Anatomy of a pressure sensing protein kinase

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Abstract

Cells respond to hydrostatic pressure to maintain cellular, organ and organism level functions, to set and respond to blood pressure, tissue perfusion, filtration rates and other processes. Pressure sensing is thought to occur at membranes where proteins can respond to mechanical cues[1,2,3] . Thus, proteins implicated as direct pressure sensors have been mainly ion channels [2,4], and more recently G-protein coupled receptors[5,6]. Here we show, contrary to expectations, that hydrostatic pressure directly induces autophosphorylation and activation of an intracellular protein kinase, With No Lysine kinase-3 (WNK3), and to a lesser extent, WNK1[7]. The pressure sensitivity is a property of the kinase domains alone of WNK1 and WNK3. The crystal structure of the unphosphorylated inactive WNK1 kinase domain (iWNK1) suggests that a dimer to monomer equilibrium and changes in hydration are central to pressure sensing. Aspects of this mechanism are supported by mutagenic analysis. We further show that hydrostatic pressure activates full-length WNK3 in *Drosophila* tubules.

The idea that WNK kinases may be pressure sensors is supported by the association of WNK1 and WNK4 with familial forms of both hypertension and hypotension [8-10]. Further, WNK1 knockout mice have low blood pressure [11-14] and reduced myogenic responses [15,16]. Thus, WNKs are effectors of the response to hydrostatic pressure changes, raising the question of whether they are sensors as well. Here

we test whether the soluble kinase domains of WNK1 and WNK3 (iWNK1 andiWNK3) exhibit autophosphorylation under pressure. iWNK3 was tested for pressure sensitivity because WNK3 knockout reduces intracranial pressure in a stroke model [17].WNK kinases are also anticipatedosmosensors (recently reviewed in [10]. Regulation by osmotic pressure and the linkage to hydrostatic pressure regulation will be discussed elsewhere.

The kinase domains of WNK3 and WNK1, phosphorylated as expressed in bacteria, were dephosphorylated to make iWNK3 and iWNK1. The unphosphorylated kinase domain of WNK1 (iWNK1)was shown previously to autophosphorylate on the activation loop serine, Ser382 (to form pWNK1), becoming active toward both specific and generic substrates[18,19].iWNK3autophosphorylates on the homologous serine, Ser308 of WNK3 [18] as well as Ser304 (Fig. s1A).

Hydrostatic pressure induces autophosphorylation of both iWNK1and iWNK3 *in vitro*(Fig. 1a).Hydrostatic pressure of190 kPa (28 psi) was applied using N_2 gas, well above the 16kPa typical of systolic blood pressure, but orders of magnitude below pressures used to study effects on proteins (200 mPa and more) [20,21]. Autophosphorylationof iWNK3increases 1.7 fold at a 7 minute time point, from 26% to 45% ($p<0.001$).iWNK1 has more basal activity (50% autophosphorylation), and the fold increase is less, 1.2 fold ($p<0.01$). To confirm that the pressure-induced autophosphorylation increased enzyme activity, we used iWNK3 and iWNK1 to phosphorylate the pan-WNK substrate OSR1 (oxidative stress-responsive-1) [22,23]. Hydrostatic pressure increased the phosphorylation of the GST-fused OSR1 peptide substrate (see Materials and Methods) about 1.2 fold (Fig. s1B).

The autophosphorylation of iWNK3 and iWNK1 was measured as a function of added pressure.iWNK3 exhibited a maximum at about 190 mPa, whereas the pressure maximum for iWNK1 was about 240 mPa (Fig. 1B,C). Autophosphorylation ofiWNK1 is inhibited by chloride [19]. The chloride opposition tothe pressure-induced autophosphorylation was measured by increasing the [Cl^-] from 50 mM to 150 mM for both enzymes. The autophosphorylation of iWNK3 was inhibited over 2-fold ($p<.001$ and $p<0.0001$) (Fig. 1B). The inhibition of pressure-induced autophosphorylationof iWNK1 was less, in the range of 20-30% ($p<0.01$) over the pressures analyzed (Fig. 1C).The chloride inhibition was similar over the pressure range studied.

The autophosphorylation of WNKs in Fig. 1 was carried out in an Amicon concentrator pressurized with N_2 gas. The effects of the method of applying pressure was tested using argon gas in the same apparatus, and the same amount of pressure was applied using weights and centrifugal force (as described in

Materials and Methods)(Fig. s2, apparatus and complete gels shown in Fig. s2B for WNK3 and Fig. s2C for WNK1). The pressure activation was similar in the different methods used (Fig. 2A), except in the microfuge where some heating affected the experiment (to be described elsewhere).

We tested whether pressure induces auotphosphorylation of other kinases from STE, TKL, and CMGC group kinases (ASK1, SPAK/OSR1, MEK6, TAK-TAB, and p38 MAP kinase)were tested[24](Fig. 2C and full gels in Fig. s3A). The MAP kinasep38 and MAP2K MEK6 do not autophosphorylate. The fusion protein TAK-TAB (TGFβ-activatedkinase-TAK1 binding protein)[25]autophosphorylates, but this activity is not enhanced by pressure. Further, ASK1 (apoptosis signaling kinase-1), a protein known to be activated by osmotic pressure in cells [26],does show modest increased phosphorylation with pressure, comparable with that observed for iWNK1 suggesting that the phenomenon may be occur in other kinases.

The effect of pressure on trans-phosphorylation of substrates of pWNK1, pWNK3, and control kinases was measured. The activity of both pWNK1 and pWNK3 toward OSR1 was enhanced by pressure, whereas the activity of MEK6 (DD), a constitutively active mutant of MEK1, and BRaf tested with their cognate substrates p38, ERK2 and MEK1, exhibit no activity enhancement with pressure (Fig. 2D, full gels in Fig. s3B).

As an additional control, we tested whether the phosphatases, PP1cγ, MAP kinase phosphatase-3 (MKP3), λ-phosphatase, and shrimp alkaline phosphatase. These enzymes showed no pressure-induced activity, as measured by the loss of phosphate from p-nitrophenyl phosphate (Fig. 2E).

To understand the mechanism of pressure-induced autophosphorylation, we looked for clues in differences between iWNK1(Ser382->A) (iWNK1A, PDB 3FPQ) (mutated to prevent phosphorylation) and pWNK1 (PDB 4PWN)[19]. The iWNK1A is an asymmetric dimer, as observed in an intimate lattice contact [27]. The dimer binds the inhibitor chloride and traps the phosphorylation site, Ser382, of one subunit in the dimer interface. pWNK1 is a monomer [19]. Thus a potential model for pressure sensing is that pressure disfavors the inactive dimer, and promotes anautophosphorylation-competent monomer (Fig. 3A).

The iWNK3 probably has a similar structure as iWNK1A based on the >90% sequence identity. To test for a dimer in iWNK3, crosslinking of iWNK3 with the lysine reagent disuccinylsuberate (DSS)was analyzed by mass spectrometry (See Materials and Methods) (Fig. S4 and Table S1). Crosslinks

introduced under pressure (introducing the DSS by mixing under pressure) produced linked peptides distinct from those observed for the unpressurized protein (complete list in Table s1). Connections to K381, accessible but near the interface were more numerous in the unpressurized sample and more easily interpreted as arising from the dimer. Further, a link to K256, which is buried in the dimer interface, was only observed in pressurized sample. Crosslinks arising from the crystallographic dimer(black lines in Fig. s4) were observed in the unpressurized protein, but were fewer, which only had crosslinks that could be interpreted based on the monomer. Gel filtration and multi-angle light scattering experiments of unpressurized iWNK3 conducted at higher protein concentrations (25-125 μM)suggested dimers and higher order oligomers of iWNK3 (Fig. s5).

To test the importance of the dimer in pressure sensing, we made mutations in the dimer interface of WNK3-KDm. Mutations were made to resemble the interface of iWNK1 or that of WNK1 from a deep-sea fish *N. Corilceps* (Fig. 3B). The mutant M9 (A139G, T140M, P142N. I216T and L217V) replicates the dimer interface ofiWNK1.The mutants M7 (I216P, L217S) and M10 (A139G,I216P and L217S) converted the WNK3 interface into that of the WNK of *N. Corilceps* (Fig. 3B, see legend Fig. s6). Working at our standard conditions and 150 mM NaCl, the pressure enhancement of autophosphorylation was measured.M10 is more active compared to WNK3 and shows pressure enhancement of autophosphorylation similar to that of WNK1. On the other hand, M7 and M9 did not show any enhancement (Fig. 3C). Thermal denaturation of wild-type and mutant WNKs revealedthat pWNK1 and pWNK3showed are relatively unstable, and iWNK1 is less stable than iWNK3. These data support the observed easier activation of iWNK1 (Fig. s6B, C). The phosphorylated form of pM10 is less stable than iM10.The two inactive mutants have different thermal properties, M9 does not behave normally, apparently unfolding in two steps, and M7 is hyperstable, both properties perhaps explaining their lack of activity.

To measure the pressure activation of full-length WNKs as well as activation in cells, we expressed full-length human *WNK3* in *D. melanogaster* together with its substrate SPAK (Ste20/SPS1-related proline/alanine-rich kinase). We have previously shown that WNK-SPAK/OSR1signaling is involved in ion transport in the *Drosophila* Malpighian tubule{Wu, 2014 #4589;[28]Sun, 2018 #5464}. Isolated tubules expressing WNK3 and its substrate SPAK were subjected to hydrostatic pressure by centrifugation (Fig s7). The activation of WNK3 was measured by increased phosphorylation of the transgenicly-expressed kinase-dead SPAK, under hydrostatic pressure of 80 kPa, lower than that used *in vitro* experiments (190kPa) (Fig. 3D). The centrifugation resulted in a 2-fold increase in the ratio of pSPAK (phosphorylated) to tSPAK (unphosphorylated) ($p<0.0001$).

Dimeric iWNK1A has special features that may be linked to the pressure sensing. The Activation Switches of both subunits of the dimer are more extensively remodeled than in other protein kinases (from the DLG sequence through the APE sequence) (Fig. 3A), giving rise to an especially large cavity (Fig. 4A). The cavity volume calculated in POCASA [29]gave a volume of 1200Å^3 for WNK1-KDm as compared with the cavities in a typical active protein kinase such as PAK6 (p21 activated kinase-6, 450Å^3) (Fig. s8A).At the remodeled Activation Switch and catalytic loop, the structure features an ionic cluster (Fig. 4B) rather than the canonical hydrophobic interactions observed in typical protein kinases (Fig. s8B) [30,31]. Well-ordered water (Fig. 4C) is trapped beneath the ionic cluster (Fig. 4D). Ion pairs are also present throughout the structure of iWNK1A (Fig. S8A). The kinase domains ofWNK1 and WNK3are composed of 10% lysine and 10% glutamic acid, twice as much as average for proteins of comparable size (aspartic acid and arginine are normal)[32].

In addition to the buried waters in the structure of iWNK1A, there is more visible water overall than normal. We modeled 30 additional waters into the electron density of iWNK1A, including first and second shell waters justified by peaks 1.5 σ in the |2Fo-Fc| map (Table S2) (deposited in PDB file 6CN9). The structure shows that the largest CavA1 contains about 40 water molecules (Fig. S8B). A similar large quantity of waters is present in the active site of subunit B (Fig. S8C). The apparent involvement of water is reasonable because water on the surface of proteins is lower density than bulk water[33-36].

The data presented here demonstrate that a soluble protein kinase is directly activated by hydrostatic pressure *in vitro* and *in vivo*. iWNK3and iWNK1sense pressure, as well as chloride. A major finding concerns mechanism. The activation by hydrostatic pressure apparently involves a conformational equilibrium between an inactive dimer and an autophosphorylation-competent monomer. This mechanism of conformational equilibrium is observed in allosteric enzymes and signaling molecules sensitive only to ligands [37,38]. We also observe a potential role for bound water. Future studies will address the role of water in pressure sensing by the WNK kinase domains, larger fragments or full length WNKs, and other soluble pressure sensors [39].

The direct pressure sensing of iWNK3 or iWNK1 has not been anticipated. Theapparent regulation of WNK kinases by hydrostatic pressure may account for familial hypertension and hypotension associated with WNKs[10].Also, since WNKs are in the pathway for angiotensin II and α1-adrenergic blood pressure stimulation and myogenic responses as observed in knockout studies [15,16], WNKs may contribute to these responses along with other pressure sensors [5]. WNK3 is also involved in volume regulation and neuronal

excitability in brain [17,40-42]. Further experimentation is required to determine if WNK pressure sensing is involved in theseprocesses.In any case, the present study expands the horizon of the identity of pressure sensors from membrane bound proteins to molecules in the cytoplasm, and offers at least one soluble protein from which we can understand pressure sensing.

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Author's Contributions. R.A. made the initial discovery, planned experiments, performed data analysis and re-refinement of structure, and assisted with manuscript preparation. K.S. collected most of gel-based data presented. H.H. performed all DNA work. J.L. performed early characterization of pressure effects. J.J. designed pressure sensitivity mutants based on sequence alignments. J.M.H. performed all mass spectrometry. J.P. and J.S. conducted pressure experiments in *Drosphila* Malphigian tubules. A.R. R. planned and oversaw tubule experiments, and assisted with manuscript preparation. E.J.G. initiated and guided this work and wrote the manuscript.

Figure Legends

Fig.1.Autophosphorylation of WNK3-KDm, and WNK1-KDm as a function of hydrostatic pressure and chloride. Autophosphorylation of (**A**) WNK3-KDm and WNK1-KDmwith 0 and 190 kPa applied hydrostatic pressure (N_2), 7 minute reactions, 4μM protein concentration (optimum), standard conditions, in 150mM [Cl^-], 25 ^oC. Chloride opposition of pressure induced autophosphorylation of (**B**) WNK3-KDm and (**C**) WNK1-KDm as a function of applied pressure (N_2 gas) and [Cl^-] (concentrations indicated). The percent phosphorylation was assessed by Pro-Q Diamond phospho-protein stained SDS-PAGE gels. Data were obtained in triplicate (One of three replicate gels is shown). *, $p<0.01$ and **, $p<0.001$. Experiments performed on different days were scaled to phosphorylated and unphosphorylated WNK3-KDm and WNK1-KDm standards.

Fig. 2. **Methods of applying pressure, and pressure effects on control kinases and phosphatases.** (**A**) iWNK3and (**B**) iWNK1-KDm pressurized with nitrogen and argon in the Amicon cell, weights and centrifuge (see Fig. S2). Phosphorylation assessed with Pro-Q Diamond. The pressure applied in each modality was set to match the weight (315gm used on the syringe, corresponding to 160 kPa). (**C)**The control kinases SPAK, ASK1, MEK6, TAK-TAB, and p38 MAP kinase (4μM protein concentration) were tested for autophosphorylation along with iWNK3 and iWNK1-KDm as a function of hydrostatic pressure, 7 min reactions, 150mM [Cl^-].(**D**) Trans-phosphorylation of iWNK3, iWNK1, MEK6/DD, MEK1,and B-Raf on their respective substrates OSR1 for both WNK1 and WNK3, p38, ERK2, and

MEK1 at 1:100 enzyme:substrate ratio as a function of hydrostatic pressure, 7 min reactions, 150mM [Cl^-] (**E**) Control phosphatases MKP3, PP1cγ, λ, and shrimp alkaline phosphatase (SAP) at 4μM were tested for phosphatase activity change with pressure by tracking the change in absorbance at 405 nM due to hydrolysis of p-nitrophenylphosphate protein concentration. All experiments were conducted at 25°C. No correction for temperature effects in the centrifuge experiment was made, which may account for the difference in activity.

Fig. 3. **Model, mutants, and *in vivo* phosphorylation of WNK3.**(**A**) Model of a dimer to monomer transition based the structure 3FPQ and crosslinking (see Fig. s4A). Subunits A and B are colored in green and cyan, the Activation Switch is red**.** (**B**)Sequence alignment of WNK3, and WNK1 with WNK from three different Habitat variants. (**C**) Hydrostatic pressure-induced autophosphorylation of the WNK3 mutants M10, M7 and M9 (see Fig. s5).(**D**) Full length WNK3 together with kinase-dead rat $SPAK^{D219A}$was expressed in *D. melanogaster* renal tubules (see Fig. S7). The endogenous *Drosophila WNK* was knocked down. Pressure was applied to isolated tubules by centrifugation. SPAK phosphorylation was quantified by Western blot using anti-pSPAK and anti-total-SPAK antibodies. Ten independent experiments, with 30 tubules/condition, were performed. In each experiment, the p-SPAK/t-SPAK ratio in centrifuged tubules was normalized to account for day to day experimental variation.***, $p<0.001$, one-sample t-test to theoretical mean of 1. A sample Western blot is shown.

Fig. 4. **Cavities, ions and water in iWNK1A.** (**A**) Cavities in WNK1-KDm calculated in PyMOL. (**B**) Ionic cluster in Subunit A that form the largest cavity (a similar cavity is found in Subunit B).Catalytic loop is yellow, residues involved in the cluster are indicated, and H-bonds are shown in purple. (**C**) Water molecules in the electron density of 6CN9 (rerefined 3FPQ) (Subunit A) contoured in COOT at 1 σ.Waters are underneath the cluster. (**D**) Waters surrounding the cluster in Subunit A. The waters colored yellow correspond to those in the electron density in (**C**).

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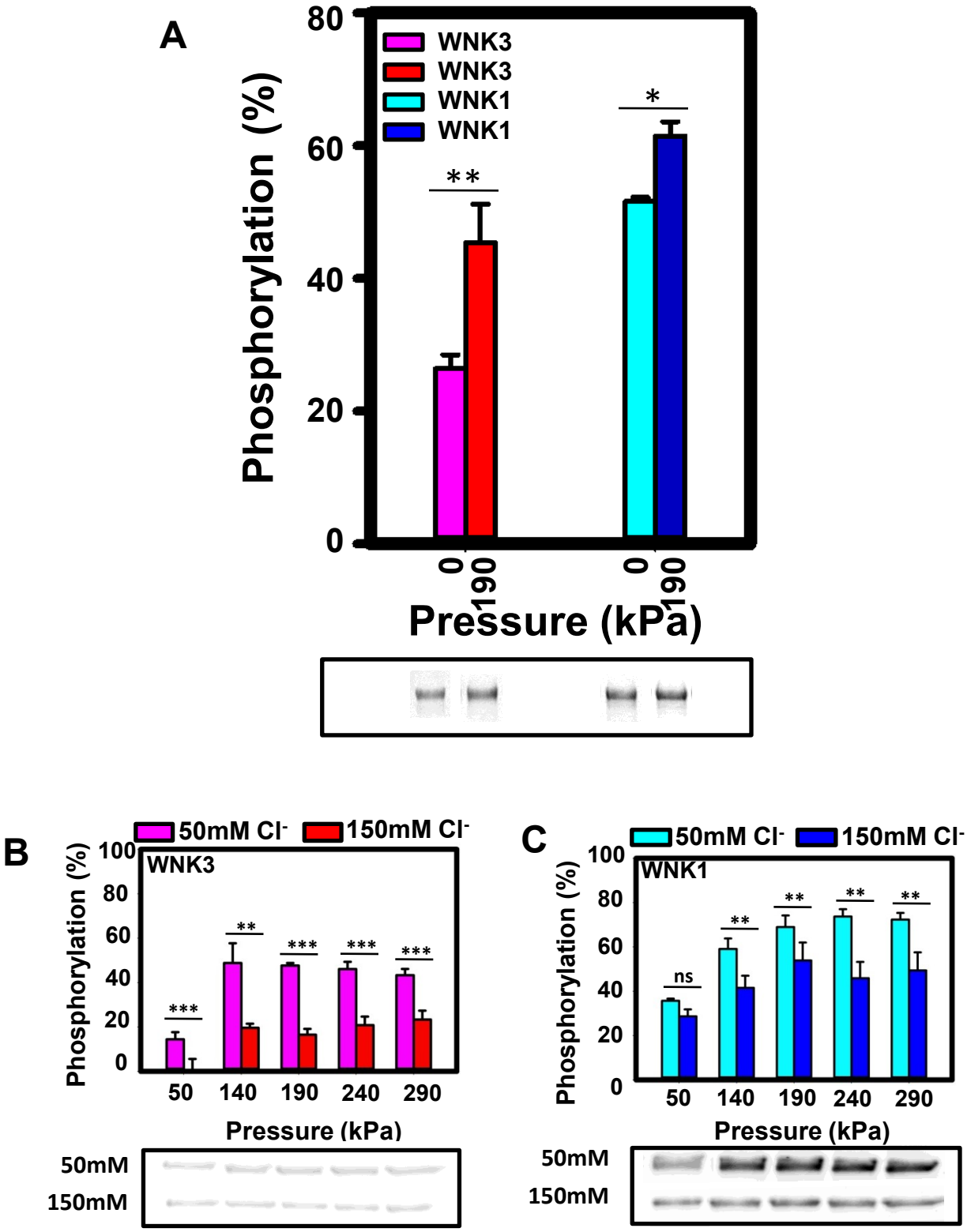
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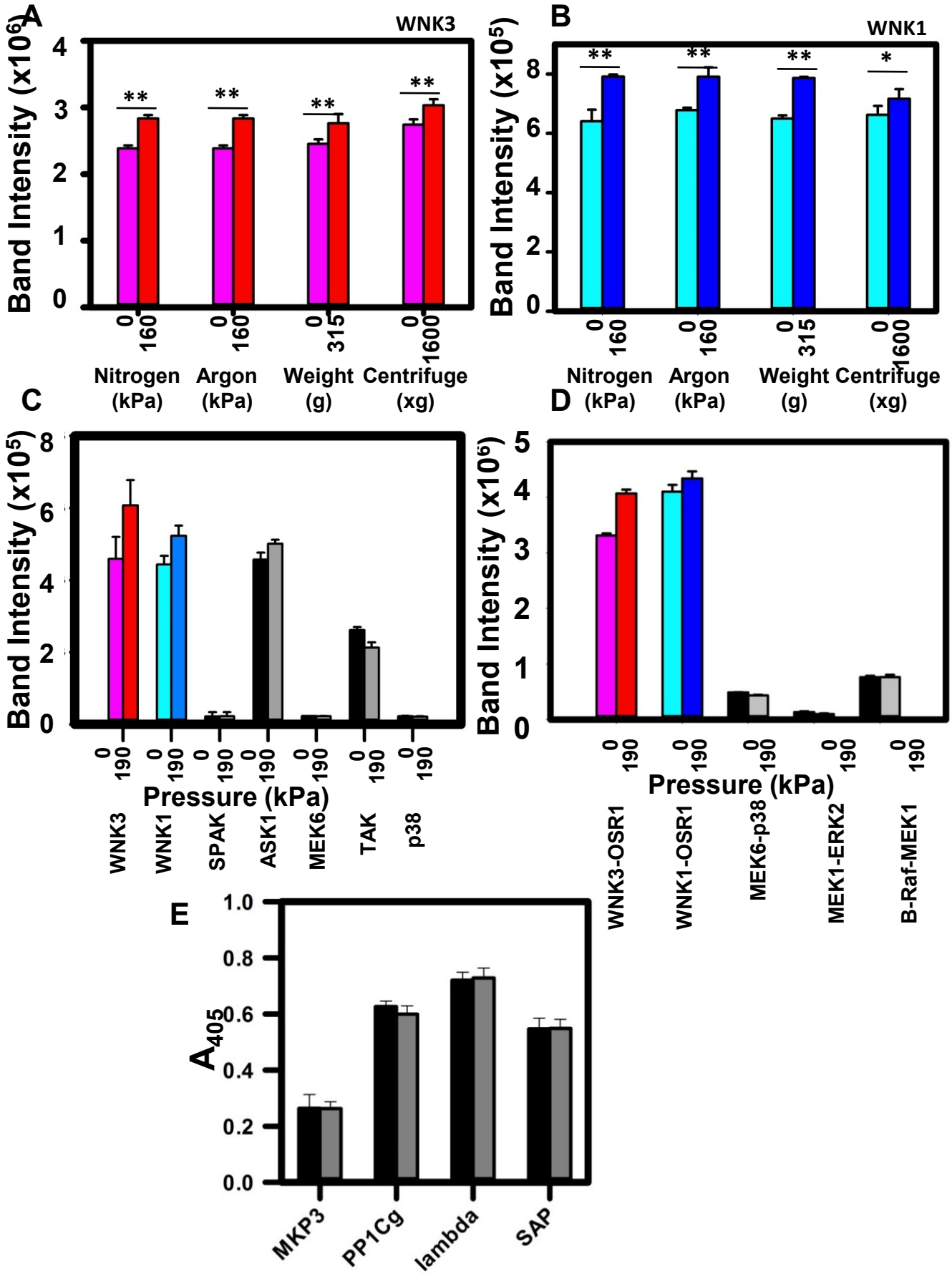
Fig. 1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190
Pressure (kPa)
WNK3-OSR1
WNK1-OSR1
MEK6-p38
MEK1-ERK2
B-Raf-MEK1
E
A405
1.0
0.8
0.6
0.4
0.2
0.0
MKP3
PP1Cg
lambda
SAP

Fig. 2

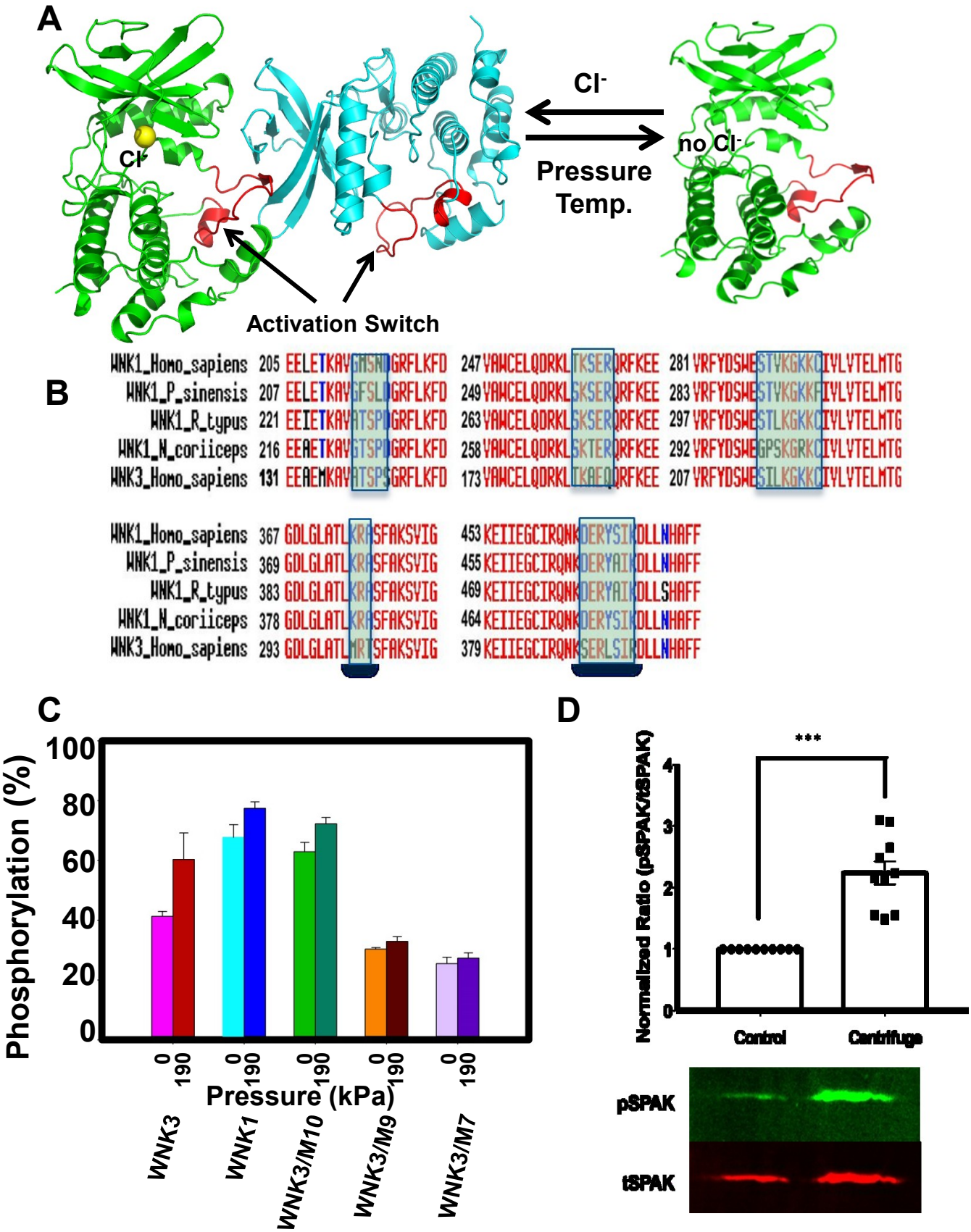

A
Cl-
Cl-
no Cl-
Pressure
Temp.
Activation Switch
B
WNK1_Homo_sapiens
WNK1_P_sinensis
WNK1_R_typus
WNK1_N_coriiceps
WNK3_Homo_sapiens
C
Phosphorylation (%)
Pressure (kPa)
WNK3
WNK1
WNK3/M10
WNK3/M9
WNK3/M7
D
Normalized Ratio (pSPAK/tSPAK)
***
Control
Centrifuge
pSPAK
tSPAK

Fig. 3

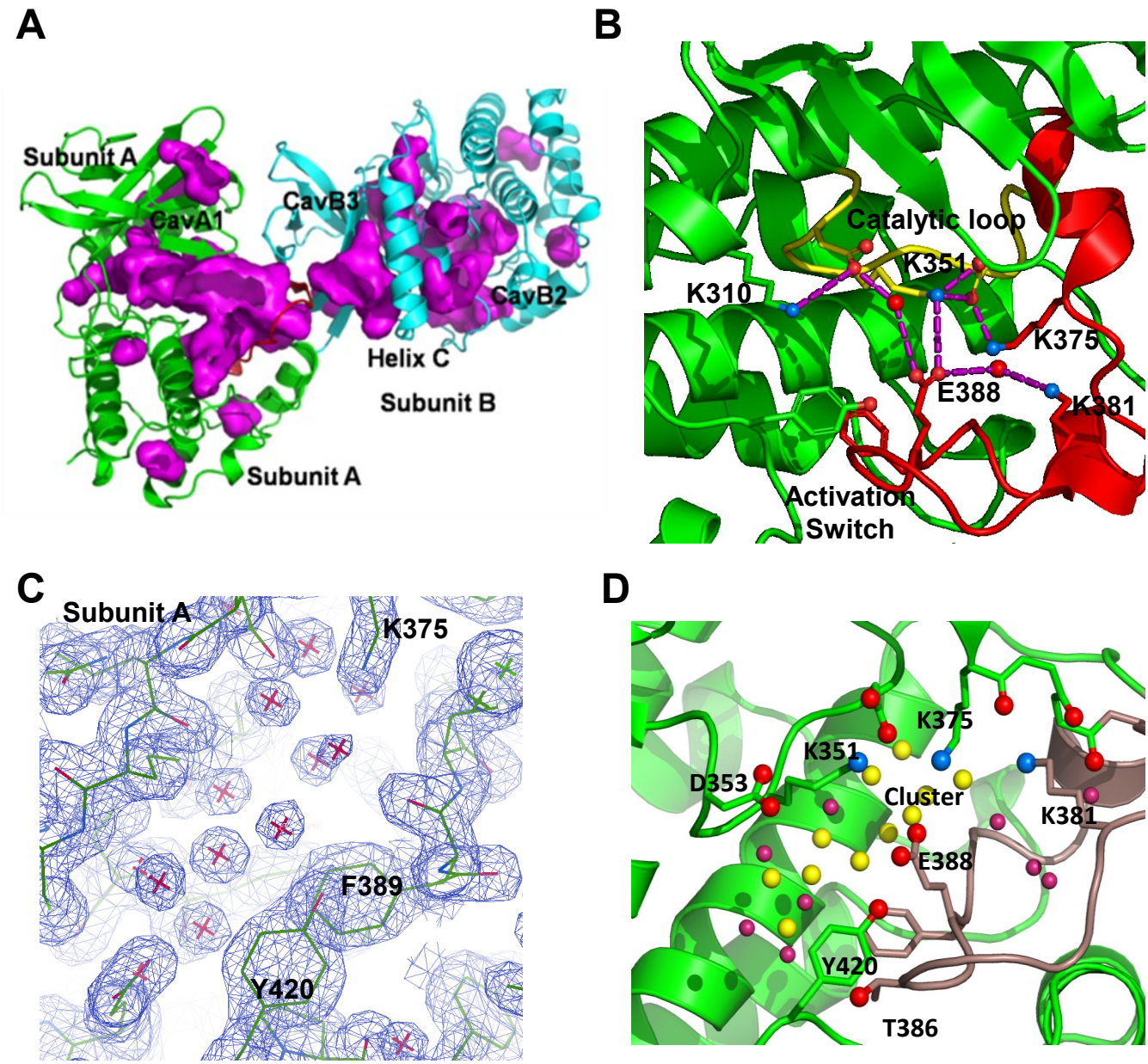
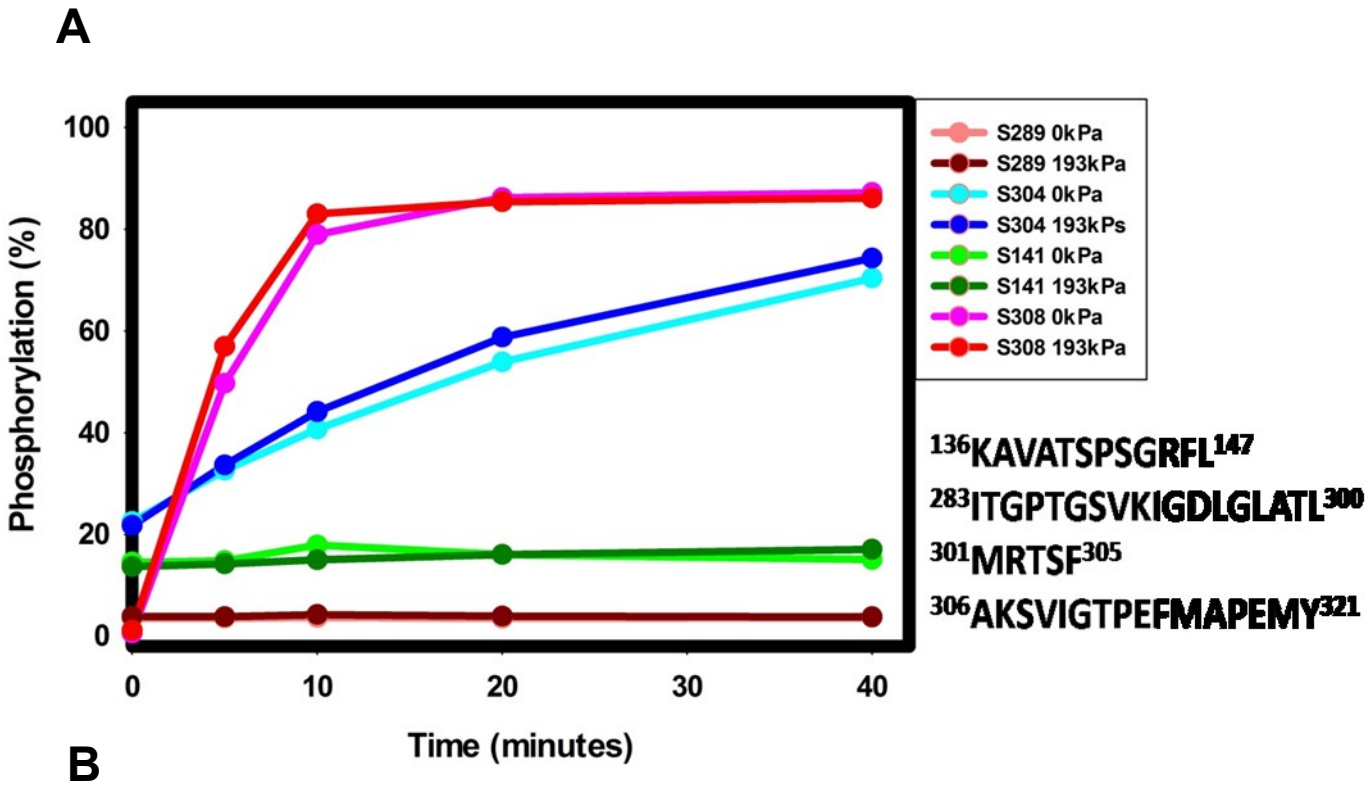

A
Subunit A
CavA1
CavB3
CavB2
Helix C
Subunit B
Subunit A
B
Catalytic loop
K351
K310
K375
E388
K381
Activation
Switch
C
Subunit A
K375
F389
Y420
D
K375
K351
D353
Cluster
K381
E388
Y420
T386

Fig. 4


A
Phosphorylation (%)
100
80
60
40
20
0
0
10
20
30
40
Time (minutes)
S289 0kPa
S289 193kPa
S304 0kPa
S304 193kPs
S141 0kPa
S141 193kPa
S308 0kPa
S308 193kPa
136KAVATSPSGRFL147
283ITGPTGSVKIGDLGLATL300
301MRTSF305
306AKSVIGTPEFMAPEMY321

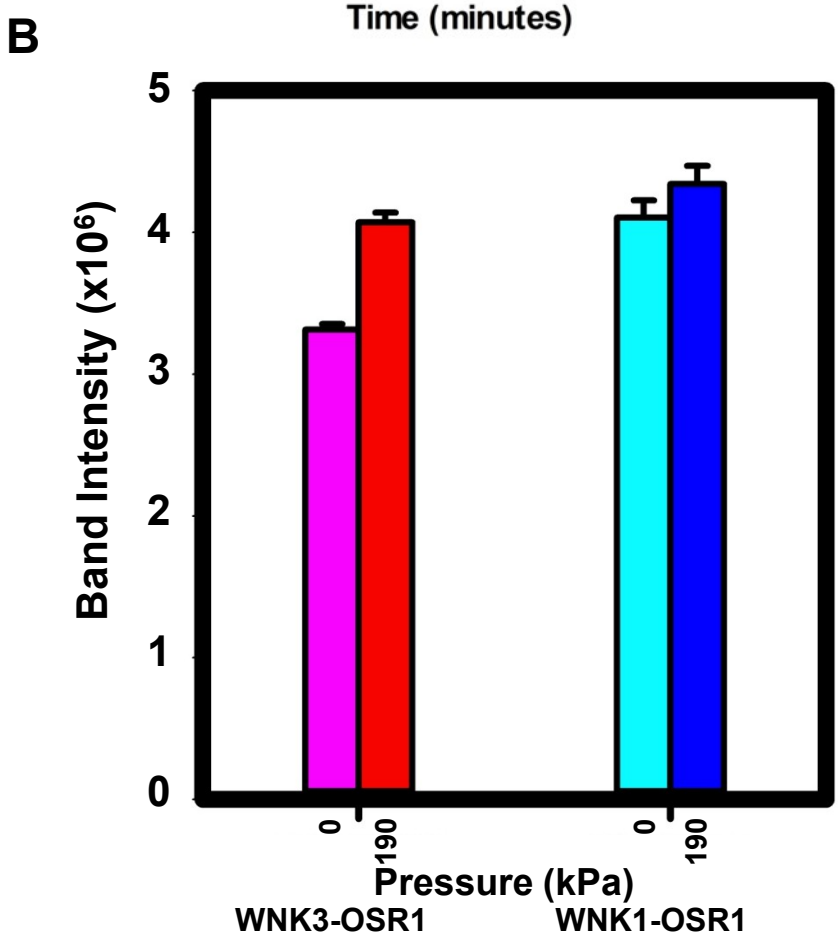
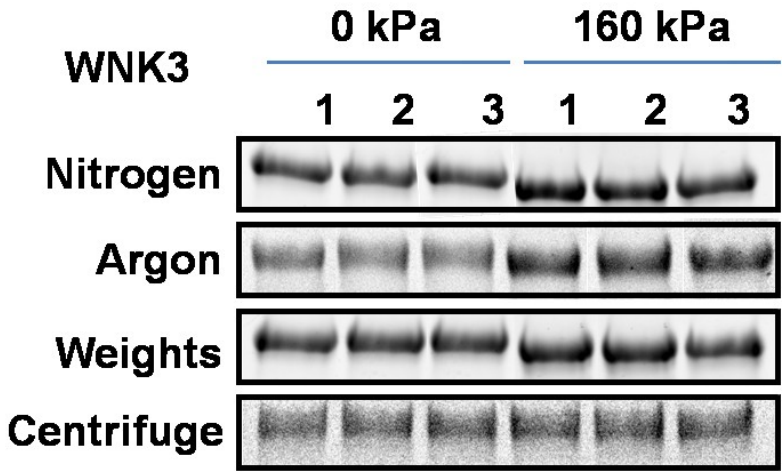

B
Band Intensity (x10⁶)
5
4
3
2
1
0
0
190
0
190
Pressure (kPa)
WNK3-OSR1
WNK1-OSR1

Fig. S1

A

A
Regulator
100% N_2
Pressure
Simicon
oppendorf
Centrifuge 5415 R

B


WNK3
0 kPa
160 kPa
1 2 3 1 2 3
Nitrogen
Argon
Weights
Centrifuge

C

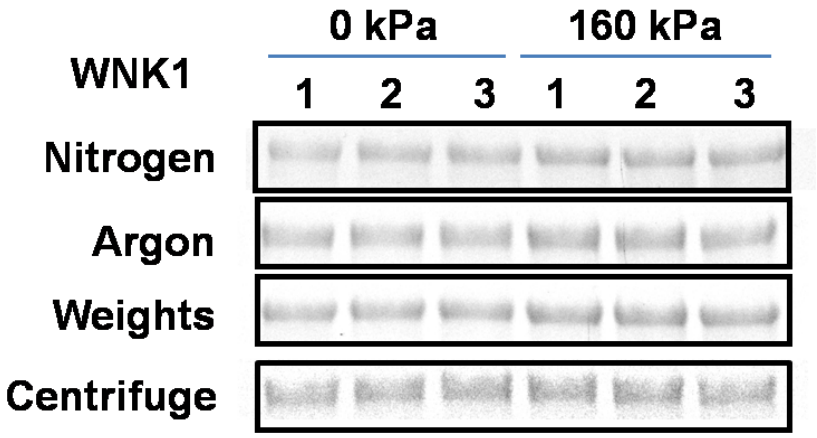

WNK1
0 kPa
160 kPa
1 2 3 1 2 3
Nitrogen
Argon
Weights
Centrifuge

Fig. S2

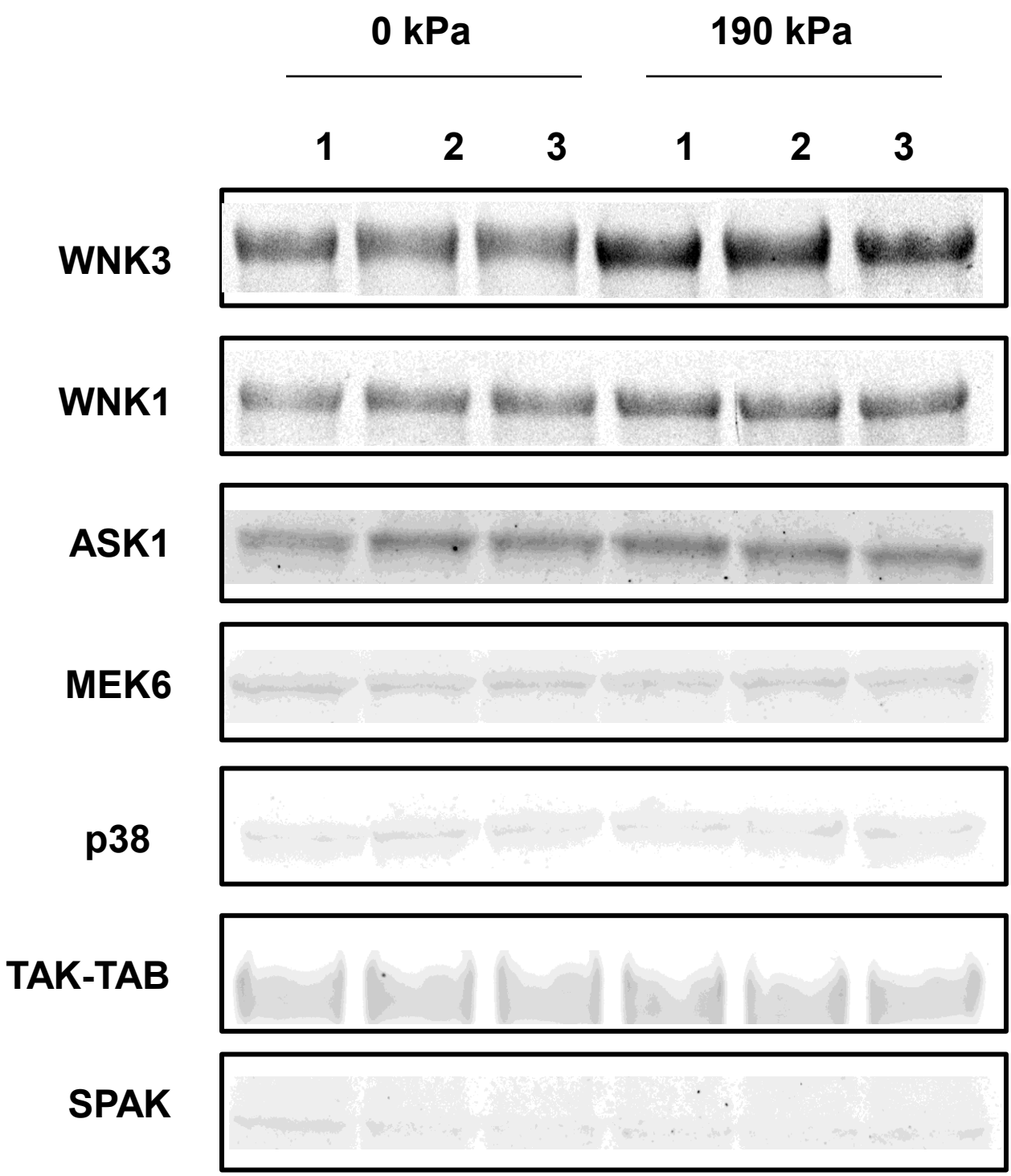


Fig. S3A

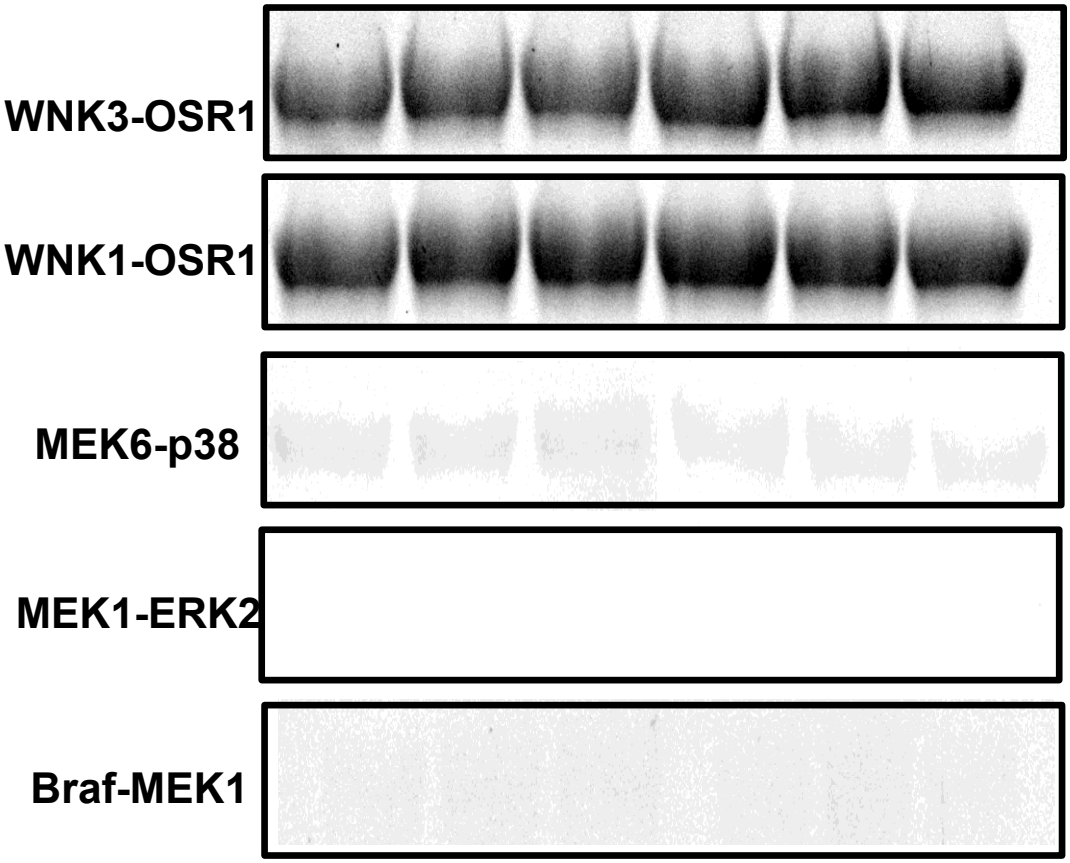
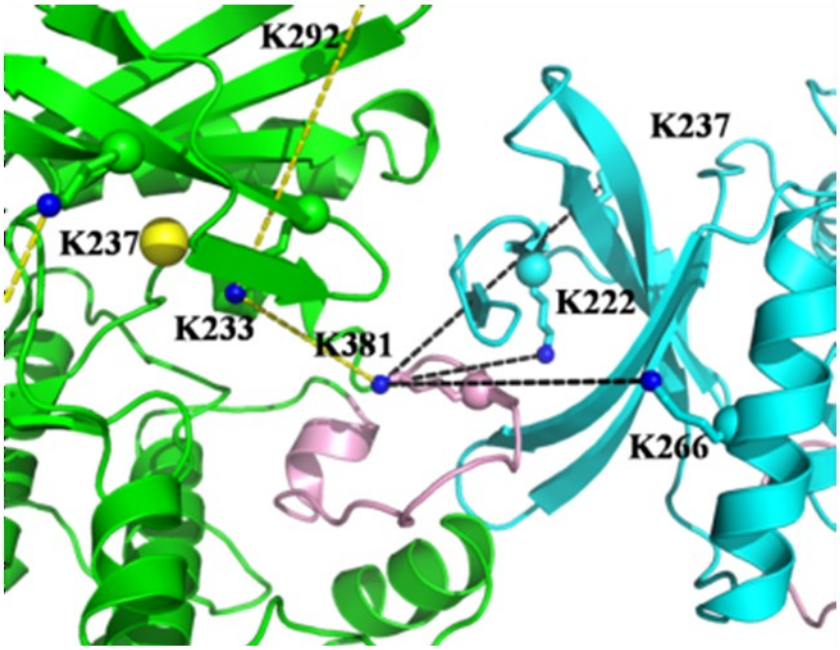
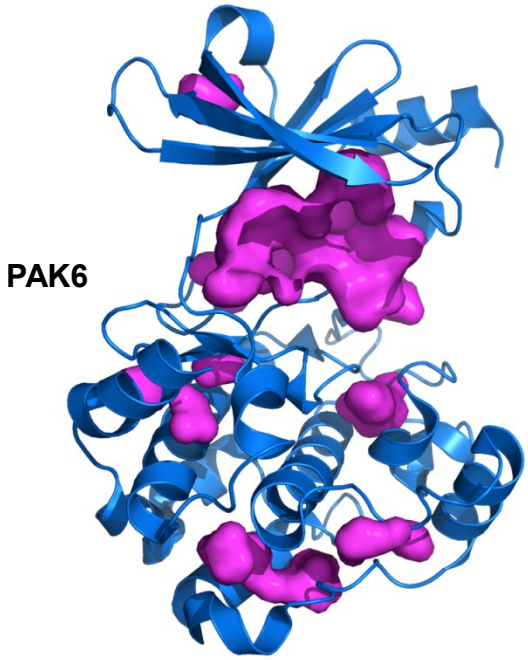


Fig. S3B

A


K292
K237
K237
K233
K381
K222
K266

B


PAK6

C

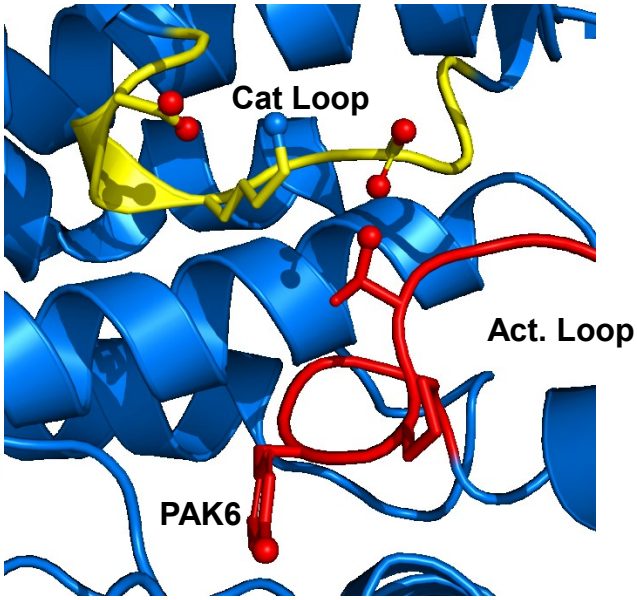
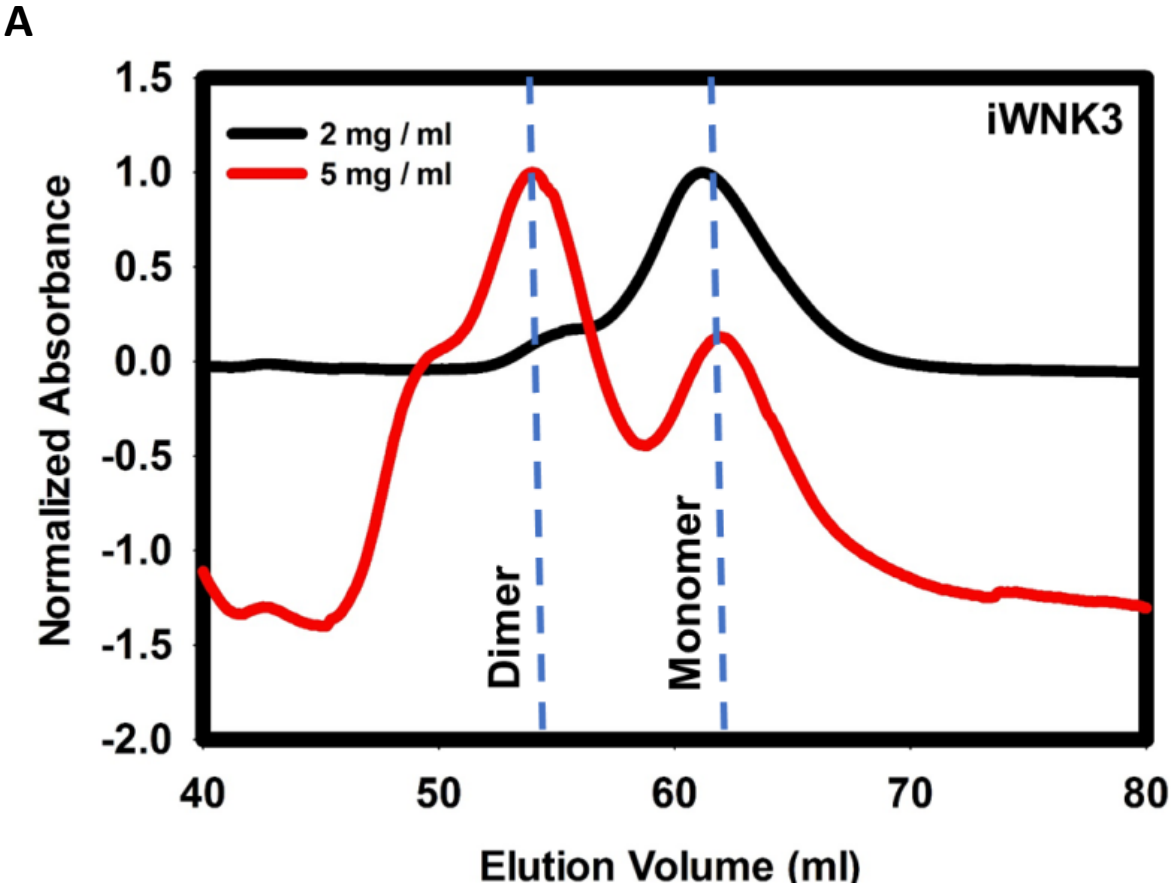

Cat Loop
Act. Loop
PAK6

Fig. S4


A
iWNK3
2 mg / ml
5 mg / ml
Dimer
Monomer
Normalized Absorbance
Elution Volume (ml)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
40
50
60
70
80

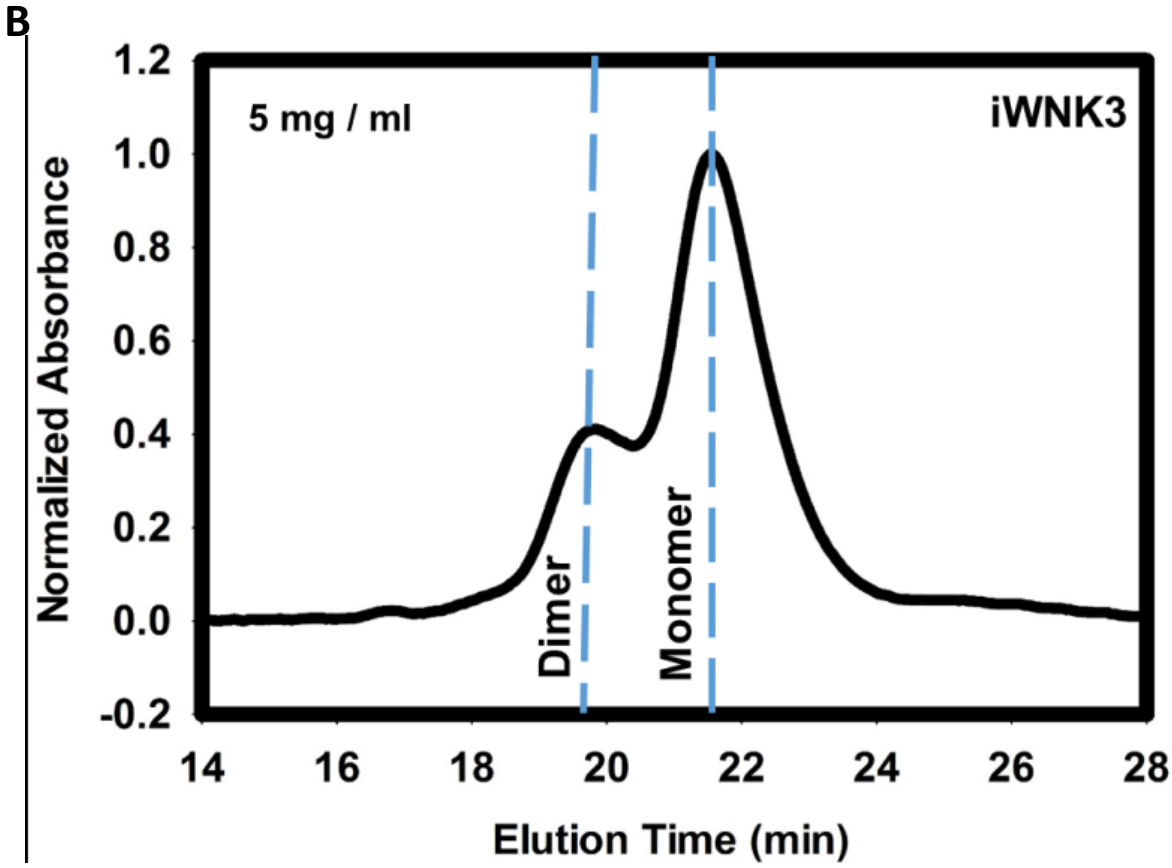

B
5 mg / ml
iWNK3
Dimer
Monomer
Normalized Absorbance
Elution Time (min)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
14
16
18
20
22
24
26
28

Fig. S5

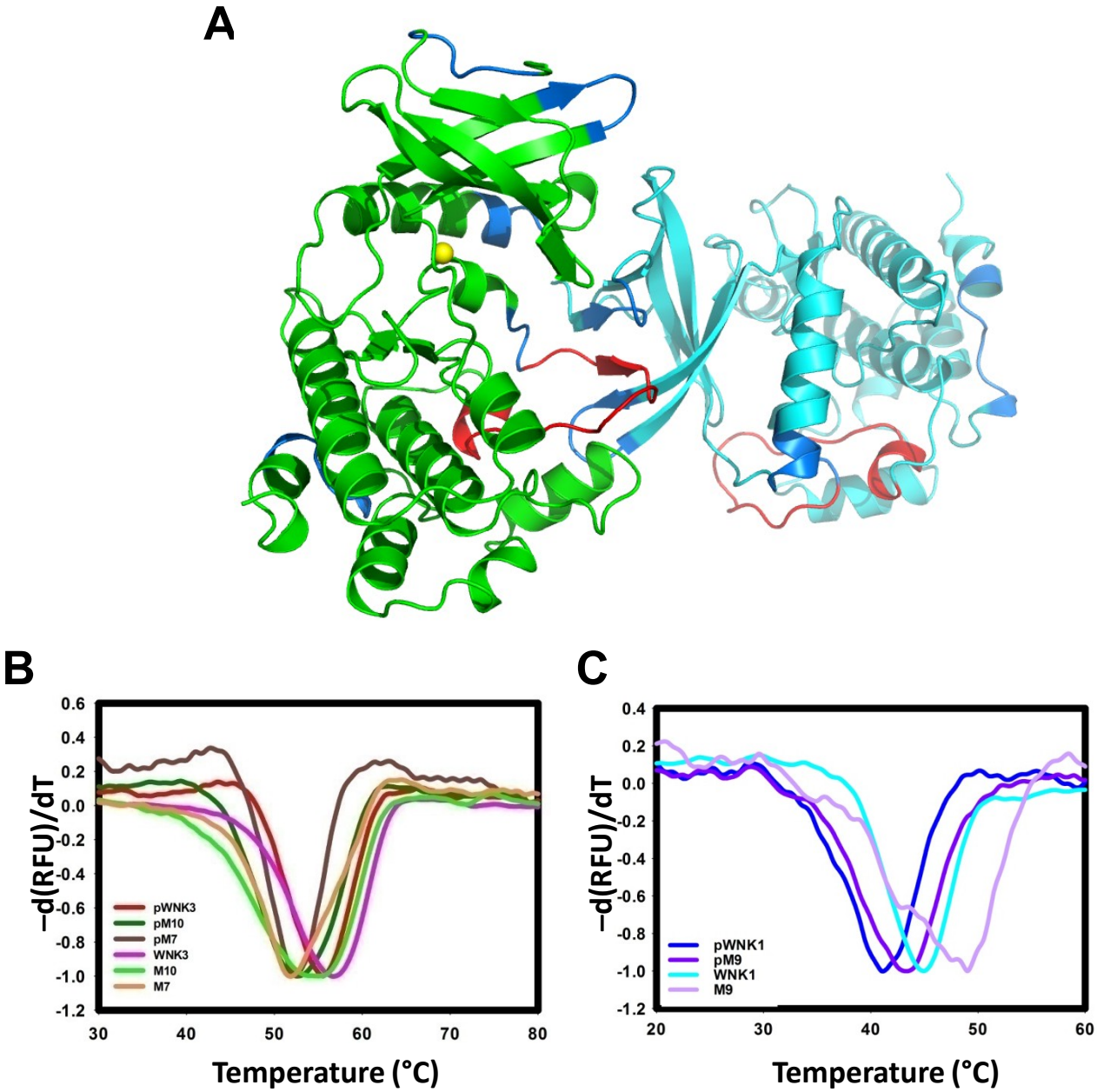

A
B
C
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
−d(RFU)/dT
pWNK3
pM10
pM7
WNK3
M10
M7
30
40
50
60
70
80
Temperature (°C)
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
−d(RFU)/dT
pWNK1
pM9
WNK1
M9
20
30
40
50
60
Temperature (°C)

Fig. S6

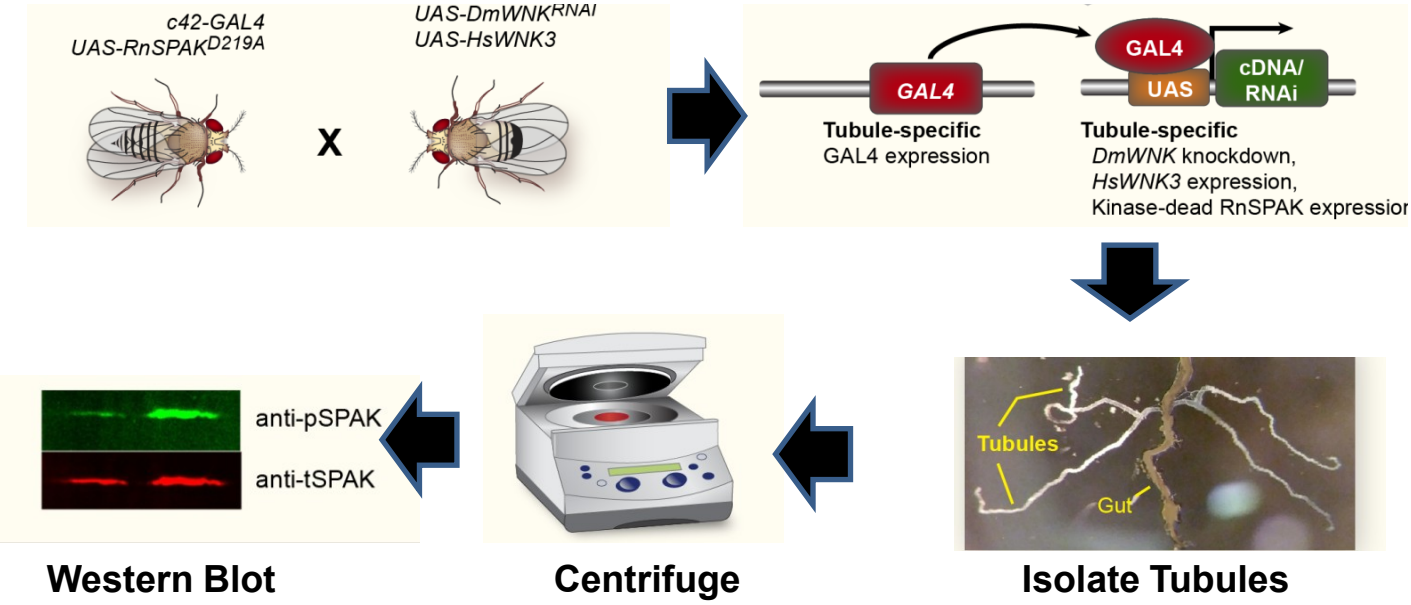

c42-GAL4
UAS-RnSPAKD219A
X
UAS-DmWNKRNAI
UAS-HsWNK3
GAL4
Tubule-specific
GAL4 expression
GAL4
UAS
cDNA/
RNAi
Tubule-specific
DmWNK knockdown,
HsWNK3 expression,
Kinase-dead RnSPAK expressior
Tubules
Gut
anti-pSPAK
anti-tSPAK
Western Blot
Centrifuge
Isolate Tubules

Fig. S7

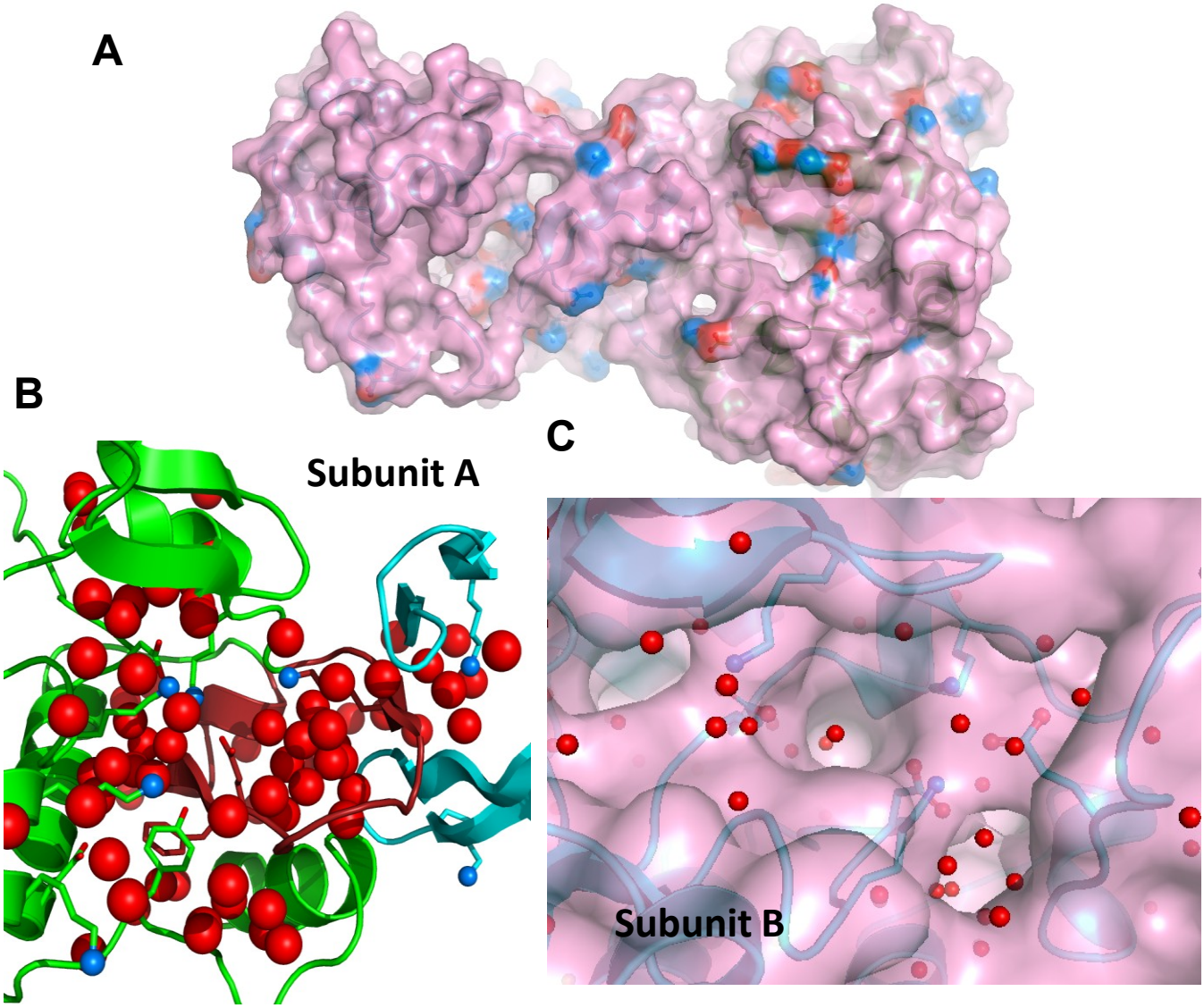


Fig. S8