

## The BAR Domain Protein PICK1 Regulates Cell Recognition and Morphogenesis by Interacting with Neph Proteins<sup>∇</sup>

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**Neph proteins are evolutionarily conserved membrane proteins of the immunoglobulin superfamily that control the formation of specific intercellular contacts. Cell recognition through these proteins is essential in diverse cellular contexts such as patterning of the compound eye in *Drosophila melanogaster*, neuronal connectivity in *Caenorhabditis elegans*, and the formation of the kidney filtration barrier in mammals. Here we identify the PDZ and BAR domain protein PICK1 (protein interacting with C-kinase 1) as a Neph-interacting protein. Binding required dimerization of PICK1, was dependent on PDZ domain protein interactions, and mediated stabilization of Neph1 at the plasma membrane. Moreover, protein kinase C (PKC $\alpha$ ) activity facilitated the interaction through releasing Neph proteins from their binding to the multidomain scaffolding protein zonula occludens 1 (ZO-1), another PDZ domain protein. In *Drosophila*, the Neph homologue Roughest is essential for sorting of interommatidial precursor cells and patterning of the compound eye. RNA interference-mediated knockdown of PICK1 in the *Drosophila* eye imaginal disc caused a Roughest destabilization at the plasma membrane and a phenotype that resembled *rst* mutation. These data indicate that Neph proteins and PICK1 synergistically regulate cell recognition and contact formation.**

Organization of cells into a specific spatial configuration is a prerequisite for the development of epithelial tissues and organs and requires selective cell adhesion and cell recognition. Multiple classes of cell surface molecules are involved in mediating cell recognition, many of which are highly conserved through evolution. Particularly important recognition molecules in humans are the Neph and nephrin proteins (15). Mutation of members of this class of adhesion proteins results in kidney failure and severe protein loss into urine due to the disruption of the kidney filtration barrier (9, 19). Neph proteins are characterized by an extracellular domain containing five immunoglobulin repeats, a transmembrane region, and an approximately 220-amino-acid cytoplasmic tail that mediates protein interactions and signaling (16). Whereas mammals contain three Neph proteins (Neph1 to -3), two Neph-like proteins can be found in *Drosophila melanogaster* (Roughest, Kirre) and at least one in *Caenorhabditis elegans* (SYG-1) (10). Neph proteins interact with the related proteins of the nephrin

family. In the mammalian kidney it could be demonstrated that signaling through these junctional proteins directs formation, maturation, and maintenance of highly specialized, interdigitating secondary processes of kidney podocytes, the visceral epithelial cells of the glomerular filtration barrier (9, 19, 46). In *Drosophila* Neph-like Roughest and Kirre mediate cell recognition and morphogenesis in the development of the fly compound eye (1, 27). The adult compound eye of *Drosophila* is composed of about 750 unit eyes, or ommatidia, that are arranged in a highly ordered crystal-like pattern. This biological crystal develops from an unpatterned single-layered epithelium, the eye imaginal disc (5, 44). In the young pupa four lens-secreting cone cells and two primary pigment cells are separated by a pool of undifferentiated interommatidial precursor cells. During pupal development this interommatidial lattice is shaped into a very regular honeycomb-like pattern of secondary and tertiary pigment cells. This morphogenetic process, which requires cell sorting and extensive cell shaping, is controlled through Neph and nephrin proteins. Interestingly, these molecular mechanisms are evolutionarily highly conserved and have been shown to largely also apply to the development of the mammalian kidney filter, making the fly eye a perfect model system to address signaling processes at the kidney filter (6).

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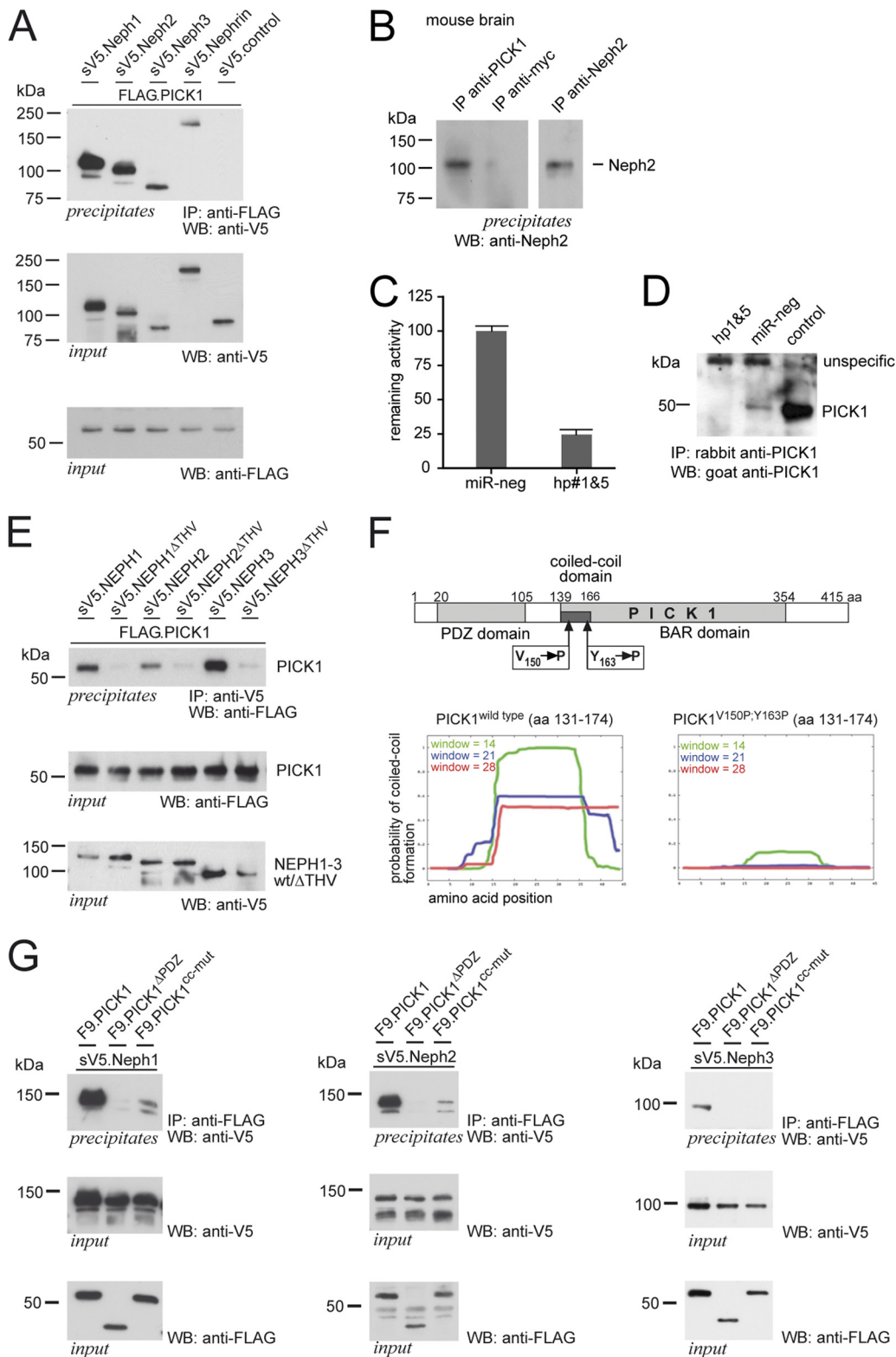


FIG. 1. PICK1 interacts with Neph/nephrin proteins via its PDZ domain. (A) Neph1-3 and nephrin coprecipitate with PICK1. FLAG-tagged PICK1 and sV5-tagged Neph/nephrin or control protein were expressed in HEK 293T cells and precipitated with anti-FLAG antibody. Western blot (WB) analysis was performed with a V5-specific antibody. Expression levels in the lysates are shown below. (B) Endogenous PICK1 interacts with Neph2 in the brain. Protein lysates from mouse brains were subjected to immunoprecipitation (IP) with a control antibody (anti-myc) or PICK1-specific antibody, washed extensively, and immunoblotted with specific anti-Neph2 antibody. The last lane depicts Neph2 expression in the brain, shown here after Neph2 precipitation due to low expression levels in the lysate. (C) To create an artificial 3' UTR for luciferase, the coding sequence of human PICK1 was cloned into a bicistronic vector, fusing this sequence with the coding sequence of *Renilla* luciferase. *Renilla*

Although much has been learned about the role of Neph proteins in controlling morphogenetic processes, the integration of adhesion and cell signaling of the cell recognition module to orchestrate cell movement, cytoskeletal reorganization, and junction remodeling is poorly understood.

Here we show that Neph proteins interact with the BAR domain protein PICK1 (protein interacting with C kinase 1) and that this interaction stabilizes Neph proteins at the plasma membrane. PICK1 was first identified as an interactor of protein kinase C alpha (PKC $\alpha$ ) (36). PICK1 harbors a PDZ domain (PSD95/DlgA/ZO-1) at the N terminus and a BAR domain (Bin/amphiphysin/Rvs) at the C terminus. BAR domains form crescent-shaped dimers and are involved in sensing and/or regulating membrane curvature, a prerequisite for vesicle formation (11, 17, 25). PICK1 has been shown to be involved in modulating the trafficking of neuronal receptors (14). We used the *Drosophila* eye imaginal disc to demonstrate that PICK1 and Neph proteins are involved in common pathways *in vivo*, suggesting a critical role for BAR domain proteins in controlling important morphogenetic processes.

#### MATERIALS AND METHODS

**Reagents and plasmids.** Phorbol myristate acetate (PMA) (tetradecanoyl phorbol acetate [TPA], catalog no. 9905; Cell Signaling Technology) was used at a final concentration of 200 nM for 30 min. Full-length PICK1 cDNA was cloned from a human podocyte cDNA library using standard cloning techniques. Mouse *Neph1*, *Neph2*, and *Neph3* cDNAs were cloned into a modified pcDNA6 expression vector coding for the CD5 signal peptide fused to the V5-tag sequence (sV5-tag) followed by restriction sites to insert the cDNA. ZO-1 plasmids have been described before (16). A membrane-bound fusion construct of the cytoplasmic domain of Neph1 (sig.7.Neph1<sup>cyt</sup>) has been described previously (31). Antibodies were from Sigma (FLAG), Serotec (MAb V5), Millipore (polyclonal antibody [pAb] V5), and Santa Cruz (H-300 goat pAb anti-PICK1). A polyclonal anti-Neph2 antibody has been described previously (12). For *Drosophila* immunofluorescence the following antibodies were used: rat anti-DE-cadherin (Developmental Studies Hybridoma Bank), rabbit anti-Pyd (kind gift from R. Cagan), mouse anti-Rst (24A5; Fischbach lab), rabbit anti-Kirre (126intra; Fischbach lab), rabbit anti-Hbs (14intra; Fischbach lab), and rabbit anti-SNS (Fischbach lab).

**Fly strains.** All crosses were kept at 25°C. The following fly lines were used: *Rsr<sup>3</sup>* (Fischbach lab), upstream activating sequence (UAS)-mCD8::GFP (Fischbach lab), *GMR-GAL4* (Fischbach lab), *GMR-GAL4*, UAS-*dicer2* and UAS-*dicer2* (gift from R. Cagan), and UAS-PICK1-RNA interference (RNAi) (Vienna Drosophila RNAi Center, strains ID22268 and ID104486) flies.

**Cell culture and coimmunoprecipitation.** HEK 293T cells were transfected with the calcium phosphate method and incubated for 24 h. Cells were then washed with phosphate-buffered saline (PBS), lysed in ice-cold IP buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 25 mM NaCl, 50 mM NaF, 15 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM Na<sub>3</sub>VO<sub>4</sub>)

on ice for 15 min and centrifuged (14,000 rpm, 4°C, 15 min). Supernatants containing equal amounts of total proteins were incubated for 1 h at 4°C with a polyclonal anti-V5 antibody (Chemicon) that was bound to protein G-Sepharose previously (1 h at 4°C) or with anti-FLAG antibody covalently coupled to agarose beads (catalog no. A2220; Sigma). The precipitates were washed three times with IP buffer, and bound proteins were resolved by 10% SDS-PAGE.

For endogenous coimmunoprecipitations, tissues were freshly prepared. Mouse brains and kidneys were homogenized using a glass potter, cleared by centrifugation, and solubilized in lysis buffer supplemented with 20 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and 3 mM ATP. After centrifugation at 15,000  $\times$  g (15 min, 4°C) and centrifugation at 100,000  $\times$  g (30 min, 4°C), cell lysates containing equal amounts of total protein were precleared with protein A-Sepharose and then incubated for 1 h at 4°C with the appropriate antibody, followed by incubation with 30  $\mu$ l of protein A-Sepharose beads for 3 h. The beads were washed extensively with lysis buffer, and bound proteins were resolved by 10% SDS-PAGE.

**RNA interference experiments.** RNA interference experiments were done as described previously (29). In brief, short hairpin RNAs (shRNAs) were designed based on the prediction of publicly available prediction programs (RNAi Designer; Invitrogen). shRNAs were cloned into the transient microRNA expression vector pcDNA6.2-GW/emGFP/miR (Invitrogen). To monitor the efficiency of shRNA-mediated knockdown, we created a luciferase reporter construct using psicheck2 (Promega) in which the coding sequence of PICK1 was fused to the coding sequence of *Renilla reniformis* luciferase as an artificial 3' untranslated region (UTR). In addition to *R. reniformis* luciferase, this construct expresses firefly luciferase for internal control. The reporter plasmid was cotransfected with the respective pcDNA6.2-GW/emGFP/miR shRNA construct into HEK 293T cells. *R. reniformis* luciferase and firefly luciferase activities were measured 24 h after transfection. Transfections and measurements were performed in triplicate. Selected hairpins (hp) (hp 1, TGCTGTCAAATACCTGGACGATATAGGTTTTGGCCACTGACTGACCTATATCGCAGGTATTGTA; hp 5, TGCTGACGACAGGTAACAACCTCAGTTTTGGCCACTGACTGACTGAGTTTTGTACCTGTCGT) were cloned into one cluster (chaining), and this cluster was Gateway cloned into pLenti6.2/V5-Dest (Invitrogen) for stable lentiviral expression in human podocytes cells.

**Human podocyte culture.** Human immortalized podocytes (AB 8 cells) were cultivated as described previously (28). In brief, cells were grown in standard RPMI 1640 medium containing 10% fetal calf serum (FCS) and supplements either at the permissive temperature of 33°C (in 5% CO<sub>2</sub>) to promote cell propagation or at the nonpermissive temperature of 37°C (in 5% CO<sub>2</sub>) to allow terminal differentiation.

**Pulldown assay.** MBP.Neph1<sup>cyt</sup> and GST.ZO-1<sup>1-111</sup> were expressed in *Escherichia coli* and affinity purified. Purified MBP.Neph1<sup>cyt</sup> was bound to amylose resin (New England BioLabs) and incubated at 4°C for 60 min with GST.ZO-1<sup>1-111</sup> that had been preincubated with buffer, recombinant PKC $\alpha$  (0.5 U; Invitrogen), or calf intestinal alkaline phosphatase (CIAP; 0.5 U; Invitrogen) for 30 min at 37°C. Bound proteins were eluted from the resin with Laemmli buffer and subjected to Western blot analysis.

**Surface labeling.** At 24 h after transfection, HEK 293T cells were starved for 3 h with serum-free medium. Cells were washed twice with PBS and surface labeled with 0.6 mg/ml EZ Link Sulfo-NHS-SS-Biotin (Pierce) for 15 min at room temperature. Cells were incubated with quenching buffer (100 mM glycine in PBS) for 10 min at room temperature, washed twice with PBS, and lysed in IP buffer. Precleared lysates were incubated with NeutrAvidin UltraLink Resin

luciferase activity, normalized to firefly luciferase activity that served as an expression control, was used to assay the efficiency of the shRNA-mediated knockdown of PICK1. The knockdown efficiencies of chained hairpins 1 and 5 are shown. Scrambled shRNA (miR-neg) was used as a control. Experiments were performed in triplicate. Error bars represent standard deviations (SD). (D) PICK1 is expressed in human podocytes. Human podocytes were stably transduced with PICK1 hairpins 1 and 5 or miReg-neg. PICK1 was precipitated from cell lysates with a rabbit anti-PICK1 antibody and immunoblotted with a goat anti-PICK1 antibody. A HEK 293T cell lysate with overexpressed untagged human PICK1 served as a control. (E) PICK1 coprecipitation with Neph proteins is dependent on the PDZ domain binding motif. FLAG-tagged PICK1 and V5-tagged Neph1-3 full-length proteins or V5-tagged Neph proteins missing the PDZ-binding motif ( $\Delta$ THV) were expressed in HEK 293T cells and precipitated with anti-V5 antibody. Western blot analysis was performed with a FLAG-specific antibody. Expression levels in the lysates are shown below. (F) Schematic representation of PICK1 showing residues V<sub>150</sub> and Y<sub>163</sub> within the predicted coiled-coil domain. Predictions using the program COILS suggest that mutation of these residues to proline impairs the formation of a functional coiled-coil domain ([http://www.ch.embnet.org/software/COILS\\_form.html](http://www.ch.embnet.org/software/COILS_form.html)). aa, amino acids. (G) Neph proteins coprecipitate with PICK1 in a PDZ domain- and coiled-coil domain-dependent manner. FLAG-tagged PICK1, PICK1 missing the PDZ domain ( $\Delta$ PDZ), and PICK1<sup>V150P,Y163P</sup> (*cc-mut*) were coexpressed with V5-tagged Neph full-length proteins and precipitated with anti-FLAG antibody. Western blot analysis was performed with a V5-specific antibody. Expression levels in the lysates are shown below.

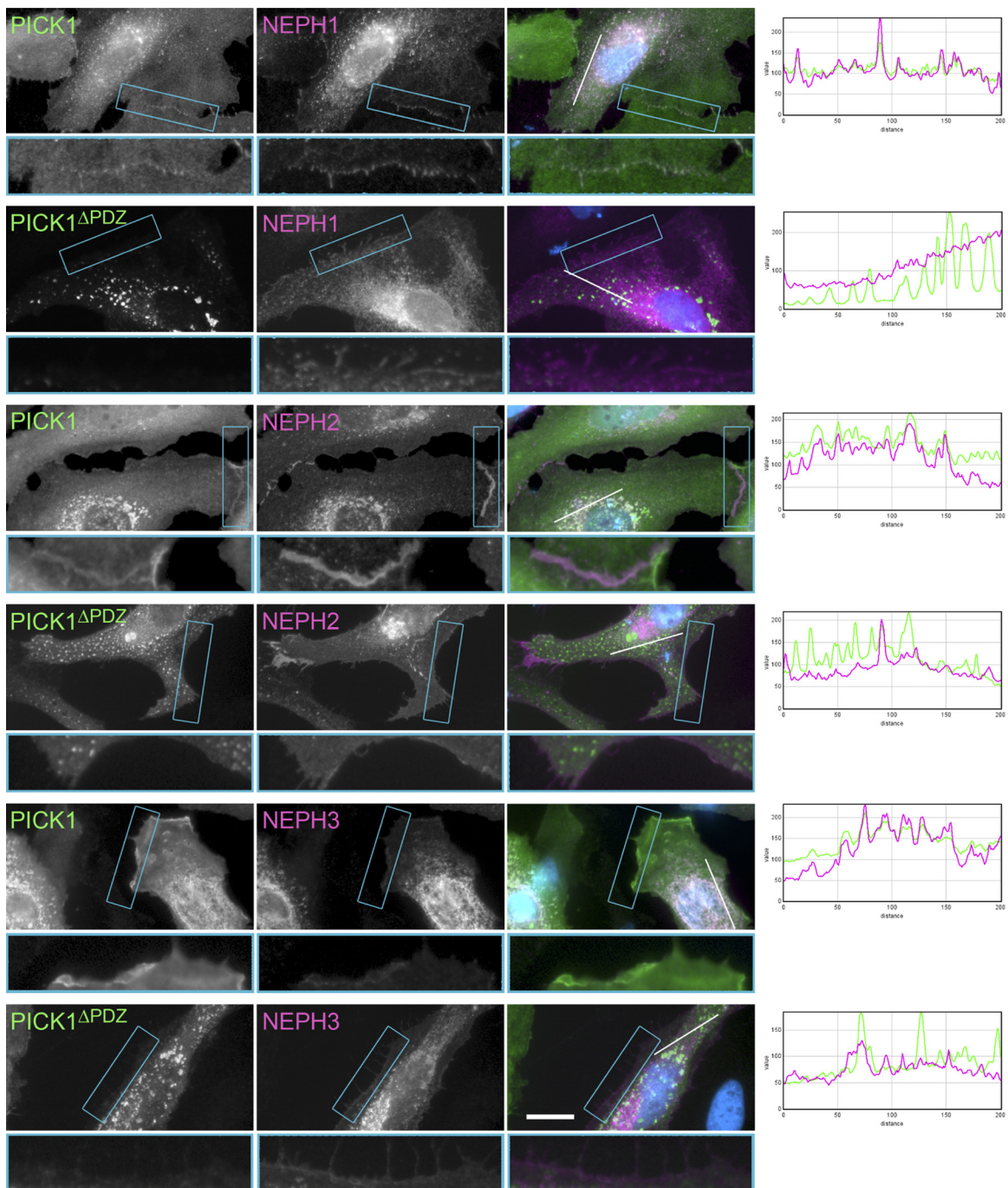


FIG. 2. PICK1 colocalizes with Neph proteins. HeLa cells were transiently transfected with the constructs indicated (Neph constructs with sV5 tag; PICK1 constructs with FLAG tag) and stained with antibodies against the tags. The full-length Neph proteins colocalize with full-length PICK1 in cytoplasmic patches and at the plasma membrane. PICK1 $\Delta$ PDZ does not colocalize with Neph proteins. The merged image shows the PICK1 channel in green and the Neph channel in magenta. Nuclei are stained with DAPI (blue). Boxed regions showing cell borders are shown in an enlarged view below each image. Intensity profiles for PICK1 (green) and Neph (magenta) along the white line in the merged image are shown to the right to assess the colocalization of PICK1 and Neph proteins and the random colocalization of PICK1 $\Delta$ PDZ and Neph proteins.

(Pierce) for 8 h at 4°C. Bound proteins were eluted from the resin with Laemmli buffer and subjected to Western blot analysis.

**In vitro phosphorylation.** His.Neph1<sup>cyt</sup> was expressed in *E. coli*, and 5  $\mu$ g of His.Neph1<sup>cyt</sup> was bound to 30  $\mu$ l of Ni-nitrilotriacetic acid agarose (Qiagen). *In vitro* phosphorylation of His.Neph1<sup>cyt</sup> was performed for 20 min at 30°C in a reaction in 60  $\mu$ l of kinase reaction buffer containing 50  $\mu$ M ATP (supplemented with 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP [Perkin Elmer]). Phosphorylation was initiated by the

addition of 0.5 U of PKC $\alpha$  purified from rat brain (Sigma) in enzyme dilution buffer, or the His.Neph1<sup>cyt</sup> was incubated in enzyme dilution buffer alone (control) or with 0.5 U of CIAP (New England BioLabs). To monitor the incorporation of phosphate, radiolabeled His.Neph1<sup>cyt</sup> was visualized by SDS-PAGE and autoradiography.

**Immunofluorescence.** HeLa cells were seeded on coverslips and transfected using Genejuice reagent (Novagen). After 24 to 48 h, cells were fixed for 10 min

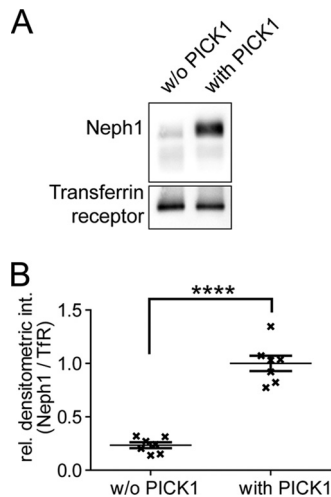


FIG. 3. PICK1 stabilizes Neph1 at the plasma membrane. (A) sV5.Neph1 was expressed in HEK 293T cells either in the presence or in the absence of PICK1. Biotinylated surface-expressed proteins were precipitated with NeutrAvidin resin, and V5-tagged Neph1 was visualized by Western blotting using V5 antibody. Staining for the transferrin receptor served as a loading control. (B) Quantitation of the blots was performed by measuring the band intensity of Neph1 compared with the intensity of the band representing transferrin receptor. Graphs represent means  $\pm$  standard errors of the means (SEM); \*\*\*\*,  $P < 0.0001$  (unpaired Student's  $t$  test) ( $n = 7$ ).

in 4% paraformaldehyde. Fixed cells were incubated for 30 min in blocking solution (5% normal donkey serum, 0.1% Triton X-100) and subsequently incubated with primary and secondary antibodies. After washing in PBS, the cells were mounted in Prolong Gold antifade (Invitrogen) and subjected to immunofluorescence microscopy with an Axiovert 200 M microscope/EC Plan-Neofluar 40 $\times$ /1.30 oil objective equipped with a charge-coupled-device (CCD) camera (all from Carl Zeiss).

For labeling of surface/endocytosed sV5.Neph1, cells were incubated at 4 $^{\circ}$ C with monoclonal antibody (MAB) anti-V5 (Serotec) diluted in full medium for 30 min. Cells were washed with medium three times and were allowed to recover at 37 $^{\circ}$ C for 30 min. Cells were then washed with PBS, fixed, but not permeabilized, with 2% paraformaldehyde for 5 min, blocked with 5% normal donkey serum in PBS, and incubated with a donkey Alexa 488-coupled anti-mouse antibody (Invitrogen). After washing, cells and bound antibodies were fixed again for 5 min before cells were incubated with permeabilizing blocking solution (5% normal donkey serum, 0.1% Triton X-100). Then, a Cy3-coupled donkey anti-mouse secondary antibody (Jackson ImmunoResearch) was applied.

*Drosophila* retina immunohistology procedures were essentially as described previously (27). Briefly, staged pupal eye discs (18 to 19 h or 41 to 42 h after puparium formation [APF]) were dissected and fixed for 15 min in 4% paraformaldehyde, blocked with normal donkey serum in PBS with 0.1% Triton X-100, and incubated with primary antibody. A fluorescent-dye-coupled secondary antibody (Cy3, DyLight 549 or DyLight 488; Jackson ImmunoResearch) was used as the secondary antibody. Preparations were embedded in Vectashield (Vector), and image acquisition was done with a Zeiss LSM710/AxioObserver Z.1 equipped with an EC Plan-Neofluar 40 $\times$ /1.30 oil objective (Carl Zeiss).

**Adult eye fluorescence.** Adult flies were frozen at  $-80^{\circ}$ C. The flies were glued on a slide, and autofluorescence was used to image the eye with a Zeiss LSM710/AxioObserver Z.1 equipped with an EC Plan Neofluar 10 $\times$ /0.3 objective (Carl Zeiss).

**Image acquisition and processing.** Fluorescent images were acquired using Zeiss Axiovision 4.8 and Zeiss ZEN 2009 (Carl Zeiss) and assembled using Adobe Photoshop and Illustrator CS4 (Adobe Inc.) or ImageJ (26). For better visibility of membrane staining (see Fig. 2), gamma was set to 0.45 in Axiovision. ImageJ software was used for fluorescence intensity measurements (see Fig. 8) and intensity profiles (see Fig. 2) (RGB line profile plug-in).

## RESULTS AND DISCUSSION

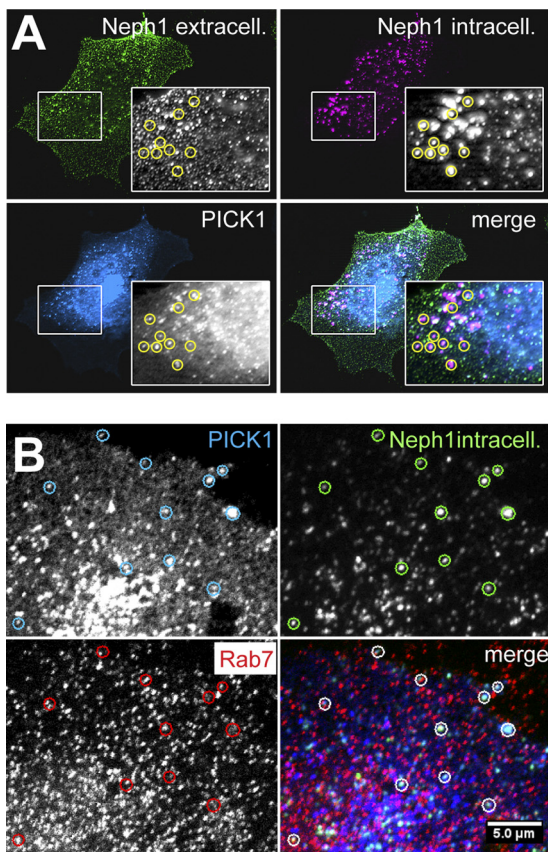
**Neph proteins bind PICK1 via PDZ domain-mediated interactions.** To identify critical regulators of the Neph/nephrin cell recognition module, we generated a cDNA library from human podocytes. The cDNA library was subjected to yeast two-hybrid screens using the intracellular domains of Neph2 and Neph3 as baits. These screens identified PICK1 as a major interactor of Neph2 and Neph3 (58% of hits in the Neph2 screen, 18% in the Neph3 screen). PICK1 is a 55-kDa cytosolic protein with several protein interaction domains (45).

To confirm the interaction, we cloned PICK1 from the human podocyte cDNA library and performed coimmunoprecipitation experiments from transiently transfected HEK 293T cells. These experiments indicated that PICK1 interacts with all three Neph proteins and confirmed the interaction with nephrin (Fig. 1A) (42). The interaction could also be demonstrated for endogenous proteins precipitated from mouse kidney and mouse brain lysates (Fig. 1B and not shown), suggesting an *in vivo* complex formation of Neph and PICK1 proteins. PICK1 is expressed in podocytes, as demonstrated in knock-down experiments using a human podocyte cell line (Fig. 1C and D).

Neph proteins contain a cytoplasmic tail that harbors a type I PDZ binding motif at the very carboxyl terminus (16). Since PICK1 contains a PDZ domain, we tested whether the Neph-PICK1 interaction is mediated via a PDZ domain-mediated mechanism. Deletion of the last three amino acids representing the PDZ domain binding motif ( $\Delta$ THV) at the carboxyl termini of Neph1, Neph2, and Neph3 strongly abrogated binding to PICK1 (Fig. 1G). Moreover, deletion of the PDZ domain in PICK1 also abrogated the interaction (Fig. 1G), indicating that the Neph-PICK1 protein complex forms via PDZ domain-mediated interactions.

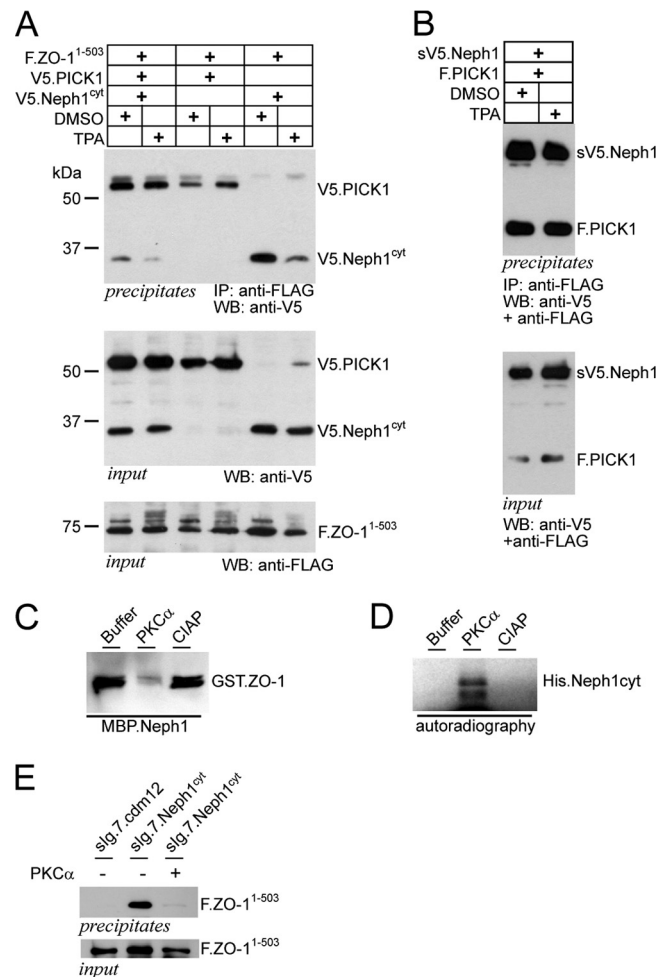
In PICK1 a short stretch at the N terminus of the BAR domain, identified as a coiled-coil motif, mediates self association (3) (Fig. 1F). To test whether dimerization of PICK1 may affect interaction with Neph proteins, we mutated valine at position 150 and tyrosine at position 163 into proline. These mutations were expected to interfere with the formation of a coiled-coil structure (Fig. 1F) and thus thought to abrogate dimerization. Coimmunoprecipitation experiments using wild-type PICK1, as well as the coiled-coil mutant PICK1<sup>V150P/Y163P</sup> (*cc-mut*), indicated that dimerization of PICK1 through the coiled-coil motif is required for the association of PICK1 with Neph proteins (Fig. 1G). Taken together, these data suggest that functional PICK1 dimers are able to bind Neph proteins and that the interaction is mediated through the PDZ domain of PICK1 and the PDZ-binding motif within the carboxyl terminus in Neph1 to -3.

**PICK1 and Neph proteins colocalize at the plasma membrane and in intracellular vesicles derived from the plasma membrane.** PICK1 has recently emerged as a central mediator in regulating endocytosis and/or recycling of neuronal receptors such as AMPA-type glutamate receptors (GluR2) and metabotropic glutamate receptors (mGluR7) (7, 14, 24, 40). In these systems PICK1 can act in either direction: GluR2 is removed from the plasma membrane in a PICK1-dependent manner, while surface localization of mGluR7 is stabilized in a PICK1-dependent manner (21, 24, 40). Given the specific in-



**FIG. 4.** (A) PICK1 colocalizes with endocytosed Neph1. HeLa cells were cotransfected with sV5.Neph1 and with FLAG.PICK1. Living cells were labeled with mouse anti-V5 antibody for 30 min at 4°C. Cells were then washed and transferred to 37°C for an additional 30 min. Next, the cells were fixed and labeled with a secondary anti-mouse antibody–Alexa 488 (green fluorescent). After washing, the bound antibodies were briefly fixed. The cells were then permeabilized and labeled with an anti-mouse secondary antibody–Cy3 (red fluorescent) to mark internalized intracellular Neph1. PICK1 was stained with a rabbit anti-FLAG antibody. PICK1 clearly colocalizes with endocytosed Neph1 in intracellular vesicles (examples are marked with circles). (B) A subpopulation of PICK1/Neph1 endocytosed vesicles is positive for the late endosome marker Rab7. HeLa cells were cotransfected and treated as in panel A, except that extracellular V5 epitopes were not labeled but blocked with a anti-mouse antibody (nonfluorescent). In addition, Rab7 was stained with a rabbit anti-Rab7 antibody. A subpopulation of the vesicles is positive for endocytosed Neph1, PICK1, and Rab7.

teraction between Neph proteins and PICK1, we speculated that PICK1 may be involved in recycling Neph adhesion proteins. To directly test this hypothesis, we performed immunofluorescence experiments. Coexpression of PICK1 and Neph proteins in HeLa cells identified a colocalization of these proteins. Besides the expected colocalization of the expressed proteins in the endoplasmic reticulum, we found a strong colocalization at the plasma membrane and in intracellular vesicles (Fig. 2). Strikingly, colocalization of Neph1 and PICK1, as well as plasma membrane staining of PICK1, was lost in a deletion mutant lacking the PDZ domain (Fig. 2 and data not shown). Recruitment of PICK1 to the membrane is a known prerequisite for the activation of the BAR domain (23).



**FIG. 5.** PKC $\alpha$  and PICK1 cooperatively regulate the Neph1/ZO-1 plasma membrane complex. (A) FLAG-tagged ZO-1<sup>1-503</sup> and V5-tagged Neph1<sup>cyt</sup> were coexpressed with PICK1 or empty vector. ZO-1 was precipitated with anti-FLAG antibody. Western blot analysis was performed with a V5-specific antibody to detect coprecipitated Neph protein. Activation of protein kinase C (PKC $\alpha$ ) with 200 nM PMA inhibits the interaction (lane 5 and 6). Coexpression of PICK1 further augments the PKC effect on the Neph1/ZO-1 interaction (lane 1 and 2) without affecting protein levels (middle and lower panels). (B) PMA does not influence the PICK1/NEHP1 interaction. Experimental setting as in panel A with the constructs indicated. (C and D) The interaction Neph1/ZO-1 is regulated through PKC $\alpha$  activity. (C) Pull-down assay of bacterially expressed GST.ZO-1<sup>1-111</sup> and MBP.Neph1<sup>cyt</sup> in the presence of buffer, PKC $\alpha$ , or calf intestinal alkaline phosphatase (CIAP). Addition of PKC $\alpha$  abrogates the Neph1/ZO-1 interaction, whereas CIAP did not affect the interaction. (D) Kinase assay of bacterially expressed His.Neph1<sup>cyt</sup> in the presence of radioactively labeled ATP and buffer, PKC $\alpha$  or CIAP. Addition of PKC $\alpha$  led to phosphorylation of Neph1<sup>cyt</sup>. Autoradiograph after *in vitro* phosphorylation of His.Neph1<sup>cyt</sup> (E) Membrane-bound Neph1<sup>cyt</sup> (sIg7.Neph1<sup>cyt</sup>) or a control plasmid (sIg7.cdm12) and FLAG-tagged ZO-1<sup>1-503</sup> were expressed in HEK 293T cells and precipitated with protein G. Western blot analysis was performed with a FLAG-specific antibody. Overexpression of PCK $\alpha$  abrogated the Neph1/ZO-1 interaction.

We next went on to test whether PICK1 may influence vesicular trafficking, targeting, or stability of Neph1 at the plasma membrane. Endocytosis assays using a pulse-chase approach did not show gross differences of overall internalization of Neph1 in the presence or absence of PICK1 (not shown).

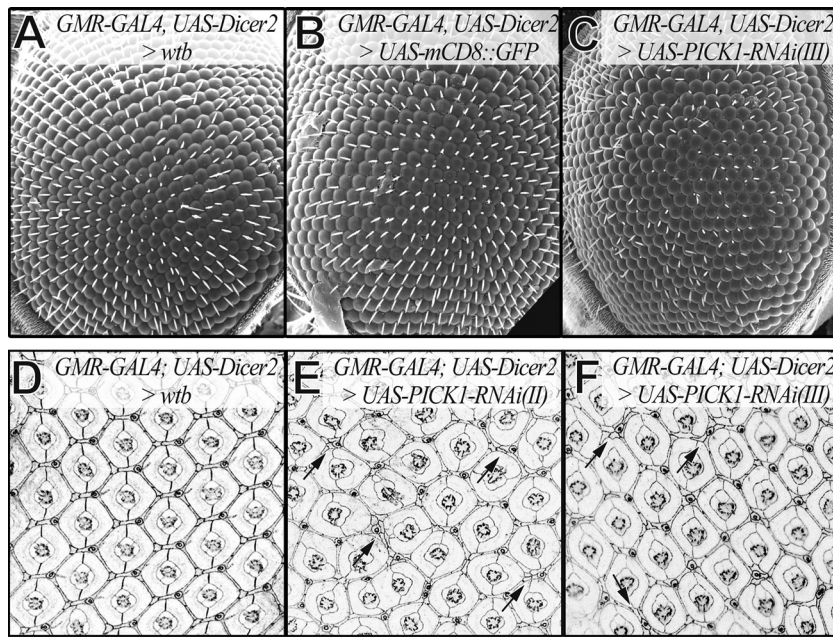


FIG. 6. Knockdown of dPICK1 causes a *rst* mutant-like phenotype. (A to C) Scanning electron micrographs of adult eyes. (A and B) Controls have a wild-type patterned eye. (C) *GMR-GAL4*-driven knockdown of dPICK1 (RNAi transgene on the third chromosome) leads to a rough eye phenotype. The RNAi enhancer *dicer2* was coexpressed. (D and E) Midpupal eye imaginal discs stained for DE-cadherin. *GMR-GAL4*-driven dPICK1 knockdown with two independent RNAi transgenes leads to the same *Rst*-like phenotype. Many ommatidia are separated not by a single row but by a double row of interommatidial cells (arrows). The RNAi enhancer *dicer2* was coexpressed. (D) Driver line control. (E) dPICK1 RNAi transgene on the second chromosome. (F) dPICK1 RNAi transgene on the third chromosome.

However, surface biotinylation experiments revealed that expression of PICK1 significantly increased the amount of plasma membrane Neph1 whereas plasma membrane targeting of a control plasma membrane protein (transferrin receptor) was not affected (Fig. 3). These data indicated that PICK1 acts to stabilize Neph1 at the plasma membrane.

BAR domains act as sensors and/or regulators of membrane curvature (17). Since BAR domain dimers bind preferentially to liposomes of specific size (25), it is not unlikely that different BAR domains have slightly different geometries, which in turn determines the type of vesicle or invaginated membrane that is bound (14). PICK1 may therefore associate with a certain subset of vesicles. To test whether PICK1 specifically associates with Neph proteins in endocytic vesicles, we performed a fluorescence-based endocytosis assay (Fig. 4A). HeLa cells were cotransfected with sV5.Neph1 and with FLAG.PICK1. Living cells were pulse-labeled with a mouse anti-V5 antibody, washed, and transferred to 37°C for 30 min to allow internalization of protein antibody complexes. After fixation, the plasma membrane pool of Neph1 and the internalized Neph1 proteins were stained in different colors. PICK1 costaining revealed a perfect colocalization with endocytosed Neph1 originating from the plasma membrane (Fig. 4A). Interestingly, a subpopulation of the Neph1-PICK1 positive vesicles stained positive for the late endosome marker Rab7, which supported the concept that PICK1 may act to recycle Neph proteins from the late endosome and related compartments (Fig. 4B).

**PKC activity controls preferential binding of PICK1 and release from the plasma membrane-associated multidomain protein zonula occludens 1 (ZO-1).** PICK1 has been shown to be important for aspects of neuronal biology such as hip-

popampal and cerebellar long-term depression, mainly by targeting neuronal receptors to endocytosis or retrieval through a PDZ domain interaction which is dependent on PKC $\alpha$  activity (18, 20, 37). Moreover, recent studies suggested that PICK1 and PKC $\alpha$  act together to stabilize mGluR7 at the plasma membrane. This stabilization required phosphorylation of mGluR7 by PKC $\alpha$  which in turn enhanced the binding of mGluR7 to PICK1 (40). Since phosphorylation of neuronal receptors disrupted their binding to the PDZ domain-containing scaffolding proteins, such as ABP/GRIP, but did not affect binding to PICK1 (22), we tested whether PKC $\alpha$  activity also influenced binding of Neph1 to the scaffolding protein zonula occludens 1 (ZO-1). We had previously demonstrated that Neph1 binds ZO-1, a large multidomain PDZ protein, similarly to the GluR2-ABP/GRIP interaction (16). ZO-1 interacts with Neph1 at the plasma membrane, providing a scaffold for Neph1 signaling activity (16, 32), and it has been suggested that Neph-dependent cell recognition requires constant remodeling of the cell junction (2, 15). Activation of PKC $\alpha$  with the phorbol ester PMA resulted in an inhibition of ZO-1 binding to Neph1 (Fig. 5A) without affecting the PICK1-Neph1 interaction (Fig. 5B). Of note, overexpressed PICK1 also coprecipitated with FLAG-tagged ZO-1 as shown in Fig. 5A. Repeated experiments revealed that this interaction was quite weak and occurred only with overexpressed proteins. To test whether ZO-1 is part of the endogenous PICK1 complex, we used a tandem affinity purification approach and characterized the interacting proteins (not shown). Although this study identified a series of specific partner proteins, including the published BAR domain protein ICA-69, ZO-1 was not among these candidates (not shown). Inhibition of ZO-1 binding to Neph1

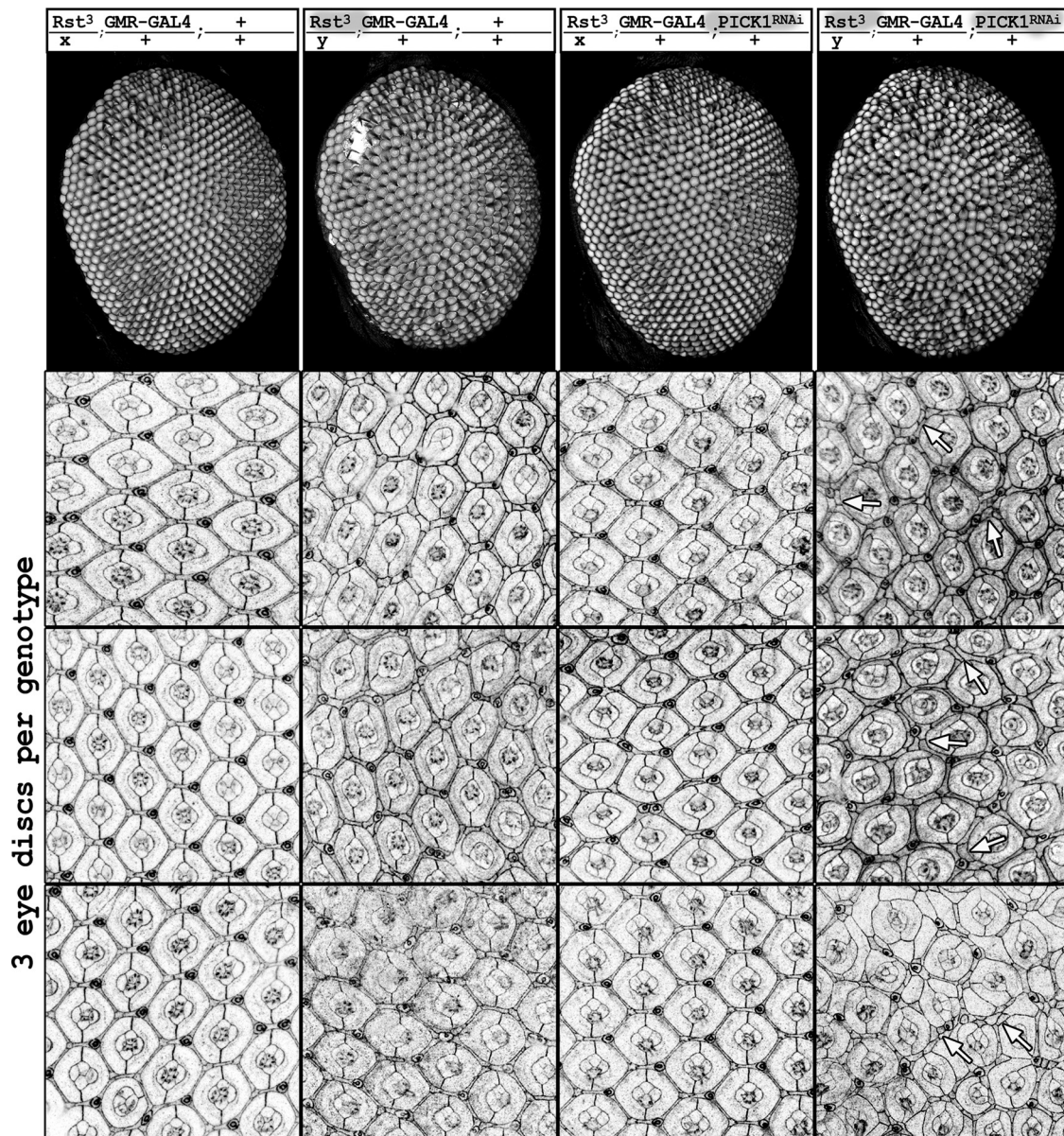


FIG. 7. Genetic interaction between *Rst* and *dPICK1*. The genotype for each column is indicated on top. First row, images of adult eyes. The weak phenotypes of the *Rst*<sup>3</sup> allele (2nd column) and *dPICK1* RNAi knockdown (3rd column) enhance each other to a severe phenotype if both lesions are present in males (rightmost column). Pupal eye imaginal discs (3 individual discs per genotype, 42 h APF) stained with anti-Pyd illustrate the same effect. Clear sorting defects are visible in the eye imaginal discs of these animals. Many ommatidia are not separated by a single row but by a double row of interommatidial cells (arrows).

was strongly enhanced in the presence of *PICK1* (Fig. 5A). To test whether *PKCα* was able to phosphorylate the cytoplasmic tail of *Neph1* to control *ZO-1* binding, *Neph1* and *ZO-1* were recombinantly expressed and purified and subjected to *PKCα* phosphorylation followed by pulldown experiments. *PKCα* treatment resulted in direct phosphorylation of the *Neph1* cytoplasmic tail (Fig. 5D) and disrupted the interaction of *ZO-1* with *Neph1* (Fig. 5C). Consistent with these findings, coexpression of *PKCα* with *ZO-1* and *Neph1* inhibited the interaction in HEK 293T cells (Fig. 5E). Taken together, these data show that the *Neph1/ZO-1* interaction is dynamically regulated by *PKCα* and *PICK1* and suggest that *PICK1* may act in

concert with *PKC* activity to control *Neph1* recycling to the plasma membrane.

***dPICK1* and *Roughest*, a *Drosophila* homolog of *Neph1*, cooperatively control patterning of the *Drosophila* compound eye.** *Drosophila* eye development involves cell sorting, cell death, and remodeling of cell contacts and shapes—processes that have been shown to be controlled by *Neph*- and *nephrin*-mediated adhesion and signaling (1, 10, 10, 13, 27). The primary pigment cells express *nephrin* proteins, *Hibris* (*Hbs*) and *Sticks-and-stones* (*Sns*), that interact with the *Neph*-like proteins, *Roughest* (*Rst*) and *Kin-of-irre* (*kirre*), present on neighboring cells. Heterophilic adhesion between *nephrin*- and



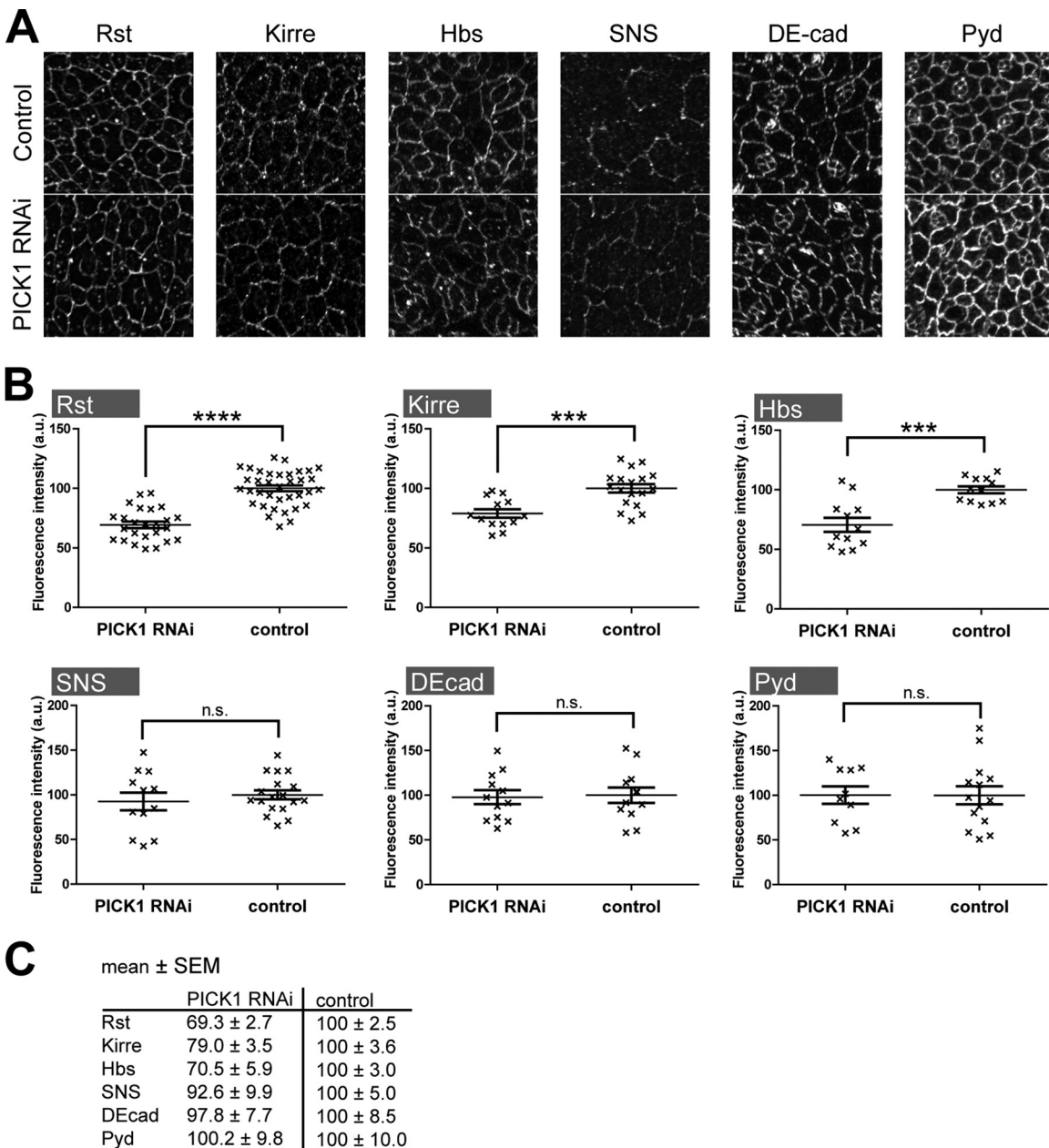


FIG. 8. Knockdown of dPICK1 does not change the subcellular distribution but the total protein level of IRM proteins. (A) Pupal eye imaginal discs (18 to 19 h APF) stained with the antibodies indicated. The subcellular localization of the stained proteins is indistinguishable between dPICK1 knockdown (*GMR-GAL4; UAS-dPICK1<sup>RNAi</sup> > UAS-dicer2*) and control (*GMR-GAL4 > UAS-dicer2*). Brightness and contrast were adjusted for optimal visibility of the localization of the proteins (B and C) Quantitation of the fluorescence intensity. Genotypes as in panel A. Quantitation of the eye discs was performed by measuring the mean fluorescence intensity in dPICK1 knockdown eye discs compared with control eye discs. Graphs represent mean fluorescence intensities of individual eye discs (each data point is for one eye disc) ± standard errors of the means (SEM); \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P \leq 0.0003$  (unpaired Student's *t* test).

Neph-like proteins results in remodeling contacts between cells to favor their contact with the pigment cells (1, 10). This sorting and culling process results in a pattern with a precise number and position of interommatidial cells (44). Double-stranded RNAs corresponding to PICK1 mRNA were expressed under the control of the eye-specific driver *GMR-GAL4*, together with the RNA interference (RNAi) enhancer *dicer2* (8). Interestingly, knockdown of dPICK1 resulted in a

rough eye phenotype closely resembling the *rst* phenotype (Fig. 6). The phenotype was confirmed using two independent RNAi lines (Fig. 6E and F). To demonstrate that the PICK1 phenotype was dependent on the Neph function, we tested for genetic interaction and made use of the weak allele *rst<sup>3</sup>* (41). The presence of one *rst<sup>3</sup>* allele in females, which have one intact copy of the *rst* gene on the X chromosome and one copy of the *GMR-GAL4* transgene on the second chromosome,

leads to a very mild phenotype. These flies demonstrated almost wild-type-patterned eyes (Fig. 7, first column). In males, however, the weak *rst*<sup>3</sup> phenotype was displayed (Fig. 7, second column). The addition of RNAi-mediated knockdown of dPICK1 using *GMR-GAL4*, without the RNAi enhancer *dicer2*, did not show any dramatic effect in females carrying one *rst*<sup>3</sup> allele (Fig. 7, third column). However, adding the PICK1 knockdown to the *rst*<sup>3</sup> allele in males (still in the absence of *dicer2*) resulted in a dramatic enhancement of the phenotype, clearly confirming the genetic interaction (Fig. 7, last column). Taken together, these data indicated that PICK1, very similarly to its action in mammals, acts to support Neph/Roughest function in the fly.

To examine the PICK1 function in the fly eye in greater detail, we analyzed Neph- and nephrin-like proteins, collectively known as IRM proteins (10), during eye development and stained pupal eye discs at a morphogenetically highly active stage (18 to 19 h APF). Interestingly, dPICK1 knockdown did not result in an obvious subcellular redistribution of IRM proteins in cells of the eye discs (Fig. 8). However, we realized that the fluorescence intensity was generally weaker in the dPICK1 knockdown eye discs than in controls. Quantification of the fluorescence intensity confirmed this effect (Fig. 8B and C). Knockdown of dPICK1 in the fly eye resulted in a significant decrease of Rst and Kirre expression at the plasma membrane but did not affect the expression of other proteins (E-cadherin, Pyd, SNS) (Fig. 8B and C). Since the IRM proteins are required for cell sorting at this stage of development (27), this reduction could well explain the rough eye phenotype.

Taken together, this work highlights the important role of PICK1 in regulating the Neph-dependent cell recognition module and Neph-mediated steps in morphogenesis. Members of the Neph/nephrin cell recognition module are conserved across species (10). They are involved in the recognition of different cell types and are mediating cell contacts of opposing cell membranes. Thus, it is highly conceivable that similar mechanisms may regulate the dynamic function of the Neph/nephrin cell recognition module in many species. We observed an increased Neph level at the plasma membrane in the presence of PICK1 in a cell culture biotinylation assay and a decreased Neph level in dPICK1 knockdown *Drosophila* eye discs. Thus, PICK1 seems to affect the rate of turnover and recycling of Neph adaptor proteins to affect their signaling abilities. In light of this hypothesis, it is important to consider that Neph proteins do not only function as adhesion molecules. They are involved in signaling for the regulation of cellular processes such as remodeling of cell contacts, organizing cell shape changes and cell sorting during *Drosophila* eye development (1, 10, 27), cell fusion in *Drosophila* muscle development (4, 38), correct wiring of the *Drosophila* brain (30, 39), localization and stabilization of presynaptic sites in *C. elegans* (34, 35), the development and maintenance of the kidney podocyte (9, 19), and the wiring of olfactory sensory neurons in the olfactory bulb of the mouse (33). It is obvious that these processes require the tightly regulated and timed expression of the Neph/nephrin proteins at the cell surface. The PDZ/BAR domain protein PICK1 has been shown to regulate the timed expression and/or recycling of associated proteins at the plasma membrane (22, 43). In conclusion, PICK1 was identified as an essential novel regulator of Neph/nephrin proteins,

most likely by influencing their availability to mediate signal transduction at the plasma membrane.

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