Identification of the WNK-SPAK/OSR1 Signaling Pathway in Rodent and Human Lenses

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Purpose. To identify whether the kinases that regulate the activity of cation chloride cotransporters (CCC) in other tissues are also expressed in rat and human lenses.

Methods. The expression of with-no-lysine kinase (WNK 1, 3, 4), oxidative stress response kinase 1 (OSR1), and Ste20-like proline alanine rich kinase (SPAK) were determined at either the transcript or protein levels in the rat and human lenses by reverse-transcriptase PCR and/or Western blotting, respectively. Selected kinases were regionally and subcellularly characterized in rat and human lenses. The transparency, wet weight, and tissue morphology of lenses extracted from SPAK knock-out animals was compared with wild-type lenses.

Results. WNK 1, 3, 4, SPAK, and OSR1 were identified at the transcript level in rat lenses and WNK1, 4, SPAK, and OSR1 expression confirmed at the protein level in both rat and human lenses. SPAK and OSR1 were found to associate with membranes as peripheral proteins and exhibited distinct subcellular and region-specific expression profiles throughout the lens. No significant difference in the wet weight of SPAK knock-out lenses was detected relative to wild-type lenses. However, SPAK knock-out lenses showed an increased susceptibility to opacification.

Conclusions. Our results show that the WNK 1, 3, 4, OSR1, and SPAK signaling system known to play a role in regulating the phosphorylation status, and hence activity of the CCCs in other tissues, is also present in the rat and human lenses. The increased susceptibility of SPAK lenses to opacification suggests that disruption of this signaling pathway may compromise the ability of the lens to control its volume, and its ability to maintain its transparency.

Keywords: lens, cell volume regulation, cataract, cation chloride cotransporters, WNK, SPAK, OSR1SPAK knock out

While lens transparency is the result of its unique tissue architecture, any failure to maintain the pseudocrystalline packing of cortical fiber cells, by either cellular swelling or dilation of the normally tight spaces between the cells, increases intralenticular light scattering. Therefore, maintaining fiber cell volume is critically important for the overall maintenance of lens transparency.[1,2] Evidence is accumulating that the lens operates an internal microcirculation of ions[3] that results in a circulating water flux that is directed into the lens at its poles and out at its equator.[4,5] This constant circulating fluid flux would make the lens susceptible to changes in cell volume. Therefore, a tightly regulated system for maintaining the volume of individual cells must be in place to preserve overall steady-state lens volume, and therefore transparency. Under isotonic conditions, it has been shown that the lens has a constitutively active Cl^- flux, which actively maintains fiber cell volume to preserve overall tissue transparency.[6,7] Furthermore, lenses placed in either hypotonic or hypertonic conditions are capable of modulating this Cl^- flux to restore lens volume either via a regulatory volume decrease (RVD), or regulatory volume increase (RVI), respectively.[8]

Histologic analysis of lenses organ cultured under isotonic conditions in the presence of a variety of Cl^- transport inhibitors revealed that blocking Cl^- transport induces distinctly different types of damage to fiber cells located at the lens periphery and deeper cortex,[6,9,10] indicating that distinct ion influx and efflux zones exist in the lens cortex that are driven by a reversal of the electrochemical gradient for Cl^{-1}. Rat lenses cultured in the presence of either the Cl^- channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB),[6] or the Na-K-Cl Cotransporter (NKCC) blocker bumetanide,10 exhibited a localized band of tissue damage that manifested as extracellular fluid accumulations between fiber cells located approximately 150 μm in from the lens capsule. In contrast, culturing rat lenses in the K-Cl Cotransporter (KCC) inhibitor [(dihydroninden-yl)oxy] alkanoic acid (DIOA) resulted in swelling of fiber cells located at the lens periphery, although some deeper extracellular space dilations were evident.[9] The two spatially distinct phenotypes observed from pharmacological experiments indicate that NKCC, KCC, and Cl^- channels all mediate ion uptake in the deeper cells, while KCC primarily mediates net ion efflux in peripheral fiber cells. Molecular experiments designed to identify the specific proteins responsible for Cl^- influx and efflux have shown that NKCC1 Cotransporter (NKCC1) is expressed in the rabbit[11] and rat lens,[10] while the

KCC isoforms, KCC1, 3, and 4 are expressed in the rat lens[9] and KCC1 and 4 in human lens epithelial cells.[12]

In a variety of cell types it has been shown that the activity of KCC and NKCC are reciprocally regulated through changes in their phosphorylation status with phosphorylation activating NKCC and inactivating KCC.[13–17] Recently, the kinases and phosphatases responsible for modulating the KCC and NKCC phosphorylation status have been identified. The key kinases include selected members of the with-no lysine kinase family (WNK 1, 3 and 4), the STE20-related proline alanine-rich kinase (SPAK, or the rat homologue PASK) and the oxidative stress response kinase 1 (OSR1).[14,18,19] WNK1 phosphorylates and activates SPAK and OSR1,[15,20–23] which in turn phosphorylate NKCC[24–26] and KCC.[27] Of the different WNK family members, WNK1 has been suggested to be a true osmosensor,[14] which can be activated by both hypertonicity[28,29] and to a lesser extent by hypotonicity.[15,21,29] WNK4 can also activate SPAK/OSR1,[20,24] while WNK3 has been shown to directly phosphorylate NKCC and KCC.[30,31] Interaction between WNK isoforms has been suggested,[29,32,33] as well as effects of SPAK/OSR1 on the WNKs.[32] In contrast, the protein phosphatases, PP1 and PP2A, have been shown to directly dephosphorylate NKCC and KCC leading to inactivation of NKCC[34–37] and activation of KCC.[17,31,38–40] PP1 can also indirectly affect the NKCC/KCC phosphorylation by inhibiting SPAK.[27,35]

While the phosphatases PP1 and PP2A are known to be actively expressed in rodent and human lenses,[41–44] less is known about the regulatory kinases. However, proteomic analysis has identified SPAK in the mouse,[45] and more recently the human lens membrane proteome.[46] In this paper, we have used a combination of RT-PCR and Western blotting to show that the regulatory kinases previously identified in other tissues to modulate KCC and NKCC phosphorylation status also exists in the rat lens. Furthermore, as a first step toward translating our work in rats to humans, we have localized these kinases in young human donor lenses. Lastly, having obtained a small number of SPAK knock-out mice, we were able to characterize the effect that knocking out SPAK has on the transparency of the lens. Taken together our results show that the WNK-SPAK/OSR1 signaling pathway exists in both rat and human lenses, and that deletion of SPAK renders the mouse lens more susceptible to opacity.

Materials and Methods

Animal and Human Tissue Collection

Twenty-one-day-old Wistar rats and 25-day-old wild-type (C57BL6) and homozygous SPAK knock-out mice[47] were euthanized in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyes were excised and lenses extracted from the globe and placed in PBS. Positive control tissues from rat brain and kidney were also obtained. Rat tissue for RNA extraction and protein purification were processed as described below. Human lenses from donors ranging in age from 16 to 82 years were collected by the New Zealand National Eye Bank in compliance with the Declaration of Helsinki using protocols approved by the Northern X Regional ethics committee (ref: NTX/07/08/079). Lenses were obtained within 72 hours post mortem and were homogenized for protein preparation as described below.

Reagents and Antibodies

The primary antibodies, and their corresponding antigenic peptides (where available), used for Western blot analysis in rat and/or human lenses are described in Supplementary Table S1.[76] Secondary antibodies used for chemiluminescent detection were donkey anti-rabbit IgG-HRP (Amersham ECL, Buckinghamshire, UK); rabbit anti-goat IgG-HRP (ZyMAX, Carlsbad, CA, USA); and sheep anti-mouse IgG-HRP (Amersham ECL). The membrane marker WGA-Alexa Fluor 594 was purchased from Life Technologies (Carlsbad, CA, USA). Phosphate buffered saline was prepared from PBS tablets (Sigma Chemical Company, St. Louis, MO, USA). Unless otherwise stated, all other chemicals were purchased from Sigma.

Reverse-Transcriptase PCR

Total RNA was isolated from rat tissues using Trizol (Gibco, New York, NY, USA) according to the manufacturer's protocols. Genomic DNA was removed from the total RNA before cDNA synthesis by a 20-minute preincubation at 20°C with 0.1 U/μL DNase I (Roche Molecular Biochemicals, Basel, Switzerland). Messenger RNA was purified using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotechnology, Piscataway, New Jersey, USA). First strand synthesis and cDNA amplification were performed with a ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA) using established protocols.[48] Amplification was performed using specific primers designed to span intron–exon borders and PCR parameters (Supplementary Table S2), with a final 10-minute period of extension at 72°C to optimize ligation conditions. Polymerase chain reaction products were run on a 0.8% agarose gel by electrophoresis and bands visualized using a Gel Doc 2000 Gel Imaging system (Bio-Rad Laboratories Ltd., Hercules, CA, USA). Bands were excised and DNA purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and submitted for sequencing to confirm PCR product identity.

Protein Fractionation

Lens protein preparations were obtained from rat, mouse, and human donor lenses using established protocols.[48,49] Rat, mouse, and human lenses were dissected out of the eye, capsule and adhering epithelial cells were separated from the remaining fiber cell mass, and both were homogenized in homogenizing solution (5 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, pH 8.0; cOmplete Protease Inhibitor Cocktail; Roche, Molecular Biochemicals) and then pelleted by centrifugation at 16,100*g* for 20 minutes. The supernatant contained the lens epithelial soluble (ES) fraction and lens fiber soluble (FS) fraction, while the pellet was washed two more times in storage solution (5 mM Tris, pH 8.0, 2 mM EDTA, 2 mM EGTA, cOmplete Protease Inhibitor Cocktail (Roche, Molecular Biochemicals) then resuspended in storage solution and retained as the lens epithelial membrane (EM) and lens fiber membrane (FM) fraction. To separate integral membrane proteins from peripheral membrane proteins in rat preparations, FM fractions were resuspended in a urea/alkaline stripping solution (5 mM EDTA, 5 mM EGTA, 5 mM Tris-HCl pH9.5, 4-M urea) and then centrifuged at 16,100*g* for 20 minutes. The supernatant was retained as the peripheral protein (PP) fraction and the pellet washed three times in 20 mM NaOH to strip away peripheral proteins and retain integral membrane proteins that were resuspended in storage buffer. This fraction is referred to as the stripped membrane (SM) fraction. Mouse protein preparations were pooled from four decapsulated lenses. Rat lens membrane proteins from the epithelium, outer cortex, inner cortex, and core regions were prepared from 10 to 15 decapsulated rat lenses pooled together. The lens epithelium remained adhered to the lens capsule so was isolated by decapsulation. The superficial layers of rat lens fiber cells were peeled away and pooled as the outer

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cortex fraction. The remaining inner cortical fiber cells were removed and retained as the inner cortex fraction. The remaining mass of tissue was retained as the core fraction. All fractions were homogenized, washed, and then prepared as either FS or FM fractions as outlined above.

For protein preparations from human donor lenses, lenses were decapsulated and the remaining fiber mass homogenized in homogenizing solution, and then centrifuged at 16,100*g* for 20 minutes at 4°C. The supernatant comprising of both soluble and insoluble proteins was processed as above. Proteins from the outer cortex, inner cortex, and core were prepared using a microscope and a pair of sharpened tweezers and dissected into distinct zones based on their physical properties. The superficial layers of fiber cells comprising of cortical differentiating fiber cells were peeled away and pooled as the outer cortex fraction. The remaining inner cortical fiber cells encompassing cortical cells in the remodelling and transition zones[50] were removed and pooled as the inner cortex fraction. The remaining hard mass of tissue that included the adult, juvenile, fetal, and embryonic nucleus[51] was retained as the core fraction. All three fractions were processed as for rat lenses. The concentration of lens proteins was determined using the BCA detection kit (Pierce, Rockford, IL, USA).

Western Blotting

Proteins (~100 μg) were separated using either 10% (SPAK and OSR1), 7.5% (WNK1 and 4) sodium dodecyl sulphate polyacrylamide gels, or Mini-PROTEAN TGX Precast Gradient Gels (4-15%) using a Mini PROTEAN Tetra cell system (Bio-Rad). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and total protein stain Ponceau S was used to ensure successful protein transfer and equal loading. Membranes were then incubated in blocking solution (5% non-fat milk or 2% ECL advanced blocking solution) for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies diluted in blocking solution (Supplementary Table S1).[76] Peptide control experiments for WNK1, WNK4, SPAK, and OSR1 followed identical procedures except that the primary antibodies were incubated with at least 10- to 50-fold excess of their antigenic peptide for 1 to 2 hours at 37°C, followed by overnight incubation at 4°C with gently rocking or rotating. The complexes were then pelleted by centrifuging at 16,100*g* for 15 minutes and the supernatant added to the membranes. Following washes in TBS/Tween, membranes were incubated in secondary antibodies (1:8000–1:15,000) for 1 hour at room temperature. After incubation, membranes were washed in TBS/Tween and labeled proteins visualized using chemiluminescence (ECL TM Plus, Advance, or Prime all obtained from GE Healthcare Life Sciences, Buckinghamshire, UK) and developed using a LAS Fujifilm 3000 or 4000 system (GE Healthcare Life Sciences, Buckinghamshire, UK).

Analysis of SPAK Knock-Out Lenses

Lenses from 20 wild-type and SPAK knock-out animals were obtained during a visit to the Delpire laboratory at Vanderbilt. Images of the external eye were taken within 1 hour of euthanizing the mice using a digital camera (Fujifilm ×10; Fujifilm, Tokyo, Japan). Eyes from both groups of animals were enucleated and weighed before the lenses were extracted from the globe and placed in prewarmed PBS. Lens transparency was then accessed by dark-field microscopy. Lenses were removed from PBS using a glass loop, placed over tissue paper to remove excess fluid, and weighed on parafilm. Lenses were then immediately processed for either protein preparation or fixed using 0.75% paraformaldehyde-PBS for 24 hours and cryoprotected by incubating lenses in 10%, and then 20% sucrose-PBS for 1 hour, followed by an overnight incubation in 30% sucrose-PBS at 4°C.[52] Lens homogenates and fixed lenses were shipped to Auckland for further processing. Western blotting was performed as described above for rat lenses. Fixed lenses were mounted in an equatorial orientation onto chucks using optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA), cryosectioned and 14- to 18-μm-thick sections collected on glass slides. Sections were washed three times in PBS. To visualize the morphology, sections were incubated with Alexa Fluor WGA-594 diluted in PBS (1:100) for 1.5 to 2 hours at room temperature, washed, and mounted in CITIFLUOR (Agar Scientific, Essex, UK) and imaged using a Leica TCS SP2 upright confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). Images were combined using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA, USA). The number of cells contained within a 100-μm region of a fiber cell column were counted in two different areas within a single equatorial section from either control or SPAK knock out lenses. The results from three lenses from each group were pooled and expressed as the mean number of cells/100 μm ± SEM. Statistical analysis was carried out with GraphPad InStat (GraphPad Software, Inc., La Jolla, CA, USA) using the Mann-Whitney *U* test comparison with 95% confidence.

Results

Since the complete WNK-SPAK/OSR1 signaling pathway has not been previously identified in the lens, we first sought to establish a molecular profile of their expression at the transcript level in the rat lens before localizing them at the protein level using Western blotting. Since the majority of our antibodies were raised against human proteins (Supplementary Table S1),[76] we took the opportunity to translate our work in rats into humans by localizing the proteins in donor human lenses. Finally, because our experiments relied on the specificity of the antibodies used, we took advantage of a SPAK knock-out mice to verify specificity of a least one class of antibodies used in this study. Although we had access to a limited number of animals, the SPAK knock-out lenses also provided some preliminary evidence for the functionality of the WNK-SPAK/OSR1 signaling pathway in the lens.

Identification of Regulatory Kinases in the Rat Lens

To first determine if WNK1, WNK3, WNK4, SPAK, and OSR1 mRNA is expressed in the rat lens, reverse-transcriptase PCR was used in combination with specifically designed primers to amplify mRNA from rat lenses (L), while rat brain (B), or kidney (K) were used as positive controls. Polymerase chain reaction products for WNK1, WNK3, WNK4, SPAK, and OSR1 of the predicted size (Supplementary Table S2) were obtained from brain and kidney mRNA (Fig. 1). A high yield of PCR products were amplified from the brain for all kinases, except OSR1, which produced a stronger product from kidney. Polymerase chain reaction products for WNK1, WNK3, WNK4, SPAK, and OSR1 were all detected in lens fiber mRNA (L). All PCR products were sequenced and found to correspond to their respective sequence contained in GenBank, thereby establishing all five regulatory kinases to be present at the transcript level in the rat lens. Two additional PCR products were obtained for OSR1, a band at approximately 900 bp and approximately 1000 bp. Sequencing revealed that both bands corresponded to OSR1, indicating that OSR1 also exists in two longer forms in the rat lens. In all reactions, there were no PCR

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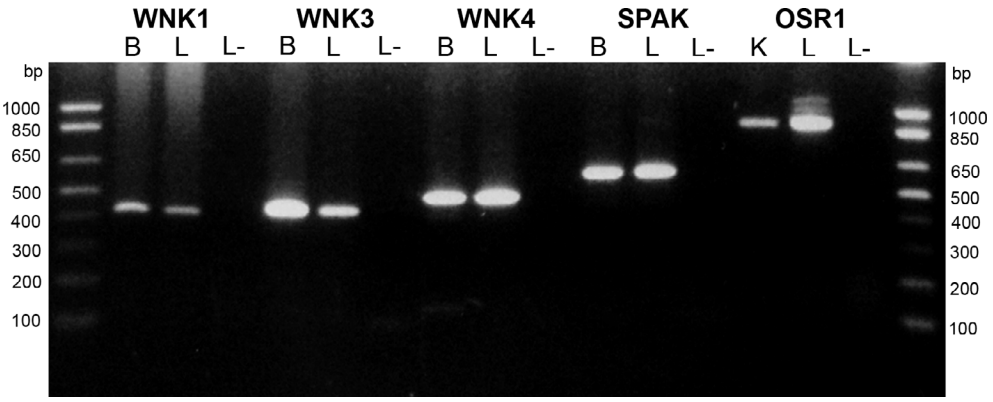

Figure 1. Identification of volume regulatory kinase transcripts in the rat lens by reverse transcriptase. PCR Agarose gel showing that WNK1, 3, 4, SPAK, and OSR1 are expressed at the transcript level in the rat lens. Single RT-PCR products for WNK1 (416 bp), WNK3 (440 bp), WNK4 (493 bp), and SPAK (610 bp) were amplified from brain (B) and lens fiber mRNA (L) using kinase specific primers. A 902 bp RT-PCR product was amplified from kidney (K) and lens fiber mRNA (L) using OSR1 primers. Two additional bands were also detected for OSR1 in lens fiber mRNA (L) approximately 900 and 1000 bp. No PCR products were seen in a control reaction using fiber cell mRNA in which reverse transcriptase was omitted (L–). A 1-kb DNA ladder is shown in the first and last lanes.

products seen in the negative controls (L–), in which the reverse transcriptase was omitted.

To confirm the expression of the regulatory kinases at the protein level, Western blotting was performed. Unfortunately, because no commercial antibodies were available to analyze WNK3 protein expression in the lens, we restricted our study to determining whether we could detect WNK1, WNK4, SPAK, and OSR1 at the protein level by Western blotting.

A review of the literature revealed that Western blotting for the different kinases in a variety of cell lines and native tissues often produced multiple bands across a range of molecular weights (Supplementary Table S3).[20,21,24,27,28,33,53,77–82] In these studies the variation in band size was attributed to differential processing of the mRNA[53] and/or posttranslational modification of the protein.[15,18,19,54,55] Because of the expected diversity in kinase band size both within and between tissues, and the potential low abundance of these regulatory kinases, we performed Western blotting using the rat brain as a positive control tissue and both rat and human lens as our target tissues. For each kinase, multiple antibodies were trialed, and the labeling conditions optimized for each antibody to reduce background and nonspecific labeling to a minimum. In addition, antibody specificity was tested by preincubation of the antibody with its antigenic peptide prior to Western blotting. The results obtained by this approach are representative of at least three experiments.

Previous studies have reported band sizes for WNK1 in the range of approximately 230 kDa to over 250 kDa.[21,28,56,57] In this current study, bands within this size range for WNK1 were obtained for the control tissue, rat brain and for rat and human lens (Fig. 2). A strong band approximately 45 kDa (Fig. 2B) was also reduced in intensity by peptide preabsorption, but was only seen by one of the antibodies, and because outside the expected size for WNK1, is likely nonspecific labeling. In the brain, a band for WNK1 that was slightly greater than 250 kDa was observed in the both the membrane and soluble fractions and this band was effectively eliminated in both fractions by preabsorption of the WNK1 antibody with its antigenic peptide (Fig. 2A). In comparison, detection of WNK1 in the rat lens was more erratic with bands in the 230- to greater than 250-kDa range only being detected in 6 of 19 experiments. In these positive experiments, the predominant band detected for WNK1 was at approximately 230 kDa. This band for WNK1 was observed in the membrane but not the soluble fraction and was confirmed to be specific for WNK1 as it was eliminated by preabsorption of the WNK1 antibody with its antigenic peptide (Fig. 2B). In three of six successful experiments using the membrane fraction, and one of five experiments using the soluble fraction, both the approximately 230- and greater than 250-kDa bands were detected (Fig. 2C) suggesting both sizes of WNK1 may exist in the rat lens, but are either present at very low abundance, or are difficult to detect using an antibody designed against the human WNK1 sequence. To investigate this latter possibility, we probed protein fractions prepared from human donor lenses by Western blotting. In these experiments a robust and reproducible approximately 230-kDa band was detected using the WNK1 antibody that was eliminated by preincubation of the antibody with its antigenic peptide (Fig. 2D). The WNK1 signal was detected in all five experiments, in three sets of lenses from donors aged 16, 64, and 85 confirming the higher sensitivity of the antihuman WNK1 antibody in human lenses. The more reproducible results obtained for WNK1 Western blots performed on human lenses strengthens the more inconsistent results obtained in the rat lens supporting the conclusion that WNK1 is expressed at the protein level in both species of lens.

Western blotting in other tissues has reported band sizes for WNK4 that ranged from 135 to 180 kDa (Supplementary Table S3).[20,21,24,27,28,33,53,77–82] Using a rabbit anti-mouse WNK4 antibody in the present study, we observed multiple distinct bands within this size range in both the membrane and soluble fractions of the rat brain that were eliminated by preincubation of the antibody with its control peptide (Fig. 3A). Of the five bands detected in the rat brain, only the approximately 135-kDa band in the membrane fraction and the approximately 90-kDa band in the soluble fraction were detected in the rat lens (Fig. 3B, arrows) and both bands were eliminated by preincubation of WKN4 antibody with its antigenic peptide. In the human lens, no bands were observed in the 135- to 180-kDa size range by the rabbit anti-mouse WNK4 antibody and the bands observed below 100 kDa were not reduced in intensity by preincubation of the antibody with its antigenic peptide (Fig. 3C). Taken together our RT-PCR and Western blot results suggest that WNK4 is expressed in the rat lens, but it will require additional experiments to confirm this and to establish whether it also expressed in the human lens.

OSR1 has two distinct translation sites,[53] which are detected as a doublet in some tissues with band sizes in the range of approximately 45 to 65 kDa (Supplementary Table S3).[20,21,24,27,28,33,53,77–82] In the rat brain, three major bands

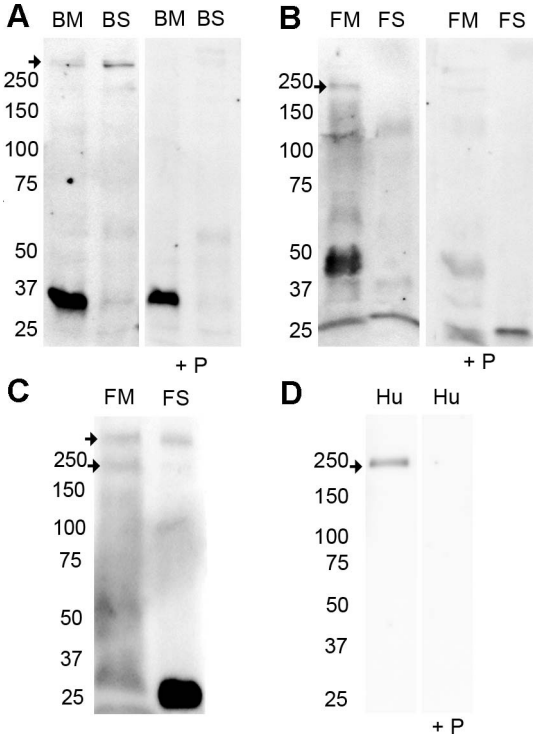

Figure 2. Identification of WNK1 at the protein level in rat and human lenses. Western blots of the protein fractions from rat brain (**A**), rat lens (**B**, **C**), and a 16-year-old human lens (**D**) probed with antihuman WNK1 antibodies, or following preincubation of the WNK1 antibody with its antigenic peptide (P+). (**A**) Detection of a WNK1 specific band above 250 kDa (*arrow*) in the membrane (BM) and soluble (BS) fractions of the rat brain. (**B**) Detection of a WNK1 specific band at approximately 230 kDa (*arrow*) in the FM fraction of the rat lenses, and a lighter band approximately 45 kDa. (**C**) Example of an experiment were both the greater than 250- and approximately 230-kDa bands were detected in the rat lens. Note the greater than 250-kDa band for WNK1 was detected in both the FM and FS fractions. (**D**) Detection of a WNK1 specific band at approximately 230 kDa (*arrow*) in the human (Hu) lens.

were detected in the membrane fraction and one in the soluble fraction, but only the lighter bands at approximately 65 kDa in both the membrane and soluble fractions were eliminated by preincubation of the antibody with its antigenic peptide (Fig. 4A). In the rat lens, four bands were detected in the membrane fraction and a very faint band was visible in the soluble fraction. Only the bands at approximately 45 and 65 kDa were deemed to be specific for OSR1 because they were eliminated by the preincubation control (Fig. 4B). In the human lens, three bands were detected, but only the two bands at approximately 55 and approximately 100 kDa were eliminated by the antigenic peptide control (Fig. 4C). The approximately 100 kDa is larger than expected and could represent a dimer of OSR1, which has been reported to exist by others.[58,59]

Like OSR1, SPAK has been repeatedly detected by Western blotting in a variety of cell types as either a doublet or a single band with molecular weights ranging from 50 to 80 kDa (Supplementary Table S3).[20,21,24,27,28,33,53,77–82] In the rat brain, four bands were evident in both the membrane and soluble fractions, but only the lower approximately 65-kDa band was within the size range and was abolished following preabsorption of the SPAK antibody with its antigenic peptide (Fig. 5A). In the rat lens, an appropriately sized doublet of approximately 65 and approximately 75 kDa was detected, but only the lighter approximately 65-kDa band, was abolished by

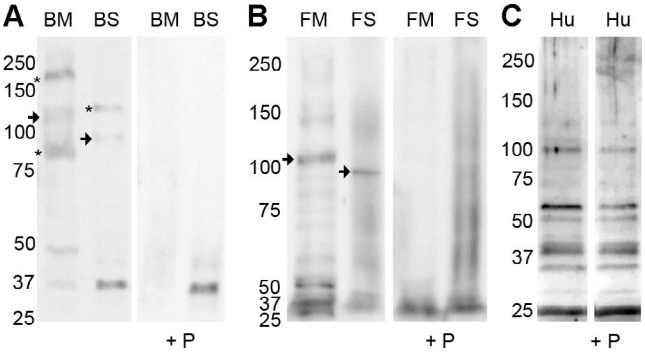

Figure 3. Identification of WNK4 at the protein level in rat and human lenses. Western blots of the protein fractions from rat brain (**A**), rat lens (**B**), and a 16-year-old human lens (**C**) were probed with anti-mouse WNK4 antibodies, or following preincubation of the WNK4 antibody with its antigenic peptide (P+). (**A**) Detection of multiple bands for WNK4 were observed in the rat brain membrane (BM) and soluble (BS) fractions (*, *arrow*), which were eliminated by a peptide control. (**B**) Of the five bands detected in the rat brain, only the approximately 135-kDa band in the rat FM and approximately 90-kDa band in the rat FS were detected (*arrows*) and eliminated with a peptide control. (**C**) In human lenses (Hu) no bands were eliminated with a peptide control suggesting that the bands detected using this antibody in the human lens are nonspecific.

preincubation of the antibody with its antigenic peptide (Fig. 5B). In the human lens, weak labeling of an approximately 65- and approximately 75-kDa doublet was also detected along with a more intense band at 37 kDa (Fig. 5C). In the human lens, all three bands were either eliminated (65 and 75 kDa) or substantially knocked down (37 kDa) suggesting that the lower band may be a degradation product of the heavier proteins. While further experimentation is required in the human lens to determine if SPAK is degraded, it would appear that SPAK is expressed in the both the rat and human lens. From our RT-PCR and Western blotting results, we are confident that we have positively identified WNK1, SPAK, and OSR1 in the rat and human lens, but the identification of WNK4 at the protein

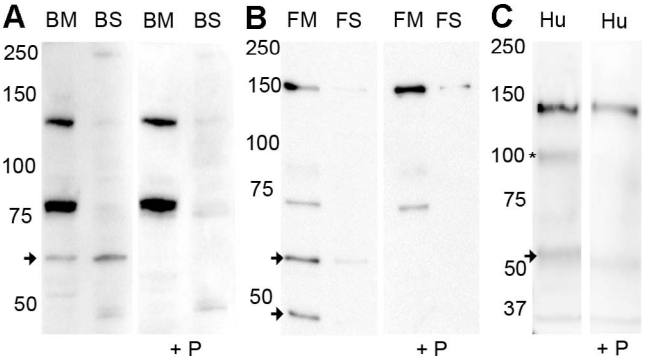

Figure 4. Identification of OSR1 at the protein level in rat and human lenses. Western blot analysis of protein fractions from rat brain (**A**), rat lens (**B**), and a 16-year-old human lens (**C**) probed with anti-human OSR1 antibodies, or following preincubation of the OSR1 antibody with its antigenic peptide (P+). ([**A**, **B**]; *arrows*) Bands for OSR1 were detected at approximately 65 kDa in all lanes, plus at approximately 45 kDa in rat FM fraction. Preabsorption of the antibodies with its corresponding peptide eliminated these two bands while other bands at approximately 150, 130, 85, and 70 kDa remained, indicating nonspecific labeling. (**C**) In the human lens (Hu) bands at approximately 55 kDa (*arrow*) and approximately 100 kDa (*) were detected, which were reduced in intensity by preabsorption with corresponding peptides, while the bands at approximately 130 kDa remained.

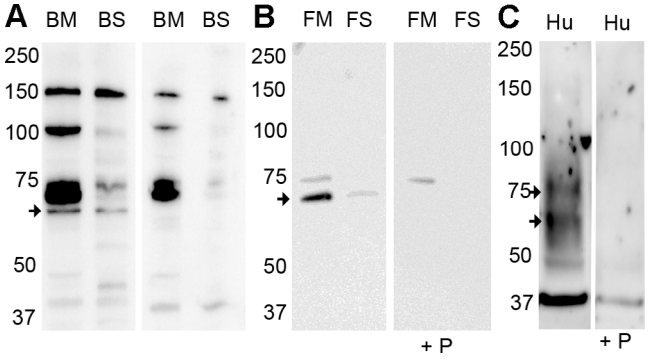

Figure 5. Identification of SPAK at the protein level in rat and human lenses. Western blots of the protein fractions from rat brain (**A**), rat lens (**B**) probed with goat anti-human SPAK antibody (Santa Cruz Biotechnology), and 16-year-old human lens (**C**) probed with rabbit anti-human SPAK (Pro-Sci) antibody, or following preincubation of the SPAK antibodies with their corresponding antigenic peptide (P+). (**A**) The SPAK antibody detected a band in both the membrane (BM) and soluble (BS) fractions of the rat brain and in the membranes (FM) and soluble (FS) fractions of the rat lens (**B**) at approximately 65 kDa (*arrow*). Bands at approximately 150, 100, and 75 kDa in BM, BS, and FM were not eliminated in the peptide control (P+), while the approximately 65-kDa band was, indicating that only the 65-kDa band was specific for SPAK. (**C**) In human lenses, bands for SPAK were detected at approximately 75, 65 kDa, which were eliminated following preabsorption of the antibody with its corresponding peptide, while another band at approximately 37 kDa decreased in intensity in the peptide control experiment.

level still needs to be definitively verified by Western blotting in the rat and human lens.

Regional and Subcellular Localization of WNK-SPAK/OSR1 in the Rat and Human Lenses

Since both SPAK and OSR1 were more consistently and reproducibly detected in the rat lens by Western blotting compared with WNK1 and WNK4, their regional and subcellular localization was further investigated. The rat lens was dissected into three regions and the resultant protein fractions analyzed by Western blotting. SPAK was detected in all lens regions (Fig. 6A, top panel), but was largely associated with the cell membranes, with the only obvious soluble fraction labeling being detected in the epithelium. In contrast, OSR1 was largely absent from the epithelium and outer cortex, but was detected predominantly in the inner cortical and core fractions (Fig. 6A, bottom panel). Interestingly, only the heavier band at approximately 65 kDa, was clearly present in the inner cortex FMs, while the lighter band at approximately 50 kDa was present in the inner cortex and core FMs. This finding was consistent with a previous study, where the lower molecular weight OSR1 band was more strongly detected than the higher molecular weight band.[53] Thus, it appears that not only are SPAK and OSR1 differentially expressed in different regions of the rat lens, but also that OSR1 may be differentially processed in the inner cortical and core regions.

To investigate the association of SPAK and OSR1 with lens membrane proteins, a crude membrane fraction prepared from whole rat lenses was exposed to an urea/alkaline stripping protocol designed to remove peripheral proteins from FM preparations to yield a stripped membrane fraction.[60,61] Subsequent analysis by Western blot analysis revealed that while both SPAK and OSR1 were predominately detected associated with fiber cell membrane fractions (Fig. 6B, arrows), both proteins were absent from stripped membrane fractions, but were strongly detected in peripheral protein fractions. This finding confirmed that SPAK and OSR1 are largely peripheral proteins that can be dissociated from their interaction with integral membrane proteins by urea/alkaline conditions that disrupt ionic bonds between proteins. This result is consistent with SPAK and OSR1 being in close proximity to the plasma membrane where they can interact with and phosphorylate integral proteins, such as NKCC/KCC.

To study the regional distribution of the three kinases definitively identified in the human lens, WNK1, SPAK, and OSR1 (Figs. 2–5) protein fractions from the outer cortex, inner cortex, and core regions from a 16-year-old donor lens were processed for Western blot analysis (Fig. 6C). Detection by the WNK1 antibody revealed that WNK1 was present in all lens regions, with a weaker signal in the lens core (Fig. 6C, top panel). SPAK was detected in all regions of the human lens with both the heavier and lighter sized bands being present in the outer and inner cortical regions, while the heavier band was the most abundant in the core (Fig. 6C, middle panel). In the 16-year-old human lens, OSR1 was also strongly detected in all regions including the core (Fig. 6C, bottom panel). However, with increasing age, the signal intensity of OSR1 decreased in the core relative to protein loaded (Fig. 6D), indicating an age-dependent processing/degradation of OSR1 was occurring in the human lens.

Analysis of SPAK Knock-Out Mouse Lenses

Our access to a limited number of SPAK knock-out animals allowed us to test the specificity of our SPAK antibodies (Fig. 7). As observed in the rat and human lenses these antibodies detected multiple bands including the expected approximately 65-kDa band. However, this band was only detected in the FS fraction using both the goat anti-human Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Fig. 7A) and the rabbit anti-human Pro-Sci SPAK antibodies (Pro-Sci, Poway, CA, USA) (Fig. 7B), directed against the N-terminus and central portion of the protein, respectively. However, an additional rabbit anti-mouse SPAK antibody obtained from the Delpire lab detected a faint approximately 65-kDa band in the membrane fraction in addition to the soluble fraction (Fig. 7C). This does not seem to be an issue with the amount loaded for each fraction since the actin loading control was similar between blots, and suggests that the Delpire antibody has a higher affinity for SPAK and that unlike the rat and human lens, in the mouse the majority of SPAK is associated with the soluble fraction. Indeed, this rabbit-anti mouse antibody detected two lighter forms of SPAK approximately 55 and approximately 45 kDa in the soluble fraction (Fig. 7C, arrows). In the blots obtained from SPAK knock out lenses, while a number of the labeled bands remained, the approximately 65-kDa band detected by all the antibodies and the approximately 55- and approximately 45-kDa bands detected by the Delpire antibody were absent. This result not only confirms the detection of SPAK in the mouse lens, but also our earlier identification of SPAK in rat and human lenses (Fig. 5) that utilized antigenic peptide controls.

The availability of a limited number of SPAK knock-out animals also afforded us the opportunity to determine whether the deletion of the SPAK affects lens transparency. No significant differences between the wet weight of either the eyes (wild-type, 14.86 ± 0.18g, $n = 20$; SPAK knock-out, 14.43 ± 0.28 g, $n = 20$) or the lenses (wild-type, 4.33 ± 0.17 g, $n = 19$; SPAK knock-out, 4.07 ± 0.14g, $n = 14$) obtained from 25 day old wild-type or SPAK knock-out animals were observed. The initial in situ examination of the lens in the eyes of the animals showed that in all the wild-type and the majority of SPAK knock-out animals the lenses appeared clear, although there was a subset of SPAK knock-out animals, which had opaque lenses within 1 hour of death (Fig. 8A). Subsequent

Investigative Ophthalmology & Visual Science

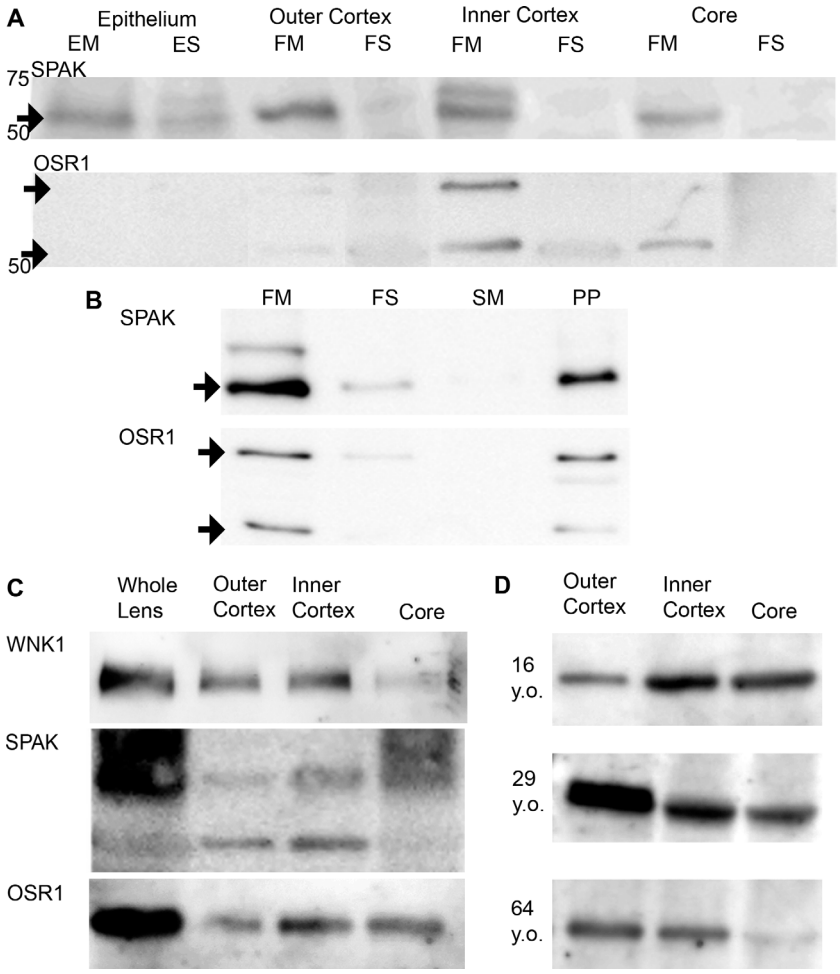

Figure 6. Regional expression of phosphoregulatory kinases in rat and human lenses. (**A**) Regional distribution in the rat lens by Western blotting detects a specific band for SPAK at approximately 65 kDa (*upper panel arrow*) in the membranes of all lens regions (FM), except for the epithelium, where it was also detected in the soluble fraction (ES). OSR1 bands were detected at approximately 65 and 50 kDa (*lower panel arrows*) in FM and FS fractions of the outer and inner cortex, the FM fraction of the core, but were absent from the epithelium (EM, ES). (**B**) Subcellular localization in the rat lens by Western blot detects a strong band specific for SPAK (*upper panel arrow*) in FM, while a fainter band is detected in the FS fraction. SPAK is absent from stripped membranes (SM) but strongly present in the peripheral protein (PP) fraction. Labeling for OSR1 (*lower panel arrows)* reveals a similar subcellular distribution. However, the two bands specific for OSR1 are present in the FM and PP, but FS contains only the heavier, approximately 65-kDa band. (**C**) WNK1 (~250 kDa), WNK4 (~100 kDa), SPAK (~75 kDa and 65 kDa), and OSR1 (65 kDa) were all present in a 16-year-old human lens. Bands were detected in the outer cortex, inner cortex, and core for all kinases, although the band for WNK1 in the core was less intense relative to the bands detected in the outer and inner cortex. Only the 75-kDa SPAK was evident in the core. (**D**) Western blotting for OSR1 in lens fractions obtained from a 16-, 29-, and 64-year donor revealed a decline in OSR1 labeling in the different regions with advancing age.

dark-field analysis of all lenses post dissection from the eye (Fig. 8B) showed an increased incidence and intensity of cortical opacities in lenses from SPAK knock-out animals relative to wild-type (Fig. 8C). While this increased incidence in opacity may be due to the dissection process, what it shows is that SPAK knock-out lenses are more fragile, and therefore more susceptible to lens opacification. In an attempt to characterize the underlying cause of the lens opacity, the morphology of the lenses was examined using confocal microscopy (Figs. 8D, 8E). No obvious differences in overall tissue architecture between wild-type and SPAK knock-out lenses was observed (Fig. 8D), supporting our initial conclusion from wet weight data that lenses can develop, grow and maintain a normal tissue architecture in the absence of SPAK. However, a closer analysis of the number of fiber cells contained within a column of cells shows that there are more cells in SPAK knock-out lenses (54.71 cells/100 μm ± 2.31; $n = 3$) than in wild-type (46.40 cells/100 μm ± 3.94; $n = 3$) lenses (Fig. 8E). This analysis reveals that cortical fiber cells are more compacted in SPAK knock-out lenses, and suggests that in these lenses volume regulation is compromised making them less able to respond to osmotic and/or mechanical stress and more susceptible to opacification.

Discussion

Previously, we have shown that the cation chloride cotransporters, KCC1, 3, 4, and NKCC1, are able to regulate fiber cell volume, control steady state lens volume, and thereby maintain rat lens transparency.[9,10] In this study, we have identified for the first time in rat and human lenses a signaling pathway known in other tissues to modulate cell volume, via the reciprocal regulation of KCC and NKCC1 activity through the modulation of the phosphorylation status of the two cotransporters. In this current study, we have shown that key kinases in this pathway WNK1, 3, 4, SPAK, and OSR1 are expressed in the rat lens at the transcript level (Fig. 1), and using a panel of

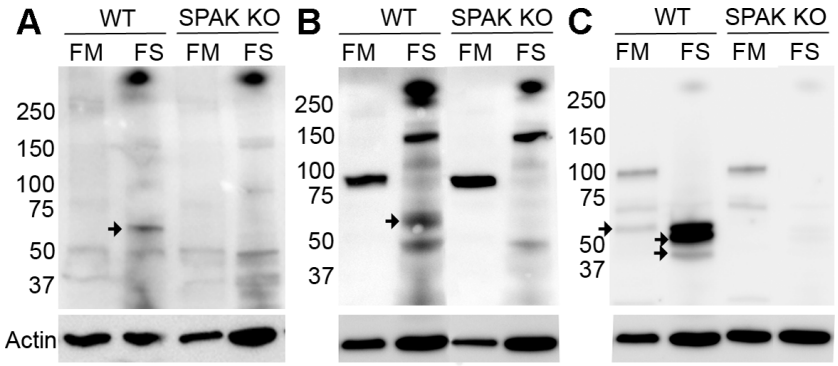

Figure 7. Confirmation of SPAK signal specificity in the lens by use of SPAK knock-out mice. (**A**) Protein fractions from the mouse lens probed with goat anti-human SPAK antibody (Santa Cruz Biotechnology), (**B**) rabbit anti-human SPAK (Pro-Sci) antibody, (**C**) and the rabbit anti-mouse SPAK antibody (Delpire). Both anti-human SPAK antibodies ([**A**, **B**]; *arrow*) labeled a band approximately 65 kDa in the soluble (FS) fraction of wild-type mice, which was absent in SPAK knock-out lenses. The anti-mouse antibody ([**C**]; *arrows*) detected the same band at approximately 65 kDa in FS, as well as in the membrane fraction (FM). Several bands of lighter molecular weight were also detected in FS (*arrows*) in the wild-type. All of the approximately 65-kDa bands were absent in the SPAK knock out lenses, confirming specificity, while nonspecific bands remained. Equal loading was confirmed by reprobing the PVDF membranes with β-actin antibody ([**A**–**C**]; *bottom panel*).

kinase specific antibodies, we have confirmed kinase (WNK1, SPAK, OSR1) expression at the protein level in rat and human lenses (Figs. 2–5). Subsequent localization of SPAK and OSR1 (Fig. 6) showed that they were distributed throughout all regions of the rat and human lens, except for OSR1, which was undetectable in the rat lens epithelium. The final effector kinases SPAK and OSR1 were also shown to be associated with fiber cell membranes as peripheral membrane proteins (Fig. 6B). This regional and subcellular localization pattern overlaps with the distribution of KCC and NKCC in the rat lens,[9,10] suggesting that the WNK-SPAK/OSR1 signaling system could influence KCC/NKCC phosphorylation status, cotransporter function, fiber cell volume regulation, and ultimately lens transparency.

Our identification and localization of this signaling pathway in different regions of the lens critically depends on the ability of a range of antibodies to specifically detect the individual components of the signaling pathway using Western blotting. While we were fortunate enough to obtain tissue from SPAK knock-out animals to test the specificity of SPAK antibodies (Fig. 7), we had to rely on the use of antigenic peptide controls to test the specificity of the other antibodies. This preincubation of the antibody with its antigenic peptide eliminated specific bands from the Western blots that were deemed to be the protein of interest. The size of the these "knocked-down" bands varied between not only the control tissue rat brain and the rat and human lens, but sometimes between the different fractions obtained from the same species of lens. In other tissues, this variation in band size has been attributed to differential processing of the mRNA or posttranslation modification of the protein (Supplementary Table S3).[20,21,24,27,28,33,53,77–82] In the lens, posttranslational modification of proteins in different regions of the lens has been proposed as a mechanism to change protein function to compensate for the inability of anucleate mature fiber cells to perform de novo protein synthesis.[62,63] Truncated functional forms of WNK1,[54] and SPAK[55] have been identified in other tissues and the phosphorylation of WNK1, WNK4, SPAK, and OSR1 at multiple sites is also known to change kinase function.[15,18,19] Therefore, it is interesting to speculate that the differences in band sizes observed for the kinases in the different lens fractions represent posttranslational modifications designed to fine tune kinase function in these regions. It is interesting to speculate whether the shorter forms of SPAK observed in the mouse lens are created by a recently identified protease aspartyl aminopeptidase (Dnpep) that was shown to cleave SPAK in the kidney.[64] While speculation on the functional significance of observed diversity of band sizes maybe premature, we are confident that we have shown that WNK1, SPAK, and OSR1 are expressed in both the rat and human lenses, and have validated a previous report of SPAK expression in the mouse lens.[45]

However, showing that WNK1, SPAK, and OSR1 are all expressed in the lens does not prove that they interact to form a signaling pathway that maintains steady-state lens volume by modulating KCC and NKCC1 activity. Indeed it is highly likely that the kinases we have identified may have additional functions in the lens. For example, in other tissues besides affecting KCC/NKCC, WNK1 has been shown to be vital for development,[16,65] growth,[28] neurite differentiation and extension,[66] and exocytosis.[67,68] While WNK4 has largely been shown to be involved in the regulation of another family member, the Na-Cl Cotransporter (NCC),[69] it has also been shown to interact with cytoskeletal elements,[70] and to inhibit TRPV5-mediated Ca^{2+} transport[71] by endocytosis.[72] SPAK and OSR1 have been shown to be involved in development and differentiation, cell transformation and proliferation, cytoskeletal rearrangement,[19] the cell cycle, and apoptosis.[73] Furthermore, a recent study has shown that SPAK is a negative regulator of SGLT1 cell membrane abundance,[74] a protein that has been identified in the rat lens.[75] The involvement of these kinases in a variety of cellular processes that are linked to development, differentiation and growth has obvious implications for a tissue like the lens, which grows throughout life by the continual differentiation of epithelial cells into fiber cells that in turn undergo extensive elongation and migration.

The genetic deletion of the final effector kinase, SPAK, however, did not appear to significantly impair lens development and growth as the size and morphology of lenses from wild-type and SPAK knock-out animals appeared similar (Fig. 8). It is plausible that the absence of SPAK may be compensated by the closely structurally and functionally related kinase, OSR1. Consistent with this view SPAK and OSR1 in the rat and human lens have distinctly different regional expression patterns (Fig. 6). While SPAK was found in all regions of the lens, OSR1 expression was higher in deeper regions of the lens that were first laid down in utero. It is therefore possible that OSR1 could play a more important role than SPAK in the embryonic development and postnatal

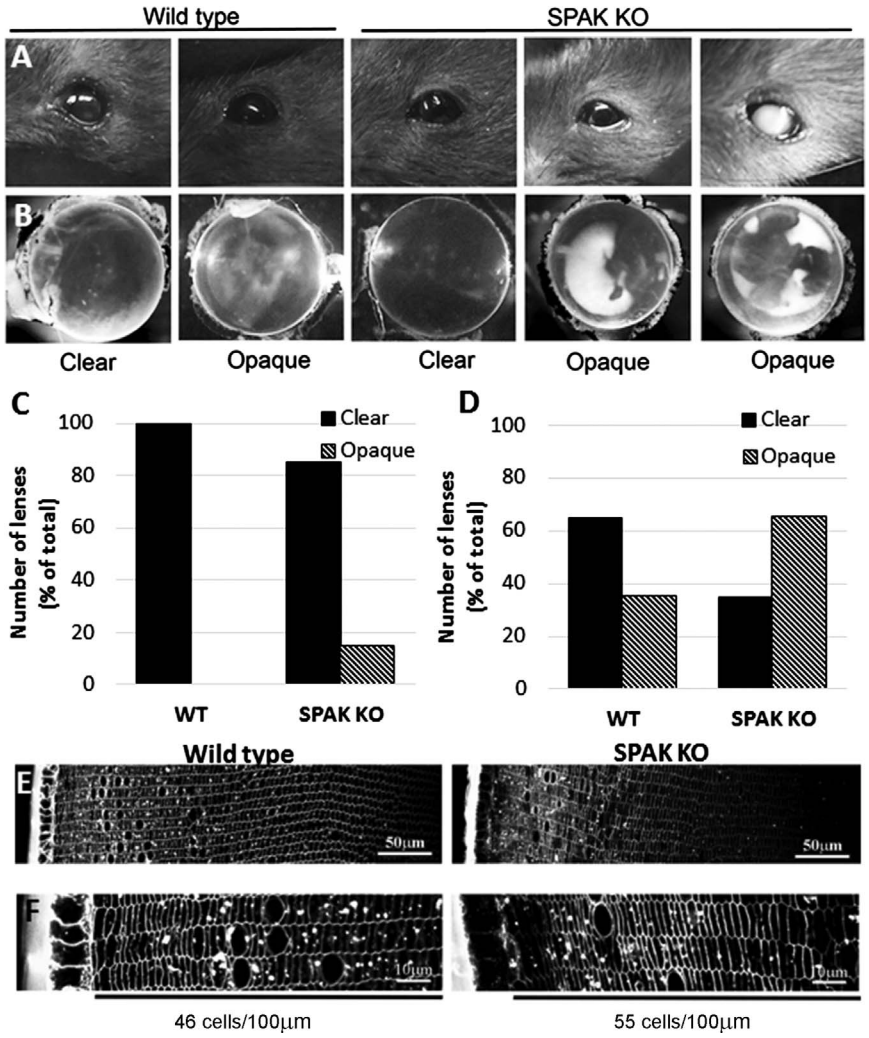

Figure 8. Characterization of SPAK knock-out lenses. (**A**) Twenty-five-day-old wild-type mice always revealed clear lenses and eyes upon external eye examination, while some SPAK knock-out mice revealed severe opacity. (**B**) Dark-field analysis of the transparency of lenses from wild-type and SPAK knock-out mice reveals clear and opaque phenotypes in lenses following dissection from the eye. (**C**) The incidence of clear and opaque lens phenotypes observed in situ in wild-type and SPAK knock-out mice. (**D**) The incidence of clear and opaque lens in wild-type and SPAK knock-out mice following dissection from the eye. (**E**, **F**) Equatorial sections from wild-type and SPAK knock-out lenses labeled with the membrane marker WGA to visualize tissue architecture (**E**) and fiber cell compaction (**F**).

growth of the lens. This observation is consistent with the finding that global OSR1 knock-out mice are embryonically lethal, while global SPAK knock-out mice are viable and display normal behavior with reduced fertility.[47]

While the majority of lenses obtained from 25-day-old SPAK knock-out animals initially appeared normal in situ, a higher percentage SPAK knock out relative to wild-type lenses exhibited intense cortical opacification within 1 hour of removal from the eye (Fig. 8D). While this observed lens opacification could simply be due to mechanical damage inflicted during lens dissection this does not explain the increased incidence of opacity seen in lenses from SPAK knock out animals. Instead it suggests that lenses lacking SPAK were less able to respond to the stresses inflicted during the dissection period causing them to be more susceptible to the formation of cataracts. This susceptibility is likely due to the compaction of fiber cells observed in SPAK knock-out lenses, which indicates that the fiber cells in these lenses are shrunken. Since the steady state volume of fiber cells is set by the reciprocal activity of CCCs,[1] we hypothesize that the knock-out of SPAK increases KCC and inhibits NKCC1 activity, thereby increasing the net efflux of ions and water from cortical fiber cells causing them to shrink. To test this hypothesis, an assessment of the change in the phosphorylation status of KCC and NKCC1 following the knock out of SPAK is required.

In conclusion, our identification of the WNK-SPAK/OSR1 signaling pathway in lens opens up new avenues for future research into the role played by this pathway in modulating the phosphorylation status of CCCs that have been previously shown to control steady-state lens volume, and thereby preserve the morphology of fiber cells that is critical for the maintenance of lens transparency. Whether this pathway is capable of sensing changes in lens volume and translating those changes into alterations to the phosphorylation status of KCC and NKCC to effect changes in ion and fluid fluxes in the lens to restore lens volume will be the focus of on-going work.

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