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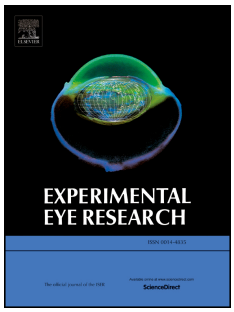


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# First insights into the expression of VAX2 in humans and its localization in the adult primate retina

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## Abstract

*VAX2* is a transcription factor specifically expressed in the ventral region of the prospective neural retina in vertebrates and is required for ventral eye specification. Despite its extensive analysis in vertebrates, the biological role of VAX2 in the human is presently unclear. This study was undertaken to investigate VAX2 in humans aiming to gain new knowledge into its involvement in retinal function. Here, we report VAX2 gene expression and protein localization in cultured cells and adult retina. RT-PCR experiments indicated that *VAX2* is enriched in neuronal tissues. Moreover, we identified a novel isoform most abundantly expressed in the retina. We termed the known transcript (NM_012476) isoform-1, and the newly identified transcript as isoform-2. Analysis of protein localization in cultured cells revealed that isoform-1 localizes to the nucleus and isoform-2 is widely expressed within the cell; partial co-localization of isoform-2 and actin filaments was also observed. In nonhuman primate retina VAX2 was seen either in the nuclear or in the cytoplasmic compartment depending on the retinal cell type. In addition, a noteworthy enrichment of the signal was observed in the outer segment of cone photoreceptors.

Overall, this study provides the first insights into the expression of VAX2 in humans and its localization in the adult primate retina. Moreover, preliminary characterization of alternative variants suggests an involvement of VAX2 in multiple cellular pathways. Our findings raise the interesting possibility for further investigation of VAX2 in the retina in health and disease.

*VAX2* belongs to a subfamily of homeobox genes closely related to Drosophila transcription factor gene empty spiracles (*ems*) and to its vertebrate homologues, the *Emx* genes (Simeone et al., 1992). *Vax2* is a transcription factor specifically expressed in the ventral region of the prospective neural retina in vertebrates and is required for ventral eye specification (Barbieri et al., 1999).

The genetic inactivation of *Vax2* in mouse produces, amongst other features, an incomplete closure of the choroidal fissure that results in an incompletely penetrant ocular coloboma (Barbieri et al., 2002; Mui et al., 2002). Considering all the similarities between the phenotype observed in *Vax2* mutant mice and the clinical features of isolated colobomata in human, *VAX2* represents a good candidate gene for this human condition. Thus far, however, no mutations affecting *VAX2* in coloboma patients have been reported. Only negative results for *VAX2* mutational screening in anophthalmia/microphthalmia patients have been published (Slavotinek et al., 2012). A meta-analysis of astigmatism in a Caucasian population identified a locus in the genomic region containing *VAX2*, suggesting a potential involvement of this gene in the pathogenesis of astigmatism (Lopes et al., 2013). Moreover, very recently a homozygous deletion at 2p13.3 encompassing all of *ATP6V1B1* and part of *VAX2* has been reported in a patient affected by Distal Renal Tubular Acidosis. Interestingly, the patient, amongst other features, showed an ocular phenotype diagnosed as bilateral rod/cone photoreceptor dystrophy and mild optic atrophy (Norgett et al., 2015). It has been shown that *Vax2* is also involved in correct intra-retinal

retinoic acid distribution, and its action extends to the mature retina, in particular to ensure appropriate gene expression in cone photoreceptor cells (Alfano et al., 2011). *Vax2* inactivation, in mouse, shows alterations in local spectral sensitivity rather than in overall cone function, at least within the range detectable by ERG (Alfano et al., 2011).

Although, the *Vax2* gene has been widely studied in vertebrates its role in humans is presently unclear. This study was undertaken with the objective to gain new knowledge into its involvement in human retinal function. Here we report VAX2 gene expression and protein localization in cultured cells and primate retina.

For this study the following methodologies were used. *VAX2* gene expression was tested by RT-PCR using primers located within exon 1 and exon 3 (Fw – GGTCAGCATGGGCGATGG; Rv- GTGGGAGTCTTAAGTGTTAGC) allowing the amplification of the full-length coding cDNA (NM_012476). Analysis was performed using human tissues (commercially available RNA, Clontech), as well as Y79 and hTERTRPE-1 (RPE-1) cell lines (RNA prepared using Trizol, Invitrogen). cDNA was prepared using both the QuantiTect® Reverse Transcription kit (Qiagen) and Superscript II (Invitrogen) with oligo dT primers according to the manufacturer's instructions. RT-PCR was performed using GoTaq® Colorless Master Mix 2X (Promega). The *HPRT* gene (Fw-GGGACATAAAAGTAATTGGTG; Rv- GCGACCTTGACCATCTTTGG) was used as an internal control. Retina cDNA PCR products were sub-cloned in pcRII TOPO Vector (Invitrogen) and clones with different inserts, discriminated by size, were analyzed by direct sequencing.

Real-time quantitative PCR (qPCR) was carried out with the StepOnePlus

System (Applied Biosystem) and performed as described by Alfano and co-workers (Alfano et al., 2005). Differences between the mean Ct values of tested genes and those of reference genes were calculated as $\Delta Ct=CT_{VAX2}$ - $CT_{HPRT}$ and represented as $2^{-\Delta Ct}$. *VAX2* full-length cDNAs were cloned at the *XhoI–KpnI* sites (Fw- ccctcgagGTCAGCATGGGCGATGG; Rv-ggggtaccGTGGGAGTCTTAAGTGTTAGC) in pEGFP-C3 (Clontech, 6082-1) and at *HindIII-KpnI* sites (Fw- ccaagcttGGTCAGCATGGGCGATGG; Rv-ggggtaccGTGGGAGTCTTAAGTGTTAGC) in p3XFLAG-*myc*-CMV™-26 (Sigma, E6401). Transfections were performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. RPE-1, SK-N-SH and HeLa cells were grown at 37 °C and 5 % $CO_2$ in DMEMF12 or DMEM, respectively, supplemented with 10 % FCS, penicillin (100U/ml) and streptomycin (50 μg/ml). Y79 cells were cultured in RPMI 1640 supplemented with 20 % FCS, penicillin (100U/ml) and streptomycin (50 μg/ml). RPE-1 cell line was purchased from Clontech, whilst the other cell lines were purchased from ATCC. Experiments were performed with cells of early passages.

For immunocytochemistry cells were fixed in 4 % PFA, blocked 2 hours with blocking reagent (6 % BSA/0.3 % Tween20) and hybridized (1-12 h) with the relevant primary antibodies. An anti-Flag antibody (F3165) was used diluted 1:10000. Endogenous expression of VAX2 protein was analyzed using a commercially available antibody (S-17, sc-79339) diluted 1:10. *Ex vivo* analysis was performed on adult monkey retina (*Macaca fascicularis,* 2 and 16.5 years old*)* and mouse eyes (C57/BL6, 2 month old). Monkey eyes were obtained from animals sacrificed in accordance with local and national ethical rules for purposes not related to this project. Eyes of 2 month old C57/BL6

mice were obtained from the UCL Institute of Ophthalmology (IOO) Biological Resources Unit (BRU); the work was approved by the IOO Institutional Animal Care and User Committee (IACUC #70/2710).

Tissues were fixed in 4 % PFA, treated with a sucrose gradient (10-30 %) and embedded in O.C.T. compound (VWR, UK). Cryosections (5-10 µm) were treated at high temperature in 0,01M Citrate buffer for antigen retrieval followed by permeabilization with 0.3 % Triton-X in PBS (5 min). Sections were blocked (2 h at room temperature) and hybridized (18 h at room temperature) with PBS containing 5 % donkey serum, 6 % BSA and 0.3 % Tween20. A goat VAX2 antibody (S-17, sc-79339) diluted 1:10 and an anti M/L-Opsin antibody (gift from Prof. Jeffery) diluted 1:500 were used. Peanut agglutinin (PNA) staining was performed using a Rhodamine conjugated PNA (Vector Lab) diluted 1:100 (1 h at room temperature). Alexa Fluor®488 (Molecular Probe, 1:400) and Cy3 conjugated AffiniPure (Jackson ImmunoResearch lab, 1:400) were used as secondary antibodies. Negative controls (-ve control) were undertaken by omitting the primary antibody. Nuclei were stained (10 min at room temperature) with 4', 6-Diamidino-2-Phenylindole Dihydrochloride (DAPI, 1:5000). Slides were mounted using Dako fluorescent medium (DAKO) and viewed on a LSM700 confocal microscope. Z-stack confocal images were analyzed using the Zen lite (black edition) Digital imaging free software (Zeiss); the final pictures of both cells and tissues were the results of maximum projections.

In the present study, we first sought to investigate *VAX2* gene expression. By performing 40 cycles PCR assays we observed that *VAX2* is enriched in the neuronal tissues (Fig. 1A, top panel); *HPRT* amplification was used as control

(Fig.1A, bottom panel). Expression was also detected in the cell lines tested (data not shown). Amongst the neuronal tissues, not including the retina, an enrichment of the signal was seen in the cerebellum. Previous studies have described *Vax2* localizing to the adult cerebellum in *Xenopus* (Liu et al., 2008). Therefore, our results suggest an involvement of VAX2 in the homeostasis of this brain compartment. Interestingly, by RT-PCR we constantly obtained two amplicons: a lower band of the expected size and an additional upper band (Fig. 1B, indicated by the red asterisk). Sub-cloning and sequencing of PCR products obtained, both from retina and RPE-1 cell line cDNAs, showed that both bands correspond to specific products. The longer amplicon is due to the amplification of a novel *VAX2* splice-variant, consisting of four exons, three of which are shared with the canonical NM_012476 transcript, plus an additional exon of 63bp (AACCAGCCCCAGTCCTGAAACTACCTAGAGATCCACCCTGAATCACCTCATTAGCA TACAACA) located between the second and third exon of the NM_012476 transcript. The newly identified transcript partially overlaps with the EST H92142 of retinal origin reported in the UCSC database (http://genome.ucsc.edu/); this EST contains the 63bp exon. We termed the canonical NM_012476 transcript isoform-1, and the newly identified transcript as isoform-2. Isoform-1 is predicted to encode a 290 amino acid (aa) protein, whereas isoform-2, a shorter protein of 150 aa. The extra-exon introduces an earlier stop codon resulting in a truncated protein which still retains, however, part of the HOX domain sequence, spanning from aa 102 to 164, according to SMART online prediction tool (http://smart.embl-heidelberg.de/). The 63bp nucleotide sequence is conserved in primates and absent in other species as

revealed by Blast alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the genome of different organisms (fish, amphibian, avian, mammals). However, experimental validations are required to assess whether or not this is a coding sequence in nonhuman primates. Suitable material for further investigation was not available in this study.

Moreover, RT-PCR suggests that the two isoforms are differentially expressed. Interestingly, isoform-2 appears highly detectable in retina compared with other tissues (Fig. 1B). Real Time qPCR experiments, using primers located within the second and the third exon of isoform-2 (Fw-GCCTGGAGATGGAGTTCC; Rv-, GTATGCTAATGAGGTGATTCAG) and therefore specific for this transcript, confirmed that isoform-2 is most abundantly expressed in the retina (Fig. 1C) suggesting a potentially important role in retinal cells.

With regards to investigating the biological function of *VAX2* isoforms, we next analyzed their *in vitro* sub-cellular localization. Transient transfection assays, performed with both constructs pEGFP-VAX2 and p3XFLAG-VAX2, showed that isoform-1 localizes to the nucleus (Fig. 1D and data not shown), whilst isoform-2 displays a widespread localization within the nucleus and the cytoplasm (Fig. 1E and data not shown). Both isoforms show a consistent localization pattern even when they are co-expressed in the same cell, highlighting that expression of each does not interfere with the other (Fig. 1F).

We next investigated VAX2 endogenous protein localization. Analysis was performed using a commercially available antibody raised against the N-terminal part of the protein, and therefore, capable of recognizing both isoforms. The specificity of VAX2 antibody was experimentally validated by its

capability of binding over-expressed VAX2 isoform-1 and -2 as shown by immunocytochemistry assays (Fig. 1G). Investigation of endogenously expressed VAX2 was carried out in RPE-1, HeLa, Y79 and SK-NS-H cell lines. Regardless of the cell type used we constantly observed VAX2 localizing within the nucleus and the cytoplasm, Alexa Fluor® 594-conjugated phalloidin (Invitrogen, 1:400) was used to highlight the cell body (Fig. 1H and data not shown). Negative controls (-ve control) were undertaken by omitting the primary antibody (Fig.1I and data not shown). Taken together, these data demonstrated that in cultured cells VAX2 isoform-1 and -2 are co-expressed and display different sub-cellular localization. The former is exclusively present in the nucleus whilst the latter localizes to the nucleus and the cytoplasm. In addition, although isoform-2 is widely expressed within the cell body, in experiments involving over-expressed protein we observed an enrichment of the signal in specific areas of the cell body likely corresponding to actin based structures such as stress fibers, filipodia, lamellipodia and actin spikes (Fig. 1J, indicated by the arrows). To assess whether VAX2 isoform-2 localizes to those structures we performed co-stainings of p3XFLAG-VAX2 isoform-2 with F-actin and an anti-actin antibody (ab179467, 1:25). Analysis carried out in RPE-1, SK-N-SH and HeLa revealed partial co-localization (as indicated by the yellow merge signal) suggesting an involvement of VAX2 isoform-2 with the actin cytoskeletal machinery (Fig. 1K, L and data not shown). The nuclear localization signal (NLS), spanning the 153-159 amino acid sequence (Kim and Lemke, 2006), is absent in isoform-2, which could explain the observed widespread cytoplasmic expression pattern. Of note, previous reports have shown homeodomain (HD) protein isoforms, lacking the

HD domain, localizing to the cytoplasm (Halleri et al., 2004; Chu and Ohtoshi, 2007). Interestingly, co-localization of cytoplasmic homeodomain variants with cytoskeleton filaments has also been reported (Haller et al., 2004). Our data, along with previous reports, suggests that homeobox genes might have as yet unidentified functions, in addition to their role as transcription factors.

In this study we also investigated VAX2 localization in the adult retina. To the best of our knowledge the expression pattern of VAX2 protein in mammalian adult retina has not yet been reported. Previous studies have only described Vax2 protein localization in embryonic and postnatal mouse eye (Kim and Lemke, 2006). It has been shown that the protein shuttles between the nucleus and the cytoplasm depending on the developmental stage analyzed. The regulated and reversible nuclear-to-cytoplasmic shuttling is an essential feature of retinal differentiation (Kim and Lemke, 2006). We therefore analyzed VAX2 protein localization in the adult retina of nonhuman primates. Analysis was performed on monkey (*Macaca fascicularis*) retina, a species whose retinal structure closely resembles that found in the human.

VAX2 signal was observed either in the nuclear or in the cytoplasmic compartment depending on the retinal cell type. Nuclear signal was observed in the ganglion cells whereas a noteworthy enrichment of VAX2 was seen in the outer segment (OS) of cone photoreceptors (Fig. 2A, D). Expression within the cone OS was validated by co-staining with Peanut agglutinin (PNA) and M/L-Opsin (Fig. 2D and data not shown). Analysis performed on young adult (Fig. 2A-D) and old (Fig. 2E-G) monkey retina led to comparable results. VAX2 was detected in both perimacular (Fig. 2E) and macular cones (Fig. 2F and data not shown). In old monkey, nuclear localization of VAX2 was

observed in the ganglion and inner nuclear layers whereas cytoplasmic localization was seen not only in the cone outer segment but also in the rod inner segment (Fig. 2G indicated by the arrows). It should be noted that part of the signal observed might reflect the expression of isoform-2. However, as reported above the expression of VAX2 isoform-2 in nonhuman primates is yet to be experimentally validated. Given that VAX2 is highly conserved throughout evolution we next sought to investigate its localization in the adult mouse retina. In the present study preliminary data indicates that Vax2 in mouse retina localizes to the cytoplasm of ganglion cells, to the outer plexiform layer and to the outer segment of cone photoreceptor cells as shown by co-stainings with M-Opsin and PNA; signal was also observed in the inner segment of rod photoreceptors (Fig. 2 H-K). Our data do not fully exclude a possible localization of VAX2 in the nuclei. It should be taken into account that proteins expressed at low levels are not detectable by immunofluorescence assays as performed in this study. Moreover, it should also be noted that in the postnatal developing eye *Vax2* gene is enriched in the ventral retina (Corbo et al., 2007), and therefore, a detailed characterization of its protein product requires analysis performed on retinal sections of known orientation (dorsal versus ventral). Taken together our results suggest that, in adult mammalian retina of primates VAX2 localizes to different retinal cells (either in the nucleus and/or in the cytoplasm), and within the photoreceptors it is enriched in the cone outer segment; the expression pattern observed in the photoreceptors is likely to be conserved throughout evolution from rodents to primates, as indicated by our data. VAX2 expression within the adult retina overall, and the novelty of its localization within the

photoreceptors, remain to be further investigated. Of note, a recent study suggests an involvement of VAX2 in the photoreceptors biology in humans (Norgett et al., 2015). This case report described a patient harbouring a homozygous deletion at 2p13.3 encompassing all of *ATP6V1B1* and part of *VAX2;* the patient was reported affected by Distal Renal Tubular Acidosis, however, amongst other features, he showed an ocular phenotype diagnosed as bilateral rod/cone photoreceptor dystrophy and mild optic atrophy (Norgett et al., 2015). Moreover, it should be mentioned that the photoreceptor outer segment is considered a specialized primary cilium and trafficking through the photoreceptor cilium is a highly regulated phenomenon (Hsiao et al., 2012; Yildiz and Khanna, 2012). In recent years it has been demonstrated that localization to the outer segment can be mediated by ciliary targeting sequences (CTS). CTSs are short amino acid sequences first identified in Rhodopsin; the two CTS motifs, which have been identified and experimentally validated, are the VxPx (x stands for any amino acid) and the FR motifs (Mazelova et al., 2009). Several other motifs have also been identified in other proteins (Hsiao et al., 2012 ; Mazelova et al., 2009). Of note, we observed a VxPx consensus sequence in the N-terminal part of the VAX2 protein (91-VLPQ-96), which is present in both isoforms. It could, therefore, be speculated that this motif is involved in the trafficking to the outer segment.

In summary, the present study provides the first insights into the expression of VAX2 in humans and brings important knowledge for further investigations. We report that *VAX2* gene is enriched in neuronal tissues (fetal and adult); studies involving analysis of *VAX2* in health and disease relating to the

nervous system may well yield significant findings. In addition, the identification of a novel VAX2 splice variant enriched in the retina represents an interesting discovery. Previous studies demonstrated that homeobox gene isoforms could play different roles, in retina as well as in the nervous system, under physiological and pathological conditions (Courtois et al., 2003; Kiselev et al., 2012). Moreover, in rodents it has been shown that the sub-cellular localization of transcription factors (nuclear or cytoplasmic) may participate in cell fate determination during particular phases of retinal development (Baas, et al., 2000; Kim and Lemke, 2006 ). Our *in vitro* analysis showed that VAX2 isoforms display a differential expression pattern within the cell body probably underlying an involvement in different molecular pathways. Similarly it could be speculated that in primates VAX2 acquired novel functions and its action is partially regulated through splice variants. Thus far, our characterization of VAX2 localization within the retina revealed signal either in the nucleus or in the cytoplasm depending of the retinal cell type. In light of our results an intriguing point to address is the localization and biological roles of the VAX2 isoforms within the retinal cells using *in vivo* models.

Furthermore, our results along with previous studies performed in mice (Alfano et al., 2011) and humans (Norgett et al., 2015) further support a likely involvement of VAX2 in the photoreceptor cell biology.

In conclusion, our findings open new avenues in the perception of VAX2 and also raise the interesting possibility for evaluating this gene as a potential candidate for retinal degeneration.

**Legends**

**Fig.1. Characterization of *VAX2* gene expression and *in vitro* protein localization in RPE-1 cells.** (A) RT-PCR analysis of *VAX2* in human tissues; *HPRT* was used as control (lower panel). (B) PCR products at high magnification showing two bands corresponding to *VAX2* isoforms, the red asterisk indicates isoform-2. (C) Real-Time PCR assays displaying *VAX2* isoform-2 transcript expression. x-axis: tissues analyzed; y-axis: relative VAX2 expression reported as $2^{-\Delta Ct}$ values; standard error is indicated by the thin horizontal bars. Green fluorescence showing the sub-cellular localization of exogenously expressed pEGFP-VAX2 isoform-1 (D) and pEGFP-VAX2 isoform-2 (E) proteins. (F) Co-expression of pEGFP-VAX2 isoform-1 and p3XFLAG- VAX2 isoform-2 in the same cell. (G) Immunocytochemistry using an anti VAX2 antibody (αVAX2) on cells transfected with pEGFP-VAX2 isoform-1 and pEGFP-VAX2 isoform-2; the antibody recognizes both isoforms (as indicated by yellow signal). (H) Endogenously expressed VAX2 is widely present within the nucleus and the cytoplasm; F-actin (red fluorescence) was used to highlight the cell body. (I) Negative control (-ve control). (J) p3XFLAG-VAX2 isoform-2 is enriched in areas likely corresponding to actin-based structures (indicated by the arrows). Co-labelling of p3XFLAG- VAX2 isoform-2 and F-actin (K) and pan actin (L) show area of partial overlay (yellow signal). Nuclei were stained using Dapi (blue). Fbr, fetal brain; Br, brain; Crb, cerebellum; Sc, spinal cord; Re, retina; He, heart; Li, liver; Ki, kidney; Ps, prostate; Te, testis; L, molecular weight marker; N, negative control. The molecular weight DNA markers are respectively 1kb and 100 bp ladder (Promega). Scale bar, 10μm.

**Fig.2. Localization of VAX2 protein in the adult retina.** (A) Immunofluorescence assays on retinal sections of young adult monkey show VAX2 expression within the retinal layers with enrichment in the cone photoreceptor outer segment. (B) Negative control (-ve control). (C) High magnification showing VAX2 nuclear localization in the ganglion cells. (D) High magnification images showing co-labelling of VAX2 and PNA; left panel shows the DIC image. Co-staining of VAX2 and PNA in the outer segments of perimacular (E) and macular (F) cones of old monkey. (G) VAX2 signal in the rod photoreceptor inner segment and nuclei of the inner nuclear layer cells as indicated by the yellow arrows. Co-labelling of Vax2 and M-opsin (H, K) and PNA (I) on mouse retina. (J) Negative control (-ve control). Nuclei were stained using Dapi (blue). RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 20μm; 10μm (C).

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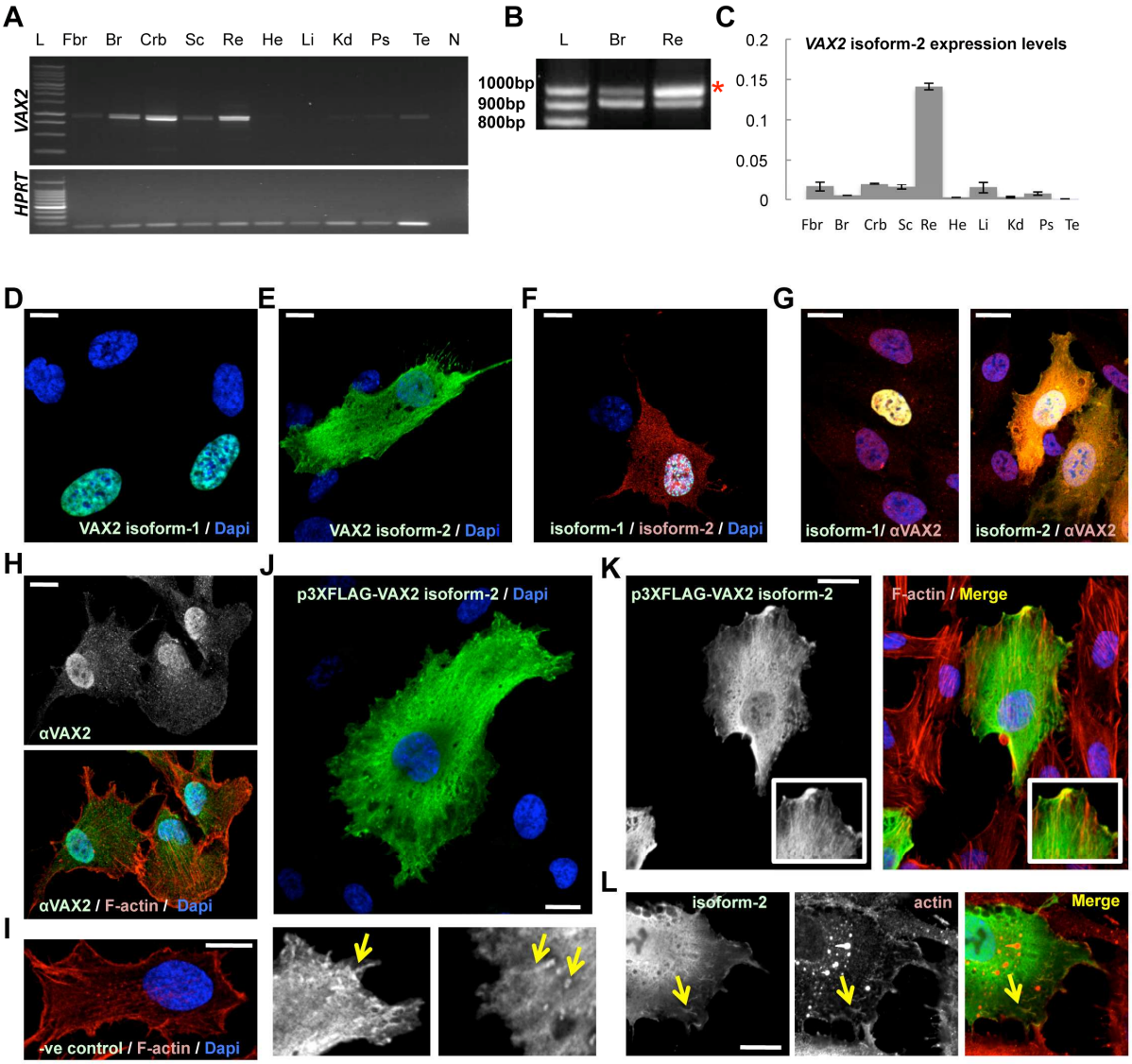
A
L Fbr Br Crb Sc Re He Li Kd Ps Te N
VAX2
HPRT
B
L Br Re
1000bp
900bp
800bp
*
C
VAX2 isoform-2 expression levels
0.2
0.15
0.1
0.05
0
Fbr Br Crb Sc Re He Li Kd Ps Te
D
VAX2 isoform-1 / Dapi
E
VAX2 isoform-2 / Dapi
F
isoform-1 / isoform-2 / Dapi
G
isoform-1/ αVAX2
isoform-2 / αVAX2
H
αVAX2
αVAX2 / F-actin / Dapi
J
p3XFLAG-VAX2 isoform-2 / Dapi
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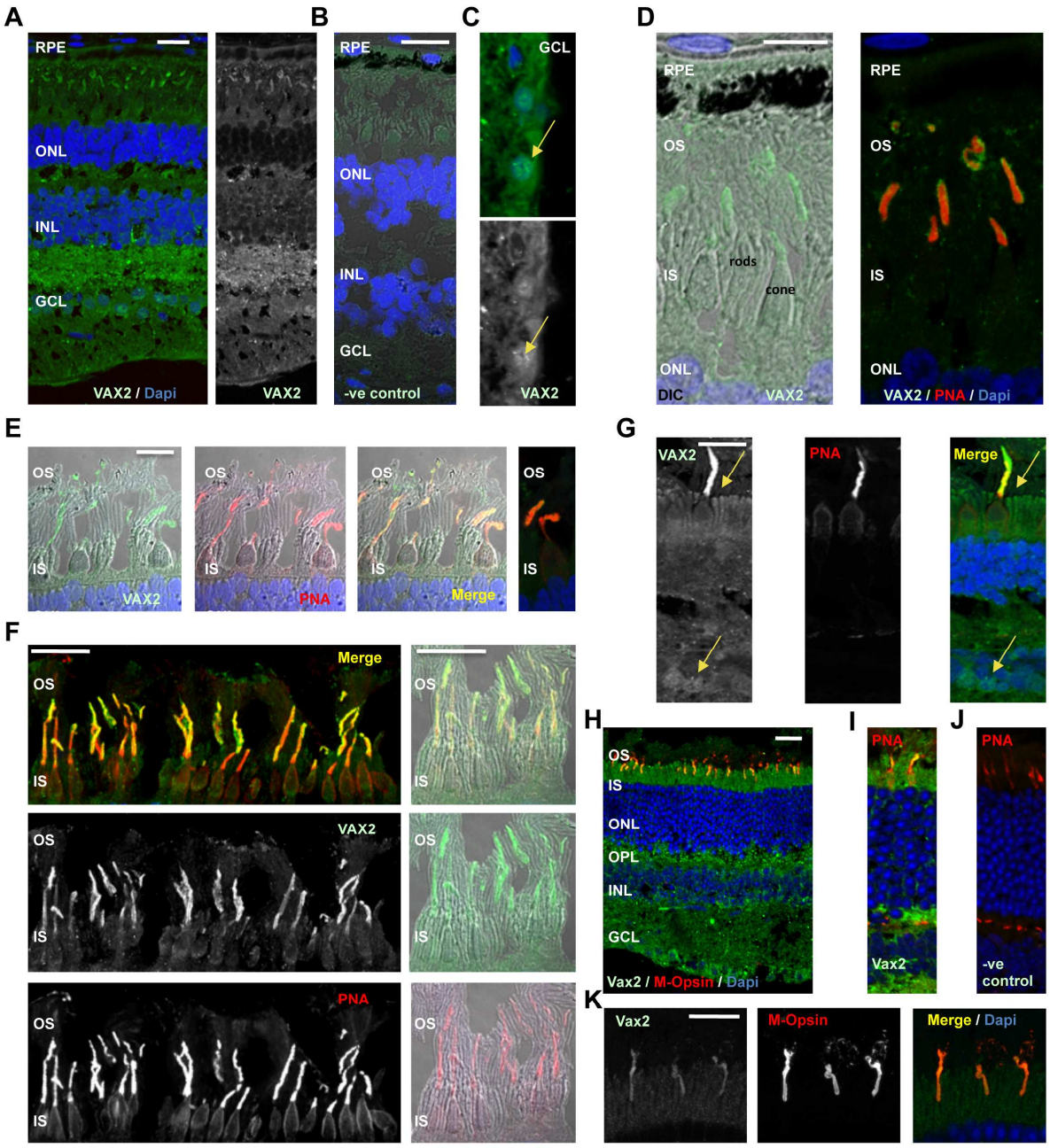
A
RPE
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RPE
ONL
INL
GCL
-ve control
C
GCL
VAX2
D
RPE
OS
rods
IS
cone
ONL
DIC
VAX2
RPE
OS
IS
ONL
VAX2 / PNA / Dapi
E
OS
IS
VAX2
PNA
Merge
F
Merge
VAX2
PNA
G
VAX2
PNA
Merge
H
OS
IS
ONL
OPL
INL
GCL
Vax2 / M-Opsin / Dapi
I
PNA
Vax2
J
PNA
-ve control
K
Vax2
M-Opsin
Merge / Dapi

ACCEPTED MANUSCRIPT

- In humans VAX2 displays two isoforms (isoform-1 and -2); isoform-2 is enriched in the retina.

-Isoform-1 localizes to the nucleus whereas isoform-2 is widespread within the cell.

-VAX2 isoform-2 partially co-localizes with actin cytoskeletal filaments.

-In retina VAX2 is present in the nucleus or in the cytoplasm depending on the retinal cell type

-An enrichment of VAX2 signal is detectable in the cone photoreceptors.

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