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.

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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 (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 inhibits transcription (7). Furthermore, PPM1B associates with Grg4, suggesting a mechanism in which Grg4-dependent recruitment of a repressor is essential for removing the adaptor PTIP, thereby switching Pax2 from an activator to a repressor.

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) were identical, nucleotide 993 was mutated from A to T using the QuikChange kit (Stratagene). Because the human and mouse cDNA sequences that are targeted by PPM1B short hairpin (sh) 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 fragment 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 Spectrometry—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 fractions were sequenced by the Harvard Microchemistry Facility. For purification with anti-Grg4 antibodies, 800 mg of nuclear extract from FLAG Grg4-transfected HEK 293 cells was fractionated on Phosphocellulose (P11, Whatman) and DEAE-Sephaloc (Pharmacia) and eluted stepwise with increasing concentrations of KCl. The peak fractions 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, DEAE-Sephaloc, 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-

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### Table 1

| Primers used in the experiments | Location On cDNA | Mismatches between Human and Mouse PPM1B |
|-------------------------------|-----------------|-----------------------------------------|
| **PRS Top**                   | 5'-GCTACCGGCTCACTAGTTCG-3' | 5 nucleotides |
| **PRS Bot**                  | 5'-TGGCAAGTGACCTGGACCTAG-3' | 0 nucleotides |
| **RAP1A Top**               | 5'-CTTAAGGCGCATCGGAACTC-3' | 1242-1262 |
| **RIP A Top**              | 5'-CTCTCCTCCCTCCCCTCCCT-3' | 1226-1246 |
| **PCR primers for ChiP** |                               | 1242-1262 |
| **PCR primers for RIP-PCR** |                               | 1226-1246 |

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% CO2, 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 used to phosphorylate 200 ng 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 as described (5). Nuclear extracts were prepared in Z buffer without Mg from 10 plates of HEK 293 cells that had the excised bands were sequenced by MS/MS.

**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 MgCl2, 0.5 mm EGTA, 12.5 mm β-glycerophosphate, 0.1 mm orthovandate, 0.5 mm NaF) containing 20 µM ATP for 30 min at 30 °C as described (28). The sequences of the PRS primer pair and for the Rap1A primer were designed using the BLOCK-IT RNAi designer tool (Life Technologies). 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).

**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-galacotosidase 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.

### TABLE 2

| 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](image-url) 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.
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 as an endogenous control to normalize targets. Primers pairs for PCR for β-actin (Life Technologies) and Rap1A (29) are described in Table 1. The ΔΔCt 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 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.
PPM1B Represses Pax2-mediated Transcription

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 from HEK293 cells transfected with FLAG-Grg4 (Fig. 1). 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. 2B). However, co-expression of Grg4 with Pax2 significantly inhibited activation of the GFP reporter (Fig. 2B), whereas Grg4 expression alone had no effect of GFP expression (Fig. 2B). ChIP experiments showed that Pax2 DNA binding was unaffected by Grg4 co-expression (Fig. 2D) and that Grg4 was localized to the PRS.
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element only in the presence of Pax2 protein (Fig. 2E). Grg4 expression was able to displace PTIP (Fig. 2F) from the PRS element and suppress the increase in H3K4me3 observed with Pax2 alone (Fig. 2H). In the presence of Grg4, PPM1B is recruited to the PRS in a Pax2-dependent manner (Fig. 2G). 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. 2I).

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 lines that were stably transduced with either a universal negative short hairpin or short hairpins targeting the coding line (Fig. 3A). 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. 3A); 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. 3C, lane 4, upper panel). As shown, the phosphatase activity was inhibited by high concentrations of EDTA (Fig. 3C, lane 8, upper panel) and fluoride (Fig. 3C, lane 9, upper panel) and was magnesium-dependent (Fig. 3C, compare lanes 4 and 11, upper panel), because substitution of Ca2+ prevented dephosphorylation of Pax2 (Fig. 3C, 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. 3C, 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 Mg2+/Mn2+-dependent serine-threonine phosphatase (31, 32), we tested the ability of recombinant PPM1B to dephosphorylate Pax2 (Fig. 3D). Indeed, PPM1B dephosphorylates Pax2 when Mg2+ is present in the buffer. Moreover, the enzymatic activity was inhibited by fluoride, EDTA, or Ca2+, 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. 3E). 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. 3A). 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. 4A). After PPM1B knockdown, Pax2 still activated the GFP reporter (Fig. 4, A and B, 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. 4A, 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. 5A, lanes 2–5 and 6–10). As described previously, Grg4 associated with chromatin only in the presence of Pax2 (Fig. 5B, lanes 3–5 and 8–10). Strikingly, PTIP was displaced by Grg4 only in the presence of PPM1B (Fig. 5D, lanes 7–9, 12–14, and 17–19).
PPM1B Represses Pax2-mediated Transcription

Displacement of PTIP correlated with reduced levels of gene expression (Fig. 4, A and B, lanes 3–5 and 9) and reduced H3K4me3 (Fig. 5E, 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. 5D, lane 5) or reduce H3K4me3 (Fig. 5E, 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. 5D, lane 10) and increased H3K4me3 (Fig. 5E, 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. 6A (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. 6B, 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. 6B, 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. 6B, compare lane 4 with lanes 9 and 14), whereas the phosphatase-deficient mutant was incapable of rescuing repression (Fig. 6B, 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. 6F) and recruited PPM1B (Fig. 6E), resulting in lower Rap1A expression (Fig. 6B, 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...
PPM1B Represses Pax2-mediated Transcription

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 blotting (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 2–5) but not in the absence of PPM1B (lanes 6–10). 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, PTP1B 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 PTP1B displacement (lane 9). G, H3K4Me3 is methylated when PTP1B is localized to this Rap1A promoter site. All bars are averages of triplicates with error bars being S.E.
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 integrant.

The results define a model for the epigenetic basis of Groucho/Grg/TLE-mediated gene silencing through the concerted actions of PPM1B and other Grg4-associated proteins that mediated transcriptional silencing.

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