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Verification and spatial mapping of TRPV1 and TRPV4 expression in the embryonic and adult mouse lens

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Abstract

The transient receptor protein vanilloid channels, TRPV1 and TRPV4, have recently been shown to be mechanosensors in the ocular lens that act to transduce physical changes in lens volume and internal hydrostatic pressure into the activation of signalling pathways in lens epithelial cells. These pathways in turn regulate ion and water transport to ensure that the optical properties of the lens remain constant. Despite the functional evidence that implicate the roles of TRPV1 and TRPV4 in the lens, their respective cellular expression patterns in the different regions of the lens has to date not been fully characterised. Using Western blotting we have confirmed that TRPV1 and TRPV4 are expressed throughout all regions (epithelium, outer cortex, inner cortex/core) of the adult mouse lens. Subsequent immunolabeling of lens cryosections confirmed that TRPV1 and TRPV4 are expressed throughout all regions of the lens, but revealed differentiation-dependent differences in the subcellular expression of the two channels in the different regions. In the epithelium and outer cortex, intense TRPV1 and TRPV4 labeling was predominately associated with the cytoplasm. In a discrete zone in the inner cortex, labeling for both proteins was greatly diminished, but could be enhanced by incubating sections with the detergent Triton X-100 to reveal TRPV1 and TRPV4 labelling that was associated with the membrane. This suggests that in this region of the lens there is a potential interacting protein that masks the binding of the TRPV1 and TRPV4 antibodies to their respective epitopes in the lens inner cortex. In the core of the lens, which contains the embryonic nucleus, TRPV1 and TRPV4 labelling was associated exclusively with fibre cell membranes. This labelling in the lens core of the adult mouse lens appeared to originate in early development as a similar membrane labelling was observed at embryonic day 10 (E10) of the cells in the lens vesicle that subsequently forms the embryonic nucleus in the adult lens. During subsequent stages of embryonic development TRPV1 and TRPV4 remained membranous in the inner cortex and core, while showing labelling that was associated with the cytoplasm in the superficial outer cortical region. The extent of cytoplasmic labelling for TRPV4, but not TRPV1, in this cortical region could however be dynamically regulated by cutting the zonules that normally attach the lens to the ciliary body. We have shown an early onset and continuous expression of TRPV1 and TRPV4 across all lens regions, and that TRPV4 can be dynamically trafficked into the membranes of differentiating fibre cells, results that suggests that these mechanosensitive channels may also be functionally active in lens fibre cells.

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Keywords

Lens; Lens development; Transient Receptor Channel expression from the vanilloid subfamily

INTRODUCTION

Transient receptor potential (TRP) proteins are a superfamily of non-selective cation channels implicated in the initiation of a number of physiological responses, in a variety of tissues, to a multitude of stimuli (O'Neil and Brown, 2003;Soboloff, et al., 2007). In the lens, two of the members from the vanilloid subfamily of TRP channels, receptor type 1 (TRPV1) and type 4 (TRPV4), have recently been shown to transduce changes in overall lens volume and hydrostatic pressure into alterations in lens transport that serve to either restore lens volume (Shahidullah, et al., 2012;Shahidullah, et al., 2018) or maintain the internal lens hydrostatic pressure gradient (Gao, et al., 2015). In terms of cell volume regulation, swelling lenses by exposure to hypoosmotic challenge has been shown to activate TRPV4 and initiate a signalling cascade which increases Na^+/K^+ ATPase activity to produce a regulatory volume decrease that restores lens volume (Shahidullah, et al., 2012). In contrast, hyperosmotic lens shrinkage activates TRPV1 and initiates a signalling cascade that phosphorylates NKCC1, thereby increasing its activity and effecting a regulatory volume increase to shift lens volume back towards normal levels (Shahidullah, et al., 2018). In addition, it has been shown experimentally that the intercellular outflow of water from the lens core to the surface occurs through gap junction channels and that this outflow of water generates a substantial hydrostatic pressure gradient (Gao, et al., 2011). Subsequently, it has recently been shown that this pressure gradient is tightly regulated by a dual feedback pathway that uses TRPV1 and TRPV4 to sense a decrease or increase in hydrostatic pressure, respectively (Gao, et al., 2015). Thus it would appear that TRPV1 and TRPV4 act as mechanosensors which are capable of transducing physical changes in lens volume/pressure into the activation of a dynamic signalling system that regulate lens transport activity to ensure that the optical properties of the lens remain constant.

Despite the importance of TRPV1 and TRPV4 channels to overall lens function, no systematic mapping of the expression patterns of the two channels throughout all regions of the lens has been undertaken to date. High levels of TRPV1 expression at the mRNA level has been reported in the rabbit lens (Martínez-García, et al., 2013). The same study showed that although the mRNA level was very high, detection of TRPV1 protein was only present in the epithelium of the rabbit and human lenses using immunohistochemistry. In porcine lenses TRPV1 and TRPV4 expression has been shown only in the epithelial cells detected with western blotting analysis (Shahidullah, et al., 2012;Delamere, et al., 2016). However, the epithelial cells of the lens comprise a single monolayer of cells that covers only the anterior surface of the lens. At the lens equator, these epithelial cells divide and differentiate into the highly specialised secondary fibre cells that comprise the bulk of the lens (Bassnett, et al., 2011). In the outer cortex of the lens, these differentiating fibre cells undergo massive elongation, express a variety of fibre specific proteins and lose their nuclei and other cellular organelles to form anuclear mature fibre cells. Since this process of fibre cell differentiation occurs throughout life, younger differentiating fibre cells surround and internalise the older

mature fibre cells that comprise the inner cortex and nucleus or core of the lens. The oldest fibre cells are laid down during embryonic development and are known as primary fibre cells (McAvoy, et al., 1999;Lovicu and Robinson, 2004). They remain a living component of the lens and becomes encapsulated in the core of the lens.

Given this unique age-dependent gradient in fibre cell differentiation, it is important that the expression of TRPV1 and TRPV4 be studied not only in the epithelium, but also in fibre cells located in the different regions of the lens. To achieve this, we have used peptide specific antibodies and Western blotting to show that TRPV1 and TRPV4 are expressed in all regions of the adult mouse lens. Furthermore, we have used immunohistochemistry to map not only how the subcellular distribution of TRPV1 and TRPV4 changes as a function of fibre cell differentiation in the adult mouse lens, but also as a function of embryonic development. Consistent with our Western blot results, immunohistochemistry confirmed that TRPV1 and TRPV4 are expressed in all regions of the mouse lens. In addition, immuno-mapping showed that the subcellular localisation of the TRPV1 and TRPV4 changed from a predominant cytoplasmic location in the epithelium and differentiating fibre cells in the outer cortex, to a membrane location in the inner cortex/core of the mouse lens, and that this subcellular distribution is established during embryonic development. Finally, we show in a discrete zone of the outer cortex, the subcellular distribution for TRPV4, but not TRPV1, is dynamically regulated by altering the tension applied to the lens by cutting the zonules that normally attach the lens to the ciliary body. These mapping experiments, by showing that TRPV1 and TRPV4 are expressed throughout all regions of the lens, suggest that these mechanosensitive channels have additional functional roles in lens fibre cells.

METHODS

Reagents

Rabbit anti-TRPV4 C-terminus polyclonal antibody (raised against a synthetic peptide consisting of amino acid residues from within 850 to the C-terminus of mouse TRPV4 protein), and its corresponding antigenic peptide, were purchased from Abcam (Cambridge, MA). Rabbit anti-TRPV1 C-terminus antibody (peptide corresponding to amino acid residues 824–838 of rat TRPV1 protein) and its corresponding antigenic peptide were obtained from Alomone Labs (Jerusalem, Israel). Goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 or Horseradish Peroxidase (HRP) markers were purchased from Life Technologies. Wheat Germ Agglutinin (WGA) conjugated to Alexa Fluor 594 was used as a membrane marker and was purchased from Molecular Probes/Thermo Fisher Scientific (Waltham, MA USA). Artificial aqueous humour (AAH) contains 125mM NaCl, 0.5mM $MgCl_2$, 4.5mM KCl, 10mM $NaHCO_3$, 2mM $CaCl_2$, 5mM Glucose, 10mM Sucrose, 10mM HEPES, pH 7.4 at 300mOsmol/L. Unless otherwise stated all other chemicals used were purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

Animals

All adult lenses used in this study were from 6–8 week old C57BL/6 mice, which were supplied by the Vernon Jansen Unit (VJU) located in the Faculty of Medical and Health Sciences at the University of Auckland. All experiments on adult mice were carried out in

accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, which were approved by the University of Auckland Animal Ethics Committee (#001303 and #001893). All embryonic tissues used in this study were obtained as tissue blocks from either Professor Frank Lovicu (University of Sydney) or Associate Professor Robb de Iongh (University of Melbourne). For immunolabeling analysis, a minimum of 10 different sections from at least three different animals at each developmental stage (adult or embryonic) were examined to identify the specific immunolabeling pattern for both TRPV1 and TRPV4.

Western blotting

Protein fractions were prepared from the mouse kidney, which served as a control tissue, and from either ten whole lenses, or twenty micro-dissected lenses that were separated into epithelium, outer cortex and inner cortex/core fractions. All tissue fractions were homogenized in an ice-cold lysis buffer (10mM HEPES, pH 8.0, 2mM EDTA, 2mM EGTA and protease inhibitor cocktail (Roche, Mannheim, Germany) using a hand held homogenizer (Ultra-Turrax, Ika, Germany). The homogenate was centrifuged at 15,000 *g* for 30 minutes at 4°C and separated into pellet and supernatant. Both the pellet and supernatant were divided in aliquots and stored at −80°C until further use. Protein samples from the supernatant and pellet were loaded and separated on a 7.5 % SDS PAGE gel and transferred to a PVDF transfer membrane. The membranes were blocked for 1 hour at room temperature with 5% low fat milk powder dissolved in TBS-Tween, pH 7.6 buffer (20mM Tris base, 137mM NaCl, 1% Tween-20). After washing, the membranes were incubated overnight at 4^0C with specific antibodies against TRPV1 or TRPV4 at 2μg/ml in antibody solution (50mM EDTA, 0.5g BSA dissolved in TBS-Tween). After washing, the membranes were incubated with secondary antibody goat anti-rabbit HRP at 1:10 000 dilution for 1 hour at room temperature. The washed membranes were developed using ECL select developing kit (Amersham/GE Healthcare Life Sciences, United Kingdom) for 5 minutes and imaged using a Fujifilm LAS 3000 Imager (Fujifilm Medical Systems, USA).

Immunohistochemistry

Embryos were obtained from superovulated FVB/N mice (E10 and E11), or wild type C57 black mice (E18.5). Embryos were fixed in 10% neutral buffered formalin (room temperature, 2 hours) then washed three times in 70% ethanol. The tissue was then dehydrated in a series of graded ethanol washes, prior to clearing in xylene. Finally, tissue was embedded in paraffin wax, and 5μm-thick axial paraffin sections collected using a microtome (Ultracut UCT, Leica Microsystems, Germany). Sections were de-paraffinised by washing with xylene followed by a wash in 100% ethanol and then water. An antigen retrieval step, using treatment in 0.01M Citrate buffer, pH 6 in a pressure cooker for 1 hour followed by 20 minutes in PBS, was applied prior to immunolabelling.

For immunolabeling we either used immediately extracted lenses from the eye globe or dissected the eye globe in two different ways to prepare the lens with zonules and lens with cut zonules conditions for organ culture incubations. To prepare the lens with cut zonules we used the following procedure. The eyeball was extracted from the eye socket immediately after euthanasia and kept in warm PBS. The globe, kept in PBS, was opened at the back by

making four incision of the sclera extending from the optic nerve towards the limbus. The four flaps were opened and the lens removed from the attaching zonules and other adherent tissues by rolling it out with a glass loop. Any remaining zonules or surrounding tissues were blotted away with paper. To prepare the lens with zonules the eyeball was extracted from the eye socket in PBS and a small opening of around 2mm diameter was cut open with a microscissor and vitreous body removed. All other surrounding tissues were left intact to maintain the zonular tension. After each preparation the samples were placed immediately in warm AAH for organ culture incubations for 120 minutes. Following the incubation period or after immediate dissection of the lens from the eye globe the lenses were fixed in 0.75% paraformaldehyde at room temperature for 24 hours.

Following fixation, all lenses were prepared for cryosectioning using the same established protocols (Jacobs, et al., 2003). Cryosections that were 14 μm in thickness were transferred onto microscope slides and where necessary, were first treated with either 0.1% Triton X-100 or 0.1% Tween 20 to permeabilize the cell membranes for 10 minutes at room temperature, following by removal of the detergent with three 5 minutes long consecutive washes of PBS. Cryosections were then incubated in blocking solution (3% bovine serum albumin, 3% normal goat serum in PBS) for 1 hour at room temperature. After blocking was completed, sections were incubated overnight at 4°C in either TRPV1, or TRPV4 primary antibodies prepared in blocking solution (1:100). Slides were washed in PBS and incubated for 1.5 hours, in the dark at room temperature, with goat anti-rabbit Alexa Fluor 488 secondary antibody in blocking solution (1:200) that contained 0.125μg/ml DAPI for fluorescent labeling of nuclei. Where necessary, sections were then incubated with WGA Alexa Fluor 594 in PBS (1:100) for 1 hour at room temperature to label cell membranes. Coverslips were mounted using VectaShield HardSet™ anti-fade mounting medium (Vector Laboratories, Burlingame, CA) and imaged using a laser scanning confocal microscope (Olympus FV1000, Tokyo, Japan) equipped with FluoView 2.0b software. For presentation purposes, labelling patterns were pseudo-coloured and combined using Adobe Photoshop software (version CS6, Adobe Systems Inc., San Jose, CA).

RESULTS

Since TRPV1 (95 kDA) and TRPV4 (98 kDA) are members of the same protein subfamily, and exhibit very similar predicted molecular weights, we have utilized commercially available peptide specific antibodies that have been successfully used in a variety of tissues to distinguish between the two proteins (Alenmyr, et al., 2014;Oklinski, et al., 2014;De Toni, et al., 2016;Kim, et al., 2016;Martínez, et al., 2016;Rizopoulos, et al., 2018). These antibodies are designed against peptide sequences in the C-terminal cytoplasmic tails of TRPV1 and TRPV4 which exhibit very low amino acid sequence similarity to each other (13.3%) to guard against the possibility of antibody cross reactivity, and the availability of the antigenic peptide means that peptide absorption controls can be performed to further validate antibody specificity.

TRPV1 and TRPV4 expression at the protein level in the adult mouse lens

To confirm TRPV1 and TRPV4 expression in the adult mouse lens, we performed Western blotting using protein fractions obtained from the pellet and supernatant of whole lens homogenates and the kidney which served as our control tissue. Two bands for TRPV1 were found around the predicted molecular weight of 100kDa (arrow) in protein fractions obtained from both the pellet and supernatant of the whole lens and kidney. An extra band (star) at ~50kDa was also evident in fractions from the pellet and supernatant of the whole lens and in the supernatant of the kidney (Figure 1A). To test the specificity of the observed bands for TRPV1 protein we have performed pre-absorption of the antibody with its corresponding antigenic peptide and applied it to the protein fraction showing the highest intensity of signal. Using this approach we found that in the supernatant fraction of the lens these bands appear specific for TRPV1 as both of the bands at ~100kDa and the band at ~50kDa mark were knocked down (Fig 1B, arrows). The knockdown of the lower 50kDa band suggest that this may represent a degraded form of the TRPV1 protein. TRPV4 was detected as a single band at its predicted molecular weight of 100kDa (arrowhead) in both the pellet and supernatant fractions in the lens and kidney, but was stronger in the pellet (Figure 2A, arrowhead). Additional bands at ~75kDa and 50kDa were found in the supernatant and pellet fractions, respectively (Figure 2A, star). Using the pellet fraction, since it is the sample with highest signal for TRPV4 protein in the lens, we found that these bands appear specific for TRPV4 as pre-absorption of the TRPV4 antibody to its antigenic peptide resulted in elimination of both the 100kDa band and the lower molecular bands (Figure 2B), which probably represent TRPV4 degradation products. Having confirmed the expression of TRPV1 and TRPV4 in the whole lens, Western blotting was then perfomed using protein fractions isolated from different regions of the lens. Bands in the predicted molecular weight range of ~100kDa were detected for TRPV1 (Figure 3A) and TRPV4 proteins (Figure 3B) in epithelium, outer cortex and combined inner cortex/core fractions isolated from the adult mouse lens. In the epithelium we detected similar level of signal intensity for TRPV1 and TRPV4 in both the supernatant and pellet fractions. In contrast, in the outer cortical and inner cortex/core regions, despite the lower concentration of proteins loaded in the pellet fractions, TRPV1/4 bands appear to be enriched in comparison to the supernatant fractions.

Mapping the subcellular distribution of TRPV1 and TRPV4 in the adult mouse lens

To investigate the subcellular distribution of TRPV1 and TPRV4 in different regions of the lens, the antibodies validated by Western blotting were used to label cryosections cut through the lens in either an axial (Figures 4 & 5) or equatoral orientation (Figures 6 & 7). A low power image montage of an axial section showing images collected from the anterior to posterior and from the capsule to central nucleus of the lens labelled with TRPV1 (Figure 4A) and TRPV4 (Figure 4B) antibodies showed continous and strong labelling for the two proteins throughout the whole lens. Pre-absorption of the antibodies with their respective antigenic peptide eliminated all TRPV1 (Figure 4C, E) labelling as shown across the entire equatorial plane. A similar knock down of TRPV4 labelling in fibre cells of the cortex and core was observed, albeit a faint signal in the epithelial cells was still evident (Figure 4D, F). Thus it appears that the antibody labelling is specific for TRPV1 and TRPV4.

Higher power images collected from sections double labelled with TRPV1/4 and the membrane marker WGA were used to map the subcellular distribution of TRPV1 and TRPV4 in the different regions of the lens. Axial sections were used to map the subcellular distribution of TRPV1 and TRPV4 in the epithelium and superficial fibre cells in the outer cortex of the lens. We found that both TRPV1 and TRPV4 exhibited a strong punctate labeling of the cytoplasm in epithelial and fibre cells located at the anterior pole (Figure 5B,E,H,K), equator (Figure 5C,F,I, L) and posterior pole (Figure 5D,G,F,M). This pattern of cytoplasmic labelling suggests that in epithelial cells and peripheral differentiating fibre cells that the majority of both TRPV1 and TRPV4 protein is associated with a cytoplamsic pool of proteins and does not associate with the plasma membrane.

Interestingly, the subcellular location for both TRPV1 and TRPV4 changed in a radial direction as a function of fibre cell differentiation. An overview of TRPV1 labelling shows that it is present from the epithelium to the core of the lens, although there is a distinct zone (~300μm wide) in the inner cortex where TRPV1 labelling intensity is greatly reduced (Figure 6A). In higher magnification images, TRPV1 labeling in the epithelium and peripheral diferentiating fibre cells showed the expected strong punctate labeling in the cytoplasm (Figure 6B,G). However, at around 30 to 40 cells in from the capsule TRPV1 labelling, although still cytoplasmic, showed a pattern of discrete punctate labelling (Figure 6C,H). With deeper distance into the lens the labelling intensity abruptly decreased in the region where the outer cortex transitions into the inner cortex (Figure 6D,I), before returning in the deeper regions of the lens, where it was now completely localised to the plasma membrane of mature fibre cells in the inner cortex (Figure 6E,J) and core of the lens (Figure 6F,K). This observed transient change in the pattern of TRPV1 labelling can be explained by some form of post translational modification to the TRPV1 protein that changes the ability of the TRPV1 antibody to bind directly to the TRPV1 protein. Alternatively this reduction in antibody binding could be due to either a complete loss of the antibody binding site on the protein, or the masking of the epitope via some interacting protein. To distinguish between these two possibilities, sections were washed in the detergents Tween 20 or Triton X-100 to see if we could unmask the antibody epitope on the TRVP1 protein. While the milder detergent Tween 20 did not change the immunolabelling signal (data not shown), pre-treatment with the stronger detergent Triton X-100 resulted in a drastic improvement of the immunolabelling of TRPV1 in the outer cortex-inner cortex transition zone, either by removing an interacting protein that was blocking the epitope or by improving epitope accessability (Figure 6L). Subsequent high magnification images of these Triton X-100 pre-treated sections revealed essentially the same subcellular pattern of TRPV1 labelling observed in the outer cortex (Figure 6M,R & N,S), inner cortex (Figure 6P,U) and core (Figure 6Q,V) of non-treated sections. However, in the outer cortex-inner cortex transition zone the unmasking of the TRPV1 epitope revelaed an area where TRPV1 labelling shifts from a predominately cytoplasmic location to one associated with the plasma membrane (Figure 6O,T).

An essentially similar pattern of labelling for TRPV4 (Figure 7) was found in the radial direction as that seen for TRPV1. TRPV4 labelling was detected from the epithelium to the core of the lens, but again there was a similar region around the outer cortex-inner cortex transition zone that exhibited a marked and localised decrease in TRPV4 labelling intensity

(Figure 7A). At higher magnification, TRPV4 labelling was predominately associated with the cytoplasm in epithelial and peripheral differentiating fibre cells of the outer cortex (Figure 7C,H). With deeper distance into the lens the labelling intensity abruptly decreased (Figure 7D,I), before returning in the deeper regions of the lens, where it was now localised to the plasma membrane of mature fibre cells in the inner cortex (Figure 7E,J) and core of the lens (Figure 7F,K). As observed for TRPV1 labelling, the pre-treatment of sections with Triton X-100 increased TRPV4 labelling in outer cortex-inner cortex transition zone (Figure 7L), and revealed that in this region the transition of TRPV4 from a predominant cytoplasmic labelling pattern to one associated with the plasma membrane (Figure 7O,T). In contrast, relative to non-treated sections, incubation of section in Triton X-100 treatment had no effects on the subcellular pattern of TRPV4 labelling observed in the other regions of the lens outer cortex (Figure 6M-V).

Taken together our Western blotting and immunolabelling experiments show that both TRPV1 and TRPV4 are expressed in all regions of the adult mouse lens, but their subcellular distribution and interaction with a ‘masking’ protein vary as a function of fibre cell differentiation. To gain further insights into when and how this distinctive pattern of TRPV1/4 labelling is established in the adult lens, we performed immunolabelling on the embryonic mouse lens.

TRPV1 and TRPV4 expression during embryonic development in the mouse lens

TRPV1 and TRPV4 expression was detected from embryonic (E) day 10, the earliest stage in eye development examined in this study (Figure 8). At E10 the lens vesicle has formed and the posterior epithelial cells have just begun the process of elongation into primary fibre cells. At this stage of lens development TRPV1 (Figure 8A) and TRPV4 (Figure 8B) labelling is pre-dominantly associated with the membranes of all cells in the lens vesicle. During the subsequent outgrowth of primary fibre cells towards the anterior pole of the lens, which results in obliteration of the lumen of the lens vesicle at E11, both TRPV1 (Figure 8C) and TRPV4 (Figure 8D) expression remained strongly membranous. This membranous expression of TRPV1 and TRPV4 continued in subsequent embryonic stages, which saw newly formed secondary fibre cells added to the peripheral regions of the growing embryonic lens (data not shown). However, towards the end of the embryonic development period, at ~E18.5, a peripheral region of cytoplasmic labelling emerged at the equator of the developing lens. At this stage of development TRPV1 and TRPV4 labelling for the first time resembles that seen in the adult mouse lens showing labelling associated with the cytoplasm in the epithelium and peripheral differentiating fibre cells for TRPV1 (Figure 8F) and TRPV4 (Figure 8G) with membrane labelling in the secondary and primary fibre cells of the inner cortex (Figure 8, TRPV1- H; TRPV4 - I) and core (Figure 8, TRPV1 - J; TRPV4 - K) of the embryonic lens. In contrast to the adult pattern of distribution, no decrease of TRPV1 or TRPV4 antibody labelling was detected in the later stages of embryonic development, which tends to indicate the observed masking of TRPV1 and TRPV4 epitopes observed in the adult lens occurs at later stage in lens develoment and growth. However, it should be noted that the antibody labelling of embryonic sections was performed on parafin embedded sections that had been subjected to antigen retrieval protocols, which would have potentially unmasked the TRPV1 and TRPV4 epitopes. Thus in summary, we can conclude that the

differentiation-dependent subcellular expression patterns observed for TRPV1 and TRPV4 in the mouse lens are established in the embryonic lens and maintained into adulthood. In the next section, we focus on the outer cortex of the lens and investigate whether the subcellular distribution of TRPV1 and TRPV4 can be dynamically modulated.

Changes in zonular tension differentially affect the subcellular distribution of TRPV1 and TRPV4

In previous studies conducted in our laboratory, we have shown that the subcellular distribution of a variety of membrane proteins in the outer cortex can be dynamically altered in response to changes in the environment under which lenses are cultured (Chee, et al., 2006;Suzuki-Kerr, et al., 2009). Since TRPV1 and TRPV4 are mechanosensitive, we hypothesised that the relative distribution of the channels between the membrane and the cytoplasm in the outer cortex could be potentially altered by changing the tension applied to the lens via the zonules. *In vivo* the lens is attached to ciliary muscle by the zonules, however, in our *in vitro* experiments the removal of the lens from the eye requires the zonules to be cut, a procedure that potentially reduces the tension applied to the lens capsule. To test whether releasing the tension applied to the lens by the zonules alters the subcellular distribution of TRPV1 and TRPV4, we compared antibody labelling patterns from adult mouse lenses, which were either left in the eye with their zonules intact, or had their zonules cut to remove the lens from the eye. In both cases, the lenses were cultured in AAH for 120 minutes and then fixed and processed for immunocytochemistry. To facilitate a comparison between lenses cultured with and without zonular attachment, the outer cortex of the lens was divided into three zones (Figure 9A) that covered the observed transition from cytoplamic to membraneous labelling observed for both TRPV1 and TRPV4. In lenses cultured for 120 minutes with their zonules cut the subcellular distribution of both TRPV1 (Figure 9B-D) and TRPV4 (Figure 9E-G) was essentially the same as that seen above for lenses removed from the eye and fixed as quickly as possible (Figures 6&7). For TRPV1 this same subcellular labelling pattern was also maintained in lenses that were retained in the eye for 120 minutes and had their zonules intact (Figure 9H-J). However, a dramatically different subcellular labelling pattern was observed for TRPV4 in lenses that were cultured with their zonules attached (Figure 9K-M). In these lenses the subcellular distribution of TRPV4 was exclusively membraneous in Region 2 of the outer cortex (Figure 9L). This result suggests that the tension applied to the lens by the zonules can modulate the association of TRPV4, but not TRPV1, with the membrane to presumably alter TRPV4 function in peripheral fibre cells. Furthermore, the differential effects that the cutting the zonules has on the subcellular labelling patterns obtained for TRPV1 and TRPV4 also helps to confer the specificity of each protein specific antibody used in this study as the effect only occurs for TRPV4.

DISCUSSION

In this study we have performed the first comprehensive mapping of the expression of TRPV1 and TRPV4 in the mouse lens. Using both Western blotting (Figures 1–3) and immunocytochemistry (Figures 4–7) we have shown that TRPV1 and TRPV4 expression is not restricted to the lens epithelium as shown previously (Shahidullah, et al.,

2012;Delamere, et al., 2016) (Martínez-García, et al., 2013), but is also expressed throughout all regions of the adult lens. The overall labelling patterns observed for TRPV1 and TRPV4 in the different regions of the lens were very similar in the adult mouse lens with these labelling patterns being established early on in embryonic development (Figure 8). Labelling was the strongest in the epithelium relative to the fibre cell layer, and the labelling in the surface epithelial and differentiating fibre cells for both TRPV1 (Figure 6) and TRPV4 (Figure 7) was predominately associated with cytoplasmic pools of the two proteins. However, in the deeper lying older fibre cells located in the inner cortex and core of the lens, TRPV1 and TRPV4 labelling was localised almost exclusively to the plasma membrane. This differentiation dependent membrane insertion of TRPV1 (Figure 6) and TRPV4 (Figure 7) became more evident if sections were pre-treated with Triton X-100 to improve access of the antibodies to their respective epitopes located on the cytoplasmic tail of each protein. In addition, to this differentiation-dependent membrane insertion in deeper fibre cells, we also observed a dynamic insertion of TRPV4, but not TRPV1 into plasma membranes of peripheral fibre cells in a discrete zone of the outer cortex of lenses that were fixed *in situ* with their zonules still attached to the ciliary body (Figure 9). Taken together these observations suggest that TRPV1 and TRPV4 may be playing distinctly different functional roles in the different regions of the lens. The implications for overall lens function of these regional differences in TRPV1 and TRPV4 expression patterns is discussed below.

Since TRPV1 and TRPV4 are both involved in dual feedback pathways that maintain cell volume (Shahidullah, et al., 2012)(Shahidullah, Mandal et al. 2018), and hydrostatic pressure (Gao, et al., 2015) in the lens, it is not surprising that TRPV1 and TRPV4 exhibit essentially similar labelling patterns not only throughout all regions of the adult lens, but also during embryonic development. A similar co-expression of TRPV1 and TRPV4 channels has been observed in some ganglion cells of the mouse and rat retina. In these cell types the elevation of intraocular pressure resulted in an increased expression of TRPV1, which was associated with protection from ganglion cell apoptosis (Sappington, et al., 2015;Lakk, et al., 2018). Interestingly, Sappington et al., found that mRNA levels of TRPV1 and TRPV4 were inversely related to changes in intraocular pressure, indicating that a similar feedback mechanism may be involved in the neuroprotection of retinal ganglion cells (Sappington, et al., 2015). In the lens epithelium and peripheral fibre cells, TRPV1 and TRPV4 are primarily located to the cytoplasm. However, the subcellular distribution of TRPV1 and TRPV4 undergoes a shift from a cytoplasmic pool to the plasma membrane with distance into the lens, as fibre cells differentiate and become internalised. A similar differentiation-dependent membrane insertion has been observed for a number of membrane proteins in the lens (Merriman–Smith, et al., 1999;Grey, et al., 2003;Chee, et al., 2006;Chee, et al., 2006;Lim, et al., 2006). This has been attributed the fact that as fibre cells differentiate they lose their cellular organelles and hence the ability to perform *de novo* protein synthesis (Bassnett, 2002). Hence, differentiating fibre cells tend to synthesis membrane proteins and store them in a cytoplasmic pool from which they can insert them into the membrane at specific stages of fibre cell differentiation (Donaldson and Lim, 2008).

The observed differentiation-dependent membrane insertion of TRPV1 and TRPV4 became more apparent when antibody labelling was performed in the presence of the detergent Triton X-100 to unmask the antibody binding sites on either TRPV1 (Figure 6) or TRPV4

(Figure 7). Both proteins contain large intracellular N- and C terminal domains. In other tissues these domains have been shown to mediate interaction between a large variety of cytoskeletal proteins (Clark, et al., 2008), junctional proteins (Akazawa, et al., 2013;Janssen, et al., 2016) and cell signalling molecules (Numazaki, et al., 2003;Rosenbaum, et al., 2004;Fischer, et al., 2013). Indeed some reports in the retina have also suggested a direct interaction between TRPV1 and TRPV4 (Sappington, et al., 2015). Hence, there is no shortage of potential interacting proteins that could be responsible for the masking of TRPV1 and TRPV4 antibody labelling observed in the inner cortex of the lens. Identifying these interaction proteins and what role they play in modulating TRPV1 and TRPV4 function specifically in the inner cortex will be the focus of future studies.

Since TRPV1 and TRPV4 function as non-selective cation (NSC) channels, it is entirely possible that they could be mediating the distinct currents observed previously in patch clamp studies performed on isolated differentiating fibre cells (Webb and Donaldson, 2008;Gunning, et al., 2012). In these studies, the physical isolation of fibre cells from the lens caused the isolated cells to swell, and activate a NSC conductance that was inhibited by Gd^{3+} (Webb and Donaldson 2008). Subsequent exposure of fibre cells isolated in the presence of Gd^{3+} to hypertonic challenge, activated a second distinct NSC conductance that was inhibited by La^{3+} (Gunning, et al., 2012). This activation of two pharmacologically distinct NSC conductances that differentially respond to osmotic challenge is consistent with more recent studies that showed cell shrinkage and swelling activated TRPV1 (Shahidullah, et al., 2018) and TRPV4 (Shahidullah, et al., 2012) channels, respectively. Since these functional studies all involved the use of cells from the lens periphery, where the observed TRPV1 and TRPV4 labelling is predominately associated with the cytoplasmic pool, it suggests that the majority of TRPV1 and TRPV4 channels are constitutively inactive in this region of the lens. Furthermore, their activation may involve dynamic trafficking to the plasma membrane in response to a stimuli, such as a change in cellular volume or hydrostatic pressure. In other tissues, membrane insertion of TRPV1/4 channels to the membrane has been demonstrated in response to a variety of external stimuli (Stein, et al., 2006; Zhang, et al., 2008; Xing, et al., 2012; Baratchi, et al., 2016). In this current study, we have shown that the membrane insertion of TRPV4, but not TRPV1, is altered by the attachment of the lens to the ciliary body via the zonules (Figure 9). This result suggests that the tension applied to the lens via the zonules may be regulating the recruitment of mechanosensitive TRPV4 channels into the membranes of fiber cells in the lens periphery. This preliminary data supports the idea that in the peripheral fibre cells TRPV4, and potentially TRPV1, exists as a pool of channels that can be dynamically recruited to the plasma membrane in response to changes in external stimuli. Determining whether that stimuli is indeed zonular tension will be the focus of future work.

In contrast to differentiating fibre cells in the outer cortex, TRPV1 and TRPV4 labelling in the inner cortex and core of the mouse lens was located exclusively in the membranes of mature fibre cells (Figure 6 and 7). The membrane location of these channels, would tend to suggest that in these deeper regions of the lens TRPV1 and TRPV4 are constitutively active NSC channels. If this was correct, then TRPV1 and TRPV4 can be considered as candidates to mediate the cation conductance of fibre cells, which to date has not been identified at the molecular level (Mathias, et al., 1997). Since TRPV1 and TRPV4 are mechanosensitive

NSC channels, it is also conceivable that in the core of the lens, the activity of channels could be responding to changes in the hydrostatic pressure gradient, and altering the Na^+ conductance of mature fibre cells. Alteration of the fibre cell Na^+ conductance would in turn change the magnitude of the circulating Na^+ current that drives water transport through the lens gap junctions to generate the hydrostatic pressure gradient (Gao, et al., 2011;Gao, et al., 2013), adding yet another component to the emerging model of how hydrostatic pressure is regulated in the lens (Gao, et al., 2015).

In summary, the mapping of the subcellular expression patterns of the mechanosensitive NSC channels TRPV1 and TRPV4 throughout different regions of the embryonic and adult mouse lens has revealed not only that the two channels are expressed in all regions of the lens, but also that their subcellular distribution changes in the different regions of the lens. Determining the functionality and regulation of TRPV1 and TRPV4 channels in the different regions of the lens will be the focus of future studies.

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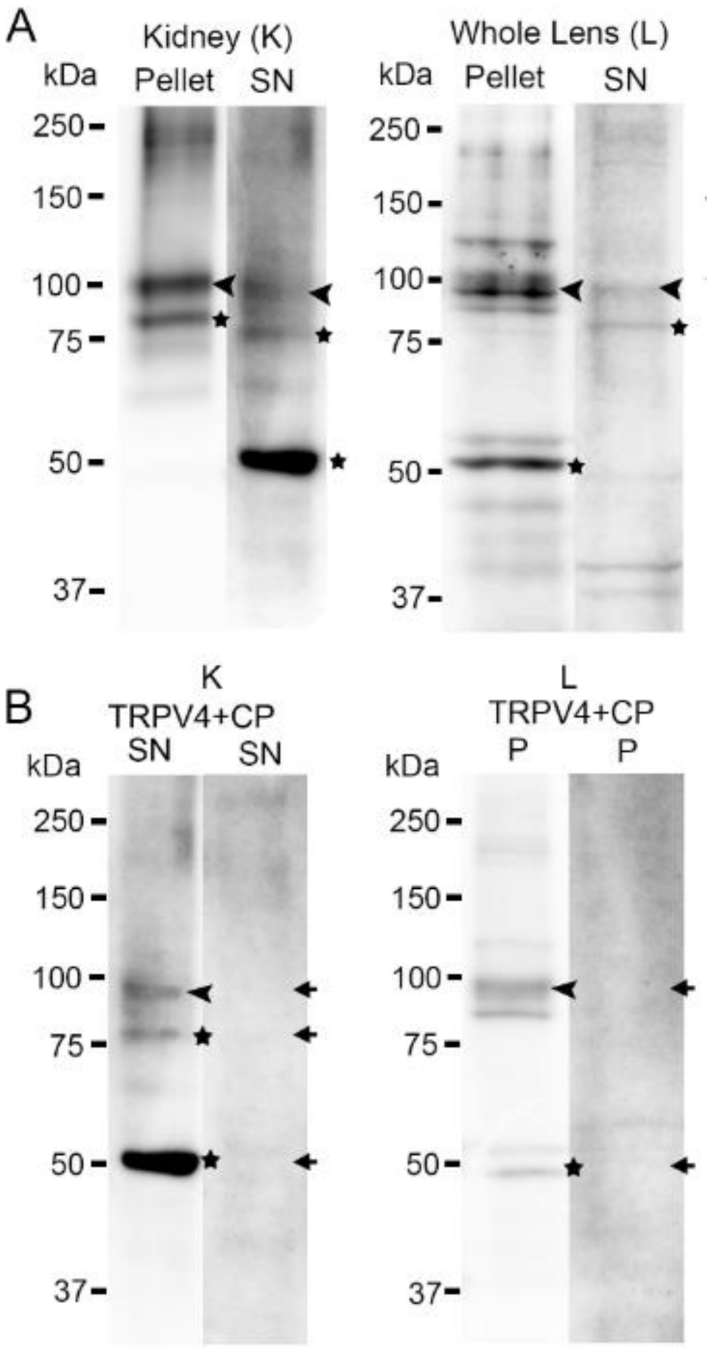

Figure 1. Identification of TRPV1 in the mouse lens.

Western blot of the protein fractions obtained from either the pellet (P) or supernatant (SN) of homogenates obtained from a mouse kidney (K), that served as a positive control, or ten whole mouse lenses (WL). Either 50 μg of kidney or 200 μg whole lens tissue were loaded in each lane. Labelling patterns obtained using a TRPV1 antibody (A) or following preincubation of the TRPV1 antibody with its coresponding antigenic control peptide (B). Arrowheads and stars indicate the expected and additional band sizes of TRPV1, respectively. Note the absence of specific bands in the presence of the control peptide (CP).

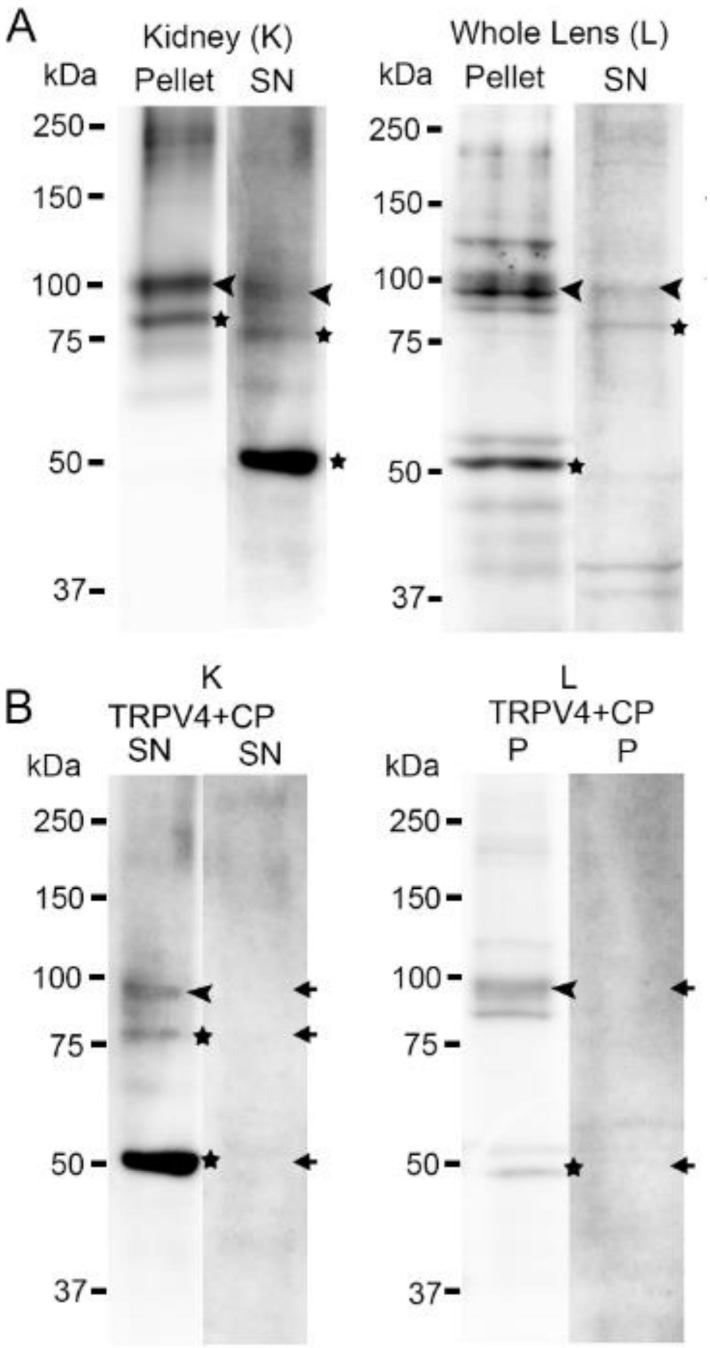

Figure 2. Identification of TRPV4 in the mouse lens.

Western blot of the protein fractions obtained from either the pellet (P) or supernatant (SN) of homogenates obtained from a mouse kidney (K), that served as a positive control, or ten whole mouse lenses (WL). Either 50 μg of kidney or 200 μg whole lens tissue were loaded in each lane. Labelling patterns obtained using a TRPV4 antibody (A) or following preincubation of the TRPV4 antibody with its coresponding antigenic control peptide (B). Arrowheads and stars indicate the expected and additional band sizes of TRPV4, respectively. Note the absence of specific bands in the presence of the control peptide (CP).

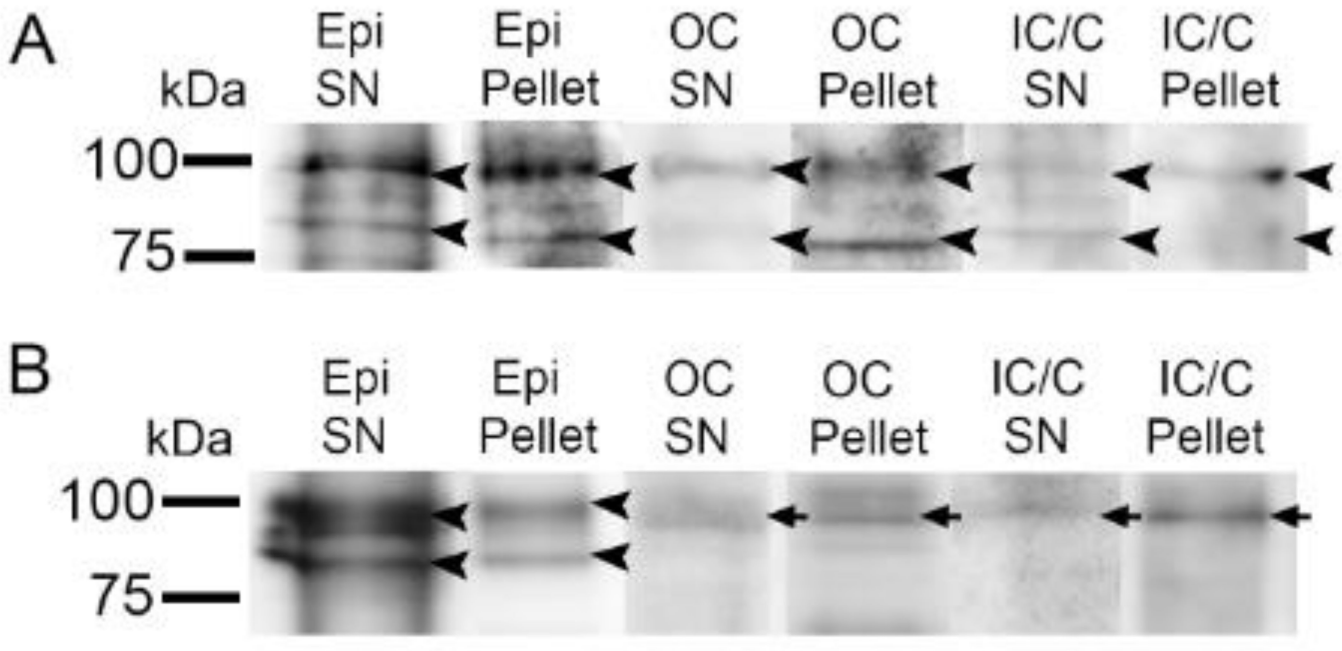

Figure 3. Regional distribution of TRPV1 and TRPV4 in the mouse lens.

Western blots of protein fractions obtained from either the pellet (P) or supernatant (SN) of homogenates obtained from the epithelium (Epi), outer cortex (OC) or combined inner cortex/core (IC/C) labelled with TRPV1 (A) or TRPV4 antibodies (B). Either 200 μg supernatant or 50 μg pellet fractions was loaded in each lane with an exception of the epithelial cell fraction where 100 μg supernatant was used. Arrowheads indicate the predicted molecular weights of TRPV1 and TPRV4.

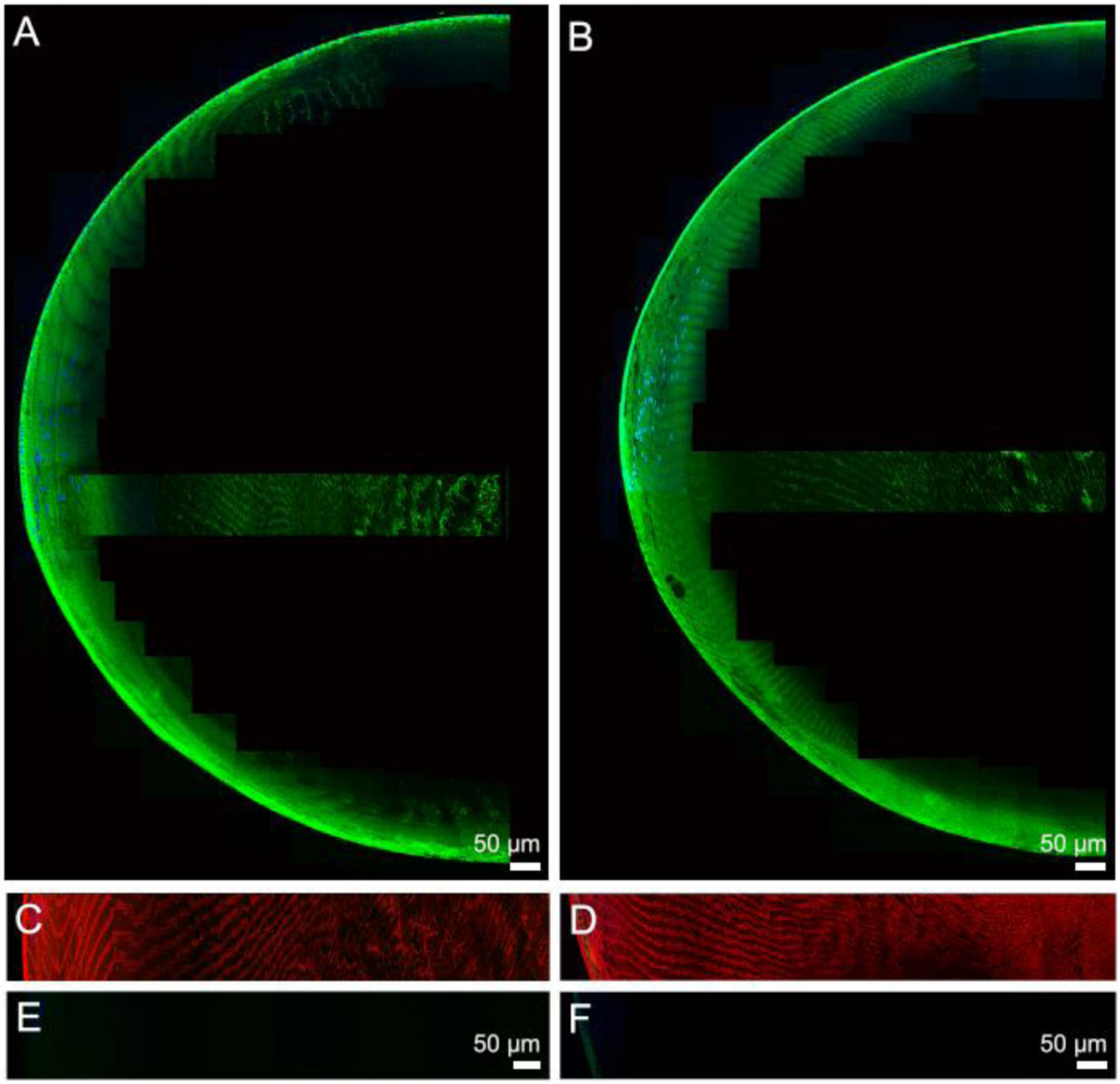

Figure 4. Overview of TRPV1 and TRPV4 expression patterns in the adult mouse lens.

Low power image montage of an axial section taken from the mouse lens and labelled with TRPV1 (A) and TRPV4 (B) antibodies showing that TRPV1 and TRPV4 labelling extends from the anterior to posterior poles and from the equatorial epithelium to the lens core. The specificity of the immunolabeling signal was verified by pre-incubation of each antibody with its corresponding antigenic peptides which resulted in a complete loss of signal for TRPV1 (C, E) and TRPV4 (D,F) from capsule to core, although there was some faint labelling for TRPV4 observed in the epithelium (D, F). In these images, cellular membranes were labelled with WGA (red), TRPV proteins were labelled with peptide specific C-terminal antibodies (green) and nuclei were labelled with DAPI (blue).

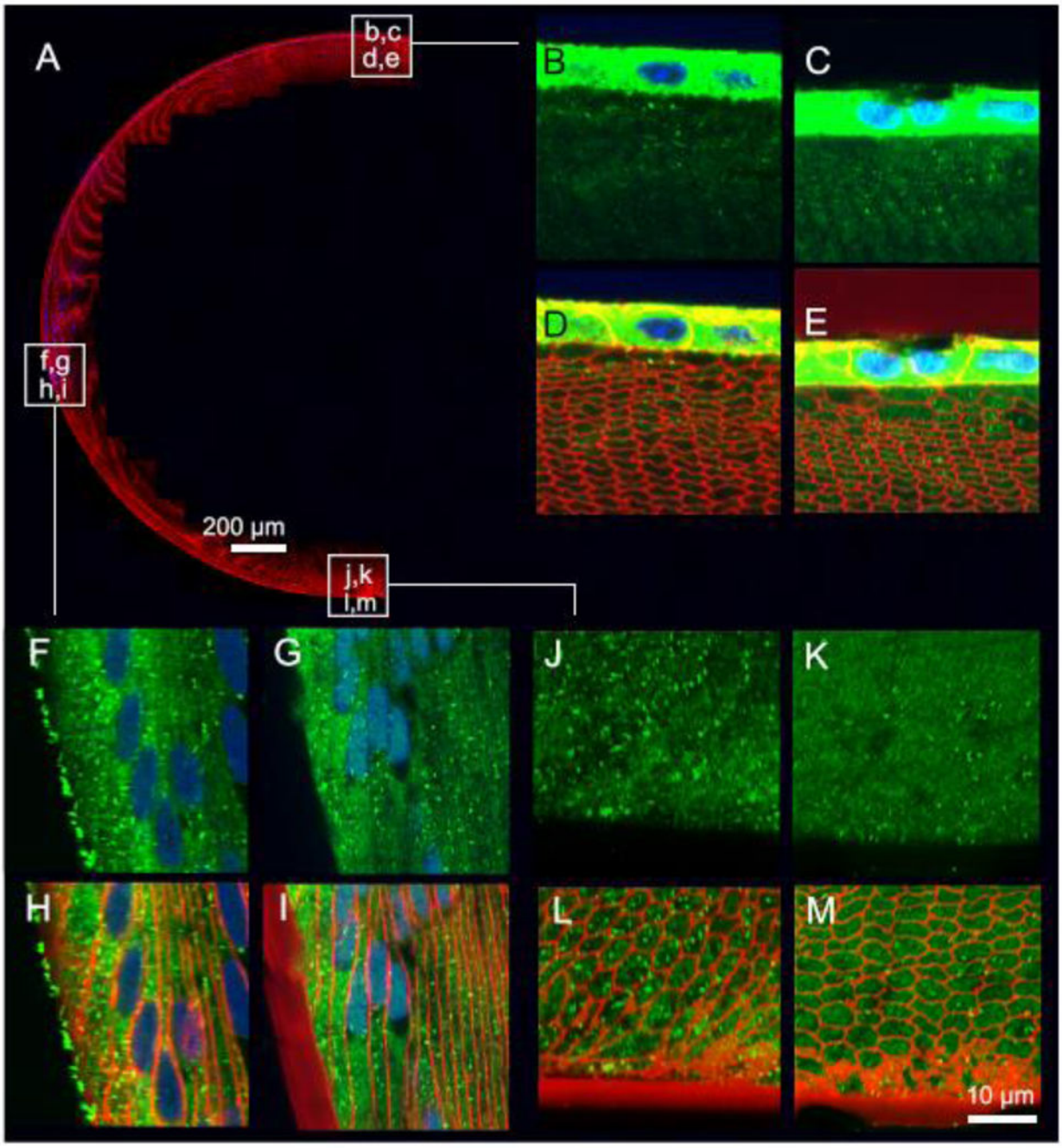

Figure 5. Subcellular distribution of TRPV1 and TRPV4 in the epithelium and peripheral fibre cells in the adult mouse lens.

(A) Image montage of a mouse axial section labelled with the membrane marker WGA (red) showing the locations (boxes) where high magnification images (B-M) were obtained. The subcellular localisation of both TRPV1 (B, D) and TRPV4 (C, E) is cytoplasmic in the epithelium and fibre cells at the anterior pole and remained cytoplasmic in the fibre cells at the equator (TRPV1 - F, H; TRPV4 - G, I) and posterior pole (TRPV1 - J, L; TRPV4 - K, M). For clarity, either only peptide specific TRPV immunolabeling is presented in green

(TRPV1 - B, F, J; TRPV4 - C, G, K), or in combination with the membrane marker WGA (red) (TRPV1 - D, H, L; TRPV4 - E, I, M). Nuclei are labelled with DAPI (blue).

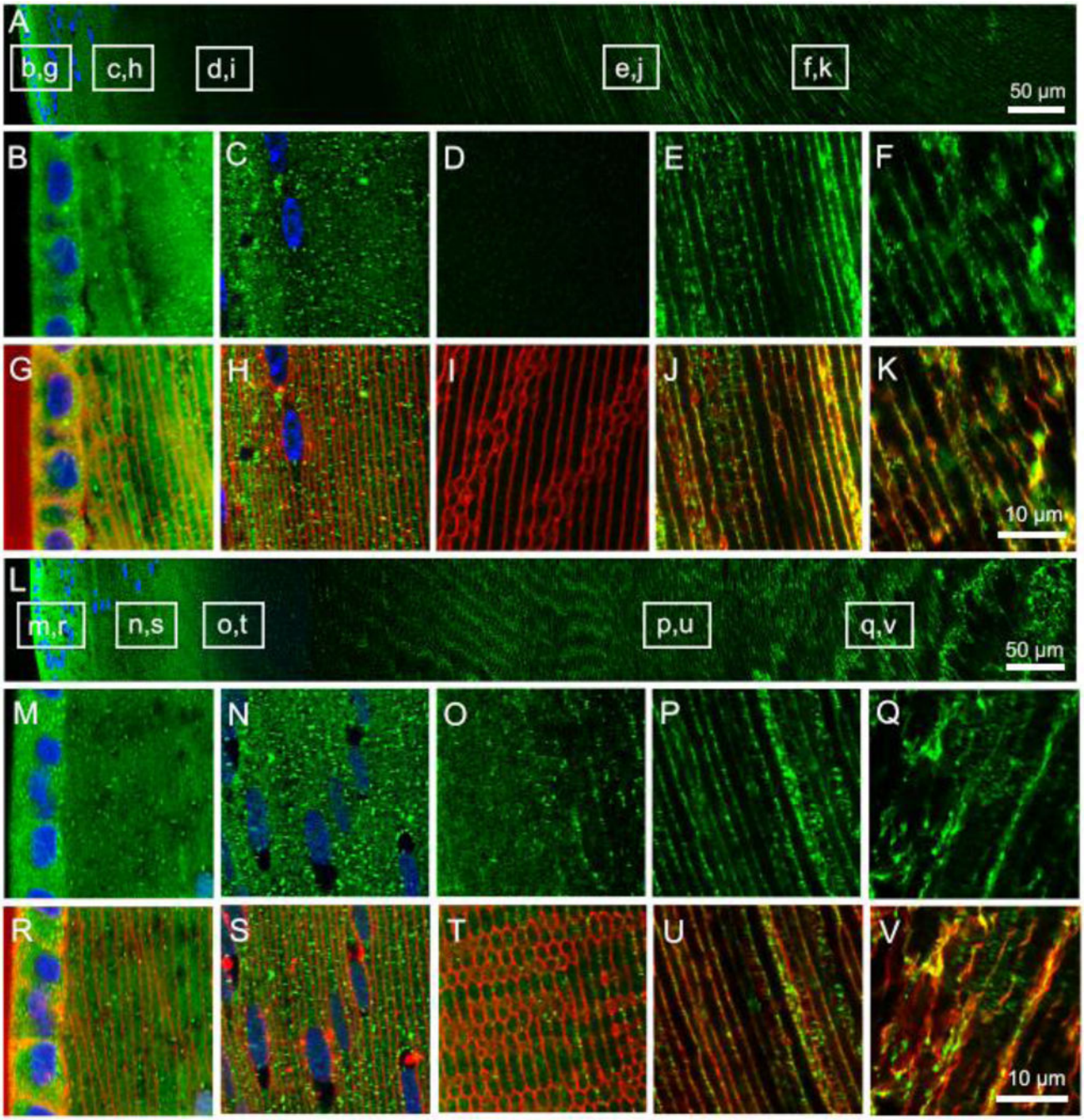

Figure 6. Subcellular distribution of TRPV1 from the lens equator to the core.

Image montage collected from the lens equator to the core in axial sections pre-incubated in the absence (A) or presence (L) of Triton X-100. TRPV1 labelling (green) was found in all regions of the lens except for a narrow region in the inner cortex where a marked localised decrease in signal was detected in the absence of pre-treatment with Triton X-100 (A). Boxes indicate the locations where high magnification images (B-K & M-V) were obtained. TRPV1 immunolabeling was strongly associated with the cytoplasm in the epithelial and differentiating fibre cells around the equator (B, G) and became discretely punctate in the

fibre cells of the deeper outer cortex (C, H). The signal abruptly decreased in the outer cortex-inner cortex transition zone (D, I) and recovered in the deeper inner cortex although was translocated to the membranes (E, J) and remained membranous in the core of the lens (F, K). The overall signal detection in the inner cortex region was improved by treatment with Triton X-100 (L) revealing a membranous subcellular localisation of TRPV1 (O, T). No additional changes in the subcellular localisation of TRPV 1 were observed with treatment of Triton X-100 in the outer cortex (M, R, N and S), deeper inner cortex (P, U) and core of the lens (Q, V). Membranes were labelled with WGA marker (red) and nuclei were labelled with DAPI (blue).

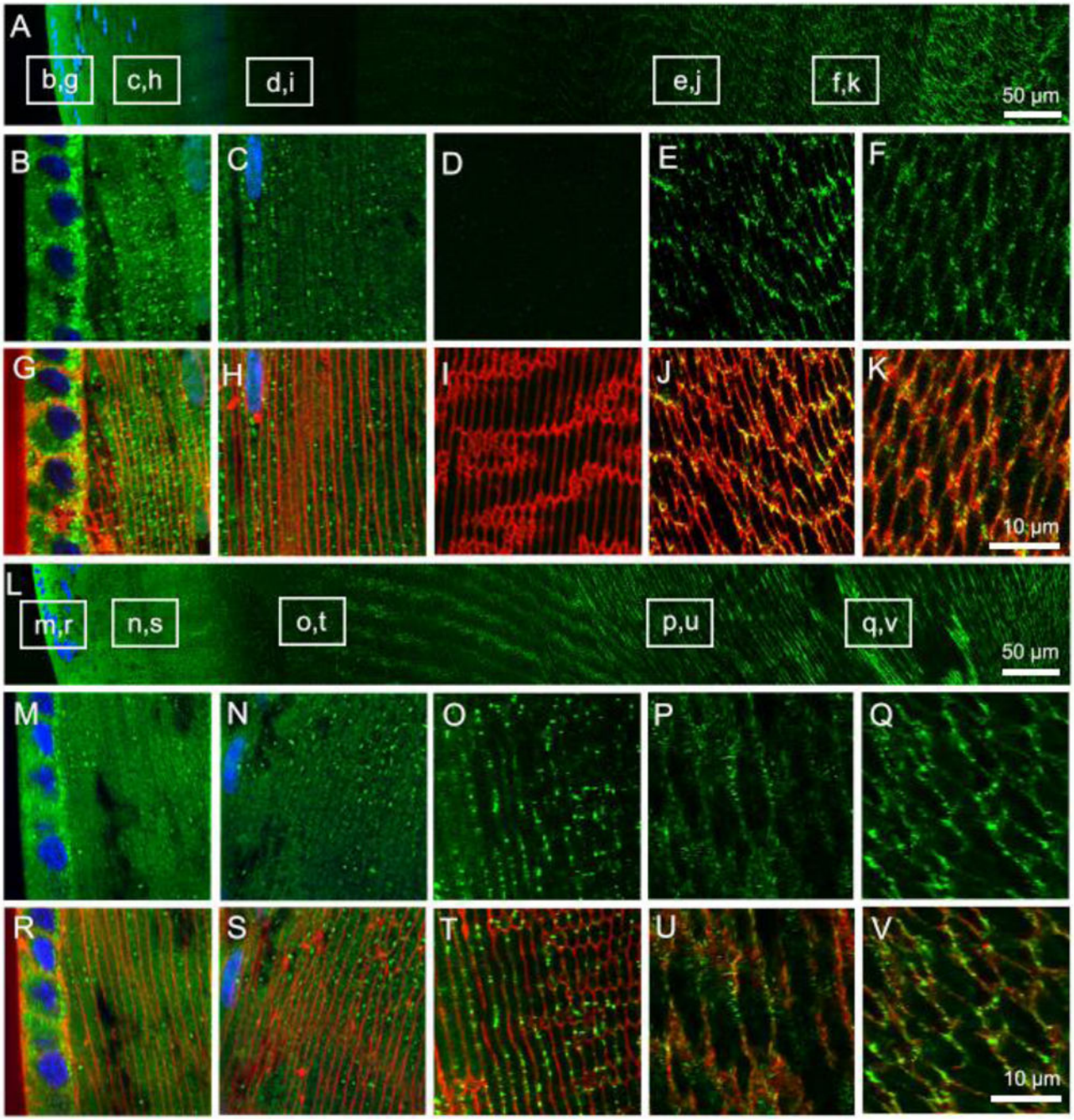

Figure 7. Subcellular distribution of TRPV4 from the lens equator to the core.

Image montage collected from the lens equator to the core in axial sections pre-incubated in the absence (A) or presence (L) of Triton X-100. TRPV4 labelling (green) was found in all regions of the lens except for a narrow region in the inner cortex where a marked localised decrease in signal was detected in the absence of pre-treatment with Triton X-100 (A). Boxes indicate the locations where high magnification images (B-K & M-V) were obtained. TRPV4 immunolabeling was strongly associated with the cytoplasm in the epithelial and differentiating fibre cells around the equator (B, G) and became discretely punctate in the

fibre cells of the deeper outer cortex (C, H). The signal abruptly decreased in the outer cortex-inner cortex transition zone (D, I) and recovered in the deeper inner cortex although was translocated to the membranes (E, J) and remained membranous in the core of the lens (F, K). The overall signal detection in the outer cortex-inner cortex transition zone region was improved by treatment with Triton X-100 (L) revealing a membranous subcellular localisation of TRPV4 (O, T). No additional changes in the subcellular localisation of TRPV4 were observed with treatment of Triton X-100 in the outer cortex (M, R, N and S), deeper inner cortex (P, U) and core of the lens (Q, V). Membranes were labelled with WGA marker (red) and nuclei were labelled with DAPI (blue).

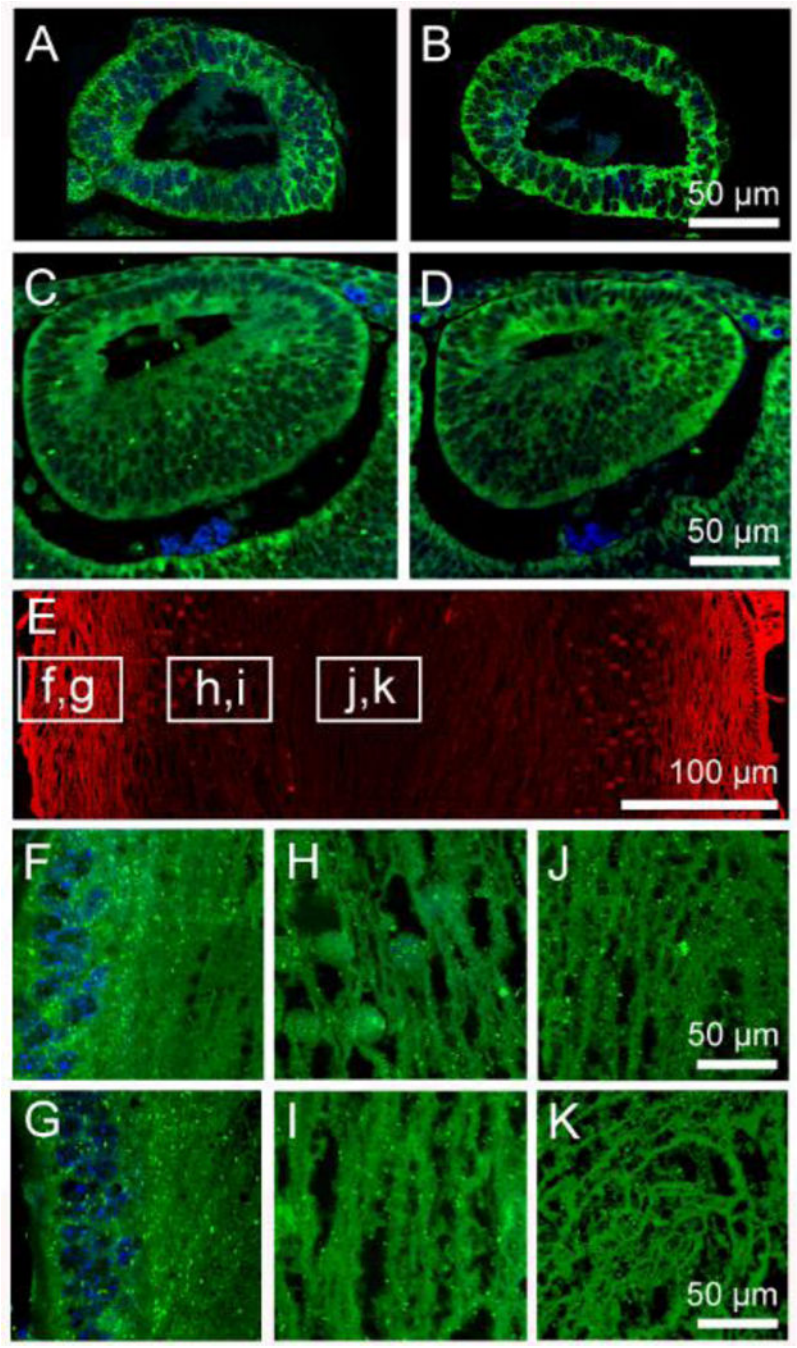

Figure 8. Cellular and subcellular distribution of TRPV1 and TRPV4 during the lens embryonic development.

At embryonic day 10 (E10) the lens vesicle shows strong membrane expression for both TRPV1 (A) and TRPV4 (B). This membrane expression is maintained at E11 day for TRPV1 (C) and TRPV4 (D). Just before birth at E18.5 the subcellular distribution of TRPV1 and TRPV4 was examined from the periphery to the core by taking high magnification images designated by the white boxes in panel E. Both TRPV1 (F) and TRPV4 (G) labelling was associated with the cytoplasm in the periphery of the outer cortex, but was located in the membrane in the inner cortex (TRPV1 - H; TRPV4 - I) and core (TRPV1 - J and TRPV4 -

K) of the lens. Nuclei are labelled with DAPI (blue), TRPV1 and TRPV4 antibodies (green) and membranes with WGA (red).

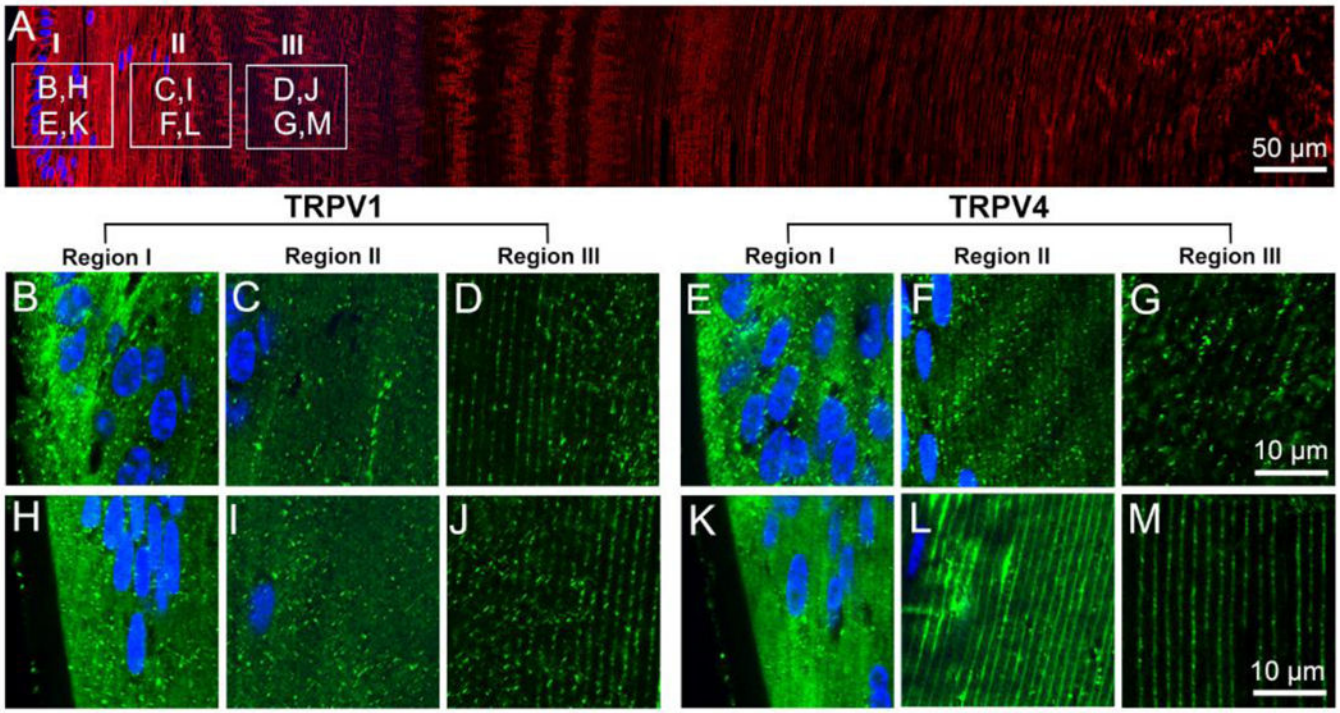

Figure 9. Dynamic insertion of TRPV4 but not TRPV1 in differentiating fibre cells.

(A) A montage of images collected from the lens equator to the core from an axial section labelled with WGA (red) showing 3 regions (boxes) from the bow region of the adult mouse lens where the subcellular distribution of TRPV1/4 transitions from being predominately cytoplasmic to membranous as the fibre cells differentiate. High power images from the 3 regions were taken from adult mouse lenses cultured for 120 minutes in AAH in which either the zonules were cut (B-G) or intact (H-M) and labelled with either TRPV1 (B-D & H-J) or TRPV4 (E-G & K-M) antibodies (green). DAPI-labelled nuclei (blue) show the bow region of the lens. Note: In region II incubation of lenses with the zonules left intact shifted the subcellular distribution of TRPV4, but not TRPV1, from cytoplamsic to membraneous.