Osmosensing by WNK kinases

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RE: Manuscript #E20-01-0089
TITLE: Osmosensing by WNK kinases

Dear Prof. Goldsmith:

As you can see the reviewers feel that the mechanism proposed is potentially interesting, but feel that more data and clarification are needed to support it. In particular the comments from both reviewers regarding the SAXS data and analysis need to be thoroughly addressed. In addition, please address the point raised by Reviewer 2 regarding the hydration mechanism and the effect of other crowding agents raised by Reviewer 1. A more thorough discussion regarding the dimer structure should also strengthen a revised manuscript.

Sincerely,

Antonina Roll-Mecak
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Goldsmith,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
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Reviewer #1 (Remarks to the Author):

Kinases in the WNK family control ion homeostasis and blood pressure through regulation of cation-chloride cotransporters. In this manuscript, Akella et al. build on previous work that established an inhibitory effect of chloride ions on WNK autophosphorylation. Here, the authors probe whether WNK (WNK1 and WNK3) could be activated by conditions that mimic osmotic stress. Indeed, WNK1 and WNK3 show increased autophosphorylation over time when incubated in buffers containing high concentrations of PEG400, a polymer that introduces osmotic stress on the purified kinases. Based on small-angle X-ray scattering, crystallography and gel filtration studies, the authors develop a model that suggests a dimer-to-monomer transition of WNK under osmotic stress being responsible for the activation of the kinase. A structural analysis indicates unusually large, water-filled cavities in the WNK1, which could sense osmotic pressure for the regulation of kinase activity. In summary, this is an interesting study that describes a plausible model for the activation of WNK under osmotic stress, an effect that is counteracted by chloride ions. The model shows parallels to osmosensing by bacterial histidine kinases (i.e., EnvZ).

However, there are several major points the authors should consider addressing in a revised manuscript:

1. The authors use a single crowding reagent to increase osmotic pressure. Is the effect unique to PEG400, or do other reagents with similar properties (e.g., dextran) introduce a similar effect on kinase activity?

2. Page 6, Figure 2C: The authors claim that WNK3 activity against the substrate gOSR1 is increased in the presence of PEG400. This result is not obvious from the figure. What is shown is that the differences in gOSR1 phosphorylation in 0% and 15% PEG400 are non-significant, the opposite to the statement in the main text. Also, there is a discrepancy between the figure legend (stating that 80 uM gOSR1 was used) and the Material and Methods section (stating that 40 uM gOSR1 was used). These points require clarification.

3. Page 6/7, SAXS analysis: To fully evaluate the SAXS data and their interpretation, it would be valuable to:
a) Provide a table summarizing SAXS data analysis with common parameters (including molecular weight determination by SAXS, Rg, Dmax, statistics regarding the modeling).
b) Present the size-exclusion chromatography profiles of the in-line measurements. This is particularly important in this case since the apo protein elutes in multiple peaks as shown in Figure 3E. Did the analysis concentrate on only one of the peaks, and if so, why? Would it be possible to model the other peak as well, which could support the proposed model?
c) Report the number of individual gnome models that were calculated and the statistics of the model averaging process.
d) include an analysis of uWNK3 (and pWNK3) in the presence of PEG400 (since it could confirm the proposed model that PEG400 shifts the equilibrium to monomeric WNK).

In addition, the figure legend uses Kratky plot and pairwise distance distribution function interchangeably. These are two different plots, and what is shown in the insets are pairwise distance distribution functions.

4. Page 7, SEC analysis: In gel filtration experiments it can be difficult to distinguish between changes in elution volume due to conformational changes or changes in oligomeric state. The analysis would be more compelling if the approach could be coupled to static multi-angle light scattering, which enables absolute molar mass determination independent of elution volume. Alternatively, analytical ultracentrifugation could be used to characterize the oligomeric state of uWNK and pWNK.

5. The authors' model proposes that PEG400 (or osmotic pressure) triggers a dimer-to-monomer transition of WNK, with monomeric WNK being the phosphorylation competent unit. While autophosphorylation of WNK1 and WNK3 increases in PEG400, the author did not test whether PEG400 changes the oligomeric state of the kinase. Given the tools and approaches available used in this study, this experiment seems doable and would address a central point in the proposed model.

6. Page 8, cavity analysis using the WNK1 crystal structure: The authors describe large, water-filled cavities in WNK1. From the figures, it appears that the analysis was conducted in the absence of nucleotide. Since some of the cavities seem to overlap with the active site of the enzyme, it would be important to consider how the cavity and water distribution would change in the native kinase in a cell that will likely be bound to ADP or ATP.

Minor point:
Page 3, 4th line from top: 'cotrasnporters' should be 'cotransporters'.

Reviewer #2 (Remarks to the Author):

This paper outlines a very simple concept regarding the WNK kinase, which regulates co-transporters and was shown previously to be inhibited by chloride. The authors here try to demonstrate that Wnk can be activated by osmotic pressure and attempt to correlate this with a monomer:dimer transition where the monomer is induced by phosphorylation of the activation loop. While this is an intriguing possibility the authors do not convincingly support the hypothesis with rigorous data.

Specific concerns:

1. A major piece of supporting data is the SAXS analysis, which is not convincing. The raw data needs to be included in Figure 3. Without this the figure is meaningless. How well does the data fit to the plots? The Kratky plots are also confusingl Once again there is no data, and they appear to be P(r) functions rather than Kratky plots. The inset says that this is a Kratky plot (pairwise distance distribution function); however, the Kratky plot and P(r) are actually different plots. The discussion of the SAXS data is superficial.
2. The gel filtration data also shown in Figure 3 shows an obvious monomer following phosphorylation; however, the unphosphorylated protein, even though the dimer is prevalent, is clearly a mixture of aggregation states that include a monomer and dimer as well as higher molecular weight aggregates. Given the aggregation state of the sample, it is not convincing to show SAXS data for this complex.
3. There needs to be quantitative stoichiometry data that shows that activation correlates with the single phosphorylation of the residue at the Activation Loop. This data should be included in Figure 1. Having an antibody to the activation loop phosphorylation would also be very helpful.
4. Is there a reason why WNK1 would not be as sensitive to osmotic pressure? Are there any obvious differences that might explain this? Is WNK1 equally sensitive to chloride?
5. The hydration data is also not convincing. The proposed mechanism of osmotic sensitivity, while intriguing, seems to be very speculative. The authors base their model on the analysis of crystal structures and cavities filled with water. Obviously osmolytes can influence water bound to the kinases, but this does not sound like a solid molecular mechanism. Can this model be supported by computational data? There is very convincing computational analyses regarding water molecules at the active site of kinases (Setny, PNAS, 2018, 2013 and J. Chem. Theory Comput., 2015). Is this applicable for the WNK kinases? The position of the lysine is a very important part of Setny's model.
6. Does the unusual position of the lysine, which is the reason for the naming of this kinase, have anything to do with making this kinase, as opposed to other kinases, sensitive to osmotic pressure?
7. In general, the dimer structure is unusual and the authors do not speculate on this. The N-lobe of one kinase faces into the open active site of the other, and both are in almost identical inactive conformations. The dimer is not symmetrical and do they propose that this is a way of stabilizing two inactive dimers? The mechanism is not clearly defined and is highly speculative.
8. What are the temperature factors for the crystal structure?

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Valerie Weaver, Editor
David Drubin, Editor
Molecular Biology of the Cell

Dear Editor,

We want to thank the editor and reviewers for their comments.

We addressed your comments with new experiments. Here we first summarize changes to the figures and two of the largest changes in the text, then respond to the individual editor and reviewer comments.

Most importantly, while collecting data in response to the reviewer comments, we found that the previously reported gel filtration data in Figure 3 was dependent on the amount of reducing agent present as well as the backpressure. Using sufficient reducing agent to break covalent oligomers, it was necessary to go to a gravity-pressurized gel filtration column to see the dimer to monomer equilibrium as a function of osmolyte for uWNK3 (kinase domain). Further, as requested by the reviewers, we have collected SEC-MALS and AUC data, but these experiments also involve elevated hydrostatic pressure and consistently showed uWNK3 to be monomeric even in the absence of crowding agent. Thus, these experiments do not add to the present discussion of osmotic effects and have not been included here.

The backpressure effects need to be thoroughly studied, and are outside the scope of the present paper.

The overall conclusion of this paper has not changed: osmolytes and crowding agents affect uWNK3 dimer-to-monomer equilibrium, autophosphorylation, and activity. However, the data presented for gel filtration and SAXS in Figure 3 (and now S2) is new. The new data include SAXS of uWNK3 in the presence of PEG400 which provides evidence of conformational changes. We have also added a survey of crowding agents and osmolytes as requested, and discovered an osmolyte that induces autophosphorylation.

Below, Monitoring Editor and Reviewer comments are in black. Our responses and comments are in red. Minor changes are identified in italics throughout the revised manuscript.

Sincerely,

Elizabeth J. Goldsmith

MAJOR CHANGES TO THE FIGURES

Figure 1. A panel has been added surveying the effects of multiple crowding agents, now Figure 1B. This experiment resulted in the identification of an additional agent, ethylene glycol, that enhances WNK3 autophosphorylation.

Figure 2. We eliminated Fig. 2C, since the PEG400-induced changes in uWNK3 phosphorylation observed by Pro-Q Diamond stain was not significant in this experiment.

Figure 3. SEC-SAXS data. This figure contains new data that more directly addresses the effect of crowding agents on the structure of uWNK3. Dr. Chakravarthy at the Advanced Photon Source was able to collect SEC-SAXS data on uWNK3 -/+ PEG400. Panel A is the SEC profile. The uWNK3 protein elutes as a single peak -/+ PEG400. PEG400 shifts the uWNK3 to higher elution volumes (we do not have standards here, but do have standards in Figure S2, described below). The pair-wise distribution functions (D) are significantly different with and without PEG400. In PEG400, there are more short distances in the distribution. The Kratky plot of uWNK3 in buffer (E) shows that it is well-folded. Figure 3F presents calculated scattering curves based on the dimeric or monomeric structures of uWNK1, and calculations presented in the text describe a better Goodness of Fit for the dimer to the observed scattering curve. Figure 3G is the overlay of the WNK1 dimer on the DAMMIF-derived envelope. Figure 3H shows the Kratky plot for uWNK3 in PEG400, which indicates partial unfolding.

Figure S2. New figure. New gel filtration data. We conducted gel filtration experiments and determined that the gel filtration shown in the original Figure 3E was influenced by very low amounts of reducing agent. In multiple repetitions of the gel filtration at the back pressures on our Akta and Sephadex 75 column (290 kPA) in 2 mM TCEP, uWNK3 eluted as a monomer. This suggests that the dimer and tetramer observed originally may have arisen from covalent effects. We found that running gel filtration on a gravity only column revealed a mixture of dimers and monomers in rapid equilibrium based on standards run in the same conditions. Further, ethylene glycol (a newly discovered osmolyte that induces autophosphorylation and activity) induced a shift to higher elution volumes, relative to standards. These data are presented in Fig. S2, (A) no ethylene glycol, (B) with ethylene glycol, (C) table of elution volumes.

Figure 4. Figure 4C has been added to show details of the dimer interface as requested by one of the reviewers.

MAJOR CHANGES TO TEXT INVOLVING SAXS

The following text has been added to the Materials and Methods:

Size exclusion chromatography with small angle x-ray scattering (SEC-SAXS) (Brosey et al., 2019) data was collected at the BIOCAT (sector18-ID) beamline at Argonne National Laboratory (http://www.bio.aps.anl.gov/pages/about-biocat.html). 300 μ L of 5mg mL^{-1} sample of uWNK3 was injected on a 24 mL Superadex-200 (GE Healthcare) column equilibrated with 50 mM HEPES pH 7.4, 150 mM NaCl, 1mM EDTA and 1 mM TCEP (Tris(2-carboxyethyl)phosphine) *in line with a SAXS flow cell. SAXS data were collected in 0.5 second exposures every second. Scattering intensity was recorded using a Pilatus3 1M (Dectris) detector which was placed ~ 3.5 m from the sample giving us access to a q-range of ~0.004 $Å^{-1}$ to 0.4 $Å^{-1}$. Frames flanking the peak were averaged to obtain the buffer background for subtraction. Data were reduced using BioXTAS RAW 1.6.0 (Hopkins,et al., 2017). Data analysis was carried out using the ATSAS package (Version 3.0.1) (Franke et al., 2017). Data processing including buffer subtraction, merging, extrapolation intensity at zero concentration (I_0), curve fitting and evaluation of radius of gyration (R_g) were performed using the PRIMUS module (Konarev, et al., 2003). The GNOM module (Svergun, et al., 1992) was used to obtain I_0, R_g, the distance distribution P(r), maximum dimension (D_{max}), Porod volume (V_p), and excluded volume (V_e). R_g and I_0 calculated by PRIMUS and GNOM were compared. Protein molecular weights were estimated from the Porod volumes (V_p). 15 bead-models comprised of spheres within a radius of $D_{max}/2$ were generated and refined against the scattering curve in DAMMIF (Franke, et al., 2009). DAMAVER and DAMFILT were used to generate an average envelope (Volkov, et al., 2003). CRYSOL was used to calculate the Goodness of Fit between the scattering curve and crystallographic dimer or monomer models as described (Svergun, et al.,1998).*

The results section now reads:

Size exclusion chromatography in line with small angle x-ray scattering (SEC-SAXS) was used to assess the oligomeric state of uWNK3 in solution. Data were collected both in the absence and presence of 15% PEG400. The uWNK3 SEC profiles gave single peaks -/+ 15% PEG400 (Figure 3A). The raw SAXS scattering profile and Guinier plot for uWNK3 are shown without PEG400 in Figure 3B, and with PEG400 in Figure 3C. Data collection and derived structural parameters are given in Table S2. The scattering profiles were truncated at s= 0.25 $Å^{-1}$, and used in the pairwise distribution function (P(r)) and Kratky plot calculations. The linearity of the Guinier plots indicate absence of aggregates (Putnam, et al., 2007). Figure 3D shows the P(r) verses r for uWNK3 (black) and uWNK3 in 15% PEG400 (red). The P(r) shows a greater percentage of short distances in the presence of PEG400. Consistent with the effect of PEG400 in activating uWNK3, the change in P(r) shows that PEG400 has altered the structure of uWNK3.

The Kratky plot derived from the scattering profile for uWNK3 in buffer (Figure 3E) suggests a compact and well-folded particle (Brosey et al., 2019, Rambo, et al., 2011). Calculated scattering curves based on dimeric WNK1/S382A (WNK1SA, phosphorylation site mutated, PDB file 3FPQ) and monomeric uWNK1 are shown in Figure 3F. To determine if either calculated scattering curve fits the observed uWNK3 data (Figure 3B) the CRYSOL module was used to measure the Goodness of Fit (χ^2). The dimer-derived curve gave a χ^2 of 8, compared with 29 for the monomer. Thus, uWNK3 in buffer is similar in structure to the crystallographically observed dimer of WNK1SA. DAMMIF was used to generate multiple ab initio models, giving the average envelope shown in Figure 3G. The crystallographic dimer of WNK1SA fits well with this envelope. The D_{max} of 95 Å (Table S2) also is consistent with a dimer model. However, the Rg and molecular weight calculated from the Porod volume was smaller than predicted from the dimer. This result may be due to lower than expected density for the protein (Rambo, et al., 2011), or due to a mixture of dimer with monomer.

*Analysis of the uWNK3 scattering data in the presence of PEG400 gave different results. The Kratky plot for uWNK3 in PEG400 plateaus at high q (Figure 3H), suggestive of partial unfolding and conformational heterogeneity (Brosey et al., 2019, Rambo, et al., 2011). Apparently, PEG400 has induced a large structural change, again consistent with the observed uWNK3 activation (Figure 1B,C). Experimental SAXS data for uWNK3 in PEG400 gave a poor CRYSOL-derived χ^2 (Table S2) to either dimer or monomer models. The lack of fit is expected from the Kratky plot divergence at high **s**. The R_g for uWNK3 in the presence of PEG400 was also small, and the D_{max} large (Table S2), again indicating potential disorder.*

MAJOR CHANGES TO TEXT INVOLVING GRAVITY GEL FILTRATION

The methods section now reads:

Gel filtration was conducted under gravity. Superdex-75 resin was packed into a 20 mm x 240 mm column (25 ml bed volume). The column void volume, measured with blue dextran 2000, was 4.5 ml. The column was calibrated using standards from the GE Healthcare calibration kit (albumin (67 kD) and ovalbumin (43 kD). The gravity column was run with 0.4 mls of 5 mg/ml uWNK3 -/+15% ethylene glycol. The flow rate was 0.2 mL/min in buffer, 0.1 ml/min in ethylene glycol.

The methods section now reads:

Gel filtration

As an additional test of uWNK3 oligomerization, gel filtration was carried out on a Sephadex 75 high flow gravity column (Figure S2A). Two standards, albumin (67 kD) and ovalbumin (43 kD), close to the molecular weight of dimeric WNK3 (80 kD) and monomeric WNK3 (40 kD), eluted at 16.0 ml and 17.0 ml, respectively. WNK3, applied to the column at 5 mg/ml, eluted as a single peak at 16.3 ml, suggestive of a mixture of dimer and monomer in rapid equilibrium.

Response to Monitoring Editor comments:

As you can see the reviewers feel that the mechanism proposed is potentially interesting, but feel that more data and clarification are needed to support it. In particular the comments from both reviewers regarding the SAXS data and analysis need to be thoroughly addressed. In addition, please address the point raised by Reviewer 2 regarding the hydration mechanism and the effect of other crowding agents raised by Reviewer 1. A more thorough discussion regarding the dimer structure should also strengthen a revised manuscript.

1. SAXS. New SAXS data has been introduced in Figure 3, as noted above. Srinivas Chakravarthy, who collected the SAXS data, is now included as an author and has reviewed our data, analysis, and presentation. Detailed comments concerning SAXS are in responses to Reviewer 1, comment 3.

2. Hydration mechanism. The main comment concerned the fact that our model is speculative and that building water structure is subjective. We have responded to this with several changes, discussed in detail in Response to Reviewer 2 comment 6. The changes include a reorganization of the discussion to put water model building last, past the discussion of cavity volumes which is more straightforward, and changing the paragraph heading to be less emphatic.

The reviewer wanted comparisons to other water structures in protein kinases, including PKA, and this analysis has been added, with references.

3. Crowding agents. As noted above, we surveyed more crowding agents/osmolytes. We found an osmolyte, ethylene glycol, which induces autophosphorylation and activation of WNK3. This was useful, because we were able to use this reagent in gel filtration experiments, now in Fig. S2. We also introduced another assay, ADP-Glo®, that has improved the statistics of our results over the Pro-Q Diamond stained gels (now Figure 1B).

4. Dimer structure discussion. An illustration, now Figure 4C, shows the interface in greater detail. We have also added two corresponding paragraphs of dimer description and discussion as explained in Responses to Reviewer 2 comment 7a,b.

Reviewer #1 (Remarks to the Author):

Kinases in the WNK family control ion homeostasis and blood pressure through regulation of cation-chloride cotransporters. In this manuscript, Akella et al. build on previous work that established an inhibitory effect of chloride ions on WNK autophosphorylation. Here, the authors probe whether WNK (WNK1 and WNK3) could be activated by conditions that mimic osmotic stress. Indeed, WNK1 and WNK3 show increased autophosphorylation over time when incubated in buffers containing high concentrations of PEG400, a polymer that introduces osmotic stress on the purified kinases. Based on small-angle X-ray scattering, crystallography and gel filtration studies, the authors develop a model that suggests a dimer-to-monomer transition of WNK under osmotic stress being responsible for the activation of the kinase. A structural analysis indicates unusually large, water-filled cavities in the WNK1, which could sense osmotic pressure for the regulation of kinase activity. In summary, this is an interesting study that describes a plausible model for the activation of WNK under osmotic stress, an effect

that is counteracted by chloride ions. The model shows parallels to osmosensing by bacterial histidine kinases (i.e., EnvZ).

However, there are several major points the authors should consider addressing in a revised manuscript:

1. The authors use a single crowding reagent to increase osmotic pressure. Is the effect unique to PEG400, or do other reagents with similar properties (e.g., dextran) introduce a similar effect on kinase activity?

To address this concern, we have added a new data figure, now Figure 1B. We tried several crowding agents and an osmolyte, PEG200, PEG400, ethylene glycol, Dextran 40 (40 kD), Dextran 70 (70 kD), and Ficoll 70 (70 kD). PEG400 and ethylene glycol were the most effective agents tested in inducing WNK3 autophosphorylation. We introduced a new reagent, ADP-Glo® Max, to track the disappearance of ATP. This reagent allowed us to make measurements at the ATP concentration required for unphosphorylated WNK3 (5 mM) activity. The ADP-Glo® Max allowed us to make multiple measurements and improve accuracy.

The following text has been added to the Materials and Methods:

ADP-Glo®

ADP-Glo® reagent (Promega Inc.) was used as a readout for autophosphorylation and activity measurements in the presence of crowding agents, such as polyethylene glycols which are incompatible with mass spectrometry. 50 µL reactions contained 40 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 4 µM pWNK, and 40 µM gOSR1. Final chloride concentration was maintained at 150 mM. The reaction was started by the addition of 5.2 mM ATP. Reactions were stopped after 15 minutes by addition of 50 µL of ADP-Glo® reagent. Manufacturers protocol was followed for the remaining steps of ATP depletion (40 min), conversion of ADP to ATP (1hour). 100 µL aliquots from each reaction were transferred to a 96-well plate which was centrifuged for 2 min. at 800 rpm. Luminescence was read on a CLARIOstar plate reader and data analyzed using MARS software (both reader and software, BMG Labtech, Ortenberg, GER). Data was analyzed using GraphPad-Prism software to obtain P-values.

The following text has been added to the Results and Discussion:

To determine if demands on solvent promote WNK autophosphorylation and activity in vitro, we used gOSR1 as a substrate with ADP-Glo® as the readout. Multiple crowding agents or osmolytes were surveyed (Figure 1B): ethylene glycol (62 Da), PEG200 (200 Da), PEG400 (400 Da), Dextran 40 (40 kD), Dextran70 (70 kD), and Ficoll70 (70 kD). Both PEG400 and ethylene glycol enhanced activity by about 30% whereas the other tested agents showed minimal effects.

2. Page 6, Figure 2C: The authors claim that WNK3 activity against the substrate gOSR1 is increased in the presence of PEG400. This result is not obvious from the figure. What is shown is that the differences in gOSR1 phosphorylation in 0% and 15% PEG400 are non-significant, the opposite to the statement in the main text. Also, there is a discrepancy between the figure legend (stating that 80 uM gOSR1 was used) and the Material and Methods section (stating that 40 uM gOSR1 was used). These points require clarification.

We eliminated this figure. Figure 1B, as noted above, surveys different crowding reagents with a better readout (ADP-Glo®).

3. Page 6/7, SAXS analysis: To fully evaluate the SAXS data and their interpretation, it would be valuable to:

a) Provide a table summarizing SAXS data analysis with common parameters (including molecular weight determination by SAXS, R_g, D_{max}, statistics regarding the modeling).

As requested, a Table of the SEC-SAXS analysis is now included in the supplemental materials:

Table S2. SAXS Analysis

Data Collection at Beamline BioCAT (18-ID)		
Parameter	*uWNK3*	*uWNK3 + 15% PEG400*
Wavelength (Å)	*1.0*	*1.0*
s-range ($Å^{-1}$)	*0.0-0.25*	*0.0-0.25*
Exposure time (sec)	*0.5*	*0.5*
Concentration (mg/mL)	*5*	*5*
Results		
I_0	*0.00008*	*0.005*
Rg from P(r) (Å)	*28.2*	*27.1*
Rg from Guinier plot (Å)	*27.3*	*26.8*
Dmax (Å)	*95*	*108*
Porod Volume ($Å^3$)	*68000*	*67000*
Molecular weight (kD)		
From Porod volume	*47*	*13*
From Vc	*44*	*15*
From sequence	*40*	*40*
Crysol Goodness of fit (χ^2)		
Dimer	*8*	*17*
Monomer	*29*	*19*

b) Present the size-exclusion chromatography profiles of the in-line measurements. This is particularly important in this case since the apo protein elutes in multiple peaks as shown in Figure 3E. Did the analysis concentrate on only one of the peaks, and if so, why? Would it be possible to model the other peak as well, which could support the proposed model?

We think the original Figure 3E data, as described above, was influenced by low concentrations of reducing agent leading to multiple high molecular weight peaks in the gel filtration elution profile. We now have new data with higher reducing agent where we observe a single monomeric peak (Figure S2A). We have since collected new SEC-SAXS data, as described above, focusing on uWNK3 -/+ PEG400. The new SEC profiles are shown in Figure 3A. There was only a single peak in each SEC run. We have removed the original Figure 3E (showing dimers and higher order aggregates).

In repeating the SEC experiments, we obtained different elution profiles under varying back pressures, as discussed above. We think this pressure effect may be real, with hydrostatic pressure altering the WNK dimer-monomer equilibrium (and potentially the activity). We are pursuing this idea, and plan to have sufficient data concerning pressure effects on WNKs for a separate publication. This concept is outside the scope of the present research.

Please note the major change to the text concerning gel filtration noted above.

c) Report the number of individual gnome models that were calculated and the statistics of the model averaging process.

Greater detail has been offered as noted above in the revised paragraph to Materials and Methods in response to (b).

d) include an analysis of uWNK3 (and pWNK3) in the presence of PEG400 (since it could confirm the proposed model that PEG400 shifts the equilibrium to monomeric WNK).

We have recently published a paper that concerns the effects of the crowding agents PEG and sucrose on the structure and activity of pWNKs (Akella et al., 2020).

In addition, the figure legend uses Kratky plot and pairwise distance distribution function interchangeably. These are two different plots, and what is shown in the insets are pairwise distance distribution functions.

Thank you for catching this mistake. Figure 3 now provides a thorough analysis of our SAXS data. See the major changes to the text concerning SAXS noted above.

4. Page 7, SEC analysis: In gel filtration experiments it can be difficult to distinguish between changes in elution volume due to conformational changes or changes in oligomeric state. The analysis would be more compelling if the approach could be coupled to static multi-angle light scattering, which enables absolute molar mass determination independent of elution volume. Alternatively, ultracentrifugation could be used to characterize the oligomeric state of uWNK and pWNK.

We carried out analytical ultracentrifugation experiments (AUC). These data showed a monomer even in the absence of PEG. As discussed in point 3b above, we posit that the hydrostatic pressures associated with both SEC-MALS and especially AUC alters WNK dimer-monomer equilibrium. We intend to publish these data in a separate paper.

5. The authors' model proposes that PEG400 (or osmotic pressure) triggers a dimer-to-monomer transition of WNK, with monomeric WNK being the phosphorylation competent unit. While autophosphorylation of WNK1 and WNK3 increases in PEG400, the author did not test whether PEG400 changes the oligomeric state of the kinase. Given the tools and approaches available used in this study, this experiment seems doable and would address a central point in the proposed model.

We have conducted uWNK3 SEC-SAXS -/+ PEG400. The Kratky plot derived from SAXS data suggests that uWNK3 is partially unfolded by PEG400 (Figure 3H). See response to Reviewer 1 (1B), above and the major changes to the text concerning SAXS.

As a second approach, gravity SEC was conducted -/+ ethylene glycol as a low-viscosity SEC compatible osmolyte (Figure S2). This experiment further confirmed that uWNK3 shifts toward a monomer in the presence of osmolyte.

6. Page 8, cavity analysis using the WNK1 crystal structure: The authors describe large, water-filled cavities in WNK1. From the figures, it appears that the analysis was conducted in the absence of nucleotide. Since some of the cavities seem to overlap with the active site of the enzyme, it would be important to consider how the cavity and water distribution would change in the native kinase in a cell that will likely be bound to ADP or ATP.

We agree with the reviewer that these cavities encompass the active site. However, the inactive form, uWNK1SA, does not bind ATP. Some discussion of the water structure in uWNK1SA

compared with canonical kinases is now included, see comment below to Reviewer 2, comment 5.

Minor point:
Page 3, 4th line from top: 'cotrasnporters' should be 'cotransporters'.
Thank you.

Reviewer #2 (Remarks to the Author):

This paper outlines a very simple concept regarding the WNK kinase, which regulates co-transporters and was shown previously to be inhibited by chloride. The authors here try to demonstrate that Wnk can be activated by osmotic pressure and attempt to correlate this with a monomer:dimer transition where the monomer is induced by phosphorylation of the activation loop. While this is an intriguing possibility the authors do not convincingly support the hypothesis with rigorous data.

Specific concerns:

1. A major piece of supporting data is the SAXS analysis, which is not convincing. The raw data needs to be included in Figure 3. Without this the figure is meaningless. How well does the data fit to the plots? The Kratky plots are also confusingl Once again there is no data, and they appear to be P(r) functions rather than Kratky plots. The inset says that this is a Kratky plot (pairwise distance distribution function); however, the Kratky plot and P(r) are actually different plots. The discussion of the SAXS data is superficial.

Please see the reply to Reviewer 1 points 3 and 4 as well as the large changes in text concerning SEC-SAXS. We thank both reviewers for pointing out that we needed to better analyze the SAXS data. As noted above, we have recollected the data, directly addressing the issue of PEG400 effects on uWNK3 structure. We have included new data in Figure 3 and Table S2 and present a more detailed data analysis.

2. The gel filtration data also shown in Figure 3 shows an obvious monomer following phosphorylation; however, the unphosphorylated protein, even though the dimer is prevalent, is clearly a mixture of aggregation states that include a monomer and dimer as well as higher molecular weight aggregates. Given the aggregation state of the sample, it is not convincing to show SAXS data for this complex.

Figure 3E has been replaced with Figure S2. As discussed above, the data originally submitted as Figure 3E was confounded by a lack of sufficient reducing agent. Please see response to Reviewer 1 Point 4, and the major changes to the text regarding gel filtration.

3. There needs to be quantitative stoichiometry data that shows that activation correlates with the single phosphorylation of the residue at the Activation Loop. This data should be included in Figure 1. Having an antibody to the activation loop phosphorylation would also be very helpful.

This paper concerns whether WNK autophosphorylation is influenced by crowders or osmolytes. We do not address whether the activity is specifically aligned with phosphorylation of Ser382 in WNK1 or Ser308 in WNK3.

However, the Cobb lab addressed the importance of specific phosphorylation sites in WNK1 activity and showed that S382 (equivalent to S308 in WNK3) is most important (Xu et al., 2002) and more important than S378 (S304 in WNK3).

Table S1 gives the mass spectrometric data for the WNK3 phosphorylation state before dephosphorylation and after rephosphorylation. After rephosphorylation, the primary activation loop phosphorylation site, S308, is ~80% phosphorylated, and the minor site, S304 is ~50% phosphorylated. The present paper does not address the state of phosphorylation of the activation loop, other than the data provided in Table S1. We added another reference on the WNK phosphorylation sites (Zagorska et al., 2007).

4. Is there a reason why WNK1 would not be as sensitive to osmotic pressure? Are there any obvious differences that might explain this?

Looking at the sequence, there isn't anything obvious to us. To our knowledge, this is the first paper on osmosensing in unphosphorylated WNKs. It is the first paper on the structural basis for osmosensing in any metazoan sensor, despite numerous papers on the identity of pathways and processes influenced by osmotic pressure. We hope to do a lot more on this interesting problem. Future projects are: 1) full length WNKs (quite challenging), 2) analysis of the osmotic sensitivities of all 4 human WNK isoforms, and 3) make mutants following structure-based hypotheses (and more).

Is WNK1 equally sensitive to chloride? Yes. We added a sentence in the results section referring to Figure S1E:

"WNK3 has similar sensitivity chloride as WNK1 in vitro (Terker et al., 2016)"

5. The hydration data is also not convincing. The proposed mechanism of osmotic sensitivity, while intriguing, seems to be very speculative. The authors base their model on the analysis of crystal structures and cavities filled with water. Obviously osmolytes can influence water bound to the kinases, but this does not sound like a solid molecular mechanism. Can this model be supported by computational data? There is very convincing computational analyses regarding water molecules at the active site of kinases (Setny, PNAS, 2018, 2013 and J. Chem. Theory Comput., 2015). Is this applicable for the WNK kinases? The position of the lysine is a very important part of Setny's model.

a. hydration data is also not convincing. We agree that the amount of water in the structure is subjective and reflects the extent of model-building. In contrast, cavity volumes, clusters of charges, and specific buried water molecules can be quatified . Therefore, we changed the order of presentation putting the paragraph on bulk water after the discussion of cavities, clusters, and specific bound water. Further, we added the sentences:

The additional waters over the 350 waters in the original refinement did not raise the R-free (0.22) and raised the overall B-factor only slightly from 24 to 27Å^2.

Then a few lines below:

Thus there appears to be more waters in uWNK1 than in proteins of comparable size (Carugo et al., 2017).

b. while intriguing, seems to be very speculative.
(1) We changed the heading "Molecular Mechanisms" to *"Cavities and conserved water."*
(2) We changed the sentence from "The structure of uWNK1 provides insights into mechanisms of osmosensing" to *"The structure of uWNK1 has unique features that may be involved in osmosensing."*

We have used the word “propose” throughout. At the bottom of page 7, in discussing buried waters in Cav 1A, we point out the conservation of these waters in Subunit B and in the Novartis structure of inactive WNK1 complexed with an inhibitor as follows:

The same ion pairs in Subunit A (Figures 5A, B) are present in subunit B and in the complex of WNK1SA with an inhibitor (5DRB, PDB file 5DRB) (Yamada et al., 2016b). Both Subunit B and 5DRB (a monomer) also have similar buried waters in this cavity (Cav1A).

c. There is very convincing computational analyses regarding water molecules at the active site of kinases (Setny, PNAS, 2018, 2013 and J. Chem. Theory Comput., 2015). Is this applicable for the WNK kinases

We thank the reviewer for pointing out this relevant paper, which referred to an even more relevant paper. We added sentences in the same place as above (Reviewer 2 Point 5b):

Water conservation in the active sites of canonical active protein kinases has been described, and reports two waters in positions similar to the WNK1SA ionic cluster {Knight, 2009 #6026}. Our W2 (shown in Figure 5C) overlaps with Dw defined by Knight et al., and W1 overlaps with an un-named water in PKA (PDB file 3fjq). The water structure is completely distinct from another conserved water cluster involved in cooperative substrate binding in PKA (Setny et al., 2018).

d. The model is speculative. Yes, our model is speculative. We have added reference to a Norma Allewell paper that considered similar ideas that water movement is involved in osmotic effects on enzymes with a phrase in the discussion:

aligns with studies of osmotic effects on enzymes (LiCata and Allewell, 1997).

6. Does the unusual position of the lysine, which is the reason for the naming of this kinase, have anything to do with making this kinase, as opposed to other kinases, sensitive to osmotic pressure?

We are sure that the lysine position is required for chloride recognition, which we explained in the Piala et al., 2014 paper. But otherwise, we do not have a clear model for Lys233 involvement in osmotic pressure sensing. We believe the main players are a cluster of charged amino acids extending from the catalytic loop and the activation loop. Again, this is a unique structure, and one that traps water. See reply to Reviewer 2 Comment 5. Lys233 is in an ion pair, however, so there may be a role for Lys233 in osmosensing. We did not have much to say here so we have not changed anything in response to this comment.

7. In general, the dimer structure is unusual and the authors do not speculate on this. The N-lobe of one kinase faces into the open active site of the other, and both are in almost identical inactive conformations. The dimer is not symmetrical and do they propose that this is a way of stabilizing two inactive dimers? The mechanism is not clearly defined and is highly speculative.

a. Describe the dimer. We agree with the reviewer that the dimer is interesting.
A new paragraph and Figure, 4C, have been added under the heading **Unique Features of the WNK1 dimer that may contribute to osmosensing:**

The WNK dimer interface is unique. The inactive dimer forms between the Activation Loop of one subunit and the N-terminal domain β-sheet of another subunit (Piala et al., 2014, Min et al., 2004). In the dimer, the Activation Loop (residues F379-S382A) of Subunit A becomes the 7th strand of a 7-stranded β-barrel, between strands β0 (residues V212-M214) and β4 (S289-V291) of Subunit B (Figure 4C). The β-barrel is irregular with fewer hydrogen bonds than normal. The

phosphorylation site (S382A, alanine in this structure) is trapped in the interface. Thus, this dimeric configuration is unlikely to support autophosphorylation.

The dimer interaction stabilizes large cavities which can be seen in surface representations (standard view Figure 4D, 180° about y, Figure 4E). Cav 1A contains a cluster of charges described below. Cav2A,B and Cav3A,B arise from displacement of helix C from the body of the kinase (Piala et al., 2014). Cav3A also makes a channel between the two subunits. In Figure 4E, the Subunit B β-barrel appears structurally isolated, with cavities on either side, separating it both from the C-terminal domain of Subunit B and from Subunit A.

b. The dimer is not symmetrical and do they propose that this is a way of stabilizing two inactive dimers? The mechanism is not clearly defined and is highly speculative.

Yes. We think the dimer is a mechanism for forming two inactive subunits and burying the phosphorylation sites. Another important role of the asymmetric dimer is to promote the formation of the very large cavity Cav 1A. The paragraph has changes throughout and now reads:

Unusual interactions span the largest cavity (Cav1A) between the Activation Loop and the Catalytic Loop (Figure 5A, alternative view in Figure S3B). K375, K381 and E388 in the Activation Loop make charged interactions with conserved catalytic residues, D349 and K351, forming an ionic cluster. Although the Cα's of E388 in the Activation Loop and K351 in the Catalytic Loop are 11Å apart, the side-chains are in contact through a water-mediated hydrogen-bonding network. In contrast, canonical active kinases lack a similar cavity because the backbones of the Activation Loop and Catalytic Loop contact each other (Figure S3C) (Taylor et al., 1999, Goldsmith et al., 2007). A similar but non-identical cluster is present in Subunit B (not shown). Charged residues in the Activation Loop that participate in the ionic cluster are conserved among WNKs, including K381 and E388.

8. What are the temperature factors for the crystal structure?

We added a sentence about B-factors in the water modeling paragraph on page 14:

The additional waters over the 350 waters in the original refinement did not raise the R-free (0.22 %) and raised the overall B-factor only slightly from 24 to 27Å^2.

RE: Manuscript #E20-01-0089R
TITLE: "Osmosensing by WNK kinases"

Dear Dr. Goldsmith,

As you can see the reviewers are split as to the solidity of your data to support your model for osmosensing for WNK kinases. Given the potential high novelty of the mechanism you propose, as Reviewer #2 also remarks, I would like to invite you to submit a second revision of the manuscript. Please make sure to address all the concerns raised by Reviewer #2 as well as some of those raised by Reviewer #3, especially the request for a more quantitative measure of the monomer/dimer equilibrium. The in vivo experiments are not necessary, although the text should state clearly the fact that the relevance of the dimer needs to be validated in cells and with the full-length construct. Also, please clarify how the contributions in this manuscript differentiate it from the study published in Biochemistry.

Sincerely,
Antonina Roll-Mecak
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Goldsmith,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires further revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office

mbc@ascb.org

Reviewer #2 (Remarks to the Author):

Overall, the revised manuscript addressed several points raised during the initial review. The changes improved the manuscript significantly. There are, however, several points concerning the revised data, which the authors should discuss and/or revise:

1. Page 9 and Table S2: The text states goodness of fit values of 8 and 47 for the crystallographic dimer and monomer of uWNK3, respectively. Table S2 states 8 and 29 for the same comparison. Please clarify.
Also, a goodness of fit of 8 is still fairly poor. Have the authors attempted modeling of the SAXS data using the crystallographic models as the input in an attempt to generate a more accurate structural interpretation of SAXS data? Likewise, the gel filtration experiments may suggest oligomeric mixtures - have the authors attempted to assess the presence of different oligomeric species based on the SAXS data (e.g., by using OLIGOMER in the ATSAS software package)? If the protein is present as a monomer-dimer mixture, the envelope modeling will likely be affected by it and hence be fairly inaccurate.

2. The observation that PEG400 destabilizes the uWNK3 fold could also indicate a less specific event where the PEG400 chemically denatures part of the structure, which could also lead to a higher activity of the catalytic domain. Whether such an effect is physiologically relevant is uncertain. Here, it is worth noting that not all crowding agents/osmolytes increase the activity of uWNK3 (see Figure 1B), suggesting the effect is not generalizable. Please discuss.

3. Figure S2: It is premature to conclude that uWNK3 forms an oligomeric mixture since the hydrodynamic radius of the protein can have an effect on its elution time. Likewise, it is conceivable that partial unfolding in ethylene glycol changes the radius without an effect on the protein's quaternary structure. Without a direct measurement of the molecular weight of the protein (and hence its oligomeric state), the interpretation of the SEC-SAXS and SEC data with regard to oligomerization are subjective and speculative.
Also, since PEG400 was used in the SEC-SAXS analysis and ethylene glycol in Figure S2, it is difficult to compare the two experiments.

Reviewer #3 (Remarks to the Author):

I find this revised manuscript to be a bit confusing and do not think that it provides sufficient new and valid information to warrant publication. A major concern from both reviewers in the initial review related to the SAXS data and many of these issues have been addressed. However, that is the main new method used in this paper to promote the dimer/monomer equilibrium and on its own, even with gel filtration, it is unconvincing - especially with regard to its physiological relevance. If there is really a reliable monomer/dimer equilibrium in this affinity range then the authors should be able to provide some quantitative Kd numbers. The system seems quite amenable to Surface Plasmon Resonance or even Analytical Ultracentrifugation. Even better would be to do BRET analysis of the full-length proteins in cells. I am not convinced that the dimer interface is physiological and crystallography certainly does not validate a dimer interface without some other validation. This is only the kinase domain and so is this physiologically relevant? I am not convinced. Do the full-length proteins form a dimer in cells? And if so do mutations at this interface break the dimer? Do mutations of key residues at the predicted dimer interface of the two kinase domains break the dimer? At the very least this should be tested.
The authors also need to clarify the novelty of this paper compared to the recent similar paper that they published in Biochemistry.
One of the points discussed by the authors in the rebuttal letter is the importance of having sufficient reducing reagent. This led me to think that the disulfide bonding might be extremely relevant and could at least identify surfaces that are in close proximity. In going back to compare the sequences of WNK1 and WNK3 and searching in particular for conserved cysteines it is striking to appreciate that there is actually a Cys in the Catalytic Loop and that the canonical Lys in beta strand 3 is actually replaced with a Cys. These are highly unusual and not likely to be just a coincidence. These regions are in close proximity in the structure. Is this a redox sensor? Where do the disulfide bonds form and is the dimer stabilized in the presence of oxidizing agents such as diamide?
The data on phosphorylation is more convincing with WNK3 and yet there is no correlation with specific sites. There are actually 4 putative phosphorylation sites in the activation loop. Which ones are phosphorylated? And can this be validated by mutagenesis? If this has already been done then it should be clarified. The induced phosphorylation of WNK1 is unconvincing while that data for WNK3 needs to be a bit more rigorous. I think it would also be extremely useful at least in the Supplement, to show the sequences of the active site regions of WNK1 and WNK3 and highlight the putative Serines and threonines in the Activation loop as well as the conserved Cysteines in the kinase core.
Even though the SAXS and phosphorylation data are most convincing for WNK3, the crystallization data is for WNK1. Clearly there are isoform differences, and these could be very important. With regard to the well-defined cavities is there any evidence that these simply provide docking sites for other motifs that lie in the other parts of the protein? The kinase is at the N-terminus,

which in itself is interesting as usually the kinase domain in most proteins is at the C-terminus, and these are relatively large proteins. Is there any evidence that in the full-length protein these cavity regions are not occupied by another part of the protein? Are there disease mutations that might shed light on this?
The figures are not even numbered. This is obviously a minor point.

Summary: Overall I think that this paper opens more questions than it answers. I am not convinced of the physiological relevance of the data, and I think that the data should be more rigorous. For these reasons I would not recommend publication.

Re: E20-01-0089R

Second Rebuttal letter to MBoC February 10, 2021

Responses to Monitoring Editor and Reviewers 2 and 3

General response: (My responses are in blue)

Described here are new data in this revision, clarifications of intellectual advances, relationships to data in our recently-published Biochemistry paper on phosphorylated WNKs, and consistency with concepts in the prior literature.

I. New data.

A) Most significant, and in support of our dimer-to-monomer transition model for osmosening by unphophosphorylated WNKs, we have added static light scattering data (SLS) on uWNK3 in the absence and presence of the osmolyte ethylene glycol. These data clearly show a shift in molecular weight from a dimer to a mixture of dimer and monomer in osmolyte. The SLS data also informed on dimer/monomer Kd, as the uWNK3 concentration that is 50/50 dimer/monomer. Chad Brautigam collected this data and has been added to the author list. The SLS data is superior to the gel filtration data we included originally. We eliminated the gel filtration data.

B) As suggested by Reviewer 3, we analyzed the dimer/monomer fraction in the SAXS scattering curves using OLIGOMER, and obtained the fraction of each in the presence and absence of PEG400.

C) We improved χ^2 to 2 between the scattering curve and the crystallographic dimer model of uWNK1 by collecting new SAXS data in the absence of PEG400, and by using a new version of CRYSOL, CRYSOL3, which explicitly models water into cavities (rather than generating envelopes).

II. Intellectual advances.

A) WNKs have been shown previously to auto-phosphorylate in vitro. This is the first demonstration that WNKs or any Ser/Thr protein kinases, exhibit osmotic stress enhanced auto-phosphorylation in vitro. (Many studies report on osmotic stress activation of kinases in cells.) As is customary, the in vitro osmotic stress was induced using osmolytes or crowding agents. Not all of the agents tested enhanced autophosphorylation, however.

B) This is the first demonstration of an overall mechanism for osmotic-stress induced autophosphorylation in any Ser/Thr kinase, namely, de-dimerization. (Additional SLS data has been collected and included that strengthens the concept that osmotic stress induces de-dimerization, as noted above.)

C) Our crystallographic evidence reveals changes in bound water between inactive and active forms of WNK1. Thus, we are able to propose a meaningful molecular mechanism for osmotic stress induced autophosphorylation for the first time. Namely, there is a conformational equilibrium between structures differing in hydration, with osmotic stress favoring the more dehydrated structure. Our structural data is much more interpretable than the crystallographic

and NMR data available on osmosensing histidine kinases. We now better-state the concepts and data available from studies of histidine kinases in the introduction.

III. Relationship to Biochemistry paper on pWNKs

Our recently published Biochemistry paper on phosphorylated WNK1 (Akella *et al.*, 2020) makes none of the points above, and instead concerns the effects of osmolytes and crowding agents on the structure of the phosphorylated form of WNKs. The structural changes presented in the Biochemistry paper suggest multistage activation of WNKs. The present paper was submitted before the Biochemistry paper, and is more conclusive on potentially significant WNK activation mechanisms.

IV. Consistency with concepts in the prior literature

Our results are consistent with two concepts in the literature concerning kinase signaling and osmosensing.

A) Brian Crane (Airola *et al.*, 2013) has proposed the general concept of conformational equilibrium in signaling through histidine kinases. Conformational equilibrium is a key feature of our proposed mechanism for osmosensing by WNKs.

B) Norma Allewell (LiCata and Allewell, 1997) interpreted the effects of osmolytes on a conformational equilibrium between two differentially hydrated states in aspartate transcarbamylase. Similar ideas were used to interpret the effects of polyethylene glycols on hexokinase (Reid and Rand, 1997). This idea is used here.

Please note the rewritten Introduction, new sentences in italics, better describing our motivation for this study. For example, we discuss the 30 year old hypothesis of volume-regulated “V”-kinases, now accepted to be WNKs.

Specific replies to review comments:

Responses to Monitoring Editor

As you can see the reviewers are split as to the solidity of your data to support your model for osmosensing for WNK kinases. Given the potential high novelty of the mechanism you propose, as Reviewer #2 also remarks, I would like to invite you to submit a second revision of the manuscript. Please make sure to address all the concerns raised by Reviewer #2 as well as some of those raised by Reviewer #3, especially the request for a more quantitative measure of the monomer/dimer equilibrium.

The new static light scattering data allow us to approximate dimer equilibrium in the absence (~0.8 mg/ml, 20 μM) and presence (~2 mg/ml, 50 μM) of osmolyte.

We anticipate a follow-up paper that focuses on WNK oligomeric equilibria and its effectors (osmotic pressure, chloride, and now potassium (just published: (Pleinis *et al.*, 2021)).

The in vivo experiments are not necessary, although the text should state clearly the fact that the relevance of the dimer needs to be validated in cells and with the full-length construct.

A paragraph has been added to the conclusion: Oligomerization of WNKs has been observed in HEK293 cells and other cells (Lenertz *et al.*, 2005), data indicating high molecular weight complexes. How our concept of a dimer to monomer equilibrium involving interactions of the kinase domains relates to these higher order assemblies requires further study. The conformational regulation of WNKs is no doubt highly complex, given the oligomeric size observed in vivo, and the presence of multiple protein-protein interaction domains (Dbouk *et al.*, 2016; Shekarabi *et al.*, 2017).

Also, please clarify how the contributions in this manuscript differentiate it from the study published in Biochemistry.

This is discussed in the General Response.

Reviewer #2

1. Page 9 and Table S2: The text states goodness of fit values of 8 and 47 for the crystallographic dimer and monomer of uWNK3, respectively. Table S2 states 8 and 29 for the same comparison. Please clarify.

Thank you for finding this typographical error in the text. The text has been modified to match the Table. The χ^2 values for uWNK3 in the absence of PEG400 also improved.

Also, a goodness of fit of 8 is still fairly poor. Have the authors attempted modeling of the SAXS data using the crystallographic models as the input in an attempt to generate a more accurate structural interpretation of SAXS data? Likewise, the gel filtration experiments may suggest oligomeric mixtures - have the authors attempted to assess the presence of different oligomeric species based on the SAXS data (e.g., by using OLIGOMER in the ATSAS software package)? If the protein is present as a monomer-dimer mixture, the envelope modeling will likely be affected by it and hence be fairly inaccurate.

These comments were helpful. We recollected the SAXS data. A better Goodness of Fit of 2 was obtained for the uWNK3 dimer (now in Table S3). This improvement comes from better data, and the use of the CRYSOL3 module of ATSAS. CRYSOL3 incorporates an explicit model for bound water and cavities (rather than envelope-based CRYSOL) —appropriate for cavity-rich uWNKs.

The reviewer also suggested OLIGOMER, software that tests ratios of two known conformers against scattering data. OLIGOMER gave a dimer/monomer ratio of 70/30 for the new uWNK3 data and 40/60 for uWNK3 in PEG400 based on the scattering curves. We think the improved goodness of fit to the recollected SAXS data and the 70/30 ratio of dimer to monomer estimated by OLIGOMER justify presenting the superposition of the ab initio envelope generated by DAMMIF and the crystallographically derived uWNK1 dimer.

2. The observation that PEG400 destabilizes the uWNK3 fold could also indicate a less specific event where the PEG400 chemically denatures part of the structure, which could also lead to a higher activity of the catalytic domain. Whether such an effect is physiologically relevant is uncertain. Here, it is worth noting that not all crowding agents/osmolytes increase the activity of uWNK3 (see Figure 1B), suggesting the effect is not generalizable. Please discuss.

Our data do not prove physiological relevance. Very few papers describe the effects of crowding agents to induce autophosphorylation in biological signaling molecules, with the work from the

Kenney group on histidine kinases a notable exception (Wang *et al.*, 2012). We were happy to identify two agents that induce autophosphorylation in vitro. (The idea that WNKs are activated by osmotic stress in cells derives from a history of papers postulating the existence of a volume-regulated protein kinase (V-kinase) to account for the osmotic stress activated and chloride inhibited phosphorylation of NKCCs. The further insight that a familial form of hypertension is linked to WNKs and is treated with antihypertensive agents targeting NKCCs made WNKs likely candidates for the V-kinase. Those discoveries prompted us to pursue the present study. The link of WNKs to NKCC regulation is now better discussed in the introduction.)

We cannot fully interpret the actions of PEG400 on uWNK3, but we do see short distances in the pairwise distribution function in the presence of PEG400 (Figure 3D), which, as with the Kratky plot, indicates denaturation or unfolding as suggested by the reviewer. But our claims are minimal in saying that PEG400 does something to the structure of WNK3. We have added a sentence “uWNK3 in the presence of PEG400 exhibit short distances in the P(r) curve (Figure 3D) and a Kratky plot that diverges at high **s**, both indicators of some unfolding.“

We hope to obtain structural data in the future on effects of osmolytes and crowders on uWNKs, as we did for pWNKs (Akella *et al.*, 2020).

3. Figure S2: It is premature to conclude that uWNK3 forms an oligomeric mixture since the hydrodynamic radius of the protein can have an effect on its elution time. Likewise, it is conceivable that partial unfolding in ethylene glycol changes the radius without an effect on the protein's quaternary structure. Without a direct measurement of the molecular weight of the protein (and hence its oligomeric state), the interpretation of the SEC-SAXS and SEC data with regard to oligomerization are subjective and speculative.

We have added new static light scattering (SLS) data collected in buffer and in ethylene glycol. The SLS data gives a direct measure of molecular weight. These data clearly indicate a shift from a dimer in the absence of ethylene glycol to a dimer-monomer mixture in the presence of ethylene glycol. Given the high quality of the SLS data, we decided to eliminate the gravity gel filtration originally included in the Supplemental Materials. The SEC profile from the uWNK3 SEC-SAXS data also shows a shift to higher retention in PEG400, indicating a lower molecular weight.

Also, since PEG400 was used in the SEC-SAXS analysis and ethylene glycol in Figure S2, it is difficult to compare the two experiments.

PEG400 would interfere with SLS, so we used osmolytes (originally identified in assays to find an autophosphorylation inducing osmolyte, Figure 1B). We agree that the PEG SEC-SAXS and ethylene glycol SLS data are not completely apples to apples. However, they belong together in the same paper because they are two different chemical agents inducing autophosphorylation and changing the uWNK dimer/monomer equilibrium.

Reviewer #3 (Remarks to the Author):

I find this revised manuscript to be a bit confusing and do not think that it provides sufficient new and valid information to warrant publication.

Please see the general comments above that outline the advances presented in this paper. We demonstrate the phenomenon of osmotic activation in vitro, present a plausible mechanism, de-

dimerization, and present a molecular level structure, showing changes in hydration, and show how the kinase is organized to be sensitive to osmotic pressure.

A major concern from both reviewers in the initial review related to the SAXS data and many of these issues have been addressed. However, that is the main new method used in this paper to promote the dimer/monomer equilibrium and on its own, even with gel filtration, it is unconvincing - especially with regard to its physiological relevance. If there is really a reliable monomer/dimer equilibrium in this affinity range then the authors should be able to provide some quantitative Kd numbers.

New SLS data allows Kd's to be estimated. SLS data on uWNK3 gives a 50/50 mixture at about 0.8 mg /ml (20 microM). SLS data collected in the presence of ethylene glycol gives a 50/50 mixture at 2 mg/ml (50 microM).

This is the first paper studying effects of osmolytes/crowding agents on uWNKs in vitro. A potential mechanism for these effects in WNKs, namely a dimer to monomer transition, is presented. Future studies will address variables affecting the equilibrium, and will study this in the context of mechanism-inspired mutants (eliminating charges described in Figure 5), and more complex full length WNKs.

The system seems quite amenable to Surface Plasmon Resonance or even Analytical Ultracentrifugation.

We have available to us SLS, and have used that to demonstrate a change in oligomeric state in uWNK3 in the presence of ethylene glycol. Further, we used OLIGOMER to analyze the SAXS scattering profile as suggested by Reviewer 2 and discussed above. SLS and OLIGOMER clearly demonstrate a change in the dimer/monomer ratio in ethylene glycol or PEG400. We do not have SPR. We collected analytical ultracentrifugation data. The data indicate a monomeric species for uWNK3 at the high pressures present in the AUC experiment. These data are to be published elsewhere.

Even better would be to do BRET analysis of the full-length proteins in cells. I am not convinced that the dimer interface is physiological and crystallography certainly does not validate a dimer interface without some other validation. This is only the kinase domain and so is this physiologically relevant? I am not convinced. Do the full-length proteins form a dimer in cells? And if so do mutations at this interface break the dimer? Do mutations of key residues at the predicted dimer interface of the two kinase domains break the dimer? At the very least this should be tested.

Please note the response to the Editor. We have added a sentence in the introduction pointing to a paper describing oligomerization of WNKs in cells (Lenertz *et al.*, 2005). We have added a paragraph to the conclusion section on the fact that the conformational regulation of WNKs is likely to be highly complex. This is our second paper on effects of osmotic pressure on WNKs, and we anticipate more, especially to make mechanism based mutants and to analyze full length WNKs. Please note the General Response section on intellectual advances.

The authors also need to clarify the novelty of this paper compared to the recent similar paper that they published in Biochemistry.

This is discussed in the General Response.

One of the points discussed by the authors in the rebuttal letter is the importance of having sufficient reducing reagent. This led me to think that the disulfide bonding might be extremely relevant and could at least identify surfaces that are in close proximity. In going back to compare the sequences of WNK1 and WNK3 and searching in particular for conserved cysteines it is striking to appreciate that there is actually a Cys in the Catalytic Loop and that the canonical Lys in beta strand 3 is actually replaced with a Cys. These are highly unusual and not likely to be just a coincidence. These regions are in close proximity in the structure. Is this a redox sensor? Where do the disulfide bonds form and is the dimer stabilized in the presence of oxidizing agents such as diamide?

Testing WNKs as potential redox sensors is an intriguing idea. However, this is outside the scope of this paper.

The data on phosphorylation is more convincing with WNK3 and yet there is no correlation with specific sites. There are actually 4 putative phosphorylation sites in the activation loop. Which ones are phosphorylated? And can this be validated by mutagenesis? If this has already been done then it should be clarified.

The LC-MS phosphorylation site data is presented in Table S1. This manuscript presents all of the phosphorylation sites we observed in pWNK3, both as it comes out of cells and on uWNK3 re-phosphorylation (autophosphorylation), including the sequences of the peptides being phosphorylated. Table S1 is discussed in the first paragraph in the Results and Discussion. Only two activation loop sites are phosphorylated. We added a phrase to the discussion of Table S1 as follows: *"as well as a second site in the activation loop (S378 in WNK1 and S304 in WNK3)."*

Multiple papers have addressed WNK phosphorylation site mass spectrometry and mutagenesis, finding multiple sites and their role in WNK activation via mutagenesis (Xu *et al.*, 2002; Zagorska *et al.*, 2007; Thastrup *et al.*, 2012).

The induced phosphorylation of WNK1 is unconvincing while that data for WNK3 needs to be a bit more rigorous.

We agree that the effects are small, but the significance is indicated, and we think it is worthwhile including these gels because they offer a visual for phosphorylation.

I think it would also be extremely useful at least in the Supplement, to show the sequences of the active site regions of WNK1 and WNK3 and highlight the putative Serines and threonines in the Activation loop as well as the conserved Cysteines in the kinase core.

The sequences around the sites of phosphorylation are presented in Table S1. The topic of conserved cysteines is outside the scope of this paper.

Even though the SAXS and phosphorylation data are most convincing for WNK3, the crystallization data is for WNK1. Clearly there are isoform differences, and these could be very important.

The sentence "WNK1 and WNK3 exhibit 94% sequence identity in their kinase domains (Xu *et al.*, 2000; Verissimo and Jordan, 2001)" has been added to the introduction in support of using the WNK1 structural model to compare to the SAXS data.

With regard to the well-defined cavities is there any evidence that these simply provide docking sites for other motifs that lie in the other parts of the protein?

No.

The kinase is at the N-terminus, which in itself is interesting as usually the kinase domain in most proteins is at the C-terminus, and these are relatively large proteins. Is there any evidence that in the full-length protein these cavity regions are not occupied by another part of the protein?

These questions have not been studied to our knowledge. We are presently attempting to express full length WNKs from lower organisms to address osmotic effects in the context of full length proteins and hopefully obtain structural information.

Are there disease mutations that might shed light on this?

Disease mutations have been extensively studied and reviewed, and so far none have been identified in the kinase domain (Shekarabi *et al.*, 2017).

The figures are not even numbered. This is obviously a minor point.

We are unclear what is wanted here. We think we followed the instructions.

Summary: Overall I think that this paper opens more questions than it answers. I am not convinced of the physiological relevance of the data, and I think that the data should be more rigorous. For these reasons I would not recommend publication.

As noted in the General Response, this paper put forth several advances, 1) direct in vitro osmotically enhanced autophosphorylation of a soluble Ser/Thr kinase, 2) a proposed overall mechanism of a conformational equilibrium between an inactive dimer and auto-phosphorylation competent monomer, and 3) a crystallography based near-atomic level mechanism showing osmotic pressure favors a more dehydrated state for WNKs. We are aware that these studies need to be validated in the context of full-length proteins and in cells. See Responses to Monitoring Editor.

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RE: Manuscript #E20-01-0089RR
TITLE: "Osmosensing by WNK kinases"

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Monitoring Editor
Molecular Biology of the Cell

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