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Letter to the Editor

A homozygous *SIX6* mutation is associated with optic disc anomalies and macular atrophy and reduces retinal ganglion cell differentiation

To the Editor:

We report on a consanguineous family with three children who had optic disc anomalies and macular atrophy without microphthalmia or cataracts (Table 1 and Fig. 1a,b). Clinical examinations were otherwise unremarkable. Levels of thyroid hormones, gonadotropins, testosterone, estradiol, liver enzymes, kidney functions, glucose and electrolytes, CBC, and urinalysis were within normal limits. Physical and ophthalmological examinations were normal in all unaffected siblings and in parents.

Genotyping eight family members via Affymetrix 5.0 SNP-chips showed an autozygous region at chr14: 59247009-75949378 (Hg19) (Fig. 1b). Two genes in this interval, *CHX10* (MIM142993) and *SIX6* (MIM606326), were previously reported to cause microphthalmia or anophthalmia in humans (1). Sanger sequencing of both genes showed that all three affected individuals were homozygous for a novel variant, c.110T>C (p.L37P) (NM_007374.2, NP_031400.2), in *SIX6* (Fig. 1c). This variant co-segregated with the phenotype in the entire family as a recessive trait and was negative in 342 ethnicity-matched controls. It is also absent from the Exome Variant Server and dbSNP137. Leucine at position 37 is completely conserved in different species (Fig. S1, Supporting Information). None of the loci containing a gene known to cause recessive macular disease, including *PRPH2* (MIM179605), *ABCA4* (MIM601691), and *CNGB3* (MIM605080) co-segregated with the phenotype. Two other autozygous regions >1 MB did not contain a gene known to cause an eye disease (Table S1, Supporting Information).

We built and compared structural models of $SIX6^{WT}$ and $SIX6^{L37P}$, using the crystal structure of SIX1 as a template (PDBID: 4EGC). SIX6 belongs to the SIX family of transcription factors with the TA-DB modular architecture, where TA is the N-terminal transactivation domain and DB is the C-terminal DNA-binding homeodomain (2). The transactivation of SIX family proteins depends upon their TA domain binding other nuclear proteins such as EYA2 and DACH2 (3). Our models suggest that the p.L37P mutation is located within the N-terminal α-hairpin, comprised the first two α-helices of the TA domain of SIX6 (Fig. 1d). In $SIX6^{WT}$, this α-hairpin is maintained with a van der Waals contact between V14 and L37 at its open end. Introduction of the p.L37P mutation would lead to unfolding of the N-terminal α-helix within this α-hairpin by about one turn due to the inability of proline to participate in intramolecular backbone hydrogen bonding (Fig. 1d). This together with the limited conformational flexibility of proline would result in the loss of an intramolecular van der Waals contact with V14, thereby compromising the integrity of the α-hairpin that would potentially drive the assembly of SIX6 with its ligands.

We further investigated for potential molecular and cellular mechanisms through which the p.L37P mutation could induce ocular phenotypes, using embryonic day 14 (E14) mouse retinal progenitor cells (RPCs) as a model. We first asked whether the p.L37P mutation affects nuclear localization by expressing $SIX6^{WT}$ and $SIX6^{L37P}$ fused to GFP tags. Both the normal and mutant SIX6 localized in the nucleus (Fig. 1e), suggesting that the mutation does not disrupt protein localization. The comparable levels of GFP-fusion protein expression and transduced cells across conditions also suggested that the $SIX6^{L37P}$ mutation did not greatly destabilize the protein or lead to significant cell toxicity.

We next hypothesized that the mutation may affect retinal ganglion cell (RGC) differentiation during early development. E14 mouse RPCs were cultured and transduced with GFP, $SIX6^{WT}$ and $SIX6^{L37P}$ (this time with bicistronic fluorescent protein markers rather than fusion proteins), allowed to differentiate over 5 days in culture, and then immunostained for the RGC marker Brn3, GFP (to mark and quantify transduced cells), and EdU (to mark and quantify cells derived from progenitors that underwent at least one cell division *in vitro*) (Fig. S2). $SIX6^{WT}$ was not sufficient to further promote the differentiation of RGCs (Fig.1f), consistent with the expression of SIX6 in embryonic RPCs (4). However, the $SIX6^{L37P}$ mutant showed a reduction in the number of differentiated RGCs (Fig. 1f). While this effect could reflect differences between the human and mouse cells, relative levels of gene expression between expressed and endogenous alleles, or contextual milieus,

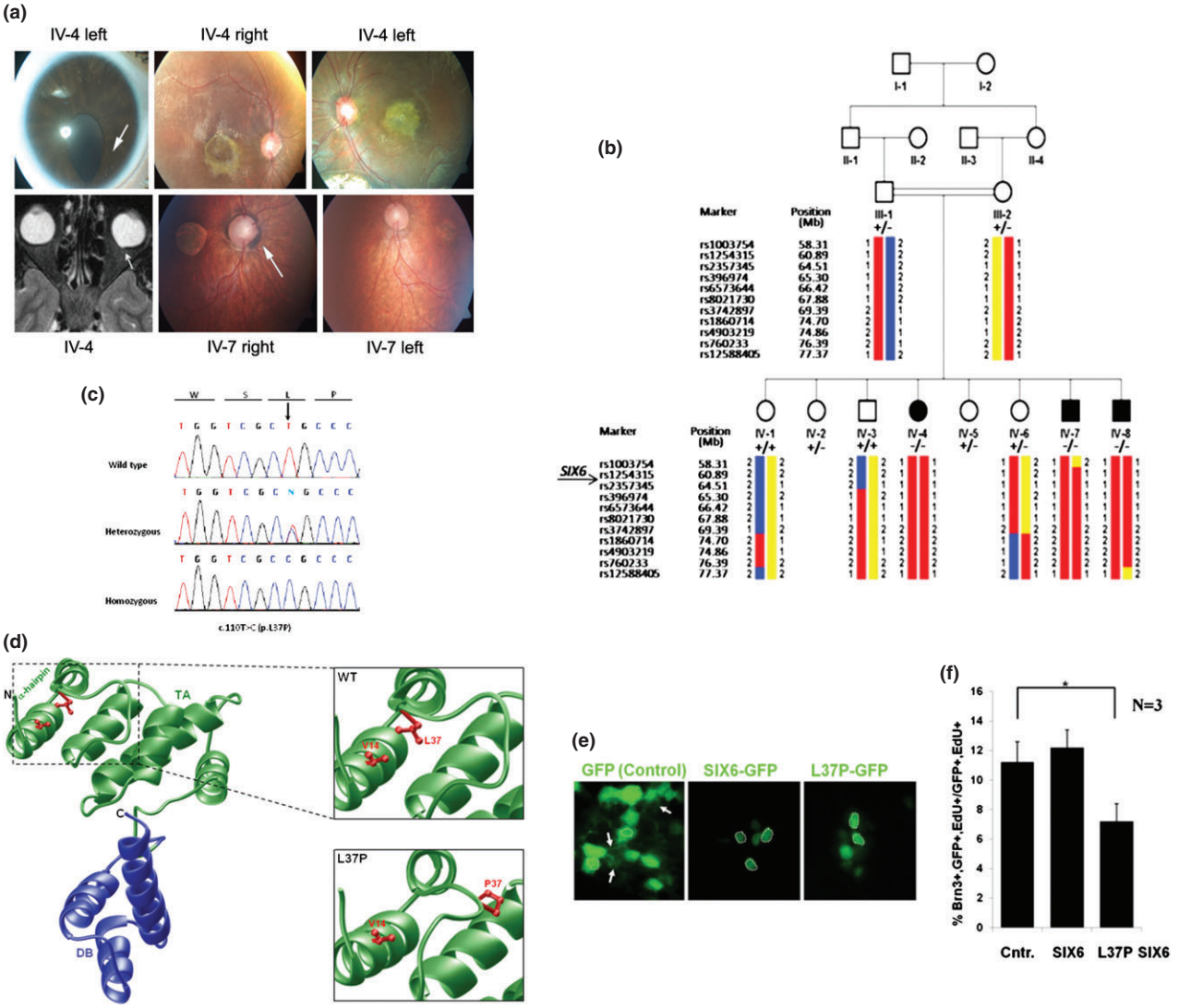

Fig. 1. Clinical, structural and functional effects of the SIX6 p.L37P mutation. (**a**) Iris coloboma (arrow) in the left eye of individual IV-4 (upper left image); colobomatous optic disc anomaly (excavated disc), straight retinal vessels arising from the periphery of the optic disc, mild peri-papillary chorioretinal atrophy, and irregularly bordered, oval-shaped, pigmented macular atrophy in both eyes and chorioretinal coloboma in the inferior region of the left eye of individual IV-4 (upper middle and right images); in an axial T2-weighted MR image, coloboma shown as a focal, posterior outpouching defect of the vitreous centered at the optic nerve head (arrow) in individual IV-4 (lower left image); excavated optic disc together with peripheral, raised retinal vessels and pigmentary chorioretinal (arrow) changes surrounding the optic disc in both eyes of individual IV-7; oval-shaped, sharp-bordered and pigmentary macular atrophy in the right eye of individual IV-7 (lower middle and right images). (**b**) Pedigree and haplotypes that co-segregate with the phenotype. +/+, wildtype; +/−, heterozygous; −/−, homozygous for p.L37P. (**c**) Sanger sequencing shows c.110T>C (p.L37P) mutation in *SIX6*. (**d**) The TA and DB domains of SIX6 are respectively colored green and blue. The sidechain moieties of L37/P37 residues and their putative counterpart V14 located within the α-hairpin are colored red. The expanded windows reveal a close-up view of the potential effect of p.L37P mutation on the structure of the TA domain. (**e**) p.L37P mutation does not affect SIX6 nuclear localization. Mouse E14 RPCs were transduced with GFP, SIX6-GFP or $SIX6^{L37P}$-GFP fusion proteins, as labeled, and counterstained with DAPI to mark nuclei (example nuclear outlines in dotted white lines). In GFP-transfected cells, the GFP is localized throughout the nuclei, cytoplasm and even into cytoplasmic cellular extensions (arrows). GFP was nuclear localized after both SIX6-GFP or $SIX6^{L37P}$-GFP fusion protein expression. F) $SIX6^{L37P}$ overexpression reduces the number of differentiated RGCs (Brn3+,Edu+) compared with $SIX6^{WT}$ overexpression. Asterisk indicates $p < 0.05$.

it is possible that the mutation located in the TA domain of SIX6 interferes with the functions of its binding partners.

Six6 has been shown to regulate early progenitor cell proliferation during mammalian retinogenesis and pituitary development (5, 6). The *Six6* knock-out mouse shows optic nerve anomalies and pituitary dysfunction, decreased fertility, and aberrant development of the suprachiasmatic nucleus (5, 6). Two *SIX6* mutations associated with microphthalmia have been reported (7, 8). Here, we report a homozygous missense mutation associated with a unique eye phenotype characterized by optic disc anomalies, macular atrophy, and coloboma of the iris and chorioretina. These data point to an involvement of SIX6 specifically in RGC and more broadly in eye development.

Table 1. Clinical Findings of the Studied Family

Family members		IV:4	IV:7	IV:8
Current age (years)		14	5	2
Bone age (years)		13.6	4.6	2
Height (SD)		154.1 cm (–0.74)	104 cm (–1.05)	87.5 cm (0.53)
Weight (SD)		45.7 kg (–1.08)	16.6 kg (–0.56)	12 kg (–0.14)
Puberty stage (Tanner)		P5	P1	P1
Menstrual periods		Regular; menarche at 12 years	NA	NA
Presentation		Poor visual acuity and horizontal jerk nystagmus bilaterally since birth	Poor visual acuity and horizontal nystagmus bilaterally since birth	Horizontal nystagmus bilaterally since birth
Eye globe dimensions (anteroposterior × transverse)		28 mm × 26 mm	25 mm × 24 mm	23 mm × 22 mm
Right eye	Visual acuity	20/200	20/400	Pursuing light and objects
	Anterior segment	Normal	Normal	Normal
	Fundus	Colobomatous optic disc anomaly, retinal vessels arising from the peripheral optic disc, peripapillary chorioretinal changes, and pigmented macular atrophy	Colobomatous optic disc anomaly, retinal vessels arising from the peripheral disc, pigmented macular atrophy, peripapillary chorioretinal atrophy and pigmentary changes	Colobomatous optic disc anomaly, retinal vessels arising from the peripheral disc and macular atrophy
Left eye	Visual acuity	20/400	20/400	Pursuing light and objects
	Anterior segment	Iris coloboma	Normal	Normal
	Fundus	Colobomatous optic disc anomaly, retinal vessels arising from the peripheral optic disc, peripapillary chorioretinal changes, and pigmented macular atrophy, chorioretinal coloboma	Colobomatous optic disc anomaly, retinal vessels arising from the peripheral disc, and pigmented macular atrophy	Colobomatous optic disc anomaly, retinal vessels arising from the peripheral disc and macular atrophy
Brain/Hypotalamic-Pituitary axis MRI		Normal	Normal	Normal
Orbital MRI		Optic disc colobomas bilaterally	Normal	Normal
Kidney, pelvic, and thyroid ultrasound		Normal	NA	NA

Supporting Information

The following Supporting information is available for this article:

Appendix S1. Supplemental materials and methods.

Additional Supporting information may be found in the online version of this article.

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