SUPPLEMENTAL METHODS

Animals

The *Erbb4*$^{Flox/Flox}$, [1] *Pax8-Cre*[2, 3] and *Rosa26LacZ*[3] mice were maintained and genotyped as previously reported. All mouse experiments were approved by Finnish national legislation, the European Convention (ETS 123), and EU Directive 86/609/EEC. For urine volume measurement, four-month old *Erbb4*^{Flox}/*Pax8-Cre* mice were kept separately in metabolic cages for 24 hours. One to three urine collections were made from four *ErbB4*$^{Flox/Flox}$/*Pax8-Cre*$^{+}$ mutant and five wild-type mice. CD-1 mice were used to examine *Erbb4* expression in wild-type embryos and newborn mice (Figure 1) while all the mutant mice were maintained and bred in C57BL/6 background.

Real-time RT-PCR

Total RNA was extracted using TRIsure (Bioline, London, UK) or RNeasy Mini Kit (QIAGEN, Hilden, Germany), and cDNA synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada). Real-time RT-PCR was carried out with ABI PRISM 7500 or 7900HT Sequence Detectors (Applied Biosystems, Foster City, CA, USA), as previously described.[4] Expression of human *ERBB4* isoforms was analyzed using primers and probes previously described.[4] Expression of mouse *Erbb4* isoforms, and *Nrg-1* was analyzed using primers and probes listed in Table S1.

In situ hybridization

Mouse-specific *Erbb4 in situ* probe was cloned by PCR from P0 mouse kidney cDNA using primers *E4m-insitu1* and *E4m-insitu2* (Table S1). The amplified fragment was digested with *Eco*RI and *Hind*III and inserted into Bluescript II SK (-) vector (Stratagene, Garden Grove, CA, USA). Antisense *Erbb4* RNA probe was synthesized using T3 RNA polymerase (Promega, Fitchburg, WI, USA), and used for *in situ* hybridization at Max-Planck-Institute of Biophysical Chemistry (Gene Paint database; http://www.genepaint.org, Gottingen, Germany). To analyze *Slc3A1* expression, *in situ* hybridization was carried out as previously described.[5]

Immunostaining

Paraffin sections were stained with hematoxylin and eosin as described.[6] Proliferation was assessed using PCNA Staining Kit (Invitrogen, Carlsbad, CA, USA). Scoring of PCNA-positive cells was carried out manually from microscopic images using Adobe Photoshop (Adobe Systems, San Jose, CA, USA) from three high magnification views of six *R26ERBB4*$^{+/-}$/*Pax8-Cre*$^{+}$ and four *R26ERBB4*$^{-/-}$/*Pax8-Cre*$^{-}$ kidneys.

ErbB4 immunohistochemistry was carried out, as previously described,[4] except that sc-283 (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody. HIF-1α immunohistochemistry was done with H1alpha67 (1:100) (Abcam, Cambridge, UK) primary antibody using HistoMouse-MAX kit (Invitrongen). To identify fibrotic tissue, paraffin sections were stained with Masson trichrome.[7] The amount of fibrosis around glomeruli was quantified from three high magnification views of three independent samples using MetaMorph morphometry software (Molecular Devices, Sunnyvale, CA, USA).

For immunofluorescence staining of paraffin sections, the sections were deparaffinized and rehydrated, and incubated for 10 min at 90 °C in 10 mM trisodium citrate (pH 6). Non-specific binding was blocked by overnight incubation at 4 °C in 10% goat serum in PBS. The sections were incubated overnight at 4 °C with primary antibodies against aquaporin-1 (1:200) (Millipore, Brillerica, MA, USA), aquaporin-2 (1:400) (Sigma-Aldrich, St. Louis, MO, USA), thiazide-sensitive NaCl cotransporter (NCC) (1:200) (Millipore), phospho-histone H3 (1:300) (Millipore), acetylated α–tubulin (1:200) (Sigma-Aldrich), *Dolichos biflorus* agglutinin (DBA; rhodamine labeled) (1:100) (Vector Laboratories, Burlingame, CA, USA), *Lotus tetragonolobus* lectin (LTL; fluorescein labeled) (1:200) (Vector laboratories), anti-ZO-1 (1:100) (Invitrogen), or E-cadherin (1:100) (BD Biosciences, Franklin Lakes, NJ, USA). To detect unconjugated primary antibodies, the sections were incubated with Alexa Fluor 488- or 546-conjugated secondary antibodies (1:800) (Invitrogen) and DAPI (1:10000) (Sigma Pharmaceuticals), and mounted with Immu-Mount (Thermo Scientific, Waltham, MA, USA).

Immunofuorescence staining of MDCK cells was carried out, as previously described,[8] using the following primary antibodies: anti-E-cadherin (rrl; 1:100),[9] anti-podocalyxin (gp135; 1:150),[10] anti-ZO-1 (1:100) (Invitrogen), and anti-Ki-67 (1:100) (Invitrogen). Anti-podocalyxin and anti-E-cadherin were kind gifts from Dr. Kai Simons (MPI-CBG, Dresden, Germany). The primary antibodies were detected using Alexa Fluor 488- or 546-conjugated secondary antibodies (1:800) and the nuclei stained with DAPI (1:10000). Olympus Fluoview 1000 laser confocal microscope (Olympus, Tokyo, Japan) and Olympus Fluorview 1.6a software were used for imaging.

Morphometry and analysis of mitotic orientation

Morphometric analyses were carried out from hematoxylin/eosin counterstained high power micrographs by an expert on murine developmental biology of the kidney using Adobe Photoshop. For *R26ERBB4*/*Pax8-Cre* mice, cell density was quantified from six high magnification views and the collecting duct lumen diameter from a minimum of 10 collecting ducts of five independent samples. For *Erbb4*^{Flox}/*Pax8-Cre* mice, cell density was quantified from six high magnification views and the collecting duct lumen diameter from a minimum of 25 collecting ducts of three independent samples. Each collecting duct was measured for one representative diameter.

To evaluate the orientation of cell division, sections of newborn *R26ERBB4*/*Pax8-Cre* mouse kidneys were immunostained with anti-phospho-histone H3 and anti-E-cadherin antibodies. Mitotic division axis of dividing collecting duct epithelial cells was defined manually from stacks of confocal optical sections and scored as longitudinal or radial in relation to duct orientation. To ascertain the direction and validity of the division event, only cells in anaphase were included in quantification. To avoid misinterpretation of cell division direction resulting from branching tubules, cortical collecting ducts were excluded. Mitotic divisions were quantified from three independent mutant and three wild type mice.

Western blotting

Proteins were extracted from embryonic and newborn kidneys and analyzed by Western blotting using anti-ErbB4 (E200; 1:1000) (Abcam) anti-phospho-ErbB4 (#4757; 1:500) (Cell Signaling, Danvers, MA, USA), anti-Akt (sc-1618; 1:1000) (Santa Cruz Biotechnology), anti-phospho-Akt (#9271; 1:1000) (Cell Signaling), anti-Erk (#9102; 1:1000) (Cell Signaling), anti-phospho-Erk (#9101; 1:1000) (Cell

Signaling), anti-β-actin (sc-1616; 1:1000) (Santa Cruz Biotechnology), and anti-GAPDH (1:2000) (Millipore) antibodies, as previously described.[11] Western data were quantified by densitometry using MCID Image Analyser (Imaging Research, St. Catharines, ON, Canada).

3D culture of MDCK cells

MDCK cells were transduced with retroviral constructs encoding *ERBB4 JM-a CYT-2* (pBABE-puro*ErbB4JM-aCYT-2*) or an empty vector [12]. The transduced cells were cultured for five days in Matrigel (BD Biosciences) and fixed with 4% paraformaldehyde.[13] The cysts were imaged and their morphology scored using Olympus Fluoview 1000 laser confocal microscope. The proportion of Ki-67-positive cells out of DAPI-stained cells, and the number of lumens per cyst were scored (n = 20 for Ki-67 quantification; n = 32 for lumen quantification). Similar data with significant differences between vector control and ErbB4 overexpressing cells were obtained from three (Ki-67 quantification) or five (lumen quantification) independent experiments.

Tissue culture

For kidney mesenchyme tubule induction assays, E11.5 mesenchymes were separated from the ureteric buds and cultured for 0, 48, 96 or 120 hours in combination with a heterologous tubule inducer tissue, a dorsal piece of E11.5 embryonic spinal cord [14]. The spinal cord was removed and the cultured kidney mesenchymes were pooled for RNA extraction. The explants that were processed for immunohistochemistry were not pooled.

Targeting of human *ERBB4* cDNA into the *Rosa26* locus to generate a mouse model for conditional expression

Human *ERBB4 JM-a CYT-2* cDNA was amplified by PCR (Table S2, PCR I) from a vector pcDNA3.1*JM-aCYT-2*,[15] using primers *attB1-A2* and *attB2-A2* (Table S1) for Gateway (Invitrogen) -mediated cloning. The amplified PCR fragment was inserted into the donor vector pDONR-221 using BP Clonase (Invitrogen). The insert and the cloning sites of the plasmid (pDONR-221-*hE4A2*) were verified by sequencing, and the *ERBB4 JM-a CYT-2* cDNA inserted with LR Clonase into the destination vector pRosa26-DEST.[16] The obtained pRosa26-DEST-*hE4A2* vector was linearized with *Bcg*I and electroporated into SV-129 mouse embryonic stem (ES) cells. Targeting of the Rosa26 locus (Figure S1A) was identified by Southern blotting and PCR (Table S2, PCR II; Figure S1B) and confirmed by sequencing.

Selected, correctly targeted ES cells were injected into the blastocyst stage C57BL/6 mouse embryos and transferred to pseudopregnant mothers to obtain chimeras. The chimeric mice were screened for germline transmission to obtain F_1-progeny of the *R26ERBB4*$^{+/-}$ mice that were heterozygous for the *ERBB4 JM-a CYT-2* cDNA under a floxed transcriptional stop cassette inserted to the *Rosa26* locus. The F_1 mice were bred with C57BL/6 mice to produce F_2-progeny. The heterozygous *R26ERBB4*$^{+/-}$ mice were further crossed with the *Pax8-Cre*-recombinase-positive mice,[2] to obtain *R26ERBB4*/*Pax8-Cre* mice (Figure S2).

The genotyping of mutant mice and embryos was carried out with DNA isolated from samples of ear, head or tail using primers *E4cDNA1*, *E4cDNA2*, *Rosa prom* and *SA-2* (Tables S1 and S2, PCR II and III). *Cre* genotyping was carried out as described.[3] Kidneys isolated from CD-1 mice or *R26ERBB4*/*Pax8-Cre* littermate mice

that had not inherited both *ERBB4* and *Pax8-Cre* were used as controls and considered as wild-type.

Statistical methods

Student's *t*-test was used to statistically analyze morphometrical and immunohistochemical data, and urine and blood biochemistry. Chi-Square test was used to determine whether the number of $ErbB4^{Flox/Flox}/Pax8\text{-}Cre^{+}$ pups and embryos deviated from the expected Mendelian ratios.

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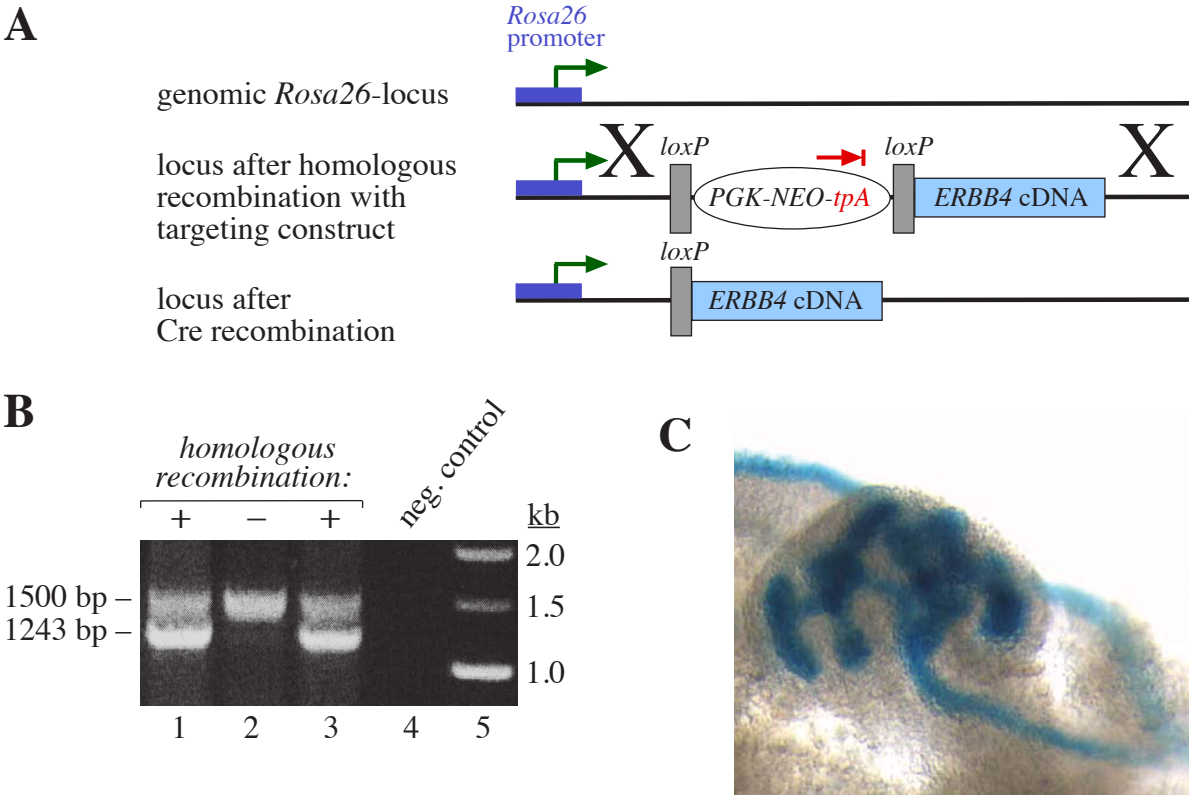
LEGENDS FOR SUPPLEMENTAL FIGURES

Supplemental Figure 1. Generation of *R26ERBB4* mice. (A) Schematic presentation of the targeting of the *Rosa26* locus. After homologous recombination, human *ERBB4 JM-a CYT-2* cDNA was integrated into the *Rosa26* locus downstream of a floxed cassette including a phosphoglycerate kinase promoter (*PGK*)-driven neomycin resistance gene (*NEO*) and a triple polyadenylation sequence (*tpA*). Removal of the *PGK-NEO-tpA* cassette by Cre-recombination enabled *ERBB4* transcription. (B) PCR screening of ES cell clones. A PCR product of 1243 bp indicates a successful homologous recombination. (C) Wholemount β-galactosidase staining of E12.5 *Rosa26LacZ*$^{+/-}$/*Pax8-Cre*$^{+}$ kidney demonstrating *Pax8* promoter-driven *Cre*-recombination in the ureter and in ureteric branches.

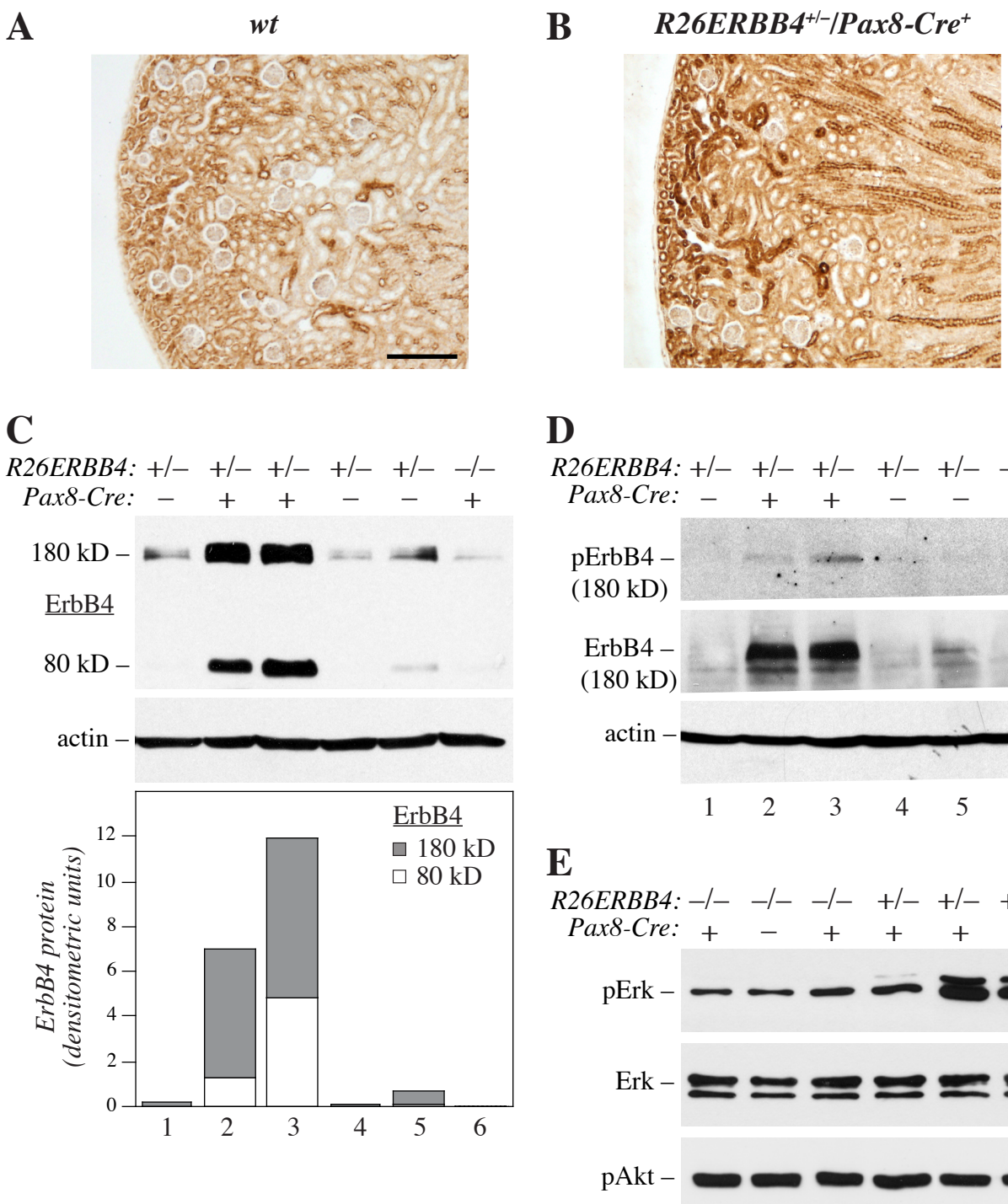
Supplemental Figure 2. ErbB4 expression and signaling in *R26ERBB4* mice**.** (A and B) Immunohistochemical analysis of ErbB4 expression in the kidney of a P10 wild-type control mouse (*wt*; *R26ERBB4*$^{-/-}$/*Pax8-Cre*$^{-}$) (A), and a mouse with *Rosa26*-targeted *ERBB4* after *Pax8-Cre*-mediated recombination (*R26ERBB4*$^{+/-}$/*Pax8-Cre*$^{+}$) (B). Mice with *Pax-Cre*-mediated *ERBB4* overexpression demonstrate enhanced ErbB4 protein expression in epithelial structures. (C and D) Western analyses of ErbB4 protein expression and the amount of phospho-ErbB4 in the kidneys of newborn mice with *Rosa26*-targeted *ERBB4* after *Pax8-Cre*-mediated recombination (*R26ERBB4*$^{+/-}$/*Pax8-Cre*$^{+}$), as compared to littermates lacking *R26ERBB4* transgene (*R26ERBB4*$^{-/-}$/*Pax8-Cre*$^{+}$) or the *Pax8-Cre* recombination (*R26ERBB4*$^{+/-}$/*Pax8-Cre*$^{-}$). The membranes were reblotted with actin (C and D) and ErbB4 (D) to control for loading and total ErbB4 protein levels, respectively. A mean increase of 10.1-fold was observed in total ErbB4 protein and a mean increase of 2.6-fold in the amount of active phosphorylated ErbB4, respectively. Columns in C represent total ErbB4 Western levels (expression of full-length 180 kD receptor plus the 80 kD processed fragment) quantified by densitometry. Expression of human *ERBB4* mRNA was first

detected in *R26ERBB4*$^{+/-}$/*Pax8-Cre*$^{+}$ mutant mice at E11.5, reached a peak of 4.5% of *β-actin* mRNA at E12.5, and reduced at later time points to 1.5% (E16.5) and 0.6% (newborn) of *β-actin* (data not shown). (E) Western analyses of phospho-Erk (pErk), Erk, phospho-Akt (pAkt), and Akt expression in kidney tissues of newborn mice with *Rosa26*-targeted *ERBB4* after *Pax8-Cre*-mediated recombination (*R26ERBB4*$^{+/-}$/*Pax8-Cre*$^{+}$), as compared to littermates lacking *R26ERBB4* transgene (*R26ERBB4*$^{-/-}$/*Pax8-Cre*$^{+}$), or both the transgene and recombination (*R26ERBB4*$^{-/-}$/*Pax8-Cre*$^{-}$). A clear increase in the amount of phosphorylated Erk but not of phosphorylated Akt can be seen in the *R26ERBB4*$^{+/-}$/*Pax8-Cre*$^{+}$ mutant kidneys. These findings demonstrate that the *R26ERBB4*/*Pax8-Cre* mice provide a gain-of-function model for examining the function of ErbB4 during kidney development. Scale bar: (A) 200 μm.

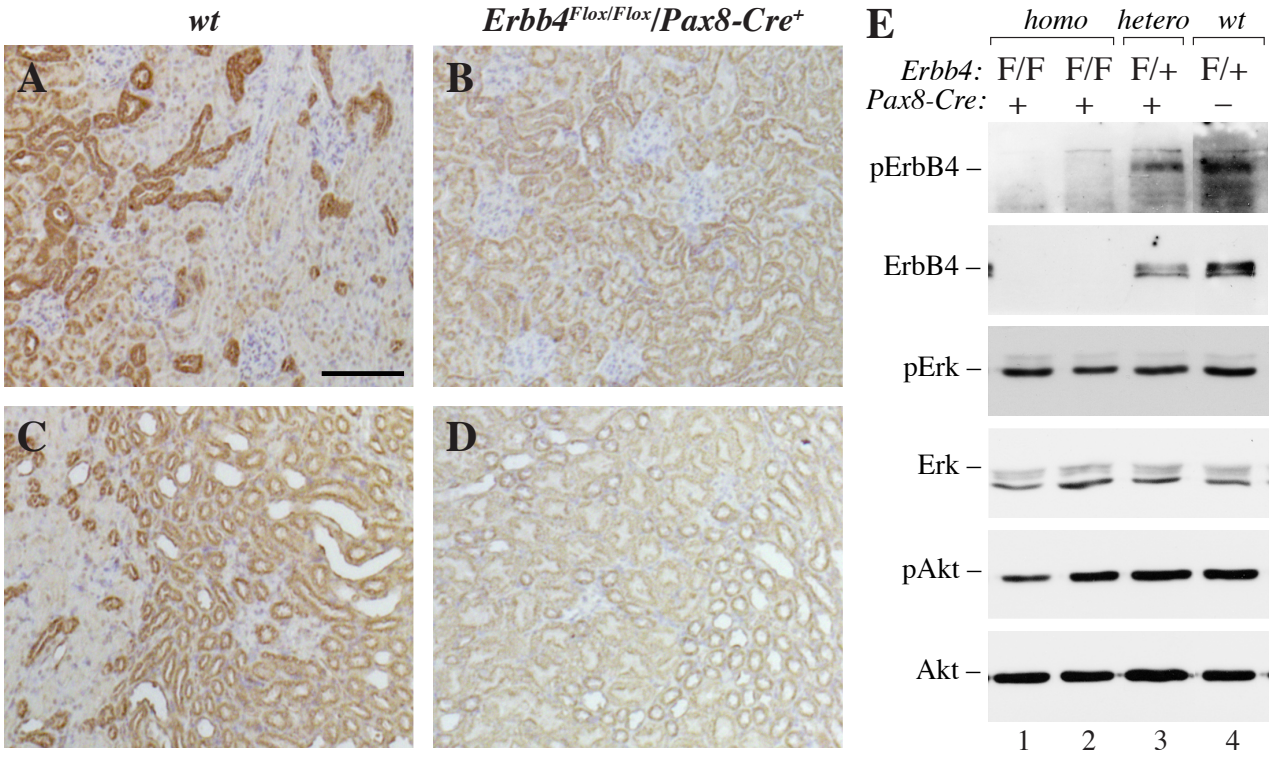
Supplemental Figure 3. *Erbb4*^{Flox}/*Pax8-Cre* mice. (A–D) Immunohistochemical analysis of ErbB4 expression in adult kidneys demonstrate significantly reduced or lost ErbB4 expression in epithelial structures of mutants with floxed *Erbb4* alleles (*Erbb4*$^{Flox/Flox}$/*Pax8-Cre*$^{+}$) as compared to littermate controls lacking *Pax8-Cre* recombination (*wt*; *Erbb4*$^{Flox/Flox}$/*Pax8-Cre*$^{-}$). (E) Western analyses of phospho-ErbB4 (pErbB4), ErbB4, phospho-Erk (pErk), Erk, phospho-Akt (pAkt), and Akt expression in kidney tissues of newborn mice homozygous (*Erbb4*$^{Flox/Flox}$/*Pax8-Cre*$^{+}$) or heterozygous (*Erbb4*$^{Flox/+}$/*Pax8-Cre*$^{+}$) for, or lacking (*Erbb4*$^{Flox/Flox}$/*Pax8-Cre*$^{-}$) *Erbb4* targeting. The analyses demonstrate the lack of ErbB4 expression and activity, and a moderate decrease in Akt phosphorylation in the kidneys of knock-out mice. Scale bar: (A) 100 μm.


A
Rosa26
promoter
genomic Rosa26-locus
locus after homologous
recombination with
targeting construct
locus after
Cre recombination
X
loxP
PGK-NEO-tpA
loxP
ERBB4 cDNA
X
loxP
ERBB4 cDNA
B
homologous
recombination:
+
–
+
neg. control
kb
2.0
1.5
1.0
1500 bp –
1243 bp –
1
2
3
4
5
C

Suppl. Fig. 1


A
wt
B
R26ERBB4+/–/Pax8-Cre+
C
R26ERBB4: +/– +/– +/– +/– +/– –/–
Pax8-Cre: – + + – – +
180 kD –
ErbB4
80 kD –
actin –
ErbB4 protein (densitometric units)
ErbB4
180 kD
80 kD
0 2 4 6 8 10 12
1 2 3 4 5 6
D
R26ERBB4: +/– +/– +/– +/– +/– –/–
Pax8-Cre: – + + – – +
pErbB4 – (180 kD)
ErbB4 – (180 kD)
actin –
1 2 3 4 5 6
E
R26ERBB4: –/– –/– –/– +/– +/– +/–
Pax8-Cre: + – + + + +
pErk –
Erk –
pAkt –
Akt –
1 2 3 4 5 6

Suppl. Fig. 2


wt
Erbb4Flox/Flox/Pax8-Cre+
A
B
C
D
E
homo
hetero
wt
Erbb4: F/F F/F F/+ F/+
Pax8-Cre: + + + –
pErbB4 –
ErbB4 –
pErk –
Erk –
pAkt –
Akt –
1 2 3 4

Suppl. Fig. 3

Name	Sequence
mJM-a/b fw	5′-TTGCCATCCAAACTGCACC-3′
mJM-a/b rev	5′-TCCAATGACTCCGGCTGC-3′
mJM-a FAM	5′-CATGGACGGGCCATTCCACTTTACCA -3′
mJM-b FAM	5′-TTCAAGCATTGAAGACTGCATCGGCCT-3′
mCYT-1/2 fw	5′-TCCTCCCATCTACACATCCAGAA-3′
mCYT-1/2 rev	5′-GGCATTCCTTGTTGTGTAGCAA-3′
mCYT-1 FAM	5′-TGAAATTGGACACAGCCCTCCTCCTG-3′
mNRG-1 fw	5′-TGGGACCAGCCATCTCATAAAG-3′
mNRG-1 rev	5′-TGAGGGGTTTGACAGGTCCTT-3′
mNRG-1 FAM	5′-AACTTTCTGTGTGAATGGAGGCGAGTGCTT-3′
E4m-*insitu*1	5′-GTTTGGAGCTATGGCGTCAC-3′
E4m-*insitu*2	5′-CACCACAGTATTCCGGTGTC-3′
*att*B1-A2	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAGAGTCGACGCCACCATG-3′
*att*B2-A2	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTACACCACAGTATTCCGGTGTCT-3′
Rosa Prom	5´-CCTAAAGAAGAGGCTGTGCTTTGG-3´
SA-2	5′-CATCAAGGAAACCCTGGACTACTG-3′
E4cDNA1	5′-TGGAAACAGAGTTGGTGGAA-3′
E4cDNA2	5′-TCCAACATTTGACCATGACC-3′

Supplemental Table 1. Primers and probes. Rosa Prom and SA-2 primers have been described by Soriano.[17]

PCR I		**PCR II**		**PCR III**	
Program	Reagents	Program	Reagents	Program	Reagents
1) 30 sec 98 °C	Primer 1: *att*B1-A2	1) 30 sec 98 °C	Primer 1: Rosa Prom	1) 3 min 94 °C	Primer 1: E4cDNA1
2) 10 sec 98 °C	Primer 2: *att*B2-A2	2) 15 sec 98 °C	Primer 2: SA-2	2) 30 sec 94 °C	Primer 2: E4cDNA2
3) 30 sec 55 °C	Phusion	3) 45 sec 60 °C	Phusion	3) 45 sec 60 °C	Dynazyme II
4) 1 min 72 °C		4) 1 min 72 °C	5% DMSO	4) 1 min 72 °C	5% DMSO
5) -> 2) x 3		5) -> 2) x 34		5) -> 2) x 34	2.5mM MgCl
6) 10 sec 98 °C		6) 10 min 72 °C		6) 10 min 72 °C	
7) 45 sec 72 °C		7) hold 4 °C		7) hold 4 °C	
8) -> 6) x 25					
9) 10 min 72 °C					
10) hold 4 °C					

Supplemental Table 2. PCR programs and reagents used in cloning the *ERBB4 JM-a CYT-2* insert for the targeting vector pRosa26-DEST-*ERBB4* (PCR I), analysis of homologous recombination of the vector into the genomic *Rosa26* locus in ES cells (PCR II), and genotyping the mice after Cre-mediated recombination of floxed sequences of the vector (PCR III). For sequences of the primers, see Supplemental Table 1. Dynazyme II and Phusion polymerases were purchased from Finnzymes (Espoo, Finland).