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Loss of *Tbx2* delays optic vesicle invagination leading to small optic cups

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Abstract

Tbx2 is a T-box transcription factor gene that is dynamically expressed in the presumptive retina during optic vesicle invagination. Several findings implicate *Tbx2* in cell cycle regulation, including its overexpression in tumours and regulation of proliferation during heart development. We investigated the role of *Tbx2* in optic cup formation by analysing mice with a targeted homozygous mutation in *Tbx2*. Loss of Tbx2 caused a reduced presumptive retinal volume due to increased apoptosis, and a delay in ventral optic vesicle invagination leading to the formation of small and abnormally shaped optic cups. *Tbx2* is essential for maintenance, but not induction of expression of the dorsal retinal determinant, *Tbx5*, and acts downstream of *Bmp4*, a dorsally expressed gene implicated in human microphthalmia. The small retina showed a hypocellular ventral region, loss of *Fgf15*, normally expressed in proliferating central retinal cells, and increased numbers of mitotic cells in the dorsal region, indicating that *Tbx2* is required for normal growth and development across the D-V axis. Dorsal expression of potential regulators of retinal growth, Cyp1b1 and Cx43, and the topographic guidance molecule ephrinb2, was increased, and intra-retinal axons were disorganised resulting in a failure of optic nerve formation. Our data provide evidence that *Tbx2* is required for proper optic cup formation and plays a critical early role in regulating regional retinal growth and the acquisition of shape during optic vesicle invagination.

Introduction

The developing embryonic eye undergoes a complex process of morphogenesis and is patterned by polarised gene expression along its dorso-ventral, naso-temporal and proximo-distal axes. At 9.5 days post coitus (dpc) in the mouse, the optic vesicle forms as an extension of the forebrain neuroepithelium and invaginates upon contact with the surface ectoderm. Invagination initiates in the dorsal optic vesicle and gives rise to an optic cup, consisting of an expanded inner layer, the presumptive neural retina, and a thin outer layer, the presumptive retinal pigmented epithelium (Chow and Lang, 2001). Initially, the dorsal region of the forming

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optic cup is larger and developmentally more advanced, while formation of the ventral region of the optic cup lags in invagination, expansion, and neurogenesis (Coulombre et al., 1965; Romanoff, 1960). This results in an asymmetrically shaped optic cup by 11.5 dpc, that has an opening across the ventral surface, termed the optic fissure. The site at which future retinal ganglion cell (RGC) axons exit from the eye, termed the optic disc, is located ventrally at the proximal end of the optic fissure. By 13.5 dpc, upon closure of the optic fissure, the optic cup appears spherical with the optic disc positioned centrally. As a possible driving force for this shape alteration, the dorsal neural retina has been shown to possess a lower proliferative index compared to the ventral neural retina in the early optic cup, which later levels out (Behesti et al., 2006; Calvente et al., 1988; Morcillo et al., 2006). It is currently not known which genes regulate differential rates of cell division and contribute to the control of optic vesicle to optic cup morphogenesis.

Patterning of the D-V axis by asymmetrical gene expression is known to be critical for correct topographic mapping of retinal RGC axons from the neural retina to the brain (Koshiba-Takeuchi et al., 2000; Mui et al., 2002; Schulte et al., 1999). The idea that early D-V patterning may also be important for optic vesicle invagination and growth is supported by reports which show that mutations in the ventrally expressed homeobox genes, *PAX2/Pax2*, *Vax2*, and *Vax1*, cause coloboma, an eye malformation associated with blindness due to failure of optic fissure closure (Barbeiri et al., 2002; Bertuzzi et al., 1999; Eccles and Schimmenti, 1999; Mui et al., 2002; Torres et al., 1996). By contrast, little is known about the role of dorsally expressed genes. The earliest genes to be specifically expressed in the dorsal region of the developing eye include members of the evolutionarily conserved T-box transcription factor gene family (Bollag et al., 1994; Chapman et al., 1996; Sowden et al., 2001). *Tbx2*, *Tbx3*, and *Tbx5*, which share a high degree of amino acid sequence homology to the *Drosophila optomoter blind* (*omb*) gene, the ortholog of *Tbx2* and *Tbx3* (Papaioannou, 2001) are expressed in the distal layer of the optic vesicle and later in a nested fashion in the dorsal neural retina of the early optic cup (Behesti et al., 2006; Sowden et al., 2001). *omb* null mutants display abnormal optomotor behaviour due to the absence of lobula-plate giant nerve fibres, causing visual impairment (Pflugfelder et al., 1992). Misexpression of *Tbx5* by electroporation in the developing chick eye leads to retinotectal misprojections, the expansion of the dorsally expressed *ephrinB1* and *ephrinB2* genes into the ventral neural retina, and the repression of the ventral genes, c*Vax* and *Pax2* (Koshiba-Takeuchi et al., 2000). Injection of synthetic *Tbx2* or *Tbx3* RNA into 2-cell stage *Xenopus* embryos has been reported to cause loss of ventral RPE pigmentation, medial displacement of the optic cup, and fused eyes in the most severe cases (Takabatake et al., 2002). In the zebrafish embryo, morpholino-mediated knock-down of *Tbx2* resulted in a transient block of retinal cell types other than the RGCs, specifically in the dorsal neural retina (Gross and Dowling, 2005). However, the effect of loss of T-box gene function in mammalian eye development has not been investigated.

Current knowledge of *Tbx2* points towards a role in cell cycle control and tissue growth. As well as being amplified in a subset of primary human breast tumours, in pancreatic tumour cell lines, and being overexpressed in melanoma cell lines (Bärlund et al., 2000; Jacobs et al., 2000; Mahlamaki et al., 2002; Sinclair et al., 2002; Vance et al., 2005), Tbx2 has been shown to function as an anti-senescence factor *in vitro* (Carlson et al., 2001; Jacobs et al., 2000; Vance et al., 2005). Furthermore, it has been shown to repress the p14ARF (*Cdkn2a*) promoter and those of other cyclin-dependant kinase inhibitors such as p21 *in vitro* (Lingbeek et al., 2002; Prince et al., 2004). These data, combined with the observation that Tbx2 expression is tightly regulated during the cell cycle have led to the proposal that in development Tbx2 may coordinate proliferation and cell cycle exit (Bilican and Goding, 2006). *In vivo*, during development, evidence for a role for Tbx2 in cell cycle control or tissue growth is currently sparse. However, it interacts with the closely related *Tbx3* in maintenance and growth of the fetal mammary glands (Jerome-Majewska et al., 2005). In the developing heart, ectopic

Tbx2 represses the expression of *Nmyc* in mice with a targeted deletion of *Tbx20* (Cai et al., 2005).

We recently showed that Bmp4, a member of the TGFβ superfamily, regulates expression of *Tbx2* in the early optic cup (Behesti et al., 2006), while mutation of the human *BMP4* gene underlies anophthalmia, microphthalmia and brain anomalies in two families (Bakrania et al., 2008) and deletion of *Bmp4* in mice disrupts lens and optic cup development (Furuta and Hogan, 1998). Although it has been reported that Tbx2 acts both as an activator and a repressor of gene expression depending on promoter context (Carreira et al., 1998; Habets et al., 2002; Paxton et al., 2002), the transcriptional activity and downstream targets have not been identified in eye development. Connexin 43 (*Cx43*), a gap-junction protein, is a candidate target of *Tbx2*, as it is co-expressed with *Tbx2* in several developing tissues, including the chamber myocardium of the heart, the coronal sutures, and in the eye (Borke et al., 2003; Janssen-Bienhold et al., 1998; Yancey et al., 1992). The *Cx43* promoter contains T-sites, the consensus T-box transcription factor binding sequence, and is repressed by *Tbx2* in transfection assays (Borke et al., 2003; Chen et al., 2004).

To address the role of *Tbx2* in mammalian optic cup formation, we analysed the phenotype of $Tbx2^{tm1Pa}/Tbx2^{tm1Pa}(Tbx2^{-/-})$ homozygous mutant embryos. These mice carry a targeted deletion of part of the T-box binding domain. They die by 14.5 dpc presumably due to cardiac defects (Harrelson et al., 2004). We explored the hypotheses that (i) *Tbx2* regulates optic vesicle invagination and regionalised cell proliferation during formation of the optic cup, and (ii) *Tbx2*, acting downstream of Bmp4, regulates the expression of other D-V patterning genes. We found that $Tbx2^{-/-}$ embryos show abnormal optic vesicle morphogenesis with a delay affecting the formation of the ventral region of the optic cup. The mutant optic cups display several abnormal features. The mutant presumptive neural retinas are smaller than in wild-type controls due to increased apoptosis, and show altered expression of proliferation markers phospho-histone H3 and *Fgf15*, indicating that *Tbx2* is necessary for normal regional growth of the invaginating optic vesicle. Later they show disturbances in RGC axonal projections towards the optic disc. We found that *Tbx2* is not required for induction, but is required for the maintenance of the expression domains of several D-V patterning genes that are critical for normal eye development.

Materials and Methods

Collection of embryos, genotyping, and histology

Heterozygous timed matings were set up to generate wild-type and homozygous embryos for the $Tbx2^{tm1Pa}$ allele on a mixed (129/C57BL/ICR) genetic background. 47 pregnant females yielded a total of 363 embryos including 75 live embryos homozygous for the mutated *Tbx2* allele. Dying or dead embryos, which displayed pericardial sac oedema and no heart beat or circulation at the time of dissection, were not included in the analysis. Embryos were genotyped from yolk sac DNA as previously described (Harrelson et al., 2004). Homozygous $Chx10^{orJ}/Chx10^{orJ}$ embryos were obtained from timed matings between $Chx10^{orJ}/Chx10^{orJ}$ homozygous mice on the 129/Sv background, while wild-type embryos were obtained from matings between 129/Sv mice. Embryos were dissected in phosphate buffered saline (PBS) pH 7.4 and fixed overnight in 4% w/v paraformaldehyde (Sigma, UK) in diethylpyrocarbonate (DEPC)-treated PBS at 4°C before processing for *in situ* hybridisation, immunohistochemistry and retinal volume analysis. For the histological analysis, embryos were fixed in Bouin's fixative and then embedded in paraffin. Transverse sections (8 μm) were prepared and stained with Haemotoxylin and Eosin (H&E).

Estimation of head length and retinal volume

To quantify the early optic cup size phenotype, retinal volume was estimated from measurements of retinal area on vibratome sections. Embryos were in embedded in a mix of 0.45% w/v gelatin, 28% w/v egg albumin, 18% w/v sucrose in PBS (all from Sigma, UK) and the block was hardened with 2.5% glutaraldehyde (Sigma, UK) at room temperature for one hour. Vibratome sections of 50 μm thickness were prepared using a Vibratome series 1000 sectioning system (Agar scientific Ltd, UK). Sections were mounted with 50% glycerol in PBS. Images were digitally captured on an Axiophot 2 microscope (Zeiss, Germany) fitted with Differential Interference Contrast (DIC) objectives and a Leica DC500 camera (Leica, Germany). Only specimens with a complete set of sections through the eye were included. Retinal area was measured on each section using OpenLab software and retinal volume obtained by multiplying retinal area by the thickness of the section and adding the values of sections per eye. Head length was measured using OpenLab software on images of whole mount embryos captured on a Leica MZ FLIII microscope (Leica, Germany) and a Leica DC500 camera (Leica, Germany). To assess generalised growth for each embryo, the length from the anterior groove of the forebrain to the posterior-most point of the hindbrain (roof of the fourth ventricle) was measured on the left and the right sides and the values averaged per embryo. Data were compared between mutant and wild-type embryos with the independent samples t-test.

Whole mount and section *in situ* hybridisation

Whole mount in situ hybridisation was performed on wild-type and mutant embryos stage matched by somite number using standard protocols and the following probes: *Tbx2, Tbx3, Tbx5* (Behesti et al., 2006), *Bmp4* (Furuta et al., 1997), *Msx2* (1000 bp in pCR2.1, kindly provided by P. Sharpe, King's College, London), *ephrinB2* (*Efnb2*; 3800 bp in pBSSK, kindly provided by D. Wilkinson, NIMR, London), *Cyp1b1* (Stoilov et al., 2004), *Vax2* (Schulte et al., 1999), *Silver* (Baxter and Pavan, 2003), *Fgf15* (McWhirter et al., 1997), *Jagged-1* (Mitsiadis et al., 1997).

Immunohistochemistry and TUNEL

Frozen sections (12 μm thick) were prepared from wild-type and mutant somite-matched embryos. Sections were blocked in 10% v/v heat-inactivated FCS (Sigma, UK), 1% BSA (Sigma, UK), 0.1% detergent. The primary antibodies used were as follows: phosphohistone H3 (pH3, 1:100, 0.1% Tween-20; Upstate, USA); Connexin43 (1:100, 0.1% Triton-X100, kindly provided by Dr D. Becker, UCL, (Wright et al., 2001); RMO270 (1:1500, 0.1% Triton-X100, Zymed, USA); VC1.1 (1:100, 0.1% Triton-X100, Sigma, UK). The secondary antibodies used were FITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit (1:100, Jackson Immunoresearch Labs, USA). The TUNEL assay (Roche, UK) was carried out according to manufacturer's description. Sections were counterstained with 1 μg/ml propidium iodide (Sigma, UK) and mounted with CITIFLUOR (Citifluor Ltd., UK).

Quantification of the relative levels of Connexin43 immunohistochemistry was performed using ImageJ (NIH) on blinded digital images of sections from age matched wild type (n=2) and mutant (n=3) embryos captured under identical exposure and contrast settings using a fluorescent Axiophot 2 microscope (Zeiss, Germany) and OpenLab software.

Quantification of cell number, cell proliferation and cell death

Sections were divided into a dorsal and a ventral region as demarcated in Fig. 3E for 10.5 dpc retina. The presumptive neural retinal region lying in close proximity to the surface ectoderm was analysed for 9.5 dpc sections. Mitotic indices and total cell number were calculated per section and the data were checked for normal distribution. The number of pH3-positive mitotic

cells per section (14 sections from 6 mutant eyes and 8 sections from 4 wild-type eyes at 10.5 dpc, and 25 sections from 6 mutant eyes and 32 sections from 8 wild-type eyes at 9.5 dpc) in each region were analysed with a one-way ANOVA, while the mitotic indices were analysed with an ANCOVA with the total number of cells per region or per section included as a covariate. The number of apoptotic nuclei were counted on 25 sections from 8 wild-type eyes and 24 sections from 6 mutant eyes at 9.5 dpc, and 16 sections from 4 wild-type eyes and 24 sections from 6 mutant eyes at 10.5 dpc and the TUNEL index was compared with the Mann-Whitney test for nonparametric data.

Results

Aberrant optic vesicle morphogenesis and optic cup development in *Tbx2*$^{-/-}$ mutants

In wild type embryos *Tbx2* expression is first detected at 9.5 dpc in the distal neuroepithelium of the optic vesicle, in close proximity to the overlying surface ectoderm, extending along the length of the dorso-ventral (D-V) axis (Fig. 1A, B). *Tbx2* is then restricted to the dorsal region of the presumptive neural retina during invagination of the optic vesicle (Behesti et al., 2006). By 11.5 dpc, *Tbx2* expression is confined to the dorsal retina in the developing optic cup (Fig. 1C, D). To assess the requirement for *Tbx2* in optic vesicle invagination and growth, *Tbx2*$^{-/-}$ embryos were analysed between 9.5-12.5 dpc. Amongst the live embryos obtained from heterozygous matings, 20% (75/363) were homozygous for the mutated *Tbx2* allele, which is consistent with the previously described embryonic survival rate of homozygous embryos (Harrelson et al., 2004).

Tbx2$^{-/-}$ embryos displayed normal optic vesicle formation (data not shown). However by 10.5 and 11.5 dpc, the invaginating optic vesicle and the optic cup were smaller in mutants than in wild-type littermates (Fig. 1G-J). Lens vesicle invagination initiated normally but a marked reduction in the size of the optic cup and lens vesicle was apparent by 11.5 dpc (Fig. 1E-H, K, L). The subretinal space in the naso-ventral optic cup often appeared abnormally large in mutants, and the RPE pigmentation was delayed compared to the wild type (Fig. 1G, H). Although optic vesicle invagination was underway at 10.5 dpc in the dorsal region (Fig. 1I, J), it was delayed in the ventral region in mutants compared to somite-matched wild-type littermates (Figs. 3A-D, dashed lines and 4E, F).

By 11.5 and 12.5 dpc, the optic cup was markedly shorter along the proximo-distal (P-Di) axis (Fig. 1K-N), indicating delayed morphogenesis and growth of the neural retina, RPE and lens. Moreover at 12.5 dpc, the eye lid grooves, which can normally be distinguished at this stage in wild-type embryos (Fig. 1O, arrows) had not formed in mutants (Fig. 1P). The mutants also displayed an unusually dense hyaloid vasculature, a small lens vesicle (Fig. 1K, L), and primitive primary lens fibre elongation compared with advanced elongation in stage-matched wild-type embryos (Fig. 1M, N). Finally, the nerve fibre layer and optic nerve were not distinguishable in mutants on H&E stained sections, while fibres were present and exited the optic cup in wild-type littermates at 12.5 dpc (Fig. 1M, N). The severity of these phenotypes was variable in mutant embryos. At 12.5 dpc, the full phenotype was observed in 8/12 eyes, whereas 4/12 eyes manifested a milder phenotype showing only reduced eye size and a slight decrease in the P-Di axial length (data not shown). Mutant eyes with the severe phenotype protruded much less than wild-type counterparts (Fig. 1O, P, compare solid black lines). These data indicate that targeted disruption of *Tbx2* results in delayed growth and invagination of the presumptive neural retina of the optic vesicle leading to abnormal development of the optic cup. The abnormalities observed by E12.5 affect several tissues external to the site of *Tbx2* expression and are likely a downstream consequence of the loss of Tbx2 during optic vesicle invagination.

Retinal volume is abnormally small in *Tbx2*$^{-/-}$ embryos

To quantify the early effect of loss of Tbx2, the presumptive retinal volume was compared between mutant and wild-type embryos at somite stages (ss) 33-35 (10.5 dpc). The optic vesicle and the lens vesicle invaginate between ss 26 and 33 (Behesti et al., 2006). Measurements of head length, used as a measure of growth during early embryogenesis (Brown et al., 1990), served to test for a generalised growth delay in mutants. Mutant presumptive retinae were significantly smaller in volume than wild-type retinae (p= 0.007; Fig. 2A). By contrast, head length measurements did not differ significantly between mutant and wild-type embryos (p= 0.352, Fig. 2B). These data indicate that the lack of *Tbx2* causes an early small retina phenotype that does not correlate with a general developmental delay. Reduced retinal volume, largely due to failure of development of the ventral retina was apparent as early as E10.5, prior to closure of the lens vesicle (Fig. 1I, J; 4E, F).

A comparative analysis of cell number in the dorsal and ventral retina (as demarcated in Fig. 3E) was performed on sections of mutant and wild-type embryos. This confirmed that the ventral retina is indeed hypocellular by 10.5 dpc whereas the dorsal retina was unaffected (Fig. 2C). These data show that *Tbx2* expression is required for normal growth across the D-V axis during optic vesicle invagination.

Altered expression of markers of retinal cell proliferation and cell death in the *Tbx2*$^{-/-}$ optic cup

To examine a role for apoptosis in causing the small retina phenotype in *Tbx2*$^{-/-}$ embryos the TUNEL assay, which detects apoptosis, was performed on somite-matched embryos at ss 30-31 (10.5 dpc) during optic vesicle invagination, when the ventral retina first showed signs of underdevelopment. TUNEL labelling revealed a normal pattern of cell death in mutants that resembled previously published apoptosis data in the 10.5 dpc mouse retina (Trousse et al., 2001) (Fig. 3A, B; Table 1, P= 0.358).

We next examined cell division in *Tbx2*$^{-/-}$ mutants by pH3 immuno-labelling on somite-matched embryos. Labelled retinal progenitors in M-phase were correctly located at the ventricular surface of the neural retina (Sidman and Smelser, 1961; Dhomen et al., 2006) in both mutant and wild-type embryos (Fig. 3C, D). Comparisons of pH3 cell counts and mitotic indices (pH3 cells/total cells) throughout the neural retina (global) showed a trend of an increase in the numbers of mitotic cells in mutants compared to wild types at ss 30-31 (Table 1; Fig. 3C, D; *P*= 0.10). Cell division in the dorsal retina only was also compared. Significantly more cells were in M-phase in mutants compared to wild-type embryos in the dorsal neural retina (Fig. 3E; *P*= 0.04). However, there was no difference in the mitotic index of the ventral retina (data not shown).

As the increased level of dorsal mitotic cells does not readily provide an explanation for the delayed optic vesicle invagination and hypocellular ventral retina (Fig. 2C), we also performed TUNEL and pH3 analysis at an earlier stage of development at ss 20-24 (9.5 dpc) prior to optic vesicle invagination. At this stage, *Tbx2* expression is not yet restricted to the dorsal retina, and is instead expressed in both the dorsal and ventral regions of the presumptive retina of the optic vesicle (Fig. 1B). A significantly increased level of TUNEL labelling index was detected in the mutant retina compared with somite-matched wild types at 9.5 dpc (Fig. 4A, B, C).

In contrast to the reduced retinal cell number observed one day later, at this earlier stage, an increased overall cell number was observed, (Fig. 4D). There were also significantly more mitotic cells present in the mutant presumptive retina compared to wild types at 9.5 dpc (Fig. 4E). Thus, in the absence of *Tbx2*, an increased mitotic index was observed at sites normally expressing *Tbx2*, namely the distal optic vesicle at 9.5 dpc and the dorsal neural retina at 10.5

dpc. An increased number of pH3 positive cells were also detected in the ventral mesenchyme between the hypoplastic ventral neural retina and the surface ectoderm, where *Tbx2* expression is normally observed (Fig. 1D, arrowhead and Fig. 3C, D, arrowheads).

Together these data show that loss of *Tbx2* at optic vesicle stage, leads to increased cell death and dysregulation of cell proliferation. These changes occur prior to a visible morphological abnormality in the mutants and coincide with the earliest phase of *Tbx2* expression in wild types. Hence, cell death at 9.5 dpc causes the small retinal volume observed by 10.5dpc, and altered regional cell proliferation and cell death cause the abnormal optic vesicle morphogenesis observed by E10.5. The hypocellularity of the ventral retina therefore results from the failure of normal growth and invagination across the D/V axis. As reduced lens vesicle size (Figs. 1L and 3B, D) was also observed, non cell autonomous signalling dependent on *Tbx2* expression must be required for growth of the lens tissue.

Fgf15 has been implicated in dorsal midbrain growth (Ishibashi and McMahon, 2002; Saitsu et al., 2005). It is first expressed, like *Tbx2*, in the distal optic vesicle at 9.5 dpc (Kurose et al., 2004; McWhirter et al., 1997) and then as *Tbx2* expression is restricted to the dorsal neural retina, *Fgf15* continues to be expressed in dividing retinal progenitor cells in the optic cup during retinal histogenesis (Blackshaw et al., 2004; Rowan et al., 2004). Moreover, its reduced expression in two mouse models, the $Chx10^{orJ/orJ}$ ocular retardation mutant (Rowan et al., 2004) and $Bmpr1a^{-/-}/Bmpr1b^{-/-}$ mutants (Murali et al., 2005) correlates with a failure of normal optic cup growth. Notably, $Bmp4^{-/-}$ optic vesicles also showed loss of *Fgf15* expression, that could be restored by application of exogenous BMP4 (Murali et al., 2005). At 10.5 dpc in wild types, *Fgf15* and *Tbx2* showed complementary expression patterns in central and dorsal presumptive retina respectively (Fig. 5A, C). In the *Tbx2* mutants we found that at early optic vesicle stage, *Fgf15* was correctly expressed (data not shown), whereas after invagination at ss 35, *Fgf15* expression was absent in 4/6 mutant retina and weakly expressed in the other two (n= 4/6 eyes; Fig. 5A, B and data not shown). The loss of *Fgf15* expression supports the conclusion that Tbx2 is required for normal regional proliferation across the D-V axis.

Analysis of *Tbx2* expression in $Chx10^{orJ/orJ}$ mutants, revealed that loss of *Chx10* did not affect *Tbx2* expression (n= 6/6 eyes; Fig. 5C, D) suggesting that *Tbx2* is not regulated by *Chx10*. In $Chx10^{orJ/orJ}$ mutants that lack Chx10 function, pigment genes normally expressed only in the RPE of the optic cup are expanded into the hypoplastic neural retina (Burmeister et al., 1996; Horsford et al., 2004; Rowan et al., 2004). To investigate whether a similar loss of retinal specification linked to reduced proliferation, was occurring in $Tbx2^{-/-}$ embryos, we examined the expression of the pigment gene, *Silver* (Baxter and Pavan, 2003). In contrast to the $Chx10^{orJ/orJ}$ mutants, in the $Tbx2^{-/-}$ optic cups, *Silver* expression was similar to that of wild-type embryos and was restricted to the RPE (Fig. 5 E, F), suggesting that the neural retina/RPE boundary is not compromised in $Tbx2^{-/-}$ embryos. Together these data indicate that while retinal specification occurs without *Tbx2*, it is required for regulation of proliferating progenitors during formation of the optic cup.

Cx43 is a putative downstream target of *Tbx2* in other tissues and has also been implicated in retinal growth (Becker and Mobbs, 1999; Kjaer et al., 2004; Paznekas et al., 2003; Vitiello et al., 2005). *CX43* mutation in humans results in oculo-dento-digital dysplasia, which is characterised by microphthalmia, cataract, and abnormalities of the iris, together with syndactyly and craniofacial skeletal defects, (Kjaer et al., 2004; Paznekas et al., 2003; Vitiello et al., 2005). Furthermore, it has been shown that the RPE, a site of Cx43 expression, regulates proliferation in the underlying neural retina by the release of ATP through gap junction Cx43 hemichannels (Pearson et al., 2005a; Pearson et al., 2005b). Immunohistochemistry in somite-matched $Tbx2^{-/-}$ mutant and wild-type retinae at 10.5 dpc during optic vesicle invagination showed Cx43 protein was distributed in the dorsal RPE, but not in the neural retina. However,

in mutants the Cx43 expression domain appeared increased in the dorsal, nasal, and temporal RPE, compared to weak or barely detectable labelling in equivalent regions in wild-type eyes (n= 6/6 eyes; wild type = 74.3 +/- 28.7; mutant =112 +/- 24.7 fluorescent intensity units; Fig. 5 G-J). Therefore, while the appropriate expression of *Silver* indicates that RPE specification is not affected by the loss of *Tbx2*, the upregulated Cx43 expression in the RPE of mutant eyes shows that *Tbx2* plays a role in mediating interactions between the neural retina and RPE during optic vesicle morphogenesis that are known to be important for the regulation of proliferation. Altered Cx43 expression across the D-V axis provides a possible mechanism to regulate regional proliferation by modulating ATP release.

Tbx2 is necessary for the maintenance of correct spatial expression of D-V markers

Previously we identified *Tbx2* as a downstream target of Bmp4 in the developing optic cup (Behesti et al., 2006). Bmp4 is thought to be a primary mediator of dorsal specific gene expression (Koshiba-Takeuchi et al., 2000) and its mutation in humans was recently shown to cause a spectrum of eye abnormalities including microphthalmia (Bakrania et al., 2008). Here we investigate whether *Tbx2*, acting downstream of Bmp4, is responsible for the regulation of other D-V patterning genes. Whole mount *in situ* hybridisation was carried out at 10.5-11.5 dpc of the dorsal markers *Tbx3, Tbx5, Msx2, ephrinB2* and *Cyp1b1*, and the ventral marker *Vax2*.

We first investigated whether the population of cells in which *Tbx2* would normally be expressed are present in their normal location in the mutants. Labelling of cells using a full-length *Tbx2* probe, which recognizes the mutant RNA, confirmed that lack of Tbx2 does not result in death nor in migration of these cells from the dorsal optic cup (n= 4/4 eyes, Fig. 6 A, B). The expression of *Bmp4* in the dorsal neural retina was also normal at both 10.5 and 11.5 dpc (Fig. 6C-F), indicating that *Tbx2* does not feed back into the regulation of *Bmp4* expression in the optic cup.

The Bmp-responsive genes *Msx2, Tbx3*, and *Tbx5* were all expressed dorsally in the developing eye of *Tbx2*$^{-/-}$ embryos, however, although *Msx2* and *Tbx3* expression was normal in mutants (n= 4/4 eyes per probe; Fig. 6G-J), the expression domain of *Tbx5* was markedly reduced in size in comparison to somite-matched wild-type littermates at 10.5 and 11.5 dpc (n= 6/6 eyes; Fig. 6K-N). In contrast to the reduction in *Tbx5* expression dorsally, *ephrinB2* appeared expanded to cover a wider region of the dorsal optic cup (n= 5/6 eyes; Fig. 6O, P). Furthermore, the expression of *Cyp1b1* in the dorsal-most region of the neural retina appeared expanded as compared with stage-matched wild-type littermates (n= 5/6 eyes; Fig. 6Q, R). We also observed that while *Vax2* expression was induced normally in the ventral retina of the *Tbx2*$^{-/-}$ embryos, by 10.5 dpc the *Vax2* expression domain in the ventral optic cup was reduced (n= 6/6 eyes; Fig. 6S, T), correlating with the reduced size of the ventral neural retina (Figs. 3A-D, dashed lines and 5E, F and 2C). Together, these analyses show that *Tbx2* is not required for the onset of expression of these D-V patterning genes and hence D-V polarisation of the optic vesicle. Rather, *Tbx2* is required to maintain correct spatial expression of the key dorsal retinal determinant, *Tbx5*, during optic cup formation. In addition, *Tbx2* is needed to delimit the normal dorsal expression domain of the topographic guidance molecule ephrinb2 and the retinoic acid (RA) generating enzyme, Cyp1b1 (Chambers et al., 2007). The fact that these dorsally expressed genes are affected differently by loss of *Tbx2* (*Tbx5* reduced, *ephrinB2* and *Cyp1b1* increased, *Msx2, Bmp4* and *Tbx3* unchanged) argues against the observed changes simply reflecting the altered patterns of growth occurring without *Tbx2*. All of these downstream genes are known to be critically important for eye development. Mutations in the human *CYP1B1* gene cause primary congenital glaucoma (Stoilov et al., 1997); MIM601771) whereas the *Tbx5* and *ephrinB2* genes are implicated in D-V patterning, axon guidance and topographic mapping of RGCs from the retina to the brain.

Tbx2 and early retinal cell differentiation

Finally we examined whether loss of *Tbx2* and the delayed ventral morphogenesis and altered gene expression patterns observed by 10.5dpc in the Tbx2 mutants affected the early stages of retinal differentiation in the optic cup. Fibroblast growth factor signalling in the central neural retina is known to trigger retinal cell differentiation in chick and zebrafish embryos (Martinez-Morales et al., 2005) and we observed loss of *Fgf15* expression in the *Tbx2*$^{-/-}$ optic cups. Moreover, the altered expression of *Tbx5* and *ephrinB2* in the mutant eyes may affect RGC axonal projections. Immunohistochemistry was carried out on sections of 12.5 dpc mutant and wild-type littermate eyes with the RMO270 and the VC1.1 antibodies. RMO270 labels RGCs from the point of becoming post-mitotic at the ventricular surface of the neural retina, allowing detection of newly born cells migrating to the vitreal surface and their axonal projections (McCabe et al., 1999). VC1.1 detects amacrine and horizontal cells, and at 12.5 dpc labels precursors destined to differentiate into amacrine and horizontal cells (Alexiades and Cepko, 1997).

RMO270 labeling revealed that RGC differentiation in the centro-dorsal retina was underway in both mutant and wild types. Normally the central retina differentiates first and the peripheral region last (Dräger, 1985), and this pattern was maintained in the mutants (Fig. 7A, B), therefore excluding premature differentiation as a factor contributing to the small optic cup phenotype. In both genotypes, RGCs migrated to the correct location adjacent to the vitreous. By 12.5 dpc in wild-type eyes an organised nerve fibre layer was present in the central neural retina, dorsal to the optic disc (Fig. 7A). A similar spatial pattern of labelling was detected in mutant eyes (n= 4 eyes; Fig. 7B), though in contrast to wild-type eyes, no axons projected through the poorly defined optic disc (Fig. 7E, F), consistent with observations on H&E sections (Fig. 1M, N). In comparison to the neat layer of RGC axons in wild-type retinae, axons were extremely disorganised in mutant retinae, where the majority appeared to project into the vitreous chamber rather than follow the vitreal surface towards the optic nerve head (Fig. 7C-F).

VC1.1 immunohistochemistry at 12.5 dpc labelled cell bodies and their processes in the central neural retina in a similar pattern in both mutant and wild-type eyes (n= 4; Fig. 7G-L), suggesting that commitment to amacrine and horizontal cell fates also occurs normally in *Tbx2*$^{-/-}$ mutants. Together these data suggest that the onset of retinal histogenesis and the migration of cells to adopt a correct position across the laminar axis of the developing retina are not affected by the loss of Tbx2. However, intraretinal RGC axon guidance and optic nerve development appear impaired.

Discussion

Tbx2 in optic cup formation

We show that without *Tbx2*, presumptive neural retinal volume is reduced and invagination of the ventral optic vesicle is delayed resulting in the formation of small and abnormally shaped optic cups in *Tbx2*$^{-/-}$ mutants. This is the first report to demonstrate that Tbx2 is required for proper optic cup formation. Investigation of premature cell differentiation, and expansion of the RPE at the expense of the neural retina, excluded these mechanisms as the cause of the small optic cup phenotype. Instead we observed increased cell death, and an abnormal increase in the number of mitotic cells, in the mutant retina, coincident with the dynamic change in *Tbx2* expression. We also found abnormal expression of Cx43, a known regulator of retinal proliferation, as well as a loss of expression of the retinal progenitor cell marker *Fgf15* in the central retina. These findings support the conclusion that *Tbx2* acts to modulate apoptosis and regional levels of proliferation across the presumptive neural retina as its expression is restricted dorsally during optic vesicle to optic cup transition.

Tbx2 is initially expressed across the D-V axis in the distal optic vesicle. Our data indicate that this early phase of *Tbx2* expression followed by development of the high dorsal retinal expression of *Tbx2* is needed for coordination of growth and morphogenesis across the D-V axis (Fig. 8). Without *Tbx2* expression in the distal optic vesicle increased cell death occurs prior to initiation of invagination causing a hypocellular ventral retina apparent one day later. Restriction of *Tbx2* to the developmentally-advanced dorsal retinal region during invagination is essential to suppress dorsal growth and this action appears to be required for the acquisition of the globe shape of the optic cup. Our data therefore indicates that *Tbx2* regulates growth and morphogenesis by modulating levels of apoptosis and facilitating regional differences in levels of proliferation across the presumptive neural retina.

Several *in vitro* studies have shown that Tbx2 acts to promote cell cycle progression/cell division, although clear evidence for a similar role during development has not been found. Instead, analysis of the developing heart (Cai et al., 2005; Ribeiro et al., 2007) and eye, point to an opposite role, where *Tbx2* slows down regional cell proliferation. In zebrafish, the pattern of proliferation along the looping heart tube is dynamic and changes from a pattern of homogenous proliferation, to a heterogeneous one in which dividing cells are more concentrated in the future chambers. This process appears to be regulated by *Tbx3* and *Tbx2*, as overexpression caused a decrease in the number of dividing cells, whereas fish embryos injected with morpholino against *tbx3b* and/or *tbx2a* showed an increased number of dividing cells, and in both cases regionalisation of proliferating cells was lost (Ribeiro et al., 2007). Recently, the *Tbx2* ortholog *omb* was proposed to act as a repressor of retinal growth acting to delimit the extent of the eye field in *Drosophila*. *omb* mutants display an increase in ommatidial number, while ectopic over expression of *omb* caused reduced eye size, or even the complete loss of retinal tissue (Porsch et al., 2005). Our data are in agreement with this emerging role of *Tbx2* as a regulator of regional growth during development and these findings highlight the importance of controlling cell proliferation as a driving force of morphogenesis.

Several of the phenotypes observed in *Tbx2*$^{-/-}$ embryos are similar to those observed in retinoic acid (RA) deficient embryos. Certainly aspects of the *Tbx2*$^{-/-}$ phenotype are consistent with Tbx2 regulation of growth factor signalling that influences surrounding tissue non cell autonomously. RA deficiency in zebrafish embryos results in lack of ventral neural retina formation (Marsh-Armstrong et al., 1994) and in mammals causes microphthalmia and anophthalmia (Kalter and Warkany, 1959), while retroviral expression of a dominant negative form of the RA receptor α (*RARα*) in the chick results in a small eye phenotype (Sen et al., 2005). Other remarkably similar abnormalities were recently detected in *Aldh1a1; Aldh1a3* compound null mutants (which lack retinaldehyde dehydogenase activity), including shortening of the ventral neural retina, expansion of the ventral extra-ocular mesenchyme and failure of eye lid groove formation (Molotkov et al., 2006). This latter study concluded that RA sources external to the optic cup, guide eye morphogenesis via paracrine signalling but are unnecessary for retinal dorsoventral patterning as both *Tbx5* expression and *Vax2* expression were unaffected (Molotkov et al., 2006). Notably, we found that loss of *Tbx2* caused an increase in dorsal *Cyp1b1* expression. CYP1B1 synthesises both all-trans-retinal and all-trans-RA independently of the major RA synthesising enzymes (RALDH1-3), and contributes to D-V patterning in the chick neural tube (Chambers et al., 2007). Whether increased CYP1B1 activity affects retinal growth is not known. However, the similarities in these phenotypes suggest that investigation of the interplay between *Tbx2* and the network of enzymes and receptors that modulate RA signalling, will offer further insight into the relationships between these pathways in optic cup formation.

Tbx2 in the regulation of D-V patterning and RGC intraretinal projections

Bmp4, expressed in the dorsal optic cup, is essential for the expression of several other dorsally expressed genes including *Tbx2* (Behesti et al., 2006) and the repression of the ventrally expressed gene *Vax2* (Koshiba-Takeuchi et al., 2000; Murali et al., 2005). Here we have shown that *Tbx2* is not required for *Bmp4* expression, nor for the onset of expression of several other D-V patterning genes. Instead, it is required for the maintenance of *Tbx5*, and the restriction of *ephrinB2* and *Cyp1b1* expression in the dorsal optic cup. Whereas chick *Tbx5* misexpression ectopically induced *ephrinB2* expression (Koshiba-Takeuchi et al., 2000), our genetic analysis showed that changes in *ephrinB2* expression can occur independently of *Tbx5* in the mouse. In fact *ephrinB2* expanded ventrally in $Tbx2^{-/-}$ mutants while *Tbx5* was reduced. Furthermore, although *Bmp4* is essential for the induction of *Tbx5* expression (Murali et al., 2005) and can induce ectopic *Tbx5* expression (Behesti et al., 2006; Koshiba-Takeuchi et al., 2000) here we show reduced *Tbx5* expression while *Bmp4* expression is unaffected. Together, these data indicate that *Tbx2* is required to maintain *Tbx5* expression in the dorsal retina, whereas the other Bmp4-responsive genes, *Msx2* and *Tbx3* are unaffected by loss of Tbx2. Our study clearly positions *Tbx2* as a downstream effector of Bmp4 signalling; loss of *Tbx5* and *Fgf15* expression is seen in both $Bmp4^{-/-}$ (Murali et al., 2005) and $Tbx2^{-/-}$ optic cups, whereas *Bmp4* expression is unchanged in the *Tbx2* mutants. However, the fact that *Vax2* expression expands into the dorsal retina in the $Bmp4^{-/-}$ optic cup (Murali et al., 2005), whereas the ventral *Vax2* expression domain is reduced in the *Tbx2* mutant, shows that *Tbx2* functions not to induce, or to repress *Vax2*, rather it is needed for generation of the ventral tissue that expresses *Vax2*.

Functional studies have previously shown that *Tbx5* and *ephrinB2* expression domains are required for D-V patterning and topographic mapping of RGC axons from the dorsal neural retina to the brain (Koshiba-Takeuchi et al., 2000; Mann et al., 2002) and here we show that disruption of these gene expression domains is associated with intraretinal RGC axon guidance problems in *Tbx2* mutant optic cups. In our study the simplest explanation for the RGC guidance problems is that they are secondary to the patterning and morphological defects in the optic cup, although a direct requirement for *Tbx2* in RGCs has not been excluded. Significantly, in the chick following *Vax2* (*cVax*) misexpression, dorsal and ventral RGC axons exhibited defects in intraretinal pathfinding near the optic disc (Muhleisen et al., 2006). A number of other different sets of positive and negative factors cooperate in the process of intraretinal guidance (Bao, 2008). These include BMP7, possibly secreted by the periocular mesenchyme, which is needed for the generation of the optic disc and normal RGC routing (Morcillo et al., 2006). However, loss of *Bmp7* was associated with reduced apoptosis and normal *Tbx5* expression, both not seen in the *Tbx2* mutants, suggesting distinct mechanisms.

Morpholino-mediated knock-down of *Tbx2b* in the zebrafish embryo resulted in a temporary block to differentiation only in the dorsal retina (Gross and Dowling, 2005). Markers of amacrine, and rod and cone photoreceptors cells were downregulated in the zebrafish dorsal retina, whereas RGCs were less affected and axons projected to the optic disc to form the optic nerve (Gross and Dowling, 2005). Here, our findings on retinal differentiation in the $Tbx2^{-/-}$ mouse are contrary to the zebrafish knockdown, although this may be due to the later stages of retinal development examined in the zebrafish. We provide evidence for the existence of a dorsal differentiating cell population in the $Tbx2^{-/-}$ mouse retina, by immunolabelling with VC1.1 which labels precursors of amacrine and horizontal cells (Alexiades and Cepko, 1997) and by labelling for RGCs which show a normal spatial onset of differentiation. Therefore in the mouse, dorsal retinal progenitors are, at least initially, fated towards distinct cell types.

Conclusion

We propose a model for the function of *Tbx2* during optic cup formation (Fig. 8) in which *Tbx2* acts downstream of the dorsal determinant *Bmp4* and is required for modulating retinal growth during optic vesicle invagination. *Tbx2* controls both cell death and regionalised cell proliferation, and is essential to maintain expression of *Tbx5* and *Fgf15* across the D-V axis, and to restrict dorsal expression of Cx43, *Cyp1b1* and *ephrinb2*. *Tbx2* is initially expressed in the entire distal optic vesicle, where it represses proliferation. As *Tbx2* expression becomes restricted to the dorsal presumptive neural retina, it slows growth regionally, allowing the ventral retinal region to proliferate more and "catch-up" in terms of growth with the dorsal neural retina. This coordination of growth across the D-V axis is required for normal morphogenesis of the optic vesicle to form the optic cup.

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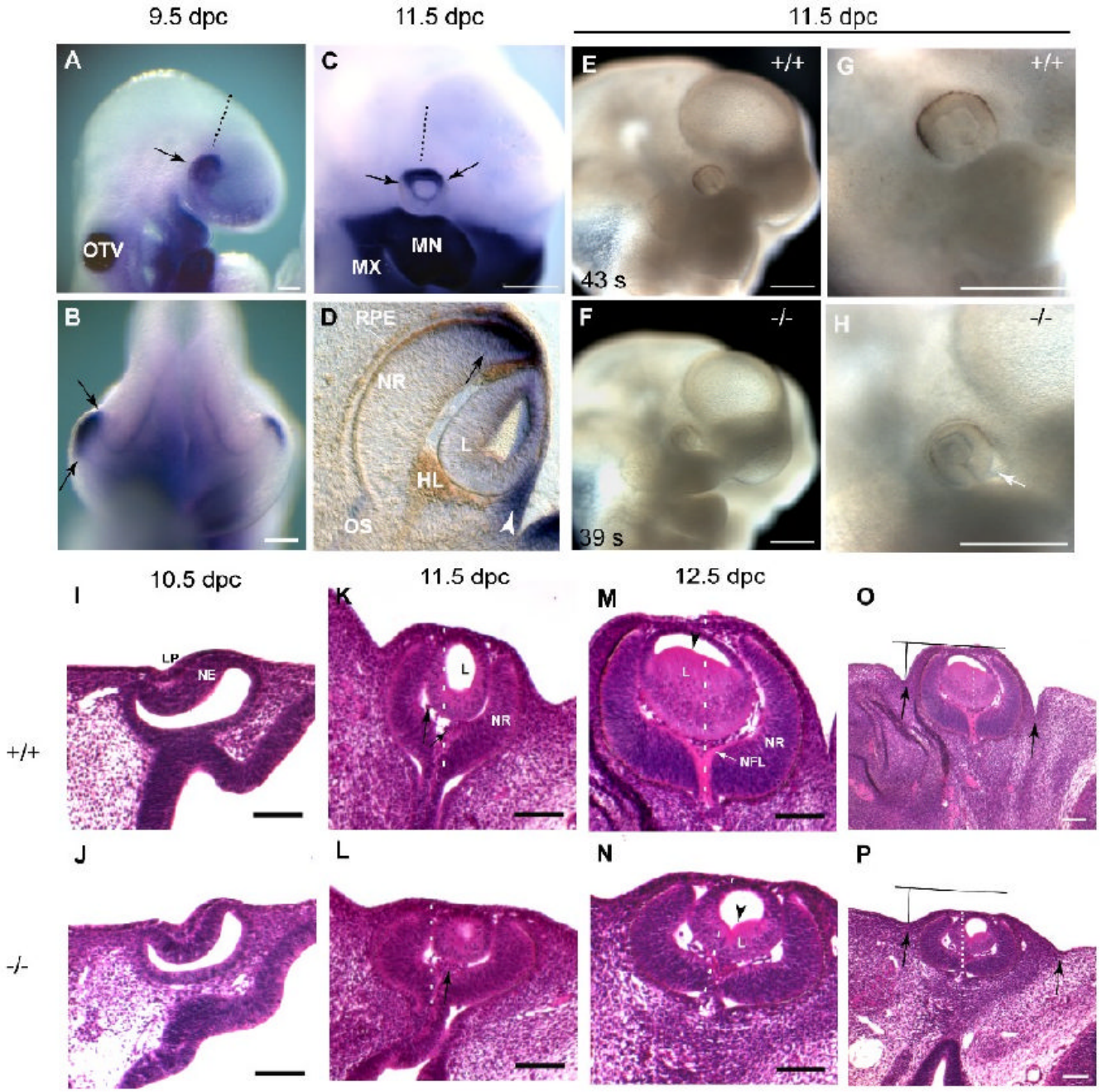

Fig. 1. Delayed ventral optic cup invagination and reduced optic cup growth in *Tbx2*$^{-/-}$ embryos, 9.5-12.5 dpc

(A, B) *Tbx2* expression along the D-V axis of the optic vesicle at 9.5 dpc. **(B)** Frontal view of the head shows *Tbx2* localised to the distal (presumptive neural retina) optic vesicle (arrows). **(C)** Restriction of *Tbx2* expression to the dorsal optic cup at 11.5 dpc (arrows). Dotted lines in **A** and **C** show the D-V axis in relation to the head. (**D**) *Tbx2* expression in the dorsal optic cup (arrow) and in ventral mesenchyme (arrowhead). **(E-H)** Lateral views of wild-type (+/+) and mutant (-/-) eyes, at 11.5 dpc, **G, H** show high magnification views. Note the small optic cup size and abnormally large subretinal space in the ventro-nasal optic cup of the mutant (arrow in H). **(I-P)** Transverse H&E stained sections of 10.5-12.5 dpc eyes, **(I, J)** at 10.5 dpc, **(K,**

L) through the ventral region at 11.5 dpc showing reduced growth and length along the proximo-distal (P-Di) axis (dotted lines) of the optic cup in mutants, arrows **(K, L)** point to the dense hyaloid vasculature in mutants compared to wild-type littermates, **(M, N)** at 12.5 dpc, sections show the lack of a nerve fibre layer (arrow) and delayed lens fibre differentiation (arrowheads) in mutants, **(O, P)** compare the small P-Di length (dotted lines) and the reduced protrusion (solid lines) of the optic cup in mutants to wild-type littermates. Arrows indicate the lack of eye lid groove formation.

Abbreviations: L, lens; LP, lens placode; OTV, otic vesicle; MN, mandibular process of the first branchial arch; MX, maxillary process of the first branchial arch; NE, neural retina; NFL, nerve fibre layer; NR, neural retina

Scale bars: A,B 100 μm; D, 50 μm; E-J, 500 μm.

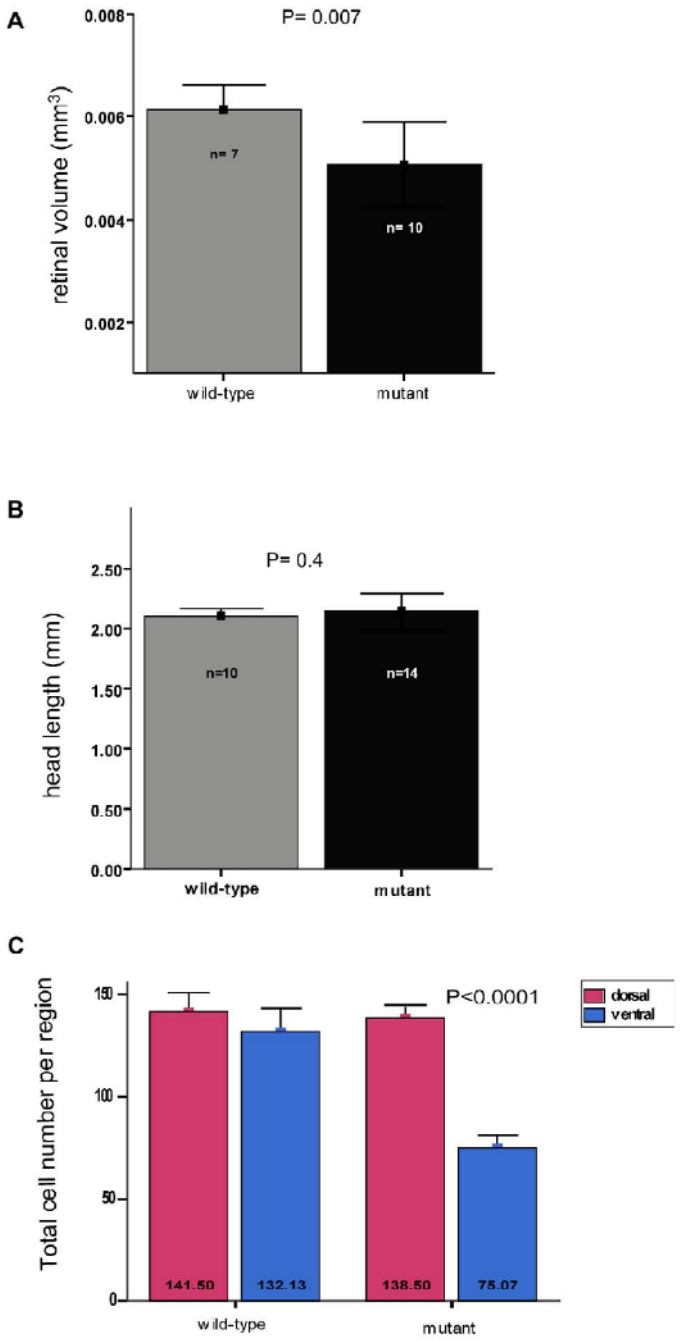

Fig. 2. Small retinal volume in *Tbx2*$^{-/-}$ embryos at 10.5 dpc (A)

Bar chart of retinal volume in wild-type and mutant embryos at ss 33-35 (10.5 dpc), showing smaller retinal volume in mutants. **(B)** Bar chart of head length in wild-type and mutant embryos at ss 33-35 (10.5 dpc), showing comparable growth. (C) Bar chart of retinal cell number per region in wild-type and mutant embryos at 10.5dpc showing reduced cell number in the ventral neural retina in mutants. Bars represent mean ± 1 s.d in A, B and ± 1 SE in C; n= number of embryos, P-value by independent samples t-test.

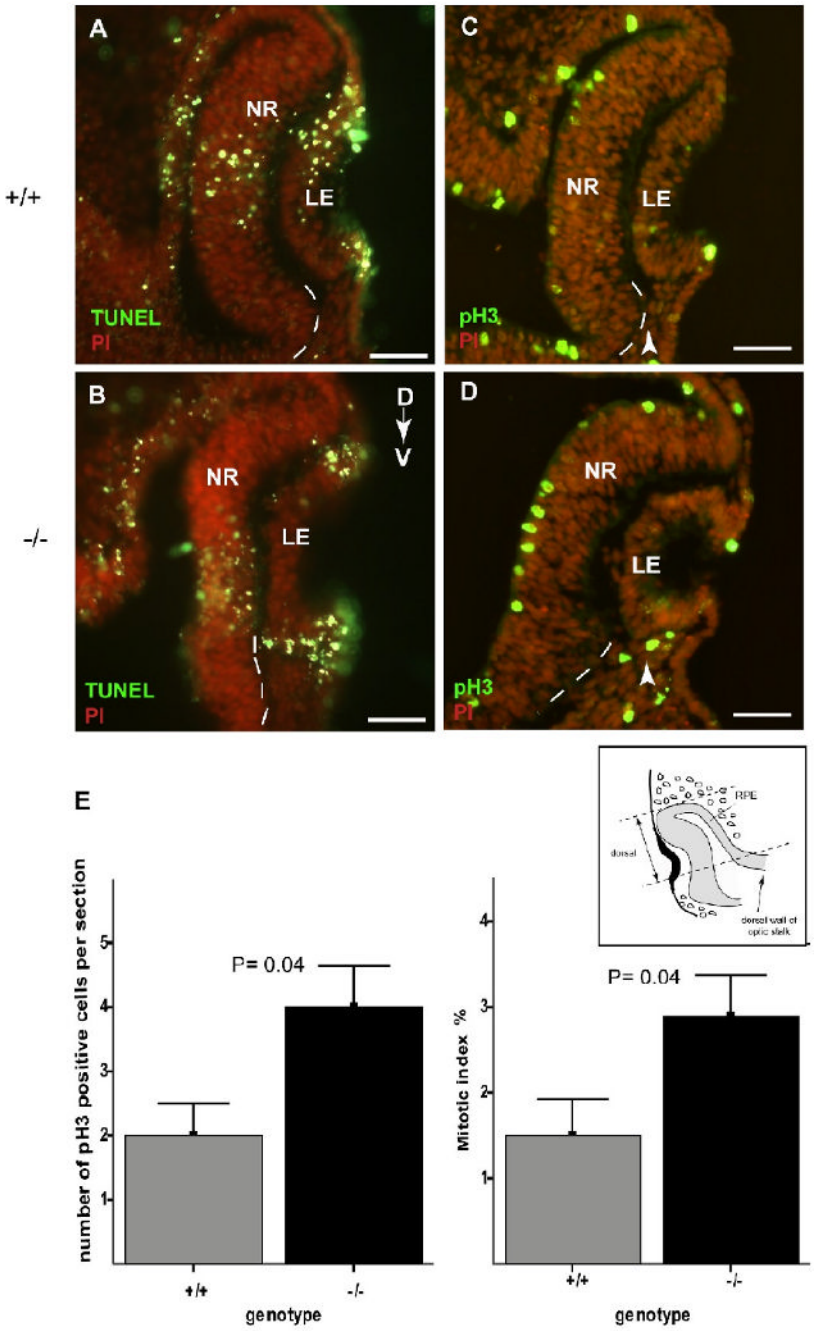

Fig. 3. Altered regional cell division, but not cell death, in the neural retina in the absence of *Tbx2* at 10.5dpc

(A, B) TUNEL labelling (green) of apoptotic nuclei on coronal sections of 10.5 dpc optic cups from somite matched (ss 30-31) wild-type and mutant eyes showing no change in the pattern of apoptosis. **(C, D)** Immunolabelling with anti-pH3 (green) showing increased number of mitotic figures in mutants (-/-) compared to somite matched wild-type embryos (+/+) at 10.5 dpc. Dotted lines demarcate the limits of the ventral optic cup; note lack of invagination in the mutant and the increase in the number of mitotic cells in the ventral extra-ocular mesenchyme (arrowheads). Sections were counterstained with propidium iodide (red) **(E)** Increased number of dividing cells, and mitotic indices (the number of mitotic cells per total number of cells)

detected in the dorsal neural retina of mutants. pH3-labelled cells were counted in 14 midline sections from 6 mutant eyes, and 8 sections from 4 wild-type eyes. P-values calculated by ANOVA for number of pH3+ cells per section and ANCOVA for mitotic indices. Bars represent means ± 1 S.E. The schematic indicates the dorsal retinal region included in counts. Abbreviations: LE, lens vesicle; NR, neural retina.
Scale bars: 50 μm

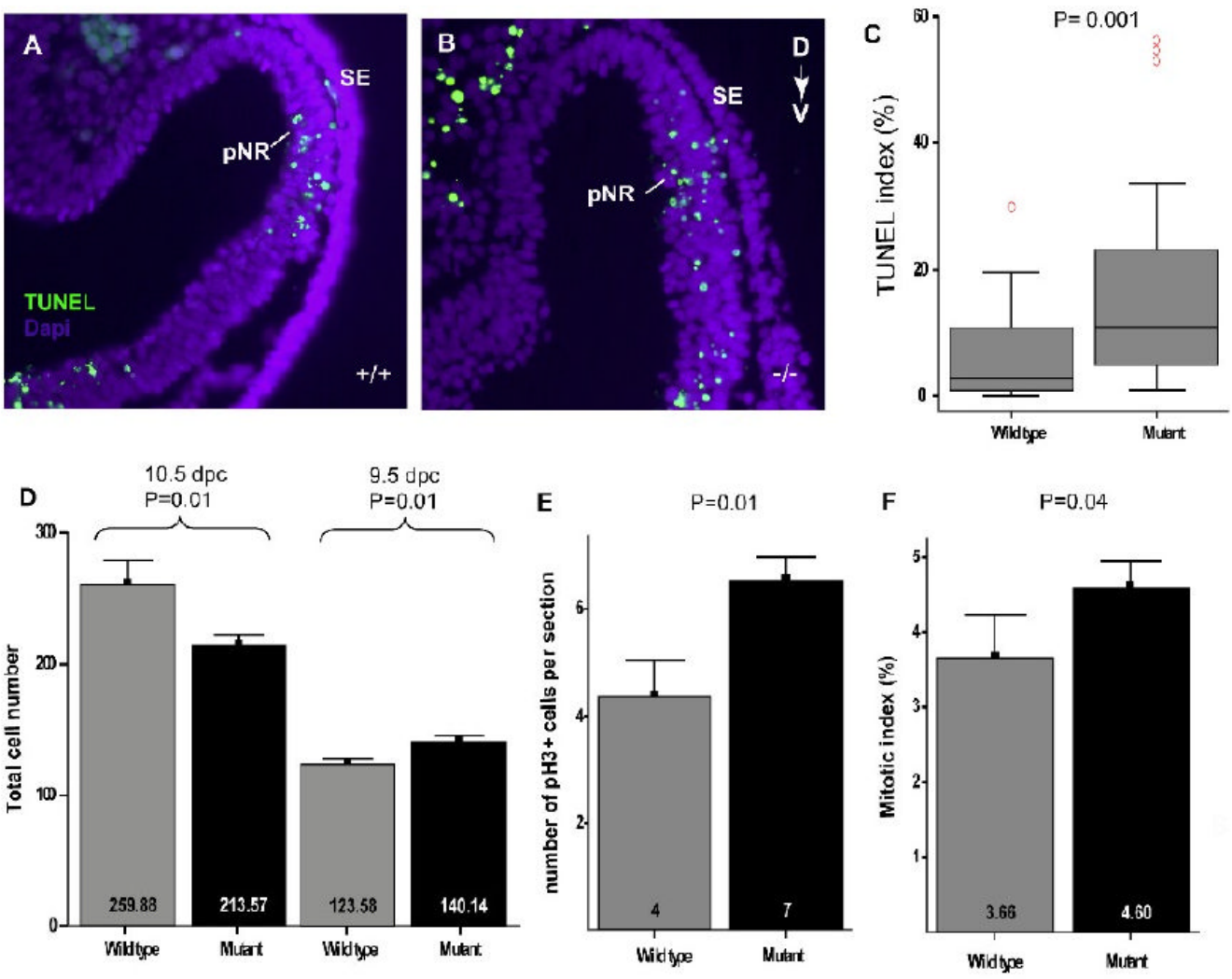

Fig. 4. Increased cell division and cell death, in the neural retina in the absence of *Tbx2* at 9.5 dpc

(A, B) TUNEL labelling (green) of apoptotic nuclei on coronal sections of 9.5 dpc optic cups from somite matched (ss 20-24) wild-type and mutant eyes. Sections were counterstained with Hoescht. (blue). **(C**) Box plot showing increased level of TUNEL index in mutants compared to wild types at 9.5 dpc. (**D**) Bar chart of total retinal cell number in wild-type and mutant embryos at 9.5 dpc and 10.5dpc. Increased cell numbers were present in the mutant retina at 9.5 dpc, whereas the retina is hypocellular compared with the wild type by 10.5 dpc. **(E)** Increased number of dividing cells, and **(F)** mitotic index (the number of mitotic cells per total number of cells) detected in the presumptive neural retina of mutants at 9.5 dpc. pH3-labelled cells were counted in 6 mutant eyes (25 sections) and 8 wild-type eyes (32 sections) and TUNEL-labelled cells were counted in 6 mutant eyes (24 sections) and 8 wild-type (25 sections). P-values calculated by ANOVA for number of pH3+ cells per section, ANCOVA for the mitotic index and Mann Whitney for the TUNEL index. Bars represent means ± 1 S.E. Mean values are indicated on bars. Abbreviations: SE, surface ectoderm; pNR, presumptive neural retina.

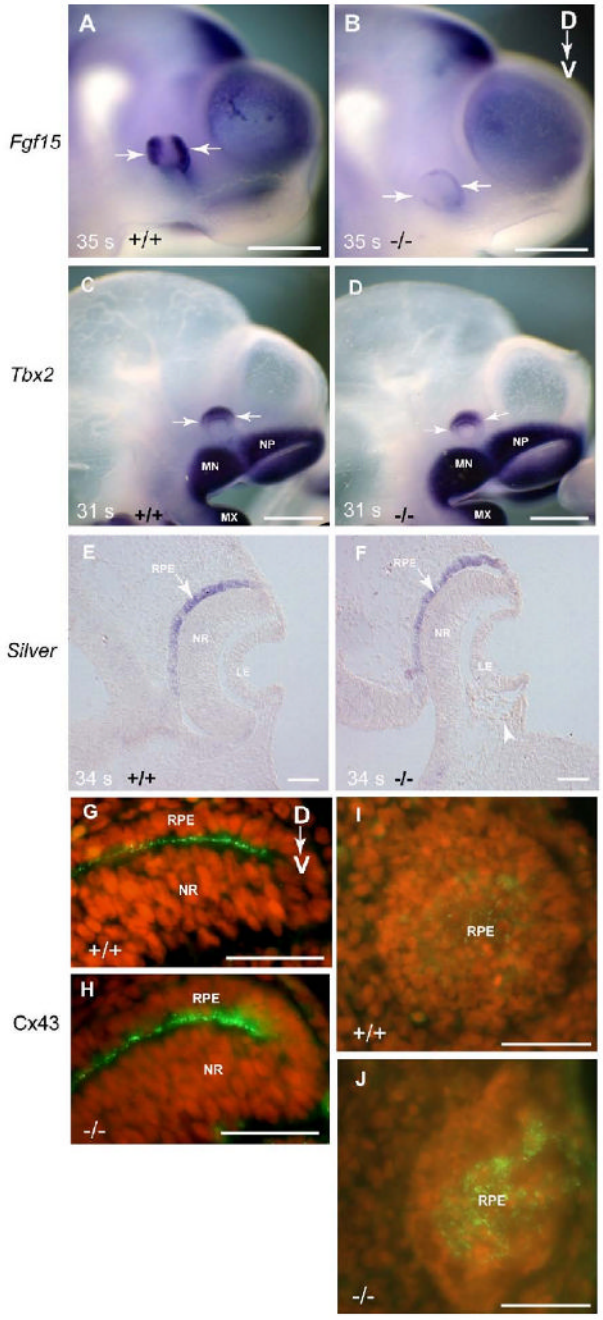

Fig. 5. *Tbx2* regulates the expression of the *Fgf15* signalling molecule in the neural retina and Cx43 in the RPE

(A, B) Lateral views of somite matched wild-type (+/+) and *Tbx2*$^{-/-}$ embryos at 10.5 dpc showing loss of *Fgf15* expression in the mutant. **(C, D)** Lateral views of somite matched wild-type (+/+) and *Chx10*$^{orJ/orJ}$ embryos at 10.5 dpc showing normal *Tbx2* expression. **(E, F)** Coronal sections of wild-type (+/+) and *Tbx2*$^{-/-}$ eyes showing normal *Silver* expression in the RPE. **(G, H)** High magnification of the dorsal optic cup on coronal sections showing Cx43 immunolabelling (green) in the dorsal RPE in the wild-type (**G**), higher labelling intensity in *Tbx2*$^{-/-}$ (**H**). (**I, J**) High levels of Cx43 detected in nasal RPE in *Tbx2*$^{-/-}$ optic cups (**J**) compared to low levels in matched wild type sections (**I**).

Abbreviations: NR, neural retina; RPE, retinal pigmented epithelium
Scale bars: 500 μm in A-D; 50 μm in E, F; 100 μm in G-J

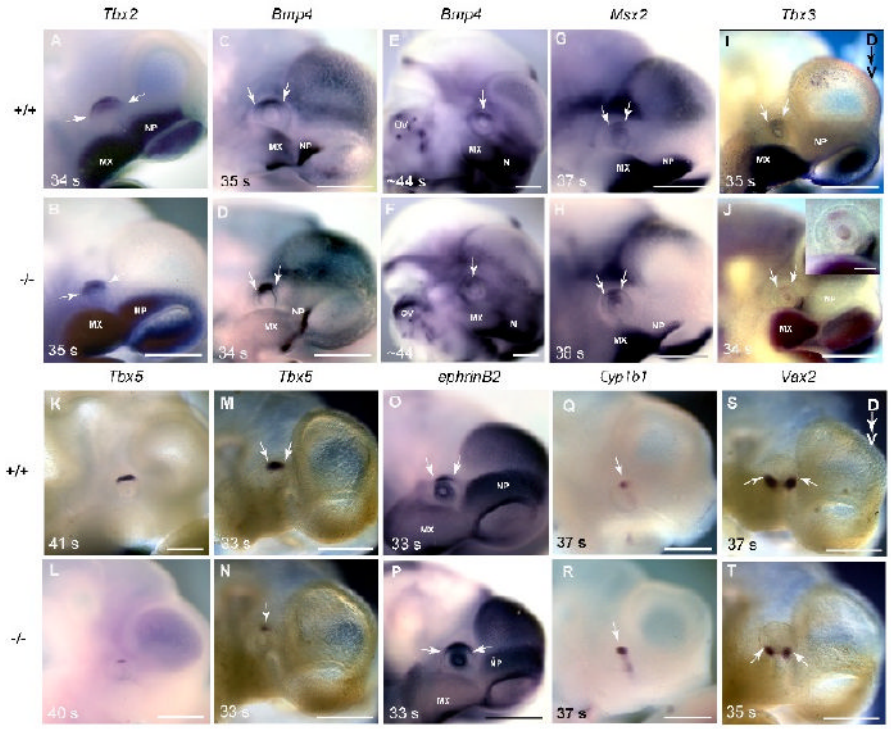

Fig. 6. *Tbx2* regulates *Tbx5* and *ephrinB2* expression but is not required for the initiation of expression of D-V patterning genes

Lateral views of somite matched mutant and wild-type embryos hybridised with probes for **(A, B)** *Tbx2*, **(C-F)** *Bmp4*, **(G, H)** *Msx2*, **(I, J)** *Tbx3*, **(K-N)** *Tbx5*, **(O, P)** *ephrinB2*, **(Q, R)** *Cyp1b1*, **(S, T)** *Vax2*. There was a reduction in *Tbx5* and *Vax2* expression domains and expansions in *ephrinB2 and Cyp1b1* expression.

Abbreviations: NP, nasal primordium; MX, maxillary process of the first branchial arch; D, dorsal; V, ventral. Scale bars: 0.5 mm

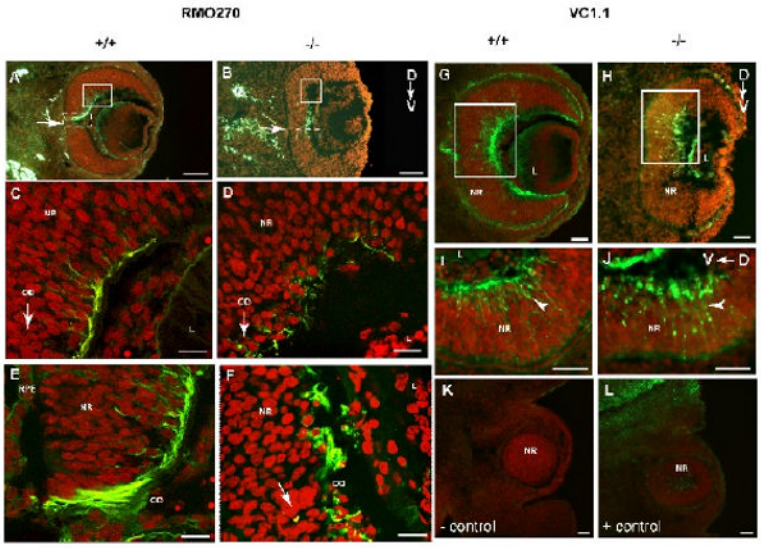

Fig. 7. Lack of an organised nerve fibre layer in *Tbx2*$^{-/-}$ embryos

Coronal midline sections of wild-type (+/+) and mutant (-/-) eyes immunolabelled with RMO270 for RGC bodies and processes (green) (A-F), and VC1.1 for inner retinal neurons (G-L), and counterstained with propidium iodide (red). **(A, B)** Low magnification showing the nerve fibre layer dorsal to the optic disc (OD; position indicated by arrows) in both genotypes. Solid boxes demarcate the dorsal regions magnified in **C, D**, and dotted boxes demarcate the optic disc region magnified in **E, F**. In the mutant **(D, F)** the RGC axons were disorganised, often protruding into the vitreous instead of following the vitreal surface towards the optic disc and no axons were seen to exit the optic disc. **(G, H)** Coronal sections showing VC1.1 labelling in the dorso-central neural retina. Boxed regions are magnified in **I, J**. Arrowheads indicate labelled cell bodies. **(K, L)** Negative (no primary) and positive controls for labelling on adjacent sections of retinal tissue at 12.5 dpc.

Abbreviations: L, lens; NR, neural retina; OD, optic disc

Scale bars: 100 μm in A, B; 20 μm in C-F, 50 μm in G-L

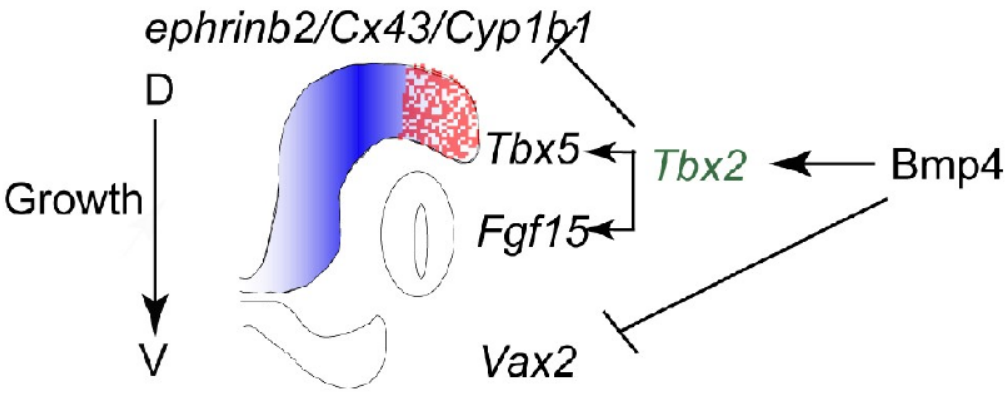

Fig. 8. A model for the function of *Tbx2* during optic cup formation

Only interactions indicated by genetic evidence from this study and Murali *et al.*, 2005 are shown. *Tbx2* acts downstream of the dorsal determinant *Bmp4* and is essential to maintain expression of *Tbx5* and *Fgf15* across the D-V axis, and to modulate cell death and regionalised cell proliferation necessary for proper optic cup formation. Restriction of *Tbx2* to the dorsal presumptive neural retina (red) occurs as dorsal *Bmp4* levels decline during invagination. The proliferating central retina expresses *Fgf15* (blue) and neural retinal growth extends along the D-V axis. *Tbx2* maintains *Tbx5* expression in the dorsal retina and restricts dorsal expression of regulators of retinal growth and topographic guidance molecules (Cx43, Cyp1b1, ephrinb2). *Tbx5* and *Fgf15* expression is lost in *Bmp4* (Murali *et al.*, 2005) and *Tbx2* mutants, whereas *Bmp4* expression is unchanged by loss of *Tbx2*. *Bmp4* represses *Vax2* expression (Murali *et al.*, 2005). Both *Vax2* induction in the ventral retina and repression in the dorsal retina is independent of *Tbx2*.

Table 1

Quantification of the number of mitotic and apoptotic cells in *Tbx2*$^{-/-}$ mutant and wild-type neural retinal sections at early 10.5 dpc

	Mean ± 1 s.d.	
	wild-type (4 eyes)	**mutant (6 eyes)**
global pH3 positive cells	3.8 ± 2.4	6.1 ± 3.9
global mitotic index	1.4 ± 0.9	2.8 ± 1.8
dorsal pH3 positive cells	2 ± 1.4	4 ± 2.4
dorsal mitotic index	1.5 ± 1.2	2.9 ± 1.8
Tunel positive cells	24 ± 22	17 ± 15