grg4, and 0.2 μg of mutant ΔMyc-PPM1B as described (15).

TABLE 2
Mass spectrometry of Flag-Groucho4-associated PPM1B

IP antibody	PPM1B peptides	Protein coverage
		%
Mouse anti-FLAG	24	36.2
Goat anti-Grg4	38	56.6

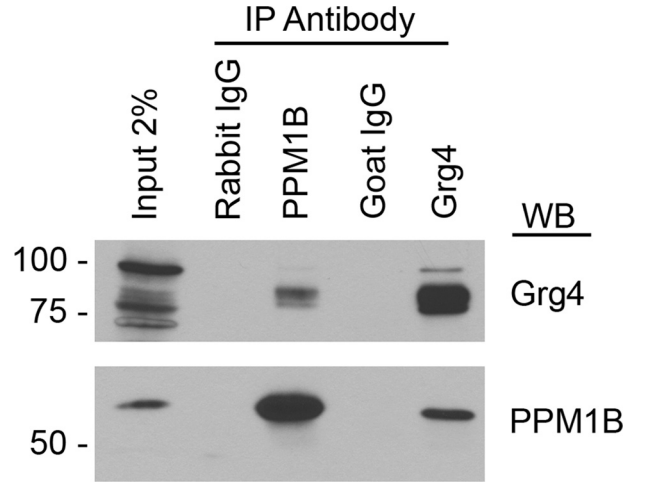

FIGURE 1. **Grg4 and PPM1B co-IP.** Rabbit anti-PPM1B or goat anti-Grg4 were used to IP proteins from nuclear extracts directly in the presence of EtBr and Western blotted (*WB*) as indicated. Controls are rabbit IgG for PPM1B and goat IgG for Grg4.

ChIP and Real Time PCR—GFP reporter cells were harvested 48 h after transfection. ChIP was performed exactly as described previously (10). The precipitated DNA was reconstituted in sterile water, and real time PCR quantitation of precipitated genomic DNA relative to inputs was performed in triplicate using IQ SYBR GREEN with ROX mastermix (Bio-Rad) in a 7900HT real time PCR system (Applied Biosystems). The sequences of the PRS primer pair and for the Rap1A primer set are listed in Table 1. ChIP data are presented as percentages of input after subtraction of nonspecific binding to either rabbit IgG or Goat IgG. In all experiments, nonspecific binding was less than 0.02% of input.

RNA Preparation, Reverse Transcription, and Real Time PCR of RAP1A—Stable sh negative, PPM1B sh1 and PPM1B sh4 GFP reporter cell lines were transfected with 1 μg of empty CMV vector, 1 μg of CMV-PAX2, 1 μg of CMV-Pax2, 2 μg of FLAG-Grg4, 1 μg of CMV-Pax2, 2 μg of FLAG-Grg4, and 0.2 μg of Myc-PPM1B or with 1 μg of CMV-Pax2, 2 μg of FLAG-Grg4, and 0.2 μg of mutant ΔMyc-PPM1B. 0.1 μg of pCMV-β-galactosidase was co-transfected to monitor for transfection efficiency. Experiments were performed in duplicate. 48 h after transfection, cells were harvested in PBS. Whole cell lysates were prepared from half of the cells in Promega lysis buffer (Promega). β-Galactosidase activity was measured as described

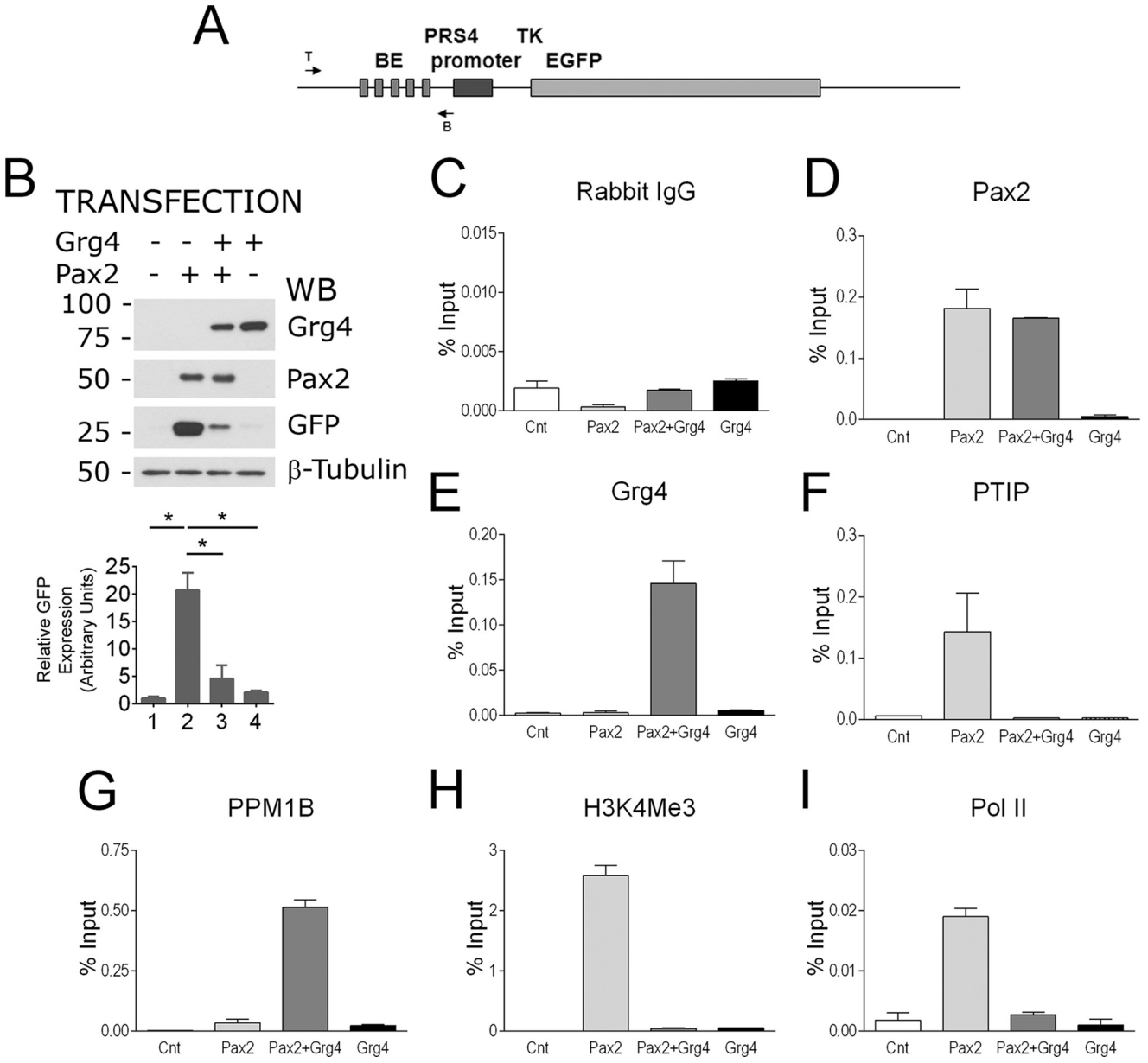

FIGURE 2. **Grg4 displaces PTIP to inhibit Pax2-dependent reporter gene expression.** *A*, a schematic of the Pax-responsive reporter gene integrated into HEK293 cells. *TK*, minimal HSV-TK promoter; *EGFP*, green fluorescent protein coding; *arrow T*, forward primer; *arrow B*, reverse primer used for real time PCR. *B*, HEK293 cells containing the integrated PRS-GFP reporter were transfected with control vectors (*lane 1*), Pax2 alone (*lane 2*), Pax2 and Grg4 (*lane 3*), or Grg4 alone (*lane 4*). Whole cell lysates were blotted for the proteins indicated. The *lower panel* shows GFP expression quantitated by scanning densitometry. GFP expression in the CMV empty vector alone (*lane 1*) was normalized to 1, and expression in other lysates are relative to this control. Note that the significant increase in GFP expression with Pax2 alone (*lane 2*) is suppressed by co-expression of Pax2 and Grg4 (*lane 3*). Grg4 alone has no effect on GFP expression (*lane 4*). Note comparisons are made for each transfection, and significant comparisons are shown. *, $p < 0.01$. *C–I*, ChIP assays from HEK293 cells transfected as in *B*. The antibodies used for each ChIP are indicated above the graph. Primer pairs flank the PRS. *D* and *E*, note that Pax2 binds to PRS independent of Grg4 (*D*) and Grg4 localizes to PRS in a Pax2-dependent manner (*E*). *F*, PTIP binds to PRS in a Pax2-dependent manner but is displaced upon Grg4 co-expression. *G*, PPM1B is localized to PRS in a Pax2/Grg4-dependent manner. *H*, Pax2/Grg4 expression suppresses H3K4me3 at the Pax2 binding element. *I*, DNA polymerase II (*Pol II*) does not accumulate at PRS4 with Pax2/Grg4 co-expression. Averages from triplicates are shown with *error bars* representing 1 S.E.

(26) and was used to normalize protein loading for Western blotting for Grg4, PPM1B, and Pax-2. RNA was prepared from the other half of the harvested cells by the TRIzol RNA isolation system (Life Technologies). 2 μg of total RNA was reverse-transcribed into cDNA with SuperScript first strand kit (Life Technologies) using random hexamers as described by the manufacturer. The cDNA products were diluted 20-fold, and quantitative real time PCR was performed to determine mRNA levels with the iTaq SYBR Green master mix (Bio-Rad) in a 7900HT real time PCR system (Applied Biosystems). Each sample was performed in triplicate. β-Actin was used an endogenous control to normalize targets. Primers pairs for PCR for β-actin (Life Technologies) and Rap1A (29) are described in Table 1. The $\Delta\Delta C_T$ method (ABI User Bulletin #2) (29) was used to analyze mRNA expression. The normalized expression of Rap1A in the empty CMV vector transfected cells was set to 1.0, and expression in the other transfected cells is relative to each respective control.

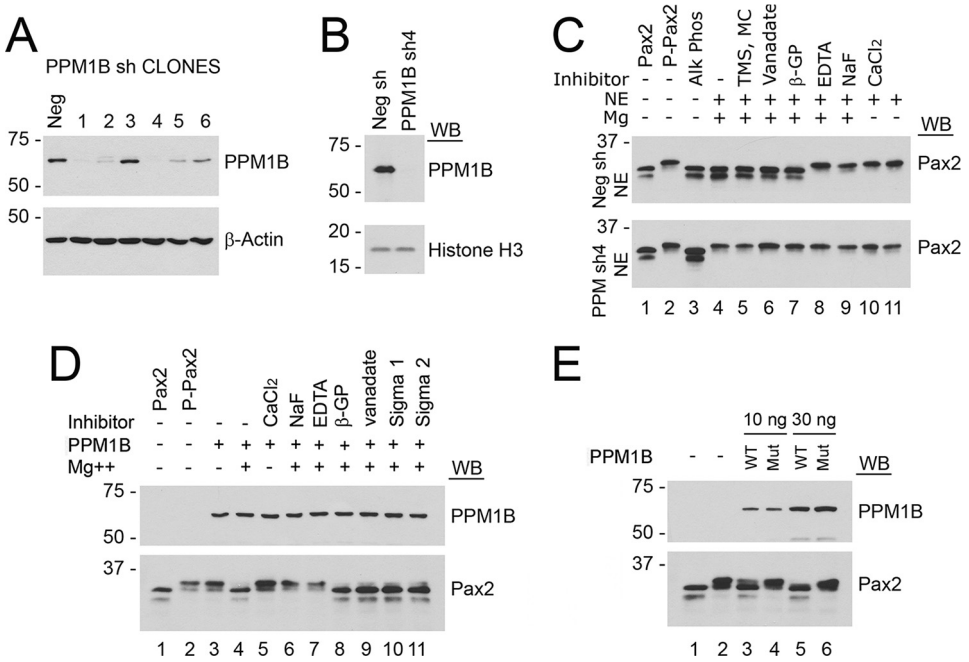

FIGURE 3. **Recombinant PPM1B dephosphorylates Pax2.** *A*, expression of PPM1B in the control shRNA cell line (*Neg*) and six shRNA stable reporter cell lines. Note PPM1B expression is markedly attenuated in cells transduced with shRNA 1, 2, and 4. *B*, PPM1B expression is markedly reduced in 20 μg of nuclear extracts from cells transduced with PPM1B sh4 *versus* sh negative. Histone H3 is used to control for protein loading. *C*, recombinant purified His-Pax2-(197–415) phosphorylated with recombinant JNK, and ATP was incubated with 20 μg of nuclear extract prepared from negative sh cells or from cells transduced with PPM1B sh4 in the presence or absence of magnesium and various inhibitors of phosphatases. The *upper panel* is for Pax2 in reactions from negative sh cells, and the *lower panel* represents reactions from PPM1B sh4 cells. *Lane 1*, unphosphorylated Pax2. *Lane 2*, phosphorylated Pax2 (*P-Pax2*), note the mobility shift. *Lane 3*, positive control, Pax2 incubated with calf intestinal alkaline phosphatase. *Lane 4*, no inhibitors. *Lane 5*, 5 mM tetramisole and 5 μM microcystin-LR. *Lane 6*, sodium orthovanadate 1 mM. *Lane 7*, 10 mM β-glycerophosphate. *Lane 8*, 6 mM EDTA. *Lane 9*, 20 mM sodium fluoride. *Lane 10*, 5 mM calcium chloride instead of magnesium. *Lane 11*, NE without inhibitors or Mg. Note dephosphorylation in *lanes 2–7*, with mobility shift down to that of unphosphorylated Pax2 in the reactions with negative sh extract. There is no dephosphorylation of Pax2 in the presence or absence of Mg and any inhibitors in the reactions with PPM1B sh4 extract. NE required magnesium for dephosphorylation activity. *D*, recombinant purified His-Pax2-(197–415) phosphorylated with recombinant JNK and ATP was incubated with 30 ng of recombinant His-PPM1B. Concentrations of inhibitors are as in *C*. Sigma phosphatase inhibitor sets 1 and 2 were used at the recommended concentrations. Note that PPM1B dephosphorylation activity requires magnesium (compare *lane 1* with *lane 5*). Fluoride and EDTA inhibit PPM1B activity (*lanes 6* and *7*). β-Glycerophosphate (*lane 8*), orthovanadate (*lane 9*), and sigma inhibitors (*lanes 10* and *11*) had little effect on PPM1B activity. Western blots (*WB*) for PPM1B and Pax2 are shown. *E*, recombinant purified His-Pax2-(197–415) phosphorylated with recombinant JNK and ATP was incubated with 10 or 30 ng of recombinant wild-type His-PPM1B (*WT*) or phosphatase-deficient PPM1B (*Mut*) in the presence of Mg. Western blot for Pax2 shows an increase in mobility to a lower molecular weight in the incubations with wild-type enzyme denoting loss of phosphorylation. Western blot for PPM1B is shown in the *upper panel*.

Scanning Densitometry—GFP expression was quantitated utilizing ImageJ software as described. The expression of GFP in the empty CMV vector transfected cells was set to 1.0, and expression in the other transfected cells is relative to each respective control.

Statistics—Statistical significance was determined by analysis of variance followed by the Tukey's post hoc test for multiple comparisons performed by using GraphPad (GraphPad Software, San Diego, CA). $p < 0.05$ was considered significant in all cases.

RESULTS

Recruitment of Grg4 to Chromatin Displaces PTIP—To determine how Grg4 suppressed Pax2-dependent gene activation, displaced the PTIP/KMT2C (lysine methyl transferase 2C) complex, and prevented H3K4me3, we purified Grg4 and its associated proteins (15). Flag-tagged Grg4 expressed in HEK293 cells was purified by conventional chromatography and goat anti-Grg4 immunoprecipitation. Mass spectrometry identified proteins associated with Grg4, including the arginine methyltransferase PRMT5, a protein phosphatase PPM1B, and proteins WDR77 (WD repeat domain 77) and PICLN (chloride channel nucleotide-sensitive 1A) (15). As shown in Table 2, 24 unique PPM1B peptides were identified in the FLAG IP, and 36 unique peptides were identified in the Grg4 IP. Protein coverage was 36.2 and 56.6%, respectively. To confirm the interaction of Grg4 and PPM1B, we immunoprecipitated these proteins from nuclear extracts that were prepared from HEK293 cells transfected with FLAG-Grg4 (Fig. 1). Although control antibodies did not pull down any proteins, Grg4 co-precipitated with PPM1B in nuclear extracts prepared with 200 μg/ml ethidium bromide. Reciprocally, antibodies against PPM1B co-precipitated Grg4.

To understand how Grg proteins repress transcription, we have used an integrated, chromatin-based reporter gene to characterize histone methylation patterns in response to Pax2 or Pax5 DNA-binding proteins (10, 30). HEK293 cells carrying a Pax response sequence (PRS) driving a GFP reporter gene were transfected with Pax2 and Grg4 (Fig. 2). Transient expression of Pax2 significantly increased GFP (Fig. 2*B*). However, co-expression of Grg4 with Pax2 significantly inhibited activation of the GFP reporter (Fig. 2*B*), whereas Grg4 expression alone had no effect of GFP expression (Fig. 2*B*). ChIP experiments showed that Pax2 DNA binding was unaffected by Grg4 co-expression (Fig. 2*D*) and that Grg4 was localized to the PRS

element only in the presence of Pax2 protein (Fig. 2*E*). Grg4 expression was able to displace PTIP (Fig. 2*F*) from the PRS element and suppress the increase in H3K4me3 observed with Pax2 alone (Fig. 2*H*). In the presence of Grg4, PPM1B is recruited to the PRS in a Pax2-dependent manner (Fig. 2*G*). Note that there is no enrichment for PPM1B in the Pax2 transfected cells. Pax2 expression results in PTIP and DNA polymerase II localization to the PRS4 element (Fig. 2, *F* and *I*). However, Grg4 co-expression prevents accumulation of DNA polymerase II at this site (Fig. 2*I*).

PPM1B dephosphorylates Pax2 in Vitro—To characterize the phosphatase activity of PPM1B, we first developed a set of cell lines that were stably transduced with either a universal negative short hairpin or short hairpins targeting the coding sequence of human PPM1B in the HEK293 GFP reporter cell line (Fig. 3*A*). As shown, the short control negative short hairpin does not affect PPM1B expression. PPM1B sh1, sh2, and sh4 markedly attenuated the expression of PPM1B (Fig. 3*A*); sh5 and sh6 were weak; and sh3 had no effect on PPM1B expression. Nuclear extracts were prepared from negative sh cells and PPM1B sh4 cells. We then examined these nuclear extracts for their ability to dephosphorylate Pax2 that was phosphorylated by JNK *in vitro*. Incubation of phosphorylated Pax2 in the presence of 20 μg of nuclear extract derived from the negative sh cells was adequate to dephosphorylate Pax2 (Fig. 3*C*, *lane 4*, *upper panel*). As shown, the phosphatase activity was inhibited by high concentrations of EDTA (Fig. 3*C*, *lane 8*, *upper panel*) and fluoride (Fig. 3*C*, *lane 9*, *upper panel*) and was magnesium-dependent (Fig. 3*C*, compare *lanes 4* and *11*, *upper panel*), because substitution of Ca^{2+} prevented dephosphorylation of Pax2 (Fig. 3*C*, *lane 10*, *upper panel*). These data suggested that a protein of the PP2C family was the nuclear phosphatase that could account for Pax2 dephosphorylation. As shown in the *lower panel* of Fig. 3*C*, nuclear extract prepared from cells deficient in PPM1B was unable to dephosphorylate Pax2 (compare *lane 2* and *lanes 4–11*).

Because PPM1B is a known Mg^{2+}/Mn^{2+}-dependent serine-threonine phosphatase (31, 32), we tested the ability of recombinant PPM1B to dephosphorylate Pax2 (Fig. 3*D*). Indeed, PPM1B dephosphorylates Pax2 when Mg^{2+} is present in the buffer. Moreover, the enzymatic activity was inhibited by fluoride, EDTA, or Ca^{2+}, whereas β-glycerophosphate, orthovanadate, and inhibitors of alkaline phosphatase, acid phosphatase, PP1, and PP2 were ineffective in inhibiting PPM1B activity. The phosphatase mutant form of PPM1B also was unable to dephosphorylate Pax2 *in vitro* (Fig. 3*E*). Taken together, these data suggest that PPM1B may be the phosphatase responsible for Pax2 dephosphorylation.

PPM1B Is Necessary for Pax2-Grg4 Mediating Silencing—To test the need for PPM1B in Pax2-Grg4-mediated gene silencing, we first generated a series of GFP reporter cells that contained a stable shRNA knockdown of PPM1B. We tested six different shRNAs and found three (shRNAs 1, 2, and 4) that maintained an effective knockdown of PPM1B (Fig. 3*A*). These stable cells and control negative sh cells were subsequently transfected with Pax2, Grg4, or Pax2 and Grg4 in the presence or absence of exogenous PPM1B (Fig. 4*A*). After PPM1B knockdown, Pax2 still activated the GFP reporter (Fig. 4, *A* and *B*,

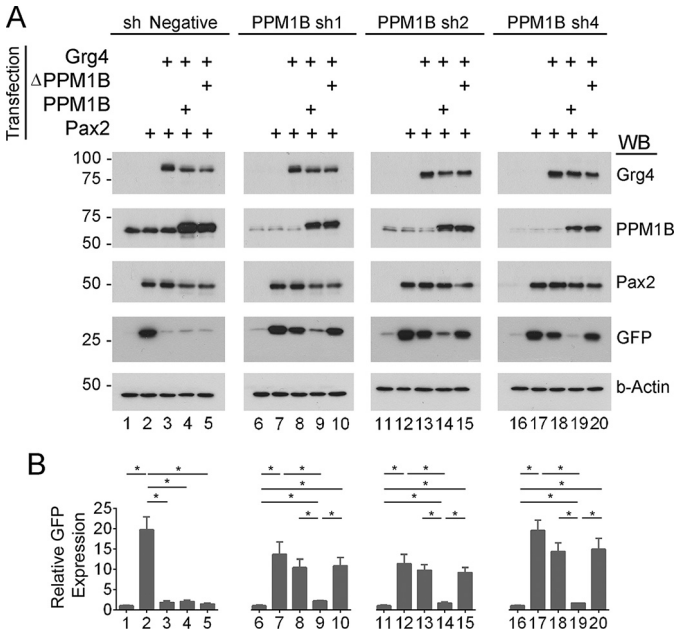

FIGURE 4. **PPM1B is required for Pax2- and Grg4-mediated gene repression.** *A*, control negative sh GFP reporter cells or GFP reporter cells with shRNA 1, 2, or 4 were transfected with Grg4, Pax2, wild-type, or phosphatase-deficient PPM1B as indicated. Whole cell lysates were Western blotted (*WB*) for the indicated proteins with β-actin used as a loading control. Note that GFP expression increased upon Pax2 transfection (*lanes 2*, *7*, *12*, and *17*) in all cell lines; this activation was inhibited by Grg4 (*lane 3*) in control cells but not in PPM1B knockdown cells (*lanes 8*, *13*, and *18*). The Grg4-mediated inhibition was rescued with wild-type PPM1B in the knockdown cells (*lanes 9*, *14*, and *19*) but not mutant enzyme (*lanes 10*, *15*, and *20*). *B*, scanning densitometry for GFP expression quantitated by ImageJ is shown. GFP expression in the CMV empty vector alone (*lanes 1*, *6*, *11*, and *16*) are normalized to 1, and expression in other lysates is relative to the respective control. Note that GFP expression is completely suppressed in the control sh cells transfected with Pax2/Grg4 in the presence or absence of PPM1B or mutant PPM1B (compare *lane 2* with *lanes 3–5*). In the knock-out cells, co-expression of Pax2/Grg4 did not repress GFP expression (compare *lanes 7* and *8*; *lanes 12* and *13*; and lanes *17* and *18*). Wild-type (*lanes 9*, *14*, and *19*) but not mutant PPM1B (*lanes 10*, *15*, and *20*) restores repression of GFP. All *bars* are averages of duplicates with *error bars* indicating S.D. Note comparisons are made between all groups of transfections in each cell line, and significant comparisons are shown. *, $p < 0.01$.

lanes 2, *7*, *12*, and *17*), but Grg4-mediated repression was attenuated in all three clones that had diminished PPM1B expression (Fig. 4, *A* and *B*, compare *lane 3* to *lanes 8*, *13*, and *18*). PPM1B activity was rescued in these cell lines with a wild-type mouse cDNA that was resistant to the effect of the short hairpins or a phosphatase-deficient mutant form of PPM1B as described under "Experimental Procedures" (Fig. 4*A*, WB for PPM1B). The wild-type enzyme was able to significantly repress GFP expression (Fig. 4, *A* and *B*, compare *lanes 8* and *9*, *lanes 13* and *14*, and *lanes 18* and *19*), whereas the mutant enzyme was unable to mediate Grg4 repression (Fig. 4, *A* and *B*, compare *lanes 8* and *10*, *lanes 13* and *15*, and *lanes 18* and *20*). Thus, PPM1B phosphatase activity is necessary for mediating part of the Grg4-dependent repression of a Pax2 reporter gene.

To characterize the role of PPM1B at the level of chromatin, antibodies were used for ChIPs under the same conditions as in Fig. 2. Grg4 or PPM1B expression levels did not affect Pax2 binding to the PRS (Fig. 5*A*, *lanes 2–5* and *6–10*). As described previously, Grg4 associated with chromatin only in the presence of Pax2 (Fig. 5*B*, *lanes 3–5* and *8–10*). Strikingly, PTIP was displaced by Grg4 only in the presence of PPM1B (Fig. 5*D*, *lanes*

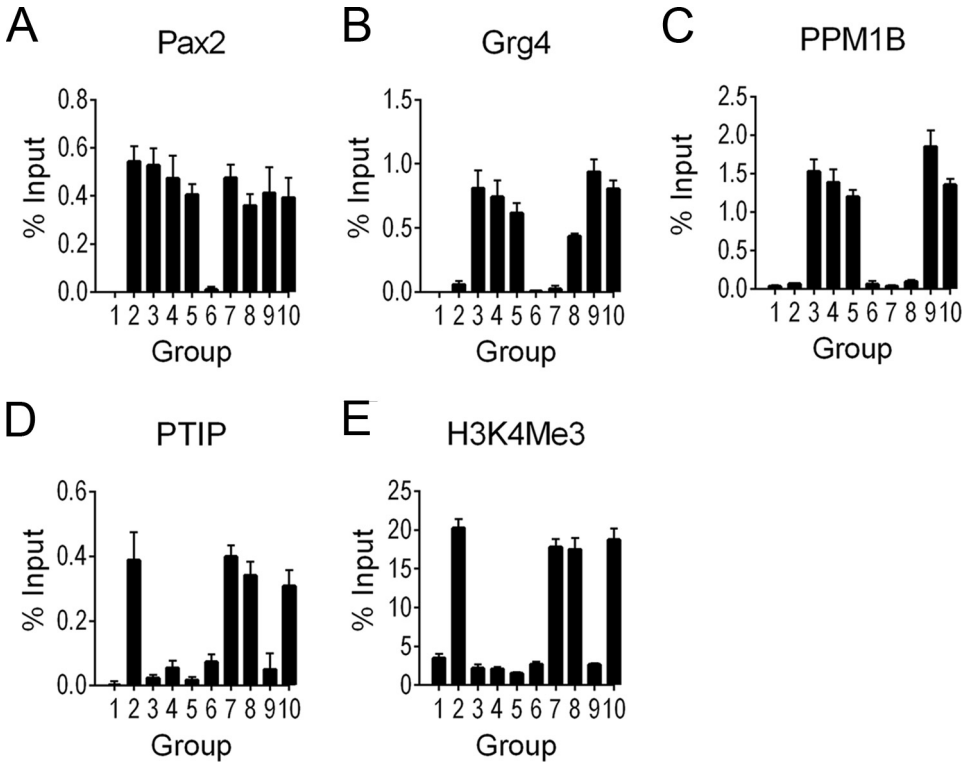

FIGURE 5. **Pax2/Grg4-mediated PTIP displacement is dependent on PPM1B.** *A–E*, ChIP assays were performed with the indicated antibodies from negative sh cells and PPM1Bsh1 cells. The lanes correspond to transfections shown in Fig. 4. *A*, note that Pax2 binds to the PRS element in all cells transfected with Pax2 (*lanes 2–5* and *7–10*). *B*, Grg4 localizes to PRS only when co-transfected with Pax2 (*lanes 3–5* and *8–10*). *C*, PPM1B localizes to PRS4 when Pax2 and Grg4 are co-expressed (*lanes 3–5*, *9*, and *10*). *D*, PTIP localizes to the PRS when Pax2 is expressed (*lanes 2* and *7*) and when wild-type PPM1B expression is attenuated in the presence of Pax2 and Grg4 (*lane 8*) or when mutant PPM1B is used in the rescue (*lane 10*). Wild-type PPM1B rescue restores Pax2- and Grg4-mediated PTIP displacement (*lane 9*). *E*, H3K4 is methylated when PTIP is localized to PRS. All *bars* are averages of triplicates with *error bars* indicating S.E.

3–5 and *9*). Displacement of PTIP correlated with reduced levels of gene expression (Fig. 4, *A* and *B*, lanes *3–5* and *9*) and reduced H3K4me3 (Fig. 5*E*, *lanes 3–5* and *9*). Overexpression of phosphatase-deficient mutant PPM1B did not function as a dominant negative in these experiments (Fig. 4, *A* and *B*, *lane 5*) and did not displace PTIP (Fig. 5*D*, *lane 5*) or reduce H3K4me3 (Fig. 5*E*, *lane 5*) or reporter gene expression. The phosphatase activity of PPM1B is necessary for mediating Pax2/Grg4-dependent gene repression, because the mutant is unable to repress gene expression (Fig. 4, *A* and *B*, *lane 10*) and allows for recruitment of PTIP (Fig. 5*D*, *lane 10*) and increased H3K4me3 (Fig. 5*E*, *lane 10*).

Although the PRS-GFP reporter was useful in defining the patterns of histone modification upon Pax2 or Pax2/Grg4 binding, it was not clear whether such modifications can occur at endogenous loci. HEK293 cells are immortalized embryonic kidney fibroblasts that presumably have a stable pattern of epigenetic marks. These cells do not express Pax2, which is specific for the epithelial lineage in the developing kidney. We previously identified the gene Rap1A to be up-regulated by Pax2 and repressed by Pax2/Grg4 in HEK293 cells (15). We also identified a Pax2 binding site close to the transcription start site of Rap1A and characterized the effects of Pax2 and Grg4 at the Rap1A promoter. We repeated the transfection experiments as in Fig. 4 in control negative sh cells and PPM1B sh1 and sh4 cells. Western blots for Grg4, PPM1B, and Pax2 are shown in Fig. 6*A* (after normalization for transfection efficiency as monitored by β-galactosidase activity). Upon Pax2 expression, Rap1A mRNA is increased in all three cell lines transfected (Fig. 6*B*, *lanes 2*, *7*, and *12*). Co-expression of Grg4 significantly attenuates the Pax2-dependent Rap1A message in control cells, but not in the two PPM1B knockdown cells (Fig. 6*B*, compare *lanes 3* with *lanes 8* and *13*). Similar to our GFP reporter gene, wild-type PPM1B co-expression with Pax2/Grg4 rescued the repressive effects on Rap1A (Fig. 6*B*, compare *lane 4* with *lanes 9* and *14*), whereas the phosphatase-deficient mutant was incapable of rescuing repression (Fig. 6*B*, compare *lane 5* with *lanes 10* and *15*).

We then characterized the effects of Pax2 and Grg4 at the Rap1A promoter region with and without PPM1B (Fig. 6, *C–G*). The overall patterns of histone methylation and protein recruitment were similar to those observed with the integrated PRS-GFP reporter. Pax2 expression increased PTIP and H3K4me3 at the promoter (Fig. 6, *F* and *G*, *lanes 2* and *7*). Pax2/Grg4 displaced PTIP (Fig. 6*F*) and recruited PPM1B (Fig. 6*E*), resulting in lower Rap1A expression (Fig. 6*B*, *lane 3*). In the absence of PPM1B, Pax2/Grg4 did not displace PTIP, and H3K4 was highly methylated (Fig. 6, *F* and *G*, *lane 8*). The PPM1B knockdowns could be rescued by the wild-type enzyme (Fig. 6, *E–G*, *lanes 9*) but not the mutant enzyme (Fig. 6, *E–G*, *lane 10*). These data further underscore the significance of PPM1B in mediating Grg4-dependent repression at an endogenous gene.

DISCUSSION

We have previously identified the critical steps and associated proteins that mediate Groucho4-dependent gene silencing

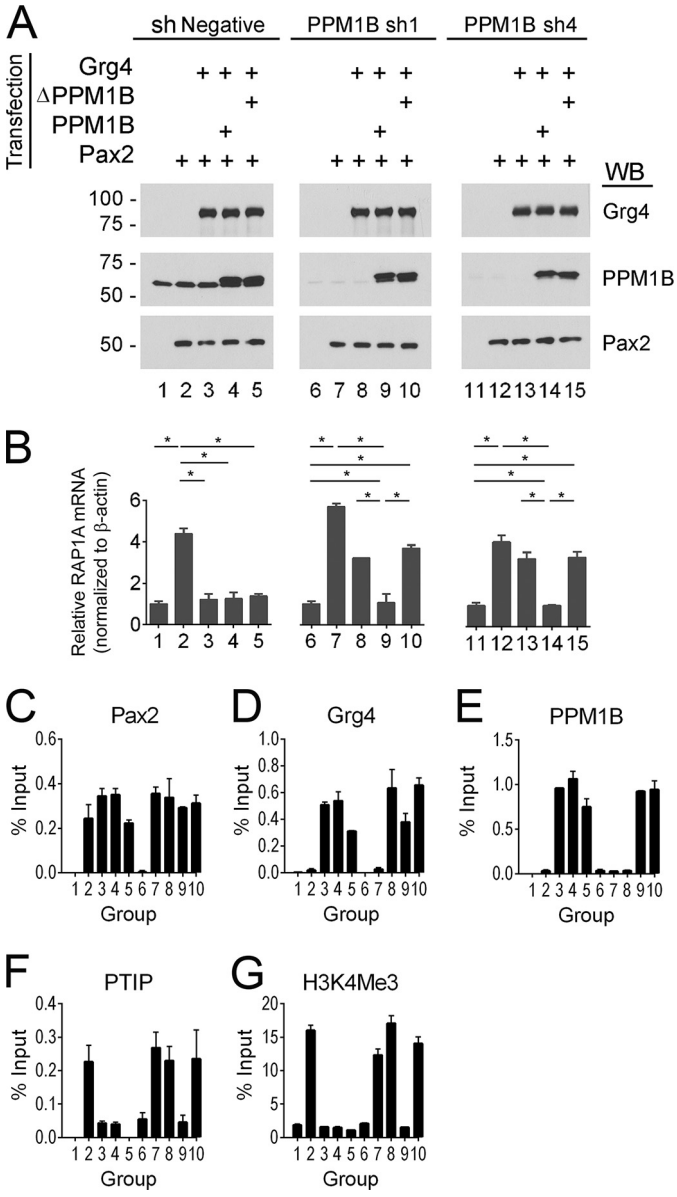

FIGURE 6. **Pax2/Grg4-mediated chromatin remodeling at the Rap1A locus is PPM1B-dependent.** *A*, GFP reporter cells with the control sh or cells with PPM1B shRNA 1 and shRNA 4 were transfected as noted and Western blotted (*WB*) for Grg4, PPM1B, and Pax2. Loading was normalized for β-galactosidase activity. *B*, RAP1A mRNA expression in the transfected cells from *A*. Rap1A expression in the CMV empty vector alone (*lanes 1*, *6*, and *11*) are normalized to 1 and expression in other lanes are relative to the respective control. Note that in all cell lines, Pax2 stimulates Rap1a expression (*lanes 2*, *7*, and *12*). In control cells, Pax2-Grg4 co-expression represses Rap1A expression (*lane 3*) but not in the absence of PPM1B (*lanes 8*,13). Rescue with wild-type PPM1B (*lanes 9* and *14*) but not phosphatase-deficient PPM1B (*lanes 10* and *15*) restores repression of Rap1A expression in the knock-out cells. All *bars* are averages of two experiments with PCR done in triplicate and *error bars* indicating S.D. Note comparisons are made between all groups of transfections in each cell line, and significant comparisons are shown. *, $p < 0.01$. *C–G*, ChIP assays were performed with the indicated antibodies. *Lanes 1–5*, control cells; *lanes 6–10*, PPM1B sh1 knockdown cells. *C*, note that Pax2 binds to the Rap1a promoter in all cells transfected with Pax2 (*lanes 2–5* and *7–10*). *D*, Grg4 localizes to the Rap1A promoter when co-transfected with Pax2 (*lanes 3–5* and *8–10*). *E*, PPM1B localizes to this site when Pax2 and Grg4 are co-expressed (*lanes 3–5*, *9*, and *10*). *F*, PTIP localizes to the Rap1A promoter when Pax2 is expressed (*lanes 2* and *7*) and when wild-type PPM1B expression is attenuated in the presence of Pax2 and Grg4 (*lane 8*) or when mutant PPM1B is used in the rescue (*lane 10*). Wild-type PPM1B rescue restores Pax2- and Grg4-mediated PTIP displacement (*lane 9*). *G*, H3K4 is methylated when PTIP is localized to this Rap1A promoter site. All *bars* are averages of triplicates with *error bars* being S.E.

at a Pax DNA-binding site (15). Here, we identified an essential phosphatase, PPM1B, in the Grg4 silencing complex that is essential for displacing PTIP from the Pax2 protein. Grg4 is able to switch Pax2 from activator to a repressor and initiate Polycomb-mediated gene silencing. In the absence of Grg4, Pax2 recruits the adaptor protein PTIP and associated Mll3/4 (KMT2C/D) complexes to chromatin (10), which increases H3K4me3 an epigenetic mark associated with gene expression and accessible chromatin. PTIP is required for maintaining high levels of H3K4me3 in ES cells (33) and in early embryonic development (10). PTIP-mediated H3K4me3 is observed at the germ line transcript promoters of the heavy-chain Ig locus upon activation of B cells to undergo class switch recombination (34), where it promotes Pax5-dependent changes in transcription and chromatin looping (30). PTIP is also needed in adult cardiomyocytes (35) to maintain the normal pattern of gene expression and in glomerular podocytes (36). Thus, the ability of Grg4 to recruit PPM1B and displace PTIP at a Pax2-binding site is likely a critical step toward gene silencing. Because PTIP is widely expressed in development, it is likely to interact with other serine phosphorylated DNA-binding proteins such as Pitx2 (37) and receptor-activated Smad proteins (38). Unlike PTIP, Grg4 levels are regulated in the developing kidney and in the neural tube, where they are low in progenitor cells and high in more terminally differentiated neurons and glomerular podocytes (6). Our data may suggest a more general mechanism for how Groucho proteins, and an associated phosphatase may be essential for switching an activator complex to a repressor complex as development proceeds.

The serine-threonine phosphatase PPM1B associates with Grg4 in a Pax2-independent manner, but recruitment to DNA was Pax2- and Grg4-dependent. Upon PPM1B knockdown, we observed no PTIP displacement, despite recruitment of Grg4. This suggests that Grg4 is not simply competing with PTIP for Pax2 binding. The displacement of PTIP required wild-type PPM1B, suggesting that a dephosphorylation event may be crucial for the displacement. In the rescue with mutant PPM1B, PTIP was not displaced. Indeed, the terminal two BRCT domains of PTIP have been shown to be phosphoserine-binding proteins (39–41). Previous data suggest that Pax2 is highly phosphorylated at serine and threonine residues in the carboxyl-terminal domain (5). Moreover, previous data from the lab suggested that DNA-bound Pax2 was not phosphorylated by JNK in the presence of Grg4 (6). In addition, Pax2 point mutants that are unable to bind DNA and still phosphorylated by JNK cannot be dephosphorylated by the Grg4 complex (6). These data and the current studies suggest that Grg4 recruits the phosphatase PPM1B to DNA-bound Pax2, dephosphorylating Pax2, and preventing an interaction with PTIP. Loss of PTIP prevents recruitment of the H3K4 methyltransferase complex, thereby preventing the imprinting of active epigenetic marks.

PPM1B controls numerous cellular functions by dephosphorylation of serine and threonine residues. In yeast and human cells, PPM1B dephosphorylates cyclin-dependent protein kinases and plays a crucial role in progression through the cell cycle (28). PPM1B negatively regulates the TAK1 signaling pathways by dephosphorylating and inactivating TAK1, a protein in the MAPK signaling pathway (42). PPM1B has been

shown to down-regulate cytokine-induced NF-KB activation by altering IKK activity by dephosphorylation of the IKKβ subunit (25). PPM1B superactivates EKLF at the β-globin and BKLF promoters (43). Recently, PPM1B has been shown to be a PPARγ-interacting protein, dephosphorylates PPARγ at a particular serine residue, and thereby increases the transcriptional activity of PPARγ (44).

The effects of Pax2 on chromatin have been difficult to study *in vivo* because of the small number of renal epithelial progenitor cells, making biochemical and ChIP analysis unfeasible. We have developed a cell culture assay to analyze the effects of Pax and Grg proteins on chromatin in a controlled system. Although transfection of Pax2 into HEK293 cells dramatically affected the GFP reporter, its impact on endogenous gene expression was minimal. This suggests that HEK293 cells are not competent to respond to Pax2, perhaps because their chromatin landscape is already fixed, and most potential Pax2 target genes are either already on or are silenced and inaccessible. This underscores the utility of a cell system modeled on the PRS-GFP reporter gene because it enabled selection for an integrated element that was responsive and accessible. The Rap1A locus was one of few responsive genes in HEK293 cells and showed identical changes in PTIP displacement and loss of H3K4me3 upon Pax2 and Pax2/Grg4 expression. Subsequent recruitment of PRMT5, Ezh2, and the Polycomb repressor 2 complex finalizes the repressive epigenetic imprints (15). These results define a model for the epigenetic basis of Groucho/Grg4/TLE-mediated gene silencing through the concerted actions of PPM1B and other Grg4-associated proteins that mediated transcriptional silencing.

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