RESEARCH ARTICLE

MarvelD3 regulates the c-Jun N-terminal kinase pathway during eye development in *Xenopus*

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**ABSTRACT**

Ocular morphogenesis requires several signalling pathways controlling the expression of transcription factors and cell-cycle regulators. However, despite a well-known mechanism, the dialogue between those signals and factors remains to be unveiled. Here, we identify a requirement for MarvelD3, a tight junction transmembrane protein, in eye morphogenesis in *Xenopus*. MarvelD3 depletion led to an abnormally pigmented eye or even an eye-less phenotype, which was rescued by ectopic MarvelD3 expression. Altering MarvelD3 expression led to deregulated expression of cell-cycle regulators and transcription factors required for eye development. The eye phenotype was rescued by increased c-Jun terminal Kinase activation. Thus, MarvelD3 links tight junctions and modulation of the JNK pathway to eye morphogenesis.

**KEY WORDS:** MarvelD3, *Xenopus* eye, Tight junction, C-Jun N-terminal Kinase

**INTRODUCTION**

Eye morphogenesis is an evolutionarily conserved feature of most organisms to ensure an efficient communication with the environment (Gilbert, 2000). Eye development is a complex multi-step process, which involves cell proliferation, migration, cell-fate determination, survival and differentiation (Fuhrmann, 2008; Gilbert, 2000; Harada et al., 2007). After the neural antero-posterior patterning of the embryo, several signals, such as bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and non-canonical Wnt, promote the expression of eye-field transcription factors (EFTFs). EFTFs specify a single eye-field in the most anterior region of the neural plate. In this region, inhibition of cell-cycle activators occurs to favour EFTF expression, while duration of the expression of the transcription factors is established by cell-cycle-independent factors (Bilitou and Ohnuma, 2010). In the optic vesicle, the fate of the retinal precursors is determined during the last-division step before their final differentiation (Sasai, 2010). In the optic vesicle, the fate of the retinal precursors is determined during the last-division step before their final differentiation (Sasai, 2010). A non-canonical Wnt signal is then subsequently required for the final differentiation of the retinal progenitors into the cell types that make up the inner and outer retinal cell layers: glia, ganglion cells, interneurons, photoreceptors and the retinal pigment epithelium (RPE) (Gilbert, 2000; Harada et al., 2007; Maurus et al., 2005).

Most of the main eye components are derived from epithelia. Epithelial integrity is preserved by tight junctions (TJ), adherens junctions (AJ) and desmosomes (Balda and Matter, 2008; Eckert and Fleming, 2008; Fleming et al., 2000a,b; Ripley et al., 2004; Wei and Huang, 2013). MarvelD3 (MD3), a TJ transmembrane protein, belongs to the tight junction-associated MARVEL domain protein family, which also includes occludin and tricellulin (Cording et al., 2013; Raleigh et al., 2010; Shen et al., 2011; Steed et al., 2009). In the eye, occludin and tricellulin are thought to contribute to the compartmentalisation of the ocular micro-environments by controlling the flux through the retinal-blood barrier (Erickson et al., 2007; Huet et al., 2011; Iwamoto et al., 2014; Muthusamy et al., 2014; Tserentsosodol et al., 1998). Our previous work showed MD3 expression in corneal and RPE-derived cell lines (Steed et al., 2009). Additionally, MD3-mediated c-Jun N-terminal kinase (JNK) inhibition modulates cell proliferation, migration and survival in vitro (Steed et al., 2009). However, the function of MD3 in eye development has not been explored. Given the role of MD3 in the regulation of the JNK pathway, analysing its function in eye morphogenesis may lead to new insights into eye morphogenesis and the role of TJ-associated signalling mechanisms. We speculated that MD3 plays a role in the establishment of the complex regulatory systems that mediate eye morphogenesis and in the spatio-temporal regulation of the interplay between expression of EFTFs and cell-cycle regulators (Bilitou and Ohnuma, 2010; Zuber, 2010). Here, we asked whether MD3 is involved in eye morphogenesis in *Xenopus*. We show that MD3 is expressed in the eye region similarly to JNK and that MD3 depletion disrupted the normal expression pattern of EFTFs, the balance between cell proliferation and survival, and led to the development of smaller eyes or to an
eye-less phenotype. Similarly, MD3 overexpression affects the expression patterns of EFTFs, cell proliferation and survival but, contrary to MD3 depletion, overexpression led to no visible eye phenotype except a more compact lamination of the retina. These findings support a new role for MD3 as a regulator of ocular morphogenesis. Unexpectedly, the eye phenotype was not caused by increased JNK signalling but could be rescued by stimulating the JNK pathway in MD3-depleted embryos. Our data suggest that MD3 acts as a linker between the signals inducing eye morphogenesis, specification of the eye field by EFTFs, and survival and differentiation of multiple ocular cell types.

RESULTS
Structure and expression of MarvelD3 in the eye field

*Xenopus* MD3 (GenBank accession number, BC 068841) encodes a 420 amino acid (AA) protein with an amino-terminal intracellular domain followed by four transmembrane domains, two extracellular loops and an 18 AA cytoplasmic carboxy-terminal domain (Fig. 1A). *Xenopus* MD3 closely resembles isoform 2 of human MD3 (Steed et al., 2009). We first analysed the spatial distribution of MD3 expression by whole-mount *in situ* hybridization (WISH) and found that the MD3 transcript is broadly expressed in the animal pole before gastrulation (Fig. S1A,B). At the neurula stage, MD3 is enriched in the anterior region of the embryo, similarly to Rx1 (Retina homeobox 1), an eye marker (stage 15; Fig. 1B,C). Consistently, at tail-bud stages, MD3 is expressed in the head region (stage 25; Fig. S1C and Fig. 1D,D′), partially overlapping with Rx1 expression (Fig. 1E). To further substantiate our data on MD3 expression in the eye, we performed a RT-PCR analysis of the transcript using RNA purified from isolated optic vesicles (stage 25) and eyes (stage 42). Consistently with the results from the spatial analysis of MD3 expression, the transcript was detected in RNA derived from the eye field at both stages (Fig. 1F).

We previously showed that MD3 acts as an inhibitor of the JNK pathway (Steed et al., 2014), and MD3 distribution in *Xenopus* embryos is reminiscent of that of JNK and JNK activators (Fuhrmann, 2008; Hirai et al., 2005; Kibardin et al., 2006; Kim et al., 2005; Yamanaka et al., 2002). To determine in which region of the eye MD3 is expressed, we sectioned labelled embryos and detected MD3 expression in the lens, and in the inner and outer plexiform layers (Fisher, 1976) (Fig. 1G). MD3 is thus expressed in the head and, particularly, in specific tissues and cell layers of the developing eye.

MD3 is required for the eye morphogenesis

To determine the functional importance of MarvelD3 for eye development, we designed two morpholino (MO) antisense oligonucleotides that block MD3 translation, one targeting the 5′-UTR (untranslated region; MD3A MO) and the other at the start of the ORF (open reading frame; MD3B MO) of MD3. Inhibition of MD3 expression by MD3 MO was confirmed by immunoblotting (Fig. S2A).

First, we asked whether MD3 MOs cause morphological changes during embryogenesis by injecting the dorsal animal region of a blastomere at the 2-cell stage. During gastrulation and neurula stages, no apparent morphological defects were observed (Fig. S2B-C). In contrast, at tailbud stage, injection of MO either individually or together (MD3A MO, 85.4±2.66%; MD3B MO, 93.9±1.04%; MD3AB MO, 88.7±2.96%) showed a patchy or absent retinal pigmentation, while control (CT) MO-injected embryos (91.5±1.26%) presented an intact ocular pigmentation (Fig. 2A-C and F), supporting the specificity of the MO-induced eye phenotype. To
further substantiate the specificity of the observed phenotype, we performed rescue experiments by co-injecting MO with corresponding non-targeted mRNA. For MD3A MO, which targets a sequence in the 5′-UTR, we co-injected mRNA representing the coding region of MD3 (MD3 FL); and with MD3B MO, which binds at the start of the coding sequence, the mRNA encoded a mutated form carrying silent mutations in the MD3B MO targeting sequence (7mut-MD3). Both rescue approaches resulted in recovery of normal retinal pigmentation (97.4%±0.43 and 78.1%±1.63, respectively), suggesting the restoration of apparently normal eye development (Fig. 2D–E and F). Thus, the phenotype induced by MD3 MOs is specific and indicates that MD3 is required for eye development.

We then performed a more detailed investigation of the ocular structure to identify cell types affected in MD3 morphants. Histological analysis revealed a strong disorganisation of the overall eye structure, loss of retinal lamination, and absence of RPE (black pigmented ring) in MD3 MO injected embryos (Fig. 2G–H).

Fig. 2. MD3 KD disrupts eye morphogenesis. Alteration of eye morphology was analysed (A–E) and quantified (F) in non-injected (NI), CT, MD3A, MD3B, MD3A MO+MD3 FL RNA, MD3B MO+7-mut-MD3 RNA MO-injected embryos and embryos co-injected with MD3A MO and FL MD3 mRNA or MD3B MO and 7-mut MD3 mRNA. Lateral (A–E) and dorsal view (A’–E’) of the embryo; white arrow, level for dorsal view; black rectangle, eye; red asterisk, injected side of the embryo in all figures. Numbers indicate the embryos analysed. Crop of the eye (G–H) and head transversal section without (G–H′) or with a hematoxylin & eosin (H&E) staining (G′–H′) were analysed in wild-type (wt) and MD3B MO embryos. Red arrow, retinal pigment epithelium (RPE); white arrowhead, eye region; dotted red line, remaining eye tissue. Analysis by immunohistochemistry on eye sections from wt and MD3B MO-injected embryos was performed for lens cells (β-crystallin, I–I′), rod photoreceptors (Rhodopsin; J–J′) and ganglion cells (Hu-C/Hu-D, K–K′), and the number of positive cells for each eye marker was quantified as described in the Materials and methods (L); numbers indicate the eye sections quantified. Statistical significance was assessed by Student’s t-test in F (P values, CT MO=0.14; MD3A MO=8.99×10^{-5}; MD3B MO=2.21×10^{-5}; MD3AB MO=2.74×10^{-6}; MD3A MO+MD3 FL RNA=0.18; MD3B MO+7mut-MD3 RNA=6×10^{-2}; NS: non significant; **P<0.001), and Mann–Whitney test in L (P values, β-crystallin=1.59×10^{-2}; Rhodopsin=1×10^{-4}; Hu-C/Hu-D=1.92×10^{-2}, *P<0.05 and **P<0.01). Scale bars: 500 µm (A–E,G,H) and 100 µm (I–K). Embryos were analysed at stage 42.
Staining of sections for markers of specific ocular cell types further corroborated a retinal defect (Fig. 2I–K). Quantification of such images revealed reduced numbers of lens epithelial cells positive for β-crystallin (15.99%±6.7), ganglion cells positive for Hu-C/Hu-D (60.9%±15.38), and rod photoreceptors positive for rhodopsin (8.75%±4.6). Moreover, the remaining rod photoreceptors formed mislocalized clusters (Fig. 2I–L). MD3 is thus required for eye development and, in particular, for the formation of a laminated retina and differentiated lens.

As a complementary approach, we injected FL MD3 mRNA to overexpress MD3. MD3 overexpression did not have any evident morphological effects on gastrula, neurula and early tailbud development (Fig. S3). Additionally, MD3 overexpressing tadpoles did not present any visible eye phenotype (Fig. 3A–C).

Fig. 3. MD3 overexpression changes eye lamination. Eye morphology was analysed (A–B′) and quantified (C) in non-injected (NI), GFP or FL MD3 mRNA-injected embryos (P values in C, GFP RNA=0.89; FL MD3 RNA=0.26; NS: non significant). Lateral (A,B) and dorsal view (A′,B′) of the embryo; GFP mRNA was used as a control. Red asterisk, injected side of the embryo. (D,E). The eye size was quantified for GFP or FL MD3 mRNA-injected embryos, as described in the schemes and in the Materials and methods. Numbers indicate the embryos analysed; NS, non significant. P values in D, GFP RNA=0.66; FL MD3 RNA=0.91; P values in E, GFP RNA=0.46; FL MD3 RNA=0.52; NS, non significant. (F,G) Histological analysis by H&E staining was performed on eye sections from GFP and FL MD3 mRNA-injected embryos and the number of cells was quantified as described in the Materials and methods. (H) Number of cell were quantified per eye section stained by H&E derived from GFP or FL MD3 mRNA-injected embryos. Numbers indicate the eye sections quantified; P values, β-crystallin=0.19; Rhodopsin=0.87; Hu-C/Hu-D=0.74; NS, non significant. Statistical significance was assessed by Mann–Whitney (C,L) and Student’s t-test (D,E,H). Scale bars: 500 µm (A,B) and 100 µm (I–K). Embryos were analysed at stage 42.
such as altered retinal pigmentation, lens:retina ratio or eye size (Fig. 3D,E). In sections, however, the retina appeared more compact because of an increased number of cells in the MD3 overexpressing-embryos (Fig. 3F-H). However, this change in cell numbers did not affect lens epithelial cells, ganglion cells and rod photoreceptors (Fig. 3I-L). Altogether, these results suggest that MD3 plays a functionally important role in *Xenopus* eye development and morphogenesis.

**MD3 regulates the optic fate of cells-derived from blastomeres A1 and A3**

The active Spemann organizer favours normal eye morphogenesis (Bailey et al., 2004; Kim et al., 2005) and convergent extension (Itoh and Sokol, 1999); consequently, the MD3 MO-induced eye phenotype might be due to a dysfunctional mesodermal signal. A mesoderm contribution is unlikely, however, as no shortening of the antero-posterior axis was evident in MD3 morphants (Fig. 2). To rule out the mesoderm involvement, MD3 MOs were injected in a dorsal animal blastomere at the 8-cell stage. This injection also led to the reduced eye phenotype (93%±0.62; Fig. 4A). Hence, the eye phenotype of MD3 depleted embryos is mesoderm-independent.

At 32-cell stage, the eye only derives from nine blastomeres in *Xenopus*, among which the main contributors in the animal pole are (from dorsal to ventral): A1 and A3 for retinal cells, and A1, A3 and A4 for lens cells (Moody, 1987). In addition to their ocular contribution, A1, A3 and A4 blastomeres give rise mainly to the neural ectoderm, neural crest and epidermis, respectively (Fig. 4B). Thus, we asked which of these blastomeres participates in the MD3 MO-eye phenotype by injecting them with MD3 MOs. Fig. 4C-E shows that A1- and A3-injected embryos presented defective eye morphogenesis, but not A4-injected embryos. This suggests that A1 and A3 blastomeres, which mainly develop into neural and neural crest-derived cell types, are responsible for the observed eye phenotype. Hence, MD3 plays an important role during the development of the neural lineage that is essential for eye development.

**MD3 controls cell proliferation and survival in the eye field**

Given the contribution of the anterior neural plate region in the eye phenotype, we asked whether MD3 might be necessary for early eye-field formation as a step towards identifying its temporal involvement in eye development. We first analysed by WISH the expression patterns of the EFTHs: retinal homeobox 1 (Rx1), Paired box 6 (Pax6), and Orthodenticle homeobox 2 (Otx2) in neurulas (stage 17) and tadpoles (stage 30) depleted for MD3. Rx1 is a specific eye-field marker, whereas Pax6 is also expressed in the spinal cord and Otx2 at the forebrain/midbrain boundary (Maurus et al., 2005). Rx1 (Fig. 5A-B), Pax6 (Fig. 5C-D) and Otx2 (Fig. 5E-F) expression domains were initially enlarged in the eye-field region of neurulas and then subsequently reduced in tadpoles. These data suggest that MD3 depletion might deregulate the balance between proliferation and death during early eye-field morphogenesis. To validate this hypothesis, we first established the expression pattern of the cell-cycle marker cyclin D1 and then measured cell proliferation and apoptosis. The cyclin D1 expression domain was enlarged in 91% of embryos at stage 17 and reduced in the eye field of 89.5% of embryos at stage 30 (Fig. 6A-B), indicating that down-regulation of MD3 affected proliferation. Consistently in MD3 morphants, an increase in the number of dividing cells was observed in neurulas (612%±88.52 BrdU-positive cells; Fig. 6C), followed by a massive apoptotic wave in tadpoles (1216.7%±142.6 TUNEL-positive cells; Fig. 6D).

To reinforce the role of MD3 during early eye morphogenesis, we analysed the impact of MD3 gain-of-function on the expression domains of the EFTHs in neurulas (stage 17), tailbuds (stage 25) and tadpoles (stage 30). Rx1, Pax6 and Otx2 expression domains were mildly enlarged at neurula stage (Fig. 7A,C and E), indicating that down-regulation of MD3 affected proliferation. Consistently in MD3 morphants, an increase in the number of dividing cells was observed in neurulas (612%±88.52 BrdU-positive cells; Fig. 6C), followed by a massive apoptotic wave in tadpoles (1216.7%±142.6 TUNEL-positive cells; Fig. 6D).

MD3 activates the JNK pathway for eye morphogenesis

Building an eye requires complex regulation of multiple signalling pathways (Zuber, 2010).
Our previous in vitro study demonstrated that MD3 acts as a modulator of the JNK pathway and controls gene expression, cell proliferation and survival (Steed et al., 2014). The expression pattern of MD3 in vivo is similar to that of JNK and thus raises the question whether MD3 and JNK act together to regulate eye morphogenesis (Garriock et al., 2005). Increased JNK/AP1 activity in MD3 morphants is compatible with the enhanced proliferation observed in the early eye field, however, normal eye morphogenesis is thought to require JNK signalling in Xenopus due to activation of a non-canonical Wnt pathway (Lee et al., 2006; Maurus et al., 2005; Valesio et al., 2013). Hence, it could be that MD3 plays a more complex role in JNK pathway regulation than that of a simple repressor.

To determine the role of JNK downstream of MD3 in eye morphogenesis, we first tested whether inhibition of JNK affects ocular development. We added increasing concentrations of the JNK inhibitor SP600125 before eye field specification at stage 11 and then removed it at stage 22, when the eye field is defined. Modulation of JNK signalling with low concentrations of SP600125 indeed affected eye morphogenesis: the lens:retina ratio increased in a dose-dependent manner (0.25 to 1 μg ml⁻¹; Fig. 8). Higher concentrations of the inhibitor disrupted convergent extension, a process in which JNK plays a key role. However, ocular development seems exquisitely sensitive to JNK inhibition as it was already affected at low concentrations of SP600125 that did not seem to affect convergent extension.

Our previous studies indicate that MD3 attenuates the JNK pathway in vitro (Steed et al., 2014). Thus, we first treated MD3 MO-injected embryos at concentrations of SP600125 that did not

**Fig. 5. MD3 KD affects early eye-field formation.** Eye-field specification was analysed by WISH against the eye markers Rx1, Pax6, Otx2 in neurulas (stage 17; anterior view; A, A', C, C', E, E') and tailbuds (stage 30; B, B', D, D', F, F') injected with CT or MD3 MOs. Black dotted line, midline of embryo; arrowhead, enlarged (A', C, E') and reduced (B', D', F') expression domain of the eye markers; red asterisks, injected side of the embryo; numbers indicate the embryos analysed. Scale bar: 500 μm.

**Fig. 6. MD3 KD stimulates cell proliferation and apoptosis in the eye field.** Eye-field proliferation was analysed by WISH against cyclinD1 in neurula (stage 17; anterior view; A-A') and tailbud (stage 30; B-B') injected with CT or MD3 MOs. Black dotted line, midline of embryo; arrowhead, enlarged (A') and reduced (B') expression domain of cyclinD1; red asterisks, injected side of the embryo; numbers indicate the embryos analysed. Scale bar: 500 μm. Cell proliferation in the anterior region of the neurula was quantified with a BrdU staining (C) and cell death with a TUNEL assay in tailbud (D) as described in the Materials and methods; Student’s t-test (P values, BrdU in C, CT MO=0.16, MD3AB MO=9.4*10⁻⁶; TUNEL in D, CT MO=7*10⁻², MD3AB MO=5.66*10⁻⁹). The numbers indicate the embryos analysed (A-B', D) or the sections counted (C). NS, non significant; ***P<0.001.
affect convergent extension from stage 11 to 22. JNK inhibition did not rescue the eye phenotype in MD3 morphants, in contrast, the phenotype deteriorated even further (Fig. S5). Hence, a simple model in which MD3 attenuates the JNK pathway during eye development does not appear to be correct.

We next hypothesised that MD3 morphants might have a reduced activity of the JNK pathway perhaps due to JNK deregulation during earlier steps of development. We first asked whether overexpression of MD3 can enhance activity of AP1, a transcription factor stimulated by JNK, in a reporter gene assay in *Xenopus* embryos at stage 12. Fig. 9A shows that overexpression of both c-Jun, used as a positive control, and MD3 increased AP1 activity. Hence, increased MD3 expression can stimulate AP1 activity in some cellular contexts.

If the eye phenotype is caused by reduced activity of the JNK pathway, it should be possible to rescue normal eye development by stimulating JNK signalling. Therefore, we next asked whether co-injecting wild-type JNK (wt JNK) or constitutively active JNK (CA-JNK) could rescue the eye phenotype in MD3 morphants. Indeed, wt JNK and CA-JNK mRNAs rescued the eye phenotype in MD3 morphant embryos (Fig. 9B-E). These data thus suggest that reduced JNK activity in MD3 morphants underlies the eye morphogenesis phenotype.

**DISCUSSION**

During *Xenopus* embryogenesis, MD3 is enriched in the lens and plexiform layers, key components for eye morphogenesis. MD3 depletion affected the formation of both the lens and the retina by reducing the number of lens epithelial cells, ganglion cells and photoreceptors, respectively. In the early eye-field, MD3 modulates the balance between cell proliferation and survival, and eye morphogenesis requires activation of the JNK pathway downstream of MD3.
MD3 is required for eye morphogenesis through JNK pathway activation

MD3 expression strongly recalls the spatial distribution of JNK in Xenopus (Yamanaka et al., 2002). The activators of JNK, mitogen-activated protein kinase kinase 7, Plenty of SH3s (POSH), MAP kinase upstream kinase and metastasis-associated kinase (MAK) also present a similar expression pattern (Fuhrmann, 2008; Hirai et al., 2005; Kibardin et al., 2006; Kim et al., 2005; Yamanaka et al., 2002). This similar spatial distribution and the in vitro function of MD3 as a JNK regulator support the idea that MD3 also regulates the JNK pathway in vivo during the eye morphogenesis.

JNK signalling is activated during embryonic development following exposure to growth or differentiation factors (Valesio et al., 2013). Consequently, several studies linked JNK to eye cell differentiation and eye morphogenesis induced by such factors. Wnt4 depleted embryos lose the expression of EFTFs and develop smaller eyes through the inactivation of the non-canonical Wnt/JNK pathway (Maurus et al., 2005). Interestingly, Kibardin et al.

Fig. 8. JNK inhibition disrupts eye morphogenesis. (A-E) The morphology of the eye was analysed in wild-type embryos treated from stage 11 to 22 with DMSO or increasing concentrations of SP600125, a JNK inhibitor. Scale bar: 500 µm. (F) The eye size was quantified from the embryos (A-E) as described in the Materials and methods; Mann–Whitney test (P values, 0.25 µg ml⁻¹=0.93; 0.5 µg ml⁻¹=7.8*10⁻⁵; 1 µg ml⁻¹=1.02*10⁻⁶; 2.5 µg ml⁻¹=1.07*10⁻⁶). The numbers indicate the embryos analysed. Note, the embryos show reduced eye formation in a dose-dependent manner. NS, non significant; ***P<0.001.

Fig. 9. MD3-induced JNK activation is required for eye morphogenesis. (A) Activity of the reporter plasmid AP1-Luc (JNK pathway) was determined by Luciferase assay from whole-embryos (stage 12) co-injected with Renilla (as a control), c-Jun (as positive control) or FL MD3 mRNA. The morphology of the eye was analysed at stage 42 (B–C’), quantified (D) and the eye size determined (E) in embryos injected with MD3 MOs, co-injected with wt JNK or CA-JNK mRNA. Scale bars: 500 µm. The numbers indicate the embryos analysed. Statistical significance was assessed by Student’s t-test (P values in A, positive CT=2.6*10⁻³; MD3 RNA=3.5*10⁻³) and Mann–Whitney test (P values in D, MD3AB MO=0.02; MD3AB MO+wt JNK RNA=0.06; MD3AB MO+CA-JNK RNA=0.19; P values in E, MD3AB MO=1.65*10⁻⁹; MD3AB MO+wt JNK=1.4*10⁻³; MD3AB MO+CA-JNK=4.37*10⁻⁶). *P<0.05; **P<0.01; ***P<0.001; NS, non significant.
field size by manipulation of MD3 expression is thus mirroring a
and Fig. S4), correlating with a small increase in proliferating cells
and a reduced eye field (Fig. 5B-F) at stage 30. Interestingly,
(Fig. 6A,C); followed by increased apoptotic activity (Fig. 6B,D)
Fig. 5A-E) and hyper-proliferation characterized MD3 morphants.
Further insight into the role of the MD3/JNK pathway during eye
development will be required to determine the up- and down-stream
signalling mechanisms governing MD3 expression and to unveil the
spatio-temporal distribution of active JNK in the eye field.

MATERIAL AND METHODS

Xenopus laevis and drugs
Animals were purchased from Nasco (Salida, California), used under Act
1986 (UK Home Office) according to the UK Home Office ethical approval
and guidance and University Animal Welfare Committee. The embryos
were staged using Nieuwkoop tables (Nieuwkoop and Faber, 1994). DMSO
(dimethyl sulfoxide; Sigma) or SP600125 (Tocris) was added at the
indicated concentrations during the growth of embryos (stage 11 to 22).

Semi-quantitative RT-PCR
To determine xMD3 expression, total RNA from 40 optic vesicles [stage 25;
without staining as described in McDonough et al. (2012)] and 50 eyes
(stage 42; removal of epidermis inrostro-caudal manner and eye isolation)
was extracted following the manufacturer instructions (RNeasy mini kit,
Qiagen) and a semi-quantitative PCR was performed for MD3 and GAPDH,
with the following primers respectively: MD3 Fw, ACAATGAGAGATT-
1639

biology Open 5, 1631-1641 doi:10.1242/bio.018945
DNA constructs
MD3 antisense probe was generated by amplifying the MD3 ORF 3'-end using the following primers, Fw: CCCGGGGATCCATGAGAAGATCT-GTTACAGAC, Rv: CGGTGCGGCGGTCAAGCTAGGATCAGTACGC and inserted in pBluescript BamH1/Eagl restriction sites. MD3 overexpression and rescue constructs were obtained by subcloning GFP tagged-full-length MD3 (GFP FL MD3) in pCS2+ (BamH1/EcoR1; Fw: GTTCGCCGATCCGCCCACTATGGTGAAGAAGGCGAG; Rv: CTTCC-GGAATTCCTCAACATGGTTGTGTGTTTCTTTTATAC) and a mutant MD3 (mutant MD3) in pCS2+ (BamH1/EcoR1; Fw: GTTCGCCG-GATCCGCCCACTAGGTGACGTACGAGAAATCCGCCG; Rv: CTTCCGGAATTCCTCAGGAAATGGTTGTGTGTTCCTTTTAAC; mutated bases are in lower cases).

Whole-mount in situ hybridization (WISH) and morpholinos
The embryos were fixed in MEMFA [0.1 M MOPS (3-N-morpholino) propane-sulfonic acid], 2 mM EGTA (ethylene glycol tetra-acetic acid), 1 mM MgSO4, 3% formaldehyde. X-gal staining and WISH were performed as described in Luehders et al. (2015). Control (CT), Morpholino (MO) and Marveldl (MO) MoRs were purchased from Gene Tools (CT MO: CTCCTTTACCTCGTAAATTTTATA; MD3A MO: AGGCCCAA-ATCTCCCTTGTGGCC; MD3B MO: CCCCTGTATACAGATCTCTCATT). MD3A MO was designed to target the 5'-UTR (untranslated region) of MD3, while MD3B MO sequence overlapped the start codon of MD3 ORF (open reading frame).

Histology, immunocytochemistry and immunoblot
For Hematoxylin and Eosin (H&E) histological staining, embryos (stage 42) were fixed 1 h at room temperature in MEMFA and equilibrated in 30% sucrose, before embedding in OCT compound (Tissue Plus, Scigen, USA) for cryo-sectioning (thickness: 8 μm; Leica). For immunocytochemistry, the embryos were rinsed once in water, fixed overnight in 4% PFA at room temperature for β-crystallin staining or 1 h in MEMFA at room temperature, followed by an overnight incubation at −20°C in Dent’s fixative (4 volumes methanol:1 volume DMSO) for Rhodopsin and Hu-C/Hu-D staining. Next, the embryos in Dent’s fixative were dehydrated, equilibrated in 30% sucrose at room temperature, and embedded in OCT compound before cryosectioning (thickness: 8 μm; Leica). The eye sections were post-fixed in 4% paraformaldehyde, blocked in PBST [0.3% Triton X100 in PBS (phosphate buffer saline)] with 10% goat serum or bovine serum albumin (BSA), then incubated with the following primary antibodies: monoclonal (phosphate buffer saline) [20 μg/mL] with 10% goat serum or bovine serum albumin in 4% paraformaldehyde, blocked in PBST [0.3% Triton X100 in PBS (phosphate buffer saline)] with 10% goat serum or bovine serum albumin (BSA), then incubated with the following primary antibodies: monoclonal β-crystallin (Abcam; ab90379), mouse anti-Hu-C/Hu-D (Life Technologies; A21271) and polyclonal rabbit anti-Rhodopsin (H-100; sc-15382, Santa Cruz) antibodies. For immunoblots, 20 ACs were homogenized in RIPA lysis buffer (50 mM Tris-HCl pH8, 150 mM NaCl, 1% Nonidet 40, 0.5% sodium deoxycholate, 0.1% SDS, cocktail of protease inhibitors). Protein expression was then determined by polyclonal anti-GFP (Invitrogen) and anti-Actin (A2066, Sigma Aldrich) antibodies.

Proliferation and apoptosis assays
To assess cell proliferation, the embryos were injected with BrdU (30 nl of 10 mM 5-Bromo-2'-deoxyuridine) in the anterior neural tube, harvested 2 h later (stage 17) in MEMFA and processed for cryo-sectioning. Sections were performed as described in Luehders et al. (2015). Control (CT), Morpholino (MO) and Marveldl (MO) MoRs were purchased from Gene Tools (CT MO: CTCCTTTACCTCGTAAATTTTATA; MD3A MO: AGGCCCAA-ATCTCCCTTGTGGCC; MD3B MO: CCCCTGTATACAGATCTCTCATT). MD3A MO was designed to target the 5'-UTR (untranslated region) of MD3, while MD3B MO sequence overlapped the start codon of MD3 ORF (open reading frame).

Quantification and statistics
Specific cell types in the eye were quantified by determining the ratio of the number of cells positive for a given eye marker divided by the number of nuclei in the same eye cell. The total number of cells corresponds to the number of nuclei per eye visualized by Hoechst staining. The eye size corresponds to the ratio of the injected side/height×width/Non-injected side/height×width in Fig. 3D, and ratio of the injected side/length×retina/Non-injected side/length×retina in Figs 3E, 7F, 9E, and Fig. S5D. The height and width correspond to the dorso-ventral and the antero-posterior length of the retina, respectively. The lens and retina were measured along the antero-posterior axis of the eye. The eye-field transcription factors expression domain area corresponds to the ratio of the injected side/non-injected side in Fig. S4. All measurements were performed with ImageJ. We quantified the number of BrdU-positive cells per section of the anterior region of the embryo for the proliferation assay (Figs 6C & 7G) and the number of TUNEL-positive cells per embryo for the apoptotic assay (Figs 6D & 7H). Error bars indicate s.e.m.; P values (NS, non-significant: *P<0.05; **P<0.01; ***P<0.001) were determined based on three independent experiments using Mann–Whitney or Student’s t-test.

Acknowledgements
The authors thank Dr Hidehiko Inomata (Riken, Japan) for the wt-JNK plasmid and University College London, Institute of Ophthalmology for use of the confocal imaging facilities.

Competing interests
The authors declare no competing or financial interests.

Author contributions
B.V. performed the experiments designed by B.V., N.S., R.M. with technical help from E.S.-H., E.S. performed pilot experiments; K.M., M.S.B. and S.-I.O. conceived the project; B.V., N.S., R.M., K.M. wrote the manuscript; all authors approved the manuscript.

Funding
The work was supported by the Biotechnology and Biological Science Research Council [grant no. BB/J015032/1 to K.M., M.S.B. and S.-I.O.] and Moorfields Eye Charity [grant no. R170006A to B.V. and K.M.].

Supplementary information
Supplementary information available online at http://bio.biologists.orglookup/doi/10.1242/bio.018945.supplemental

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Figure S1

(A-B) Spatial analysis of MD3 transcript expression performed by WISH at 4-cells stage (animal view, A; vegetal view, B) and in tailbuds (stage 25; C). (D) MD3 sense probe was used as a negative control. Scale bar, 500 μm.
**Figure S2**

(A) Analysis by western blot of MD3 protein level was performed at stage 15 on animal caps injected with GFP FL MD3 RNA, GFP FL MD3 RNA + CT MO, GFP FL MD3 RNA + MD3A MO. Actin was used as a control; kDa: kilo Dalton. Morphological analysis on early gastrula (stage 8; B-C, animal and B’-C’, vegetal view) and neurula (stage 15; B”-C”) injected with CT or MD3 MO. Scale bar, 500 μm.
Figure S3
Morphological analysis on gastrula (stage 10; A-B, animal and A'-B', vegetal view) and tadpole (stage 25; C-D) embryos injected with FL MD3 or GFP mRNA. GFP mRNA is used as a control. Scale bar, 500 μm (A-D).
Figure S4

Eye field specification was analysed by WISH against the eye markers Rx1, Pax6, Otx2 in tailbuds (stage 30) injected with GFP or FL MD3 RNAs (A-C'). Numbers indicate the embryos analysed. Scale bar 500µm. Student’s t-test, (p values, A, 9*10⁻³; B, 6*10⁻⁴; C, 2*10⁻²). (A’-C’)

Ratio of the area of expression of EFTF between injected and non-injected side. Mann-Whitney test (p values, A”, Rx1=1.89*10⁻⁶; B”, Pax6=3*10⁻⁴; C”, Otx2=2*10⁻⁴).
A-B”, The alteration of eye morphology was determined and quantified (C) by the comparison of the non-injected and the injected side in MD3 MO-embryos treated from stage 11 to 22 with DMSO (A-B), 0.5 (A’-B’) or 1 μg.ml⁻¹ (A”-B”) SP600125, a JNK inhibitor. Scale bar, 500 μm. D, The eye size from (A-B”) was measured and quantified as described in the methods. Statistical significance was assessed by Student’s t-test (p values, C, CT MO + 0.5 μg.ml⁻¹= 6*10⁻⁴; MD3AB MO DMSO=1*10⁻⁴; MD3AB MO + 0.5 μg.ml⁻¹ stronger decreased eye= 0.87*10⁻³; MD3AB MO + 1 μg.ml⁻¹ stronger decreased eye=1*10⁻²) and Mann-Whitney test (p values, D, MD3AB MO + 0.5 μg.ml⁻¹=2.9*10⁻²; MD3AB MO + 1 μg.ml⁻¹=1*10⁻⁴).

Figure S5