Supporting Information

Morris and McClatchey 10.1073/pnas.0902031106

SI Materials and Methods

Animals and Animal Procedures. Animals of both genders were used for all experiments. Genotyping by PCR was performed as described for *Nf2* (1), *Cre* recombinase, *Mx1*, and *GtRosa26* (Jackson Laboratory). BrdU (Sigma) was injected i.p. at 100 mg/kg body weight. In BrdU pulse experiments mice were killed 3 h after injection; in pulse-chase label-retaining experiments mice were injected daily for 1 week and killed 2 weeks later. Activation of the *Mx1* promoter was induced via 2 i.p. injections of 250 μg of polyIC (polyinosinic-polycytidylic acid; Sigma) at 48-h intervals.

Histology and Immunohistochemistry. Unless otherwise indicated, all reagents were from Sigma-Aldrich. Freshly dissected tissues were fixed in 3.7% formaldehyde-PBS, processed, and paraffin embedded. Sections (5-μm) were dewaxed, rehydrated, and stained with hematoxylin and eosin or further processed for immunohistochemistry. Antigen retrieval was performed by boiling in 10 mM citrate buffer (pH 6.0) (20 min) for pAkt, pMAPK, Ki67, β-catenin, and ezrin detection; boiling in 1 mM EDTA (pH 8.0) (15 min) for pSTAT3 detection; or incubation at 37 °C (10 min) in 0.5 M Tris (pH 2.0) with 1 mg/mL pepsin for vimentin detection. Sections were incubated for 10 min in 0.3% H_2O_2-methanol and blocked for 1 h in PBS-1% BSA-10% normal goat serum before overnight incubation with primary antibody.

For whole-mount LacZ staining, freshly dissected tissues were fixed (0.2% glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$, and 0.1 M $NaPO_4$ buffer, pH 7.3), washed 3 times in wash buffer [2 mM $MgCl_2$, 0.02% Nonidet P-40, 0.01% deoxycholate, and 0.1 M $NaPO_4$ buffer, (pH 7.3)], stained overnight in wash buffer (1 mg/mL X-gal, 5 mM K-ferricyanide, and 5 mM K-ferrocyanide) at 37 °C with gentle agitation, and postfixed in 3.7% formaldehyde–PBS. Some specimens were further processed and paraffin embedded, and 5-μm sections were counterstained with nuclear fast red.

Immunofluorescence. Paraffin sections (5 μm) were dewaxed, rehydrated, and blocked for 1 h in PBS-1% BSA-10% normal goat serum before incubation with primary antibody. Antigen retrieval was performed by boiling in 10 mM citrate buffer (pH 6.0) (20 min) for PH3 and NHERF-1 detection; boiling in 1 mM EDTA (pH 8.0) (15 min) for pY100 detection; or incubation at 37 °C (10 min) in PBS with 1 mg/mL trypsin for ZO-1detection. For cryosections, tissues were dehydrated through increasing concentrations of sucrose and embedded in OCT mounting media. Cryosections (7 μm) were fixed in 1:1 methanol/acetone for 10 min at −20 °C, air dried, rehydrated in PBS + Tween 20, and blocked in 0.1% Triton PBS with 1% BSA-10% normal goat serum before incubation with primary antibody.

Immunoblotting. Kidney tissue was homogenized at 4 °C in ≈10 volumes (wt/vol) of either 0.1% or 1.0% SDS RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM sodium fluoride, 10 mM β-glycerophosphate, and 10 μg/mL each of aprotinin, pepstatin, and leupeptin] using a teflon Potter-Elvehjem tissue grinder. Insoluble debris was cleared by centrifugation at 10,000 × *g* for 10 min at 4 °C, and lysates were normalized using the DC protein assay (BioRad). Proteins were separated by SDS-PAGE, transferred to PVDF, and blocked in Tris-buffered saline + Tween 20 with 5% BSA (1 h at room temperature) before incubation with primary antibody.

Primary Cells. To isolate primary cells, cortical tissue was dissected away from the renal capsule in cold PBS, minced, and suspended in primary cell media [50:50 DMEM/F12 (Gibco), 20 mM $NaHCO_3$, 15 mM Hepes (pH 7.4), 0.1 mg/mL kanamycin, 0.3 IU/mL penicillin, 5 μg/mL bovine insulin, 5 μg/mL human transferrin, 5×10^{-8} M hydrocortisone, 5×10^{-12} M triiodothyronine, 25 ng/mL prostaglandin E1, and 10 ng/mL EGF (Peprotech)] with 1 mg/mL soy trypsin inhibitor and 1 mg/mL collagenase. This suspension was rotated (15 min) at 37 °C, pelleted (500 × *g* centrifugation), and resuspended by rapid pipetting in a fresh aliquot of the same solution. After repeating this twice, cells were resuspended in 0.25% Trypsin/EDTA (3 min) at 37 °C, pelleted, washed twice in PBS with 1 mg/mL soy trypsin inhibitor, and plated in primary cell media.

1. Giovannini M, et al. (2000) Conditional biallelic Nf2 mutation in the mouse promotes manifestations of human neurofibromatosis type 2. *Genes Dev* 14:1617–1630.

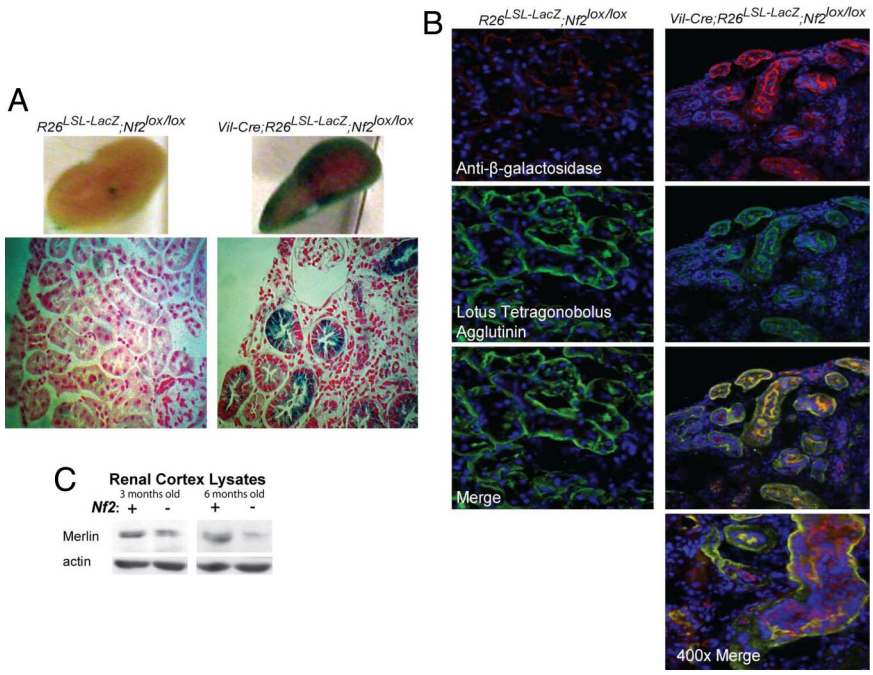

Fig. S1. *Vil-Cre;Nf2*$^{lox/lox}$ mice exhibit targeted loss of *Nf2* in the PCT of the kidney. (*A*) Whole-mount staining of *Vil-Cre;R26*$^{LSL\text{-}LacZ}$*;Nf2*$^{lox/lox}$ illustrated that β-galactosidase activity was grossly limited to the renal cortex and further confined to tubule epithelia in paraffin-embedded tissue sections. (*B*) Immunofluorescent labeling of *R26*$^{LSL\text{-}LacZ}$*;Nf2*$^{lox/lox}$ control (*Left*) and*Vil-Cre;R26*$^{LSL\text{-}LacZ}$*;Nf2*$^{lox/lox}$ (*Right*) kidneys with an antibody against the β-galactosidase enzyme and with the PCT epithelial cell-specific lectin marker *Lotus tetragonolobus* agglutinin demonstrated specific targeting of the PCT by *Vil-Cre* recombinase expression. This confirms results previously reported for *Vil-Cre* mice (56). This pattern held true in the tumors that developed in *Vil-Cre;R26*$^{LSL\text{-}LacZ}$*;Nf2*$^{lox/lox}$ mice (×400 merge, *Bottom Right*). (*C*) Immunoblot of renal cortex lysates confirmed decreased expression of Merlin in *Vil-Cre;Nf2*$^{lox/lox}$ kidneys. These data are representative of multiple sections from several mice.

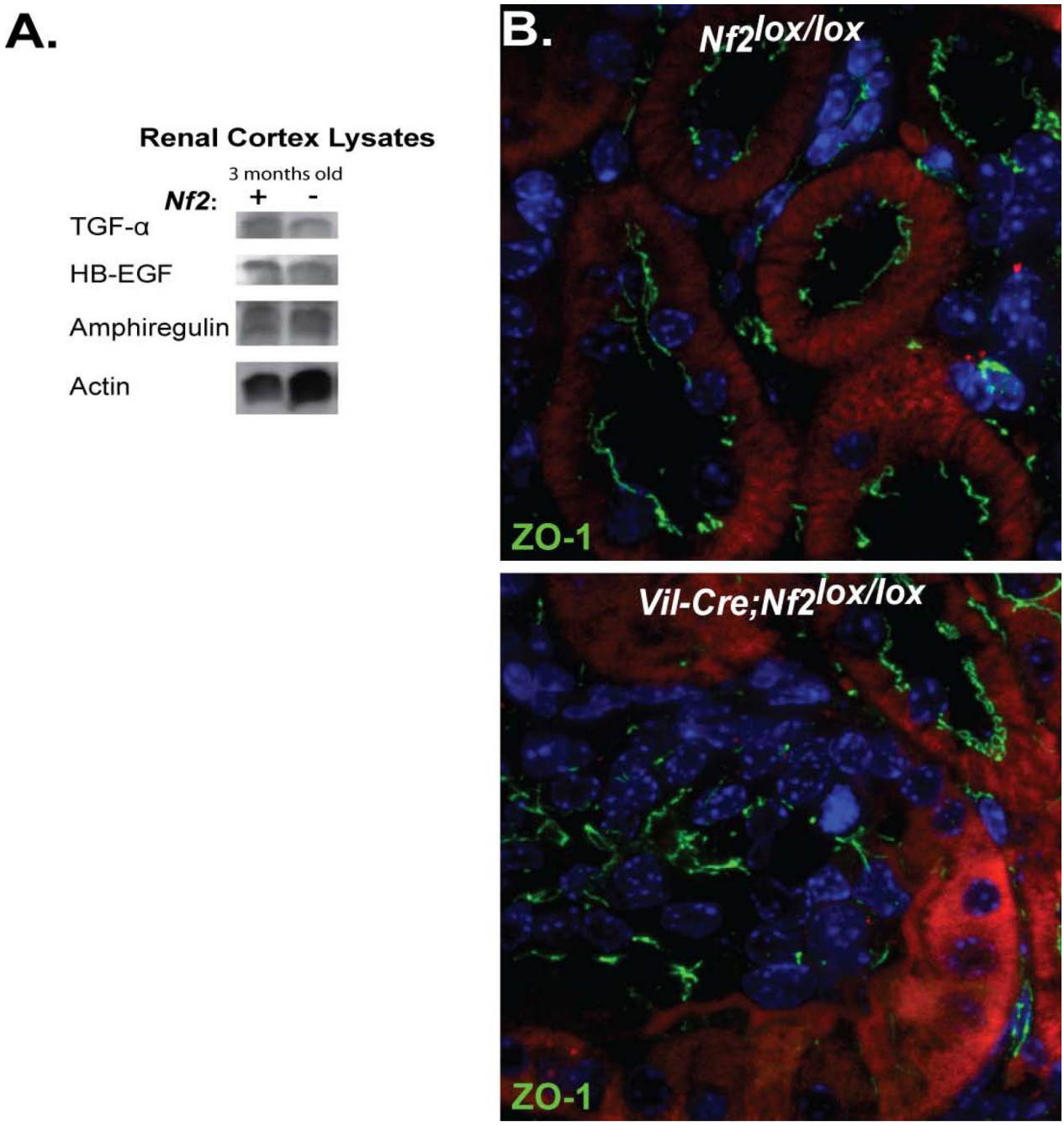

Fig. S2. EGF ligand expression and ZO-1 localization in *Vil-Cre;Nf2*$^{lox/lox}$ tumors. (*A*) No major differences were detected in the levels of the EGFR ligands TGF-α, HB-EGF, and amphiregulin in lysates from 3-month-old *Vil-Cre;Nf2*$^{lox/lox}$ kidney cortex relative to *Nf2*$^{lox/lox}$ control. (*B*) Like other markers of polarity, the tight junction marker ZO-1 was mislocalized or lost in renal tumors of 3-month-old *Vil-Cre;Nf2*$^{lox/lox}$ mice.

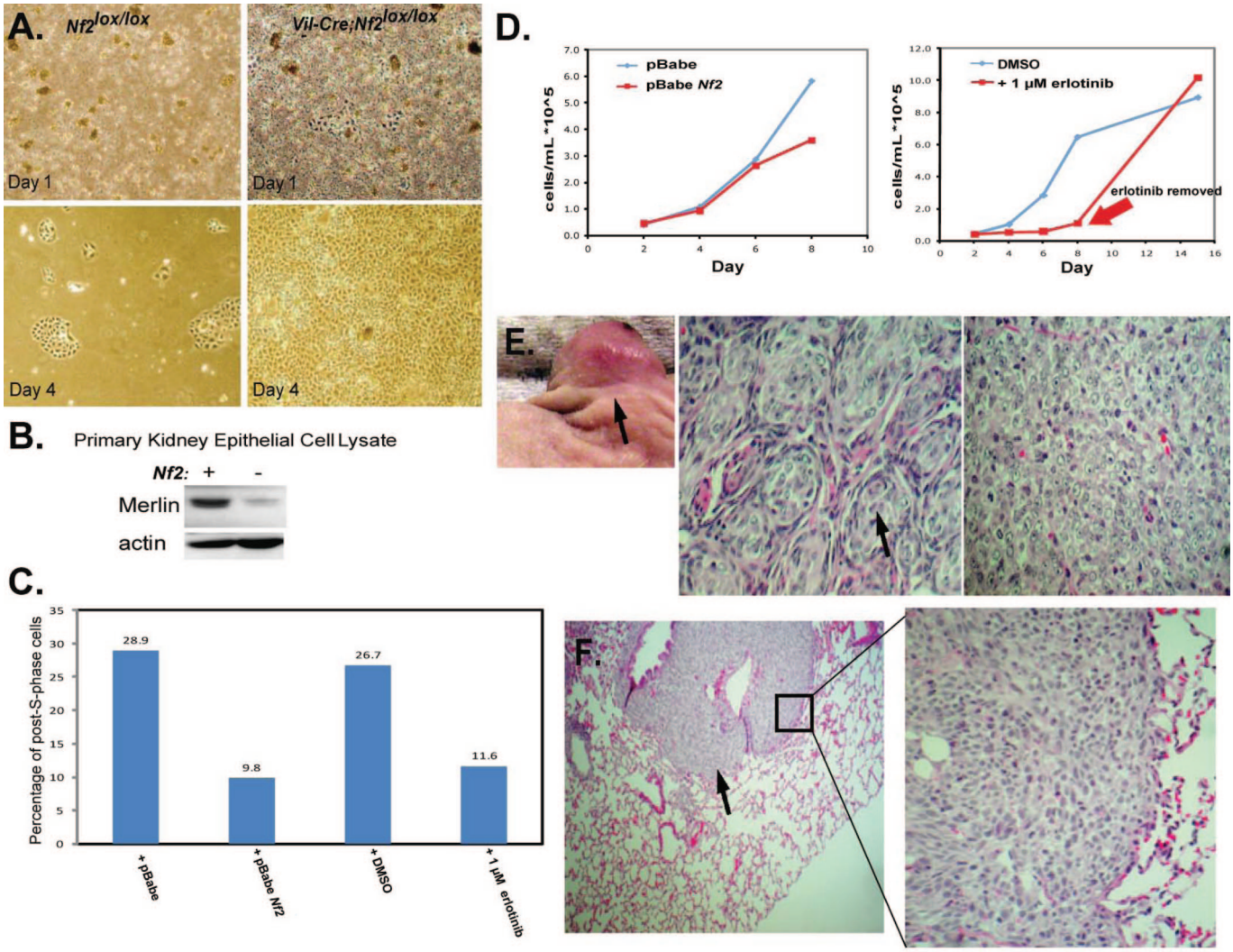

Fig. S3. Primary cultures of *Vil-Cre;Nf2*$^{lox/lox}$ epithelial kidney cells do not undergo contact-inhibition of proliferation, require EGFR signaling for growth in culture, and form malignant tumors in nude mice. (*A*) Renal epithelial cells derived from renal cortex of *Vil-Cre;Nf2*$^{lox/lox}$ mice grew to confluence and were capable of being passaged more than 12 times (*Right*). A few colonies of epithelial cells grew from *Nf2*$^{lox/lox}$ renal cortex, but they did not reach confluence and could not be passaged (*Left*). (*B*) Western blot analysis of cell lysate from these cultures confirmed the loss of Merlin in *Vil-Cre;Nf2*$^{lox/lox}$ samples. (*C*) The percentage of post-S-phase nuclei was determined in confluent (passage 8) *Vil-Cre;Nf2*$^{lox/lox}$ renal epithelial cells by propidium iodide staining and FACS analysis. Reintroduction of *Nf2* or treatment with 1 μM erlotinib greatly reduced the percentage of cells that had progressed through S-phase. (*D*) Growth curve analyses revealed a loss of contact-dependent inhibition of proliferation in *Vil-Cre;Nf2*$^{lox/lox}$ renal epithelial cells that was reversed by *Nf2* re-expression. Erlotinib treatment reversibly blocked proliferation of *Vil-Cre;Nf2*$^{lox/lox}$ renal epithelial cells. These curves reflect a constitutive antagonism of EGFR signaling by erlotinib as compared with the contact-dependent regulation of EGFR by Merlin. (*E*) Renal epithelial cells derived from both 3-week- and 9-month-old *Vil-Cre;Nf2*$^{lox/lox}$ mice were injected s.c. into the hip flank of nude mice (1×10^6 cells). Tumors developed from these injections regardless of the age of the source and grew slowly over the course of 4–6 months. Pathologic examination of tumors revealed mixed histology, with cells frequently recapitulating a tubular carcinoma appearance (arrow, *Center*) within masses of otherwise uniform tumor cells (*Right*). (*F*) Subcutaneous tumors in nude mice were malignant, and large metastases were observed in the lungs.