Supporting Information

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SI Experimental Procedures

Constructs and Screening. Cloning of full-length rat WNK1, non-neuronal form of human WNK3 (GenBank accession no. AJ409088) and their kinase-dead mutants WNK1(KD), WNK3(KD) to pCMV5-Myc vectors were described previously (1, 2). Brain isoform of human WNK3 (WNK3B, GenBank accession no. AY352048) was amplified from a cDNA provided by Lucy Raymond (Cambridge Institute for Medical Research, Cambridge, UK) and cloned into pCMV5-Myc. Small fragments encoding WNK1(1857C), WNK1 (1804C), WNK3(1474–1743), WNK3(1–411), WNK3(1–411/KD), and WNK3B(1521–1800) were amplified and subcloned into pCMV5-Myc for mammalian expression. WNK3 fragments 1–405, 406–750, 751–990, 991–1365, 1366–1743, 991–1412, 1413–1800 were generated and transferred to pGEX-KG vector for GST pull-down. Mouse Fox-1 cDNA (GenBank accession no. AY659954) was purchased from Open Biosystems, and mouse Fox-2 cDNA (GenBank accession no. AY659951) was a gift from Mariano Garcia-Blanco (Duke University Medical Center, Durham, NC). Both of the full-length proteins and Fox-1 small fragments including 1–326, 117–396, 1–196, 1–253, 1–291, 253–396, and 196–326 were amplified to subclone into p3XFLAG-CMV-7.1 (Sigma) or pCMV5-Myc.

The FMNL3 sequence including exon 25a, its upstream 132 nt, and downstream 713 nt was amplified from human genomic DNA (a gift from Kristen Lynch, University of Pennsylvania, Philadelphia, PA) then inserted between beta-globin constitutive exons 1 and 2 in the pcAT7-Glo1 parental minigene vector (also a gift from Kristen Lynch) to construct FMNL3 minigene. A neonatal mouse brain cDNA library was used for screening the interaction partners of various WNK3 baits (1474–1743, 1–146, and 490–782) in yeast strain L40. The assay was performed as described previously (3).

Antibodies. Anti-Myc antibody (9E10) was from the National Cell Culture Center and used at a dilution of 1:1,000 for immunoblotting and 1:100 for immunoprecipitation. For immunofluorescence, anti-myc antibody was purchased form Santa Cruz. Anti-Flag antibody was from Sigma and used at 1:10,000 for immunoblotting, 1:1,000 for immunoprecipitation, and 1:10,000 for immunostaining. Anti-RBM9 and anti-WNK3 antibodies were purchased from Bethyl Laboratories and Santa Cruz, respectively, and used at 1:1,000 for immunoblotting. Anti-EEF1A1 and anti-EEF2 antibodies were from Millipore.

Cell Culture, Transfection, Immunoblotting, Immunoprecipitation, and Immunofluorescence. HEK293 cells (ATCC) were grown in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) FBS, 1 mM L-glutamine, and 100 units/mL penicillin/streptomycin at 37 °C under 5% CO_2. The cells were reverse-transfected with Fugene 6 (Roche) and then harvested for analysis 36–48 h later, unless specified. For RNAi, the cells were transfected with siFox-2 oligonucleotides (sense, gguucguaacuuucgagaatt; antisense, uucucgaaaguuacgaacccg) with Lipofectamine RNAiMax (Invitrogen) following the manufacturer's protocol. For immunoblotting, cultured cells were lysed with 25 mM Hepes (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM EDTA, 50 μg/mL RNase A, 0.5 mM sodium orthovanadate, 20 μg/mL aprotinin, 10 μg/mL pepstatin A, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride) on ice for 45 min. Insoluble material was removed by centrifugation at 15,000 × *g* for 15 min at 4 °C. Protein concentration was determined by Bradford assay using BSA as standard. Soluble proteins from each sample were resolved by SDS/PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk for 1 h at room temperature and then incubated with primary antibody. For immunoprecipitation, lysates were precleared by incubation with 50 μL of 50% slurry of protein A/G-Agarose (Santa Cruz) for 1 h at 4 °C. Cleared lysates were incubated with M2-agarose (Sigma) or indicated antibody and 30 μL of 50% slurry of protein A/G-Agarose at 4 °C overnight. The agarose beads were washed three times with lysis buffer, resolved by SDS/PAGE, and blotted as above. Cells were grown on glass chamber slide (Lab-Tek) for immunofluorescence. The process was done as previously described (4).

Phosphorylation Site Mapping. Kinase reactions were performed as noted in *Experimental Procedures*,*In Vitro Kinase Assays*. Proteins were separated on gels and visualized through colloidal Coomassie blue staining. Proteins were extracted from the gel and subjected to trypsin digestion. Phosphorylation sites on the digested proteins were identified by precursor ion scanning and nanoelectrospray tandem mass spectrometry (MS/MS) as described previously (5).

1. Xu B, et al. (2000) WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. *J Biol Chem* 275:16795–16801.
2. Dorwart MR, Shcheynikov N, Wang Y, Stippec S, Muallem S (2007) SLC26A9 is a Cl(-) channel regulated by the WNK kinases. *J Physiol* 584:333–345.
3. Lee BH, et al. (2004) WNK1 phosphorylates synaptotagmin 2 and modulates its membrane binding. *Mol Cell* 15:741–751.
4. Tu SW, Bugde A, Luby-Phelps K, Cobb MH (2011) WNK1 is required for mitosis and abscission. *Proc Natl Acad Sci USA* 108:1385–1390.
5. Shu H, Chen S, Bi Q, Mumby M, Brekken DL (2004) Identification of phosphoproteins and their phosphorylation sites in the WEHI-231 B lymphoma cell line. *Mol Cell Proteomics* 3:279–286.

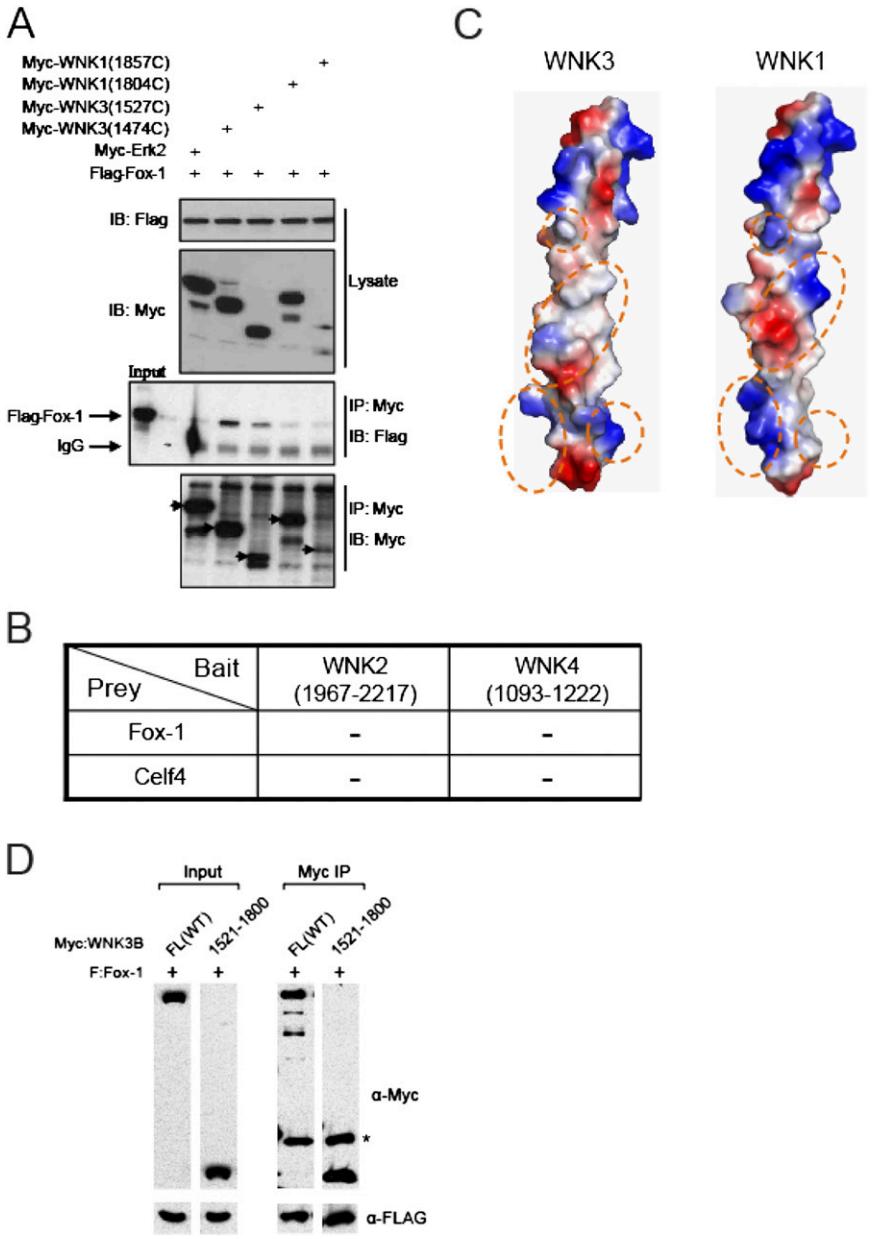

Fig. S1. Fox-1 binds to WNK3. (*A*) FLAG-tagged Fox-1 (F:Fox-1) was transfected to HEK293 cells with Myc-tagged proteins as indicated. Protein complexes were coimmunoprecipitated with anti-Myc antibody and analyzed by immunoblotting. Arrows in bottom panel indicate migration of Myc-tagged proteins. (*B*) Summary of yeast two-hybrid assays with the WNK C terminus including a coiled-coil region. "-" is no detectable binding. (*C*) The C-terminal coiled-coil domains of WNK3 and WNK1 were modeled to compare potential charged surfaces. Blue, positively charged regions; red, negatively charged regions; white, uncharged regions. Dotted lines indicate regions of charge difference between WNK3 and WNK1. (*D*) F:Fox-1 was expressed in HEK293 cells with Myc-tagged WNK3 as indicated. The protein complex was coimmunoprecipitated with anti-Myc antibody and analyzed by immunoblotting. Asterisk indicates position of IgG.

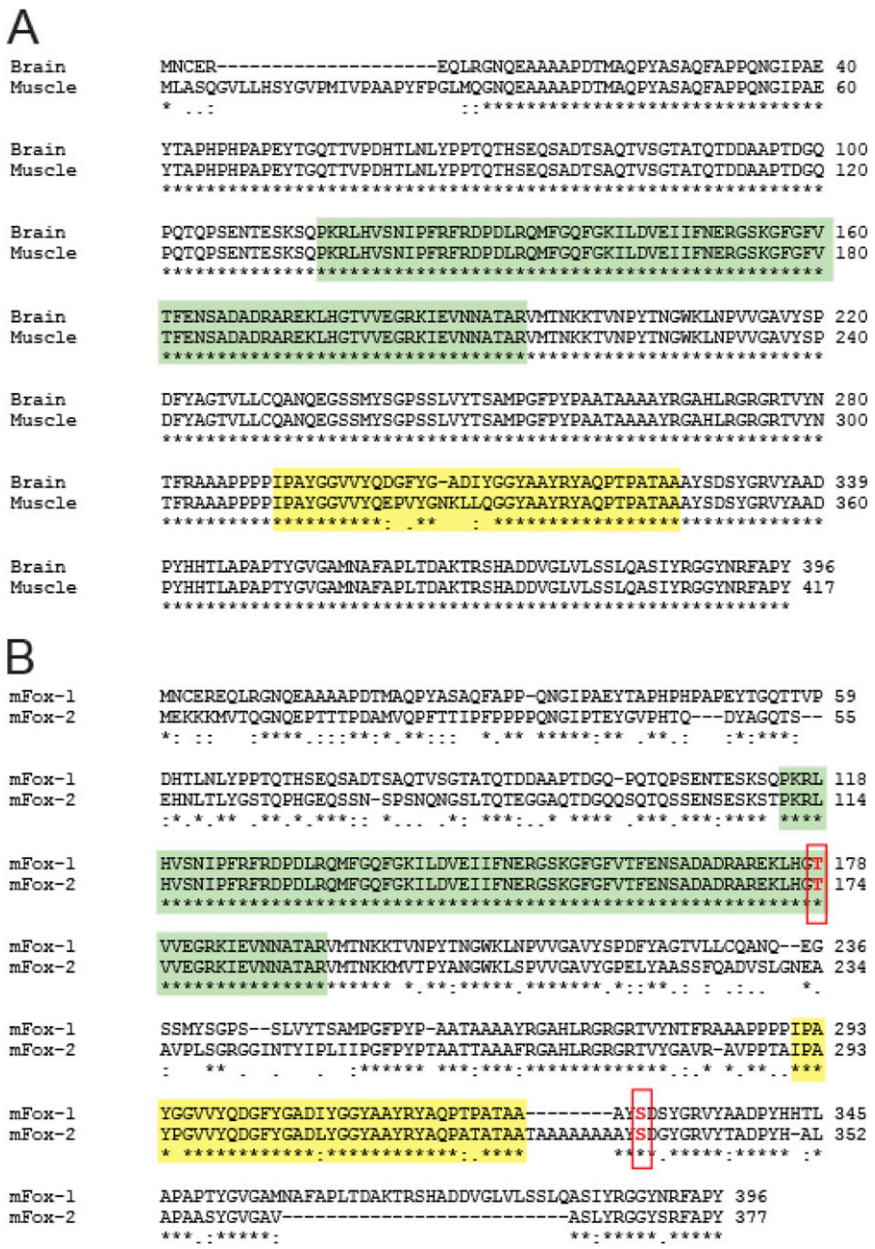

Fig. S2. Amino acid sequence alignment of Fox proteins. (*A*) Amino acid sequence alignment of brain- and muscle-specific isoforms of mouse Fox-1. The RRM is colored green and WNK3-binding region is in yellow. (*B*) Amino acid sequence alignment of mouse Fox-1 and Fox-2. The RRM is colored green and WNK3-binding region is in yellow. WNK3 phosphorylation sites identified by mass spectrometry are boxed in red.

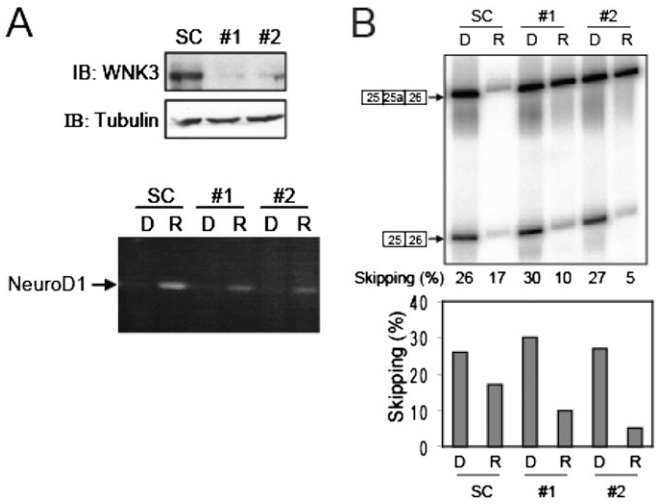

Fig. S3. The effect of endogenous WNK3 on FMLN3 splicing in neuronal system. (*A*) WNK3-stable knock-down cells were established with NTERA-2 cl.D1 cell line, a pluripotent embryonal carcinoma. SC represents the cell line carrying control shRNA and 1 and 2 indicate two different WNK3 knock-down cells. These cells were induced with retinoic acid for neuronal differentiation. The differentiation was confirmed through RT-PCR and agarose gel electrophoresis by monitoring induction of Neuro D1 (a neuronal differentiation marker). (*B*) Endogenous FMNL3 splicing assay with stable cell lines tested in *A*.

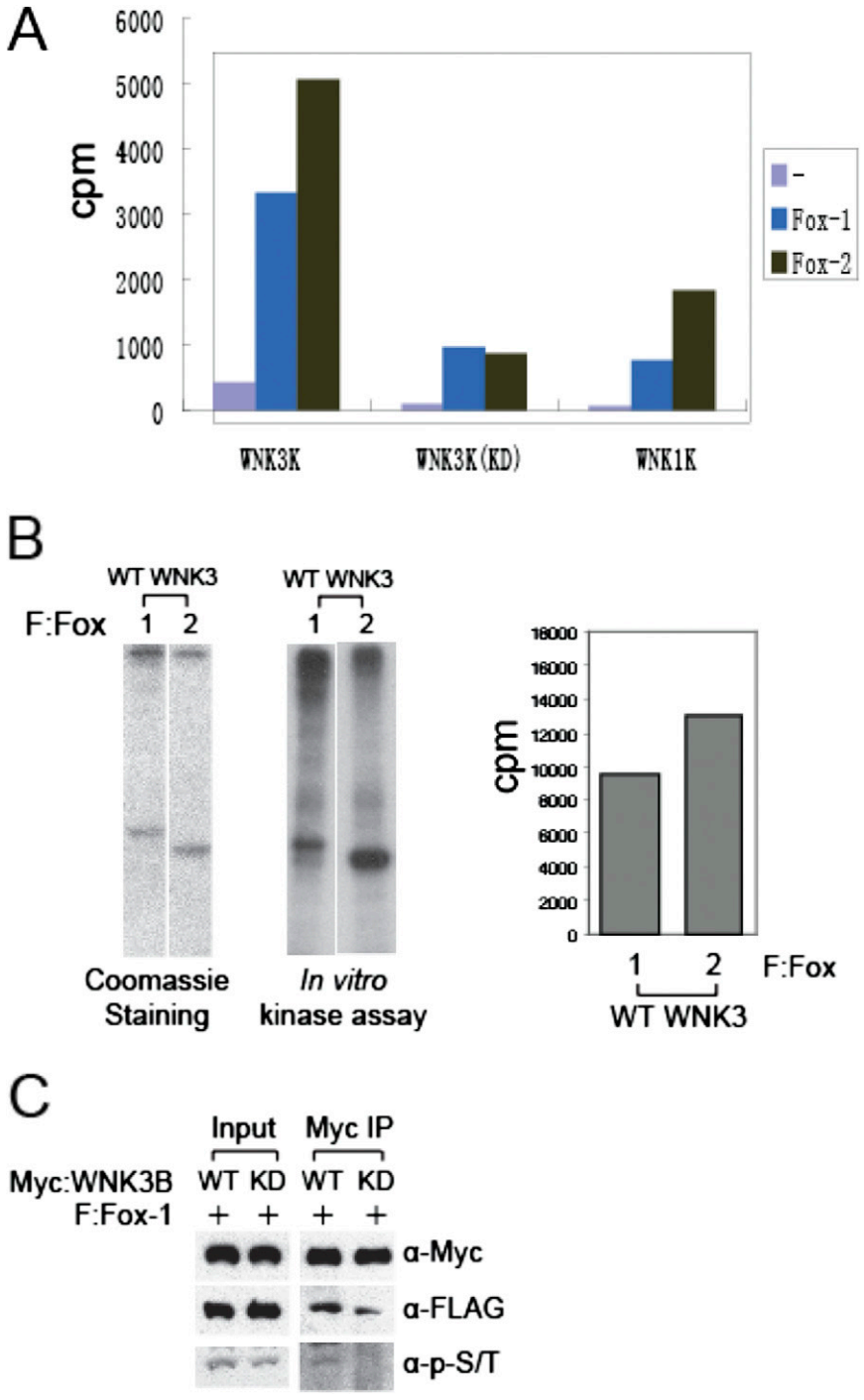

Fig. S4. WNK3 phosphorylates Fox-1. (*A*) Measurement of phosphate incorporation into Fox by phosphorylation. (*B*) Full-length WNK3 (WT WNK3) was incubated with purified Fox-1 and 2 for kinase assay. (*C*) F:Fox-1 was expressed in HEK293 cells with Myc-tagged WNK3 as indicated. The protein complex was coimmunoprecipitated with anti-Myc antibody and analyzed by immunoblotting.

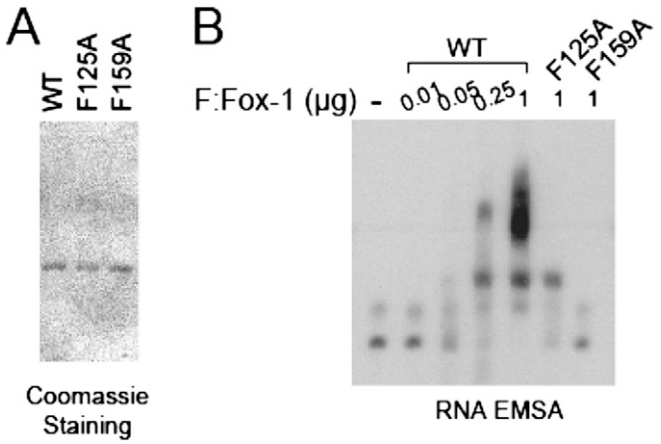

Fig. S5. Fox-1 recognizes (U)GCAUG elements through its RRM. (*A*) Coomassie blue-stained gel of FLAG-Fox-1 (F:Fox) proteins purified from bacteria. WT indicates wild-type. Both F125A and F159A are mutants that inactivate RNA binding ability. (*B*) The indicated amounts of F:Fox-1 proteins were incubated with RNA template with a (U)GCAUG element for RNA EMSA. The reaction was visualized using chemiluminescence detection as described in *Experimental Procedures*.

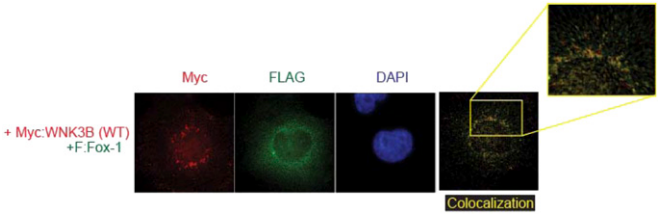

Fig. S6. Colocalization of WNK3 and Fox-1. Colocalization of Myc:WNK3 and F:Fox-1 was analyzed through Imaris software and shown as yellow. The magnified region is marked with a yellow box.

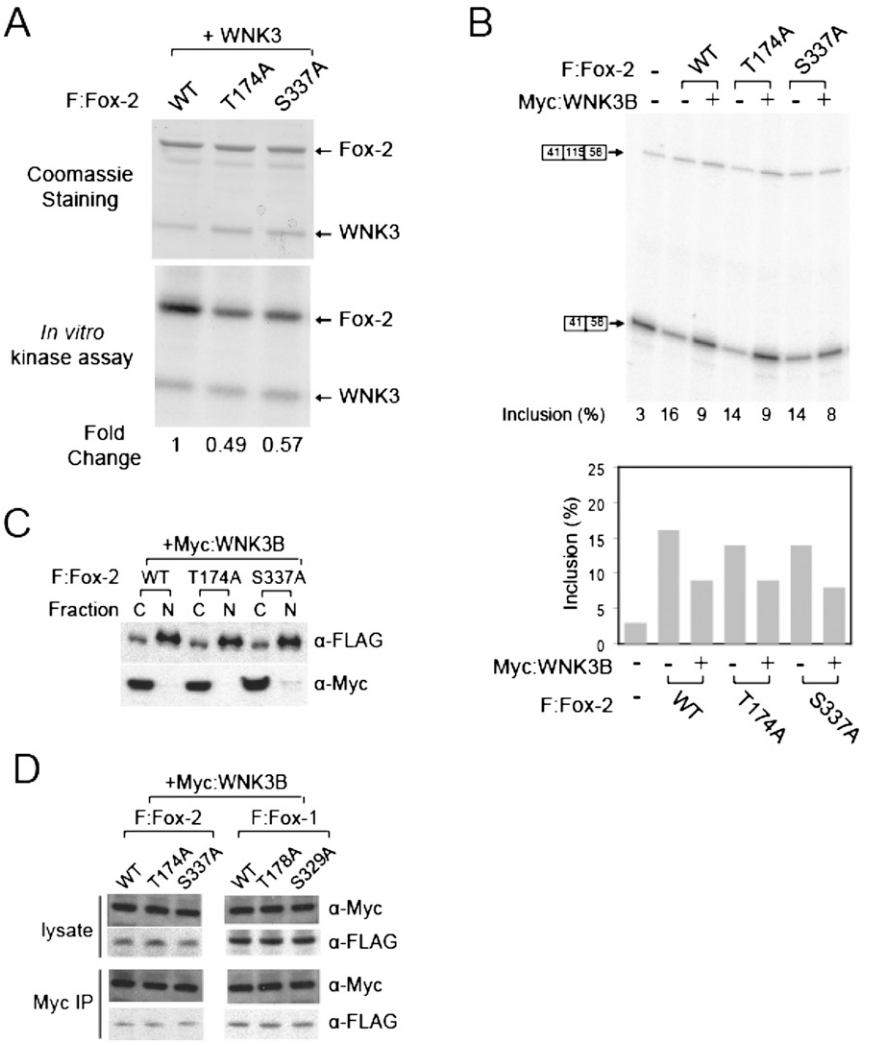

Fig. S7. Identification of WNK3 phosphorylation sites on Fox-2. (*A*) Wild-type (WT) and phosphorylation site mutants (T174A and S337A) of Fox-2 were incubated with the WNK3 kinase domain to be phosphorylated. (*B*) Minigene splicing assays with F:Fox-2 and Myc-tagged WNK3 as indicated. (*C*) F:Fox-2 proteins were coexpressed with Myc-tagged WNK3 proteins in HEK293T cells and then subjected to subcellular fractionation. C and N represent cytoplasmic and nuclear fractions, respectively. (*D*) F:Fox-1 and 2 proteins were expressed in HEK293 cells with Myc-tagged WNK3B as indicated. The protein complexes were coimmunoprecipitated with anti-Myc antibody and analyzed by immunoblotting.

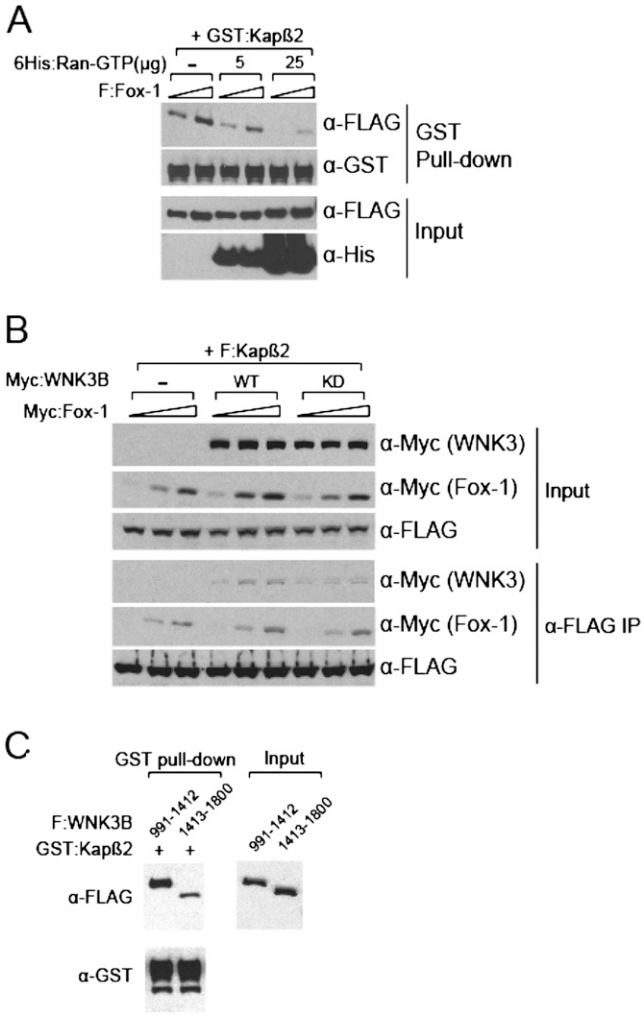

Fig. S8. Fox-1 binds to Kapβ2. (*A*) Increasing amounts (50 and 200 ng, indicated by a triangle) of purified F:Fox-1 were incubated with immobilized GST-tagged Kapβ2 in the absence or presence of GTP-loaded Ran. Binding was visualized by immunoblotting with the indicated antibodies. (*B*) Myc-tagged Fox-1 and FLAG-tagged Kapβ2 were transfected in HEK293 cells with or without WNK3B proteins [wild-type (WT) or kinase-dead mutant (KD)]. Protein complexes were coimmunoprecipitated with anti-FLAG antibody and analyzed via immunoblotting with the indicated antibodies. (*C*) GST pull-down assays with GST-tagged Karyopherin β2 and bacterially purified F:WNK3 proteins.