

## RESEARCH ARTICLE

# Meis1 coordinates a network of genes implicated in eye development and microphthalmia

Séverine Marcos<sup>1,2</sup>, Monica González-Lázaro<sup>3</sup>, Leonardo Beccari<sup>1,2</sup>, Laura Carramolino<sup>3</sup>, Maria Jesus Martin-Bermejo<sup>1,2</sup>, Oana Amarie<sup>4</sup>, Daniel Mateos-San Martín<sup>3</sup>, Carlos Torroja<sup>5</sup>, Ozren Bogdanović<sup>6,7</sup>, Roisin Doohan<sup>3</sup>, Oliver Puk<sup>4</sup>, Martin Hrabě de Angelis<sup>4</sup>, Jochen Graw<sup>4</sup>, Jose Luis Gomez-Skarmeta<sup>6</sup>, Fernando Casares<sup>6</sup>, Miguel Torres<sup>3,\*</sup> and Paola Bovolenta<sup>1,2,\*</sup>

## ABSTRACT

Microphthalmos is a rare congenital anomaly characterized by reduced eye size and visual deficits of variable degree. Sporadic and hereditary microphthalmos have been associated with heterozygous mutations in genes fundamental for eye development. Yet, many cases are idiopathic or await the identification of molecular causes. Here we show that haploinsufficiency of *Meis1*, which encodes a transcription factor with evolutionarily conserved expression in the embryonic trunk, brain and sensory organs, including the eye, causes microphthalmic traits and visual impairment in adult mice. By combining analysis of *Meis1* loss-of-function and conditional *Meis1* functional rescue with ChIP-seq and RNA-seq approaches we show that, in contrast to its preferential association with Hox-Pbx BSs in the trunk, *Meis1* binds to Hox/Pbx-independent sites during optic cup development. In the eye primordium, *Meis1* coordinates, in a dose-dependent manner, retinal proliferation and differentiation by regulating genes responsible for human microphthalmia and components of the Notch signaling pathway. In addition, *Meis1* is required for eye patterning by controlling a set of eye territory-specific transcription factors, so that in *Meis1*<sup>−/−</sup> embryos boundaries among the different eye territories are shifted or blurred. We propose that *Meis1* is at the core of a genetic network implicated in eye patterning/microphthalmia, and represents an additional candidate for syndromic cases of these ocular malformations.

**KEY WORDS:** Developmental disorders, Notch signaling, Patterning, TALE transcription factors, Microphthalmia, Mouse

## INTRODUCTION

Eye formation initiates with the specification of the retinal field in the anterior neural plate followed by morphogenetic rearrangement of retinal progenitors to form the optic vesicles. Subsequent interaction of the optic neuroepithelium with the surrounding tissue generates the optic cup, which is concomitantly patterned along its three main axes: proximodistal, nasotemporal and dorsoventral. This results in the

formation of the neural retina, retinal pigment epithelium (RPE), optic stalk and lens (Martinez-Morales et al., 2004), the proliferation and differentiation of which generate a mature eye (Esteve and Bovolenta, 2006). Disruption of any of these events leads to ocular malformations, including anophthalmia (complete absence of the ocular globe) or microphthalmia (significant reduction of the globe axial length), which, in turn, cause severe visual deficits that, for microphthalmia, account for up to 11% of infant blindness in developed countries (Bardakjian and Schneider, 2011).

Cases of anophthalmia and microphthalmia have been associated with homozygous and heterozygous mutations in genes at the core of forebrain regulatory networks (Beccari et al., 2013), such as the transcription factors (TFs) *SOX2* (Fantes et al., 2003), *OTX2* (Ragge et al., 2005), *PAX6* (Glaser et al., 1994), *VSX2* (*CHX10*) (Ferda Percin et al., 2000), *RAX* (Voronina et al., 2004), *FOXE3* (Reis et al., 2010) and perhaps *SIX6* (Gallardo et al., 2004); in key components of cell to cell communication, including *SHH* (Schimmenti et al., 2003) and *BMP4* (Reis et al., 2011); or in genes involved in retinal progenitor proliferation and survival such as *STRA6* (Pasutto et al., 2007; White et al., 2008), *BCOR* (Ng et al., 2004), *HCCS* (Indrieri et al., 2013; Morleo et al., 2005) and *SMOC1* (Abouzeid et al., 2011; Okada et al., 2011). Yet, only a minor proportion of patients receive an accurate molecular diagnosis of the pathogenesis of their ocular malformation (Bardakjian and Schneider, 2011; Williamson and FitzPatrick, 2014), indicating that additional causative genes need to be identified. Given that anophthalmia and microphthalmia frequently occur in association with other birth defects, most commonly involving anomalies of the limbs, face, ears and skeletal muscle system (Slavotinek, 2011), genes implicated in multiple aspects of embryonic development, such as *Meis1*, are good candidates to be explored.

*Meis1*, its *Drosophila* homolog *homothorax* (*hth*) and the related *Meis2* and *Meis3*, belong to a subfamily of TALE (three amino acid loop extension) homeodomain-containing TFs (Longobardi et al., 2014). *Meis* proteins form stable heteromeric complexes with other transcriptional regulators, enhancing their affinity and specificity of binding to DNA sites in the target gene locus (Penkov et al., 2013; Slaterry et al., 2011). For example, together with *Pbx1*, *Meis1* plays a major role as a co-factor for the TFs of the Hox complex, which, in turn, have a pivotal and evolutionarily conserved role in orchestrating embryonic trunk development (Duboule, 2007; Mallo and Alonso, 2013). In accordance with this notion, loss of *Meis1* function impairs the formation of *Meis1*-expressing trunk organs and systems, such as the limbs, heart, blood and vasculature (Azcoitia et al., 2005; Erickson et al., 2010; Hisa et al., 2004; Mercader et al., 1999, 2009; Zhang et al., 2002).

Members of the *Meis* subfamily are however also expressed in the brain and sensory organs (Schulte and Frank, 2014), which are Hox-

<sup>1</sup>Centro de Biología Molecular “Severo Ochoa”, CSIC-UAM, c/Nicolás Cabrera, 1, Madrid E-28049, Spain. <sup>2</sup>CIBER de Enfermedades Raras (CIBERER), c/Nicolás Cabrera, 1, Madrid E-28049, Spain. <sup>3</sup>Departamento de Desarrollo y Reparación Cardiovascular, Centro Nacional de Investigaciones Cardiovasculares (CNIC), c/Melchor Fernández Almagro, 3, Madrid E-28029, Spain. <sup>4</sup>Institute of Developmental Genetics Helmholtz Center Munich, Neuherberg D-85764, Germany. <sup>5</sup>Bioinformatics Unit, Centro Nacional de Investigaciones Cardiovasculares (CNIC), c/Melchor Fernández Almagro, 3, Madrid E-28029, Spain. <sup>6</sup>Centro Andaluz de Biología del Desarrollo (CABD), CSIC-UPO, Carretera de Utrera Km1, Sevilla E-41013, Spain. <sup>7</sup>ARC Center of Excellence in Plant Energy Biology, School of Chemistry and Biochemistry, Faculty of Science, The University of Western Australia, Perth, Western Australia 6009, Australia.

\*Authors for correspondence (mtorres@cnic.es; pbovolenta@cbm.csic.es)

free embryonic regions (Duboule, 2007; Mallo and Alonso, 2013). In particular, *Meis1* is expressed in the vertebrate forebrain and sensory organ primordia, including the eye, being essential for the specification of part of these structures. Indeed, genetic inactivation of *Meis1* in mice causes lens reduction and abnormal retinal morphology (Hisa et al., 2004). Cardiovascular-related embryonic lethality of *Meis1*<sup>-/-</sup> embryos (Azcoitia et al., 2005; Hisa et al., 2004) and the virtual lack of information on the alternative and Hox-independent transcriptional mechanisms that *Meis1* must adopt in the head region (Longobardi et al., 2014) have presented hurdles to understanding why and how these eye defects arise.

Here, we have begun to address these issues by taking advantage of *Meis1* loss-of-function and conditional *Meis1* functional rescue in mice, combined with ChIP-seq and RNA-seq approaches. Our results indicate that *Meis1*, by binding to ‘*Meis*-only’ binding sites in the DNA, regulates (directly and indirectly) the expression of genes involved in patterning, proliferation and differentiation of the neural retina, including components of the Notch signaling pathway. *Meis1* is also at the core of a genetic network implicated in mammalian microphthalmia, and its haploinsufficiency suffices to cause microphthalmic traits in adult mice, suggesting that *Meis1* itself represents an additional candidate for this ocular malformation.

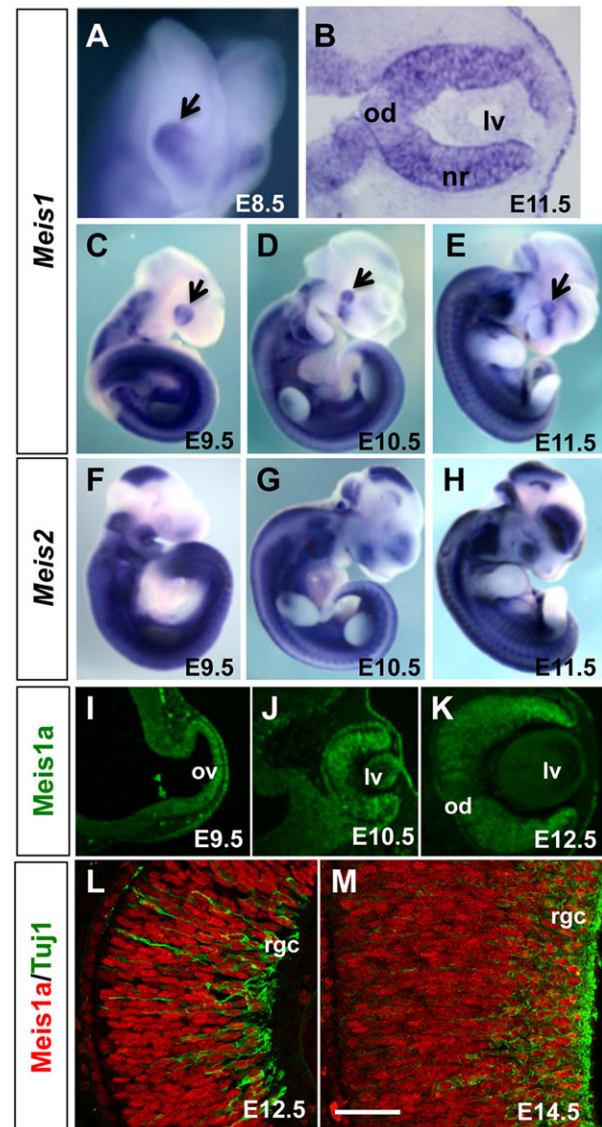
## RESULTS

### *Meis1* deficiency causes embryonic microphthalmia

*Meis1* is uniformly expressed in the zebrafish and chick eye primordium and progressively retracts from the central retina following the wave of retinal cell differentiation (Bessa et al., 2008; Heine et al., 2008). In both species, *Meis1* regulates the expression of cyclin D1 (*Cnd1*), thereby promoting G1-S transition of retinal cells and thus the generation of sufficient numbers of retinal progenitors (Bessa et al., 2008; Heine et al., 2008). Accordingly, interference with *Meis1* expression causes eye hypoplasia (Bessa et al., 2008; Heine et al., 2008).

We reproduced these observations in mice. *Meis1* mRNA localized to the eye field (Fig. 1). Its expression (Fig. 1A–E) and that of its protein (Fig. 1I–K) was thereafter maintained in the optic neuroepithelium and the overlying surface ectoderm throughout eye formation, according to the distribution detected with a *lacZ* reporter (Hisa et al., 2004). *Meis1* expression was also maintained in retinal neurons as defined by its co-expression with the neuronal differentiation marker Tuj1 (Tubb3) (Fig. 1L,M). Although *Meis2* has been reported to be transiently expressed in E9.5 optic vesicles (Heine et al., 2008), we could not detect *Meis2* expression at any early stages of eye development (Fig. 1F–H). Thus, early mouse eye development seems to depend mostly on *Meis1* function, in contrast to what has been observed in zebrafish and chick, where *Meis2* is instead clearly detected (Bessa et al., 2008; Heine et al., 2008).

Complete inactivation of *Meis1* caused lens vesicle reduction, as described using a different *Meis1*<sup>-/-</sup> mouse line (Hisa et al., 2004), but we did not observe the reported retinal duplication (Hisa et al., 2004). Instead, we noticed a significant reduction of the optic cup compared with wild-type (wt) littermates. This reduction was first apparent at E11 (Fig. 2) and became accentuated with development, especially in the ventral side, so that E13 *Meis1*<sup>-/-</sup> eyes were roughly half the size of those of wt (Fig. 2S). This was associated with a significant decrease in BrdU incorporation and *Cnd1* expression (Fig. 2A–C,T; supplementary material Fig. S1D–F), although there was no statistically significant difference in the mitotic index [calculated as the number of cells in M phase (phospho-histone H3<sup>+</sup>)/area] among wt and *Meis1* null embryos in both the neural retina and the RPE (Fig. 2U).



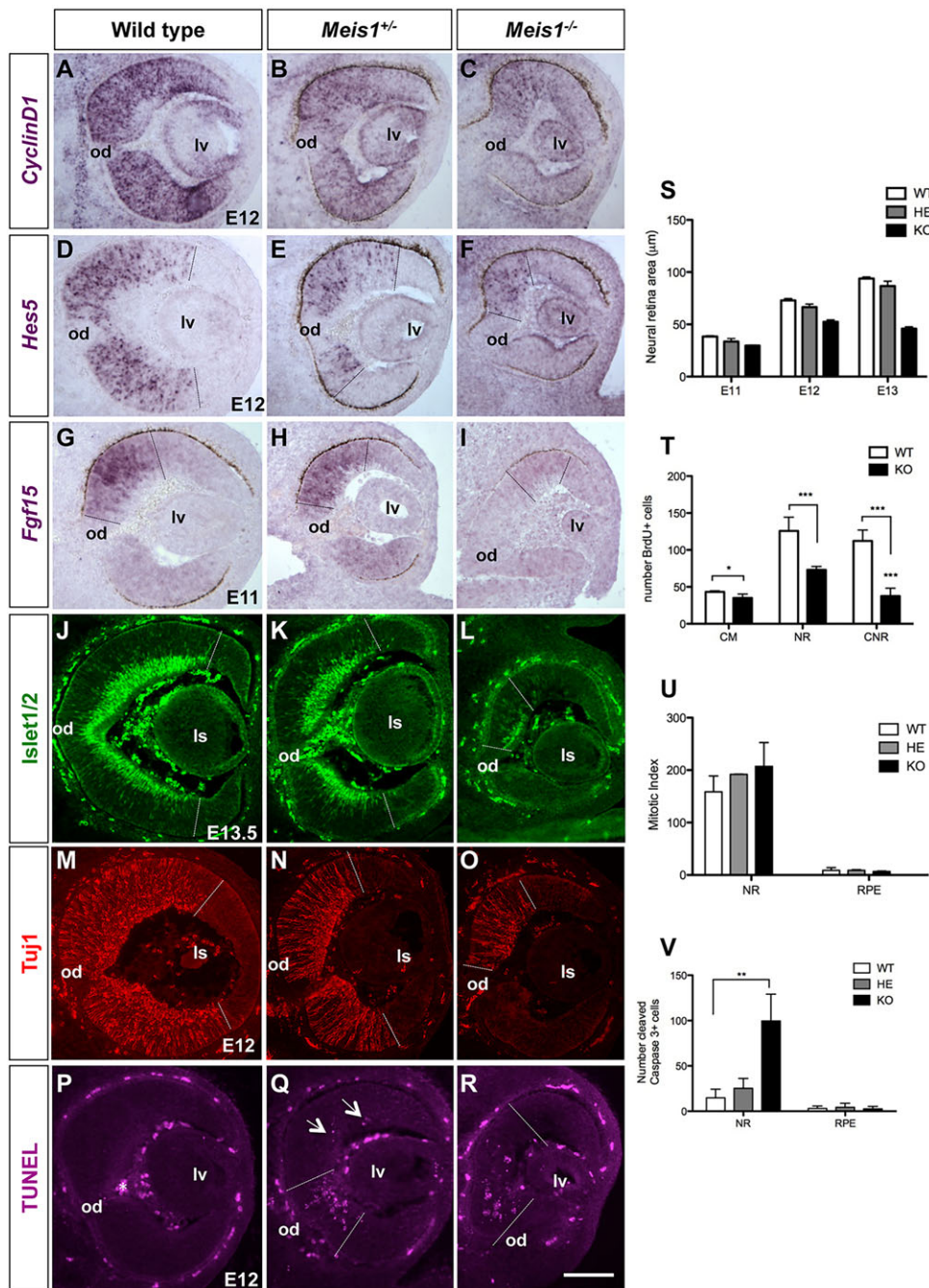
**Fig. 1. Embryonic expression of *Meis1* and *Meis2*.** (A–H) Frontal (A) and lateral (C–H) views of mouse embryos at stages between E8.5 and E11.5 hybridized *in toto* with probes specific for *Meis1* and *Meis2*. (B) Frontal paraffin section through the optic cup of an E11.5 mouse embryo hybridized with a probe against *Meis1*. Note that *Meis1* is strongly expressed in the eye field (arrow in A) and its expression is maintained as the optic cup forms (arrows in C–E). The expression is particularly abundant in the neural retina (B). *Meis2* is not expressed in the developing eye (F–H) but is strongly expressed in the mesencephalon and spinal cord. (I–M) Frontal cryostat sections of mouse embryos at stages between E9.5 and E14.5 were immunostained with antibodies against *Meis1a*, (one of the *Meis1* isoforms) or co-immunostained with the neuronal differentiation marker Tuj1 (L,M). Note that *Meis1a* is detected in the entire optic vesicle and overlying ectoderm, in the developing optic cup and in differentiated neurons. lv, lens vesicle; nr, neural retina; od, optic disc; ov, optic vesicle; rgc, retinal ganglion cells. Scale bar: 25  $\mu$ m.

Together, these observations indicate that the proposed Hth/*Meis1*-mediated control of retinal progenitor proliferation is conserved in mice and complete loss of *Meis1* function drastically affects ocular development, causing microphthalmia.

### Microphthalmia is associated with decreased neurogenesis and increased apoptosis

Previous studies in *Drosophila* and zebrafish retina have shown that *Meis1*/Hth expression is turned off in differentiating cells and its





**Fig. 2. *Meis1* loss of function causes dose-dependent alterations in retinal neurogenesis leading to microphthalmia.** (A-R) Frontal sections of E11-E13.5 wt, *Meis1*<sup>+/-</sup> and *Meis1*<sup>-/-</sup> optic cups processed for the indicated markers. Compared with the wt, note the decreased proliferation (less *Ccnd1* expression) and the impaired onset (*Fgf15*, *Hes5*) and progression (*Isl1/2*, *Tuj1*) of neuronal differentiation in the *Meis1* mutants, associated with increased apoptosis (P-R). Absence of one *Meis1* allele suffices to induce these defects. Dotted lines delineate the extent of marker staining. Arrows (Q) indicate apoptotic cells. (S-V) Quantification of neural retina area (S), number of BrdU<sup>+</sup> cells (T), mitotic index (phospho-histone H3<sup>+</sup>/area, U) and number of cleaved caspase 3<sup>+</sup> cells (V) in E12.5 optic cups. Total areas were determined from DAPI-stained sections. Error bars are s.e.m. of counting all sections from both eyes of at least three different embryos (*n*=3). There is no statistical difference in the mitotic index of the different genotypes (U). \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 (Student's *t*-test). ls, lens; lv, lens vesicle; od, optic disc; HE, *Meis1*<sup>+/-</sup>; KO, *Meis1*<sup>-/-</sup>; CM, ciliary margin; CNR, central neural retina; NR, neural retina; RPE, retinal pigmented epithelium. Scale bar: 25 μm.

forced maintenance prevents the acquisition of a differentiated neuronal fate (Bessa et al., 2002, 2008). This downregulation was not observed in the embryonic mouse retina, in which differentiated neurons are still *Meis1* positive (Fig. 1M). We thus examined whether the reduced eye size of *Meis1* null mouse embryos is also associated with abnormal neurogenesis.

We first compared the onset of Fgf signaling, which triggers retinal neurogenesis (Martinez-Morales et al., 2005), in E11.5 wt and mutant embryos. *Fgf15* was strongly expressed in the wt central retina but was visibly reduced in both level and extent in *Meis1*<sup>-/-</sup> embryos (Fig. 2G-I). Furthermore, the number of Otx2<sup>+</sup> retinal progenitors (supplementary material Fig. S1G,I) (Bovolenta et al., 1997) and that of Tuj1<sup>+</sup> or islet 1/2 (*Isl1/2*)<sup>+</sup> differentiating neurons (Esteve et al., 2011) was reduced in E12/E13 *Meis1*<sup>-/-</sup> retinas (Fig. 2J,L,M,O). In contrast to what has been reported in zebrafish

and chick (Bessa et al., 2008; Heine et al., 2008), in *Meis1* null embryos, but not in wt, the prospective neural retina at E12.5-E13.5 showed a significant number of TUNEL<sup>+</sup> and cleaved caspase 3<sup>+</sup> apoptotic cells (Fig. 2P-R,V). The majority of apoptotic cells were concentrated in the regions of ongoing neuronal differentiation (compare Fig. 2O with 2R), suggesting a link between the two events.

#### Haploinsufficiency of *Meis1* causes microphthalmic traits in adult mice

In humans, microphthalmia is often caused by dominant heterozygous mutations, especially when mutations hit genes fundamental for eye development (Williamson and FitzPatrick, 2014). We thus investigated if loss of one *Meis1* allele suffices to impair eye growth. Indeed, the size of the eye in *Meis1*<sup>+/-</sup> embryos

was slightly reduced in all of the embryos analyzed (30/30) as compared with wt (Fig. 2A,B,D,E,G,H,S) and associated with an evident decrease in *Cnd1* expression (Fig. 2B). Furthermore, the domain of expression of markers implicated in neuronal differentiation, including *Fgf15*, *Otx2*, *Isl1/2* and *Tuj1*, was smaller than that observed in wt littermates but not as reduced as that in *Meis1*<sup>-/-</sup> embryos (Fig. 2G–O; supplementary material Fig. S1G–I). As in homozygous mutants, the retinas of *Meis1*<sup>+/-</sup> embryos presented an increased number of apoptotic cells in the region of active neurogenesis (Fig. 2Q).

Altogether, these observations suggested that heterozygous embryos present a milder version of the ocular phenotype seen in *Meis1* null mice. To confirm this and exclude the possibility that reduced *Meis1* function might simply delay eye development, we examined whether the observed embryonic defects persisted into adulthood, as *Meis1*<sup>+/-</sup> mice, in contrast to homozygous mice, are viable and fertile.

Scheimpflug imaging revealed no anterior segment abnormality in adult *Meis1*<sup>+/-</sup> animals as compared with wt littermates (supplementary material Fig. S2). Likewise, optical coherence tomography (OCT) showed no defects in the number and distribution of the main blood vessels when adult wt and *Meis1* heterozygous animals were compared (Fig. 3A,B). By contrast, non-invasive analysis of left and right eye morphometrics and histological analysis of the retina showed that in *Meis1*<sup>+/-</sup> mice the axial length of the optic globe and the thickness of the neural retina were significantly decreased (Fig. 3C–H). This reduced thickness seemed to affect, albeit slightly, all nuclear layers (Fig. 3E,F) and

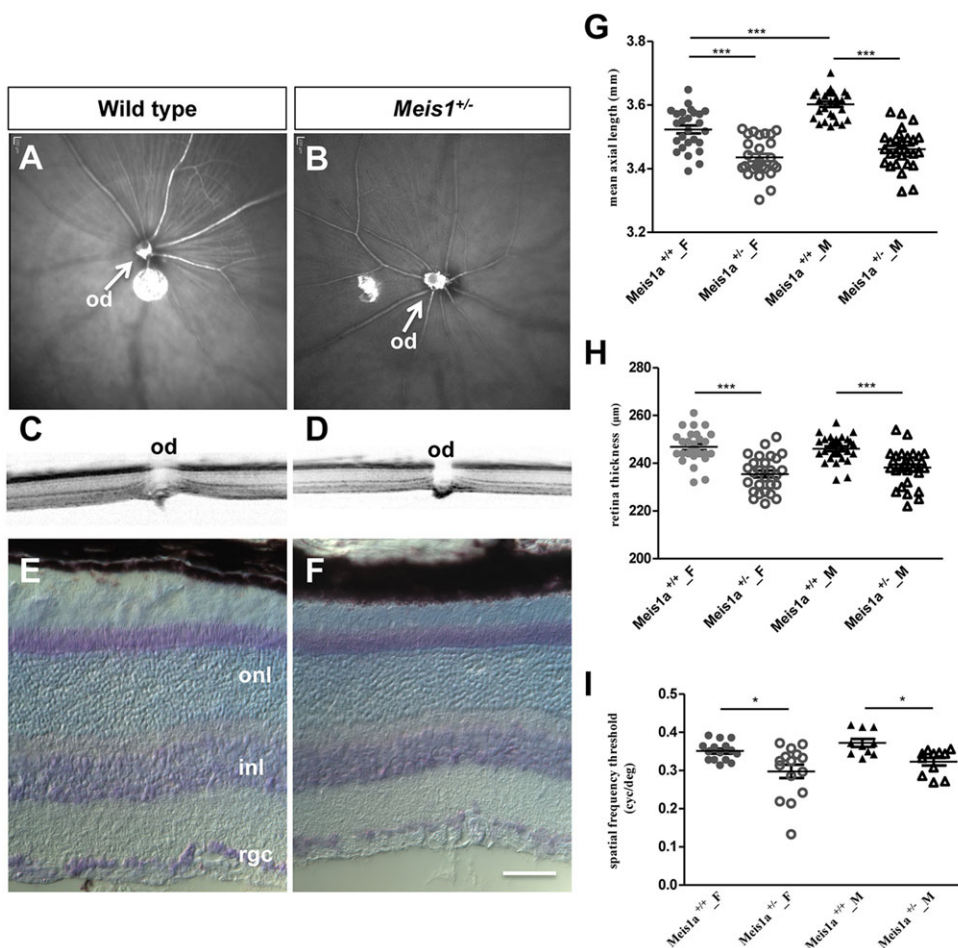
could result from the decrease in neurogenesis observed in the heterozygous embryos coupled to the increase in apoptosis. Notably, these morphological changes were associated with a significant loss of visual performance (Fig. 3I), as determined by the virtual drum vision test (Prusky et al., 2004).

Thus, haploinsufficiency of *Meis1* causes morphological and functional defects characteristic of microphthalmia (Williamson and FitzPatrick, 2014). Microphthalmia is likely to be a direct consequence of *Meis1* requirement in the retinal neuroepithelium since blood vessels and lens, which may both influence retinal development, formed normally.

### Microphthalmia is not a consequence of *Meis1* function in the vascular system

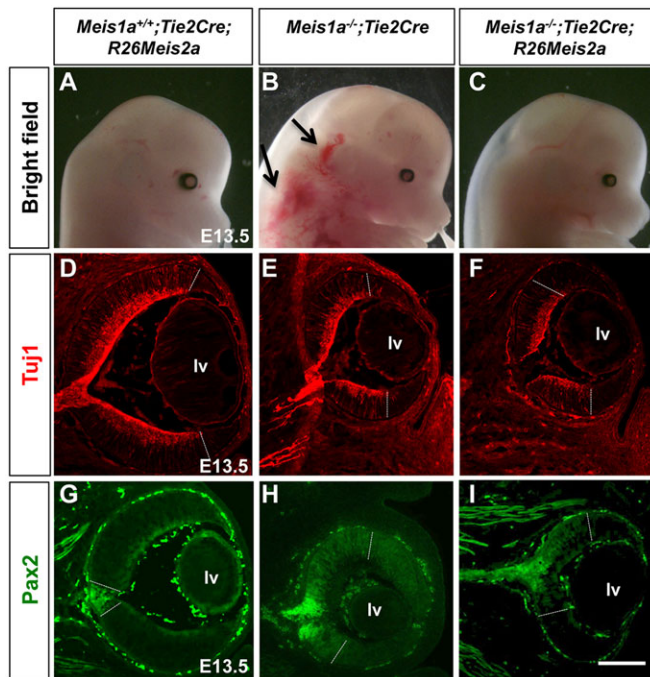
To further confirm the idea that the microphthalmia observed in *Meis1* mutant embryos is independent from the abnormal development of the hematopoietic/vascular system characteristic of *Meis1* null embryos (Azcoitia et al., 2005; Hisa et al., 2004), we analyzed a mouse line with a targeted rescue of *Meis1* function in the hematopoietic and vascular system in a *Meis1a*<sup>-/-</sup> background [*Meis1a*<sup>-/-</sup>; *Tie2Cre*; *R26Meis2a* (Rosello-Diez et al., 2014)].

In contrast to the evident rescue of the hemorrhage that is usually present in *Meis1* null embryos (Fig. 4A–C) (Rosello-Diez et al., 2014), the eye size of all of the analyzed *Meis1a*<sup>-/-</sup>; *Tie2Cre*; *R26Meis2a* embryos (13/13) was still reduced and comparable to that observed in *Meis1a*<sup>-/-</sup>; *Tie2Cre* littermates (Fig. 4A–C). Indeed, at E13 the area of the neural retina of *Meis1a*<sup>-/-</sup>; *Tie2Cre*; *R26Meis2a* and *Meis1a*<sup>-/-</sup>; *Tie2Cre* embryos was on average 47%



**Fig. 3. Haploinsufficiency of *Meis1* causes microphthalmic traits in adult mice.** (A,B) Retina fundus of wt and *Meis1*<sup>+/-</sup> adult eye obtained by OCT. No difference in the vasculature organization was detected between heterozygous ( $n=5$ ) and wt ( $n=5$ ) littermates. (C,D) Images of wt and *Meis1*<sup>+/-</sup> adult central retinas obtained by OCT. (E,F) Frontal cryostat sections of wt and *Meis1*<sup>+/-</sup> adult central retinas stained with Cresyl Violet. Note the slight difference in thickness of the various layers, leading to an overall reduced thickness of the retina in *Meis1*<sup>+/-</sup>. (G) Eye size measurements by LIB revealed significantly reduced axial eye length in mutants of both sexes: females (F) and males (M). (H) Retinal thickness in both females and males was significantly decreased in *Meis1a*<sup>+/-</sup>. (I) Virtual drum vision testing showed a reduced response in both female ( $P=0.012$ ) and male ( $P=0.01$ ) mutants. Error bars are s.e.m. of  $n=15$  mice per group. \* $P<0.05$ , \*\*\* $P<0.001$  (Student's *t*-test). od, optic disc; onl, outer nuclear layer; inl, inner nuclear layer; rgc, retina ganglion cells. Scale bar: 25 μm.





**Fig. 4. Microphthalmia is not linked to *Meis1* function in the hematopoietic/vascular system.** (A–C) Bright field lateral view of E13.5 *Meis1a*<sup>+/+</sup>;Tie2Cre;R26Meis2a, *Meis1a*<sup>-/-</sup>;Tie2Cre and *Meis1a*<sup>-/-</sup>;Tie2Cre;R26Meis2a embryonic heads. Note that the hemorrhage evident in *Meis1a*<sup>-/-</sup> (arrows in B) is no longer observed in rescued embryos (C), although the eye size is still reduced (B,C) as compared with control littermates (A). (D–I) Frontal cryostat sections of the optic cup immunostained with antibodies against Tuj1 or Pax2. Note that the rescue of *Meis1a* expression in the vasculature does not improve neuronal differentiation and optic cup patterning defects. Dotted lines delineate the extent of marker labeling. Iv, lens vesicle. Scale bar: 25 μm.

and 48.2% smaller, respectively, than that of the *Meis1a*<sup>+/+</sup>;Tie2Cre;R26Meis2a control littermates. This reduction is very similar to that seen in *Meis1*<sup>-/-</sup> embryos (Fig. 2S).

Likewise, in both *Meis1a*<sup>-/-</sup>;Tie2Cre and *Meis1a*<sup>-/-</sup>;Tie2Cre;R26Meis2a E13.5 retinas, the number of Tuj1<sup>+</sup> differentiating neurons was similarly reduced (Fig. 4D–F) and the expression of optic cup patterning markers altered. For example, and as observed in *Meis1* null embryos (supplementary material Fig. S1J–L; and see below), the distribution of the TF Pax2, which is normally restricted to cells of the optic disc at E12/E13 (Morcillo et al., 2006) (supplementary material Fig. S1J; Fig. 4G), was instead expanded in the ventral and dorsal retina of both *Meis1a*<sup>-/-</sup>;Tie2Cre and *Meis1a*<sup>-/-</sup>;Tie2Cre;R26Meis2a embryos (Fig. 4H,I), comparably to that observed in *Meis1* mutant embryos (supplementary material Fig. S1K,L).

### **Meis1 interacts with a set of enhancers specifically involved in eye development using Hox/Pbx-independent binding sites**

Altogether, these data indicate that a full dose of *Meis1* is required for the progression of retina development. Reduced *Meis1* levels prevent retinal progenitors from undertaking a normal proliferative and differentiation program, ultimately leading to the death of some cells. As previously noted (Heine et al., 2008), *Ccnd1* downregulation by itself is unlikely to explain this severe phenotype because retinal differentiation is normal in *Ccnd1*<sup>-/-</sup> mice (Sicinski et al., 1995) and overexpression of *Ccnd1* only partially rescues the effect of loss of *Meis1* function (Heine et al., 2008). Thus, besides

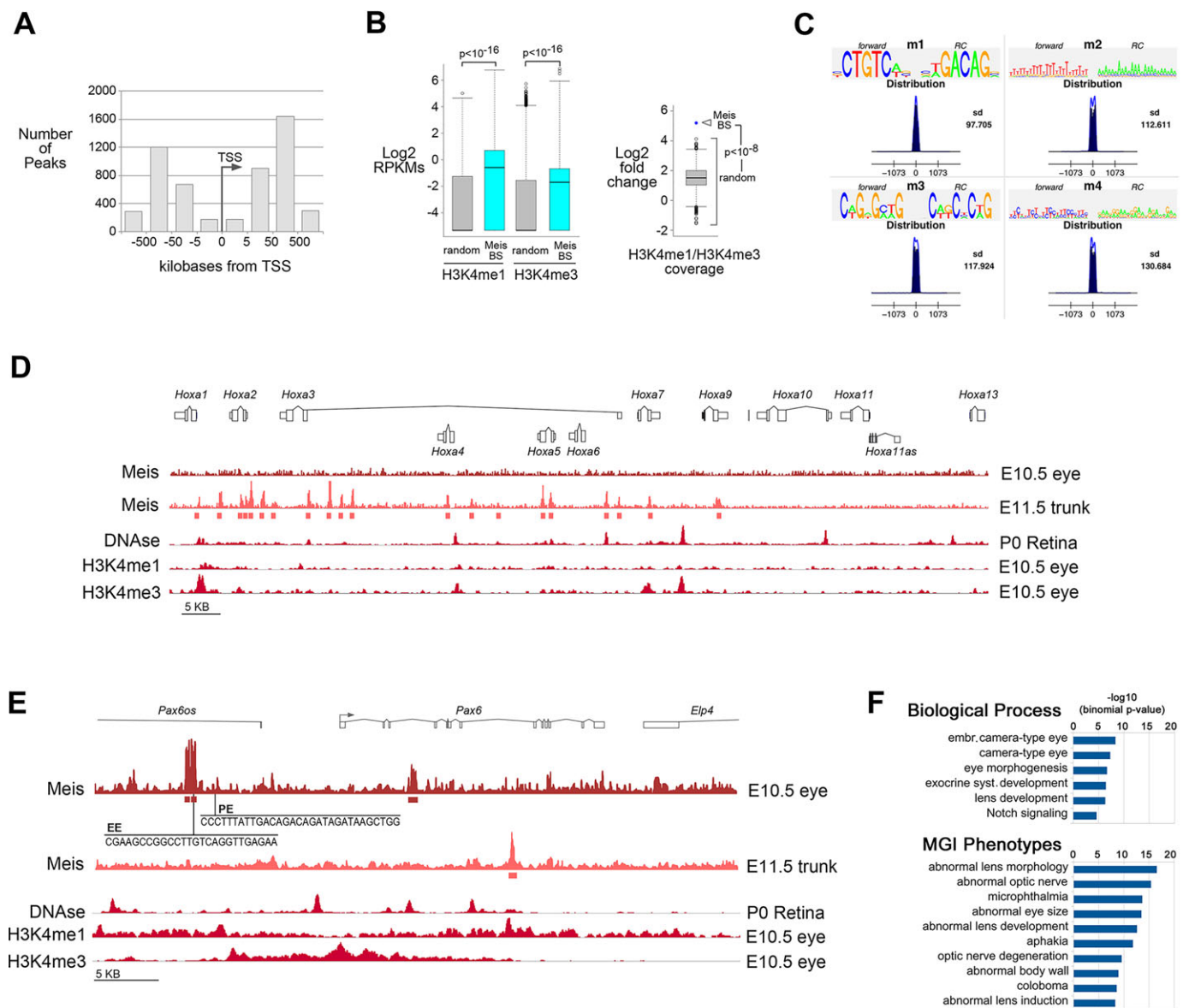
*Ccnd1*, *Meis1* is likely to regulate additional neurogenic pathways. This regulation must take place via a Hox/Pbx-independent mechanism because Hox genes, which are well-known partners of *Meis* in the trunk, are not expressed in the head (Schulte and Frank, 2014). Furthermore, there is no indication that Pbx genes contribute to early eye formation, even though compound knockout mice have been generated and extensively studied (Capellini et al., 2011; Stankunas et al., 2008).

To address this question and identify the binding sites (BSs) of endogenous *Meis1* protein in the developing eye, we performed ChIP-seq analysis using isolated E10.5 optic cups, shortly before the detection of overt *Meis1*<sup>-/-</sup> eye defects. We identified a total of 5361 *Meis1* BSs in the genome and a collection of 3182 genes with a transcription start site (TSS) closest to any *Meis1* BS (GEO GSE62786). As previously reported (Penkov et al., 2013), most *Meis1* BSs were located in regions remote from their closest associated TSS (Fig. 5A).

To study the functional relevance of *Meis1* BSs we performed further ChIP-seq analysis of the E10.5 optic cup to determine histone modification marks that identify promoter (H3K4me3) and enhancer (H3K4me1) regions. *Meis1* BSs associated very significantly with both H3K4me1 and H3K4me3 marks, indicating a preference for enhancer and promoter region binding (Fig. 5B). When comparing *Meis1* preference for binding to H3K4me1 and H3K4me3 marks, as reported in other tissues (Penkov et al., 2013), we found that *Meis1* preferentially binds to enhancer regions (Fig. 5B). *Meis1* selects two main sequences in the embryonic trunk: the Pbx-Hox binding sequence (A/TGATNNAT), to which it binds indirectly, and a direct BS (TGACAG) (Penkov et al., 2013). To determine binding preferences in the developing eye, we identified consensus sequences in the E10.5 eye *Meis1* BS collection. We found only one consensus sequence showing a unimodal distribution, with the maximum mapping to the center of the *Meis1* BS and therefore representing the *Meis1*-bound core sequence (m1 sequence, Fig. 5C). We identified three additional consensus sequences showing a bimodal distribution, with maxima mapping at a certain distance from the *Meis1* BS center, which are likely to represent cooperating sequences not directly bound by *Meis1* (m2–m3, Fig. 5C). The m1 consensus is a very close variant of the *Meis1* direct binding sequence identified in the trunk (Penkov et al., 2013), whereas m2–m3 sequences are low-complexity or rather relaxed sequences that we could not correlate to previously described consensus binding motifs.

These results suggest that in the eye *Meis1* selects BSs and sequences unrelated to the Hox-Pbx network. In support of this view, the prominent pattern of *Meis1* binding to the *HoxA* cluster seen in the trunk is completely absent in the E10.5 eye (Fig. 5D). We then looked for previously described *Meis1*-regulated enhancers in the developing eye. Within the *Meis1* BS collection, we found the previously described *Meis1* BS in the *Pax6* lens ectoderm enhancer (Zhang et al., 2002) and an additional peak in the *Pax6* third intron, but not the *Pax6*-associated *Meis1* BS reported after embryonic trunk ChIP-seq (Penkov et al., 2013) or those found in a pancreatic enhancer (Zhang et al., 2006) (Fig. 5E). The eye-specific *Meis1* BS coincided with H3K4me1<sup>high</sup>/H3K4me3<sup>low</sup> marks typical of enhancer regions (Fig. 5E).

Remarkably, despite the distance between *Meis1* BSs and TSSs, the analysis of ‘biological process’ and ‘MGI phenotypes’ Gene Ontology (GO) classes for the *Meis1* BS-associated genes identified eye development classes as the most overrepresented, with a predominance of ‘eye size’ and ‘eye morphology’ categories (Fig. 5F). Thus, the *Meis1* binding profile reveals its functional



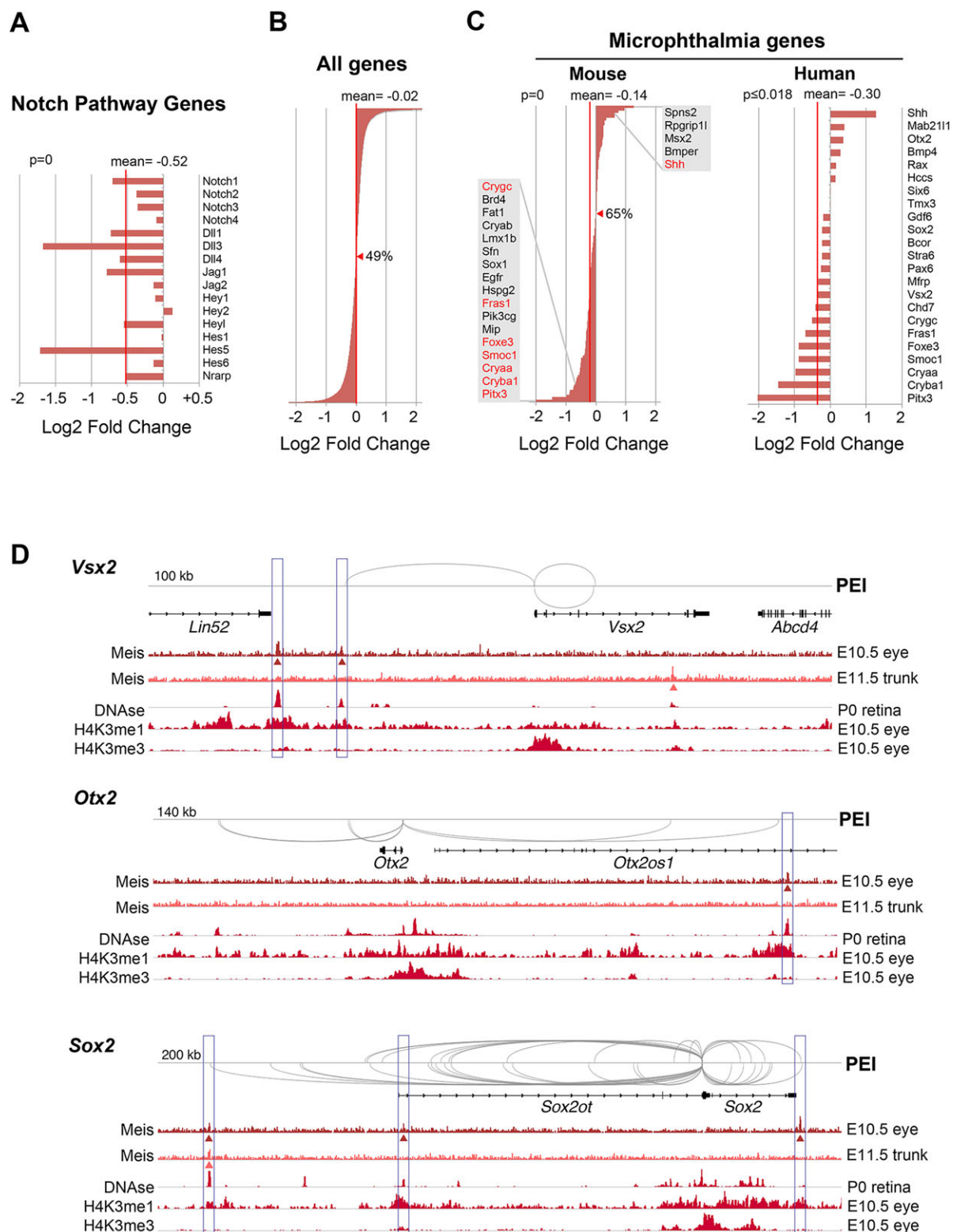
**Fig. 5. ChIP-seq analysis of Meis1 function in the developing eye.** (A) Distribution of Meis1 binding sites (BSs) by their position with respect to the nearest transcription start site (TSS). GREAT analysis (see Materials and Methods). (B) (Left) Distribution of H3K4me1 and H3K4me3 coverage in Meis BSs compared with that in a collection of randomly chosen equivalent genomic DNA segments. (Right) Comparison of the H3K4me1/H3K4me3 coverage ratio in Meis BSs (blue dot) versus that in a series of randomly chosen equivalent genomic DNA segments. (C) *De novo* identification of consensus sequences in the Meis BSs. Four motifs were identified (m1-m4). Forward and reverse complementary (RC) sequences and the distribution of the positions relative to the BS center are shown for each motif. (D,E) The *HoxA* complex and *Pax6* genomic regions showing the Meis1 ChIP-seq read profile from E10.5 eye (this study) and E11.5 trunk (Penkov et al., 2013), the P0 DNA-seq profile [from The ENCODE Project Consortium (2012), GEO:GSM1014188] and the H3K4me1 and H3K4me3 ChIP-seq profiles (this study, GEO GSE62786). Detected Meis1 BSs are shown by boxes below the read profiles. (E) The sequences bound by Meis1 (EE) and pancreatic (PE) enhancers (Zhang et al., 2002, 2006) are indicated in the eye ChIP-seq profile of the *Pax6* genomic region. (F) The overrepresented 'biological process' and 'MGI phenotypes' GO classes are shown in order of significance by their binomial *P*-value.

association with enhancers involved in eye development, in contrast to what was observed in the embryonic trunk following a similar analysis (Penkov et al., 2013) as used here for comparison. Besides the 'eye development' categories, the 'Notch signaling' class appeared enriched in Meis1 BS-associated genes (Fig. 5F) in GO analysis.

#### Meis1 regulates the expression of Notch pathway genes and of selected genes involved in mammalian microphthalmia

To correlate the Meis1 BS pattern with actual gene expression regulation, we compared E10.5 wt and *Meis1*<sup>-/-</sup> eye cup transcriptomes by RNA-seq, identifying 406 downregulated and

242 upregulated transcripts (supplementary material Tables S1 and S2). The expression of transcripts encoding the core factors of the Notch pathway was extensively downregulated in *Meis1* mutants (Fig. 6A), indicating that this pathway is a major target of Meis1 regulation in the developing eye. To determine whether some of the genes encoding core components of the Notch pathway could be direct targets of Meis1, we examined the occurrence of Meis1 BSs in the enhancer-promoter units (EPUs) described in the ENCODE project (Shen et al., 2012). We found Meis BSs associated with the enhancer regions of *Notch2*, *Jag1*, *Hes2* and *Hes5*, coincident with enhancer histone marks in the E10.5 eye (supplementary material Fig. S3). A clear downregulation of *Hes5* mRNA, a major effector



**Fig. 6. Meis1 regulates components of the Notch signaling pathway and genes involved in microphthalmia.** (A) RNA-seq expression level changes in genes encoding core components of the Notch signaling pathway detected in E10.5 *Meis1*<sup>-/-</sup> versus wt eyes. (B,C) Representations of changes in gene expression detected in *Meis1*<sup>-/-</sup> for all genes (B), mouse microphthalmia genes and mouse orthologs of human microphthalmia genes (C). Genes are ordered according to their expression change. A red line indicates the mean of expression variations. Gray boxes in C indicate genes with a log<sub>2</sub> fold change >±0.5. Genes highlighted in red have a human ortholog associated with microphthalmia. The complete list of mouse orthologs of human genes analyzed in C is shown to the right. (A,C) p indicates the familywise error rate. (D) *Vsx2*, *Otx2* and *Sox2* genes showing their described promoter-enhancer interactions (PEI) according to Shen et al. (2012), the Meis1 ChIP-seq read profile from E10.5 eye (this study) and E11.5 trunk (Penkov et al., 2013), the P0 DNase-seq profile [from The ENCODE Project Consortium (2012), GEO:GSM1014188] and the H3K4me1 and H3K4me3 ChIP-seq profiles (this study). Detected Meis1 BSs are shown by arrowheads below the read profiles. Boxes highlight the E10.5 eye Meis BS regions and their coincidence with histone modification marks and described enhancer-promoter interactions.



of Notch signaling, was further confirmed by comparative *in situ* hybridization (ISH) analysis in wt and *Meis1* mutants (Fig. 2D–F). These results suggest that *Meis1* controls Notch pathway activity at various levels.

In addition, the association of *Meis1* BSs with genes involved in eye size regulation, together with the reduced eye size of *Meis1* mutants, further suggested a relationship between *Meis1* function and the direct or indirect regulation of microphthalmia genes. RNA-seq analysis that was focused on 121 mouse genes linked to microphthalmia confirmed this association (Fig. 6B,C). Interestingly, the human orthologs of the five most downregulated genes belonging to this class, *Pitx3*, *Smoc1*, *Cryba1*, *Cryaa* and *Foxe3*, have been associated with human microphthalmia (Fig. 6C) and a specific survey of all the mouse orthologs of human microphthalmia genes showed a very significant trend to downregulation in *Meis1* mutants (Fig. 6C). These results identify *Meis1* as a major regulator of microphthalmia-associated genes.

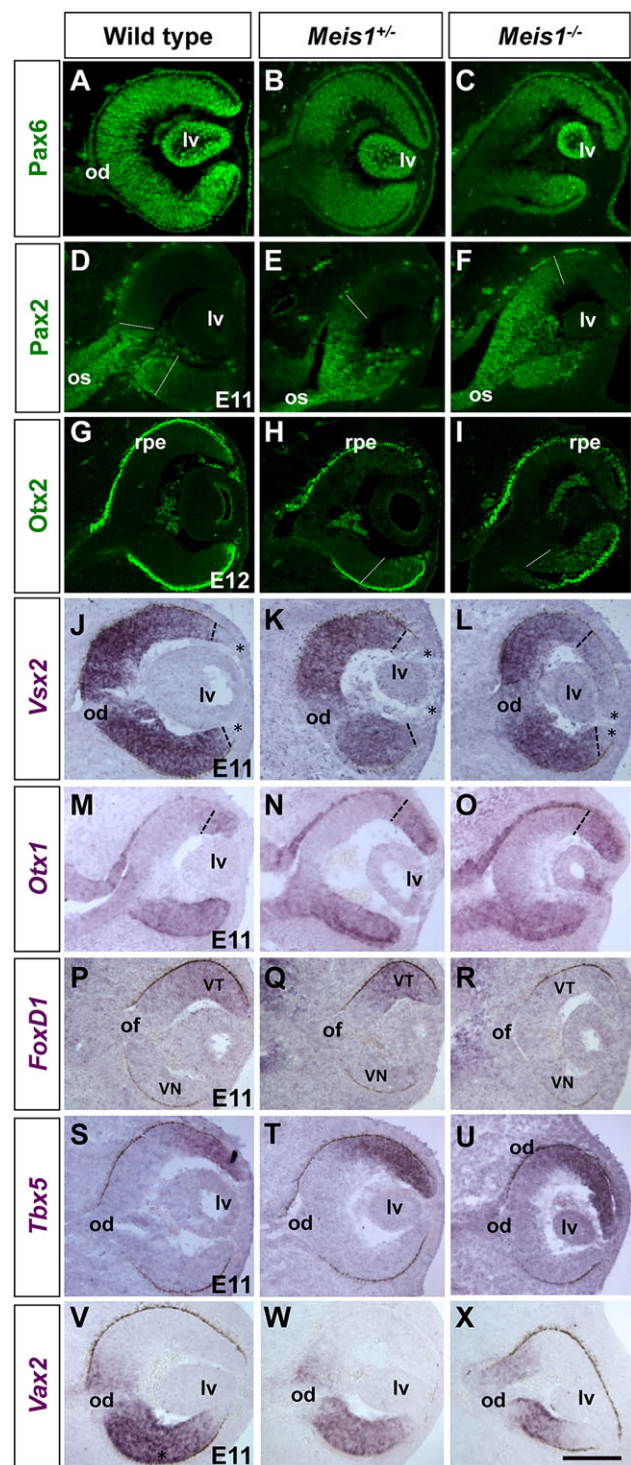
To analyze possible direct targets within genes that change their expression levels, we determined the presence of *Meis* BSs in the EPU of the 35 microphthalmia-associated genes that showed the strongest change in expression. We found that 11 out of these 35 genes contained one or more *Meis* BSs in their EPUs in coincidence with enhancer histone marks in E10.5 eyes (Fig. 6D; supplementary material Fig. S4; data not shown). Interestingly, 12 of the 35 genes showing altered expression and 8 of the 11 genes with *Meis1* BSs in their EPUs encode TFs, including some of those more frequently associated with human microphthalmia (*Otx2*, *Vsx2*, *Sox2*; Fig. 6D). These results suggest that *Meis1* controls eye size at various levels but predominantly by orchestrating the regulation of microphthalmia-associated TFs.

### ***Meis1* is required to sharpen the boundaries between the different eye territories**

Because eye development gene classes were overrepresented in *Meis1* ChIP-seq analysis (Fig. 5F), developmental processes other than neurogenesis were likely to be affected in *Meis1* mutant eyes. To test this possibility we analyzed the distribution of well-established markers of eye patterning. Although most of these markers were detected, their distribution was generally shifted and with blurred boundaries: for example, the border between *Pax2*, a marker of the proximal eye (optic stalk), and *Pax6*, a marker of the distal eye, was distally shifted, with a clear increase in *Pax2* expression in *Meis1* mutants versus wt embryos (Fig. 7A–F); this is also in agreement with the enhanced expression of *Shh* in mutants (Fig. 6C), which is known to expand the optic stalk. Similarly, *Otx2*, an RPE marker (Martinez-Morales et al., 2004), was abnormally extended in the ventrodorsal retina (Fig. 7G–I), in accordance with RNA-seq analysis and the presence of *Meis* BSs in its locus (Fig. 6C,D). The *Otx1*<sup>+</sup> peripheral retina invaded the *Vsx2*<sup>+</sup> central retina (Fig. 7J–O), again in agreement with our RNA-seq and ChIP-seq analysis indicating that *Meis1* might activate *Vsx2* (Fig. 6C,D). Patterning along the dorsoventral axis was also abnormal, with an expansion of the *Tbx5*<sup>+</sup> dorsal region and a reduction of the *Vax2*<sup>+</sup> ventral retina, whereas the expression of *Foxd1*, a temporal retinal marker, was considerably reduced (Fig. 7P–X). Notably, blurring of all boundaries was dose-dependent, as these defects were milder in *Meis1*<sup>+/-</sup> eyes (Fig. 7).

### **DISCUSSION**

In mammals, *Meis1* is crucial for the formation of the heart, vasculature and hematopoietic system (Azcoitia et al., 2005; Hisa et al., 2004). During the development of these structures, as well as



**Fig. 7. *Meis1* is required to define proper patterning of the optic cup along its principal axes.** Frontal (A–O,S–X) and horizontal (P–R) cryostat sections of E11 wt, *Meis1*<sup>+/-</sup> and *Meis1*<sup>-/-</sup> optic cups processed for the indicated markers. Note the allele-dependent expression shift of the various markers in *Meis1* mutant optic cups. Asterisks (J–L) indicate the tip of the ciliary margins. Dashed lines delineate the extent of marker staining. lv, lens vesicle; od, optic disc; of, optic fissure; os, optic stalk; rpe, retinal pigmented epithelium; VN, ventronasal; VT, ventrotemporal. Scale bar: 25 μm.

that of the limbs, *Meis1* acts as a co-factor for Hox proteins, often in cooperation with the related Pbx TFs (Penkov et al., 2013). Our study demonstrates that *Meis1* is also crucial for the progressive



formation of the eye, but, in this case, its function is mediated by Hox/Pbx-independent BSs on the DNA. In the absence of *Meis1*, the expression of the main patterning determinants of the optic cup is altered and the boundaries between the proximodistal, dorsoventral and nasotemporal domains of the cup are shifted or blurred. Retinal neurogenesis is also affected because *Meis1*, directly or indirectly, controls the expression of components of the neurogenic cascade mediated by the Notch receptor. Furthermore, *Meis1* is required for the expression of a set of genes involved in mammalian microphthalmia. Accordingly, *Meis1* haploinsufficient adult mice present morphological and functional defects characteristic of this congenital defect. Therefore, our data, together with previous studies showing that *Meis1* controls lens development and *Ccnd1*-mediated retinal proliferation (Bessa et al., 2008; Heine et al., 2008; Zhang et al., 2002), indicate that *Meis1* is a global regulator of eye development.

At least in mammals, this key function does not seem to be shared by the related *Meis2* or *Meis3*. *Meis3* is expressed in the eye only when the first retinal ganglion cells begin to differentiate (Gray et al., 2004). Both *Meis2* and *Meis1* contribute to lens specification by binding to the same BS present in a lens-specific enhancer of the *Pax6* locus (Zhang et al., 2002), but only *Meis1* is strongly and continuously expressed in the retinal neuroepithelium, according to our data and the distribution previously reported (Zhang et al., 2002). *Meis2*<sup>-/-</sup> mice have no evident early eye alterations (M.T., unpublished observations), suggesting that the reported *Meis2* expression in the optic vesicle (Heine et al., 2008) is either too transient to be consistently detected and/or dispensable in the gene regulatory network that controls early eye formation. This predominant role of *Meis1* in the mammalian eye primordium differs from that reported in chick and fish, in which alteration of *Meis2* levels also perturbs eye development (Conte et al., 2010; Heine et al., 2008). Furthermore, in contrast to its role in zebrafish where it is limited to neurogenesis (Bessa et al., 2008), *Meis1* is also crucial for patterning and neuronal differentiation in the mouse retina. This latter function is supported by the maintenance of *Meis1* expression in differentiated neurons, which are very much reduced in number in *Meis1*<sup>-/-</sup> mouse embryos. A similar role has been observed in chick, in which interference with *Meis* function compromises the appearance of retinal differentiation markers (Heine et al., 2008), including *Foxn4*, a TF directly regulated by *Meis1* and important for the generation of horizontal and amacrine neurons (Islam et al., 2013). As shown here, *Meis2* rescues *Meis1* deficiency in the vascular system, indicating that the two proteins are functionally similar and can show redundancy during eye development. Differential evolution of the regulatory elements controlling *Meis1* and *Meis2* expression, rather than *Meis1/2* protein functional specialization, might thus underlie their different requirement in fish, avian and mammalian eye development.

Our expression and genomic analyses indicate that *Meis1* must act upstream in the gene regulatory network controlling eye formation, as several of its targets are themselves TFs at the core of the network, such as *Sox2*, *Otx2* and *Pax6* (Beccari et al., 2013). Genetic inactivation of *Meis1* does not prevent the formation of the eye primordium, although it has, however, a fuzzy pattern. This ‘fuzziness’ affects its main tissues – optic stalk, neural retina and RPE – as well as its entire axes, indicating that *Meis1* is crucial to consolidate boundaries between the different eye domains. *Meis1* could, for example, render the activity of each of the tissue determinant genes [e.g. *Otx2* for the RPE (Martinez-Morales et al., 2001)] more efficient. There are various and not mutually exclusive mechanisms by which this could occur. In a view based around its

cooperation with Hox/Pbx in the trunk (Duboule, 2007; Penkov et al., 2013), *Meis1* could act as a co-factor with an as yet undefined predominant partner, perhaps binding to the m2-m3 core sequence that we have identified. This interaction would make the putative factor more efficient, allowing the correct expression of target genes. Alternatively, *Meis1* could interact with a wide variety of TFs in the eye, including its own putative targets such as *Sox2*, *Pax6* and *Otx2*, reinforcing their activity. The latter possibility is supported by the observation that the related *Meis2* has been shown to interact at least with *Otx2*, *Pax3*, *Pax6* and *Pax7* (Agoston et al., 2014, 2012; Agoston and Schulte, 2009). Alternatively, and based on the predominant presence of a ‘*Meis1*-only’ targeted sequence (m1, Fig. 5) in the eye chromatin, we favor the hypothesis that *Meis1* directly binds on enhancers of many determinant genes. Its binding would be necessary to achieve sufficient levels of target expression, and indispensable for regulating the extent of each domain. This mechanism could be illustrated by possible direct regulation by *Meis1* of *Pax6* in the retina and the establishment of proximodistal patterning of the optic vesicle. Indeed, the boundary between the proximal and distal optic vesicle is known to depend on a cross-regulatory loop between *Pax6* expressed distally and *Pax2* expressed proximally (Schwarz et al., 2000). We have identified a *Meis1* BS on a *Pax6* enhancer that is different to that known to mediate lens development (Zhang et al., 2002). In the absence of *Meis1*, retinal *Pax6* expression is strongly reduced, probably allowing *Pax2* upregulation. This should result in a weak cross-repressive loop between the two TFs and thus in a shifted proximodistal boundary, which is indeed observed in *Meis1* mutant embryos. This mechanism could be reinforced by a possible direct repression of *Meis1*, as we identified by ChIP-seq *Meis* BSs in the *Pax2* locus. Similar considerations could apply for other TFs known to act in a cross-regulatory loop during boundary establishment, as with *FoxG1* and *FoxD1* in the specification of the nasotemporal domains of the retina (Hatini et al., 1994; Huh et al., 1999). Notably, we identified by ChIP-seq *Meis* BSs also in the *Foxd1* locus.

A similar potential direct regulation could also be relevant in the control of components of the Notch signaling pathway, including the Notch2 receptor and the Notch signaling effectors *Hes2* and *Hes5*. For other members of the pathway, the decreased expression identified in our RNA-seq comparison might instead be indirect. Nevertheless, whether direct or not, the poor Notch pathway activation, in conjunction with decreased expression of *Ccnd1* and other microphthalmia-associated genes, could explain the *Meis1*<sup>-/-</sup> microphthalmic phenotype. Indeed, Notch signaling controls the number of progenitors entering retinal differentiation: loss of Notch function forestalls retinal neurogenesis (Jadhav et al., 2006), whereas abnormal Notch receptor activation transiently increases retinal proliferation and differentiation (Esteve et al., 2011). Notably, *Meis1* action on the Notch pathway and on microphthalmia-related genes could be linked since *Sox1*, *Sox2* and Notch signaling have been shown to regulate each other’s activity in various contexts (Genethliou et al., 2009; Kan et al., 2004; Neves et al., 2011). Most notably, *Sox2* regulates the Notch signaling pathway in retinal progenitor cells in a concentration-dependent manner, so that the levels of *Sox2* directly correlate with the levels of Notch1 (Taranova et al., 2006), a correlation that we have also observed between *Meis1* levels and *Hes5* expression.

Our RNA-seq analysis reveals a strong association between *Meis1* function and genes linked to microphthalmia. As expected from the use of complete E10.5 eye cups, we identified genes expressed only in the lens, such as the TF *Foxe3* and the crystallins (Graw, 2009), or in the neural retina, including the TF *Vsx2* and

*Smoc1* (Liu et al., 1994; Okada et al., 2011), or in both, such as *Sox1/2* and *Otx2* (Fuhrmann, 2010; Lang, 2004). This finding not only supports a pleiotropic function of *Meis1* in eye formation but also indicates a direct role of *Meis1* in the development of the retinal neuroepithelium. Our analysis of mutants with a conditional rescue of *Meis1* expression in the vasculature indicates that the microphthalmia observed is unlikely to derive from abnormal vasculature formation. The relative impact of *Meis1* loss-of-function on the lens or retina in the microphthalmic phenotype cannot be precisely dissected in our analysis. However, *Meis1* haploinsufficiency in the adult mouse eye has no consequence for blood vessel or lens development but it affects the expression of patterning and neurogenic genes, indicating that the retinal neuroepithelium is more sensitive to the levels of *Meis1* expression, directly implicating *Meis1* in retina development.

In conclusion, our data support that *Meis1* has a crucial and previously unreported role in integrating patterning and neurogenesis of the developing eye through the regulation of signaling pathways and patterning genes. *Meis1* seems to be at the core of a genetic network implicated in human microphthalmia, itself representing an additional candidate for syndromic cases associated with this ocular malformation. In this respect, eye developmental defects, including bilateral microphthalmia, have been linked to alterations in chromosome 2 (Waters et al., 1993; Weaver et al., 1991), at a location that may include the extensive *MEIS1* regulatory region (Royo et al., 2012), raising the possibility that reduced *MEIS1* levels could contribute to the phenotypic traits.

## MATERIALS AND METHODS

### Animals

*Meis1a* heterozygous mice were generated as described (Azcoitia et al., 2005). Embryos were obtained from timed (vaginal plug as E0.5) mating of *Meis1a*<sup>+/-</sup> mice or outbred CD1 mice. Animals were treated according to institutional or national guidelines for the use of animals in scientific research.

### BrdU incorporation

BrdU (10 mg/ml; Roche) was injected (50 µg/g body weight) into pregnant mice intraperitoneally. Embryos were sacrificed and collected 1 h later.

### TUNEL

Staining was performed using the In Situ Cell Death Detection Kit (Roche) on cryosections following the manufacturer's instructions.

### In situ hybridization

E10.5–13.5 embryos were immersion fixed in 4% paraformaldehyde in PBS for 3 h. Tissue was processed for cryosectioning in the frontal or horizontal plane and ISH was performed as previously described (Causseret et al., 2004) using the following digoxigenin-labeled mouse-specific antisense riboprobes were used: *Fgf15*, *Ccnd1*, *Hes5*, *Tbx5*, *Vax2*, *Foxd1*, *Otx1*, *Vsx2*.

### Immunohistochemistry

Cryosections were incubated with 0.1% Triton X-100 in PBS (PBT) and immunofluorescence was performed in PBT with 1% normal goat serum. For *Otx2*, *Pax6* and *Pax2* staining, sections were heated at 110°C for 2 min in 10 mM citrate buffer (pH 6) using a decloaking chamber (Biocare Medical). The following primary antibodies were used (at 1:1000 unless stated otherwise): rabbit anti-*Meis1a/2a* (Mercader et al., 2005), anti-*Otx2* (Abcam, ab21990), anti-cleaved caspase 3 (Cell Signaling, 9661), anti-*Pax2* (Invitrogen, 71-6000), anti-*Pax6* (Covance, PRB278P); mouse anti-phospho-histone H3 (Millipore, 06-570), anti-BrdU [1:4000; DSHB, G3G4(AntiBrdUrd)], anti-*Isl1/2* (1:500; DSHB, 39.4D5) and anti-Tuj1 (βIII tubulin, Babco, MMS-435P). Secondary antibodies were conjugated to

Alexa 488 or Alexa 594 (1:1000; Molecular Probes). Counterstaining was with DAPI (1 µg/ml; Vector Labs).

### RNA-seq

For RNA-seq library production, RNA of intact E10.5 optic cups (thus including the lens) from eight homozygous and wt embryos (out of 41 embryos from seven litters) was isolated using standard procedures, quantified (260 nm in a NanoDrop) and checked for integrity (Agilent Bioanalyzer). Total RNA was processed with the TruSeq RNA Sample Preparation v2 Kit (Illumina) to construct index-tagged cDNA libraries. The quality, quantity and the size distribution of the Illumina libraries were determined using the DNA-1000 Kit (Agilent Bioanalyzer). Prepared cDNA libraries were applied to an Illumina flow cell for cluster generation (TruSeq SR Cluster Kit V2 cBot, Illumina) and sequence-by-synthesis single reads of 75 bp using the TruSeq SBS Kit v5 (Illumina) were generated on the Genome Analyzer IIx (Illumina) following the standard RNA sequencing protocol. Sequencing adaptor contaminations were removed from reads using cutadapt software (<http://code.google.com/p/cutadapt/>) and the resulting reads were mapped and quantified on the transcriptome (Ensembl gene build 70) using RSEM v1.2.3 (Li and Dewey, 2011). Only genes with at least five counts per million in at least one sample were considered for statistical analysis. Data were then normalized and differential expression tested using the Bioconductor package edgeR (Robinson et al., 2010). Genes were considered differentially expressed when they presented a fold change ≥40%. Data were analyzed using Gene set enrichment and Ingenuity pathway software (Biobase International). Mouse microphthalmia genes were obtained by searching the Jackson Laboratory Mouse Genome Informatics database for the term 'microphthalmia' in the field 'mouse phenotypes and mouse models of human disease' of the 'genes and markers' query. Data are deposited in the NCBI GEO database under accession number GSE62786.

### ChIP-seq

ChIP assays to determine the histone methylation marks were performed using ~100 eyes from E10.5 mouse embryos. Chromatin was cross-linked with 1% formaldehyde for 15 min and fragmented to obtain DNA in the range 200–500 bp. DNA was divided in three pools (of 10 µg) and precipitated with 2 µg anti-H3K4me1 (CS-037-100, Diagenode) or anti-H3K4me3 (pAB-033-050, Diagenode). Immunoprecipitated DNA was purified with QIAquick columns (Qiagen). Data are deposited in the NCBI GEO database under accession number GSE62786. ChIP data for *Meis1* were obtained from ~200 eyes of E10.5 CD1 embryos. Two pools of ~20 µg total chromatin were immunoprecipitated with 4 µg anti-*Meis1* antibody (Mercader et al., 2005) and the immunoprecipitated DNA was purified and pooled together. ChIP-seq and bioinformatic processing were performed as described (Penkov et al., 2013). Chromatin was cross-linked with 1% formaldehyde for 15 min and fragmented to 300–500 bp. Data have been deposited in the NCBI GEO database under accession number GSE62786. Annotation of *Meis1* BSs and identification of *Meis1* BS-associated genes or overrepresentation in GO and phenotype-association databases was performed with the 'genomic regions enrichment of annotations tool' (GREAT) (McLean et al., 2010). For the identified peaks, 'de novo motif discovery' was run to identify consensus sequences enriched in the selected regions versus the whole genome using rGADEM (Li, 2009).

### Morphometric and functional analysis of the eye

The visual acuity and eye morphology of *Meis1a* mutant mice were evaluated at 15 weeks of age by virtual optokinetic drum, Scheimpflug imaging, OCT and laser interference biometry (LIB). For LIB and OCT, the eyes were treated with 1% atropine to ensure pupil dilation and mice were further anesthetized with 137 mg ketamine and 6.6 mg xylazine per kg body weight. For all tests, previously published protocols were followed: virtual drum vision test (Prusky et al., 2004), Scheimpflug imaging (Puk et al., 2013a), OCT (Puk et al., 2013b) and LIB (Puk et al., 2006).

### Quantification and statistical analysis

Area measurements and cell counting were performed with a Leica DM 5000M fluorescence microscope and a Leica DFC 500 camera. All



statistical analysis was performed using a minimum of 3 or 6 embryos or eyes per genotype, using ImageJ software (NIH). Differences between calculated averages were considered significant when  $P < 0.05$  by Student's  $t$ -test. For each of the ISH probes or immunohistochemical markers used in this study, analysis was performed on a minimum of three embryos for each genotype. For each embryo, all sections from both eyes were photographed and compared using sections at the same axial level.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

P.B., M.T. and S.M. conceived the study; J.G. and M.H.d.A. conceived the phenotypic tests; S.M., M.G.-L., L.B., L.C., R.D., O.P., O.A. and M.J.M.-B. performed experiments; S.M., L.B., O.B., J.G., D.M.-S.M., C.T., J.L.G.-S., F.C., M.T. and P.B. analyzed the data; M.T. and P.B. wrote the paper. All authors approved the manuscript.

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#### Supplementary material

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