

Ptf1a Directly Controls Expression of Immunoglobulin Superfamily Molecules Neph3 and Neph1 in the Developing Central Nervous System^{*[5]}

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Ptf1a, a basic helix-loop-helix transcription factor, plays an indispensable role for cell fate specification of subsets of neurons in the developing central nervous system. However, downstream molecules induced by Ptf1a during neural development have not been well characterized. In the present study, we identified immunoglobulin superfamily molecules, Neph3 and Neph1, as direct downstream targets of Ptf1a. First, the expression domains of *Neph3* and *Neph1* closely resembled those of *Ptf1a* in the developing retina, hypothalamus, cerebellum, hindbrain, and spinal cord. Second, Ptf1a bound directly to a PTF-binding motif in the 5'-flanking region of *Neph3* and *Neph1* genes. Third, Ptf1a activated transcription driven by the 5'-flanking region of these genes. Finally, the expression of *Neph3* and *Neph1* was lost in *Ptf1a*-null mice, whereas ectopic expression of *Neph3* and *Neph1* was induced by forced expression of *Ptf1a*. We provided further evidence that Neph3 and Neph1 could interact homophilically and heterophilically, suggesting that Neph3 and Neph1 might regulate certain developmental aspects of Ptf1a-positive neurons as homo- or heterooligomers.

Ptf1a,³ which encodes a basic helix-loop-helix transcription factor, was first identified as a cell fate determinant of the pancreas (1, 2). Mutations in the human and mouse *Ptf1a* result not only in the malformation of the pancreas but also in cerebellar agenesis, indicating its involvement in neuronal development (3, 4). Recent studies demonstrated that Ptf1a is expressed in

neuronal progenitors in the retina, cerebellum, hindbrain, and spinal cord, most of which are fated to be subsets of GABA (γ -aminobutyric acid)-ergic and glycinergic inhibitory neurons (4–10). In the *Ptf1a*-null cerebellum, Ptf1a-positive progenitors, which are normally fated to inhibitory neurons, trans-fate to excitatory granule cells (8). Cell fate changes in *Ptf1a*-null mice were also reported in the retina, hindbrain, and spinal cord (5–7, 9). Conversely, ectopic expression of *Ptf1a* in neural progenitors of the dorsal telencephalon, which are normally fated to glutamatergic excitatory neurons, confers inhibitory GABA characteristics to these neurons (4). These loss- and gain-of-function experiments suggest that Ptf1a acts as a cell fate switch in the central nervous system. However, how Ptf1a operates to fulfill such a critical role is not known because of a lack of understanding of its downstream targets.

In the present study, we searched for direct downstream targets of Ptf1a, which is likely to be responsible for cell fate specification of Ptf1a neurons. We identified Neph3 and Neph1 as candidates of Ptf1a target molecules. Neph3 and Neph1 are transmembrane proteins of the immunoglobulin superfamily. *Neph3* was originally isolated as a gene responsible for the congenital nephrotic syndrome of the Finnish type (11). Neph3 belongs to Neph family, which is composed of Neph1, Neph2, and Neph3 (also called as Kirrel, Kirrel3/mKirrel, and Kirrel2/Filtrin, respectively), and Neph family members are structurally related to Neph3 (12). Neph3 and Neph family members can interact homophilically, and in some cases heterophilically, which in turn triggers cell adhesion or signal transduction (13–19). Recent reports showed expression of Neph3 and Neph family molecules in the developing central nervous system (17, 18, 20–22). Although their function in vertebrate neural development is still unclarified, their invertebrate homologs appear to play indispensable roles in synapse formation or axon guidance during development (23, 24).

Here, we provide evidence that Ptf1a directly controls the expression of *Neph3* and *Neph1*. Moreover, Neph3 and Neph1 interacted homophilically and heterophilically. These findings suggest that Neph3 and Neph1, via their interaction, play important roles in Ptf1a-positive neuronal progenitors.

EXPERIMENTAL PROCEDURES

Vectors—*pCAG*, *pCAG-EGFP*, *pCAG-mCherry*, *pCAG-IRES-EGFP*, and *pEF-RL* vectors were described previously

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–6.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB513652.

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³ The abbreviations used are: Ptf1a, pancreas transcription factor; AP, alkaline phosphatase; DIG, digoxigenin; E followed by number, embryonic day; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; EGFP, enhanced GFP; HA, hemagglutinin; GABA, γ -aminobutyric acid.

Nephrin and Neph3 Are Downstream Targets of Ptf1a

(25–29). Full-length *NephrinB* cDNA was cloned from mouse E13.5 cerebellum, and its sequence was deposited into DDBJ with accession number AB513652. For construction of expression vectors of *NephrinA* (87–3857 of NM_019459), *NephrinB* (1–3729 of AB513652), and *Neph3* (130–2232 of BC052773), cDNAs were subcloned into *pCAG* vector. A HA or FLAG sequence was inserted between 200 and 201 of *NephrinA* (NM_019459), between 72 and 73 of *NephrinB* (AB513652), and between 189 and 190 of *Neph3* (BC052773). For construction of expression vectors of *Ptf1a* (199–1173 of NM_018809), *E47* (87–2033 of AF352579), *RBPJ* (217–1716 of NM_009035), *N-Twist* (66–572 of NM_033522), *Math1* (196–1251 of NM_007500), and *Neurogenin1* (232–966 of NM_010896), cDNAs were subcloned into *pCAG* or *pCAG-IRES-EGFP*. A HA, FLAG, or Myc tag was added in their N termini by PCR. *pGL2-basic* vector was obtained commercially (Promega). The 5'-flanking region of *Nephrin* and *Neph3* genes was amplified by PCR from genomic DNA of ICR mice and subcloned into *pGL2-basic*. The following primers were used for PCR amplification: primer 1 (5'-ATG CAA GCT TCA GAG TGC CAG GAA AGG G-3'), primer 2 (5'-GAC CAA GCT TTG TGT GTA CCC CAA GAT C-3'), and primer 3 (5'-GAC CAA GCT TGT TTG TTG CTG TC-3'). For amplification of the 1.6-kb sequence upstream of the *Neph3* start codon (see Fig. 4A), primers 1 and 2 were used as forward and reverse primers, respectively. For the 1.6-kb sequence upstream of the *NephrinB* start codon or 3.3 kb upstream of the *NephrinA* start codon (see Fig. 4B), primers 2 and 1, or primers 2 and 3 were used as forward and reverse primer, respectively. The E-box and the TC-box mutations were introduced by PCR-based site-directed mutagenesis.

Antibodies—Anti-Ptf1a antibody was described previously (9, 30). Anti-FLAG (M2, Sigma), anti-Myc (9E10, Santa Cruz Biotechnology), anti-HA (3F10, Roche Applied Science), alkaline phosphatase (AP)-conjugated anti-mouse IgG (Bio-Rad), AP-conjugated anti-rat IgG (Jackson Laboratories), AP-conjugated anti-digoxigenin (DIG, Roche Applied Science), peroxidase-conjugated anti-DIG (Roche Applied Science), anti-GFP (Molecular Probes), and biotin-conjugated anti-rabbit IgG (Jackson Laboratories) antibodies were obtained commercially.

In Situ Hybridization and Immunohistochemistry—The *Ptf1a^{cre}* mouse line was described previously (1). *In situ* hybridization was performed using DIG-labeled RNA probes of *Nephrin* (2738–3764 of NM_019459) and *Neph3* (1037–2129 of BC052773). For fluorescent detection, samples were incubated with a peroxidase-conjugated anti-DIG antibody, biotinylated-tyramide (PerkinElmer Life Sciences), and Alexa Fluor 594-streptavidin (Molecular Probes). To couple with immunostaining, sections were first hybridized with DIG-RNA probes and immunostained with an anti-Ptf1a (30) or an anti-GFP antibody.

In Utero Electroporation—*In utero* electroporation into the cerebral cortex of E14.5 mouse embryos was described previously (26). Expression vectors were introduced into E14.5 ICR mice, and pregnant mice were killed 1 or 2 days later for further analyses.

Chromatin Immunoprecipitation Assay—Cerebellum from E11.5 ICR mice was dissected out, and chromatin immunoprecipitation assay was performed as described previously (31).

Endogenous Ptf1a was immunoprecipitated with an anti-Ptf1a antibody (9). The amount of co-precipitated DNA fragments was analyzed by quantitative PCR using a LightCycler (Roche Applied Science). Primers used in quantitative PCR were as follows: PTF1 motif forward (5'-GCC AGG AGT TCA GAT TTA GGT G-3') and reverse (5'-AGG ATG GGA AAA CCA ACA GAC-3'); 1.5 kb 5' of the PTF1 forward (5'-TCC CTC TCA CCC ACT CAC AG-3') and reverse (5'-AGT TCA CAC TGG GTC CAA GC-3'); 1.5 kb 3' of the PTF1 forward (5'-TAC ACA CAC TCG GGA TGC TG-3') and reverse (5'-CCA AAC AGG GCT GTA ATG GAC-3').

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed following the manufacturer's protocol in the Light-Shift Chemiluminescent EMSA kit (Pierce). Nuclear extracts of *HA-Ptf1a*-, *FLAG-E47*-, or *Myc-RBPJ*-transfected COS7 cells were prepared as described previously (32). Forward and 3'-biotinylated reverse oligonucleotides were purchased commercially (GeneDesign). The sequence of oligonucleotides is shown in Fig. 3B. Nuclear extracts were incubated with 200 fmol of annealed biotinylated oligonucleotides. For the supershift assay, the nuclear extracts were preincubated with anti-HA, anti-FLAG, or anti-Myc antibody. Biotinylated oligonucleotides were detected by streptavidin-AP (Jackson Laboratories) and an AP substrate, CDP star (Roche Applied Science), and chemiluminescence was analyzed by RAS3000 mini (Fuji film).

Luciferase Reporter Assay—Luciferase reporter assay was performed following the manufacturer's protocol in the Dual Luciferase Reporter Assay system (Promega). *pGL2-basic* vector constructs and *pEF-RL* with or without *pCAG-Ptf1a* were transfected into COS7 cells by FuGENE 6 (Roche). 48 h later, the activity of the firefly and *Renilla* luciferases was analyzed by Luminometer (Promega).

Affinity Probe in Situ Assay—An affinity probe *in situ* assay using Neph3-AP was performed as described previously (18). Binding of Neph3-AP was detected by AP activity using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrates. To measure the binding affinity of Neph3-AP to COS7 cells transfected with *HA-Neph3* or *HA-NephrinA* expression vector, cells were incubated with an AP substrate, *p*-nitrophenyl phosphate, for 2 h. Increase of A_{405} in each sample relative to that in mock-transfected samples was analyzed.

Immunoprecipitation and Western Blotting—COS7 cells were transfected with expression vectors by FuGENE 6 or a standard calcium phosphate method. Immunoprecipitation and Western blotting were performed as described previously (13).

RESULTS

Nephrin and Neph3 Genes Have a PTF1-binding Motif in Their 5'-Flanking Region—Ptf1a is a component of a trimeric transcription factor, PTF1, which interacts with the PTF1-binding motif, comprising an E-box (CAG/CCTG) and a TC-box (TTTCCC) separated by one or two helical turns (33–35). In an attempt to identify novel downstream targets of Ptf1a, we took advantage of a data base, DBTSS, to screen for genes containing PTF1 motif in their 5'-flanking regions. As a result, we found a PTF1 motif located in the shared 5'-flanking region of *Nephrin* and *Neph3* genes on mouse chromosome 7 (Fig. 1).

Mouse chromosome 7

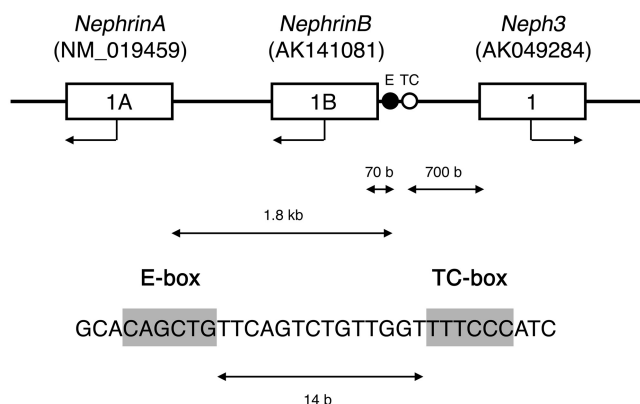


FIGURE 1. PTF motif found in the 5'-flanking region of *Nephrin* and *Neph3* gene. Black and white circles on the upper line indicate E-box and TC-box of the PTF1 motif, respectively. The sequence of the PTF motif is shown in the bottom.

The *Nephrin* gene has two isoforms, *NephrinA* and *NephrinB*, resulting from utilizing alternative first exons, 1A and 1B. Thus, the PTF1 motif is located 700 bases upstream of the first exon of *Neph3*, and 70 bases and 1.8 kb upstream of exon 1B and exon 1A of *Nephrin*, respectively.

Nephrin and *Neph3* Are Expressed in *Ptf1a*-positive Domains in the Developing Central Nervous System—If *Nephrin* and *Neph3* are downstream targets of *Ptf1a*, they would be expressed in *Ptf1a*-positive domains. To examine this possibility, we first analyzed the expression of *Nephrin* and *Neph3* in the developing central nervous system. *Nephrin* and *Neph3* were expressed in overlapping regions where *Ptf1a* is expressed, in the cerebellum and hindbrain at E12.5, in the spinal cord at E10.5, and in the retina and hypothalamus at E13.5 (Fig. 2, supplemental Fig. 1, and data not shown). Many *Ptf1a*-positive cells appeared to co-express *Nephrin* and *Neph3* (Fig. 2). Moreover, *Nephrin* and *Neph3* were co-expressed in the same cells, which was confirmed by double labeling of *Nephrin* and *Neph3* (data not shown). We further examined the expression of two isoforms of *Nephrin* in the developing cerebellum by quantitative PCR. Consistent with the previous report (36), comparable amounts of endogenous *NephrinA* and *B* were expressed in the developing cerebellum, whereas adult kidney predominantly expressed *NephrinA* (supplemental Fig. 2). These results raise the possibility that *Ptf1a* may control the expression of *Nephrin* and *Neph3*.

Ptf1a Binds to the Promoter Region of *Nephrin* and *Neph3* Genes That Contains PTF1 Motif—Because *Nephrin* and *Neph3* genes contain the PTF1 motif in their 5'-flanking region, it is highly possible that *Ptf1a* controls the transcription of these genes by direct binding to the PTF1 motif. We examined this possibility by chromatin immunoprecipitation assay to test whether *Ptf1a* in the developing cerebellum associates with the 5'-flanking region of *Nephrin* and *Neph3* genes *in vivo*. Anti-*Ptf1a* antibody immunoprecipitated DNA fragments centered on the PTF1 motif, but not 1.5 kb upstream or downstream of the motif (Fig. 3A). This interaction could also be detected in *Ptf1a*-overexpressed cerebral cortex (supplemental Fig. 3). We further examined whether the interaction could be reconsti-

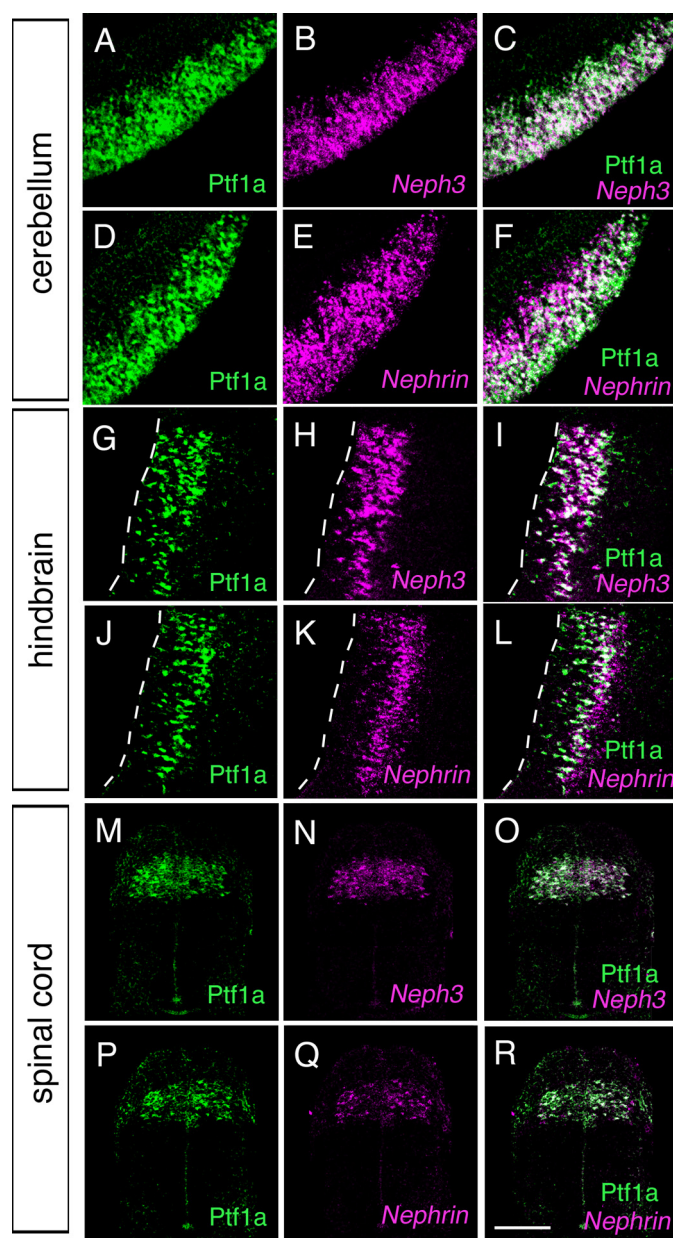


FIGURE 2. Expression of *Ptf1a*, *Neph3*, and *Nephrin* in the developing cerebellum, hindbrain, and spinal cord. Immunostaining by anti-*Ptf1a* (A, C, D, F, G, I, J, L, M, O, P, and R), together with *in situ* hybridization of *Neph3* (B, C, H, I, N, and O) or *Nephrin* (E, F, K, L, Q, and R) on coronal sections of E12.5 cerebellum (A–F), E12.5 dorsal hindbrain (G–L), and E10.5 spinal cord (M–R) are shown. Dorsal is upward. In the cerebellar sections (A–F), midline is toward the right, and lateral is toward the left. In the sections of the hindbrain (G–L), medial is toward the left. Scale bar, 100 μm.

tuted *in vitro*. Beres *et al.* (37) reported that PTF1 complex in the pancreas comprised *Ptf1a*, a class A basic helix-loop-helix protein, such as E47 or HEB, and RBPL. In the developing central nervous system, RBPJ instead of RBPL is expressed and interacts with a PTF1-binding motif *in vitro* (37, 38). Thus, we performed EMSA using oligonucleotides containing the PTF1 motif and a combination of *Ptf1a*, E47, and RBPJ (Fig. 3B). RBPJ or an E47 dimer could bind the PTF motif alone. However, addition of all three proteins resulted in a new shifted band associated with a heterotrimeric complex composed of *Ptf1a*/E47/RBPJ. The presence of *Ptf1a*/E47/RBPJ trimer in the

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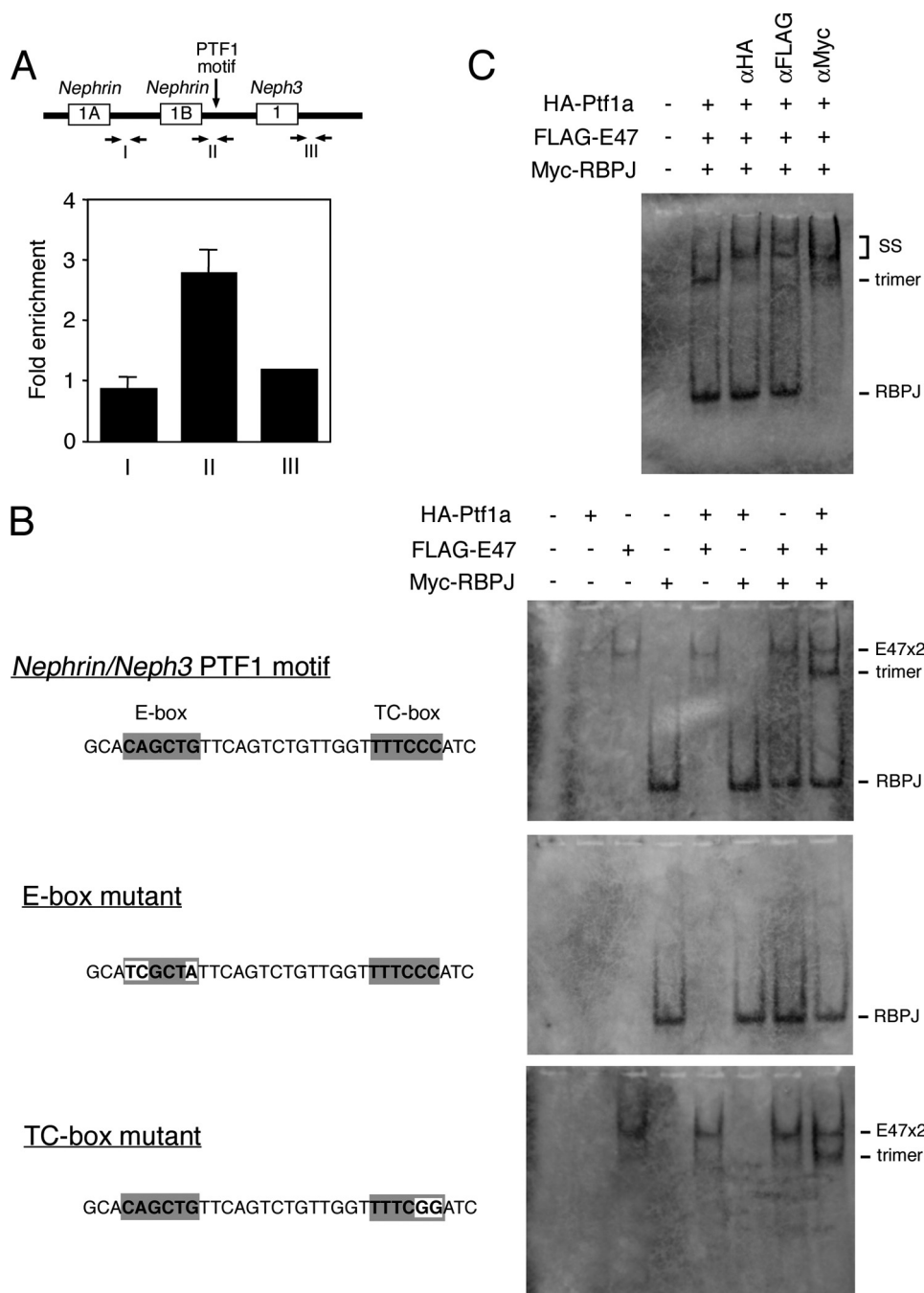


FIGURE 3. Interaction of Ptf1a with the 5'-flanking region of *Nephrin* and *Neph3* genes. *A*, Ptf1a chromatin immunoprecipitation assay was performed on chromatin isolated from E11.5 cerebellum. The amount of precipitated DNA fragments around the 5'-flanking region of *Nephrin* and *Neph3* genes was analyzed by quantitative PCR. PCR primers encompassing the PTF1 site (*II*) or at a distance of 1.5 kb from this site (*I* and *III*) were used. The amount of PCR products in immunoprecipitates with anti-Ptf1a relative to those without antibodies is shown. Values are the means \pm S.E. (error bars) of three samples. *B*, EMSA using oligonucleotides of wild type PTF1 motif (*top panel*), E-box mutant (*middle panel*), and TC-box mutant (*bottom panel*), in the presence of HA-Ptf1a, FLAG-E47, and/or Myc-RBPJ. Sequences of the oligonucleotides are shown on the left. *C*, EMSA supershift analysis. EMSA was performed using oligonucleotides with wild type PTF1 motif, HA-Ptf1a, FLAG-E47, and Myc-RBPJ, in the presence of an anti-HA, an anti-FLAG, or an anti-Myc antibody.

shifted band was confirmed by the fact that incubation with an antibody for each component all led to a supershift of the band (Fig. 3C). Mutations in E-box abolished Ptf1a binding, whereas the mutated TC-box had no effect in its binding, suggesting that E-box is necessary for the binding of Ptf1a to the PTF1 motif (Fig. 3B). Taken together, these results demonstrated *in vivo*

and *in vitro* that Ptf1a binds to the promoter region of *Nephrin* and *Neph3* genes and this binding is dependent on the PTF1 motif.

Ptf1a Activates Transcription from the *Nephrin* and *Neph3* Promoter—We next tested whether Ptf1a binding to the promoter region of *Nephrin* and *Neph3* leads to trans-activation of downstream genes by using a luciferase reporter system. Expression of Ptf1a in COS7 cells strongly activated a luciferase reporter driven by the 5'-flanking region of *Nephrin* and *Neph3* containing the PTF1 motif in both directions (Fig. 4, *A* and *B*). Note that transcriptional activation by Ptf1a could take place from both promoter fragments upstream of exon 1A and exon 1B of the *Nephrin* gene, albeit weaker in the former. Mutations in the E-box or TC-box, particularly the E-box, notably reduced the level of Ptf1a-dependent transactivation, suggesting that activation of the promoter depends on the successful binding of Ptf1a to the PTF1 motif. Some other members of basic helix-loop-helix, such as *Math1*, *Neurogenin1*, or *N-Twist*, did not enhance Ptf1a transactivity, and Ptf1a did not activate *c-fos* promoter (Fig. 4, *C* and *D*, and data not shown). Furthermore, co-expression of Ptf1a and E47 caused synergistic enhancement of *Neph3* promoter activation (supplemental Fig. 4). To confirm the dependence of *Neph3* promoter activation on Ptf1a *in vivo*, we performed a luciferase assay in the developing cerebellum and cerebral cortex, which are Ptf1a-positive and Ptf1a-negative areas, respectively. As shown in supplemental Fig. 4, *Neph3* promoter was highly activated in the developing cerebellum compared with the cerebral cortex, indicating a correlation of the presence of Ptf1a and high activation of the *Neph3* promoter. These results suggest that

Ptf1a activates transcription from *Nephrin* and *Neph3* promoter.

Ptf1a Is Essential for Expression of *Nephrin* and *Neph3* *In Vivo*—Ptf1a interacted with the PTF1 motif, thereby inducing trans-activation of the *Nephrin/Neph3* promoter, raising the possibility that Ptf1a controls the expression of *Nephrin* and *Neph3* in

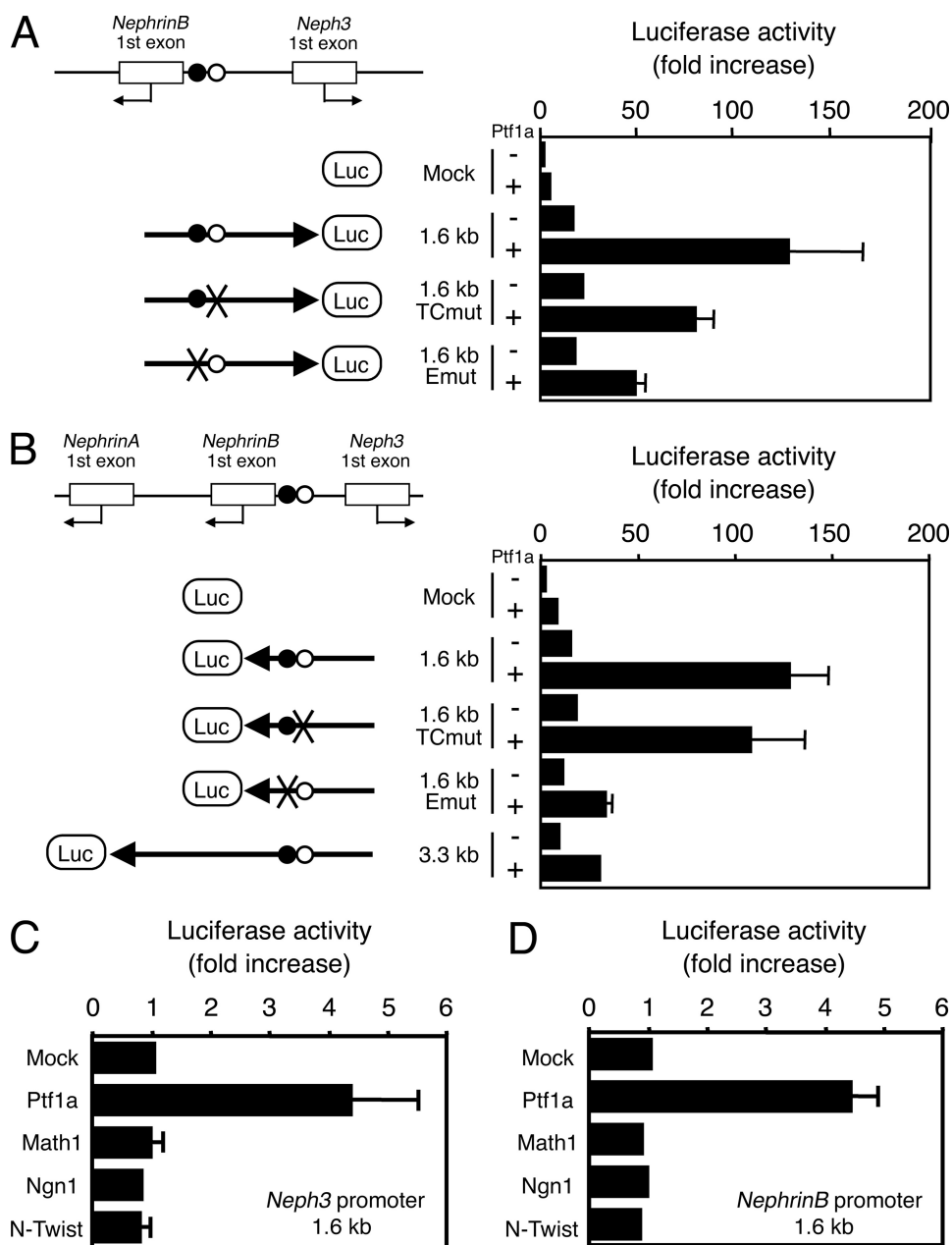


FIGURE 4. Luciferase reporter assay of the 5'-flanking region of *Nephrin* and *Neph3* genes. *A* and *B*, promoter activity of 5'-flanking region of *Neph3* (*A*) or *Nephrin* (*B*) driven by *Ptf1a* was analyzed in COS7 cells. The luciferase activities were normalized against those of co-transfected *Renilla* luciferase. The results are presented as fold increase relative to mock-transfected sample. Black and white circles on the left indicate the E-box and the TC-box of the PTF1 motif, respectively. The E-box and the TC-box mutations were introduced the same as in Fig. 3. *C* and *D*, luciferase reporter constructs together with *Ptf1a*, *Math1*, *Neurogenin1*, or *N-Twist* expression vector were transfected into COS7 cells, and luciferase activities in the lysates were analyzed. Values are the means \pm S.E. (error bars) of three to five independent transfections.

in vivo. To assess this possibility, we first examined the expression of *Nephrin* and *Neph3* in *Ptf1a*-null mice (Fig. 5, *A–L*, and supplemental Fig. 1). The expression of *Nephrin* and *Neph3* was lost in the developing cerebellum, hindbrain, spinal cord, retina, and hypothalamus of the homozygotes. We next performed gain-of-function experiments by ectopically expressing *Ptf1a* in the developing cerebral cortex, where *Ptf1a*, *Nephrin*, and *Neph3* are not endogenously expressed (data not shown). We found that ectopic *Ptf1a* triggered a notable induction of *Nephrin* and *Neph3* expression (Fig. 5, *M–T*), but not the other Neph

family members *Neph1* and *Neph2* (data not shown). Because *Ptf1a* activated transcription from exon 1A and 1B of the *Nephrin* gene (Fig. 4*B*), we further examined whether both *Nephrin* isoforms are induced by ectopic expression of *Ptf1a* in the cerebral cortex. As expected, we found induction of *NephrinA* and *B* by *Ptf1a* (supplemental Fig. 2). These results together suggest that *Ptf1a* controls the expression of two isoforms of *Nephrin* and *Neph3* in the developing central nervous system.

Interaction between *Nephrin* and *Neph3*—So far we have shown that *Ptf1a* simultaneously activates the transcription of *Nephrin* and *Neph3* in subsets of neural precursors. This raises the possibility that *Nephrin* and *Neph3* may interact and in turn control certain aspects of the fate of these cells. Indeed, heterophilic interactions between *Nephrin*, and *Neph1* or *Neph2* have been previously demonstrated (13–16). However, a *Nephrin*-*Neph3* interaction has not been reported. We therefore investigated any potential interaction between *Nephrin* and *Neph3* by co-immunoprecipitation experiments in COS7 cells. As shown in Fig. 6*A*, FLAG-*NephrinA* was co-precipitated with co-expressed HA-*Neph3* or HA-*NephrinA*, but not with HA-*NephrinB*. To examine whether *NephrinA* and *Neph3* interact in *cis* on the same cell, or in *trans* on different cells, we performed a similar experiment using a mixture of cells transfected with a mixture of cells transfected with each construct separately (Fig. 6*B*). However, *NephrinA* was co-precipitated with neither *Neph3* nor itself in this condition. These results imply that *NephrinA* interacts with *Neph3* as well as itself in *cis*. To

investigate whether *NephrinA*-*Neph3* may interact in *trans*, we performed an affinity probe *in situ* assay using *Neph3*-AP fusion protein (Fig. 6, *C* and *D*). Consistent with a previous report (18), *Neph3*-AP bound to *Neph3*-expressing cells, but not to *NephrinA*-expressing cells.

It has been shown in invertebrate that trans-interactions between *Nephrin* and *Neph* family mediate cell-cell adhesion (23, 24). We thus examined whether *NephrinA* and *Neph3* could mediate cell adhesion. As expected, only *Neph3*-expressing cells formed cell aggregates, but no aggregation was

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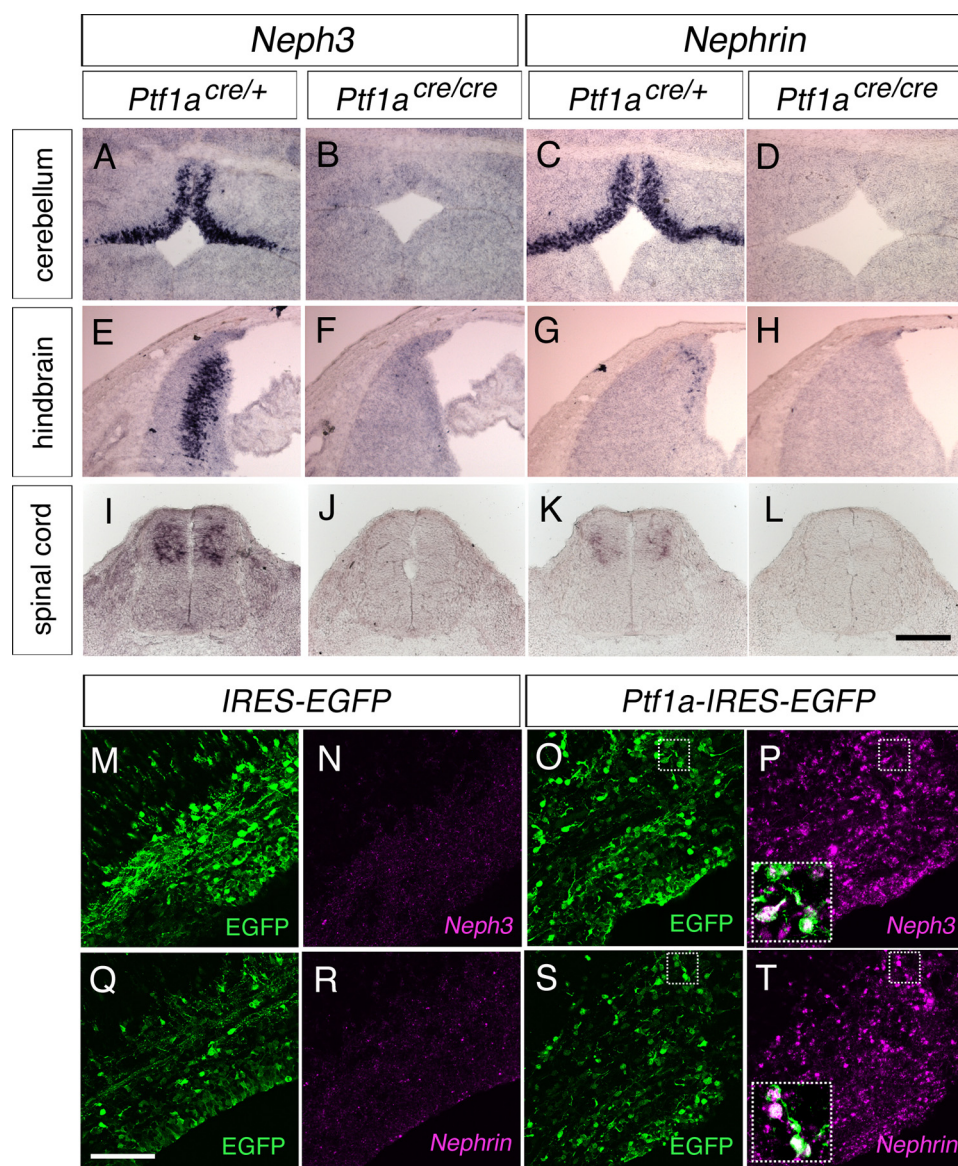


FIGURE 5. Nephrin and Neph3 expression in *Ptf1a*-null mice and *Ptf1a*-misexpressed cerebral cortex. A–L, expression of *Nephrin* and *Neph3* in *Ptf1a*-null mice. Expression of *Neph3* (A, B, E, F, I, and J) and *Nephrin* (C, D, G, H, K, and L) was analyzed by *in situ* hybridization on coronal sections of E13.5 cerebellum (A–D), E13.5 dorsal hindbrain (E–H), and E10.5 spinal cord (I–L). *Ptf1a*^{cre/+} mice (A, C, E, G, I, and K) and *Ptf1a*^{cre/cre} mice (B, D, F, H, J, and L) were used. Dorsal is upward. Scale bar, 100 μ m. M–T, expression of *Nephrin* and *Neph3* in *Ptf1a*-overexpressed cerebral cortex at E16.5. Expression of *Neph3* (N and P) and *Nephrin* (R and T) were analyzed by *in situ* hybridization on coronal sections of IRES-EGFP-transfected (M, N, Q, and R) or *Ptf1a*-IRES-EGFP-transfected (O, P, S, and T) cerebral cortex. Expression of EGFP on the same sections was analyzed by immunostaining by anti-EGFP (M, O, Q, and S). Insets in P and T are high magnification views of merged images outlined in O, P, S, and T. Dorsal is upward. Scale bar, 100 μ m.

detected between *Neph3*- and *NephrinA*-expressing cells and between *NephrinA*-expressing cells (supplemental Fig. 5). These data, taken together, showed that *NephrinA*-*Neph3* interactions take place in *cis* but not in *trans*, whereas *Neph3* could interact homophilically in *trans*, thereby mediating cell adhesion.

DISCUSSION

In the present study, we identified *Nephrin* and *Neph3* as direct downstream targets of *Ptf1a* in the developing central nervous system. Our finding provides an important clue to unravel as yet unclarified molecular mechanisms underlying

cell fate specification of *Ptf1a*-neurons. Henke *et al.* (39) recently reported *Neurog2*, itself a basic helix-loop-helix transcription factor, as a first example of downstream target of *Ptf1a* in the developing spinal cord. However, in contrast to *Ptf1a*-null mice, neurogenesis of *Ptf1a*-neurons is not affected in *Neurog2* knock-out mice, raising the possibility that *Ptf1a* downstream targets other than *Neurog2* are also involved in the development of *Ptf1a*-neurons (7, 40). Compared with *Neurog2*, *Nephrin* and *Neph3* as transmembrane proteins more likely mediate the effect of *Ptf1a* by directly influencing the cellular behavior of *Ptf1a*-neurons. Moreover, *Nephrin* and *Neph3* were more specifically expressed in the *Ptf1a*-positive domains, implicating their specific function in *Ptf1a*-neurons (Fig. 2 and supplemental Fig. 1). *Ptf1a* might also control *Nephrin*/*Neph3* expression outside the nervous system, such as islet cells in the pancreas (20, 41–43).

Ptf1a required co-factors for binding to the promoter region of *Nephrin* and *Neph3* and to induce full promoter activation (Fig. 3 and supplemental Fig. 4). Our *in vitro* data suggest that E47 facilitates binding of *Ptf1a* to the promoter via the E-box, and in turn activates *Nephrin*/*Neph3* transcription. The wide expression of E47 in the ventricular zone of the developing central nervous system supports the possibility that it acts as a co-factor for *Ptf1a* *in vivo* (data not shown). RBPJ is another factor that is necessary for the binding of *Ptf1a* to the promoter of *Nephrin* and *Neph3* *in*

vitro. However, differing from E47, RBPJ interacted with the TC-box of the PTF1 motif, and this interaction was not required for *Ptf1a* binding to the PTF1 motif (Fig. 3). It is possible that a direct interaction between RBPJ and *Ptf1a* enhances association of *Ptf1a* to the E-box. Indeed, this possibility is implied by an *in vivo* study showing that the ability of *Ptf1a* to interact with RBPJ is crucial for its role in the specification of GABA-ergic neurons in the dorsal spinal cord (38). *Ptf1a*/E47/RBPJ may not be the only proteins that trans-activate *Nephrin* and *Neph3* genes. For example, *NephrinA* expression in the adult kidney is controlled by a zinc finger protein, *Wt1*, which interacts with the 5'-flanking region of exon 1A of

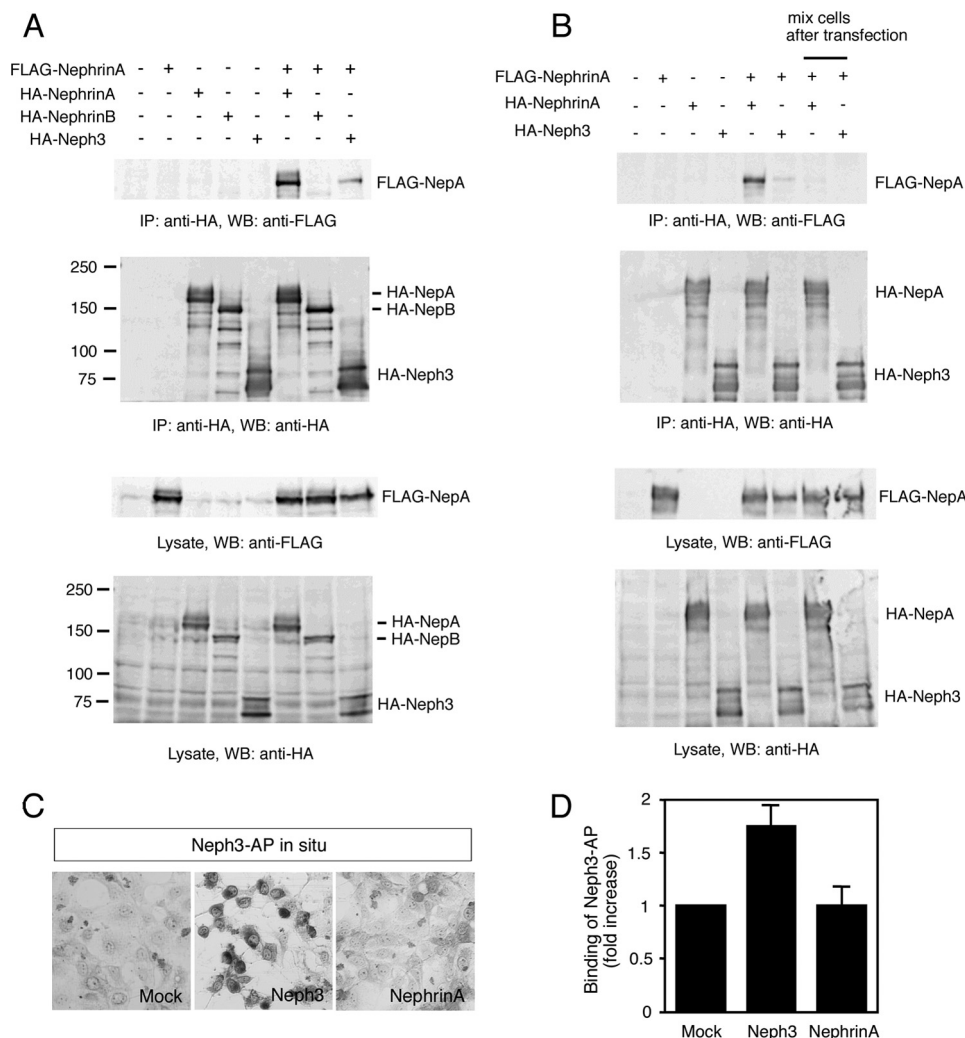


FIGURE 6. Homophilic and heterophilic interaction of Nephrin and Neph3. *A*, interaction of FLAG-NephrinA with HA-NephrinA, HA-NephrinB, or HA-Neph3 by co-immunoprecipitation experiments in COS7 cells. *B*, interaction between NephrinA and Neph3. *Left six lanes*, 2.5 μ g of the indicated vectors was co-transfected into COS7 cells by FuGENE 6. 24 h later, cells were detached, spread onto the same plates again, and cultured for another 24 h for co-immunoprecipitation assay. *Right two lanes*, 5 μ g of FLAG-NephrinA, HA-NephrinA, or HA-Neph3 was transfected into COS7 cells. 24 h later, the cells were collected, and half of FLAG-NephrinA-cells were mixed with half of HA-NephrinA-cells or HA-Neph3-cells and incubated for another 24 h for co-immunoprecipitation assay. Note that expression of each protein in the lysate in the *left six lanes* is comparable with that in the *right two lanes*. *C*, affinity probe *in situ* assay of Neph3-AP. Binding of Neph3-AP to mock-, HA-Neph3-, or HA-NephrinA-transfected COS7 cells is shown. *D*, binding affinities of Neph3-AP to COS7 cells transfected with mock, HA-Neph3, or HA-NephrinA vector. All values are the means of five to six independent transfections \pm S.E. (error bars).

Nephrin gene (44). Transcription factors NF- κ B and Sp1 are key regulators of *Neph3* expression in the kidney podocytes (45).

In the present study, we characterized homophilic and heterophilic interaction of Nephrin and Neph3, which provides insights into their mechanistic cellular function. To our surprise, we failed to detect an interaction between NephrinA-expressing cells, which has been demonstrated in a previous study (19). A possible reason for this discrepancy may be ascribable to differences in the type of cells being used. Conformation of NephrinA necessary for trans-homophilic interaction might be regulated by its interaction with cell-type specific cytoplasmic proteins, such as CD2AP, which is expressed in the kidney podocytes but not in the brain (46).

The function of Nephrin and Neph3 in the development of Ptf1a-neurons has yet to be determined. So far, no anatomical or morphological abnormalities have been demonstrated in the brains of *NephrinA*-null mice, and knock-out mice of *NephrinB* and *Neph3* have not been reported (20). Neph3 is transiently expressed in early postmitotic neurons of the developing spinal cord, and intracellularly Neph3 is localized at the adherens junction between apical processes (17). Miyata *et al.* (47) reported that early postmitotic neurons maintain their apical processes before departing the ventricle to undergo radial migration. Therefore, one may speculate that Neph3 mediates the adhesion between the Ptf1a-progenitors, which in turn is necessary for their later development, such as neuronal migration, maturation, or differentiation. Alternatively, Neph3 trans-homophilic interaction may induce intracellular signals required for further development of Ptf1a-neurons. The ability of Neph family members in triggering intracellular signaling pathway has been best demonstrated in the case of Neph1. Neph1 overexpression activates the AP-1 promoter, and this activation is further stimulated by phosphorylation of Neph1 by tyrosine kinases (12, 48). Moreover, clustering of Neph1 results in its own tyrosine phosphorylation and its interaction with cytoplasmic proteins (49). Interestingly, the same study showed that Neph1 clustering elicits tyrosine phosphor-

ylation of NephrinA and the recruitment of cytoplasmic proteins to the NephrinA complex (49). If Neph3 acts similarly to Neph1, we may speculate that in Ptf1a-neurons, Neph3 clustering triggered by its trans-homophilic binding may induce intracellular signals both via its own cytoplasmic domain and via its cis-interaction with NephrinA in the same cells. We showed that the other isoform of Nephrin, NephrinB, is equally expressed in the cerebellum. However, its function seems less clear because NephrinB does not possess a signal peptide cleavage site in its amino-terminal region and does not localize in the plasma membrane when overexpressed in COS7 cells (36) (supplemental Fig. 6). One may hypothesize that it competes with NephrinA for cytoplasmic targets, sequestering them from the membrane to the cytosol, acting as a negative modulator for the effect mediated by NephrinA. Deeper understanding of molec-

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ular mechanism underlying Ptf1a-controlled neuronal cell fate specification must await clarification of signaling cascades downstream of Nephrin and Neph3 and functional characterization of two isoforms of Nephrin.

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