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### **Visual impairment in FOXG1-mutated individuals and mice**

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Title: Visual impairment in FOXP1-mutated patients and mice

Article Type: Research Paper

Section/Category: Disease-Oriented Neuroscience

Keywords: Rett syndrome, Autism, West syndrome, visual cortex, inhibitory interneurons, cortical blindness

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First Author: Elena M Boggio

Order of Authors: Elena M Boggio; Laura Pancrazi; Caterina Lo Rizzo; Francesca Mari; Ilaria Meloni; Francesca Ariani; Anna Panighini; Elena Novelli; Enrica Strettoi; Joussef Hayek; Alessandra Rufa; Tommaso Pizzorusso, Ph.D.; Alessandra Renieri; Mario Costa

Abstract: The Forkhead Box G1 (FOXP1) gene encodes for a DNA-binding transcription factor, essential for the development of the telencephalon in mammalian forebrain. Mutations in FOXP1 have been reported to be involved in the onset of Rett Syndrome, for which sequence alterations of MECP2 and CDKL5 are known. While visual alterations are not classical hallmarks of Rett syndrome, an increasing body of evidence shows visual impairment in patients and in MeCP2 and CDKL5 animal models. Herein we focused on the functional role of FOXP1 in the visual system of animal models (Foxp1+/Cre mice) and of a cohort of patients carrying FOXP1 mutations or deletions. Visual physiology of Foxp1+/Cre mice was assessed by visually evoked potentials, which revealed a significant reduction in response amplitude and visual acuity with respect to wild-type littermates. Morphological investigation showed abnormalities in the organization of excitatory/inhibitory circuits in the visual cortex. No alterations were observed in retinal structure. By examining a cohort of FOXP1-mutated patients by means of a panel of neuro-ophthalmological evaluations, we found that all of them exhibited visual alterations compatible with high level visual dysfunctions. In conclusion our data show that Foxp1 haploinsufficiency results in an impairment of mouse and patient visual function.

Response to Reviewers: Pisa February 27th 2016

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**Highlights**

- 1) *Foxg1* haploinsufficiency causes impairment of mouse and human visual function.
- 2) *Foxg1*<sup>+/*Cre*</sup> mice show defects in the visual cortex excitatory/inhibitory circuits.
- 3) *FOXG1*-related visual alterations are compatible with the blind-sight syndrome.



## Visual impairment in *FOXP1*-mutated individuals and mice

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4 Elena M Boggio<sup>1,£,#</sup>, Laura Pancrazi<sup>1,2#</sup>, Mariangela Gennaro<sup>1,6</sup>, Caterina Lo Rizzo<sup>3,4</sup>, Francesca  
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32 # both authors equally contributed to the work

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## Abstract

The *Forkead Box G1* (*FOXG1* in humans, *Foxg1* in mice) gene encodes for a DNA-binding transcription factor, essential for the development of the telencephalon in mammalian forebrain. Mutations in *FOXG1* have been reported to be involved in the onset of Rett Syndrome, for which sequence alterations of *MECP2* and *CDKL5* are known. While visual alterations are not classical hallmarks of Rett syndrome, an increasing body of evidence shows visual impairment in patients and in *MeCP2* and *CDKL5* animal models. Herein we focused on the functional role of *FOXG1* in the visual system of animal models (*Foxg1*<sup>+/*Cre*</sup> mice) and of a cohort of subjects carrying *FOXG1* mutations or deletions. Visual physiology of *Foxg1*<sup>+/*Cre*</sup> mice was assessed by visually evoked potentials, which revealed a significant reduction in response amplitude and visual acuity with respect to wild-type littermates. Morphological investigation showed abnormalities in the organization of excitatory/inhibitory circuits in the visual cortex. No alterations were observed in retinal structure. By examining a cohort of *FOXG1*-mutated individuals with a panel of neuro-ophthalmological assessments, we found that all of them exhibited visual alterations compatible with high level visual dysfunctions. In conclusion our data show that *Foxg1* haploinsufficiency results in an impairment of mouse and human visual cortical function.

Keywords: Rett syndrome, Autism, West syndrome, visual cortex, inhibitory interneurons, cortical blindness

1 Rett Syndrome (RTT) is a neurodevelopmental disorder representing one of the most common  
2 causes of intellectual disability in girls. Beside the classical form due to *MECP2* mutations, two  
3 other forms have been associated to specific molecular defects, namely the early-onset seizure  
4 variant, mostly due to *CDKL5* mutations, and the congenital variant, mostly due to *FOXG1*  
5 mutations. The association between the *FOXG1* gene (OMIM#164874) and the congenital variant  
6 of RTT is relatively recent (Ariani et al., 2008), and since its discovery an increasing number of  
7 patients with *FOXG1* point mutations have been reported. The human *FOXG1* gene is located in the  
8 14q12 chromosome and encodes for a phylogenetically well conserved DNA-binding transcription  
9 factor of 489 aa. The mechanisms by which *FOXG1* mutations cause RTT are still unknown,  
10 however the presence of *FOXG1* on an autosomic chromosome suggests haploinsufficiency as a  
11 candidate for the aetio-pathological mechanisms of this RTT variant (Shoichet et al., 2005, De  
12 Filippis et al., 2012). Furthermore, a physical interaction between MeCP2 and FOXG1 has been  
13 demonstrated (Dastidar et al., 2012) suggesting that, at least in the Central Nervous system (CNS),  
14 an impairment in FOXG1-MeCP2 interaction could be critical for the development of RTT.  
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16 Intriguingly, a recent report indicates that Foxg1 displays, together with a nuclear localization, a  
17 specific targeting to mitochondria. This finding sheds new light on the etiology of FOXG1-RTT and  
18 on the original mitochondrial dysfunction hypothesis for the RTT pathogenesis (Pancrazi et al.,  
19 2015).

20 Foxg1 presence is essential for the embryonic development of the telencephalon in mammalian  
21 forebrain (Xuan et al., 1995). Its expression is abundant since the early development, persisting at  
22 lower levels in the adult cortex including the visual areas (Shen et al., 2006). This pattern suggests  
23 that specific aspects of the RTT associated with *FOXG1* mutations might involve visual cortical  
24 circuits. Foxg1 is also necessary for the correct formation of the inner ear and the olfactory system  
25 (Pauley et al., 2006, Duggan et al., 2008) and the appropriate crossing of retinal ganglion cell axons  
26 during development (Pratt et al., 2004). Interestingly, recent data show that Foxg1 overexpression is  
27 associated with neurodevelopmental pathologies and autism (Mariani et al., 2015).

28 *Foxg1* null mice were first obtained in 1995: while *Foxg1*<sup>+/-</sup> animals were believed to have a normal  
29 phenotype, *Foxg1*<sup>-/-</sup> mutants showed a dramatic reduction in the size of the cerebral hemispheres  
30 and died at birth. Due to the lethal phenotype, functional studies in null animals were initially  
31 restricted to the determination of the mechanisms influencing the forebrain size and the  
32 development of the telencephalon. More in depth analysis of the *Foxg1*<sup>+/-</sup> model showed  
33 microcephaly, hyperlocomotion, impaired habituation in the open field and a severe deficit in  
34 contextual fear conditioning, recapitulating some features of disorders derived from deregulation of  
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1 *FOXG1* expression such as the congenital variant of RTT and West syndrome (Shen et al., 2006,  
2 Eagleson et al., 2007, Siegenthaler et al., 2008). These features suggested the possibility to use  
3 *Foxg1*<sup>+/-</sup> mutants as models for these diseases (Shen et al., 2006, Ariani et al., 2008, Bahi-Buisson et  
4 al., 2010, Mencarelli et al., 2010, Philippe et al., 2010, Le Guen et al., 2011a, Le Guen et al., 2011b,  
5 Striano et al., 2011, Tohyama et al., 2011).  
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7 Recent work showed that the visual system is altered in mouse models of RTT (Tropea et al., 2009,  
8 Durand et al., 2012, Krishnan et al., 2015) providing the opportunity to use the visual system to  
9 investigate the patho-physiology of RTT and to improve objective patient evaluation. Indeed, a  
10 specific cortical processing deficit has been discovered using pattern reversal VEPs in RTT patients.  
11 Importantly, amplitude and latency of VEP waveforms are related to disease stage, clinical severity  
12 and MECP2 mutation (LeBlanc et al., 2015). The hypothesis that *FOXG1* mutations affects visual  
13 development is also supported by data showing that in West syndrome patients, altered VEPs and  
14 poor visual behavior are hallmarks of the pathology (de Freitas Dotto et al., 2014). These data  
15 prompted us to analyze whether visual deficits were also present in *Foxg1* RTT patients and in  
16 *Foxg1*<sup>+/-</sup> mice. *FOXG1* subject's visual behavior suggested a high level visual dysfunction  
17 compatible with the blind-sight syndrome occurring in individuals with extensive damage of  
18 primary visual cortex and consisting in loss of awareness for visual stimuli. Similarly, mouse  
19 studies point to a severe alteration in the development of cortical inhibitory and excitatory  
20 circuits. The results of this study show that cortical visual impairments occur in both patients and  
21 animal model of RTT.  
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## Experimental procedures

### Subjects

*FOXG1* subjects: Eight subjects (5 females and 3 males) with a *FOXG1* mutation were enrolled in the study. Age ranged from 9 months to 22 years (Table 1). All subjects had a clinical diagnosis of the congenital variant of RTT, with severe intellectual disability with early regression phase, severe microcephaly (range -3SD/-8SD) mostly postnatal, stereotypic hand and tongue movements. One subject showed generalized epileptic seizures. Sleep disturbances were present in 5 out of 6 individuals in whom the information was collected. Brain MRI was available for 6 cases. It was typical in one while the other 5 showed corpus callosum agenesis or hypoplasia and other brain asymmetries (Table 1). Menstrual disorders are not reported in the only girl in post-pubertal age (patient 8).

Four subjects showed a 14q12 micro-deletion involving the *FOXG1* gene ranging in size from 1.1Mb to 6.73Mb; four showed a point mutation predicted to lead to a truncation of the protein (Table 1).

Controls were 20 subjects (10 males and 10 females, mean age 7.8 years; range 12 months-22 years). None of them reported neurological problems, seizures, visual system diseases or history of pre- or perinatal illness. Subjects with refractive errors exceeding  $\pm 1.5$  diopters and strabismus were excluded. Regular neuro-ophthalmologic assessments were performed in each subject, including tests of visual acuity, color sensitivity, pupil reactivity, ocular alignment, fixation and motility, slit lamp evaluation, confrontation visual field testing, fundoscopy and recording of standard flash VEPs (Celesia, 1982, Lenassi et al., 2008).

1) Patient clinical assessment: Genetic counseling and clinical assessment were performed for 5 out of 8 subjects by the same clinical geneticists (AR and MF) and child neuropsychiatrist (JH). Clinical data of the remaining 3 patients were collected through clinical reports provided by the families (patients 1,2,5).

2) Visual Assessment: Clinical neuro-ophthalmologic data of each subject, evaluated by an experienced neuro-ophthalmologist (AR), were collected, together with an extensive parents' interview, specific for visual abilities impairment. Patients were approached gradually by planning several sections of examination or observation. The following visual tests were performed: refractive errors measurement (by cycloplegia and correction with appropriate lenses); an estimate of visual acuity and color vision (obtained using a modified form of forced choice-preferential

1 looking method); ocular alignment and fixation; oculo-cephalic reflexes; optokinetic nystagmus  
2 (when possible), cranial nerve examination; pupil reactivity evaluation; slit lamp examination;  
3 dilated fundoscopy. An estimation of visual field extension and subject's interest towards colored or  
4 familial stimuli compared to unfamiliar or non-colored stimuli was obtained during the  
5 examination; finally, visual engagement and visual-manual coordination were also evaluated.  
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7 Brain MRI, EEG and flash VEP data were collected whenever the examination was possible.  
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## 10 11 12 **Animals**

13 The *Foxg1*-cre line was created by replacing the intron-less *Foxg1* coding region (expressed  
14 specifically in the telencephalic cells) with cre recombinase, resulting in a *Foxg1* heterozygous  
15 mouse with reduced expression of *Foxg1* (Hebert and McConnell, 2000).  
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17 *Foxg1*<sup>+/*Cre*</sup> mice founders were a generous gift from Dr. Vania Broccoli. Animals were housed in a  
18 12-h light/dark cycle with free access to food and water. All the experiments were carried out in  
19 accordance with the directives the European Community Council (86/609/EEC) and approved by  
20 the Italian Ministry of Health. All mice used in this study were generated by heterozygous wild type  
21 matings of the original *Foxg1*<sup>*Cre*</sup> background (C57BL/6J) male with a wild type C57BL/6J female.  
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23 This breeding scheme was used since the heterozygous animals show behavioral abnormalities in  
24 the parental care. C57BL/6J (wild type) mice were purchased from the Jackson Laboratories (Bar  
25 Harbor, ME, USA).  
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## 36 **Statistics and data collection.**

37 Data were collected and analyzed by investigators blind to the genetic and treatment status of the  
38 animal. Only animals with significantly altered EEG or breath rate during VEP recordings were  
39 discarded. Sample size was estimated by power analysis using data present in the literature on VEP  
40 and inhibitory circuit alterations in mouse models of neurodevelopmental disorders. Animals were  
41 randomly assigned to the various experimental groups caring that littermates were divided in the  
42 different experimental groups. To analyze data we used the paired t-test (to compare two repeated  
43 measures on the same subjects) and the t-test (to compare between different subjects). ANOVA was  
44 used to compare many groups. P=0.05 was assumed as significance level. Statistical analysis was  
45 performed using the Sigma Stat (Systat, USA) software.  
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## 58 **Visually evoked potentials**

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1 Mice were anesthetized with an intraperitoneal injection of 20% urethane and mounted in a  
2 stereotaxic apparatus allowing a full view of the visual stimulus. After carefully removing a portion  
3 of the skull overlying the binocular visual cortex while leaving the dura mater intact, a glass-pulled  
4 recording electrode filled with NaCl (3 M) was inserted into the visual cortex perpendicularly to the  
5 stereotaxic plane. The electrode was inserted 100  $\mu\text{m}$  deep from pial surface and 2.7-3 mm lateral in  
6 correspondence to lambda. The electrical signals were amplified (10,000-fold), band-pass filtered  
7 (0.3–100 Hz), digitized, and averaged (at least 75 events in blocks of 5 each). The transient VEPs in  
8 response to an abrupt contrast reversal (1 Hz) were evaluated by measuring the peak-to-trough  
9 amplitude. The visual stimuli consisted of horizontal gratings of different spatial frequencies and  
10 contrasts generated by a visual stimulator interface (VSG2:2 card; Cambridge Research System,  
11 Cheshire, UK) and presented on a monitor (Sony model CPD-G520) placed 20 cm in front of the  
12 animal.  
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### 23 **Immunohistochemistry on visual cortex of *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+/*Cre*</sup> mice**

24 Animals were deeply anaesthetized and perfused transcardially with PBS 1X followed by 4%  
25 paraformaldehyde. Brains were removed, post-fixed in the same fixative at 4°C and cryoprotected  
26 by immersion in 30% sucrose. Forty  $\mu\text{m}$  coronal sections were cut on a freezing microtome and  
27 processed for immunohistochemistry. Free floating sections were incubated for 1-2 hours in a  
28 blocking solution (10% BSA, 0,3% Triton X-100 in PBS), then in the primary antibody solution  
29 O/N. The following day, the sections were incubated with appropriate secondary antibodies,  
30 mounted on glass slides and acquired with a Leica TCS-SP confocal microscope. The images were  
31 analyzed with ImageJ software. The following primary Abs were used: NeuN (1:500; Millipore,  
32 Billerica, MA, USA), MeCP2 (1:1000 cat. n. M 9317 Sigma Aldrich, Germany), parvalbumin  
33 (1:1000, Sigma Aldrich, Germany), calretinin (1:1000, Swant, Switzerland) and GAD67 (1:500,  
34 Chemicon, USA). Secondary antibodies used were Alexa Fluor 488 and Alexa Fluor 555 from  
35 Invitrogen.  
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### 49 **Immunohistochemistry on retinal sections of *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+/*Cre*</sup> mice**

50 Whole eyes were removed from deeply anesthetized mice (4 *Foxg1*<sup>+/*Cre*</sup> and 4 wt mice), cut at the  
51 ora serrata and fixed for 1 hr in 4% paraformaldehyde. The anterior segments were removed, the  
52 eye cups rinsed in 0.1M phosphate buffer (PB), cryoprotected in 30% sucrose, infiltrated in OCT  
53 and snap frozen in isopentane/dry ice. Afterwards, the eye cups were sectioned vertically at 12  $\mu\text{m}$   
54 on a Leica cryostat. The sections were collected on coated slides, processed for  
55 immunofluorescence using a panel of primary antibodies and reacted with appropriated secondary  
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antibodies conjugated with Alexa Fluor 488 (from Invitrogen), Rodamine RedX or Cy3 (from Jackson). Selected sections were counterstained with micromolar solutions of Ethidium homodimer-1 or BOBO 1-iodide nuclear dyes (from Invitrogen). All preparations were screened with a Zeiss Axioplan microscope equipped with a colorAxioplan camera. Selected sections were imaged at high resolution by confocal microscopy or with a Zeiss Apotome apparatus. The following antibodies were used: for photoreceptors: rhodopsin (RET-P1Sigma #O4886) and cone-specific L and M opsins (Millipore #AB5404 and #AB5407); for rod bipolar cells: PKC $\alpha$  (Sigma #P4334);for selected cone bipolar cell types: synaptotagmin2 - ZNP1 (ZIRC, Zebrafish International Resource Center); for amacrine cells:calretinin (Swant #7699/4); for cholinergic amacrine cells:ChAT (Chemicon #AB114P); for horizontal and ganglion cells:Neurofilament 200 (Sigma #N0142); for ganglion cells: SMI-32 (Covance#SMI32R) and Brn3 (Santa Cruz sc-6026); for synaptic ribbons: CtBP2 (Ribeye, BD Transduction Lab. #612044);for mitochondria: SOD2 (Invitrogen#A21990).

### Counts of cells in the ganglion cell layer (GCL)

Additional *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+/*Cre*</sup> mice were used for counting cells in the ganglion cell layer (GCL) following published protocols (Damiani et al., 2012, Rodriguez et al., 2014). Briefly, paraformaldehyde fixed retinas (n=3 per strain, each from a different animal) were isolated from eye cups, the vitreous removed and 4 partial cuts made to delimitate the dorsal, nasal, ventral and temporal quadrants. After washes in 0.01M PBS, the retinas were blocked overnight at 4°C in a solution containing 0.5% Triton X-100 and 5% goat serum. Then, they were incubated for 6 days at 4°C with an anti-RNA binding protein (RBPMS), guinea pig antibody (Phospho Solutions, CO, USA), specific for GCs (diluted 1:500), with 1% serum and 0.1% Triton X-100. After 3x20' washing in PBS, the retinas were incubated in Alexa Fluor 488-conjugated goat anti-guinea pig secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:800, washed 3x20' in PBS and counterstained for 2 hrs with Ethidium homodimer 1 (Vector Laboratories), diluted 1:1,000. After washing, retinal samples were finally mounted "ganglion cells up" in Vectashield. Retinal profiles of whole mount preparations were imaged in bright field with an Axiocam camera attached to a Zeiss microscope, using a 1.25x objective. Retinal areas were then measured on image tiff files using the edge detector tool of a Metamorph® routine. Flat-mounted retinas were imaged with a Zeiss Apotome fluorescence microscope using a 40x/1.5 n.a. Plan Neofluar oil objective. Five focal series of images (each covering a retinal area of 224 x 168  $\mu$ m) were acquired in each of the four retinal quadrants at regular intervals from the periphery towards the optic nerve head. Serial optical sections (10-20), 0.5  $\mu$ m apart, covering the thickness of the GCL were obtained. Projection images



1 were than generated and used for counting separately RBPMS positive cells (labelled green) and the  
2 nuclei of all the neurons in the layer, (labelled red), and comprising also those of displaced  
3 amacrine cells. The nuclei of blood vessel cells were excluded based on their typical elongated  
4 shape, small size and high brightness. Serial optical sections were used to count cells by navigating  
5 through z-stacks of the GCL in central retina areas characterized by elevated cell density. Total  
6 numbers of cells per retina were obtained multiplying average cellular densities by corresponding  
7 retinal areas.  
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### 14 **Dendritic spine analysis.**

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16 Animals (n=3 for each genotype) were deeply anesthetized and perfused through the heart with 4%  
17 paraformaldehyde. A block of visual cortex was sectioned in the coronal plane into 300- $\mu$ m-thick  
18 slices by using a vibratome. The lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-  
19 tetramethylindocarbocyanine (DiI) (Invitrogen) was coated onto tungsten particles (diameter 1.1  
20  $\mu$ m; Bio-Rad) according to Gan et al. (Gan et al., 2000). DiI-coated particles were delivered to the  
21 slices by using a Helios Gene Gun System (Bio-Rad). A polycarbonate filter with a 3.0- $\mu$ m pore  
22 size (Molecular Probes) was inserted between the gun and the preparation on a platform to remove  
23 clusters of large particles. Density of labeling was controlled by gas pressure (80 psi of helium).  
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25 After labeling, slices were fixed in 4% paraformaldehyde. Labeled structures were analyzed  
26 by confocal microscopy. Images of basal dendrites of layer 2/3 pyramidal cells were acquired,  
27 stacked (0.5  $\mu$ m step), and then analyzed with ImageJ. A total of 49 cells were counted; at least 6  
28 dendrites per neuron were analyzed for a total number of 8820 spines. We measured the average  
29 spine lengths and densities (number of spines per length of dendrite) for each animal and calculated  
30 the mean per group.  
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## Results

### Visual performance in control subjects

All subjects with age older than 4 years showed a best corrected visual acuity between 20/20-20/25, color vision was 15/15 (Hishihara platelets), pupils were reactive, anterior segment and lenses were clear, fixation was stable and ocular motility was normal. Younger patients (age under 4 years) showed a good engagement for near and far visual stimuli, they were able to follow light and objects and were also able to reach or grasp objects of interest even if presented in a crowded surrounding. VEPs results in control subjects showed a mean latency of the P100 of 99.19 ms (range 90-105 ms); and a mean amplitude of the N75-P100 of 7,37  $\mu$ V (range 5-10,1  $\mu$ V).

### Visual impairment in patients with *FOXG1* mutations

Considering that West syndrome patients carrying unbalanced levels of *FOXG1* show visual impairment and that RTT models show abnormal development and plasticity of the visual system (LeBlanc et al., 2015), we decided to investigate whether patients with *FOXG1* mutations displayed visual atypicalities (Castano et al., 2000). In all patients (Table 1), visual attention and engagement worsened with fatigue both when observed during examination and as reported by parents. Patients were apparently receptive to near (within reaching) visual stimuli that captured their attention. Actually, bright colored stimuli and moving objects (particularly when presented at short distance) were preferred. Patients' attention and awareness strongly decreased for far visual stimuli particularly when they were assembled with other objects (crowded surrounding). In these conditions, acoustic stimuli favored a brief visual engagement. Four patients out of eight demonstrated an inconstant ability to recognize familial faces (essentially parents), as observed during examination and reported by parents (Table 1). All patients had a variable dissociation between looking and reaching and demonstrated reduced or absent visual contact. All patients except one (#7) showed scarce visual engagement and no voluntary gazing toward surrounding visual stimuli. However, they were able to avoid obstacles or had a reaction of defense. They also showed an asymmetrical refractive defect between the two eyes. Four out of eight patients were photophobic and one showed compulsive gazing to bright light (#5). Anterior segment was typical in all patients. Pupils were reactive in seven out of eight cases. A jerk nystagmus was present in one patient (#2). Variable degrees of eyes misalignment and strabismus (exo and esotropia) were previously diagnosed in all patients. One patient presented a congenital oculomandibular syncinesia (#3). At fundoscopy, three patients showed smaller shape but typical appearing optic discs. Flash

1 VEPs, performed in three out of eight patients, were in the range of controls (see table 1). EEG  
2 showed epileptic changes particularly in occipital parietal and temporal parietal regions in two  
3 patients. These data prompted us to further characterize visual impairment and uncover the  
4 underlying neural defects exploiting a mouse model of *Foxg1* haploinsufficiency.  
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### 8 **Reduced cortical VEP acuity in *Foxg1*<sup>+/-</sup> Mice.**

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12 To investigate possible consequences of the *Foxg1* haploinsufficiency on visual function, we  
13 analyzed visual responses by recording Visually Evoked Potentials (VEP), a well-established  
14 technique for measuring overall visual function in mammals (Porciatti et al., 1999). Three *Foxg1*  
15 heterozygous models (*Foxg1*<sup>+/*tet*</sup>, *Foxg1*<sup>+/*lacZ*</sup> and *Foxg1*<sup>+/*cre*</sup>) showing similar phenotype have been  
16 described in the literature (Shen et al., 2006, Eagleson et al., 2007). Among them, we choose the  
17 widely used *Foxg1*<sup>+/*Cre*</sup> mice on a C57/BL6J background, which shows the most evident anatomical  
18 alterations of the cerebral cortex (Eagleson et al., 2007). Heterozygous mice were chosen because  
19 they better recapitulate the patient condition, insofar homozygous animals show birth lethality and a  
20 dramatic reduction in the size of the cerebral hemispheres.  
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23 As expected, VEP responses to alternating gratings decreased in amplitude in response to  
24 increments of the spatial frequency of the stimulus (Fig. 1). As shown in Fig. 1A, VEP transient  
25 amplitude is plotted against the spatial frequency log and visual acuity is taken as the spatial  
26 frequency that coincides with the extrapolation to zero amplitude of the linear regression. Visual  
27 acuity was significantly reduced in P30 *Foxg1*<sup>+/*Cre*</sup> mice compared to wild-type littermates  
28 (*Foxg1*<sup>+/*Cre*</sup>, 0.20 +/- 0.05 cycles/degree, n=9; WT, 0.51 cycles/degree, n=5; \*, P=0.01, t-test, Fig.  
29 1B). Moreover, VEP amplitudes at low spatial frequency were strongly diminished in *Foxg1*<sup>+/*Cre*</sup>  
30 mice (0.1 c/deg, wt 166.3 μV SEM 18.1, *Foxg1*<sup>+/*Cre*</sup> 18.1 μV SEM4.6; t-test, p<0.001). These data  
31 suggest that FOXG1 mutations could result in visual impairments in mice as in humans.  
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### 47 **Retinal morphology in *Foxg1*<sup>+/*Cre*</sup> and wt mice.**

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51 To assess whether *Foxg1* haploinsufficiency results into evident abnormalities in retinal  
52 morphological organization, we probed retinal sections of *Foxg1*<sup>+/*Cre*</sup> mice and wild type littermates  
53 (n=3 per mice, per strain) with a panel of antibodies, comprising well established cell-type specific  
54 markers, indicators of retinal lamination and constituents of retinal synaptic contacts (Jeon et al.,  
55 1998, tom Dieck and Brandstatter, 2006, Wassle et al., 2009, Barone et al., 2012).  
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Retinal neurons of all the five classes (photoreceptors, horizontal, bipolar, amacrine and ganglion cells), were labeled and compared among the two strains (Fig. 2, 3). None of the used retinal markers revealed obvious differences in the thickness of retinal layers, in their lamination pattern or in the morphology of individual cell types, between mutant and wt mice. Rhodopsin antibodies and ethidium nuclear staining highlighted a regular array of rod photoreceptors with no signs of degeneration (Fig.2, A-B). Cones displayed the normal, elongated morphology with outer segments of adequate length and orientation. Similarly, the main neurons of the retino-fugal (“vertical”) pathway, and namely rod bipolars (Fig. 3, A-B) and types of cone bipolars belonging to the “On” and “Off” functional varieties (C, D), had well ramified dendrites; their axonal arbors terminated in the appropriate sublaminae of the inner plexiform layer. Cholinergic processes, constituted by the dendrites of starburst amacrine cells, retained the characteristic distribution in two parallel bands precisely located at 1/3 and 2/3 of the inner plexiform layer (Fig.3, E and F). Calretinin staining revealed a third tier of processes, regularly positioned between the two cholinergic bands (Fig. 3, C and D). Ribeye, a marker of ribbons at retinal glutamatergic synapses (established by photoreceptors and bipolar cells) had the expected distribution in the outer and inner retina (Figure 3, A and B). Particular attention was paid to the analysis of the morphology and the number of ganglion cells (GCs), known to display abnormalities in axonal projections in homozygous mice (Pratt et al., 2004, Tian et al., 2008). SMI-32 anti-neurofilament antibodies highlighted a tier of large-size cell bodies corresponding to alpha-like ganglion cells, while immunolabeling for Brn3B, the transcription factor selectively expressed in these neurons (Xiang et al., 1993) showed a similar pattern of nuclear staining in the ganglion cell layer (GCL) of mutant and control retinas (Fig.2 C-D). Similarly, immunostaining with 200 kDa neurofilament antibodies showed a regular arrangement of cell bodies in the GCL, the expected bundles of axons in the optic fiber layer and profuse dendritic ramifications in the inner plexiform layer (Fig.3, E-F). The morphology of the optic nerve head in mutant mice appeared normal as well (Fig.2 A).

The GCL contains amacrine and ganglion cells, approximately in equal number in the rodent retina. Cell counts of retinal whole mounts (Fig. 4) from *Foxg1<sup>+Cre</sup>* (n=3 retinas) and *Foxg1<sup>+/+</sup>* (wt) mice (n=3) demonstrated that the two strains did not differ in the absolute number of cells in the GCL (*Foxg1<sup>+Cre</sup>* 106300, SEM 5369; *Foxg1<sup>+/+</sup>* 115475 cells SEM 4803, t-test; p=0.27; Fig.4 A-C).

Selective counts of GCs, labeled with a RBPMS cell type-specific antibody, showed overlapping numbers of these neurons in mutant and wt strains (Fig.4 D). Noticeably, the numbers obtained here are not different from previously published data accounting for the total number of cells in the GCL (Jeon et al., 1998) and of GCs (Williams et al., 1998) of C57Bl6 mice.

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In conclusion, no changes in general organization, architecture and pattern of lamination were observed in retinas of *Foxg1<sup>+/-Cre</sup>* mice, which appeared undistinguishable from those of their wt littermates. The heterozygous expression of FoxG1 does not affect the total number of cells in the innermost retinal layer.

### **Morphological analysis of the visual cortex of *Foxg1<sup>+/-Cre</sup>* mice.**

Prompted by the strong functional impairment in cortical VEPs, we investigated the architecture of excitatory and inhibitory neurons in the visual cortex of *Foxg1* haploinsufficient mice.

As a general neuronal marker, we assessed the pattern of Neu-N immunostaining in P30 *Foxg1<sup>+/-Cre</sup>* mice. As already reported (Eagleson et al., 2007), the visual cortex is thinner in this line of mutants. (wt 914.3  $\mu$ m SEM 12.6; *Foxg1<sup>+/-Cre</sup>* 747.9  $\mu$ m SEM 29.8; n=4 for each group, t-test p=0.002). To determine the occurrence of layer-specific effects of *Foxg1* haploinsufficiency on neuronal density, we quantified Neu-N positive neurons along the cortical depth. As shown in Fig. 5A, a significant reduction specific for layer II-III and layer IV was found in the mutant mice as compared to wt littermates.

To assess whether morphological defects were associated with specific neuronal populations, we first studied markers of inhibitory interneurons since disruption in interneuron circuits has been found in RTT mouse models (Durand et al., 2012, Tomassy et al., 2014, Krishnan et al., 2015), and then we investigated alterations in dendritic spines of excitatory pyramidal cells.

*Calretinin (CR), parvalbumin (PV) and GAD67 immunostaining.* Since maturation of cortical inhibition in visual areas is correlated with maturation of visual acuity and defects in cortical inhibition have been found in other mouse models of RTT (Dani et al., 2005, Lonetti et al., 2010), we decided to investigate the number of cortical inhibitory cells in *Foxg1<sup>+/-Cre</sup>* mice. Therefore, we estimated the density of CR, PV and GAD67 positive cells in the visual cortex at P30. CR immunostaining highlighted a significant increase in the interneuron density in the layers II-III and VI of the *Foxg1<sup>+/-Cre</sup>* cortex with respect to wt (Fig. 5B). By contrast, we observed a reduction of PV positive cell density in layers II-III of the visual cortex (Fig. 5C). The total number of inhibitory cells calculated using GAD67 immunohistochemistry was not affected in heterozygous *Foxg1<sup>+/-Cre</sup>* mice (Fig. 5D).

*Dendritic spine density and length.* Alterations in density and size of dendritic spines have been found in *Mecp2* mouse models of Rett Syndrome (Belichenko et al., 2009, Landi et al., 2011) and

1 also in many other models of neurodevelopmental disorders (Fiala et al., 2002). To investigate  
2 morphological alterations induced by *Foxg1* haploinsufficiency, we evaluated by diolistic labeling  
3 the spine density and length in the basal dendrites of pyramidal cells (layer 2/3) in visual cortices of  
4 *Foxg1<sup>+/-Cre</sup>* mice and wt littermates. As indicated in Fig. 6, no differences was present in spine  
5 length between the two genotypes (Fig. 6C) but a significant decrease in spine density was  
6 measured in *Foxg1<sup>+/-Cre</sup>* as compared to wt mice (Fig. 6B). No difference was present in cell body  
7 diameter (wt N= 25 cells 16.9  $\mu$ m SEM 2.3, *Foxg1<sup>+/-Cre</sup>* N=24 cells 15.8  $\mu$ m SEM 2.6; t-test  
8 p=0.76).

9 These results indicate that the severe impairment of visual acuity caused by *Foxg1*  
10 haploinsufficiency is likely to be due to defects in the morphological organization of visual cortical  
11 neurons.

## 12 Discussion

13 In this report we demonstrate for the first time the functional consequences of *Foxg1*  
14 haploinsufficiency in the visual system of *Foxg1<sup>+/-Cre</sup>* mice and a visual impairment in a cohort of  
15 Rett individuals presenting genetic alteration on *FOXG1*. Previous reports showed minor alterations  
16 in haploinsufficient animals consisting in a modestly thinner neocortex and reduced dentate gyrus  
17 size. To address this issue, we measured the visual acuity of *Foxg1<sup>+/-Cre</sup>* mice and their wild type  
18 littermates by means of Visually Evoked Potentials (VEPs). Our results show that *Foxg1<sup>+/-Cre</sup>* mice  
19 have a severe impairment in visual acuity and that the origin of such an impairment in visual  
20 function is not likely to reside in the retina, as retinal organization in these animals is normal.  
21 Indeed, retinal structure is qualitatively and quantitatively normal in *Foxg1<sup>+/-Cre</sup>* mice, and previous  
22 work showed that loss of a single *Foxg1* allele does not impair development of retinofugal axons  
23 (Tian et al., 2008). Consistently with the animal model, all the examined patients with *FOXG1*  
24 haploinsufficiency also show a visual impairment, with some common peculiarities. Such  
25 impairment seems to be principally caused by abnormal elaboration of visual signals in cortical  
26 areas.

## 27 Visual alterations in *FOXG1* mutated subjects

28 A common visual behavior reported in these subjects by relatives is the apparent lack of interest for  
29 any visual stimulus, often interpreted as low vision or narrowed visual field. This visual behavior  
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1 was better characterized in our subjects who mainly showed a scarce eye engagement, inconstant  
2 recognition of familial faces, and a general limited awareness to visual stimuli anywhere in the  
3 visual field, particularly when far and crowded. Despite a limited visual capacity, they were able to  
4 avoid obstacles and catch objects whenever positioned in neighboring space. The loss of attention  
5 and awareness for visual stimuli associated with the inability to disambiguate crowded objects or  
6 complex shapes; the inability of immediate recognition of familial faces, but the preserved interest  
7 and correct reaching or grasping toward colored stimuli and moving objects might suggest a  
8 condition resembling “blindsight”. Blindsight syndrome is a well-recognized neuro-  
9 ophthalmological defect, which occurs upon extensive damage of the primary visual cortex (V1).  
10 Blindsight refers to the ability of cortically blind patients (bearing a primary visual cortex damage)  
11 to use extra striate visual information in guiding behaviors but in the absence of conscious object  
12 identification (Weiskrantz, 2009). Human blind-sight subjects are able to orient to and even answer  
13 questions about stimuli presented to the blind visual field. However, they are entirely unaware of  
14 the stimuli to which they are responding. Another characteristic of blindsight is that subjects can  
15 discriminate simple objects on the basis of their spatial frequency, shape, texture and color (Dineen  
16 and Keating, 1981), but they have lost the ability to identify more elaborate visual attributes, such  
17 as the capacity to visually recognize complex shapes or crowded objects, foods and familial faces.  
18 Moreover, blindsight patients well maintain reflexive saccades and motion perception,  
19 suggesting that a residual network elaborating some visual information remains active even in case  
20 of extensive striate cortex lesions. The latter supports visually guided behaviors in the absence of  
21 awareness for visual stimuli. At least two separate pathways, and namely the retino-collicular and  
22 retino-geniculate pathways, reach the extrastriate cortex independently from the primary visual  
23 cortex, projecting to the motion area V5/MT in the medial temporal lobe. These connections may  
24 represent the unconscious part of the dorsal ‘where’ visual stream, whereas the conscious counterpart  
25 passes through the primary visual cortex before reaching the V5/MT area. The extrastriate blind-  
26 sight pathways could be active in the *FOXG1* patients studied here, who indeed are attracted by  
27 colored and moving targets. On the contrary, their scarce awareness to visual stimuli might indicate  
28 a functional impairment of the striate visual pathway in the absence of retino-geniculate  
29 dysfunctions. This hypothesis (although not sustained by the demonstration of a structural damage  
30 of the occipital lobe detected by MRI) would suggest a functional impairment or incomplete  
31 maturation of the striate cortex as reinforced by the present results in the murine model.  
32 Another visual system change in the human subjects studied here is the occurrence of ocular  
33 malformations, such as strabismus, oculo-mandibular syncynias and small optic discs. These  
34 anomalies (observed in three individuals), together with other cerebral malformations (i.e. corpus

callosum hypoplasia) may represent the expression of a global immaturity of the brain due to *FOXG1* mutations.

### Cortical architecture is defective in *Foxg1*<sup>+Cre</sup> mice

Retinal structure appeared normal in *Foxg1*<sup>+Cre</sup> mice, with no apparent anomalies in cell type distribution, laminar organization and numerical deficit in GCs that could explain the visual abnormalities. On the contrary, morphological studies showed significant changes in NeuN, PV and CR positive cells distribution in the visual cortex of *Foxg1*<sup>+Cre</sup> mice compared to wild type animals. PV and CR are calcium binding proteins that serve as calcium buffers and label main subpopulations of GABA-ergic cortical interneurons. PV immunoreactivity develops postnatally: in mice, it appears around eye opening at P13-P14 in intermediate layers, from which it expands to the upper and inner cortical layers at subsequent developmental stages. In the visual cortex, PV positive cell maturation occurs during the critical period of ocular dominance plasticity and manipulations that accelerate or delay the time course of the critical period correspondingly regulate developmental maturation of PV cells. The decrease in PV cell density in uppermost layers and the increase in CR positive cells of *Foxg1*<sup>+Cre</sup> cortex suggest that *Foxg1* haploinsufficiency results in an impairment in inhibitory circuitry maturation. PV+ synapses are usually perisomatic and PV+ pattern of innervation of pyramidal cells is engaged in control of the spiking pattern. Their reciprocal and extensive connections can regulate the timing of activity in the cortex. Hence, we can speculate that the epileptic phenotype observed in *FOXG1* mutated patients (Brunetti-Pierri et al., 2011, Guerrini and Parrini, 2012) may be linked to a defective PV positive cells maturation. Conversely, CR positive cells usually synapse on dendrites; thus, the observed increase in CR positive cells in the lower layers of the *Foxg1*<sup>+Cre</sup> cortex may cause a local alteration in cortical inhibition. Intriguingly, analysis of GAD67 positive neurons showed that the total number of inhibitory cells is preserved in *Foxg1*<sup>+Cre</sup> mice. Thus, the reduction of NeuN positive neurons in the superficial layers is likely due to alterations of excitatory cells.

To further analyze the excitatory phenotype in *Foxg1*<sup>+Cre</sup> primary visual cortex, we evaluated dendritic spine density and length in layer 2/3 pyramidal neurons. In the cerebral cortex, more than 90% of excitatory synapses terminate on spines. Spine density in the mouse neocortex increases during the second and third week of life and is followed by a period of major spine pruning and loss. In the primary visual cortex, dendritic spine density and morphology are developmentally regulated: they are plastic during young ages and become remarkably stable in adulthood. Our



1 results demonstrate a decrease in spine density in *Foxg1*<sup>+/*Cre*</sup> primary visual cortex compared to wild  
2 type animals.

3 Altogether, these results suggest that *Foxg1* haploinsufficiency causes alterations in both inhibitory  
4 and excitatory cells. Interestingly, alterations in inhibitory and excitatory synapses have been  
5 described in other mouse models of RTT as well as in a human disease model (iPSCs) obtained by  
6 genetic reprogramming of *FOXG1*-mutated patient fibroblasts (Patriarchi et al., 2015). In *MeCP2*-  
7 *KO* animals, the cortical excitatory input is reduced whilst the total inhibitory input is enhanced,  
8 leading to a shift of the homeostatic balance between excitation and inhibition in favor of inhibition.  
9 Moreover, *MeCP2*-deficient cortical and hippocampal neurons have fewer dendritic spines.

10 *CDKL5*, the other gene found mutated in some cases of RTT, codes for a protein that has been  
11 demonstrated to localize at excitatory synapses, where it contributes to correct dendritic spine  
12 structure and synapse activity. *CDKL5* knockdown in cultured hippocampal neurons results in  
13 aberrant spine morphology (Chen et al., 2010) and unstable dendritic spines (Della Sala et al.,  
14 2015). Intriguingly, the analysis of novel mouse models carrying *Cdkl5* deletion showed alterations  
15 in sensory evoked potentials both in the auditory and the visual cortices (Wang et al., 2012,  
16 Amendola et al., 2014).

17 In conclusion, *Foxg1* haploinsufficiency causes a severe impairment in visual function that is likely  
18 due to altered cortical mechanisms. This should be taken into account when *Foxg1*<sup>+/*Cre*</sup> mice are  
19 used in behavioral experiments and when visual function is evaluated in patients carrying *FOXG1*  
20 mutations.  
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# The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

## Legend to Figures

### Figure 1. Visual acuity in *Foxg1*<sup>+Cre</sup> mice and wild-type littermates

(A) Examples of VEP waveforms recorded in response to 0.1 c/deg gratings. (B) VEP amplitude changes in response to gratings of high contrast and of decreasing bar size (increasing spatial frequency) in a wild-type (WT) and a *Foxg1*<sup>+Cre</sup> mouse. VEP amplitude decreases by progressively increasing the spatial frequency. Visual acuity was determined by linearly extrapolating VEP amplitude to 0 V. Symbols represent data average data from all animals (WT n=9; *Foxg1*<sup>+Cre</sup> n=5). Arrows in the abscissa point to visual acuity value. Dotted line represents average noise level. (C) Spatial resolution in the visual cortex of wild-type and *Foxg1*<sup>+Cre</sup> mice. Visual acuity is significantly reduced in *Foxg1*<sup>+Cre</sup> mice compared with wild-type littermates (*Foxg1*<sup>+Cre</sup>, 0.20 cycle/degree SEM 0.05, n=9; WT, 0.51 cycle/degree, SEM 0.05 n=5; \*, P,0.01, Student's *t* test).

### Figure 2. Retinal morphology in *Foxg1*<sup>+Cre</sup> animals and wild-type littermates

Retinal morphology in *Foxg1*<sup>+Cre</sup> (A and C) and wt (B and D) mice. A, B: Low magnification images of retinal sections stained with rhodopsin antibodies (green signal), showing normal retinal laminations and bright staining of photoreceptor outer segments (OS) in both mutant and wt mice. Red: nuclear counterstaining. In A the normal morphology of the optic nerve head (ONH) can be appreciated. The insets show at 2x details of photoreceptor rows and outer segments. Here and in the following images: ONL, IPL outer nuclear and inner plexiform layer, respectively. C and D: examples of retinal ganglion cells (GC) stained by the type specific transcription factor Brn3-B (red), with nuclear localization, and by neurofilament antibodies (green), depicting their morphology in detail.

### Figure 3. Morphology of neuronal subtypes in *Foxg1*<sup>+Cre</sup> mice and wild-type littermates

Morphology of retinal neuronal subtypes in *Foxg1*<sup>+Cre</sup> (left panels) and wt mice  
Retinal neurons in *Foxg1*<sup>+Cre</sup> (left panels) and wt mice. A, B: rod bipolar cells have been labelled red by PKC antibodies. Their dendrites form dense plexa in the OPL while axonal endings (rbe) cluster in regularly in the deepest part of the IPL. Green puncta are synaptic ribbons from glutamatergic processes, regularly arranged in the two plexiform layers. Blue: nuclear counterstaining. C,D: types of cone bipolar cells stained by ZnP-1 (synaptotagmin antibodies). Both dendritic tips in the OPL and axonal arbors in the IPL have the expected fine structure. The IPL show three typical bands, revealed by calretinin staining of amacrine cells (red). E, F: Cholinergic amacrine cells, stained red by ChAT antibodies, are arranged in two mirror-

1 symmetric populations at the two margins of the IPL. Alpha-like ganglion cells, labelled by SMI-32  
2 antibodies (green) have well preserved morphologies in both mutant and wt retinas.  
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5 **Fig.4. Neurons in the GCL of *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+/*Cre*</sup> mice**

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7 A,B. Cells in the GCL have been labelled by antibodies against the RNA binding protein RBPMS  
8 that produce a cytoplasmic staining in GCs selectively (green signal). Ethidium counterstaining (red  
9 signal) show the nuclei of GCs and displaced amacrine cells (AC).The general morphology (A and  
10 B) and the total number of cells in the GC layer (C) are virtually identical in *Foxg1*<sup>+/+</sup> (A) and  
11 *Foxg1*<sup>+/*Cre*</sup> (B) mice. Quantification in C. The number of GCs proper (D) is also not different  
12 between the two strains (D).  
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20 **Figure 5. Immunohistochemistry on visual cortex of *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+/*Cre*</sup> mice**

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22 Panel A. A significant reduction of NeuN staining is present in layer II-III and IV of the visual  
23 cortex of *Foxg1*<sup>+/*Cre*</sup> mice (WT: n=4; *Foxg1*<sup>+/*Cre*</sup> = n=4; two-ways RM ANOVA genotype X cortical  
24 layer p=0.003; post-hoc Sidak wt vs. *Foxg1*<sup>+/*Cre*</sup> within layer II-III p=0.004, within layer IV  
25 p=0.006).  
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29 Panel B. Calretinin positive cells in visual cortices from WT and *Foxg1*<sup>+/*Cre*</sup> mice. Quantification of  
30 calretinin positive cells density in visual cortical layers of WT and *Foxg1*<sup>+/*Cre*</sup> mice shows a  
31 significant increase of calretinin positive cells in layer II-III and VI of the *Foxg1*<sup>+/*Cre*</sup> cortex (two-  
32 ways RM ANOVA genotype X cortical layer p=0.04; post-hoc Sidak wt vs. *Foxg1*<sup>+/*Cre*</sup> within layer  
33 II-III p<0.01, within layer VI p<0.05).  
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38 Panel C Parvalbumin positive cells in visual cortices from WT and *Foxg1*<sup>+/*Cre*</sup> mice. Quantification  
39 of parvalbumin positive cells density in visual cortical layers of WT and *Foxg1*<sup>+/*Cre*</sup> mice shows a  
40 significant decrease of parvalbumin positive cells in layer II-III of the *Foxg1*<sup>+/*Cre*</sup> cortex (two-ways  
41 RM ANOVA genotype X cortical layer p=0.03; post-hoc Sidak wt vs. *Foxg1*<sup>+/*Cre*</sup> within layer II-III  
42 p<0.01).  
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47 Panel D GAD67 positive cells in visual cortices from WT and *Foxg1*<sup>+/*Cre*</sup> mice. Quantification of  
48 GAD67 positive cells density in visual cortical layers of WT and *Foxg1*<sup>+/*Cre*</sup> mice shows that the  
49 density of GAD67 positive cells is not statistically different between wt and *Foxg1*<sup>+/*Cre*</sup> mice (two-  
50 ways RM ANOVA effect of genotype p=0.44, genotype X cortical layer p=0.33)..\*: p<0.05; \*\*:  
51 p<0.01.  
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58 **Figure 6. Dendritic spine length and density in *Foxg1*<sup>+/*Cre*</sup> animals and wild-type littermates**

1 A) Confocal microscope images of WT and *Foxg1*<sup>+Cre</sup> dendritic segment. Scale bar= 5μm. B and  
2 C) Average dendritic spine length and density (respectively) in basal dendrites of layer 4/5  
3 pyramidal cells of *Foxg1*<sup>+Cre</sup> and WT mice. A significant decrease in spine density was measured  
4 in *Foxg1*<sup>+Cre</sup> as compared to wt mice (WT: n= 7; *Foxg1*<sup>+Cre</sup>: n=7; p=0.045, Student's *t* test).  
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3 **Visual impairment in *FOXG1*-mutated individuals and mice**  
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**Abstract**

The *Forkead Box G1* (**FOXG1** in humans, *Foxg1* in mice) gene encodes for a DNA-binding transcription factor, essential for the development of the telencephalon in mammalian forebrain. Mutations in *FOXG1* have been reported to be involved in the onset of Rett Syndrome, for which sequence alterations of *MECP2* and *CDKL5* are known. While visual alterations are not classical hallmarks of Rett syndrome, an increasing body of evidence shows visual impairment in patients and in *MeCP2* and *CDKL5* animal models. Herein we focused on the functional role of FOXG1 in the visual system of animal models (*Foxg1*<sup>+/*Cre*</sup> mice) and of a cohort of subjects carrying *FOXG1* mutations or deletions. Visual physiology of *Foxg1*<sup>+/*Cre*</sup> mice was assessed by visually evoked potentials, which revealed a significant reduction in response amplitude and visual acuity with respect to wild-type littermates. Morphological investigation showed abnormalities in the organization of excitatory/inhibitory circuits in the visual cortex. No alterations were observed in retinal structure. By examining a cohort of *FOXG1*-mutated individuals with a panel of neuro-ophthalmological assessments, we found that all of them exhibited visual alterations compatible with high level visual dysfunctions. In conclusion our data show that *Foxg1* haploinsufficiency results in an impairment of mouse and human visual cortical function.

Keywords: Rett syndrome, Autism, West syndrome, visual cortex, inhibitory interneurons, cortical blindness

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4 Rett Syndrome (RTT) is a neurodevelopmental disorder representing one of the most common  
5 causes of intellectual disability in girls. Beside the classical form due to *MECP2* mutations, two  
6 other forms have been associated to specific molecular defects, namely the early-onset seizure  
7 variant, mostly due to *CDKL5* mutations, and the congenital variant, mostly due to *FOXG1*  
8 mutations. The association between the *FOXG1* gene (OMIM#164874) and the congenital variant  
9 of RTT is relatively recent (Ariani et al., 2008), and since its discovery an increasing number of  
10 patients with *FOXG1* point mutations have been reported. The human *FOXG1* gene is located in the  
11 14q12 chromosome and encodes for a phylogenetically well conserved DNA-binding transcription  
12 factor of 489 aa. The mechanisms by which *FOXG1* mutations cause RTT are still unknown,  
13 however the presence of *FOXG1* on an autosomic chromosome suggests haploinsufficiency as a  
14 candidate for the aetio-pathological mechanisms of this RTT variant (Shoichet et al., 2005, De  
15 Filippis et al., 2012). Furthermore, a physical interaction between MeCP2 and FOXG1 has been  
16 demonstrated (Dastidar et al., 2012) suggesting that, at least in the Central Nervous system (CNS),  
17 an impairment in FOXG1-MeCP2 interaction could be critical for the development of RTT.

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19 Intriguingly, a recent report indicates that Foxg1 displays, together with a nuclear localization, a  
20 specific targeting to mitochondria. This finding sheds new light on the etiology of FOXG1-RTT and  
21 on the original mitochondrial dysfunction hypothesis for the RTT pathogenesis (Pancrazi et al.,  
22 2015).

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24 Foxg1 presence is essential for the embryonic development of the telencephalon in mammalian  
25 forebrain (Xuan et al., 1995). Its expression is abundant since the early development, persisting at  
26 lower levels in the adult cortex including the visual areas (Shen et al., 2006). This pattern suggests  
27 that specific aspects of the RTT associated with *FOXG1* mutations might involve visual cortical  
28 circuits. Foxg1 is also necessary for the correct formation of the inner ear and the olfactory system  
29 (Pauley et al., 2006, Duggan et al., 2008) and the appropriate crossing of retinal ganglion cell axons  
30 during development (Pratt et al., 2004). Interestingly, recent data show that Foxg1 overexpression is  
31 associated with neurodevelopmental pathologies and autism (Mariani et al., 2015).

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33 *Foxg1* null mice were first obtained in 1995: while *Foxg1*<sup>+/-</sup> animals were believed to have a normal  
34 phenotype, *Foxg1*<sup>-/-</sup> mutants showed a dramatic reduction in the size of the cerebral hemispheres  
35 and died at birth. Due to the lethal phenotype, functional studies in null animals were initially  
36 restricted to the determination of the mechanisms influencing the forebrain size and the  
37 development of the telencephalon. More in depth analysis of the *Foxg1*<sup>+/-</sup> model showed  
38 microcephaly, hyperlocomotion, impaired habituation in the open field and a severe deficit in  
39 contextual fear conditioning, recapitulating some features of disorders derived from deregulation of  
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Comment [M2]: "Mental retardation" was replaced by "Intellectual disability" along the entire paper.

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3 *FOXG1* expression such as the congenital variant of RTT and West syndrome (Shen et al., 2006,  
4 Eagleson et al., 2007, Siegenthaler et al., 2008). These features suggested the possibility to use  
5 *Foxg1*<sup>+/-</sup> mutants as models for these diseases (Shen et al., 2006, Ariani et al., 2008, Bahi-Buisson et  
6 al., 2010, Mencarelli et al., 2010, Philippe et al., 2010, Le Guen et al., 2011a, Le Guen et al., 2011b,  
7 Striano et al., 2011, Tohyama et al., 2011).

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10 Recent work showed that the visual system is altered in mouse models of RTT (Tropea et al., 2009,  
11 Durand et al., 2012, Krishnan et al., 2015) providing the opportunity to use the visual system to  
12 investigate the patho-physiology of RTT and to improve objective patient evaluation. Indeed, a  
13 specific cortical processing deficit has been discovered using pattern reversal VEPs in RTT patients.  
14 Importantly, amplitude and latency of VEP waveforms are related to disease stage, clinical severity  
15 and MECP2 mutation (LeBlanc et al., 2015). The hypothesis that *FOXG1* mutations affects visual  
16 development is also supported by data showing that in West syndrome patients, altered VEPs and  
17 poor visual behavior are hallmarks of the pathology (de Freitas Dotto et al., 2014). These data  
18 prompted us to analyze whether visual deficits were also present in *Foxg1* RTT patients and in  
19 *Foxg1*<sup>+/-</sup> mice. *FOXG1* subject's visual behavior suggested a high level visual dysfunction  
20 compatible with the blind-sight syndrome occurring in individuals with extensive damage of  
21 primary visual cortex and consisting in loss of awareness for visual stimuli. Similarly, mouse  
22 studies point to a severe alteration in the development of cortical inhibitory and excitatory circuits.  
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30 The results of this study show that cortical visual impairments occur in both patients and animal  
31 model of RTT.  
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## Experimental procedures

### Subjects

*FOXG1* subjects: Eight subjects (5 females and 3 males) with a *FOXG1* mutation were enrolled in the study. Age ranged from 9 months to 22 years (Table 1). All subjects had a clinical diagnosis of the congenital variant of RTT, with severe intellectual disability with early regression phase, severe microcephaly (range -3SD/-8SD) mostly postnatal, stereotypic hand and tongue movements. One subject showed generalized epileptic seizures. Sleep disturbances were present in 5 out of 6 individuals in whom the information was collected. Brain MRI was available for 6 cases. It was typical in one while the other 5 showed corpus callosum agenesis or hypoplasia and other brain asymmetries (Table 1). Menstrual disorders are not reported in the only girl in post-pubertal age (patient 8).

Four subjects showed a 14q12 microdeletion involving the *FOXG1* gene ranging in size from 1.1Mb to 6.73Mb; four showed a point mutation predicted to lead to a truncation of the protein (Table 1).

Controls were 20 subjects (10 males and 10 females, mean age 7.8 years; range 12 months-22 years). None of them reported neurological problems, seizures, visual system diseases or history of pre- or perinatal illness. Subjects with refractive errors exceeding  $\pm 1.5$  diopters and strabismus were excluded. Regular neuro-ophthalmologic assessments were performed in each subject, including tests of visual acuity, color sensitivity, pupil reactivity, ocular alignment, fixation and motility, slit lamp evaluation, confrontation visual field testing, funduscopy and recording of standard flash VEPs (Celesia, 1982, Lenassi et al., 2008).

1) ~~Subjects Patient~~ clinical assessment: Genetic counseling and clinical assessment were performed for 5 out of 8 subjects by the same clinical geneticists (AR and MF) and child neuropsychiatrist (JH). Clinical data of the remaining 3 patients were collected through clinical reports provided by the families (patients 1,2,5).

2) Visual Assessment: Clinical neuro-ophthalmologic data of each subject, evaluated by an experienced neuro-ophthalmologist (AR), were collected, together with an extensive parents' interview, specific for visual abilities impairment. Patients were approached gradually by planning several sections of examination or observation. The following visual tests were performed: refractive errors measurement (by cycloplegia and correction with appropriate lenses); an estimate of visual acuity and color vision (obtained using a modified form of forced choice-preferential

**Comment [M3]:** terms normal and abnormal were replaced by typical and atypical along the entire paper when referring to human subjects.

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3 looking method); ocular alignment and fixation; oculo-cephalic reflexes; optokinetic nystagmus  
4 (when possible), cranial nerve examination; pupil reactivity evaluation; slit lamp examination;  
5 dilated fundoscopy. An estimation of visual field extension and subject's interest towards colored or  
6 familial stimuli compared to unfamiliar or non-colored stimuli was obtained during the  
7 examination; finally, visual engagement and visual-manual coordination were also evaluated.  
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9 Brain MRI, EEG and flash VEP data were collected whenever the examination was possible.  
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### 13 **Animals**

14 The *Foxg1-cre* line was created by replacing the intron-less *Foxg1* coding region (expressed  
15 specifically in the telencephalic cells) with cre recombinase, resulting in a *Foxg1* heterozygous  
16 mouse with reduced expression of *Foxg1* (Hebert and McConnell, 2000).  
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19 *Foxg1<sup>+Cre</sup>* mice founders were a generous gift from Dr. Vania Broccoli. Animals were housed in a  
20 12-h light/dark cycle with free access to food and water. All the experiments were carried out in  
21 accordance with the directives the European Community Council (86/609/EEC) and approved by  
22 the Italian Ministry of Health. All mice used in this study were generated by heterozygous wild type  
23 matings of the original *Foxg1<sup>Cre</sup>* background (C57BL/6J) male with a wild type C57BL/6J female.  
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26 This breeding scheme was used since the heterozygous animals show behavioral abnormalities in  
27 the parental care. C57BL/6J (wild type) mice were purchased from the Jackson Laboratories (Bar  
28 Harbor, ME, USA).  
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### 33 **Statistics and data collection.**

34 Data were collected and analyzed by investigators blind to the genetic and treatment status of the  
35 animal. Only animals with significantly altered EEG or breath rate during VEP recordings were  
36 discarded. Sample size was estimated by power analysis using data present in the literature on VEP  
37 and inhibitory circuit alterations in mouse models of neurodevelopmental disorders. Animals were  
38 randomly assigned to the various experimental groups caring that littermates were divided in the  
39 different experimental groups. To analyze data we used the paired t-test (to compare two repeated  
40 measures on the same subjects) and the t-test (to compare between different subjects). ANOVA was  
41 used to compare many groups. P=0.05 was assumed as significance level. Statistical analysis was  
42 performed using the Sigma Stat (Systat, USA) software.  
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### 51 **Visually evoked potentials**

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3 Mice were anesthetized with an intraperitoneal injection of 20% urethane and mounted in a  
4 stereotaxic apparatus allowing a full view of the visual stimulus. After carefully removing a portion  
5 of the skull overlying the binocular visual cortex while leaving the dura mater intact, a glass-pulled  
6 recording electrode filled with NaCl (3 M) was inserted into the visual cortex perpendicularly to the  
7 stereotaxic plane. The electrode was inserted 100  $\mu\text{m}$  deep from pial surface and 2.7-3 mm lateral in  
8 correspondence to lambda. The electrical signals were amplified (10,000-fold), band-pass filtered  
9 (0.3–100 Hz), digitized, and averaged (at least 75 events in blocks of 5 each). The transient VEPs in  
10 response to an abrupt contrast reversal (1 Hz) were evaluated by measuring the peak-to-trough  
11 amplitude. The visual stimuli consisted of horizontal gratings of different spatial frequencies and  
12 contrasts generated by a visual stimulator interface (VSG2:2 card; Cambridge Research System,  
13 Cheshire, UK) and presented on a monitor (Sony model CPD-G520) placed 20 cm in front of the  
14 animal.  
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### 22 **Immunohistochemistry on visual cortex of *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+/*Cre*</sup> mice**

24 Animals were deeply anaesthetized and perfused transcardially with PBS 1X followed by 4%  
25 paraformaldehyde. Brains were removed, post-fixed in the same fixative at 4°C and cryoprotected  
26 by immersion in 30% sucrose. Forty  $\mu\text{m}$  coronal sections were cut on a freezing microtome and  
27 processed for immunohistochemistry. Free floating sections were incubated for 1-2 hours in a  
28 blocking solution (10% BSA, 0,3% Triton X-100 in PBS), then in the primary antibody solution  
29 **over night** (O/N). The following day, the sections were incubated with appropriate secondary  
30 antibodies, mounted on glass slides and acquired with a Leica TCS-SP confocal microscope. The  
31 images were analyzed with ImageJ software. The following primary Abs were used: NeuN (1:500;  
32 Millipore, Billerica, MA, USA), MeCP2 (1:1000 cat. n. M 9317 Sigma Aldrich, Germany),  
33 parvalbumin (1:1000, Sigma Aldrich, Germany), calretinin (1:1000, Swant, Switzerland) and GAD67  
34 (1:500, Chemicon, USA). Secondary antibodies used were Alexa Fluor 488 and Alexa Fluor 555 from  
35 Invitrogen.  
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### 44 **Immunohistochemistry on retinal sections of *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+/*Cre*</sup> mice**

45 Whole eyes were removed from deeply anesthetized mice (4 *Foxg1*<sup>+/*Cre*</sup> and 4 wt mice), cut at the  
46 ora serrata and fixed for 1 hr in 4% paraformaldehyde. The anterior segments were removed, the  
47 eye cups rinsed in 0.1M phosphate buffer (PB), cryoprotected in 30% sucrose, infiltrated in OCT  
48 and snap frozen in isopentane/dry ice. Afterwards, the eye cups were sectioned vertically at 12  $\mu\text{m}$   
49 on a Leica cryostat. The sections were collected on coated slides, processed for  
50 immunofluorescence using a panel of primary antibodies and reacted with appropriated secondary  
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2 antibodies conjugated with Alexa Fluor 488 (from Invitrogen), Rhodamine RedX or Cy3 (from  
3 Jackson). Selected sections were counterstained with micromolar solutions of Ethidium  
4 homodimer-1 or BOBO 1-iodide nuclear dyes (from Invitrogen). All preparations were screened  
5 with a Zeiss Axioplan microscope equipped with a colorAxioplan camera. Selected sections were  
6 imaged at high resolution by confocal microscopy or with a Zeiss Apotome apparatus. The  
7 following antibodies were used: for photoreceptors: rhodopsin (RET-P1Sigma #O4886) and cone-  
8 specific L and M opsins (Millipore #AB5404 and #AB5407); for rod bipolar cells: PKC $\alpha$  (Sigma  
9 #P4334);for selected cone bipolar cell types: synaptotagmin2 - ZNP1 (ZIRC, Zebrafish  
10 International Resource Center); for amacrine cells:calretinin (Swant #7699/4); for cholinergic  
11 amacrine cells:ChAT (Chemicon #AB114P); for horizontal and ganglion cells:Neurofilament 200  
12 (Sigma #N0142); for ganglion cells: SMI-32 (Covance#SMI32R) and Brn3 (Santa Cruz sc-6026);  
13 for synaptic ribbons: CtBP2 (Ribeye, BD Transduction Lab. #612044);for mitochondria: SOD2  
14 (Invitrogen#A21990).

#### 23 24 **Counts of cells in the ganglion cell layer (GCL)**

25 Additional *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+Cre</sup> mice were used for counting cells in the ganglion cell  
26 layer (GCL) following published protocols (Damiani et al., 2012, Rodriguez et al., 2014). Briefly,  
27 paraformaldehyde fixed retinas (n=3 per strain, each from a different animal) were isolated from  
28 eye cups, the vitreous removed and 4 partial cuts made to delimitate the dorsal, nasal, ventral and  
29 temporal quadrants. After washes in 0.01M PBS, the retinas were blocked overnight at 4°C in a  
30 solution containing 0.5% Triton X-100 and 5% goat serum. Then, they were incubated for 6 days at  
31 4°C with an anti-RNA binding protein (RBPMS), guinea pig antibody (Phospho\_Solutions, CO,  
32 USA), specific for GCs (diluted 1:500), with 1% serum and 0.1% Triton X-100. After 3x20'  
33 washing in PBS, the retinas were incubated in Alexa Fluor 488-conjugated goat anti-guinea pig  
34 secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:800, washed 3x20' in PBS  
35 and counterstained for 2 hrs with Ethidium homodimer 1 (Vector Laboratories), diluted 1:1,000.  
36 After washing, retinal samples were finally mounted “ganglion cells up” in Vectashield. Retinal  
37 profiles of whole mount preparations were imaged in bright field with an Axiocam camera attached  
38 to a Zeiss microscope, using a 1.25x objective. Retinal areas were then measured on image tiff files  
39 using the edge detector tool of a Metamorph® routine. Flat-mounted retinas were imaged with a  
40 Zeiss Apotome fluorescence microscope using a 40x/1.5 n.a. Plan Neofluar oil objective. Five focal  
41 series of images (each covering a retinal area of 224 x 168  $\mu$ m) were acquired in each of the four  
42 retinal quadrants at regular intervals from the periphery towards the optic nerve head. Serial optical  
43 sections (10-20), 0.5  $\mu$ m apart, covering the thickness of the GCL were obtained. Projection images  
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3 were than generated and used for counting separately RBPMS positive cells (labelled green) and the  
4 nuclei of all the neurons in the layer, (labelled red), and comprising also those of displaced  
5 amacrine cells. The nuclei of blood vessel cells were excluded based on their typical elongated  
6 shape, small size and high brightness. Serial optical sections were used to count cells by navigating  
7 through z-stacks of the GCL in central retina areas characterized by elevated cell density. Total  
8 numbers of cells per retina were obtained multiplying average cellular densities by corresponding  
9 retinal areas.

### 13 14 **Dendritic spine analysis.**

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16 Animals (n=3 for each genotype) were deeply anesthetized and perfused through the heart with 4%  
17 paraformaldehyde. A block of visual cortex was sectioned in the coronal plane into 300- $\mu\text{m}$ -thick  
18 slices by using a vibratome. The lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-  
19 tetramethylindocarbocyanine (DiI) (Invitrogen) was coated onto tungsten particles (diameter 1.1  
20  $\mu\text{m}$ ; Bio-Rad) according to Gan et al. (Gan et al., 2000). DiI-coated particles were delivered to the  
21 slices by using a Helios Gene Gun System (Bio-Rad). A polycarbonate filter with a 3.0- $\mu\text{m}$  pore  
22 size (Molecular Probes) was inserted between the gun and the preparation on a platform to remove  
23 clusters of large particles. Density of labeling was controlled by gas pressure (80 psi of helium).  
24 After labeling, slices were fixed in 4% paraformaldehyde. Labeled structures were analyzed  
25 by confocal microscopy. Images of basal dendrites of layer 2/3 pyramidal cells were acquired,  
26 stacked (0.5  $\mu\text{m}$  step), and then analyzed with ImageJ. A total of 49 cells were counted; at least 6  
27 dendrites per neuron were analyzed for a total number of 8820 spines. We measured the average  
28 spine lengths and densities (number of spines per length of dendrite) for each animal and calculated  
29 the mean per group.

## Results

### Visual performance in control subjects

All subjects with age older than 4 years showed a best corrected visual acuity between 20/20-20/25, color vision was 15/15 (Hishihara platelets), pupils were reactive, anterior segment and lenses were clear, fixation was stable and ocular motility was normal. Younger patients (age under 4 years) showed a good engagement for near and far visual stimuli, they were able to follow light and objects and were also able to reach or grasp objects of interest even if presented in a crowded surrounding. VEPs results in control subjects showed a mean latency of the P100 of 99.19 ms(range 90-105 ms); and a mean amplitude of the N75-P100 of 7,37  $\mu$ V(range 5-10,1  $\mu$ V).

### Visual impairment in patients with *FOXP1* mutations

Considering that West syndrome patients carrying unbalanced levels of *FOXP1* show visual impairment and that RTT models show abnormal development and plasticity of the visual system (LeBlanc et al., 2015), we decided to investigate whether patients with *FOXP1* mutations displayed visual atypicalities (Castano et al., 2000). In all patients (Table 1), visual attention and engagement worsened with fatigue both when observed during examination and as reported by parents. Patients were apparently receptive to near (within reaching) visual stimuli that captured their attention. Actually, bright colored stimuli and moving objects (particularly when presented at short distance) were preferred. Patients' attention and awareness strongly decreased for far visual stimuli particularly when they were assembled with other objects (crowded surrounding). In these conditions, acoustic stimuli favored a brief visual engagement. Four patients out of eight demonstrated an inconstant ability to recognize familial faces (essentially parents), as observed during examination and reported by parents (Table 1). All patients had a variable dissociation between looking and reaching and demonstrated reduced or absent visual contact. All patients except one (#7) showed scarce visual engagement and no voluntary gazing toward surrounding visual stimuli. However, they were able to avoid obstacles or had a reaction of defense. They also showed an asymmetrical refractive defect between the two eyes. Four out of eight patients were photophobic and one showed compulsive gazing to bright light (#5). Anterior segment was typical in all patients. Pupils were reactive in seven out of eight cases. A jerk nystagmus was present in one patient (#2). Variable degrees of eyes misalignment and strabismus (exo and esotropia) were previously diagnosed in all patients. One patient presented a congenital oculomandibular syncinesia (#3). At fundoscopy, three patients showed smaller shape but typical appearing optic discs. Flash

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3 VEPs, performed in three out of eight patients, were in the range of controls (see table 1). EEG  
4 showed epileptic changes particularly in occipital parietal and temporal parietal regions in two  
5 patients. These data prompted us to further characterize visual impairment and uncover the  
6 underlying neural defects exploiting a mouse model of *Foxg1* haploinsufficiency.  
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### 10 **Reduced cortical VEP acuity in *Foxg1*<sup>+/-</sup> Mice.**

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13 To investigate possible consequences of the *Foxg1* haploinsufficiency on visual function, we  
14 analyzed visual responses by recording Visually Evoked Potentials (VEP), a well-established  
15 technique for measuring overall visual function in mammals (Porciatti et al., 1999). Three *Foxg1*  
16 heterozygous models (*Foxg1*<sup>+tet</sup>, *Foxg1*<sup>+lacZ</sup> and *Foxg1*<sup>+cre</sup>) showing similar phenotype have been  
17 described in the literature (Shen et al., 2006, Eagleson et al., 2007). Among them, we choose the  
18 widely used *Foxg1*<sup>+Cre</sup> mice on a C57/BL6J background, which shows the most evident anatomical  
19 alterations of the cerebral cortex (Eagleson et al., 2007). Heterozygous mice were chosen because  
20 they better recapitulate the patient condition, insofar homozygous animals show birth lethality and a  
21 dramatic reduction in the size of the cerebral hemispheres.  
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24 As expected, VEP responses to alternating gratings decreased in amplitude in response to  
25 increments of the spatial frequency of the stimulus (Fig. 1). As shown in Fig. 1A, VEP transient  
26 amplitude is plotted against the spatial frequency log and visual acuity is taken as the spatial  
27 frequency that coincides with the extrapolation to zero amplitude of the linear regression. Visual  
28 acuity was significantly reduced in P30 *Foxg1*<sup>+Cre</sup> mice compared to wild-type littermates  
29 (*Foxg1*<sup>+Cre</sup>, 0.20 +/- 0.05 cycles/degree, n=9; WT, 0.51 cycles/degree, n=5; \*, P=0.01, t-test, Fig.  
30 1B). Moreover, VEP amplitudes at low spatial frequency were strongly diminished in *Foxg1*<sup>+Cre</sup>  
31 mice (0.1 c/deg, wt 166.3 μV SEM 18.1, *Foxg1*<sup>+Cre</sup> 18.1 μV SEM4.6; t-test, p<0.001). These data  
32 suggest that FOXG1 mutations could result in visual impairments in mice as in humans.  
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### 41 **Retinal morphology in *Foxg1*<sup>+Cre</sup> and wt mice.**

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44 To assess whether *Foxg1* haploinsufficiency results into evident abnormalities in retinal  
45 morphological organization, we probed retinal sections of *Foxg1*<sup>+Cre</sup> mice and wild type littermates  
46 (n=3 per mice, per strain) with a panel of antibodies, comprising well established cell-type specific  
47 markers, indicators of retinal lamination and constituents of retinal synaptic contacts (Jeon et al.,  
48 1998, tom Dieck and Brandstatter, 2006, Wassle et al., 2009, Barone et al., 2012).  
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3 Retinal neurons of all the five classes (photoreceptors, horizontal, bipolar, amacrine and ganglion  
4 cells), were labeled and compared among the two strains (Fig. 2, 3). None of the used retinal  
5 markers revealed obvious differences in the thickness of retinal layers, in their lamination pattern or  
6 in the morphology of individual cell types, between mutant and wt mice. Rhodopsin antibodies and  
7 ethidium nuclear staining highlighted a regular array of rod photoreceptors with no signs of  
8 degeneration (Fig.2, A-B). Cones displayed the normal, elongated morphology with outer segments  
9 of adequate length and orientation. Similarly, the main neurons of the retino-fugal (“vertical”)  
10 pathway, and namely rod bipolars (Fig. 3, A-B) and types of cone bipolars belonging to the “On”  
11 and “Off” functional varieties (C, D), had well ramified dendrites; their axonal arbors terminated in  
12 the appropriate sublaminae of the inner plexiform layer. Cholinergic processes, constituted by the  
13 dendrites of starburst amacrine cells, retained the characteristic distribution in two parallel bands  
14 precisely located at 1/3 and 2/3 of the inner plexiform layer (Fig.3, E and F). Calretinin staining  
15 revealed a third tier of processes, regularly positioned between the two cholinergic bands (Fig. 3, C  
16 and D). Ribeye, a marker of ribbons at retinal glutamatergic synapses (established by  
17 photoreceptors and bipolar cells) had the expected distribution in the outer and inner retina (Figure  
18 3, A and B). Particular attention was paid to the analysis of the morphology and the number of  
19 ganglion cells (GCs), known to display abnormalities in axonal projections in homozygous mice  
20 (Pratt et al., 2004, Tian et al., 2008). SMI-32 anti-neurofilament antibodies highlighted a tier of  
21 large-size cell bodies corresponding to alpha-like ganglion cells, while immunolabeling for Brn3B,  
22 the transcription factor selectively expressed in these neurons (Xiang et al., 1993) showed a similar  
23 pattern of nuclear staining in the ganglion cell layer (GCL) of mutant and control retinas (Fig.2 C-  
24 D). Similarly, immunostaining with 200 kDa neurofilament antibodies showed a regular  
25 arrangement of cell bodies in the GCL, the expected bundles of axons in the optic fiber layer and  
26 profuse dendritic ramifications in the inner plexiform layer (Fig.3, E-F).The morphology of the  
27 optic nerve head in mutant mice appeared normal as well (Fig.2 A).

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41 The GCL contains amacrine and ganglion cells, approximately in equal number in the rodent retina.  
42 Cell counts of retinal whole mounts (Fig. 4) from *Foxg1<sup>+Cre</sup>* (n=3 retinas) and *Foxg1<sup>+/+</sup>* (wt) mice  
43 (n=3) demonstrated that the two strains did not differ in the absolute number of cells in the GCL  
44 (*Foxg1<sup>+Cre</sup>* 106300, SEM 5369; *Foxg1<sup>+/+</sup>* 115475 cells SEM 4803, t-test; p=0.27; Fig.4 A-C).  
45 Selective counts of GCs, labeled with a RBPMS cell type-specific antibody, showed overlapping  
46 numbers of these neurons in mutant and wt strains (Fig.4 D). Noticeably, the numbers obtained here  
47 are not different from previously published data accounting for the total number of cells in the GCL  
48 (Jeon et al., 1998) and of GCs (Williams et al., 1998) of C57Bl6 mice.  
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3 In conclusion, no changes in general organization, architecture and pattern of lamination were  
4 observed in retinas of *Foxg1*<sup>+/*Cre*</sup> mice, which appeared undistinguishable from those of their wt  
5 littermates. The heterozygous expression of FoxG1 does not affect the total number of cells in the  
6 innermost retinal layer.  
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### 10 11 **Morphological analysis of the visual cortex of *Foxg1*<sup>+/*Cre*</sup> mice.**

12 Prompted by the strong functional impairment in cortical VEPs, we investigated the architecture of  
13 excitatory and inhibitory neurons in the visual cortex of *Foxg1* haploinsufficient mice.  
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15 As a general neuronal marker, we assessed the pattern of Neu-N immunostaining in P30 *Foxg1*<sup>+/*Cre*</sup>  
16 mice. As already reported (Eagleson et al., 2007), the visual cortex is thinner in this line of  
17 mutants. (wt 914.3  $\mu$ m SEM 12.6; *Foxg1*<sup>+/*Cre*</sup> 747.9  $\mu$ m SEM 29.8; n=4 for each group, t-test  
18 p=0.002). To determine the occurrence of layer-specific effects of *Foxg1* haploinsufficiency on  
19 neuronal density, we quantified Neu-N positive neurons along the cortical depth. As shown in Fig.  
20 5A, a significant reduction specific for layer II-III and layer IV was found in the mutant mice as  
21 compared to wt littermates.  
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27 To assess whether morphological defects were associated with specific neuronal populations, we  
28 first studied markers of inhibitory interneurons since disruption in interneuron circuits has been  
29 found in RTT mouse models (Durand et al., 2012, Tomassy et al., 2014, Krishnan et al., 2015), and  
30 then we investigated alterations in dendritic spines of excitatory pyramidal cells.  
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34 *Calretinin (CR), parvalbumin (PV) and GAD67 immunostaining.* Since maturation of cortical  
35 inhibition in visual areas is correlated with maturation of visual acuity and defects in cortical  
36 inhibition have been found in other mouse models of RTT (Dani et al., 2005, Lonetti et al., 2010),  
37 we decided to investigate the number of cortical inhibitory cells in *Foxg1*<sup>+/*Cre*</sup> mice. Therefore, we  
38 estimated the density of CR, PV and GAD67 positive cells in the visual cortex at P30. CR  
39 immunostaining highlighted a significant increase in the interneuron density in the layers II-III and  
40 VI of the *Foxg1*<sup>+/*Cre*</sup> cortex with respect to wt (Fig. 5B). By contrast, we observed a reduction of PV  
41 positive cell density in layers II-III of the visual cortex (Fig. 5C). The total number of inhibitory  
42 cells calculated using GAD67 immunohistochemistry was not affected in heterozygous *Foxg1*<sup>+/*Cre*</sup>  
43 mice (Fig. 5D).  
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51 *Dendritic spine density and length.* Alterations in density and size of dendritic spines have been  
52 found in *Mecp2* mouse models of Rett Syndrome (Belichenko et al., 2009, Landi et al., 2011) and  
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2 also in many other models of neurodevelopmental disorders (Fiala et al., 2002). To investigate  
3 morphological alterations induced by Foxg1 haploinsufficiency, we evaluated by diolistic labeling  
4 the spine density and length in the basal dendrites of pyramidal cells (layer 2/3) in visual cortices of  
5 *Foxg1<sup>+Cre</sup>* mice and wt littermates. As indicated in Fig. 6, no differences was present in spine  
6 length between the two genotypes (Fig. 6C) but a significant decrease in spine density was  
7 measured in *Foxg1<sup>+Cre</sup>* as compared to wt mice (Fig. 6B). No difference was present in cell body  
8 diameter (wt N= 25 cells 16.9  $\mu$ m SEM 2.3, *Foxg1<sup>+Cre</sup>* N=24 cells 15.8  $\mu$ m SEM 2.6; t-test  
9 p=0.76).

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11 These results indicate that the severe impairment of visual acuity caused by Foxg1  
12 haploinsufficiency is likely to be due to defects in the morphological organization of visual cortical  
13 neurons.  
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## 21 Discussion

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23 In this report we demonstrate for the first time the functional consequences of *Foxg1*  
24 haploinsufficiency in the visual system of *Foxg1<sup>+Cre</sup>* mice and a visual impairment in a cohort of  
25 Rett individuals presenting genetic alteration on *FOXG1*. Previous reports showed minor alterations  
26 in haploinsufficient animals consisting in a modestly thinner neocortex and reduced dentate gyrus  
27 size. To address this issue, we measured the visual acuity of *Foxg1<sup>+Cre</sup>* mice and their wild type  
28 littermates by means of Visually Evoked Potentials (VEPs). Our results show that *Foxg1<sup>+Cre</sup>* mice  
29 have a severe impairment in visual acuity and that the origin of such an impairment in visual  
30 function is not likely to reside in the retina, as retinal organization in these animals is normal.  
31 Indeed, retinal structure is qualitatively and quantitatively normal in *Foxg1<sup>+Cre</sup>* mice, and previous  
32 work showed that loss of a single *Foxg1* allele does not impair development of retinofugal axons  
33 (Tian et al., 2008). Consistently with the animal model, all the examined [subjects](#) with *FOXG1*  
34 haploinsufficiency also show a visual impairment, with some common peculiarities. Such  
35 impairment seems to be principally caused by abnormal elaboration of visual signals in cortical  
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## 48 Visual alterations in *FOXG1* mutated subjects

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51 A common visual behavior reported in these subjects by relatives is the apparent lack of interest for  
52 any visual stimulus, often interpreted as low vision or narrowed visual field. This visual behavior  
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3 was better characterized in our subjects who mainly showed a scarce eye engagement, inconstant  
4 recognition of familial faces, and a general limited awareness to visual stimuli anywhere in the  
5 visual field, particularly when far and crowded. Despite a limited visual capacity, they were able to  
6 avoid obstacles and catch objects whenever positioned in neighboring space. The loss of attention  
7 and awareness for visual stimuli associated with the inability to disambiguate crowded objects or  
8 complex shapes; the inability of immediate recognition of familial faces, but the preserved interest  
9 and correct reaching or grasping toward colored stimuli and moving objects might suggest a  
10 condition resembling “blindsight”. Blindsight syndrome is a well-recognized neuro-  
11 ophthalmological defect, which occurs upon extensive damage of the primary visual cortex (V1).  
12 Blindsight refers to the ability of cortically blind patients (bearing a primary visual cortex damage)  
13 to use extra striate visual information in guiding behaviors but in the absence of conscious object  
14 identification (Weiskrantz, 2009). Human blind-sight subjects are able to orient to and even answer  
15 questions about stimuli presented to the blind visual field. However, they are entirely unaware of  
16 the stimuli to which they are responding. Another characteristic of blindsight is that subjects can  
17 discriminate simple objects on the basis of their spatial frequency, shape, texture and color (Dineen  
18 and Keating, 1981), but they have lost the ability to identify more elaborate visual attributes, such  
19 as the capacity to visually recognize complex shapes or crowded objects, foods and familial faces.  
20 Moreover, blindsight patients well maintain reflexive saccades and motion perception,  
21 suggesting that a residual network elaborating some visual information remains active even in case  
22 of extensive striate cortex lesions. The latter supports visually guided behaviors in the absence of  
23 awareness for visual stimuli. At least two separate pathways, and namely the retino-collicular and  
24 retino-geniculate pathways, reach the extrastriate cortex independently from the primary visual  
25 cortex, projecting to the motion area V5/MT in the medial temporal lobe. These connections may  
26 represent the unconscious part of the dorsal ‘where’ visual stream, whereas the conscious counterpart  
27 passes through the primary visual cortex before reaching the V5/MT area. The extrastriate blind-  
28 sight pathways could be active in the *FOXG1* patients studied here, who indeed are attracted by  
29 colored and moving targets. On the contrary, their scarce awareness to visual stimuli might indicate  
30 a functional impairment of the striate visual pathway in the absence of retino-geniculate  
31 dysfunctions. This hypothesis (although not sustained by the demonstration of a structural damage  
32 of the occipital lobe detected by MRI) would suggest a functional impairment or incomplete  
33 maturation of the striate cortex as reinforced by the present results in the murine model.  
34 Another visual system change in the human subjects studied here is the occurrence of ocular  
35 malformations, such as strabismus, oculo-mandibular syncinesias and small optic discs. These  
36 anomalies (observed in three individuals), together with other cerebral malformations (i.e. corpus  
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3 callosum hypoplasia) may represent the expression of a global immaturity of the brain due to  
4 *FOXG1* mutations.  
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### 7 **Cortical architecture is defective in *Foxg1*<sup>+Cre</sup> mice**

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10 Retinal structure appeared normal in *Foxg1*<sup>+Cre</sup> mice, with no apparent anomalies in cell type  
11 distribution, laminar organization and numerical deficit in GCs that could explain the visual  
12 abnormalities. On the contrary, morphological studies showed significant changes in NeuN, PV  
13 and CR positive cells distribution in the visual cortex of *Foxg1*<sup>+Cre</sup> mice compared to wild type  
14 animals. PV and CR are calcium binding proteins that serve as calcium buffers and label main  
15 subpopulations of GABA-ergic cortical interneurons. PV immunoreactivity develops postnatally: in  
16 mice, it appears around eye opening at P13-P14 in intermediate layers, from which it expands to the  
17 upper and inner cortical layers at subsequent developmental stages. In the visual cortex, PV positive  
18 cell maturation occurs during the critical period of ocular dominance plasticity and manipulations  
19 that accelerate or delay the time course of the critical period correspondingly regulate  
20 developmental maturation of PV cells. The decrease in PV cell density in uppermost layers and the  
21 increase in CR positive cells of *Foxg1*<sup>+Cre</sup> cortex suggest that Foxg1 haploinsufficiency results in an  
22 impairment in inhibitory circuitry maturation. PV+ synapses are usually perisomatic and PV+  
23 pattern of innervation of pyramidal cells is engaged in control of the spiking pattern. Their  
24 reciprocal and extensive connections can regulate the timing of activity in the cortex. Hence, we can  
25 speculate that the epileptic phenotype observed in *FOXG1* mutated patients (Brunetti-Pierri et al.,  
26 2011, Guerrini and Parrini, 2012) may be linked to a defective PV positive cells maturation.  
27 Conversely, CR positive cells usually synapse on dendrites; thus, the observed increase in CR  
28 positive cells in the lower layers of the *Foxg1*<sup>+Cre</sup> cortex may cause a local alteration in cortical  
29 inhibition. Intriguingly, analysis of GAD67 positive neurons showed that the total number of  
30 inhibitory cells is preserved in *Foxg1*<sup>+Cre</sup> mice. Thus, the reduction of NeuN positive neurons in the  
31 superficial layers is likely due to alterations of excitatory cells.  
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44 To further analyze the excitatory phenotype in *Foxg1*<sup>+Cre</sup> primary visual cortex, we evaluated  
45 dendritic spine density and length in layer 2/3 pyramidal neurons. In the cerebral cortex, more than  
46 90% of excitatory synapses terminate on spines. Spine density in the mouse neocortex increases  
47 during the second and third week of life and is followed by a period of major spine pruning and  
48 loss. In the primary visual cortex, dendritic spine density and morphology are developmentally  
49 regulated: they are plastic during young ages and become remarkably stable in adulthood. Our  
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3 results demonstrate a decrease in spine density in *Foxg1*<sup>+Cre</sup> primary visual cortex compared to wild  
4 type animals.

5 Altogether, these results suggest that *Foxg1* haploinsufficiency causes alterations in both inhibitory  
6 and excitatory cells. Interestingly, alterations in inhibitory and excitatory synapses have been  
7 described in other mouse models of RTT as well as in a human disease model (iPSCs) obtained by  
8 genetic reprogramming of *FOXG1*-mutated patient fibroblasts (Patriarchi et al., 2015). In *MeCP2*-  
9 *KO* animals, the cortical excitatory input is reduced whilst the total inhibitory input is enhanced,  
10 leading to a shift of the homeostatic balance between excitation and inhibition in favor of inhibition.  
11 Moreover, *MeCP2*-deficient cortical and hippocampal neurons have fewer dendritic spines.  
12 *CDKL5*, the other gene found mutated in some cases of RTT, codes for a protein that has been  
13 demonstrated to localize at excitatory synapses, where it contributes to correct dendritic spine  
14 structure and synapse activity. *CDKL5* knockdown in cultured hippocampal neurons results in  
15 aberrant spine morphology (Chen et al., 2010) and unstable dendritic spines (Della Sala et al.,  
16 2015). Intriguingly, the analysis of novel mouse models carrying *Cdkl5* deletion showed alterations  
17 in sensory evoked potentials both in the auditory and the visual cortices (Wang et al., 2012,  
18 Amendola et al., 2014).

19 In conclusion, *Foxg1* haploinsufficiency causes a severe impairment in visual function that is likely  
20 due to altered cortical mechanisms. This should be taken into account when *Foxg1*<sup>+Cre</sup> mice are  
21 used in behavioral experiments and when visual function is evaluated in patients carrying *FOXG1*  
22 mutations.  
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30 Vision Research Foundation.  
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# The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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3 **Legend to Figures**  
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5  
6 **Figure 1. Visual acuity in *Foxg1*<sup>+Cre</sup> mice and wild-type littermates**

7 (A) Examples of VEP waveforms recorded in response to 0.1 c/deg gratings. (B) VEP amplitude  
8 changes in response to gratings of high contrast and of decreasing bar size (increasing spatial  
9 frequency) in a wild-type (WT) and a *Foxg1*<sup>+Cre</sup> mouse. VEP amplitude decreases by progressively  
10 increasing the spatial frequency. Visual acuity was determined by linearly extrapolating VEP  
11 amplitude to 0 V. Symbols represent data average data from all animals (WT n=9; *Foxg1*<sup>+Cre</sup> n=5).  
12 Arrows in the abscissa point to visual acuity value. Dotted line represents average noise level. (C)  
13 Spatial resolution in the visual cortex of wild-type and *Foxg1*<sup>+Cre</sup> mice. Visual acuity is significantly  
14 reduced in *Foxg1*<sup>+Cre</sup> mice compared with wild-type littermates (*Foxg1*<sup>+Cre</sup>, 0.20 cycle/degree SEM  
15 0.05, n=9; WT, 0.51 cycle/degree, SEM 0.05 n=5; \*, P,0.01, Student's *t* test).  
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22 **Figure 2. Retinal morphology in *Foxg1*<sup>+Cre</sup> animals and wild-type littermates**

23 Retinal morphology in *Foxg1*<sup>+Cre</sup> (A and C) and wt (B and D) mice. A, B: Low magnification  
24 images of retinal sections stained with rhodopsin antibodies (green signal), showing normal retinal  
25 laminations and bright staining of photoreceptor outer segments (OS) in both mutant and wt mice.  
26 Red: nuclear counterstaining. In A the normal morphology of the optic nerve head (ONH) can be  
27 appreciated. The insets show at 2x details of photoreceptor rows and outer segments. Here and in  
28 the following images: ONL, IPL outer nuclear and inner plexiform layer, respectively. C and D:  
29 examples of retinal ganglion cells (GC) stained by the type specific transcription factor Brn3-B  
30 (red), with nuclear localization, and by neurofilament antibodies (green), depicting their  
31 morphology in detail.  
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39 **Figure 3. Morphology of neuronal subtypes in *Foxg1*<sup>+Cre</sup> mice and wild-type littermates**

40 Morphology of retinal neuronal subtypes in *Foxg1*<sup>+Cre</sup> (left panels) and wt mice  
41 Retinal neurons in *Foxg1*<sup>+Cre</sup> (left panels) and wt mice. A, B: rod bipolar cells have been labelled  
42 red by PKC antibodies. Their dendrites form dense plexa in the OPL while axonal endings (rbe)  
43 cluster in regularly in the deepest part of the IPL. Green puncta are synaptic ribbons from  
44 glutamatergic processes, regularly arranged in the two plexiform layers. Blue:  
45 nuclearcounterstaining. C,D: types of cone bipolar cells stained by ZnP-1 (synaptotagmin  
46 antibodies). Both dendritic tips in the OPL and axonal arbors in the IPL have the expected fine  
47 structure. The IPL show three typical bands, revealed by calretinin staining of amacrine cells (red).  
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49 E, F: Cholinergic amacrine cells, stained red by ChAT antibodies, are arranged in two mirror-  
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3 symmetric populations at the two margins of the IPL. Alpha-like ganglion cells, labelled by SMI-32  
4 antibodies (green) have well preserved morphologies in both mutant and wt retinas.  
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7 **Fig.4. Neurons in the GCL of *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+/-Cre</sup> mice**

8 A,B. Cells in the GCL have been labelled by antibodies against the RNA binding protein RBPMS  
9 that produce a cytoplasmic staining in GCs selectively (green signal). Ethidium counterstaining (red  
10 signal) show the nuclei of GCs and displaced amacrine cells (AC).The general morphology (A and  
11 B) and the total number of cells in the GC layer (C) are virtually identical in *Foxg1*<sup>+/+</sup> (A) and  
12 *Foxg1*<sup>+/-Cre</sup> (B) mice. Quantification in C. The number of GCs proper (D) is also not different  
13 between the two strains (D).  
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19 **Figure 5. Immunohistochemistry on visual cortex of *Foxg1*<sup>+/+</sup> and *Foxg1*<sup>+/-Cre</sup> mice**

20 Panel A. A significant reduction of NeuN staining is present in layer II-III and IV of the visual  
21 cortex of *Foxg1*<sup>+/-Cre</sup> mice (WT: n=4; *Foxg1*<sup>+/-Cre</sup> n=4; two-ways RM ANOVA genotype X cortical  
22 layer p=0.003; post-hoc Sidak wt vs. *Foxg1*<sup>+/-Cre</sup> within layer II-III p=0.004, within layer IV  
23 p=0.006).  
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26 Panel B. Calretinin positive cells in visual cortices from WT and *Foxg1*<sup>+/-Cre</sup> mice. Quantification of  
27 calretinin positive cells density in visual cortical layers of WT and *Foxg1*<sup>+/-Cre</sup> mice shows a  
28 significant increase of calretinin positive cells in layer II-III and VI of the *Foxg1*<sup>+/-Cre</sup> cortex (two-  
29 ways RM ANOVA genotype X cortical layer p=0.04; post-hoc Sidak wt vs. *Foxg1*<sup>+/-Cre</sup> within layer  
30 II-III p<0.01, within layer VI p<0.05).  
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33 Panel C Parvalbumin positive cells in visual cortices from WT and *Foxg1*<sup>+/-Cre</sup> mice. Quantification  
34 of parvalbumin positive cells density in visual cortical layers of WT and *Foxg1*<sup>+/-Cre</sup> mice shows a  
35 significant decrease of parvalbumin positive cells in layer II-III of the *Foxg1*<sup>+/-Cre</sup> cortex (two-ways  
36 RM ANOVA genotype X cortical layer p=0.03; post-hoc Sidak wt vs. *Foxg1*<sup>+/-Cre</sup> within layer II-III  
37 p<0.01).  
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40 Panel D GAD67 positive cells in visual cortices from WT and *Foxg1*<sup>+/-Cre</sup> mice. Quantification of  
41 GAD67 positive cells density in visual cortical layers of WT and *Foxg1*<sup>+/-Cre</sup> mice shows that the  
42 density of GAD67 positive cells is not statistically different between wt and *Foxg1*<sup>+/-Cre</sup> mice (two-  
43 ways RM ANOVA effect of genotype p=0.44, genotype X cortical layer p=0.33)..\*: p<0.05; \*\*:  
44 p<0.01.  
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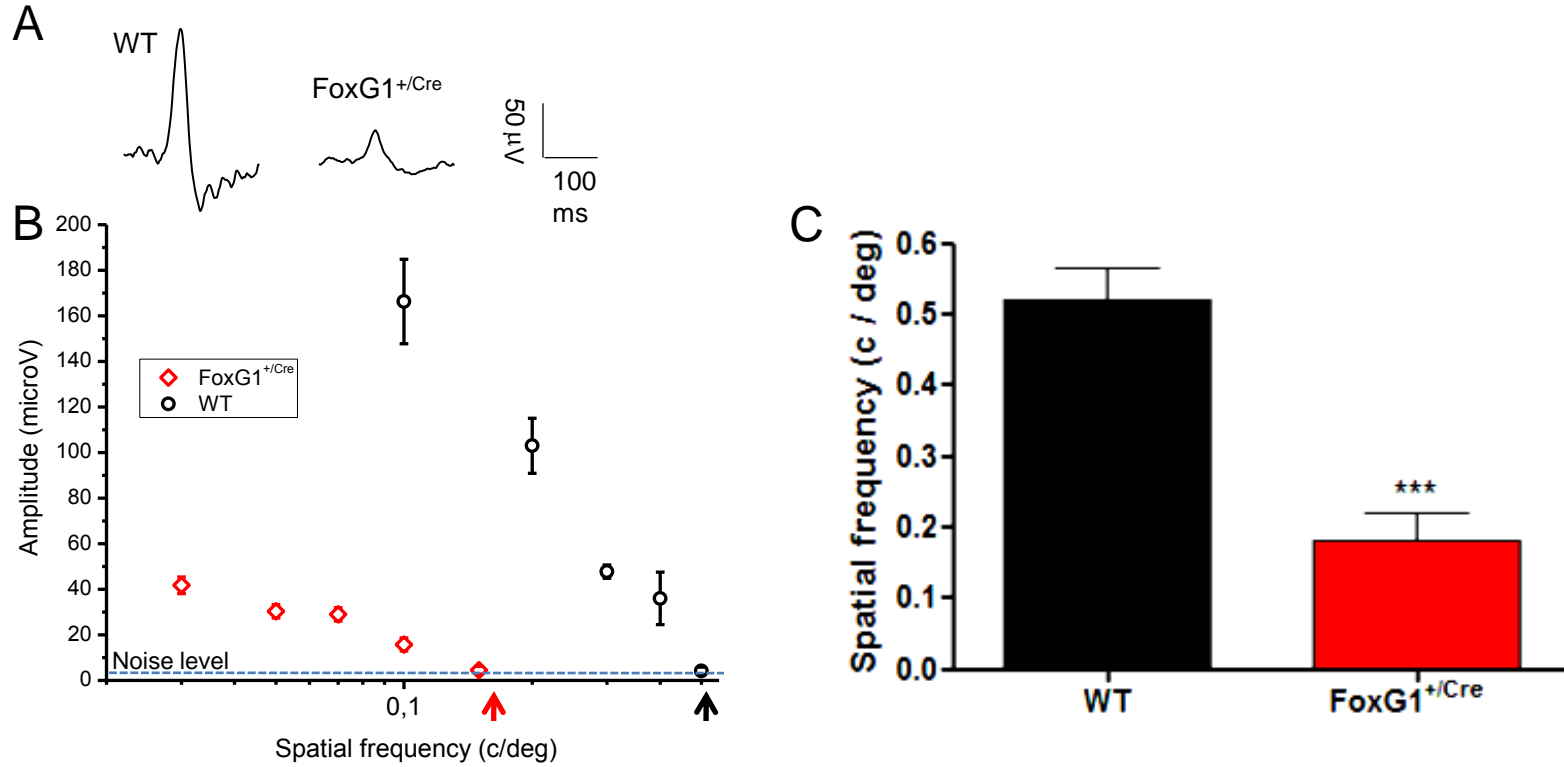
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51 **Figure 6. Dendritic spine length and density in *Foxg1*<sup>+/-Cre</sup> animals and wild-type littermates**

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A) Confocal microscope images of WT and *Foxg1<sup>+Cre</sup>* dendritic segment. Scale bar= 5µm. B and C) Average dendritic spine length and density (respectively) in basal dendrites of layer 4/5 pyramidal cells of *Foxg1<sup>+Cre</sup>* and WT mice. A significant decrease in spine density was measured in *Foxg1<sup>+Cre</sup>* as compared to wt mice (WT: n= 7; *Foxg1<sup>+Cre</sup>*: n=7; p=0.045, Student's *t* test).



**Figure 1**  
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**Fig. 1**

Figure 2  
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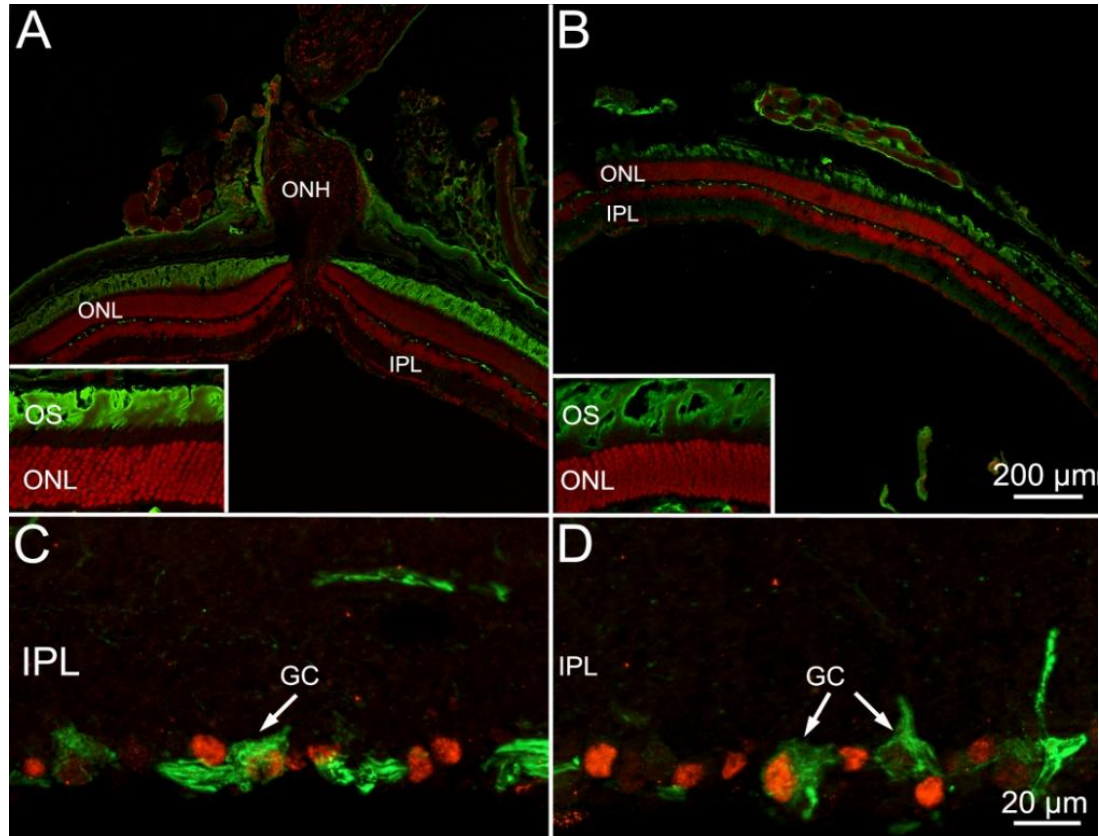
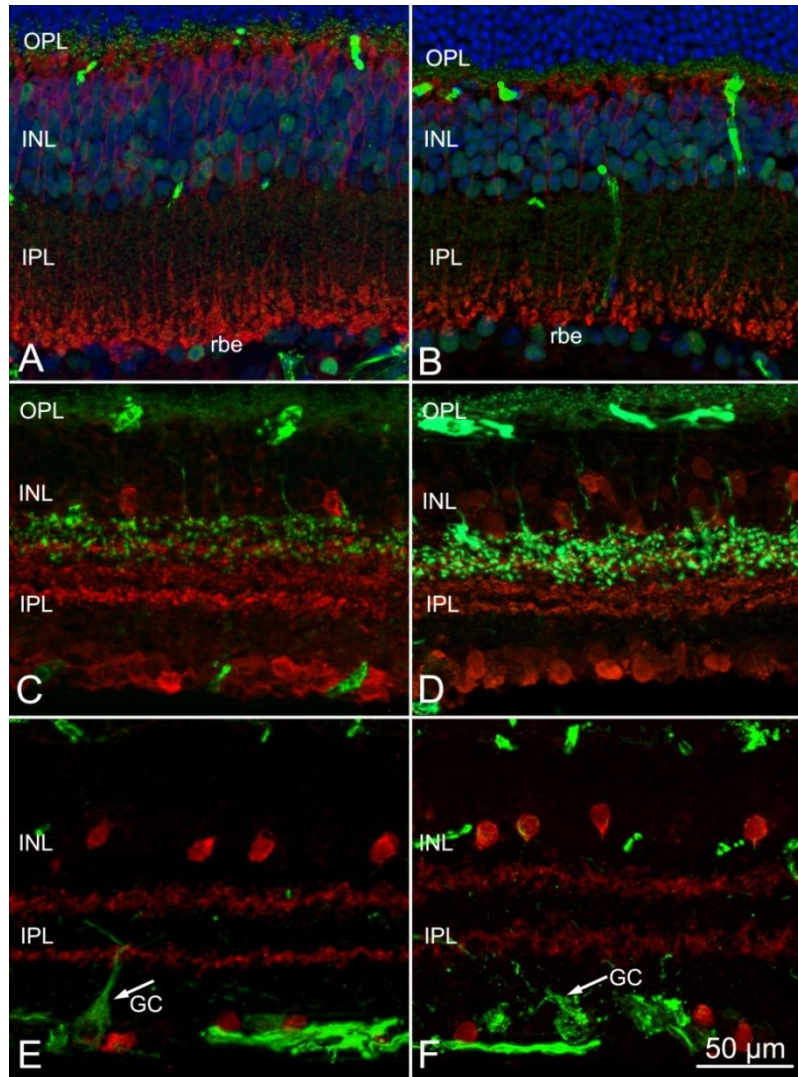


Fig. 2

**Figure 3**  
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**Fig.3**

Figure 4  
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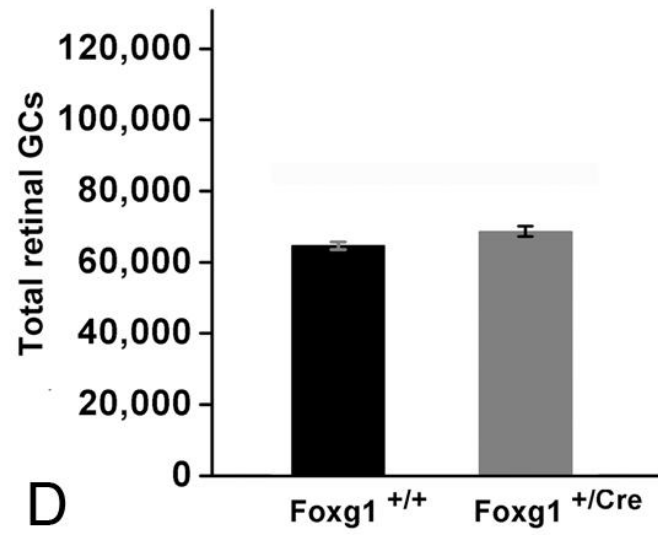
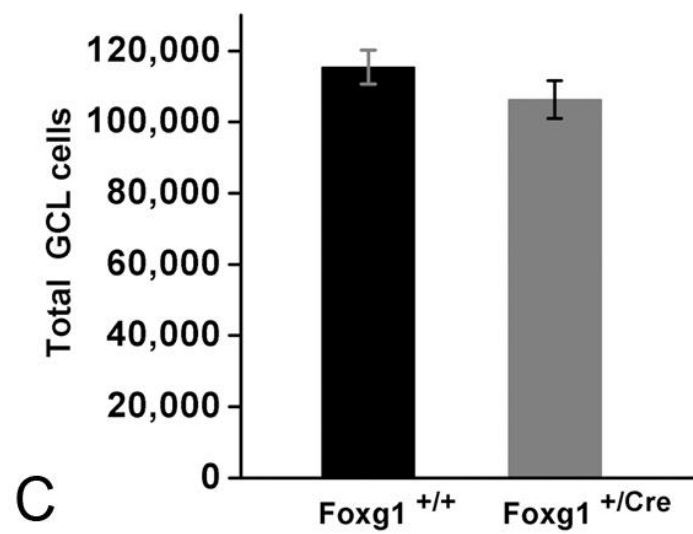
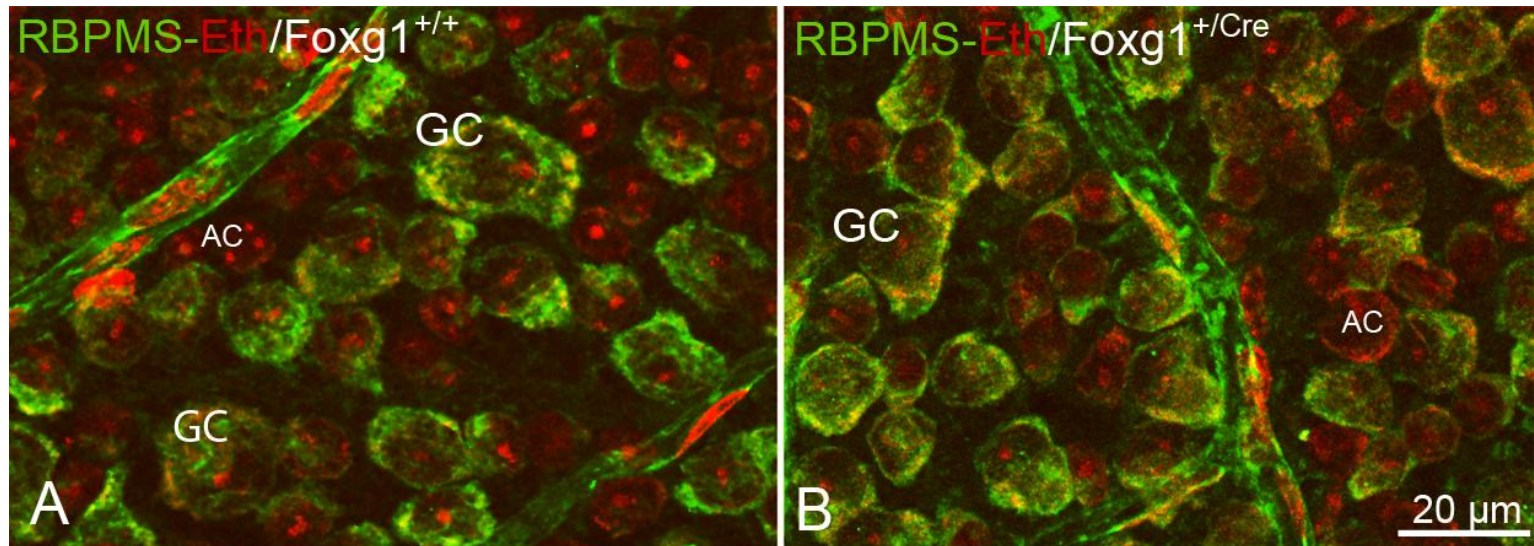
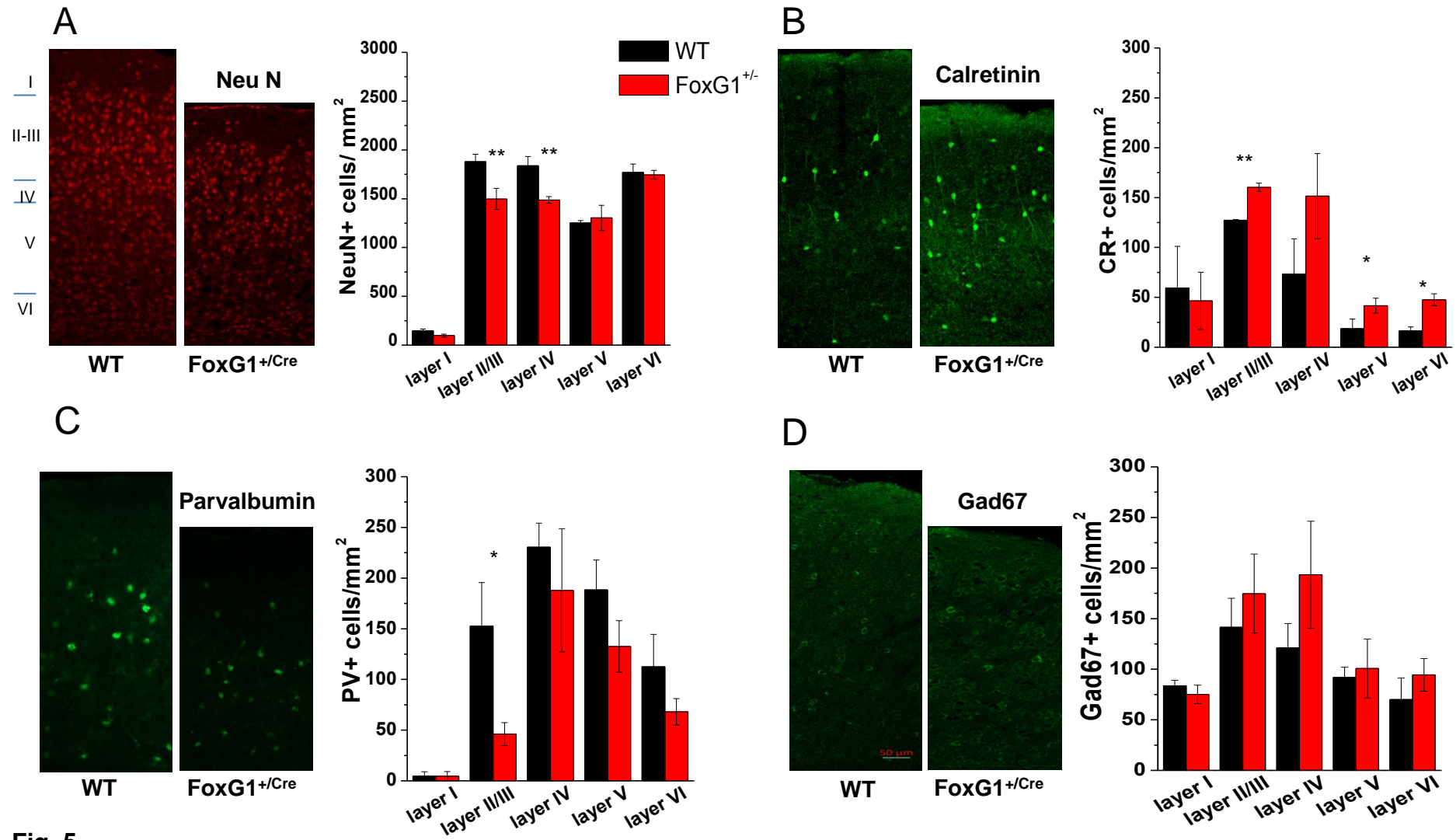
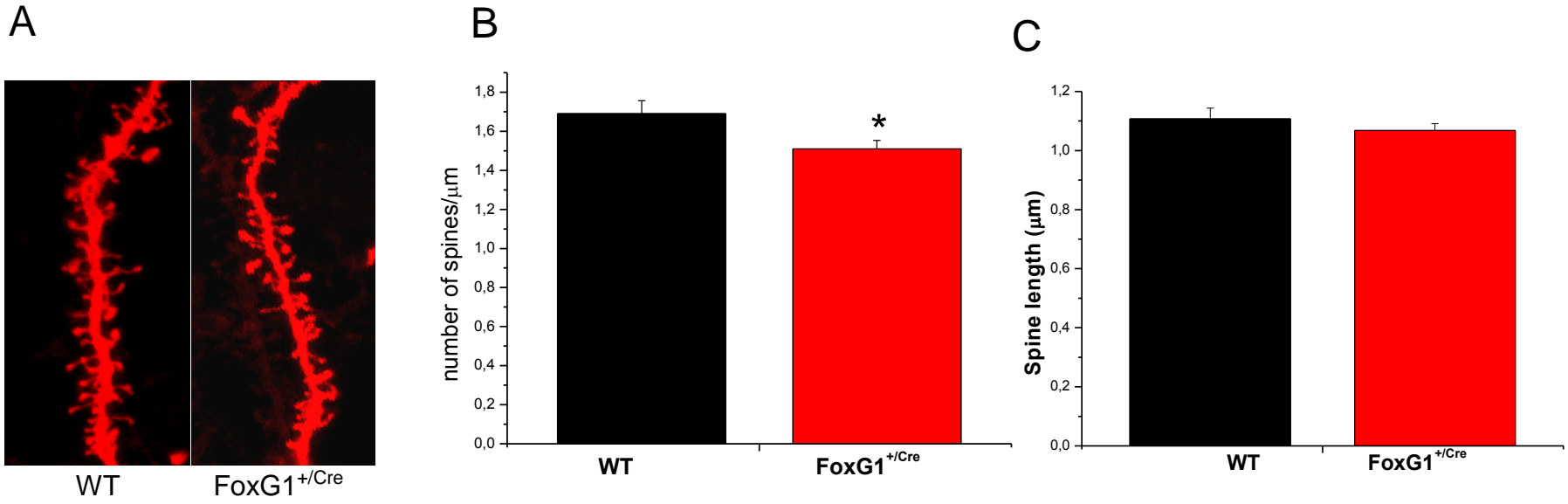


Fig. 4

**Figure 5**  
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**Fig. 5**



**Fig.6**

Table 1

Table 1. summary of patients features and clinical results

Patient	Sample ID	Sex	Age	FOXG1 mutation	Attention and awareness to visual stimuli	Deficit to distinguish far stationary crowded objects	Familiar faces recognition	Diss. between looking and reaching	Absent or reduced visual contact	Colored visual stimuli awareness	Moving target vision	Fixation and engagement	Eye movement and alignment	Photophobia or compulsive gazing to the light	Funduscopy	VEP flash	EEG	MRI
1	/	F	9m	6.73 Mb Del chr 14: 26,633,912-33,360,894	No	yes	Inconstant	yes	yes	NE	yes	scarce	Exotropia	No	small optic disks OD>OS	NE	NE	Corpus Callosum agenesis
2	/	M	1y 8m	c.762C>Gp. Tyr254*	No	yes	No	yes	yes	yes	Inconstant	scarce	Exotropia Upbeating nystagmus	Yes	Small normal appearing optic nerves	NE	Occipital-Parietal activity	NE
3	#934/2013	F	2y 7m	6.37 Mb del chr 14: 26,908,842-33,280,997	No	yes	Inconstant	yes	yes	yes	yes	poor for distance sufficient for near	Esotropia, oculomandibular syncinesia	Yes	Normal	NE	Normal	Corpus Callosum agenesis, colpocephaly, subversion of hippocampus
4	#2362	F	3y 5m	1.1 Mb Del chr 14: 27,622,465-28,725,069	No	yes	No	yes	yes	yes	yes	scarce for distance better for near	Exotropia Ocular apraxia	No	Normal	NE	NE	Corpus Callosum agenesis
5	/	M	5 y	c.651C>Gp. Y217X	No	yes	No	yes	yes	Yes	yes	poor	strabismus surgically treated	preference for bright light	small optic disk in OD	NE	NE	NE
6	#611/2013	F	1y 5m	c.460dupG, p.Glu154Glyfs*300	No	yes	Inconstant	yes	yes	Yes	yes	scarce	Exotropia	Yes	Normal	L: 102ms A: 9.7 $\mu$ V	Normal	Corpus Callosum dysgenesis, mild symplified gyral pattern
7	#1009/2013	M	2y 5m	1.8 Mb Del chr 14: 28,339,878-30,108,071	No	yes	No	yes	yes	Yes	yes	good	Esotropia	Yes	Normal	L: 104 ms A: 7.7 $\mu$ V	Generalized epilepsy	Normal
8 <sup>^</sup>	#156	F	22y	c.756G>Ap. W255X	No	yes	Inconstant	yes	yes	NE	yes	scarce	Exotropia	No	Normal	L: 100 ms A: 10 $\mu$ V	Slowing in Occipital and Temporal regions	Corpus Callosum hypoplasia, hypoplastic hemispheres

NE: not evaluated

<sup>^</sup>This case corresponds to Patient 1 in Ariani et al (1)

L: Latency of the first positive inflection peak, normally close to 100 ms after stimulus onset (range between 90-105 ms in normal controls)

A: Amplitude of the main positive peak (N75-P100) after the stimulus onset, ranged between 5.3 to 10  $\mu$ V in normal controls