The American Journal of Human Genetics, Volume *93*

Supplemental Data

Recessive Mutations in *SLC38A8* Cause Foveal Hypoplasia and Optic Nerve Misrouting without Albinism

James A. Poulter, Musallam Al-Araimi, Ivan Conte, Maria M. van Genderen, Eamonn Sheridan, Ian M. Carr, David A. Parry, Mike Shires, Sabrina Carrella, John Bradbury, Kamron Khan, Phillis Lakeman, Panagiotis I. Sergouniotis, Andrew R. Webster, Anthony T. Moore, Bishwanath Pal, Moin D. Mohamed, Anandula Venkataramana, Vedam Ramprasad, Rohit Shetty, Murugan Saktivel, Govindasamy Kumaramanickavel, Alex Tan, David A. Mackey, Alex W. Hewitt, Sandro Banfi, Manir Ali, Chris F. Inglehearn, and Carmel Toomes

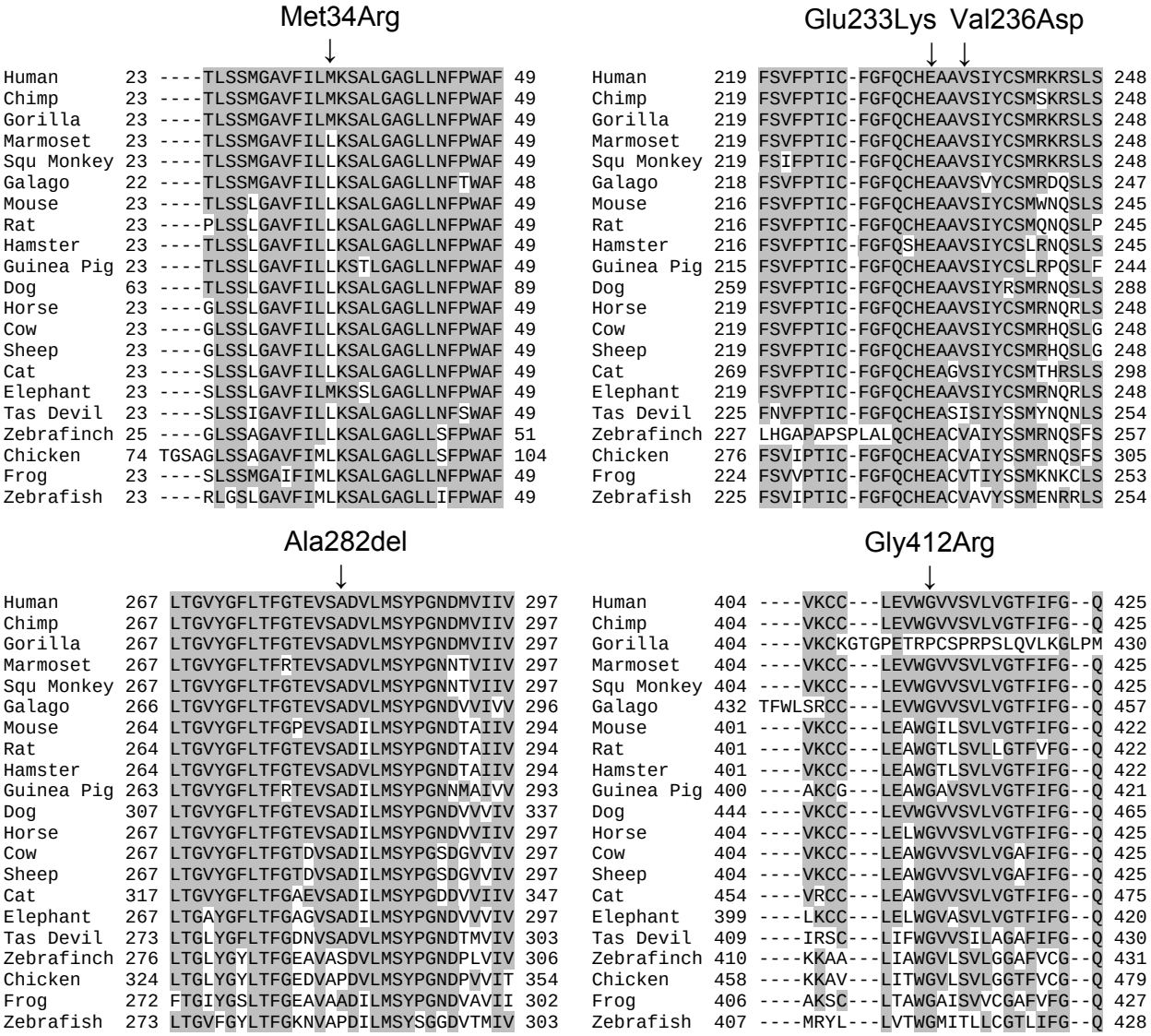


Figure S1. Protein sequence alignment of human SLC38A8 with all orthologues. Multiple protein alignments were calculated using ClustalW (http://www.ebi.ac.uk/clustalw2/) [Larkin, M.A. et al. (2007). Bioinformatics 23, 2947-8]. Thirty amino acid residues surrounding each mutation are shown. Conserved amino acid residues are shaded. The positions of the missense mutations p.Met34Arg, p.Glu233Lys, p.Val236Asp and p.Gly412Arg are indicated along with the single deleted amino acid residue p.Ala282. Protein sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/protein). Accession numbers: Human NP_001073911.1; Cow NP_001179423.1; Mouse NP_001009950.1; Hamster XP_003495171.1; Elephant XP_003418131.1; Marmoset XP_002761268.1; Frog NP_001037900.1; Tasmanian Devil XP_003758500.1; Galago XP_003791463.1; Chimpanzee XP_003315260.1; Squirrel Monkey dbsXP_003922850.1; Rat NP_001178938.1; Horse XP_003364689.1; Dog XP_546805.3; Gorilla XP_004058132.1; Cat XP_003998360.1; Chicken XP_003641956.1; Zebrafinch XP_004175047.1; Sheep XP_004014985.1; Zebrafish NP_001138287.1; Guinea Pig XP_003462017.1.

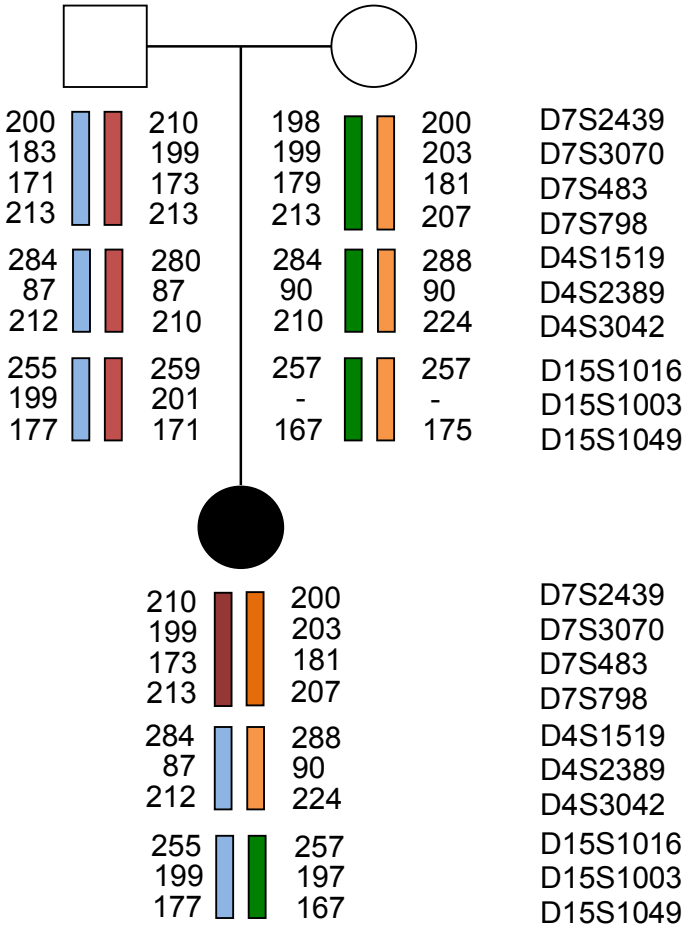


Figure S2. Paternity testing in family F3. Results are shown for 10 microsatellites markers, from chromosomes 7, 4 and 15, used to genotype DNA from individuals I:3, I:4 and II:4 from family F3 (Figure 2). Haplotypes are represented by coloured bars (each colour representing a different haplotype). The numbers alongside each haplotype bar correspond to the size of each allele amplified by the microsatellite markers, in base pairs. Mendelian inheritance is observed for all markers indicating no sample mix up or non-paternity.

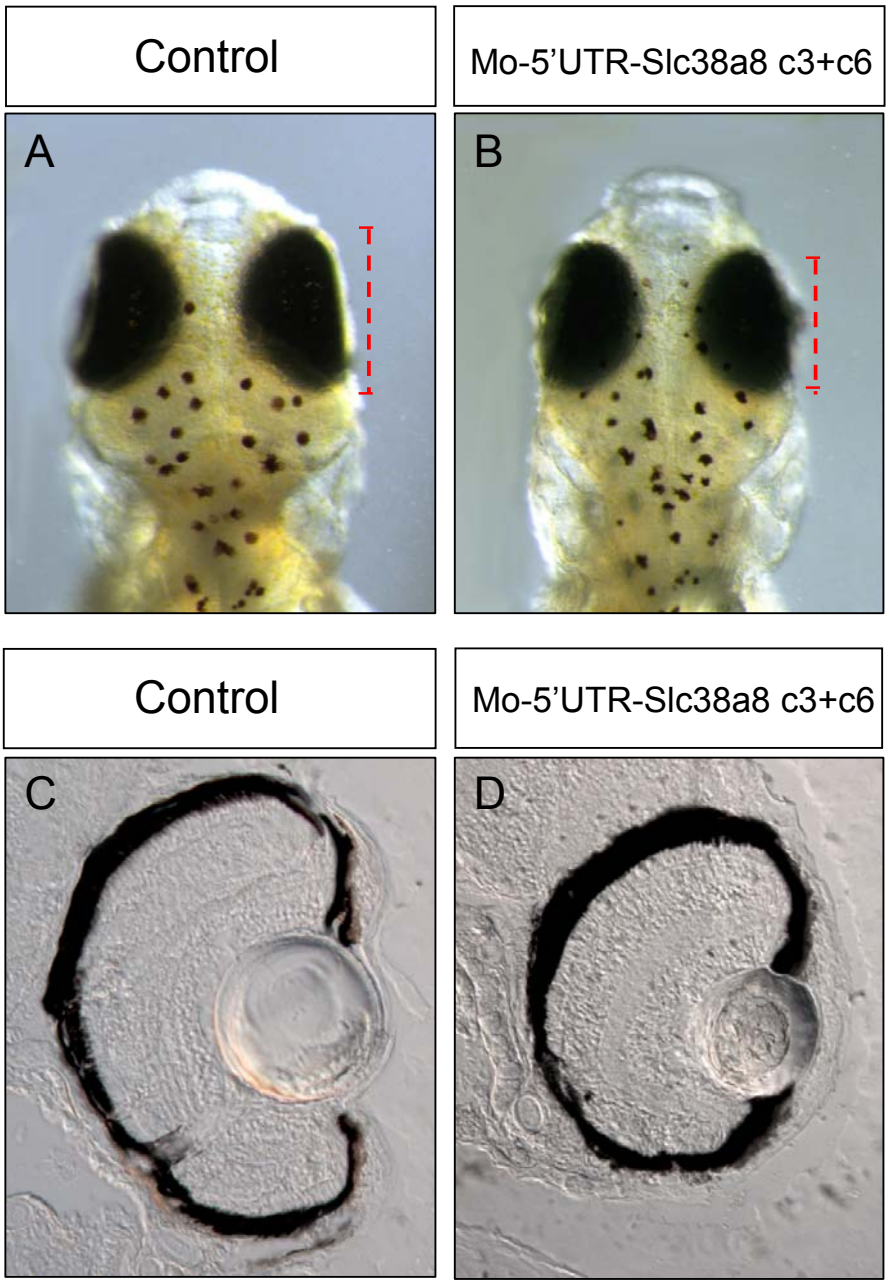


Figure S3. Knockdown of the *Slc38a8* gene does not cause melanogenesis defects in medaka. (A, B) Bright-field stereomicroscopy images of dorsal views of medaka embryos at St40. No differences were observed in tegument melanophores between control (A) and Mo-5'UTR-Slc38a8-c3 and Mo-5'UTR-Slc38a8-c6 co-injected (B) medaka embryos. Dotted red lines indicate the microphthalmic phenotype present in Mo-5'UTR-Slc38a8-c3 and Mo-5'UTR-Slc38a8-c6 co-injected embryos. (C, D) Cryostat frontal sections of the retina from St40 control (C) and Mo-5'UTR-Slc38a8-c3 and Mo-5'UTR-Slc38a8-c6 co-injected (D) medaka embryos. No significant alterations of retinal pigment epithelium (RPE) pigmentation were observed in Mo-5'UTR-Slc38a8-c3 and Mo-5'UTR-Slc38a8-c6 co-injected retina with respect to controls. Other abbreviations: R, retina; L, lens.

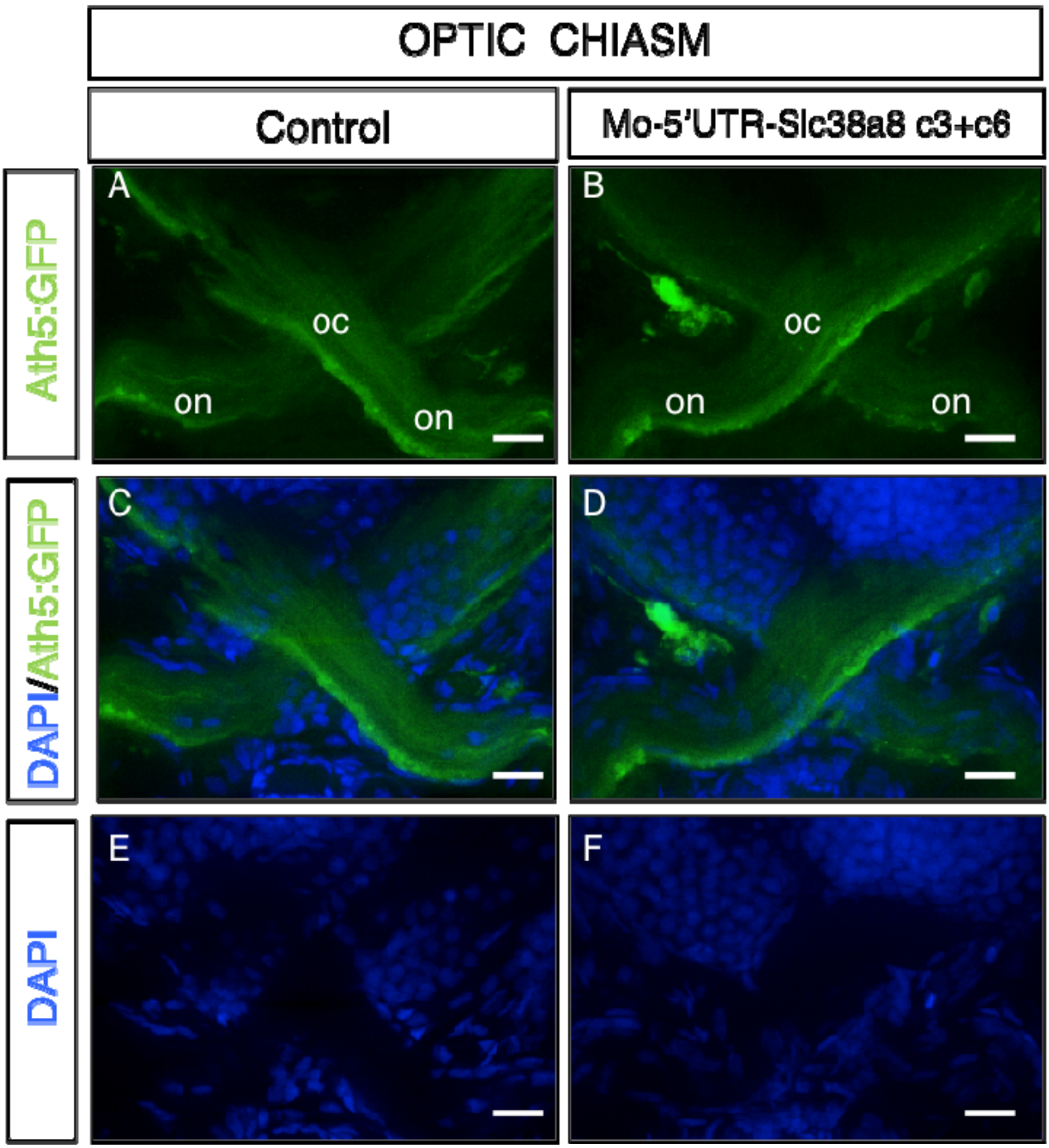


Figure S4. Analysis of the optic chiasm in medaka *Slc38a8* morpholino knockdown morphants shows no abnormalities. Confocal images of the optic chiasm from frontal cryostat sections of St38 control and Mo-Slc38a8 injected Ath5::GFP transgenic embryos. Retinal ganglion cell (RGC) axons are EGFP stained (A, B, C, D). Sections are counterstained with DAPI (blue, C, D, E, F). No major alterations in the distribution of RGC axons at the optic chiasm were observed in Mo-5'UTR-Slc38a8-c3 and Mo-5'UTR-Slc38a8-c6 co-injected morphant fish as compared with control embryos. oc, optic chiasm; on, optic nerve. Scale bars: 20µm.

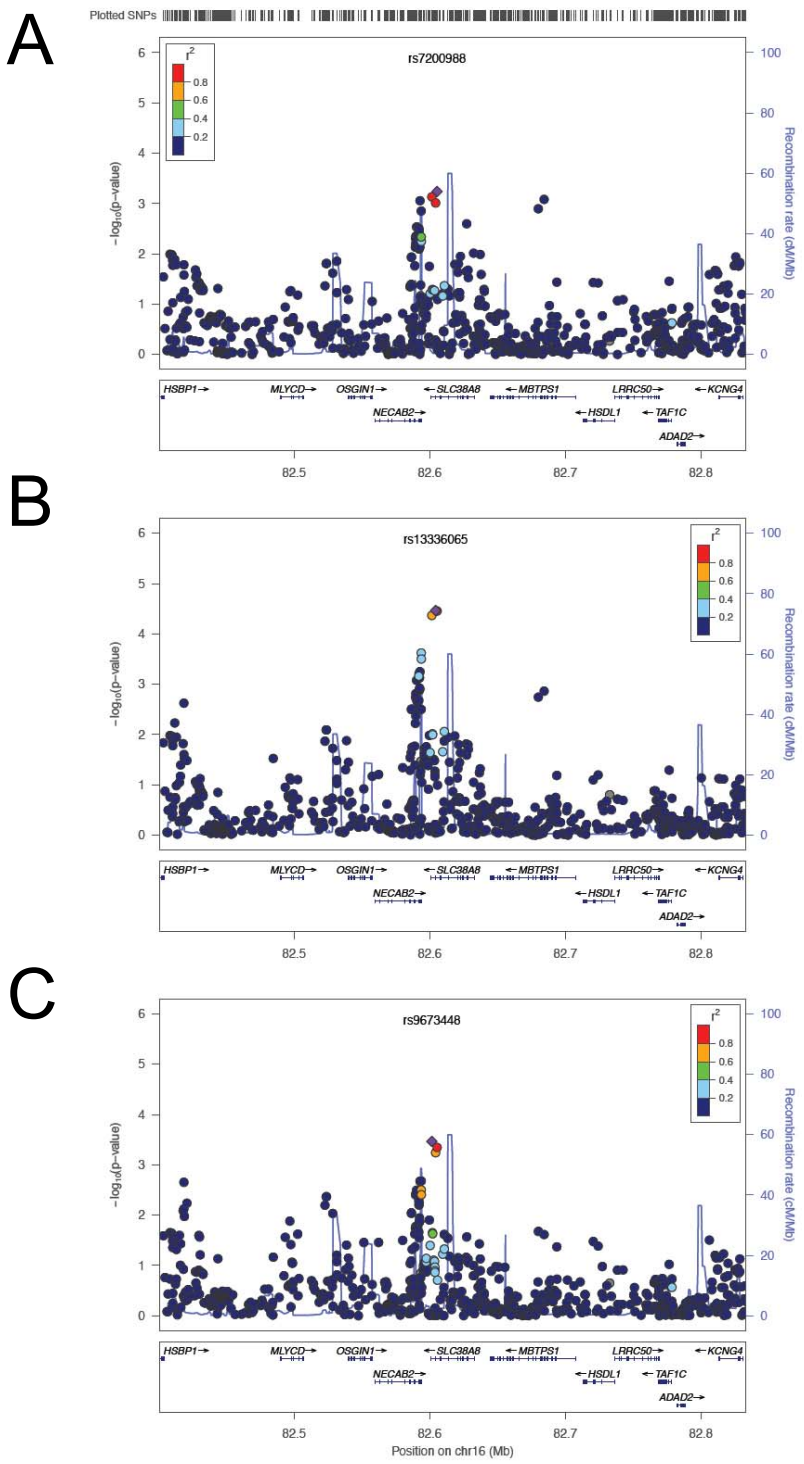
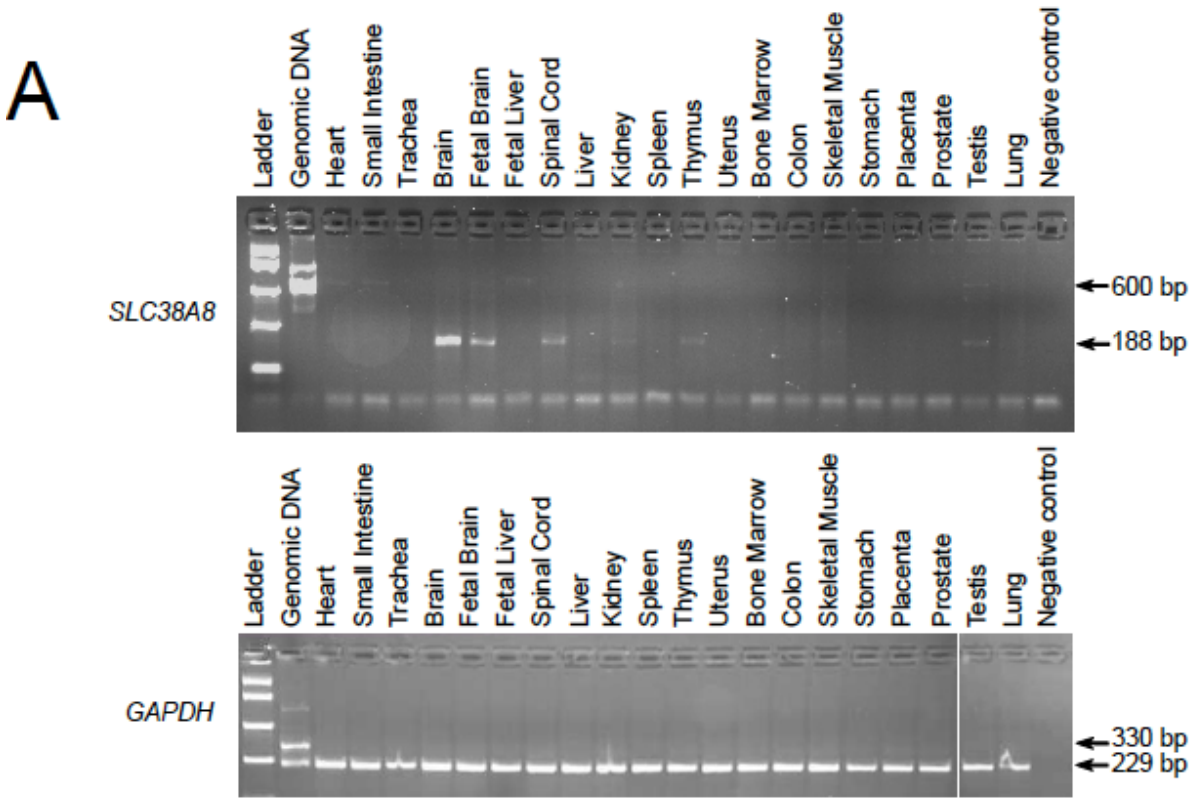
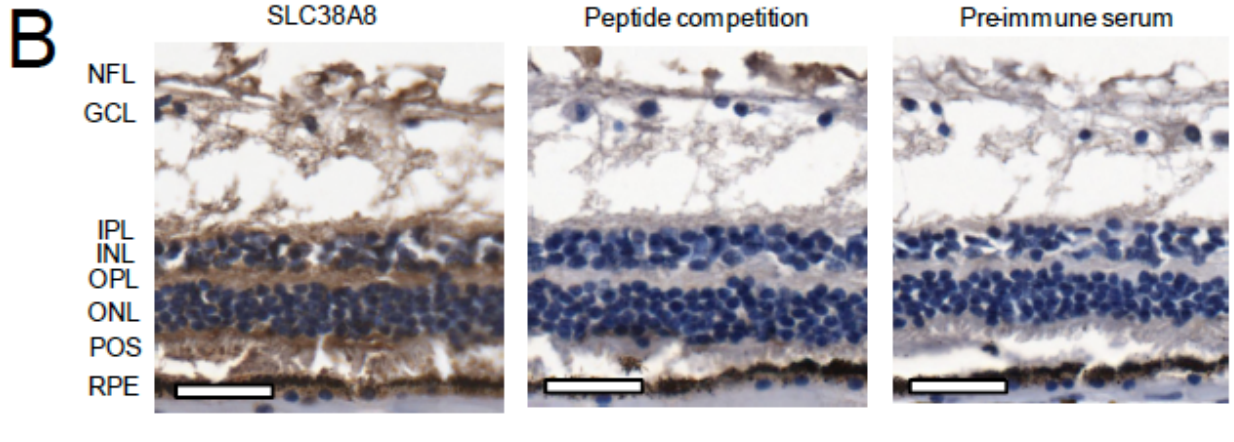


Figure S5. Role of common single nucleotide polymorphisms (SNPs) at the *SLC38A8* locus in determining foveal thickness in the general population. Plots were generated using LocusZoom [Pruim, R.J. et al. (2010). Bioinformatics 26, 2336-7] and genomic references refer to hg.18. Results from analysis of the foveal thickness (A), the fovea:parafovea thickness ratio (B), and the fovea:perifovea thickness ratio (C) are presented. SNPs are plotted as the $-\log_{10}$ of the p-value.


A
Ladder
Genomic DNA
Heart
Small Intestine
Trachea
Brain
Fetal Brain
Fetal Liver
Spinal Cord
Liver
Kidney
Spleen
Thymus
Uterus
Bone Marrow
Colon
Skeletal Muscle
Stomach
Placenta
Prostate
Testis
Lung
Negative control
SLC38A8
600 bp
188 bp
GAPDH
330 bp
229 bp


B
SLC38A8
Peptide competition
Pre-immune serum
NFL
GCL
IPL
INL
OPL
ONL
POS
RPE

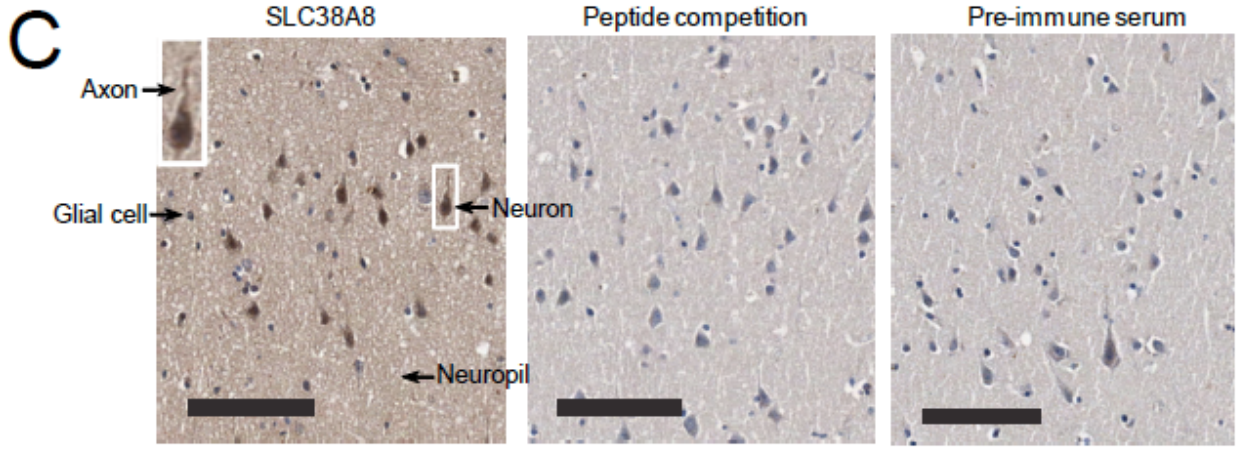

C
SLC38A8
Peptide competition
Pre-immune serum
Axon
Glial cell
Neuron
Neuropil

Figure S6. Human RNA and protein localization data for *SLC38A8*. (A) RNA expression results. *SLC38A8* expression is found predominantly in neuronal tissue (brain, fetal brain and spinal cord) with very weak expression also present in the kidney, thymus and testis. Human adult and fetal tissue total RNA was purchased from Clontech and converted to cDNA using standard protcols. A 188-bp fragment of *SLC38A8* spanning intron 7 was amplified from the cDNAs using primers cDNA(7-8)-F 5'-AATGATATGGTCATCATTGTGG-3' and cDNA(7-8)-R 5'-AGGATGGTCAGCGGCATC-3' (genomic product size was 600 bp). *GAPDH* control primers used were GAPDH-F 5'-CGACCACTTTGTCAAGCTC-3' and GAPDH-R 5'-CAAGGGTCTACATGGCAAC-3' (cDNA product size was 229 bp and genomic product size was 330 bp). (B) and (C) Protein localization of SLC38A8. Tissues were formalin fixed and paraffin embedded according to standard methods. 4μm serial sections were blocked for endogenous peroxidase activity using 3% hydrogen peroxide in methanol for 10 minutes. Blocked sections were subsequently incubated with a custom rabbit polyclonal antibody (GenScript, USA), raised against 14 amino acids located at the N-terminus of SLC38A8 (QTPGSRGLPEKPHP). Primary antibody was applied at a 1/200-250 dilution for eye tissue and 1/400-450 dilution for brain tissue in Zymed diluent (Invitrogen) and incubated overnight at 4°C. To confirm specificity of the antibody, serial sections were incubated with the pre-immune serum of the host rabbit and the custom antibody pre-incubated with the peptide (at a 1:10 ratio). Bound primary antibody was detected with the Rabbit Envision Detection System (Dako, UK) and slides counter-stained in $CuSO_4$ and Mayers haematoxylin. Finally, sections were dehydrated in increasing concentrations of ethanol (25-100%) and cleared in two changes of xylene before being mounted in DPX mounting media (Sigma). (B) Protein localization in adult human retina. SLC38A8 staining is found throughout the neuronal retina with particularly strong staining evident in the inner and outer plexiform layers and the photoreceptor cell layer. NFL - nerve fiber layer, GCL – ganglion cell layer, IPL – inner plexiform layer, INL – inner nuclear layer, OPL – outer plexiform layer, ONL – outer nuclear layer, POS – photoreceptor outer segments, RPE – retinal pigment epithelium. The scale bars represent 40 μm. (C) Protein localization in human adult brain. A section of grey matter from the occipital lobe is shown and is representative of all brain sections examined (pre-frontal cortex, mid-frontal cortex, primary motor cortex and hippocampus). The majority of the neuronal cells are stained along with the neuropil. However, many glial cells remain unstained. The scale bar represents 100 μm.

Table S1. *SLC38A8* primers for PCR/sequencing

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
1	GAGCTCTCACTGAGTTGGGG	CCTCATTAGCTGAGCGGG	437
2	TGTGGAGAGTGTGCCCG	GGAGCCTCCCTTCCCTAC	449
3	GTGGATGTCCAACCCCAG	CAAATGTGAAGGCCAATTCC	350
4	ACCTCATTGTCAAGGGCG	CTCTGCAGTGAGCCACGAGT	312
5	TTGCAGCCATGCTCTGTTAC	CCCTGACAGAGAAACCAAGG	266
6	TCAGGCTATTTCAAGAGAAACCTC	TGAGGCCTTTTCCTATGCAC	398
7	AAGGAAAATCTTAGGTGGTAAAGC	AGTCCCACAGCCGCGTAG	352
8	TGTGGAACAGGAGCACTGAC	TCCTACCCATATGTGGCTCC	442
9	CCTTCCAGCTCCACTGTGTT	CAGATGCCCTCATACTGGGT	380
10	CCTACAGGTAACTGGAAATATTCTGA	GGCATCAGTCTCTCCAGCAT	364

Primer sequences for all other genes screened are available on request.

Table S2. Summary of bioinformatics analyses undertaken to predict the pathogenic nature of the missense mutations

Mutation	PolyPhen2	MutationTaster	SIFT	Blosum62*	PROVEAN	MutPred
p.Met34Arg	Benign (score 0.225)	Disease causing (prediction probability 0.8385)	Tolerated (score 0.07)	Score -1	Deleterious (score 3.611)	Deleterious mutation (prediction probability 0.893)
p.Glu233Lys	Probably damaging (score 1.000)	Disease causing (prediction probability 1.0000)	Tolerated (score 0.34)	Score +1	Neutral (score -2.267)	Deleterious mutation (prediction probability 0.752)
p.Val236Asp	Probably damaging (score 1.000)	Disease causing (prediction probability 0.9999)	Damaging (score 0.03)	Score -3	Deleterious (score 6.274)	Deleterious mutation (prediction probability 0.846)
p.Gly412Arg	Probably damaging (score 1.000)	Disease causing (prediction probability 0.9999)	Damaging (score 0.01)	Score -2	Deleterious (score 5.317)	Deleterious mutation (prediction probability 0.857)

URLs: PolyPhen2, http://genetics.bwh.harvard.edu/pph2/ [Adzhubei, I.A. et al. (2010). Nat. Methods 7, 248-9]; Mutationtaster, http://www.mutationtaster.org/ [Schwarz, J.M. et al. (2010). Nat. Methods 7, 575-6]; SIFT, http://sift.jcvi.org/ [Ng, P.C. et al. (2003). Nucleic Acids Res. 31, 3812-4]; Blosum62 [Henikoff, S. et al. (1993). Proteins 17, 49-61]; PROVEAN, http://provean.jcvi.org/ [Choi, Y. et al. (2012). PLoS ONE 7, e46688]; MutPred, http://mutpred.mutdb.org/ [Li, B. et al. (2009). Bioinformatics 25, 2744-50]. *Blosum62 scores range from +3 to -3 and negative scores are more likely to be damaging substitutions.

Table S3. Medaka *Slc38a8* morpholino sequences

Morpholino name	Sequence (5'-3')	Concentration (mM)
Mo-5'UTR-Slc38a8-c3	GGCAGGGTTTGCAAAGAGTTGACAA	0.06
mmMo-5'UTR-Slc38a8-c3	GGtAGcGTTTcCAAAcAGTTtACAA	0.06
Mo-spl-Slc38a8-c3-ex4	GTAACTCACCCCTGGATGTGTTCTG	0.06
mmMo-spl-Slc38a8-c3-ex4	GTAAcTCACgCCTGcATtTGTTgTG	0.06
Mo-5'UTR-Slc38a8-c6	TCAGAGGGAAGATGCTCGAGGCAGC	0.06
mmMo-5'UTR-Slc38a8-c6	TCAcAGGcAAGATcCTCtAGGtAGC	0.06
Mo-spl-Slc38a8-c6-ex4	AAAACATACCCAGTGGAGTGGATCG	0.06
mmMo-spl-Slc38a8-c6-ex4	AAAAgATACgCAGTaGAGTcGATTg	0.06
Mo-p53 control	CGGGAATCGCACCGACAACAATACG	0.09

Mo = morpholino, mmMo = mismatch morpholino. c3 and c6 = chromosome 3 and 6 respectively.

Table S4. Summary of clinical phenotypes of families with *SLC38A8* mutations

Family	Ethnicity	Foveal hypoplasia	Optic nerve decussation defect	Anterior segment abnormalities	Iris transillumination	Other notes
F1	Pakistani	Present in all affected members (IV:1, IV:3, IV:4, IV:6, IV:7)	Present in all affected members tested (IV:1, IV:3)	Posterior embryotoxon in all affected members (IV:1, IV:3, IV:4, IV:6, IV:7) and Axenfeld's anomaly also present in IV:1 and IV:7	Absent in all affected members (IV:1, IV:3, IV:4, IV:6, IV:7)	
F2	Afghan	Present in both affected members (V:2 and V:5)	Present in both affected members (V:2 and V:5)	Posterior embryotoxon present in both members (V:2 and V:5)	Absent in both affected members (V:2 and V:5)	
F3	Northern European	Present in single affected case (II:4)	Present in single affected case (II:4)	None	Absent in single affected case (II:4)	Kartagener syndrome also present in II:4
F4	Pakistani	Present in single affected case (II:1)	Test inconclusive in single affected case (II:1)	None	Absent in single affected case (II:1)	
F5	Turkish	Present in single affected case (IV:1)	Present in single affected case (IV:1)	None	Absent in single affected case (IV:1)	
F6	Indian	Present in all examined affected members (IV:7, VI:1, VI:2)	Not tested	None	Absent in all examined affected members (IV:7, VI:1, VI:2)	IV:7 and VI:1 have bilateral microphthalmia and IV:7 has a unilateral retinochoroidal coloboma (right eye).
F7	Northern European	Present in both affected members (II:1, II:2)	Present in both affected members (II:1, II:2)	Axenfeld's anomaly present in only II:2	Absent in both affected members (II:1, II:2)	

Detailed descriptions of members of F1, F2, F3 and F6 have been reported previously.[4, 5, 6, 8]

Table S5. Top associated variants at the *SLC38A8* locus with foveal thickness parameters in the general population, using data from the Raine Eye Health Study

Trait	SNP	CHR	BP	Effect Allele	MAF	Beta	SE	P	HWE	IxScore
Fovea Thickness	rs7200988	16	82605474	A	0.051	-0.456	0.132	5.77E-04	0.41	0.83
Fovea:Parafovea Thickness Ratio	rs13336065	16	82604407	A	0.045	-0.596	0.143	3.44E-05	1.00	0.84
Fovea:Perifovea Thickness Ratio	rs9673448	16	82601632	C	0.056	-0.467	0.130	3.42E-04	0.72	0.84

SNP, single nucleotide polymorphism; CHR, chromosome; BP, base position on hg.18; MAF, minor allele frequency; SE, standard error; HWE, Hardy-Weinberg Equilibrium; IxScore, imputation QC score.