Supplementary Materials

Molecular Biology of the Cell

Humphreys *et al*.

Hydrostatic Pressure Sensing by WNK Kinases

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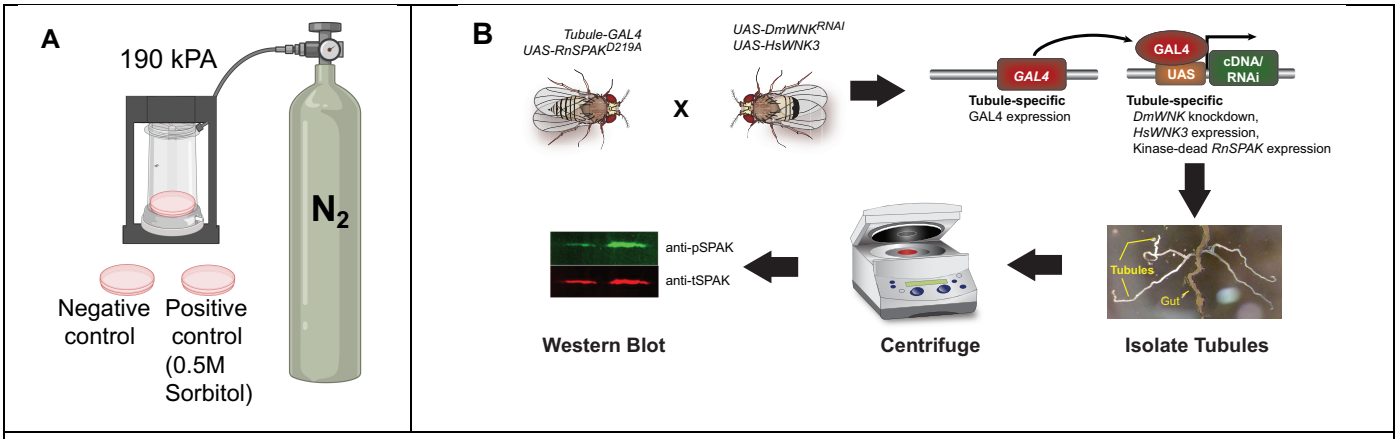

Figure S1. Measuring WNK activity in vivo. (A) Scheme for applying pressure to MDAMB231 cells using N_2 gas. Western blots for tKCC2 and pKCC2 were conducted for the pressurized cells and the positive and negative controls. (B) WNK3 expressed in *D. melanogaster* Malpighian tubules. The GAL4/UAS system was used to knock down endogenous *Drosophila* WNK in the fly Malpighian (renal) tubule and replace it with full-length human WNK3. Kinase-dead SPAK/D219A was coexpressed using the cell-specific driver c42-GAL4. Tubules were isolated and pressure applied by centrifugation. SPAK phosphorylation was quantified by using anti-pSPAK and anti-tSPAK antibodies. Band intensities were quantified using ImageJ. The effect of temperature was controlled for by imposing similar temperature increase to control tubules in a PTC-100 Programmable Thermal Controller.

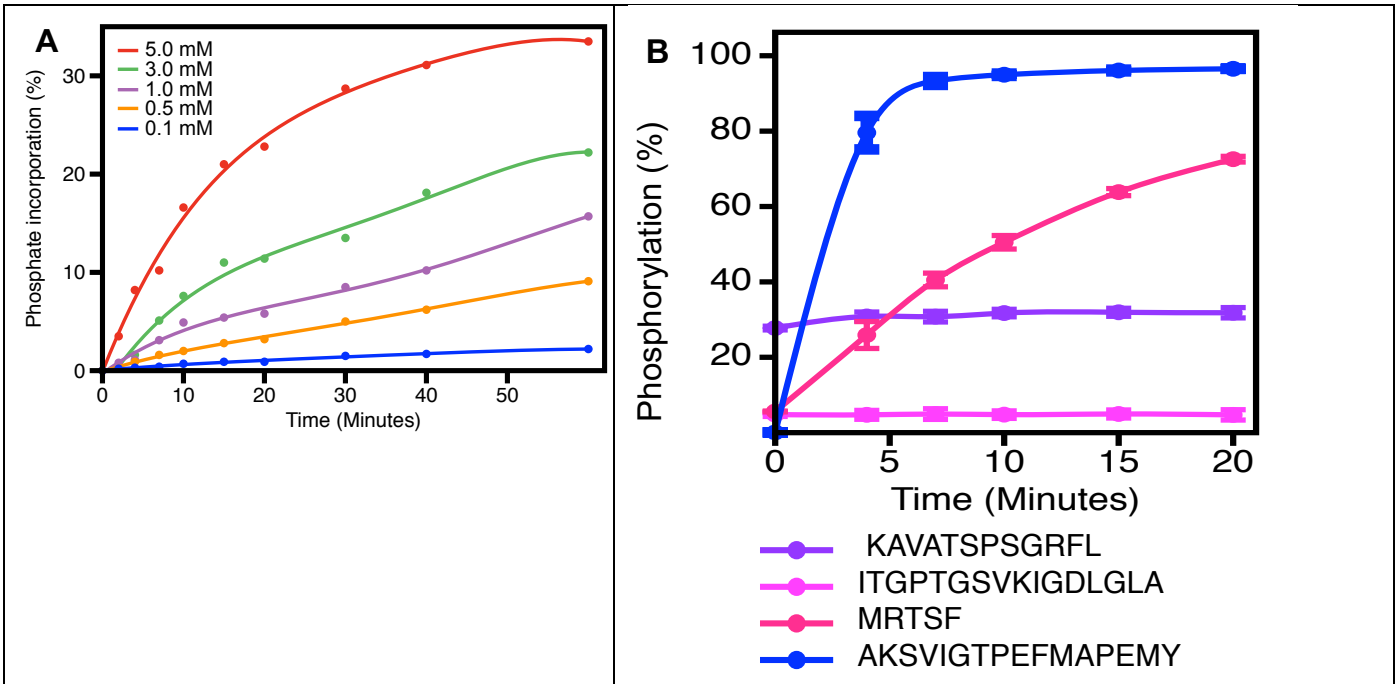

Figure S2. ATP requirements for autophosphorylation reactions and auto-phosphorylation sites. (A) ^{32}P incorporation into uWNK3 as a function of the [g-^{32}P ATP]. (B) Mass spectrometry tracking the appearance auto-phosphorylated peptides WNK3 peptides starting with dephosphorylated WNK3 (uWNK3). Standard assay conditions at 4 μM uWNK3 and 25 ˚C. Monitored peptides are indicated. Error bars indicate s.e.m.

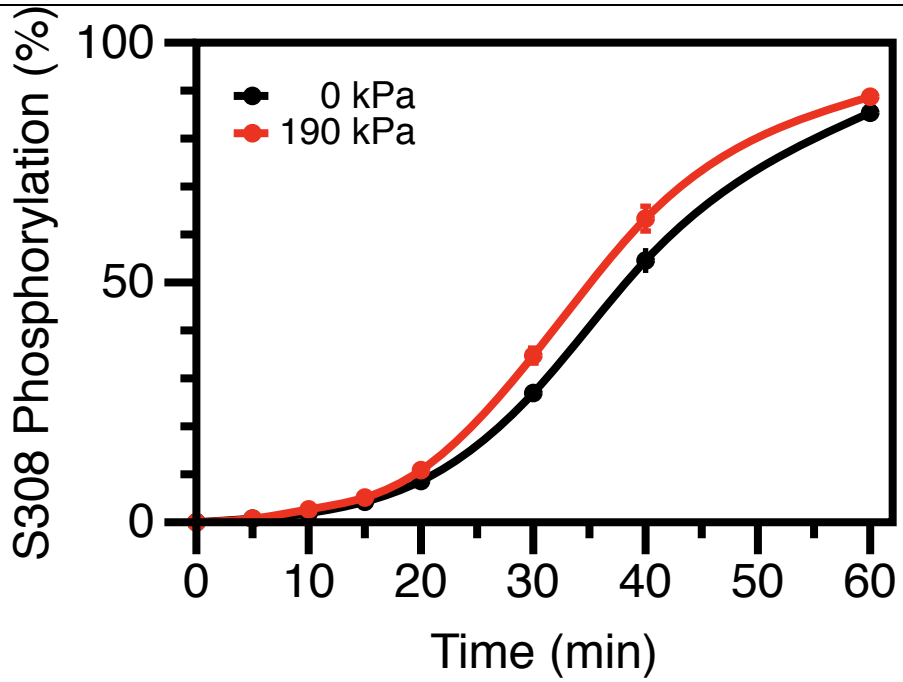

Figure S3. Triplicated hydrostatic pressure assay. Assay of 10 μM uWNK3 for hydrostatic pressure. Assay conditions as in Figure 3. Data points represent mean value. Two-tailed t-test for the 30 minute time point gives p=.0043 for the triplicated data. Error bars indicate s.e.m.

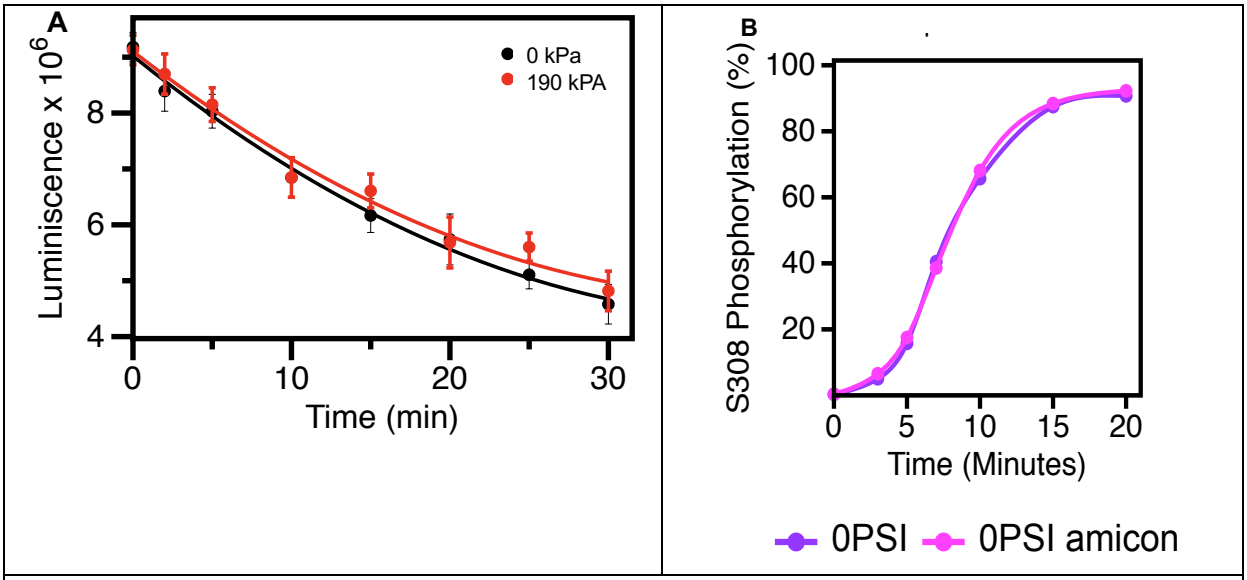

Figure S4. Additional controls. (A) Kinase-Glo ® assays of pWNK3 phosphorylation of OSR synthtide. Two-sided t-test for the midpoint (15 minutes), p=0.33 (n.s.) Data points represent mean value. Error bars indicate s.e.m. (B) Control for Amicon sample handling. Mass spectrometry tracking the appearance of pS308 peptides (purple, bench, magenta, Amicon). Standard assay conditions at 4 μM uWNK3 and 25 ˚C.

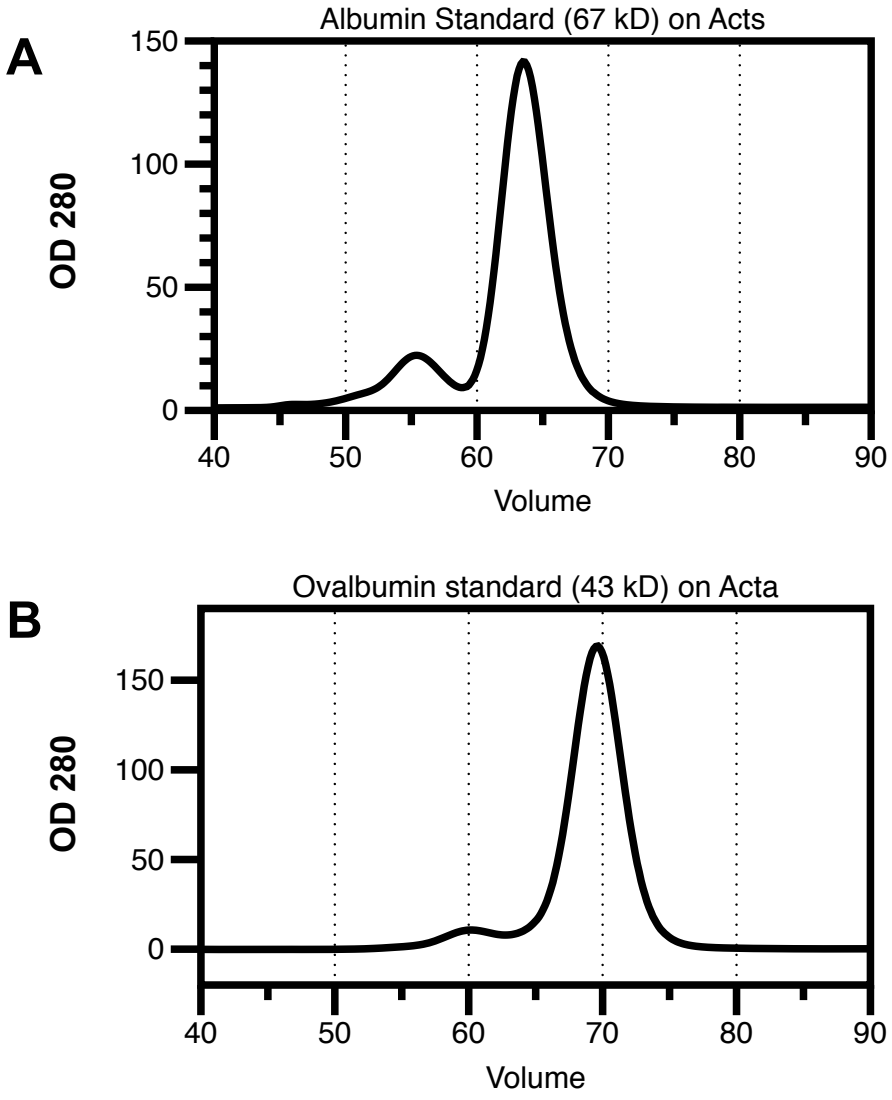

Figure S5. Gel filtration standards. (A) 67 kDa Albumin. (B) 43 kDa ovalbumin. Chromatography on an Acta FPLC using a Superdex S75 column at 290 kPa. Data compared to the uWNK3 elution volume on the same column in Figure 4A (second panel).

Table S1. Statistics for cellular assays in MDAMB231 cells

	+Pressure	0.5M Sorbitol
Theoretical mean	1.000	1.000
Actual mean	2.062	2.218
Number of values	3	3
One sample t test		
t, df (degrees of freedom)	t=10.90, df=2	t=3.997, df=2
P value (two tailed)	0.0083	0.0573
P value summary	**	ns
Significant (alpha=0.05)?	Yes	No
How big is the discrepancy?		
Discrepancy	1.062	1.218
SD of discrepancy	0.1687	0.5278
SEM of discrepancy	0.09738	0.3047
95% confidence interval	0.6425 to 1.481	-0.09309 to 2.529
R squared (partial eta squared)	0.9834	0.8887

Unpaired t test	
P value	0.008
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.997, df=4

Table S2. DynaFit script for autocatalytic autophosphorylation model

```
[task]
  data  = progress
  task  = fit
[mechanism]
  WNK3_u + WNK3_p --> WNK3_p + WNK3_p :  kphos
[constants]
  kphos = 0.04 ??
[concentrations]
  WNK3_u = 5 ?? ; values used, 40, 20, 10, 5
  WNK3_p = 0.1 ??
[data]
        directory ./WNK3_Modeling_1/Data
        sheet   data.csv
        column 2
        offset 1
        response WNK3_p = 1
[output]
 directory ./WNK3_Modelling_1/Output
[end]
```

Table S3. uWNK3 Oligomeric state by SLS, SEC-MALS and AUC

Pressure	[uWNK3] (mg/ml)	MW (kDa)
SLS (Gravity)*	0.8-2.4	60-90
SEC-MALS 250 kPa	5.0	43-74
AUC 9-19 MPa	0.8, 2.1	38.5

*Data presented in (Akella *et al*, 2021)

Table S4. Apparent uWNK3 and pWNK3 molecular weights by gel filtration

WNK3 protein	Pressure (kPa)	Elution Vol (EV) (mL)	Estimated MW (kDa)	Standard MW (kDa), EV (ml)	Ovalbumin (43 kDa) EV
uWNK3	Gravity	16.3	60	67, 16	17
uWNK3	280	70.0	40	67, 63.4	69.6
pWNK3	290	64.5	43	80, 56.6	64.5

Table S5. DSS Crosslinks in uWNK3 without added pressure observed by mass spectrometry

Peptide 1	Peptide 2	Res. no. x-link Peptide A		Res. no. x-link Peptide B		Score
		WNK3	WNK1	WNK3	WNK1	
FAEDTKLPTTENLY (tag)	AEDTKLPTTENLY (tag)	410	484	410	484	22.3
AEDTKLPTTENLY (tag)	KGLDTETW	410	484	163	237	21.1
AKSVIGTPEFMAPEMY	AEDTKLPTTENLY (tag)	307	381	410	484	20.1
AEDTKLPTTENLY (tag)	AKSVIGTPEF	410	484	307	381	17.1
FAEDTKLPTTENLY (tag)	FAEDTKLPTTENLY (tag)	410	484	410	484	15.5
DSWESILKGKKCIVLVTELMTSGTLKTY	KTVYKGLDTETWVEVAW	218	292	159	233	14.7
AKSVIGTPEF	KGLDTETW	307	381	163	237	14.6
AEDTKLPTTENLYF (tag)	AEDTKLPTTENLY (tag)	410	484	410	484	12.5
KGLDTETW	KGLDTETW	163	237	163	237	12.4
RKVTSGIKPASF	RKVTSGIKPASF	360	434	360	434	11.7
KVXKPKVLRSW	KGLDTETW	248	322	163	237	10.4
AEDTKLPTTENLYF (tag)	CRQILKGLQF	410	484	259	333	9.3
AKSVIGTPEFMAPEMY	LKFDIELGRGAFKTVY	307	381	148	222	9.2
LKFDIELGRGAF	CRQILKGLQF	148	222	259	333	8.7
LKRFKVXKPKVLRSW	AEDTKLPTTENLYF (tag)	243	317	410	484	7.5
AKSVIGTPEFMAPEMYEEHY	KEEAEXLKGLQHPNIVRF	307	381	192	266	6.5
AKSVIGTPEFMAPEMY	AKSVIGTPEFXAPEMY	307	381	307	381	5.9
AKSVIGTPEFMAPEMYEEHY	LKFDIELGRGAFKTVY	307	381	159	233	4.1

Table S6. DSS Crosslinks in uWNK3 under 190 kPa observed by mass spectrometry

Peptide 1	Peptide 2	P-site res. no. Peptide A		P-site res. no. Peptide B		Score
		WNK3	WNK1	WNK3	WNK1	
KGLDTETW	KGLDTETW	163	237	163	237	29.5
FAEDTKLPTTENLY (tag)	FAEDTKLPTTENLY (tag)	410	484	410	484	21.0
AEDTKLPTTENLY (tag)	AKSVIGTPEF	410	484	307	381	20.3
FAEDTKLPTTENLY (tag	AEDTKLPTTENLY (tag)	410	484	410	484	18.4
AKSVIGTPEFMAPEMY	AEDTKLPTTENLY (tag)	307	381	410	484	18.2
AEDTKLPTTENLY (tag)	KGLDTETW	410	484	163	237	16.4
AEDTKLPTTENLY (tag)	AEDTKLPTTENLY (tag)	410	484	410	484	16.2
KGLDTETWVEVAW	AKSVIGTPEF	163	237	307	381	14.8
KEEAEXLKGLQHPNIVRFY	DIELGRGAFKTVY	192	266	159	233	13.3
KEKNEKEMEEEAEXKAVATSPSGRF	CRQILKGLQF	122	196	259	333	11.0
KTVYKGLDTETW	RKVTSGIKPASF	163	237	360	434	10.6
KGLDTETWVEVAW	KGLDTETW	163	237	163	237	10.1
AEDTKLPTTENLYF (tag)	AEDTKLPTTENLYF (tag)	410	484	410	484	10.1
AEDTKLPTTENLY (tag)	LKFDIELGRGAF	410	484	148	222	8.5
DIELGRGAFKTVY	KVMKPKVLRSW	159	233	243	317	8.5
DSWESILKGKKCIVLVTELMTSGTLKTY	CRQILKGLQF	236	310	259	333	6.7
CELQDRKLTKAEQQRFKEEAEMLKGLQHPNIVRF	KEEAEMLKGLQHPNIVRFYDW	185	259	192	266	6.6
SECQNAAQIYRKVTSGIKPASF	CRQILKGLQ	360	434	259	333	5.9
ESILKGKKCIVLVTELMTSGTLKTYLKRF	CRQILKGLQF	221	295	259	333	5. 7
AKSVIGTPEFMAPEMY	RKVTSGIKPASF	307	381	360	434	5.6
AKSVIGTPEFMAPEXYEEHY	LKFDIELGRGAFKTVY	307	381	148	222	4.9
ITGPTGSVKIGDLGLATLMRTSF	KTVYKGLDTETWVEVAW	291	365	163	237	4.4
FAEDTKLPTTENLY (tag)	AKSVIGTPEF	410	484	307	381	3.9
ESILKGKKCIVLVTELMTSGTLKTY	LKFDIELGRGAF	236	310	148	222	3.9
ESILKGKKCIVLVTELMTSGTLKTY	DIELGRGAFKTVY	236	310	159	233	2.7

REFERENCES

Akella, R., Humphreys, J.M., Sekulski, K., He, H., Durbacz, M., Chakravarthy, S., Liwocha, J., Mohammed, Z.J., Brautigam, C.A., and Goldsmith, E.J. (2021). Osmosensing by WNK Kinases. Mol Biol Cell *32*, 1614-1623.