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The cataract-associated protein TMEM114, and TMEM235, are glycosylated transmembrane proteins that are distinct from claudin family members

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ABSTRACT

A novel gene, TMEM114, was annotated as a member of the claudin gene family and was subsequently associated as a cause of autosomal dominant cataract because of a translocation in its putative promoter. Our bioinformatic and molecular analyses of TMEM114, and the closely related TMEM235, demonstrate that these proteins are more closely related to members of the voltage dependent calcium channel gamma subunit family. TMEM114 and TMEM235 differed from claudins in terms of localisation in polarised epithelial cells and by the presence of N-linked glycans. By gene expression knockdown in *Xenopus tropicalis* we also demonstrate a role for Tmem114 in eye development.

Structured summary of protein interactions:
Claudin-2 and **ZO-1** colocalize by fluorescence microscopy (View interaction).
ZO-1 and **Tmem114** colocalize by fluorescence microscopy (View interaction).

1. Introduction

Claudins are transmembrane proteins that form the main constituent of tight junctions, which modulate the paracellular permeability of epithelial cell layers [1]. A PSI-BLAST of the human genome identified a number of predicted proteins that showed homology to claudins [2]. The protein XP_208930 was described as a 102 amino acid (aa) protein encoded by two exons. However, the full-length four exon gene was subsequently identified by virtue of its close proximity to a balanced translocation in a family with congenital cataract [3]. The encoded 223 aa protein was named transmembrane protein 114 (TMEM114) and had a predicted four transmembrane topology [3]. The 196 aa protein XP_211287 was also identified in the search and was shown to be expressed at the mRNA level in human brain [2]. XP_211287 was subsequently re-annotated as the 223 aa protein Transmembrane protein 235 (NP_001191139.1) by incorporating exon 2, which is present in other species.

Claudins form the majority of the Pfam00822 family of proteins which also includes epithelial membrane proteins-1, -2 and -3, peripheral myelin protein 22, the lens intrinsic membrane protein MP20 and the more distantly related voltage dependent calcium channel gamma subunits (CACNGs) [4]. Family members contain four transmembrane domains (TMDs) with two extracellular loops, the first of which contains a conserved W-GLW-C-C motif (Prosite motif PDOC01045). Here we report the relationship of TMEM114 and XP_946151 to the Pfam00822 proteins, characterise the proteins in an in vitro model system, and report the role of Tmem114 in eye development.

2. Materials and methods

2.1. Phylogenetic tree construction

Human protein sequences (Supplementary Table 1) were aligned using ClustalW. Phylogenetic trees were constructed with the Seaview software using the neighbour-joining method based on observed distances with gap sites ignored. Bootstrap proportions [5] were used to assess the robustness of tree with 10 000

Abbreviations: TMEM114, transmembrane protein 114; TMEM235, Transmembrane protein 235; CLDN, claudin; CACNG, voltage dependent calcium channel gamma subunit; EMP, epithelial membrane protein; PMP, peripheral myelin protein; TMD, transmembrane domain; aa, amino acid; WT, wildtype; MO, morpholino

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bootstrap replications. Bootstrap values of less than 50% were not considered significant. As recommended, an outgroup protein (clarin-1) was used in the analysis [4,6].

2.2. Cloning and site-directed mutagenesis

Murine Tmem114 coding sequence was amplified with or without a C-terminal V5-tag from the RIKEN 4930511J11 cDNA IMAGE clone. Murine Tmem235 was amplified with or without a C-terminal V5-tag from testis cDNA. PCR products were cloned into pGEM-T easy vector (Promega, UK) and subcloned into the expression vector pcDNA3.1(−) (Invitrogen, UK). Point mutations were introduced by site-directed mutagenesis using the QuikChange Lightning Kit (Stratagene, UK).

2.3. Cell Culture, immunoblotting and de-glycosylation

Madin–Darby canine kidney II (MDCK II) cells were cultured and transfected as previously described [7]. Stable expressing cells were generated by transfecting linearised plasmids with Lipofectamine LTX (Invitrogen) and isolated by selection in media containing 1 mg/ml Geneticin (Invitrogen). Lysates were resolved and blotted by standard methods using antibodies detailed in Supplementary Table 2. For deglycosylation assays, lysates were treated with PNGase F (Roche, UK) or Endo H (New England Biolabs, UK) for 2 h at 37 °C.

2.4. Immunofluorescence

Immunofluorescence experiments were performed 72 h after stable MDCK II cells grown on Transwell filters (Corning, USA) had reached confluency. Cells were fixed with methanol:acetone (1:1) at −20 °C for 5 min and then blocked, labelled with antibodies and visualised by confocal microscopy as previously described [7]. Antibodies and dilutions in Supplementary Table 2. The localisation of the Tmem114 glycosylation mutants was detected using transiently transfected MDCK II cells grown on Transwell filters analysed 48 h post-transfection.

2.5. RNA extraction and RT-PCR

RNA extracted from human eyes at Carnegie stage 21 and foetal stage (FS) 2 was obtained from the MRC-Wellcome Trust Human Developmental Biology Resource at Newcastle University, UK, whose ethical approval covers its use in all registered UK research projects. RNA was extracted from *Xenopus* tissue using the Qiashredder and RNAeasy kits (Qiagen, UK). RT-PCR primers (Supplementary Table 3) were designed to encompass multiple exons.

2.6. X. tropicalis embryo injection, culture and phenotyping

Two non-overlapping translation blocking morpholino oligonucleotides (MOs) (Supplementary Table 4) were designed against

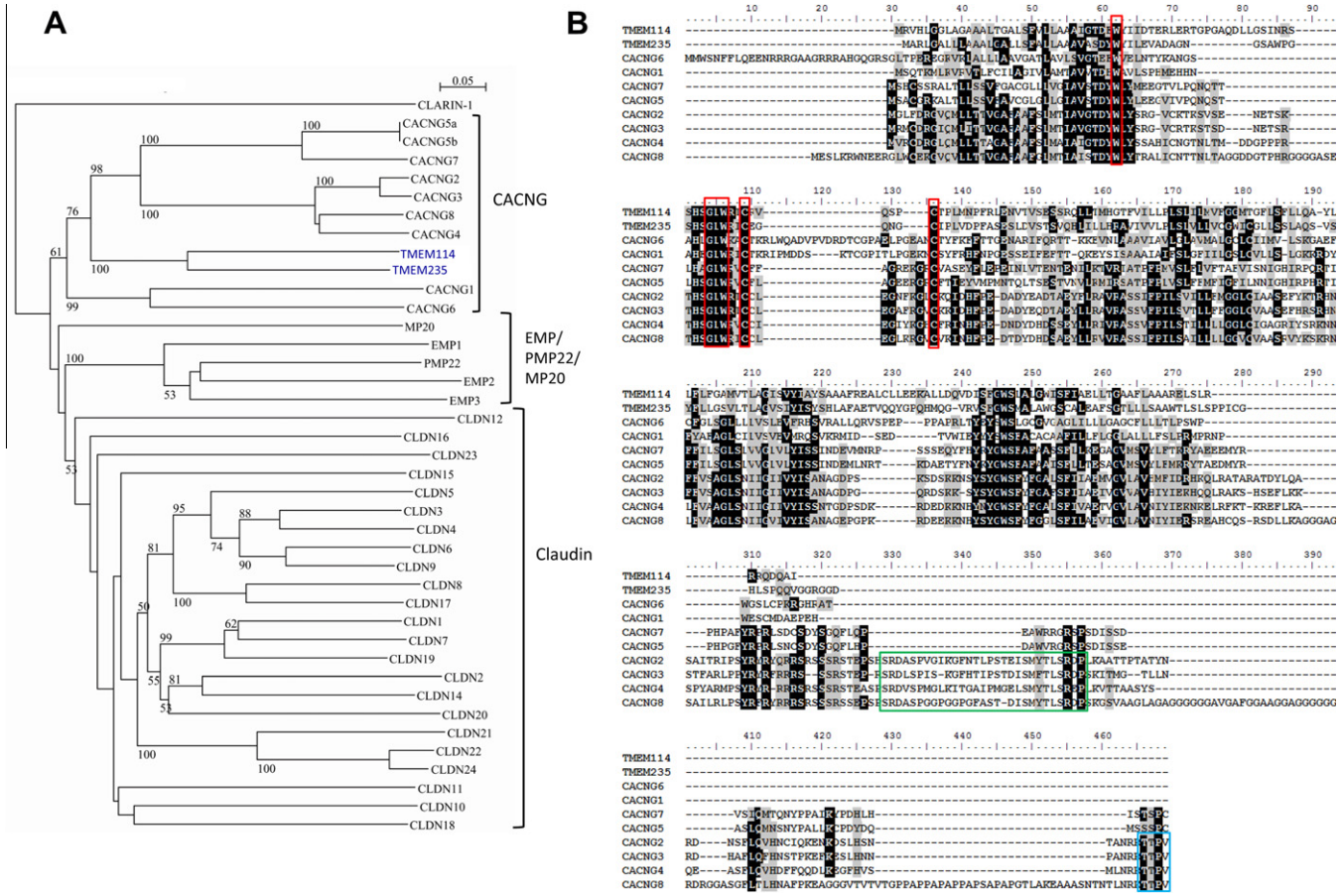

Fig. 1. (A) Phylogenetic tree of human TMEM114, TMEM235 and Pam00822 family members. A phylogram of full-length protein sequences was constructed using ClustalW and the neighbour-joining method. The protein clarin-1 was used an outgroup. The scale bar indicates the branch length that corresponds to 0.05 substitutions per position. Numbers indicate bootstrap values as the percentage of 10 000 replicates for each branch. Bootstrap values of less than 50 were considered non-significant and are not displayed. (B) Multiple alignment of TMEM114, TMEM235 and the CACNGs. Full-length amino acid sequences were aligned with ClustalW. Residues of the conserved W-GLW-C-C motif are boxed in red. The nPIST- and PDZ-binding domains of the TARPs are boxed in green and blue, respectively. Black highlight = identical amino acid present in ⩾50% of sequences. Grey highlight = similar amino acids present in ⩾50% of sequences.

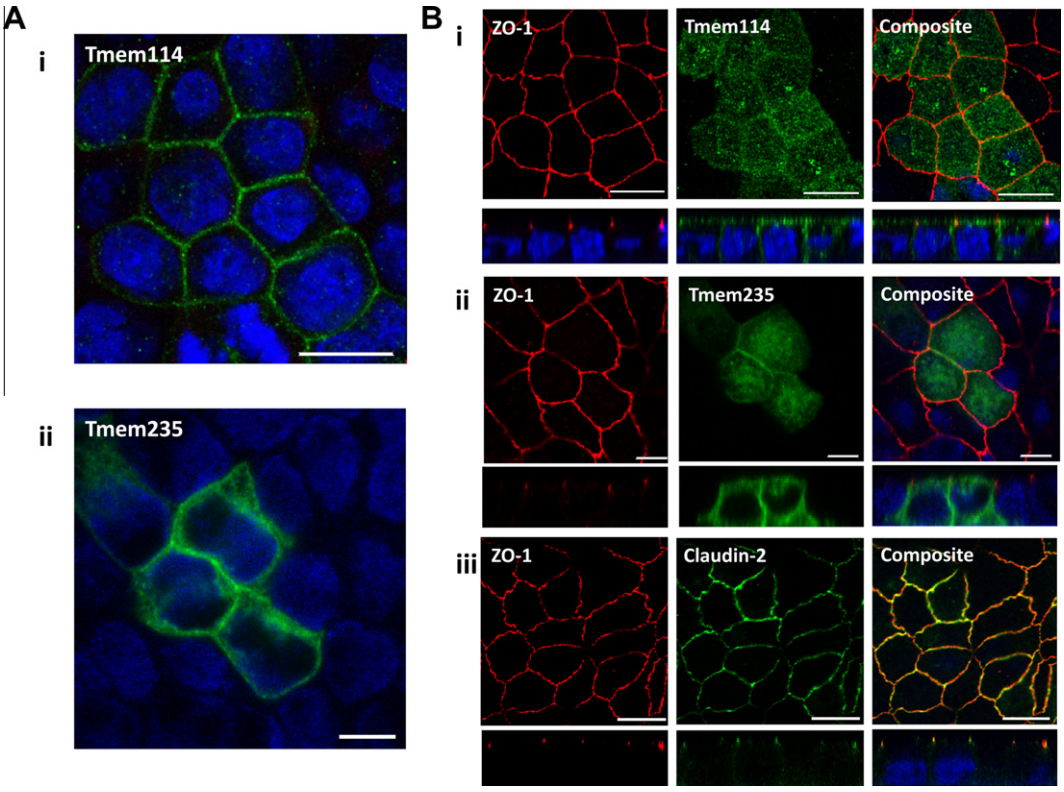

Fig. 2. Localisation of Tmem114 and Tmem235 in polarised MDCK II cells. (A) (i) Tmem114 localises to the plasma membrane. (ii) Tmem235 is mainly detected in the cytoplasm but displays some lateral membrane localization. (B) The localisation of (i) Tmem114, (ii) Tmem235 and, (iii) claudin-2 (green) in relation to the tight junction protein ZO-1 (red). Nuclei were stained with DAPI (blue). Z-stacked images are shown below each X–Y image. Scale bar = 10 μm.

xtTmem114 according to the manufacturer's guidelines (Gene Tools LLC, USA). Standard control MOs were obtained from Gene Tools. Injections and culturing were performed as described previously [8]. Phenotype scoring of MO-injected and uninjected embryos was performed at stages 41–43 [9].

3. Results

3.1. Phylogenetic relationship of TMEM114 and TMEM235 to the Pfam00822 family

In this phylogenetic tree the claudins form distinct branches from the CACNGs and the EMP/PMP22/MP20 family members (Fig. 1A), as previously described [4]. TMEM114 and TMEM235 show show the greatest similarity to each other within the CACNG branch.

3.2. Localisation of mouse Tmem114 and Tmem235 in polarised MDCK II cells

Specific custom rabbit polyclonal antibodies were generated against the final 14 aa of the intracellular C-termini of mouse Tmem114 and Tmem235 (data not shown). The custom antibodies did not label non-transfected cells which acted as a control for antibody specificity. The localisation of Tmem114 and Tmem235 proteins was determined in stably expressing polarised MDCK II cells. In polarised MDCK II cells claudin-2 co-localises with the tight junction marker ZO-1 (Fig. 2Biii). Tmem114 localised at the lateral (Fig. 2Ai) and apical (Fig. 2Bi) membranes and displayed minimal co-localisation with ZO-1 (Fig. 2Bi). Tmem235 was detected in the cytoplasm with some lateral localisation (Fig. 2Aii, Bii), distinct from ZO-1 (Fig. 2Bii). Comparable localisation was obtained for C-terminal V5-tagged versions of Tmem114 and Tmem235 when detected with an anti-V5 antibody (data not shown).

3.3. Tmem114 and Tmem235 are glycoproteins

When detected by western blot, Tmem114 was apparent as a series of bands with a higher molecular mass than predicted (24 kDa) (Fig. 3A). Predicted, and conserved, N-glycosylation sites were identified in the first extracellular loop at residues p.N54 and p.N88 (equivalent of p.N55 and p.N89 in human TMEM114). When these glycosylation sites were mutated, or when wildtype Tmem114 was treated with the de-glycosylating enzyme PNGase F, a single band of 24 kDa was observed, thus confirming that Tmem114 is N-glycosylated at p.N54 and p.N88. The presence of both glycosylation sites is required for the plasma membrane localization of Tmem114 (Fig. 3B). Human TMEM235 contains a single predicted glycosylation site in the first extracellular loop (p.N41), but murine Tmem235 lacks this consensus glycosylation motif [10]. Tmem235 contains an atypical N-X-C motif [11] and shows partial glycosylation as detected by sensitivity to PNGase F. Sensitivity to the enzyme Endo H, which cleaves N-linked mannose-rich oligosaccharides but not N-linked complex oligosaccharides, is an indicator for ER localization. All Tmem235 bands were sensitive to Endo H which suggests it is located in the ER (Fig. 3C).

3.4. TMEM114 and TMEM235 expression in the developing human

As *Tmem114* and *TMEM235* are expressed in the adult mouse eye and brain [3], and adult human brain [2], respectively, we sought to determine the expression of *TMEM114* and *TMEM235*

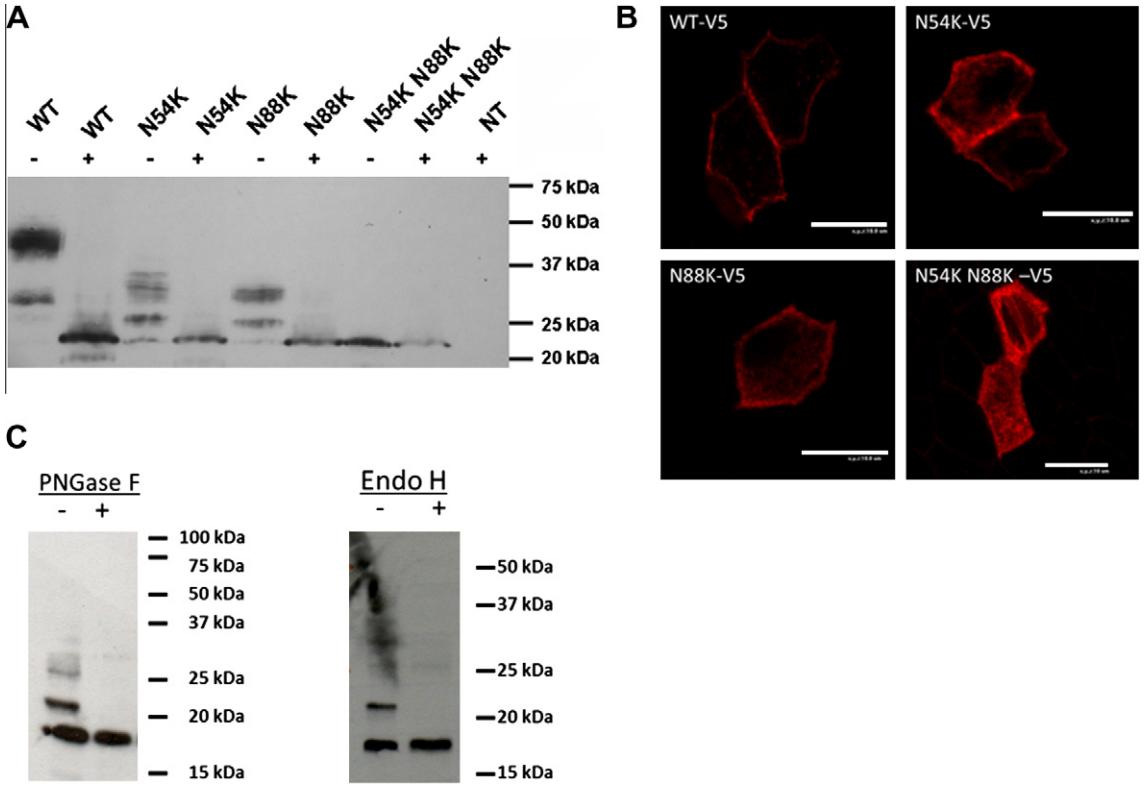

Fig. 3. Identification of the glycosylated residues of mTmem114. (A) Western blots of V5-tagged Tmem14 wildtype and glycosylation mutants using anti-V5 antibody. Single glycosylation mutants (p.N54K and p.N88K) are detected at a lower molecular mass than the wildtype (WT). Treating the wildtype and single glycosylation mutants with PNGase F, which cleaves N-linked oligosaccharides, results in a shift to the 24 kDa isoform confirming the presence of N-linked oligosaccharides. Untreated (−) and PNGase F treated (+) double mutant (p.N54K N88K) protein were also detected at 24 kDa indicating it is not glycosylated. NT=non-transfected (B) Anti-V5 immunofluorescent detection of V5-tagged WT and glycosylation mutants expressed in MDCK II cells shows the single glycosylation mutants localise to the plasma membrane but have increased amounts of intracellular protein. The double glycosylation mutant of Tmem114 is retained in the cytoplasm. (C) Western blots of V5-tagged WT Tmem235 treated with PNGase F or Endo H. Untreated Tmem235-V5 is detected as three bands ranging from ~19 to 28 kDa. Following treatment with either PNGase F or Endo H, Tmem235 is detected as a single band of 19 kDa.

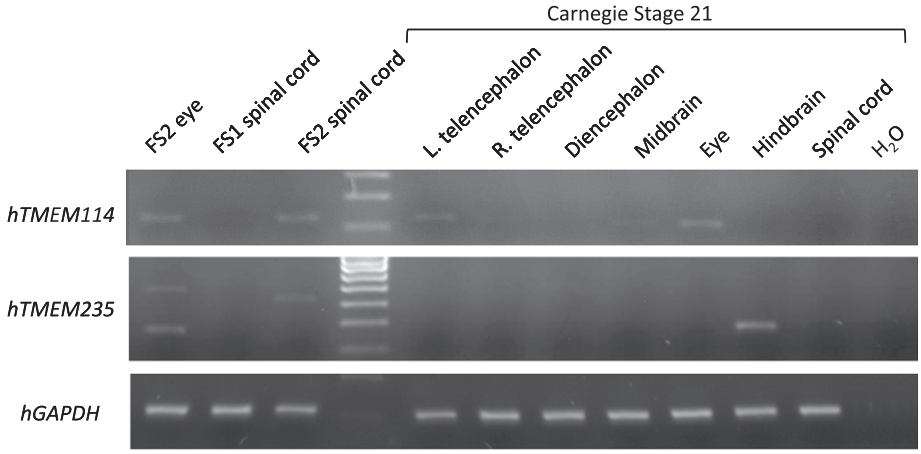

Fig. 4. RT-PCR expression profile of *TMEM114* and *TMEM235* in developing eye and neural tissues. (A) Expression of *TMEM114* was detected in the developing eye at Carnegie stage 21 (CS21) and FS 2 (FS2). Expression was also detected in the left telencephalon (CS21) and spinal cord (FS2). (B) TMEM235 was expressed in the hindbrain at CS21 in a splice isoform which lacks exons two and three. At FS2 *TMEM235* was expressed in the eye and spinal cord. Expression in the spinal cord was weak and was restricted to a splice isoform which lacks exon 2. In the eye *TMEM235* was present as two isoforms: full length *TMEM235* and *TMEM235* which lacked exons 2 and 3. H_2O indicates a no template control. Lane 4 contains a 100 bp DNA ladder. Detection of the housekeeping gene *GAPDH* was used as a control for equal cDNA loading in the RT-PCR reactions.

in the developing human eye and central nervous system. Expression of *TMEM114* was detected in the eye at Carnegie Stage 21 (CS21) (day 53–54) and Foetal Stage 2 (FS2) (week 10) (Fig. 4). Full-length *TMEM235* was detected in the eye at FS2 (week 10) but not the earlier stage of CS21 (day 53–54) (Fig. 4). An isoform which lacked exons 2 and 3 (Δexon2, 3) was also detected in the eye at FS2 as well as in the hindbrain at CS21. Expression of an isoform lacking exon 2 (Δexon2) was detected in the spinal cord at FS2 (Fig. 4).

3.5. In vivo model of Tmem114 in Xenopus tropicalis

An *X. tropicalis* ortholog of TMEM235 could not be identified. *xtTmem114* expression was detected in the developing embryo

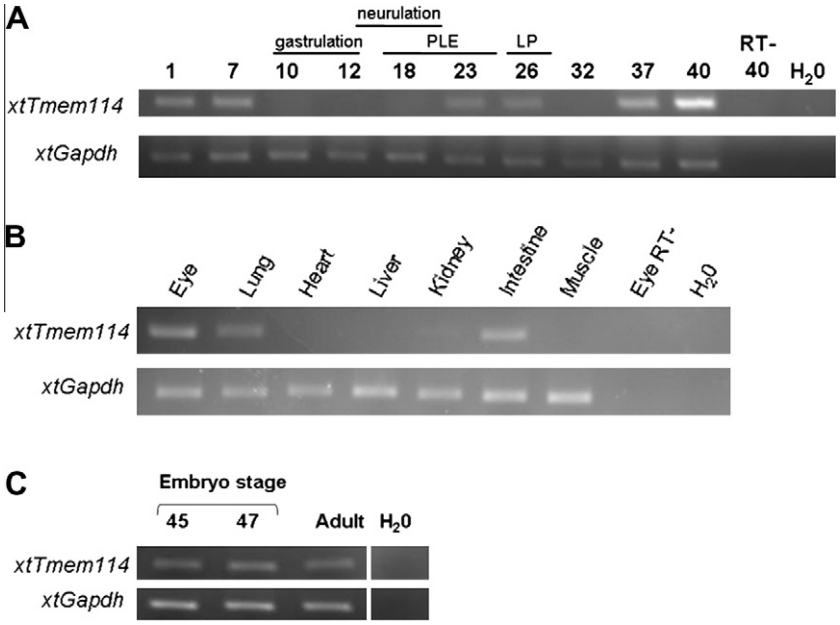

Fig. 5. RT-PCR expression of *xtTmem114* in developing and adult *X. tropicalis*. (A) Expression in the developing whole embryo. *xtTmem114* is maternally expressed at stages 1–7, and zygotic expression was detected between stages 23 and 40 (except stage 32). (B) Expression in adult tissues. Expression was detected in the adult eye, lung and intestine. (C) *xtTmem114* is expressed at similar levels in the developing and adult eye. The housekeeping gene *Gapdh* was used as a control for equal cDNA loading in the RT-PCR reactions. 'RT-' are samples to which no reverse transcriptase was added. H_2O is a no template control.

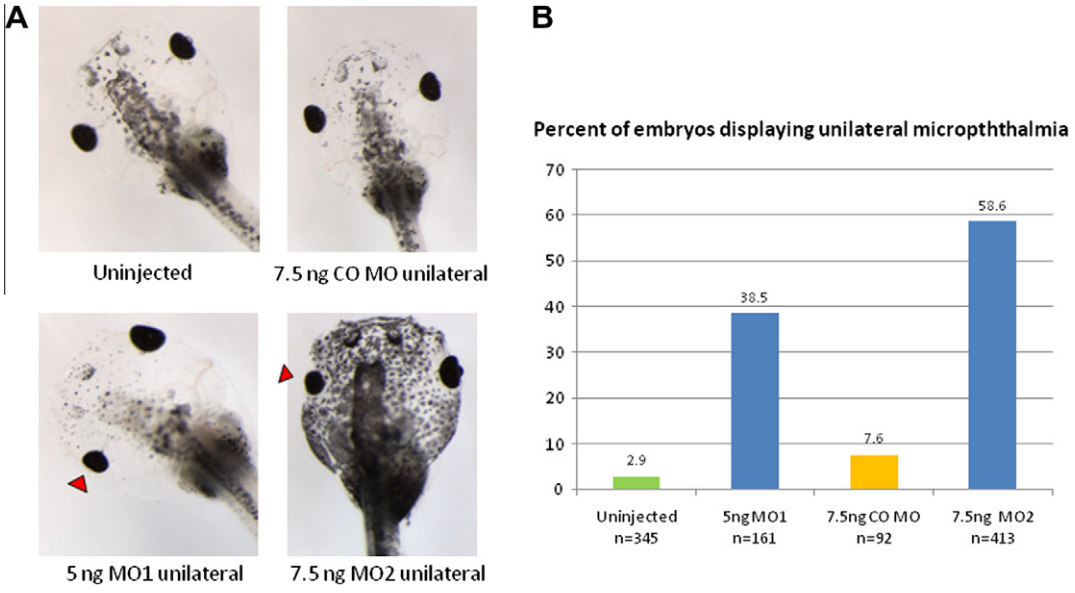

Fig. 6. Unilateral injection of anti-Tmem114 MOs results in unilateral microphthalmia. (A) Representative images of uninjected and MO injected embryos. Unilateral injection of 5 ng of MO1 or 7.5 ng of MO2 resulted in unilateral microphthalmia (arrowhead). (B) Frequency of unilateral microphthalmia observed in tadpoles unilaterally injected with 5 ng MO1, 7.5 ng MO2 or 7.5 ng control MO (CO MO). Data is from $\geqslant 3$ independent experiments.

and in the eye, lung and intestine of the adult (Fig. 5). To knock-down expression of xtTmem114, translation blocking MOs were injected unilaterally at the four cell stage. Unilateral microphthalmia was observed when 5 ng of MO1 was injected into only one side of the developing embryo (Fig. 6A). Microphthalmia was present in 39% of injected embryos compared to 2.9% in uninjected embryos (Fig. 6B). Targeting specificity against *xtTmem114* was confirmed by the use of a second non-overlapping MO (MO2). Unilateral microphthalmia was observed in 59% of stage 43 tadpoles unilaterally injected with 7.5 ng of MO2 compared to 7.6% of those unilaterally injected with a standard control MO (Fig. 6B).

4. Discussion

The original study describing TMEM114 and TMEM235 suggested that they are claudins [2]. However the analysis was biased because the phylogenetic tree was limited to claudins and PMP22. The predicted four TMD topology and motif in the first extracellular loop are also present in EMP-1, -2 and -3, PMP22, MP20 and the voltage dependent calcium channel gamma subunits (CACNGs). By extending the scope of the phylogenetic tree we have shown that TMEM114 and TMEM235 are more closely related to the CACNGs than to claudins.

There are eight voltage dependent calcium channel gamma subunits in the human genome, but only the two most outlying members (CACNG-1 and -6) have been demonstrated to modulate activity of calcium channels [12,13]. Other members, CACNG-2, -3, -4 and -8, mediate trafficking of glutamate receptors (AMPARs) to the cell surface of neurons and hence are known as TARPs (transmembrane-AMPA receptor regulating proteins) [14]. As well as modulating AMPAR activity by trafficking, TARPs also regulate the gating of AMPARs [15]. CACNG-5 and -7 can also regulate glutamate receptors [16]. In the phylogenetic tree TMEM114 and TMEM235 branch between the TARPs and the calcium channel modulating CACNGs (CACNG-1 and -6) (Fig. 1A).

Unlike most claudins and TARPs, TMEM114 does not contain a PDZ binding domain which may explain why it does not co-localise with ZO-1 at tight junctions [17,18] (Fig. 2Bi). The vast majority of the EMP/PMP22/MP20 proteins and CACNGs, but only 2/24 claudins contain predicted N-glycosylation sites [4]. The conservation of glycosylation sites in TMEM114 suggests they are functionally significant. We demonstrated that Tmem114 is glycosylated at these sites and is necessary for membrane localisation (Fig. 3B). The mouse Tmem235 ortholog does not contain the N-glycosylation site present in human TMEM235, and is probably localised to the ER, as suggested by the immunofluorescence and Endo H digestion data.

To our knowledge we report the first experimental detection of the full-length 223 aa isoform of *TMEM235*. We also detected splice isoforms which are reported in brain derived ESTs (Unigene Hs.632228). The alternatively spliced isoforms do not alter the frame of TMEM235 but result in the loss of the latter part of the first extracellular loop (Δexon2) and TMD2 and part of TMD3 (Δexon3). *CACNG6* is also detected in alternative Δexon2 and Δexon2, 3 isoforms which are predicted to result in the loss of TMD2 and TMD3 [19]. The functional significance of the alternative splicing of CACNG6 has yet to be elucidated.

TMEM114 is expressed in the developing eye in humans (Fig. 4), mouse [3] and *X. tropicalis* (Fig. 5) and knockdown of Tmem114 in *X. tropicalis* results in the developmental ocular disorder microphthalmia, suggesting a role for Tmem114 in eye development. Another Pfam00822 family member, MP20, is expressed in the lens and a homozygous missense mutation in mice causes microphthalmia and cataract [20].

In conclusion, TMEM114 and TMEM235 are developmentally expressed CACNG-like transmembrane proteins which form part of Pfam00822 family of transmembrane proteins that are phylogenetically and biologically distinct from claudins. It will be of interest to establish they are also functionally similar to the CACNGs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.05.060.

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