Groucho suppresses Pax2 transactivation by inhibition of JNK-mediated phosphorylation

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Pax proteins are DNA-binding transcription factors that regulate embryonic development through the activation and repression of downstream target genes. The Pax2 gene is absolutely required for kidney development and for patterning specific regions of the nervous system such as the eye, ear and hindbrain. The Pax2/5/8 family of proteins contains both transcription activation and repression domains. The activation domain of Pax2 is phosphorylated by the c-Jun N-terminal kinase (JNK) to enhance Pax2-dependent transcription. In this report, we demonstrate that the Groucho/TLE family protein, Grg4, interacts with Pax2 to suppress transactivation. Grg4 is able to specifically inhibit phosphorylation of the Pax2 activation domain, even in the presence of activated JNK. Furthermore, the Grg4 interaction and suppression of phosphorylation depends on Pax2 binding to its target DNA sequence and is independent of histone deacetylation. These data suggest a new model for Groucho mediated suppression of transcription through the specific inhibition of modifications in the activation domain of a transactivator.
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Introduction

The Pax family of transcription factors are important developmental regulators in a wide variety of tissues across divergent species. In mouse and man, the Pax2 gene is essential for the differentiation of epithelial cells in the urogenital tract, fusion of the optic fissure and patterning the hindbrain and inner ear (Torres et al., 1995, 1996; Favor et al., 1996; Bouchard et al., 2000). The closely related Pax2, Pax5 and Pax8 genes constitute a subfamily that encodes DNA-binding proteins with both repression and activation domains. The Pax2/5/8 activation domain is located at the carboxy terminus and is rich in serine, threonine and proline residues (Dorfler and Busslinger, 1996; Lechner and Dressler, 1996). In addition, the Pax2/5/8 proteins contain a conserved octapeptide with homology to the engrailed-type repressor sequence. The ability of Pax2 to activate transcription is enhanced by phosphorylation of its activation domain by the c-Jun N-terminal kinase (JNK) (Cai et al., 2002). Furthermore, the phenotypes of a JNK1/JNK2 double null embryos exhibit both optic nerve coloboma and kidney hypoplasia (Weston et al., 2003), which is strikingly similar to a Pax2 partial loss of function. Thus, the activity of Pax2/5/8 proteins may be regulated by external stimuli that mediate JNK signaling in the developing kidney or nervous system. However, at least in the case of Pax5, transactivation activity can also be negatively regulated through interactions with the Groucho/TLE family of co-repressors (Eberhard et al., 2000).

The Groucho/TLE family encodes modular, nuclear proteins that interact with DNA binding factors and promote formation of the repressosome, a complex implicated in short and long term repression of transcription (Courey and Jia, 2001). In yeast, the Groucho related protein Tup1 can repress transcription in vitro, presumably through interactions with subunits of the RNA polymerase II holoenzyme. However, more long term repression by Groucho may involve interactions with histone deacetylases (HDACs) and the chromatin silencing machinery (Chen et al., 1999). Expression of the Groucho proteins Grg1, 3 and 4 is widespread during development and overlaps partially with the Pax2/5/8 family in the neural tube and the kidney (Dehni et al., 1995; Koop et al., 1996; Leon and Lobe, 1997; Muhr et al., 2001). In the nervous system, Groucho-mediated repression, through direct interactions with the Nkx family of homeodomain DNA-binding proteins, is necessary for patterning of the embryonic neural tube (Muhr et al., 2001). This spatially controlled repression, used to create boundaries among sets of neural precursor cells, has been termed the derepression strategy for neural cell specification. In the pituitary gland, Groucho/TLE1 binds to the homeodomain protein Hesx1/Rspx to suppress transcription of candidate target genes such as fgf8 and Prop1 (Dasen et al., 2001). However, deacetylation of histones through potential interactions with HDACs is not the only possible mechanism of Groucho-mediated repression, as experiments with deletion mutants (Fisher et al., 1996), deacetylase inhibitors (Chen et al., 1999; Yao et al., 2001) and anti-HDAC antibodies (Dasen et al., 2001) would suggest. Given the importance of Groucho/TLE interactions in the embryo, characterizing the potential biochemical mechanisms of Groucho-mediated repression will have profound implications for understanding patterning and differentiation during development.

In this report, we examine the mechanism of the Groucho protein Grg4 to negatively regulate Pax2-dependent gene activation. Grg4 can completely suppress Pax2-dependent activation of a reporter gene even in the presence of activated JNK. With increasing amounts of Grg4 expression, phosphorylation of the Pax2 activation domain is blocked. The ability of Grg4 to interact with and block Pax2 phosphorylation requires binding of Pax2 to a
DNA target sequence. Thus, the data provide a new mechanism for Groucho-dependent suppression of activation whereby Groucho inhibits phosphorylation of the activation domain of a DNA-bound protein.

**Results**

**Grg4 interacts with the Pax2/DNA complex**

Given the known interaction of the Groucho family protein Grg4 with Pax5 (Eberhard et al., 2000), it was reasonable to postulate that Grg4 may also interact with the related protein Pax2. Within the carboxy terminal 190 amino acids, which contain the activation domain, Pax2 and Pax5 share >67% amino acid identity and >80% amino acid homology. To confirm that Pax2 and Grg4 did interact in cells, whole cell lysates from transfected 293 cells were immunoprecipitated with antibodies against epitope tagged Grg4 and immunoblotted for Pax2 (Figure 1A). Pax2 could be readily detected after immunoprecipitation for Grg4. In order to determine the effects of the Pax2 DNA binding site, PRS4, on the ability to interact with Grg4, we co-transfected the PRS4 containing plasmids prior to immunoprecipitation. The added PRS4 sequence increased the amount of Pax2 that co-precipitated with Grg4 (Figure 1A, lane 4). That Grg4 and Pax2 could interact upon the DNA template was further suggested by electrophoretic mobility shift assay (EMSA) using nuclear lysates from transfected cells (Figure 1B). With the PRS4-labeled sequence as a target, Pax2b transfected 293 cells show two specific shifted complexes (I and II), whereas the Grg4 protein does not exhibit any shifted complexes other than those observed with untransfected 293 cells. However, when Pax2 and Grg4 were present in the lysate, a third higher molecular weight shifted complex was observed (Figure 1B, III). Complex III was only observed when both Pax2 and Grg4 were present and most likely represents an interaction between Pax2, Grg4 and the Pax2 DNA binding site. Since it was difficult to definitively
determine the composition of the protein/DNA complex III, we utilized recombinant Pax2 and Grg4 proteins to confirm their ability to form a complex with the PRS4 DNA sequence (Figure 1C). Poly-His tagged Pax2b was expressed in bacteria and purified by nickel affinity chromatography under denaturing conditions and dialyzed stepwise into binding buffer, whereas GST–Grg4 was purified by glutathione affinity under native conditions. Recombinant Pax2b binds PRS4 with a major shifted complex and several smaller complex present (P, Figure 1C). These smaller complexes may be due to breakdown or cleaved products of the recombinant Pax2b, which are evident on Coomassie blue stained gels from the final, purified fraction (data not shown). Addition of recombinant GST–Grg4 results in a slower mobility complex, presumably due to increased molecular mass of the Pax2/Grg4/DNA complex (P + G, Figure 1C). Also, the addition of Grg4 appears to increase the efficiency of DNA binding in vitro, as much of the free probe is now bound in the presence of both proteins. However, recombinant Grg4 does not bind PRS4 in the absence of Pax2 (Figure 1C, lane 4).

**Grg4 suppresses JNK-dependent Pax2 transcription activation**

We next examined the ability of Grg4 to affect Pax2 mediated transcription activation. Given that the activation of the JNK pathway results in increased Pax2 phosphorylation and Pax2 dependent transactivation (Cai et al., 2002), cells were transfected with Pax2 and its reporter, PRS4-CAT, and activators of the JNK pathway (Figure 2). Three different activators of JNK were utilized. These included the upstream kinase MEKK1, the mixed lineage kinase DLK and the secreted signaling protein Wnt4. While MEKK1 is able to increase Pax2-dependent transactivation from ~10- to 50-fold, transfection of as little as 250 ng of Grg4 plasmid completely inhibits Pax2-dependent activation in this system (Figure 2A). In fact, Grg4 is better able to suppress Pax2 activation in the presence of MEKK1. Given the ability of Groucho proteins to interact with histone deactylases, we examined the effects of the HDAC inhibitor Trichostatin A (TSA) (Yoshida et al., 1995) on Grg4 suppression of Pax2-mediated activation (Figure 2A). TSA was able to increase Pax2-dependent activation ~2-fold in the absence of MEKK1. TSA had little effect when MEKK1 was co-transfected with Pax2. Upon co-expression of Grg4 with Pax2, either with or without MEKK1, inhibition of HDACs did not affect the ability of Grg4 to suppress Pax2-dependent activation (Figure 2A). These data suggest that Grg4 suppression of Pax2 activation is not mediated simply by recruitment of HDACs to the chromatin template. Similar results were obtained if Wnt4 or DLK/JNK (Figure 2B) were used to activate JNK and enhance Pax2 dependent transactivation. In all cases examined, Grg4 completely blocked Pax2-mediated activation of the PRS4-CAT reporter construct. In fact, Grg4 inhibited basal levels of Pax2 dependent activation, in the absence of exogenous JNK activators.

As a control, we examined the effects of Grg4 on the activation of Elk1 or c-Jun dependent transcription using the gal4-dbd fused to the activation domains of Elk1 or c-Jun and a luciferase reporter (Figure 2C). In both cases, co-expression of Grg4 with MEKK1 did not suppress the activation potential of c-Jun or Elk1. Thus, Grg4 did not have a general suppressive effect on transient transfection based activation assays.
Fig. 3. Grg4 inhibits phosphorylation of Pax2 in a DNA-dependent manner. (A) In vivo phospho-labeling of 293 cells after transfection with the indicated plasmids is shown. After 2 h of ^32P labeling, cells were washed and lysates were immunoprecipitated for Pax2 with the anti-HA monoclonal antibody, separated on SDS–PAGE, electroblotted and autoradiographed (top panel). The bottom panel shows the same blot probed with a rabbit anti-Pax2 antibody. Note the reduced levels of Pax2 phosphorylation upon co-expression of Grg4. (B) Steady state levels of Pax2 and P-Pax2 in cells co-transfected with the plasmids as indicated. Protein lysates were assayed by western blotting for Pax2, Grg4, P-JNK and P-c-Jun. Transfections were done in the presence of 1 µg PRS4 containing plasmid (+) or 1 µg of control vector (−), which has deleted the PRS4 sites. Note that increasing amounts of Grg4 reduce the levels of the wild-type P-Pax2 (arrow) but only in the presence of the PRS4 plasmid. The Pax2 DNA binding mutants, Q40P and C57Y, are phosphorylated in response to MEKK1 but do not exhibit reduced P-Pax2 levels with increasing Grg4 even in the presence of PRS4 vector. Grg4 has no effect on P-c-Jun levels. (C) Single point mutations in the Pax2 paired domain inhibit DNA binding. Increased amounts of nuclear extracts from cells transfected with wild-type (lanes 1–3) or Pax2 paired domain mutations (Q40P, lane 4–6; C57Y, lane 7–9) were used for EMSAs. Wild-type Pax2 shows two shifted species (I and II) and very little free probe. Only a faint shift (II) is observed with either mutant and the amount of free probe is not reduced significantly. (D) Pax2 DNA binding mutants are efficient substrates for JNK. In vitro kinase reactions using recombinant activated JNK and lysates from cells transfected with either wild-type Pax2 or DNA binding mutants are shown. The arrow points to higher migrating, phosphorylated form of Pax2. (E) The Pax2 DNA binding mutants Q40P and C57Y locate to the nucleus in transfected cells by immunostaining with anti-Pax2 antibodies.

Inhibition of Pax2 phosphorylation by Grg4 is DNA dependent

To more closely examine the mechanism of Grg4 mediated suppression, we examined the effect of Grg4 on the status of Pax2 phosphorylation (Figure 3). The level of Pax2 phosphorylation was observed in 293 cells by a 2 h in vivo labeling with ^32P after transfection (Figure 3A). As previously published, MEKK1 significantly elevated levels of phosphorylated Pax2 (P-Pax2). Co-expression of Grg4 reduced levels of P-Pax2 on average 5–10-fold under the conditions used for this assay.

To examine the dose dependence and other parameters of the effects on Pax2 phosphorylation, we used the western blotting assay to measure the relative amounts of P-Pax2 in transfected 293 cells. As previously reported (Cai et al., 2002), phosphorylation of Pax2 by JNK results in an indicative shift in apparent molecular weight. Using the upstream kinase MEKK1, the hyperphosphorylated form of Pax2 is readily observed as an ~50 kDa species migrating larger than the hypophosphorylated 48 kDa Pax2 isoform (Figure 3B). As the amount of Grg4 expression vector is increased, the amount of phosphorylated Pax2 (P-Pax2) clearly decreases. However, this decrease in P-Pax2 is only observed in the presence of excess PRS4-containing plasmid. By co-transfecting either PRS4-CAT or PRS4 cloned into Bluescript (Stratagene Inc.), Grg4 is able to inhibit P-Pax2 levels. Using vectors alone that have deleted the PRS4 sites, little decrease in P-Pax2 levels is observed. Furthermore, there is no inhibition of JNK activity as phospho-c-Jun levels are unaffected by Grg4. Similar results were obtained using DLK/JNK to phosphorylate Pax2 in the presence or absence of PRS4 (data not shown). These data indicate that Grg4 can inhibit or reduce the steady-state levels of P-Pax2 and that this effect is enhanced by the presence of excess Pax2 DNA binding sites.

If Pax2 must first bind to DNA in order for efficient interaction with Grg4 in vivo, then mutations in the Pax2 paired domain that inhibit DNA binding may not show Grg4 suppression of phosphorylation. While Pax2 point mutants that affect DNA binding have not been well described, the Pax2 and Pax8 paired domain are >95%
Fig. 4. Co-expression of Pax2 and Grg4 in the embryonic kidney and neural tube. Immunostaining with anti-Pax2 (red, A and C) or anti-Grg4 (red, B and D) and E-cadherin (green, A–D) was performed on E15.5 embryos. Sagittal sections through the kidney (A and B) and spinal cord (C and D) are shown. (A) Pax2 localizes to the peripheral mesenchyme and early polarized renal vesicles (arrowhead) and is down-regulated in the proximal part of the s-shaped body (arrow). The dashed line marks the periphery of the developing kidney, with the embryonic pancreas (P) above. (B) In an adjacent kidney section, low levels of Grg4 staining are observed in peripheral and interstitial mesenchyme, with increasing protein levels in polarized renal vesicles (arrowhead) and s-shaped bodies (arrow). (C) In the neural tube, Pax2 expression is seen in many post-mitotic interneurons along the dorsal half of the intermediate zone. (D) Adjacent section to C showing the domain of Grg4 positive cells. (E) Immunoprecipitation of Grg4 and Pax2 from kidney (Kid), neural tube (NT) and liver (Liv) nuclear extracts prepared from E16 tissues. Nuclear extracts were immunoprecipitated with either anti-Grg4 (left panels) or anti-Pax2 (right panels) as indicated. The immunoprecipitates were then blotted for Pax2 (top panels) or Grg4 (bottom panels) as indicated. Pax2 is easily immunoprecipitated from kidney and neural tube and runs just below the IgG heavy chain (top right). Pax2 can also be seen in kidney and neural tube after immunoprecipitation with Grg4 (arrow, top left). Grg4 is immunoprecipitated from all three tissues (bottom left). Grg4 can also be seen co-precipitating with anti-Pax2 in neural tube and faintly in kidney (arrow, bottom right). Note that Grg4 is not detected after immunoprecipitation of liver extracts with anti-Pax2, indicating that co-precipitation is dependent on the presence of Pax2 protein.

homologous. Inactivating mutations in the DNA binding domain of Pax8 have been characterized (Macchia et al., 1998). Thus, two DNA binding mutants were generated in Pax2, based on naturally occurring Pax8 mutations mapped in humans (Congdon et al., 2001; Vilain et al., 2001). The glutamine to proline mutation at position 40 (Q40P) or the cysteine to tyrosine mutation at position 57 (C57Y) significantly inhibited the ability of Pax2 to bind to the PRS4 sequence (Figure 3C). As predicted, neither Q40P nor C57Y were able to transactivate the PRS4CAT reporter in the presence or absence of MEK1 (data not shown). However, both mutant proteins were still phosphorylated with similar efficiency as wild-type Pax2 in an in vitro kinase assay using purified JNK (Figure 3D). Strikingly, in transfected cell lysates, using increasing amounts of Grg4 and the upstream activator MEK1, Grg4 had no effect on the levels of phosphorylated Q40P or C57Y (Figure 3B). Similar amounts of hyperphosphorylated mutant Pax2 proteins were observed even with as much as 1.0 μg of Grg4 expression plasmid. This was in contrast to the marked inhibition of wild-type Pax2 phosphorylation by Grg4 (Figure 3B). As Grg4 is a nuclear protein, we tested to make sure both Pax2 DNA binding mutants were still able to locate to the cell nucleus (Figure 3E). Taken together, the data suggest that Grg4-mediated inhibition of Pax2 phosphorylation requires binding of Pax2 to its DNA target sites.

Co-localization of Pax2 and Grg4
In order to examine the potential Pax2/Grg4 interaction in vivo, we utilized polyclonal antibodies for immunostaining and immunoprecipitation (Figure 4). Grg4 expression is widespread in the embryo, but exhibits local areas with high nuclear protein levels. In the developing kidney at E15.5, mesenchymal cells at the periphery of the tissue are aggregating around the tips of the ureteric bud epithelia and forming polarized vesicles (for review see Dressler, 2002). The polarized vesicles are the precursors of the s-shaped bodies and generate much of the glomerular, proximal tubular and distal tubular epithelia. As described (Dressler and Douglass, 1992; Ryan et al., 1995), Pax2 protein is found in the mesenchymal aggregates, the polarized vesicles and in the part of the s-shaped bodies. Grg4 is found at low levels in much of the mesenchyme at the periphery and within the interstitium. Grg4 levels are slightly higher in the polarized vesicles and are maximal in the proximal portion of the s-shaped body, just at a time when Pax2 begins to be down-regulated. Based on their characteristic position, the cells expressing maximal amounts of Grg4 are the precursors of the glomerular podocytes.

In the neural tube Pax2 expression is complex, but is generally restricted to post-mitotic neurons as they populate discrete regions of the spinal chord (Burrill et al., 1997). At E15.5, high levels of Pax2 and Grg4 protein co-localize in the dorsal half at a time when Pax2 positive interneurons have migrated from the ventricular zone (Figure 4C and D). Grg4 expression overlaps well with Pax2 in this region of the neural tube. The same antibodies were used to immunoprecipitate Pax2 or Grg4 from embryonic nuclear lysates made from E16 kidneys, neural tube or liver (Figure 4E). Immunoprecipitation with anti-Grg4 followed by western blotting with anti-Pax2 indicated Pax2 protein co-precipitating with Grg4 in both
kidney and neural tube extracts. The reciprocal immunoprecipitation with anti-Pax2 indicated Grg4 co-precipita-
tion in the neural tube and with a very weak signal present
in kidney extracts. Even though high levels of Grg4 are
present in liver, no Grg4 signal was detected after anti-
Pax2 immunoprecipitation of liver extracts indicating that
the Grg4 signal observed in neural tube was dependent of
Pax2 protein being present. These data indicate that Pax2
and Grg4 can co-localize in both neural tube and kidney,
where Pax2 activity is known to be important. Furthermore,
ate least some fraction of Pax2 and Grg4 can associate in nuclear extracts from these tissues.

**Discussion**

Previously, we have shown that activation of JNK by
upstream kinases or Wnt signaling enhanced Pax2 phos-
phorylation and transactivation potential (Cai et al., 2002).
Grg4 is able to specifically block Pax2 phosphorylation
and suppress transactivation, even in the presence of
activated JNK. In the absence of exogenous JNK activators,
Grg4 suppress Pax2-dependent activation below basal levels. The base level of Pax2 activation in
293 cells may also be due to low levels of P-Pax2, detected
after longer exposures (Cai et al., 2002), as low
endogenous JNK activity is still present. This phosphory-
alation of Pax2 by JNK may be an important regulator of
activity is further underscored by the phenotype of JK1/
JK2 double mutant mice (Weston et al., 2003), which
exhibit optic nerve coloboma and renal hypoplasia in a
manner very reminiscent of a Pax2 hypomorphic allele or
Pax2 gene dosage reduction (Sanyanusin et al., 1995;
Schimmenti et al., 1995; Torres et al., 1995, 1996; Favor
et al., 1996).

The data presented in this report point to a model
whereby Grg4 inhibits Pax2 phosphorylation once Pax2 is
bound to DNA, or recruits specific phosphatases to
deactivate Pax2. This implies that Pax2 is phosphorylated
in the nucleus by nuclear kinases, such as activated JNK.
This event must be independent of DNA binding, as the
data with our Pax2 mutants indicate. Once bound to Pax2,
Gr4 could recruit other factors known to associate with
the repressosome to block access of JNK to the Pax2
activation domain and thus inhibit Pax2 transactivation.
The ability of Gr4 to suppress the activation potential of
Pax2 provides a new level of control for regulating genes
transiently induced by Pax2, such as GDNF (Brophy et al.,
2001) and WT1 (Dehi et al., 1996). This mechanism
could account for rapid short-term repression of Pax2
target genes within the kidney or nervous system once
Gr4 is expressed. Such short term repression may be
independent of HDACs. While this presumes Pax2 to be
an exclusive activator of transcription, there may be
additional targets of Pax2 that are normally repressed
through these interactions with Groucho. The related gene
Pax5 both activates and represses different genes within
the same lymphoid precursor lineage (Nutt et al., 1998,
1999), yet it is unclear how Pax5 can distinguish among
this set of target genes within the same cell. Our data argue
that DNA binding is essential for the Pax2/Groucho
interaction, suggesting that the paired domain binding to a
DNA target alters or stabilizes the conformation of
Pax2 and allows Groucho to recognize protein–protein
interaction domains. Given the size and diversity of paired
domain DNA binding sequences, whether the Pax2/
Groucho interaction depends on the type of DNA target
sequence must be considered.

These data suggest that the Pax2/Gr4 interaction can
occur on the DNA template and is in fact strengthened
when Pax2 is bound to DNA. In our EMSAs with nuclear
lysates from Pax2 transfected cells, the identities of the
shifted species I and II are not entirely clear, though in the
presence of Gr4 the species II disappears more readily
while the levels of species I are slightly enhanced. This
could indicate preferential binding of Gr4 with the
species II complex. Given the differences in motility,
species II may represent two molecules of Pax2 bound to
the optimal PRS4 sequence and this could favor an
interaction with Gr4. Alternatively, the shifted species I
and II could be two different conformations of the Pax2
protein or the result of proteolytic processing during
preparation. That Pax proteins undergo conformational
changes upon binding to DNA has been observed
previously with Pax6 using circular dichroism spectro-
scopy (Epstein et al., 1994). The interaction of Gr4 with
Pax5 is complex and involves both the Pax5 octapeptide
and the transactivation domain (Eberhard et al., 2000).
Thus, the conformation of Pax2 protein may determine
how accessible these sites are to Gr4 interaction. We have
confirmed the Pax2/Gr4/DNA interaction using recombi-
nant proteins purified from Escherichia coli. These
*in vitro* assays with recombinant proteins also suggest that
Gr4 enhances binding of Pax2 to the PRS4 sequence, yet
this could not explain the decreased level of transcription
activation observed *in vitro*. To date, no evidence for
Groucho modification of Pax DNA binding activity has
been observed *in vivo*.

While the tissue-specific expression of Pax2/5/8 is
precisely regulated to ensure correct patterning during
development, the amount of Pax activity is also essential,
as too little or too much can be detrimental. Reduced
activity in heterozygous mice and humans is commonly
associated with hypoplasia in the kidney, eye and neural
crest (for review see, Chi and Epstein, 2002). Conversely,
increased levels of Pax6 activity in the eye, due to multiple
gene copy number, also disturbs normal development
(Shedl et al., 1996). In the kidney, down-regulation of
Pax2 expression is evident as renal epithelial progenitor
cells differentiate and exit mitosis. Ectopic or persistent
expression of Pax2 is associated with a variety of renal
diseases such as Wilms’ tumor (Dressler and Douglass,
1992; Eccles et al., 1992), renal cell carcinoma (Gnarra
and Dressler, 1995; Igarashi et al., 2001) and polycystic
kidney (Ostrom et al., 2000). The Groucho proteins could
play an important role in limiting the activity of the Pax2/
5/8 family during terminal differentiation. Thus, under-
standing the biochemical mechanisms underlying
Groucho-mediated inhibition of Pax gene function may
provide novel avenues for therapeutic interventions.

**Materials and methods**

**Plasmids**

The CMV-Pax-2HA and PRS4-CAT reported plasmid, which contains
five tandem copies of the Pax2 binding site, PRS4, cloned upstream of the
herpes simplex virus thymidine kinase promoter, were constructed as
described previously (Fickenscher et al., 1993). The CMV-MEK1 wild-type expression plasmid was a gift from Dr B. Margolis (University of Michigan). The HA-DLK, Flag-JNK, HA-epitope tagged N-terminal of JIP were provided by Dr Lawrence B. Holzman (University of Michigan). The pWnt4 cDNA (gift from A. McMahon) was inserted upstream of the CMV expression plasmid CB6. A partial Grg4 cDNA (gift from C. Lobe) was spliced with a 5’ Grg4 PCR product amplified from an embryonic kidney cDNA library and cloned into pFLAG-CMV2. All expression plasmids were sequenced for verification.

**Antibodies**

The antibodies used were anti-Pax2 (1:3000; Zymed Labs.), anti-Flag (M2 Monoclonal Antibody; Sigma, 1:2000), anti-HA (16B12; Covance, 1:2000), Anti-Myc (9E10; Covance, 1:2000), anti-Phospho-JNK (Thr183/Tyr185; Cell Signaling Technology, 1:2000) and anti-Phospho-c-Jun (Ser63; Cell Signaling Technology).

**Transient transfection**

HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium (glucose concentration, 450 mg/dl) supplemented with heat-inactivated 10% fetal bovine serum and 100 U/ml penicillin and 100 mg/ml streptomycin under humidified 5% CO₂/95% air at 37°C. Subconfluent cells were transfected using 2–3 μg of plasmid DNA and 0.6 μg of FuGene 6 as described by the manufacturer (Roche Molecular Biochemicals). For 5 × 10⁶ of 293 cells, 0.6 μg of Flag reporter plasmid was co-transfected with various doses of effector plasmid and 0.25 μg of pCH110, a β-galactosidase reporter plasmid used to monitor transfection efficiency. Forty hours after transfection, cells were harvested and extracts were prepared and normalized for transfection efficiency. CAT analysis was performed essentially as described previously (Cai et al., 2002). For HDAC inhibition, 100 ng/ml TSA (1 mg/ml stock in ethanol, Sigma) was added 20 h post transfection and the cells harvested after an additional 20 h. Percent acetylation was determined by scintillation counting, and a value of 1.0 was arbitrarily assigned to control parental plasmids. Each experiment was repeated three times and the results presented as average ± 1 SD from the mean. Luciferase assays were done with the PathDetect (Stratagene Inc.) signal transduction reporting system according to the manufacturer’s protocols.

**Immunoprecipitation, immunostaining and western blotting**

For immunoprecipitation, cells were transfected with the expression plasmids as indicated in the legends. After 48 h, lysates were prepared in IP buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 0.5% Triton X-100, 10 mM NaF, 1 mM Na₂VO₃, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture). Lysates were incubated with desired antibodies for 2 h at 4°C. Antibodies were captured with protein G-Sepharose for 1 h and washed three times with IP-wash buffer (same as IP buffer except 0.1% Triton X-100). Proteins were eluted from protein G-Sepharose by boiling in 2× SDS-PAGE sample buffer and analyzed by SDS–PAGE. Western blots were performed with equal amounts of protein obtained by lysis of transiently transfected cells in PK lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM Na₂VO₃, 50 mM NaF, 1% Triton X-100). 10% glycerol was used to mix the protease inhibitors (Roche Molecular Biochemicals, cat 1836170). The lysates were separated by SDS–PAGE and immunooblotted with antibodies indicated in the Figure legends. Horseradish peroxidase-conjugated secondary antibodies were used to detect antigen–antibody complexes, which were visualized with an ECL detection system (Amersham).

Embryonic lysates were made from microdissected tissues by first isolating nuclei with a sucrose step-gradient as described (Phelps and Dressler, 1996) and lysing nuclei in IP buffer. Incubation with primary antibodies against Pax2 or Grg4 were done overnight at 4°C followed by 2 h with protein A/G sepharose and three washes with 1 ml IP buffer.

For immunocytochemistry, embryos were dissected free of extra-embryonic tissue, fixed for 1 h in 4% paraformaldehyde/PBS washed 2 × 30 min in PBS, and perfused overnight in 0.5 M sucrose/PBS at 4°C. Tissues were frozen, sectioned and air dried. Optimal Grg4 staining was observed after a 10 min fixation in methanol at −20°C. Sections were rinsed in PBS/0.1% Tween-20 and incubated with primary antibodies for 2 h. After additional washes, fluorescent-conjugated secondary antibodies (Sigma) were used to visualize Pax2 (Dressler and Douglass, 1992), Grg4 (Yao et al., 1998), and E-cadherin (mouse monoclonal, Transduction labs). Sections were photographed with a Nikon E8000 fluorescent microscope using a digital Spot camera.

**In vivo labeling**

293 cells were transfected with expression plasmids as indicated in the legends. The cells were cultured in phosphate-free Dulbecco’s modified medium for 2 h after 40 h of transfection, and then labeled with 200 μCi/ml [3H]orthophosphate for 2 h. Lysates were prepared in IP buffer and incubated with anti-HA antibodies at 4°C for 2 h. The immunocomplexes were adsorbed to protein G-Sepharose and were subjected to SDS–PAGE followed by blotting onto nitrocellulose and autoradiography. Quantiﬁcation was achieved by using PhosphorImager. The amount of Pax2 that was immunoprecipitated was assessed by re-immunoblotting of the PVDF membranes.

**EMSAs**

A 25 bp PRS fragment was excised from the PRS4-Cat vector via SpeI digestion. The fragment was puriﬁed and isolated on a 4% Nuse gel and end labeled with [32P]dCTP via the Klenow ﬁll in reaction. Nuclear extracts from transfected 293 cells were made 40 h post transfection as described (Phelps and Dressler, 1996) and quantiﬁed with the Bradford assay. Binding reactions were performed in a total volume of 15 μl for 30 min at room temperature and contained increasing amounts of nuclear extract protein (0, 250 and 500 ng), 100 ng poly (dl-dc), 0.25-labeled probe (20 000 d.p.m.). Free DNA and DNA–protein complexes were resolved at room temperature on 4 or 6% neutral polyacrylamide gels in 0.5X TBE at 120 V.

Recombinant proteins were expressed in BL21 E. coli at 30°C after induction with IPTG. After lysis and sonication, GST fusion proteins were puriﬁed using glutathione agarose and eluted with 5 mM reduced glutathione. His-tagged proteins were puriﬁed under denaturing conditions using 6 M GHCl and nickel afﬁnity chromatography. His-tagged proteins were eluted at low pH and dialyzed stepwise into 4, 2 and 1 M urea in PBS, 0.1% NP-40 before final dialysis into Z-buffer (20% glycerol, 25 mM HEPES pH 7.8, 12.5 mM MgCl₂, 1 mM DTT, 0.1 M KCl, 0.1% NP4).

**Site-directed mutagenesis**

Two different substitutions were made in the CMV-Pax2b plasmid utilizing the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) as per the manufacturer’s directions. Speciﬁcally, a point mutation was introduced using an HPLC puriﬁed set of primers. Sequencing analysis conﬁrmed that the substitutions had been introduced.

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