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Supplemental Online Methods

Fly stocks

Fly rearing and crosses were done on standard cornmeal/yeast/molasses food (prepared in a central kitchen at University of Texas Southwestern Medical Center or the University of Utah). The following *D. melanogaster* strains were used: *w; c42-GAL4*, expressing *GAL4* in the principal cell of the main segment of the renal tubule (obtained from Julian Dow and Shireen Davies, University of Glasgow, Glasgow, United Kingdom)[1]; *w; tub-GAL80^{ts20},* a temperature-sensitive GAL4 repressor, allowing temporal manipulation of GAL4 activity[2] (obtained from Bloomington *Drosophila* Stock Center, Indiana University, Bloomington, IN, stock #7019); *w;*

UAS-DmWNK^{RNAi}, which drives expression of interfering RNA against *Drosophila WNK* (obtained from Bloomington *Drosophila* Stock Center, Indiana University, Bloomington, IN, stock #42521), *w; UAS-DmMo25^{RNAi}*, which drives expression of interfering RNA against *Drosophila Mo25*[3], obtained from the Vienna *Drosophila* Resource Center (Vienna, Austria, stock #34788), and *w; UAS-Fray^{T206E}*, in which the Thr 206 target of WNK phosphorylation is mutated to a phosphomimicking Glu[4]. The following transgenic lines were newly generated in this study and are described in more detail below: *w; UAS-ClopHensor c304*, *w; UAS-SPAK^{D219A}*, *w; UAS-DmWNK^{WT}, w; UAS-DmWNK^{L421F}, w; UAS-DmMo25* and *w; UAS-MmMo25.* Except for *w; UAS-ClopHensor c304,* which has multiple inserts, all lines were outcrossed for at least 5 generations to the Rodan laboratory *wBerlin* genetic background.

Flies expressing ClopHensor in the tubule were reared at 26°C. Other crosses were performed at 28°C, except in the case of experiments using *tub-GAL80^{ts20}*, in which case crosses were performed at 18°C. Adult females were collected within 1-2 days of eclosion and placed on regular fly food for an additional 3-5 days prior to dissection of tubules. For experiments using *tub-GAL80^{ts20}*, flies were shifted to 28°C for two days prior to experimentation.

Generation of transgenic fly lines

UAS-ClopHensor

The pcDNA3-ClopHensor plasmid[5] was obtained from Addgene (plasmid #25938). Using KpnI and XbaI restriction sites, the ClopHensor cDNA was subcloned into a modified pUASt vector, sequence verified, and injected into *w^{1118}* embryos by Bestgene Inc (Chino Hills, CA). Transgenics were identified by *w^{+}* eye color and crossed to double balancer stocks to identify chromosomal location. The *UAS-ClopHensor c304* transgenic was chosen based on robust expression in renal tubule epithelial cells when crossed to the principal cell driver, *c42-GAL4.*

UAS-SPAK^{D219A}

The UAS-rat SPAK plasmid was generated using the Gateway cloning method (ThermoFisher) with Platinum *Pfx* DNA polymerase (ThermoFisher 11708013), pENTR/D-TOPO Cloning Kit (ThermoFisher, K240020), LR Clonase II (ThermoFisher, 11791020), and the Gateway compatible destination vector pUASg.attB, obtained from Johannes Bischof and Konrad Basler (Zurich, Switzerland)[6]. Template vector (pRK5-rSPAK) was obtained from Chou-Long Huang (UT Southwestern, Dallas, TX). A rat SPAK PCR amplicon was gel purified and incorporated into the attL containing entry vector pENTR via directional TOPO cloning per manufacturer's protocol using primers 5' CACCATGGCGGAGCCGAGCGGCTC 3' and 5' GCTCACACTCAACTGGGCGAACC 3'. The full-length SPAK open reading frame was recombined into the attR containing destination vector pUASg.attB by LR clonase reaction and sequence confirmed. The D219A mutation was introduced using the QuikChange II Site-Directed Mutagenesis Kit (Agilent catalog #200523) per manufacturer's protocol and primers 5' GGATCAGTACAGATAGCAGCTTTTGGAGTAAGTGCATTC 3' and 5' GAATGCACTTACTCCAAAAGCTGCTATCTGTACTGATCC 3'. Midiprep DNA was injected into stock #24483 (M{vas-int.Dm}ZH-2A, M{3xP3-RFP.attP}ZH- 51D) by Rainbow Transgenic Flies (Camarillo, CA). Transgenic lines were generated from single male transformants, and PCR confirmation of UAS-SPAK was performed using sequence-specific primers.

UAS-DmWNK^{WT}

The *Drosophila* WNK open reading frame, previously cloned into pENTR[4], was recombined into pUASg.attB using the LR clonase II kit (ThermoFisher). Midiprep DNA was injected into stock #24481 (M{vas-int.Dm}ZH-2A, M{3xP3-RFP.attP}ZH- 22A) by Rainbow Transgenic Flies (Camarillo, CA). Transgenic lines were generated from single male transformants, and PCR confirmation of UAS-WNK was performed using sequence-specific primers.

UAS-DmWNK^{L421F}

To generate the open reading frame encoding *Drosophila* WNK^{L421F} under UAS control, pUASg.attB_DmWNK (described above) was mutated using the QuikChange II Site-Directed Mutatagenesis Kit (Agilent) and primers 5' GTGTTAAAATCGGCGACTTCGGCCTGGCAACTTT 3' and 5' AAAGTTGCCAGGCCGAAGTCGCCGATTTTAACAC 3'. Midiprep DNA was injected into stock #24481 (M{vas-int.Dm}ZH-2A, M{3xP3-RFP.attP}ZH- 22A) by Rainbow Transgenic Flies (Camarillo, CA). Transgenic lines were generated from single male transformants, and PCR confirmation of UAS-WNK was performed using sequence-specific primers.

UAS-DmMo25

A plasmid containing the open reading frame of *Drosophila Mo25* was obtained from the *Drosophila* Genomics Resource Center (clone LD09550, Indiana University, Bloomington, IN). The open reading frame was amplified using Platinum *Pfx* DNA polymerase (ThermoFisher) with primers 5' CACCATGCCACTGTTCGGGAAGTCAC 3' and 5' AGCCTCGGGCAGCGGCTTC 3' and cloned into pENTR using the pENTR/D-TOPO cloning kit (ThermoFisher). After sequence confirmation, the open reading frame was recombined into pUASg.attB using the LR clonase II kit (ThermoFisher). Midiprep DNA was injected into stock #24483 (M{vas-int.Dm}ZH-2A, M{3xP3-RFP.attP}ZH- 51D) by Rainbow Transgenic Flies (Camarillo, CA). Transgenic lines were generated from single male transformants, and PCR confirmation of UAS-Mo25 was performed using sequence-specific primers.

UAS-MmMo25

The full-length cDNA encoding mouse *Mo25* (also known as *Cab39*) (ORF accession number NM_133781) was obtained from GenScript (Pistcataway, NJ) in the Gateway compatible vector pENTR/D-TOPO. A clonase reaction (ThermoFisher) was performed to recombine the open reading frame from pENTR into the pUASg.attB vector. After sequence confirmation, midiprep

DNA was sent to Rainbow Transgenic Flies (Camarillo, CA) for microinjection into stock line #24862 (*M{vas-int.Dm}ZH-2A, PBac{y[+]-attP-9A}VK00005*). Positive transformant males were isolated by presence of 'mini-white,' and used to generate homozygous transgenic stocks.

WNK kinase autophosphorylation assays

Expression and purification of Drosophila WNK

The kinase domain of *Drosophila* WNK, residues 261-534[4] optimized by GenScript, was cloned into the pET29b vector (Novagen). Protein was expressed in BL21 (DE3) pLysS cells, which were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside at OD_{600} = 1.0 and the protein expressed for 4 hours at 18°C. The lysate was cleared by centrifugation at 35,000 x g for 1 hour. The supernatant was applied to a Ni-NTA agarose column (Qiagen) and the protein eluted with 250 mM imidazole. The fraction containing the kinase was further purified on a Mono-Q HR10/10 column (GE Healthcare) by a linear gradient. The protein was concentrated to 5 mg/ml and buffer exchanged to 50 mM HEPES pH 7.4, 150 mM NaCl.

Autophosphorylation assays using Pro-Q Diamond

Autophosphorylation assays were performed in 50 μl volume in buffers including (in mM) HEPES 20, pH 7.4, Mg gluconate 20, ATP 5, and NaCl to achieve a final chloride concentration of 50 or 150 mM. Reaction mixtures were pre-warmed for 5 minutes at 30°C. 50 μg purified WNK kinase domain (5 mg/ml) was added to start the reaction and run over a 0, 1, 2 or 4 hour timecourse at 30°C. Reactions were terminated by adding 17 μl 4x SDS sample buffer and heating the sample for 5 minutes. Reactions were then run on a 12% Mini-PROTEAN TGX Precast gel (Bio-Rad) using the Pro-Q Diamond phosphoprotein gel stain according to the manufacturer's instructions (ThermoFisher). Band intensity was quantified using ImageJ.

Autophosphorylation assays using mass spectrometry

Reactions were run as above, except that WNK autophosphorylation was terminated in 1 M guanidine-HCl for HPLC-MS analysis.

Peptide Standards for Mass Spectrometry

Isotopically labeled WNK peptide standards corresponding to phosphorylated and unphosphorylated chymotrypsin-derived activation loop peptides (AKSVIGTPEFMAPEMY and AKS*VIGTPEFMAPEMY, with S* denoting phospho-serine) were synthesized (21st Century Biochemicals). These standards were used to optimize HPLC peptide separation, determine elution times, and for relative quantitation.

Protein Digestion and HPLC Peptide Separation

Chymotrypsin protease reaction mix (containing 100 mM Tris-HCl, pH 8.3, 25 mM $CaCl_2$, and sequencing grade chymotrypsin (Roche Applied Science)) was used to digest *Drosophila* WNK. All proteolysis reactions were conducted at a 100:1 protein:protease molar ratio. Activation loop peptides were separated by an Agilent 1100 series LC system (Agilent Technologies) with an RP-C18 microbore HPLC column (Phenomenex Aeris WIDEPORE 150 2.1 mm, 3-μm particle size, 200 Å pore diameter). Peptides were eluted using a water/acetonitrile gradient with 0.2% formic acid. *Drosophila* WNK activation loop peptides eluted at 21–23% acetonitrile. Peptides were separated from denaturant on the HPLC column by diverting early eluents.

LC-MS/MS Analysis—HPLC-MS/MS analysis was performed on an LCQ DECA XP ion trap mass spectrometer (ThermoFinnigan) with the HPLC in-line to an orthogonal electrospray ionization source. Integration under ion traces corresponding to mass ranges for the activation loop peptides were used to acquire raw MS detector responses. Time courses for the appearance of peptides were conducted in triplicate, with the raw MS response scaled to

peptide standards, then converted to percent phosphorylation. MS/MS spectra were acquired in a data-dependent manner for each MS scan (ThermoFinnigan). MASCOT (Matrix Science Ltd.)[7] and MassMatrix[8] software was used for identifying peptides from tandem mass spectrometry spectra.

Measurement of intracellular chloride concentration

pH calibration

Tubules from female flies expressing ClopHensor in the principal cell (*w; c42-GAL4/UAS-ClopHensor c304*) were dissected from 3-5 day old flies in *Drosophila* saline, consisting of (in mM): NaCl 117.5, KCl 20, $CaCl_2$ 2, $MgCl_2$ 8.5, $NaHCO_3$ 10.2, NaH_2PO_4 4.3, HEPES 15, and glucose 20, pH 7.0. Tubules were attached to the bottom of 35 mm glass bottom dishes with 14 mm microwell/#1.5 cover glass (Cellvis) coated with poly-lysine, and the solution exchanged to pH varied solution containing: 100 mM Na gluconate, 50 mM K gluconate, 8.5 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM glucose, 15 mM HEPES (varied pH), 10 μM tributyltinchloride (Sigma), 5 μM nigericin (Invitrogen), 5 μM carbonyl cyanide 3-chlorophenylhydrazone (Sigma) and 5 μM valinomycin (Sigma). After equilibration for at least 1 hour, cells were imaged using a Zeiss LSM510 confocal microscope, with excitation at 488 nm (green emission) and 458 nm (cyan emission). Individual cells were then outlined and pixel intensity measured using ImageJ without image manipulation. The ratios of green/cyan vs. pH were entered into GraphPad Prism, and a sigmoidal curve interpolated using the function “sigmoidal, 4PL, X is log(concentration).” This provided the values for the following equation, used to calculate intracellular pH (pH_i) in the tubule epithelial cells[9]:

$$pH_i = pK_a - \frac{1}{p} * \log\left(\frac{B2 - B1}{R_{pH} - B1} - 1\right)$$

where R_{pH} is the experimentally derived green/cyan ratio, pK_a = 7.239, p = power (Hill slope, 2.04), and *B1* (0.716) and *B2* (2.271) are the minimum and maximum asymptotic values of R_{pH}.

Chloride calibration

Tubules expressing ClopHensor in the principal cells (*w; c42-GAL4/UAS-ClopHensor c304*) were dissected from 3-5 day old flies in *Drosophila* saline. Tubules were attached to the bottom of 35 mm glass bottom dishes with 14 mm microwell/#1.5 cover glass (Cellvis) coated with poly-lysine, and the solution exchanged to the chloride calibration solution, consisting of (in mM): 100 mM Na-Cl/gluconate, 50 mM K-Cl/gluconate, 2 mM Ca-Cl/gluconate, 8.5 mM Mg-Cl/gluconate, 20 mM glucose, 15 mM HEPES pH 7.2, 10 μM tributyltinchloride (Sigma), 5 μM nigericin (Invitrogen), 5 μM carbonyl cyanide 3-chlorophenylhydrazone (Sigma) and 5 μM valinomycin (Sigma). Cl/gluconate anions were adjusted to achieve varying chloride concentrations. After 1 hour equilibration, tubules were imaged using a Zeiss LSM510 confocal microscope, with excitation at 543 nm (red emission) and 458 nm (cyan emission). Individual renal tubule epithelial cells were outlined and pixel intensity measured in ImageJ without image manipulation. The ratios of cyan/red vs chloride were entered into GraphPad Prism, and a sigmoidal curve interpolated using a logistic dose-response sigmoidal fit. This provided the values for the following equation, used to calculate intracellular chloride ($[Cl^-]_i$)[9]:

$$[Cl^-]_i = K_d * \left(\frac{A1 - A2}{R_{Cl} - A2} - 1\right)^{\frac{1}{p}}$$

where R_{Cl} is the experimentally derived cyan/red ratio, K_d = 17.33, p = power (Hill slope 1.622), and *A1* (3.504) and *A2* (1.897) are the maximum and minimum asymptotic values of R_{Cl}.

Measurement of R_{pH} and R_{Cl}

Tubules from adult female flies expressing ClopHensor in the principal cell (*w; c42-GAL4/UAS-ClopHensor c304)* were dissected in *Drosophila* saline at 3-5 days of age. Tubules were attached to the bottom of 35 mm glass bottom dishes with 14 mm microwell/#1.5 cover glass (Cellvis) coated with poly-lysine, and then bathed in standard bathing medium (measured osmolality, 335 mosm/kg^4) for 60 minutes. Standard bathing medium consists of a 1:1 mixture of *Drosophila* saline (above) and Schneider's medium (LifeTechnologies). Schneider's medium consists of (in mM): glycine, 3.33; L-arginine, 2.3; L-aspartic acid, 3.01; L-cysteine, 0.496; L-cystine, 0.417; L-glutamic acid, 5.44; L-glutamine, 12.33; L-histidine, 2.58; L-isoleucine, 1.15; L-leucine, 1.15; L-lysine hydrochloride, 9.02; L-methionine, 5.37; L-phenylalanine, 0.909; L-proline, 14.78; L-serine, 2.38; L-threonine, 2.94; L-tryptophan, 0.49; L-tyrosine, 2.76; L-valine, 2.56; β-alanine, 5.62; $CaCl_2$, 5.41; $MgSO_4$, 15.06; KCl, 21.33; KH_2PO_4, 3.31; $NaHCO_3$, 4.76; NaCl, 36.21; Na_2HPO_4, 4.94; α-ketoglutaric acid, 1.37; D-glucose, 11.11; fumaric acid, 0.862; malic acid, 0.746; succinic acid, 0.847; trehalose, 5.85; and yeastolate, 2000 mg/L.Tubules were imaged using a Zeiss LSM510 confocal microscope, with excitation at 543 nm (red emission), 488 nm (green emission), and 458 nm (cyan emission). Standard bathing medium (measured osmolality, 335 mosm/kg^4) was then exchanged for hypotonic bath, in which water is added to standard bathing medium at a ratio of 90 μL water to 300 μL standard bathing medium (measured osmolality, 257 mosm/kg^4). The tubules were then re-imaged at varying timepoints after incubation in hypotonic medium. Individual epithelial cells were outlined in ImageJ, excluding the nucleus, and pixel intensity captured for each emission channel. The ratios of green/cyan and cyan/red were used to calculate pH and chloride as described above. The same cells were analyzed in a paired fashion in isotonic and hypotonic conditions. pH and chloride were measured for each individual cell. Results were analyzed with GraphPad Prism.

Measurement of tubule WNK activity

15 pairs of anterior renal tubules, expressing kinase-dead rat SPAK as a substrate for the endogenous *Drosophila* WNK, were dissected from adult females in standard bathing medium (SBM). Tubule pairs were transferred to 300 µl SBM in a 9 well Pyrex® dish. To prevent evaporation, Parafilm was used to cover the wells. After 1 hour equilibration in SBM, 90 µl H_2O was added to the SBM to make the hypotonic solution. Tubules were bathed in the hypotonic bath for 0-120 minutes, again with Parafilm covering the wells. Tubules were then transferred to 100 µl 1X Laemmli sample buffer and lysed by pipetting up and down several times. In a second experiment, tubules were bathed for one hour in normal potassium medium, then for an additional hour in normal, low or high potassium bathing medium prior to analysis. Normal potassium saline consisted of the same components as *Drosophila* saline (above), except that NaCl concentration was 97.5 mM and 20 mM NMDG Cl was added. KCl was omitted from the low potassium saline, and NMDG Cl concentration was 40 mM. In the high potassium saline, KCl concentration was 40 mM, and NMDG Cl was omitted. As with SBM, salines were mixed 1:1 with Schneider's medium. Osmolality was measured in triplicate samples using an Osmette II osmometer (Precision Systems). The composition of the varying salines, and individual ion concentrations of the saline/Schneider's bathing media, are shown in Supplemental Tables 1 and 2.

30-50 µl lysate was used to detect phospho-SPAK and total-SPAK by Western blotting. Lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF membrane, 0.45 µm) (Thermo Scientific, #88518) by semi-dry transfer (Bio-Rad, Model: TransBlot SD Cell). The membrane was blocked in Tris buffer saline-0.1% Tween 20 (TBST) with 3% nonfat milk for 30 minutes at room temperature, and then it was probed with antibodies to phosho-SPAK (Ser373)/phospho-OSR1 (Ser325) (Millipore, Cat. #07-2273, Lot#2689632 and Lot#2840398), total-SPAK (Cell Signaling Technology, #2281, Lot:4, Abcam anti-STK39 [2E10], #117982, or GeneTex anti-STK39 [2E10], Cat. #GTX83543, Lot#821703924) and actin

(JLA20-s) (DSHB) overnight at 4°C. For the hypotonic bath experiments, after extensive washes in TBST, the membrane was incubated with a horseradish peroxidase-conjugated anti-rabbit (Thermo Scientific, Cat. #31460, Lot #NL181270) or anti-mouse secondary antibody (Thermo Scientific, Cat. #31430, Lot #NJ178769) for 1 hour at room temperature in 5% non-fat milk-TBST, and washed again extensively in TBST. The primary antibodies were used at 1:1000 dilution, or, for the Abcam anti-STK39 antibody, 1:2000, and the secondary antibodies were used at 1:10,000 dilution. Protein bands were visualized using SuperSignal® West Pico Stable Peroxide Solution (Thermo Scientific, Cat. #1856135, Lot #ME157404A). For the varying potassium bath experiments, after extensive washes in PBS, the membrane was incubated with a fluorophore-conjugated anti-rabbit AzureSpectra 800 (VWR, Cat. #AC2134, Lot #170124-06) or anti-mouse AzureSpectra 700 (VWR, Cat. #AC2129, Lot #170403-07) secondary antibody for 1 hour at room temperature in 5% non-fat milk-PBS, and washed again extensively in PBS. The primary antibodies were used at 1:1000 dilution, and the secondary antibodies were used at 1: 2500 dilution. Protein bands were visualized using a c600 Azure Biosystems instrument. Band intensities were quantified in ImageJ by manually outlining the bands and subtracting background pixel intensities from a nearby region. Data were analyzed in GraphPad Prism, version 7.

Real-time quantitative reverse transcriptase polymerase chain reaction

Measurement of tubule DmMo25 knockdown

50 pairs of renal tubules were collected from female flies of genotypes *w; c42-GAL4/+* (control) or *w; UAS-Mo25^{RNAi}/+; c42-GAL4/+* (*DmMo25* knockdown). RNA was purified with RNeasy Mini Kit (QIAGEN Ref 74104) and quantified using a NanoDrop 2000c (Thermo Scientific, Waltham, MA). 100 ng of total RNA was used for the reverse transcriptase reaction using iScript cDNA Synthesis Kit (BIO RAD Cat. #170-8890) according to manufacturer's instructions. qPCR was performed using the CFX Connect Real-Time PCR detecting system (Bio-Rad, Hercules, CA)

with the iTaq Universal Probes Supermix (Bio-Rad). cDNA from 5 ng total RNA was used as template. The TaqMan primer/probe sets for *Drosophila Mo25* (Dm01822943_s1) and endogenous control *RpL32* (Dm02143724_m1) were ordered from Invitrogen. The PCR cycle was 95°C × 3 min, 95°C × 10 s, 60°C × 30 s (repeated × 40). Results were analyzed with Bio-Rad CFX Manager.

Measurement of tubule DmWNK knockdown

Renal tubules were collected from female flies of genotypes *w; c42-GAL4/+* (control) or *w; UAS-DmWNK^{RNAi}/+; c42-GAL4/+*. RNA was isolated and qPCR was performed as described above. The TaqMan primer/probe set for *Drosophila WNK* (Dm01792325_g1) was ordered from Invitrogen. DNA standards for absolute quantification were made by PCR. Primers were designed outside the sequence targeted by the TaqMan primers, forward primer 5' AGAAGCAACTCTCCAAGCAGCC 3' and reverse primer 5' CCAGCGGAACATTTTGAATAGG 3'. 4 standard DNAs were used from 10^5 to 10^2 with 10 fold dilution.

Measurement of transepithelial fluid secretion and potassium flux

Ramsay assay with measurement of secreted fluid potassium concentration using ion-specific electrodes was performed as previously described[4,10-12]. Briefly, renal tubules were dissected from adult females 4-6 days after eclosion and transferred to standard or hypotonic bathing medium (described above) under mineral oil. Secreted fluid droplet volume and potassium concentration were measured ~2 hours after initiation of the assay. Potassium ionophore I cocktail B (Sigma) was used in the ion-specific electrodes. Results were analyzed with GraphPad Prism.

Purification of Fray, Fray^{T206E}, DmMo25, and the N-terminus of fly NKCC (Ncc69).

The *DmMo25* open reading frame in pENTR (described above) was recombined into pDEST15 via LR clonase reaction (ThermoFisher), to allow expression of DmMo25 with an N-terminal GST tag. The pDEST15 construct was transformed into BL21(DE3) *Escherichia coli*. Protein expression and purification were undertaken as described in the GST Gene Fusion System Handbook of GE Healthcare. Briefly, cells were grown at 37°C in 2X YT medium to an A_{600} of 1 to 2 OD. Isopropyl-D-galactosidase was added to a final concentration of 0.1 mM to induce protein expression, and the cells were cultured for another 16 h at 20°C. Cells were harvested by centrifugation and lysed by sonication in PBS with SIGMAFAST™ Protease Inhibitor. GST-tagged proteins were purified from the lysates using GSTrap^{TM} FF (GE Healthcare, Dallas, TX) and eluted from the column in 50 mM Tris-HCl pH 8.0 with 10 mM glutathione. After gel electrophoresis, Coomassie blue-stained proteins were compared to a BSA standard to determine approximate protein concentrations. N-terminally GST tagged Fray, Fray^{T206E} and the N-terminus of the fly NKCC (amino acids 1-204) were similarly prepared and are described in[4].

***In vitro* kinase assay**

In vitro kinase assays were performed as described[4] with the following modifications. Approximately 2.5 µg GST-Fray or GST-Fray^{T206E}, with or without 10 µg GST-Ncc69[1-204] and 10 µg GST-DmMo25, were incubated in 50 µl kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, 10 µM ATP, and 10 µCi γ-^{32}P-ATP (3000 Ci/mmol, Perkin-Elmer, Waltham, MA). After incubation for 1h at 30°C, incorporation of phosphate was determined after electrophoresis of samples on a Novex 4-20% Tris-Glycine Gel (Life Technologies) and autoradiography of the dried Coomassie Blue-stained gels.

REFERENCES

1. Rosay P, Davies SA, Yu Y, Sözen MA, Kaiser K, Dow JA: Cell-type specific calcium signalling in a Drosophila epithelium. *J. Cell. Sci.* 110 (Pt 15): 1683–1692, 1997

2. McGuire SE, Mao Z, Davis RL: Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. *Sci. STKE* 2004: pl6–pl6, 2004

3. Dietzl G, Chen D, Schnorrer F, Su K-C, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ: A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* 448: 151–156, 2007

4. Wu Y, Schellinger JN, Huang C-L, Rodan AR: Hypotonicity stimulates potassium flux through the WNK-SPAK/OSR1 kinase cascade and the Ncc69 sodium-potassium-2-chloride cotransporter in the Drosophila renal tubule. *J. Biol. Chem.* 289: 26131–26142, 2014

5. Arosio D, Ricci F, Marchetti L, Gualdani R, Albertazzi L, Beltram F: Simultaneous intracellular chloride and pH measurements using a GFP-based sensor. *Nat Meth* 7: 516–518, 2010

6. Bischof J, Maeda RK, Hediger M, Karch F, Basler K: An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. *Proc Natl Acad Sci USA* 104: 3312–3317, 2007

7. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS: Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20: 3551–3567, 1999

8. Xu H, Freitas MA: MassMatrix: a database search program for rapid characterization of proteins and peptides from tandem mass spectrometry data. *Proteomics* 9: 1548–1555, 2009

9. Mukhtarov M, Liguori L, Waseem T, Rocca F, Buldakova S, Arosio D, Bregestovski P: Calibration and functional analysis of three genetically encoded Cl(-)/pH sensors. *Front. Mol. Neurosci.* 6: 9, 2013

10. Schellinger JN, Rodan AR: Use of the Ramsay assay to measure fluid secretion and ion flux rates in the Drosophila melanogaster Malpighian tubule. *J Vis Exp* 2015

11. Rodan AR, Baum M, Huang C-L: The Drosophila NKCC Ncc69 is required for normal renal tubule function. *Am. J. Physiol., Cell Physiol.* 303: C883–94, 2012

12. Wu Y, Baum M, Huang C-L, Rodan AR: Two inwardly rectifying potassium channels, Irk1 and Irk2, play redundant roles in Drosophila renal tubule function. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 309: R747–56, 2015

Supplemental Table 1. Composition of salines. Note that not all components of Schneider's medium are listed – see Methods for more details.

mM	Schneider's	Dros saline	Normal K	Low K	High K
NaCl	36.21	117.5	97.5	97.5	97.5
KCl	21.33	20	20	0	40
$CaCl_2$	5.41	2	2	2	2
$MgCl_2$	0	8.5	8.5	8.5	8.5
$MgSO_4$	15.05	0	0	0	0
$NaHCO_3$	4.76	10.2	10.2	10.2	10.2
NaH_2PO_4	0	4.3	4.3	4.3	4.3
Na_2HPO_4	4.94	0	0	0	0
KH_2PO_4	3.31	0	0	0	0
glucose	11.11	20	20	20	20
NMDG Cl	0	0	20	40	0
HEPES	0	15	15	15	15

Supplemental Table 2. Ion concentrations in bathing media. Standard bathing medium is a 1:1 mix of *Drosophila* saline and Schneider's. The hypotonic bath consists of 300 µl SBM + 90 µl H_2O. The normal, low and high potassium baths consist of the listed salines mixed 1:1 with Schneider's.

mM	Dros saline	Schnei-der's	SBM	Hypo	Norm K saline	Norm K bath	Low K saline	Low K bath	High K saline	High K bath
Na^+	132	50.9	**91.4**	**70.3**	112	**81.4**	112	**81.4**	112	**81.4**
Cl^-	158.5	68.4	**113.4**	**87.3**	158.5	**113.4**	158.5	**113.4**	158.5	**113.4**
K^+	20	24.6	**22.3**	**17.2**	20	**22.3**	0	**12.3**	40	**32.3**
Ca^{2+}	2	5.4	**3.7**	**2.9**	2	**3.7**	2	**3.7**	2	**3.7**
Mg^{2+}	8.5	15.1	**11.8**	**9.1**	8.5	**11.8**	8.5	**11.8**	8.5	**11.8**
HCO_3^-	10.2	4.8	**7.5**	**5.8**	10.2	**7.5**	10.2	**7.5**	10.2	**7.5**
$H_2PO_4^-$	4.3	3.3	**3.8**	**2.9**	4.3	**3.8**	4.3	**3.8**	4.3	**3.8**
HPO_4^{2-}	0	4.9	**2.5**	**1.9**	0	**2.5**	0	**2.5**	0	**2.5**
$NMDG^+$	0	0	**0**	**0**	20	**10**	40	**20**	0	**0**
measured osm (mosm/kg)			**335**	**257**		**337**		**337**		**338**

Supplemental Figure Legends

Supplemental Figure 1. High chloride decreases WNK autophosphorylation *in vitro*. A) Coomassie-stained gel of purified *Drosophila* WNK kinase domain, with size markers (M) shown in kDa. Two replicate purifications are shown. B) Ion extraction chromatograms of activation loop peptides AKSVIGTPEFMAPEMY and AKpSVIGTPEFMAPEMY were used to track DmWNK phosphorylation at Ser 434 over time. C) Purified *Drosophila* WNK kinase domain was allowed to autophosphorylate for 0, 1, 2 or 4 hours. After termination of the reaction and SDS-PAGE, the Pro-Q Diamond phosphoprotein stain was used to determine kinase autophosphorylation. Three independent experiments were performed in 50 mM vs 150 mM chloride concentration, as shown. The top bands were quantified using ImageJ (Figure 1C). Size markers are shown in kDa.

Supplemental Figure 2. Effects of hypotonic bathing medium potassium and chloride concentrations on tubule epithelial cell intracellular chloride concentration. A) Ion concentrations in bathing media. Tubules were bathed for one hour in isotonic standard bathing medium (measured osmolality, 317 mosm/kg), followed by one hour in hypotonic medium of varying ionic compositions. For the hypotonic media, salines were mixed 1:1 with Schneider's medium, and then diluted 300 μL saline/Schneider's + 90 μL H_2O. The low potassium chloride saline consisted of (in mM): NaCl 85.5, Na gluconate 32, $CaCl_2$ 2, $MgCl_2$ 8.5, $NaHCO_3$ 10.2, NaH_2PO_4 4.3, HEPES 15 and glucose 20. The high potassium saline consisted of (in mM): NaCl 107.5, KCl 30, $CaCl_2$ 2, $MgCl_2$ 8.5, $KHCO_3$ 10.2, KH_2PO_4 4.3, HEPES 15 and glucose 20. Osmolality was measured in triplicate using an Advanced Instruments Osmometer, model 303 (Norwood, MA). The final ionic concentrations are shown in the table (note that there are additional components as well, such as amino acids; see Methods). B) The decrease in intracellular chloride concentration that resulted from switching the tubules from isotonic

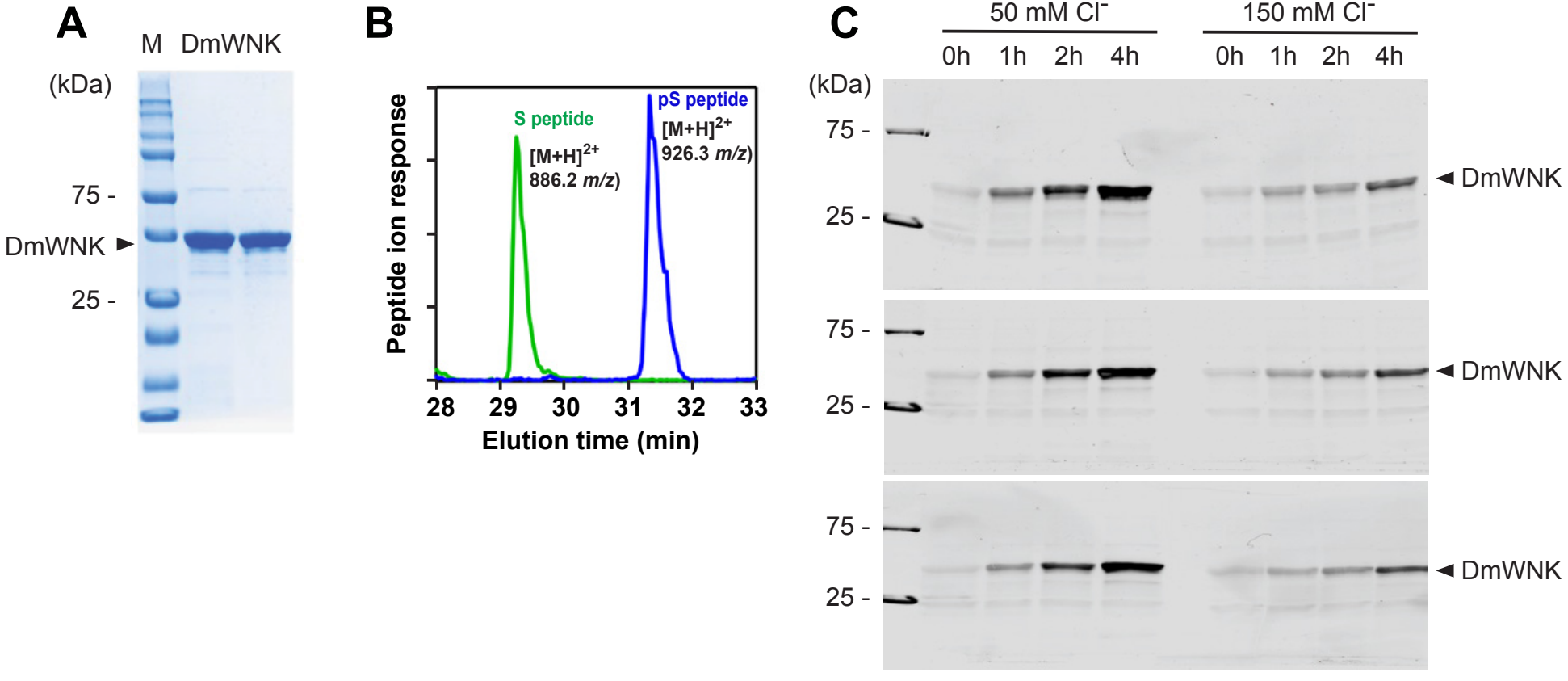
standard bathing medium to the various hypotonic media is shown. Chloride concentrations were measured using ClopHensor expressed in the principal cells (genotype: *w; c42-GAL4/UAS-ClopHensor c304*). The fall in intracellular chloride in hypotonic medium was accentuated when the hypotonic bath contained lower potassium and chloride concentrations, consistent with efflux of potassium and chloride in hypotonic conditions. n=21 cells analyzed per condition (3 cells/tubule in 7 tubules). Results were analyzed with one-way ANOVA with Tukey's correction for multiple comparisons. *, $p<0.05$; ***, $p<0.001$.

Supplemental Figure 3. Real-time quantitative RT-PCR measurement of *DmMo25* and *DmWNK* transcript in knockdown tubules. 50 pairs of renal tubules were collected from female flies of genotypes *w; c42-GAL4/+* (control) or *w; UAS-DmWNK^{RNAi}/+; c42-GAL4/+* (*DmWNK* knockdown) (A) or *w; c42-GAL4/+* (control) or *w; UAS-DmMo25^{RNAi}/+; c42-GAL4/+* (*DmMo25* knockdown) (B). Transcript level in the control tubules was normalized to 1. Note that the degree of knockdown may underestimate knockdown in the principal cells, since whole tubules were used, which consist of both cells in which the RNAi is expressed, and others in which it is not.

Supplemental Figure 4. Effects of hypotonic and varying potassium baths on *Drosophila* WNK activity. A) Kinase-dead rat SPAK was transgenically expressed in *Drosophila* renal tubule principal cells in control tubules (*w; UAS-SPAK^{D219A}/+; c42-GAL4/+*) or *Drosophila WNK* (*DmWNK*) knockdown tubules (*w; UAS-SPAK^{D219A} UAS-WNK^{RNAi}/+; c42-GAL4/+*). Phosphorylated SPAK (p-SPAK) or total SPAK (t-SPAK) were detected by Western blotting, and band intensities were quantified using ImageJ. Results for three independent experiments are shown. In each experiment, 15 pairs of tubules were included in each time point. In order to compare control and *WNK* knockdown tubules, the 0-minute time points were quantified in Figure 3A, and results for the *DmWNK* knockdown tubules were normalized to control. B)

Tubules expressing kinase-dead rat SPAK (*w; UAS-SPAK^{D219A}/+; c42-GAL4/+*) were bathed in standard bathing medium for 60 minutes, then transferred to hypotonic medium for the times shown. In some cases tubules expressing only the *c42-GAL4* driver (*w; c42-GAL4/+*) were included to demonstrate antibody specificity. Results for four independent experiments are shown, and the results are quantified in Figure 3B. In each experiment, 15 pairs of tubules were included in each time point. C) Tubules expressing kinase-dead rat SPAK (*w; UAS-SPAK^{D219A}/+; c42-GAL4/+*) were bathed in normal potassium bath for 60 minutes, then transferred to normal, low or high potassium baths for 60 minutes (see Supplemental Tables 1 and 2 for bath compositions). Results for four independent experiments are shown, and the results are quantified in Figure 3C. In each experiment, 15 pairs of tubules were included in each bath tested.

Supplemental Figure 5. Mo25 stimulates Fray activity *in vitro*. *In vitro* kinase assays were performed with purified *Drosophila* Fray (SPAK/OSR1 homolog, either wild-type or carrying the phospho-mimicking T206E mutation on the T-loop Thr phosphorylated by WNK), *Drosophila* Mo25, and the N-terminus of the *Drosophila* NKCC, Ncc69 (amino acids 1-204). Three independent experiments were performed and are shown. The top gels are the Coomassie-stained gels and the bottom gels are the autoradiographs of the same gels.



Supplement Figure 1
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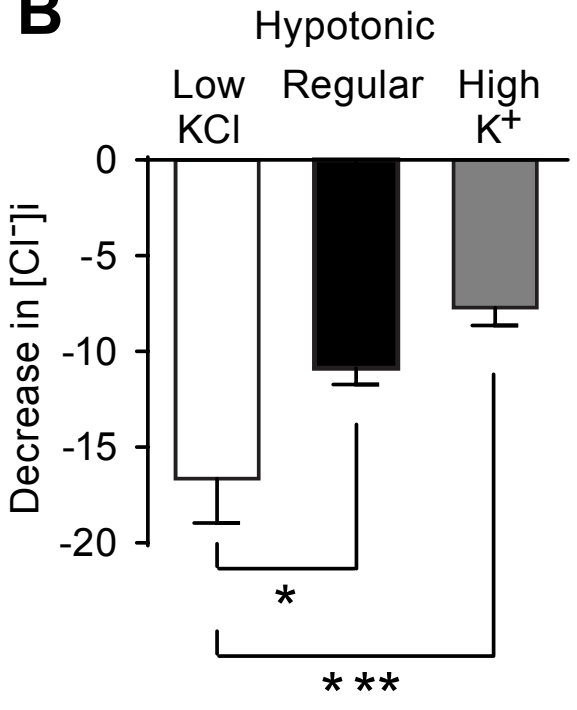
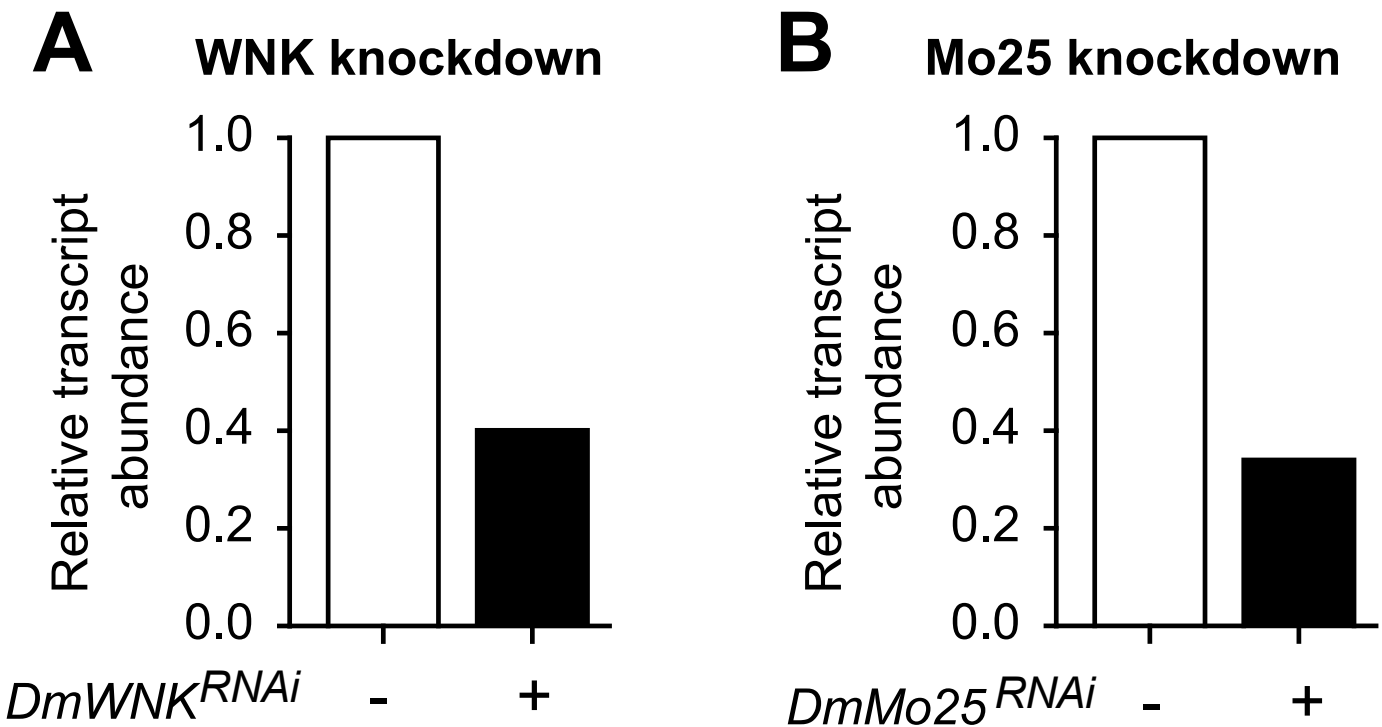
A

	Isotonic	Hypotonic	High K^+ hypotonic	Low KCl hypotonic
Na	91	69	59	69
Cl	113	85	85	66
K	22	17	26	9
Ca	4	3	3	3
Mg	12	9	9	9
HCO_3	7.5	5.6	5.6	5.6
H_2PO_4	4	3	3	3
HPO_4	2.5	2	2	2
Glucose	16	12	12	12
Gluconate				12
Osmolality (measured)	317	244	230	243

B



Supplement Figure 2
Q. Sun, et al.



Supplement Figure 3
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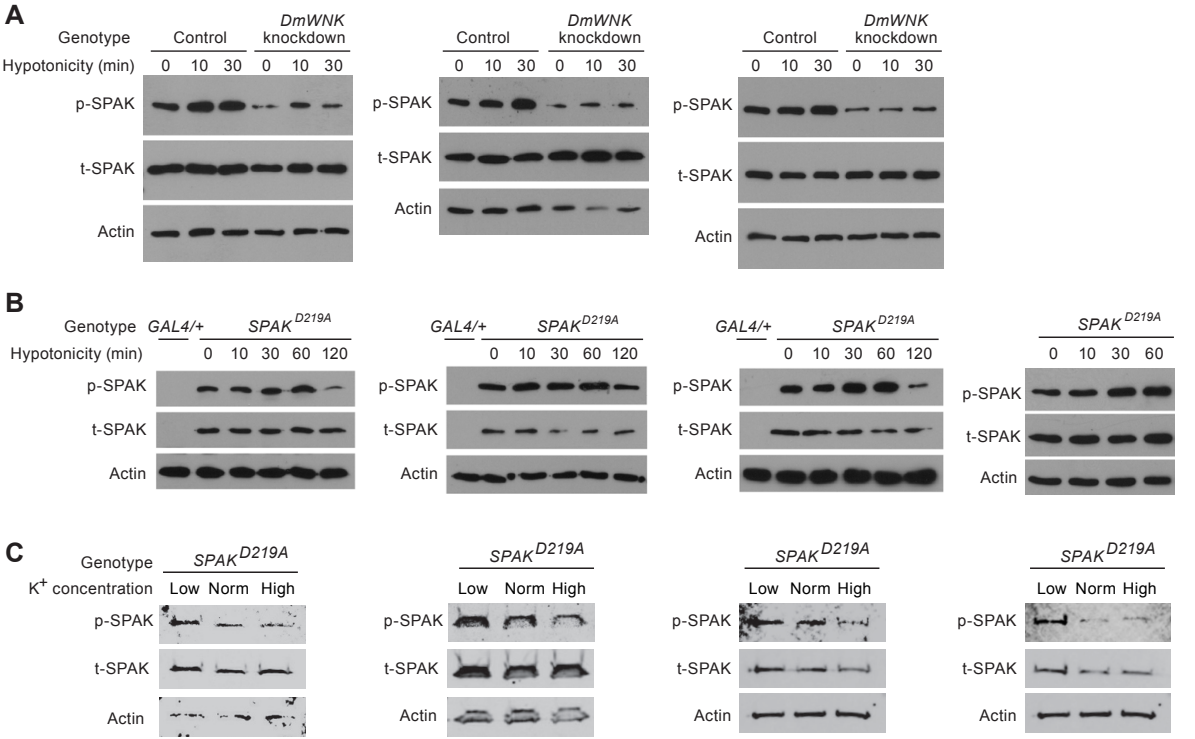

Supplement Figure 4
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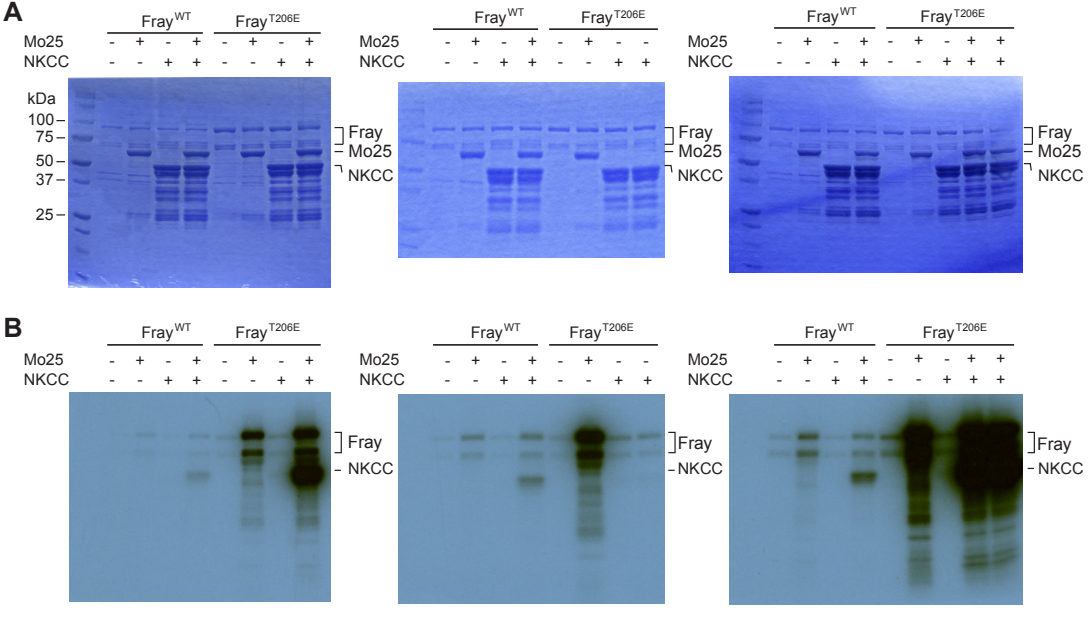

Supplement Figure 5
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