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Supplemental Data

RAC1 Missense Mutations in Developmental Disorders with Diverse Phenotypes

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Supplemental Note: Case Reports

Individual 1 (male, 13 years) - He is the second of four children born to non-consanguineous parents. The mother is of Dutch ancestry and the father of Egyptian ancestry. Family history was non-contributory. He was born after uncomplicated pregnancy and delivery. He had neonatal feeding problems and an absent suck reflex. He walked without support at the age of 18 months and spoke his first words at the age of 6 years. Psychological assessment at the age of 13 years showed severe intellectual disability with an IQ of 35. He developed epilepsy at the age of 2 years. He has a progressive scoliosis that affected his mobility and required surgical correction. At 13 years of age, his height was 137 cm (-2.5 SD), weight was 30 kg (0 SD) and occipito-frontal circumference (OFC) was 50 cm (-2.5 SD). He has small and dysplastic ears, synophrys, arched eyebrows, long palpebral fissures, broad nasal tip, short philtrum and broad canine teeth. His hands and feet are small with bilateral simian creases, 5^{th} finger brachydactyly and hyperlaxity of joints. Magnetic resonance imaging (MRI) studies of the brain at the age of 12 years showed severe dysplasia of cerebellar vermis and right cerebellar hemisphere, large retrocerebellar arachnoid cyst, mega cisterna magna, hypoplasia of splenium of the corpus callosum, bilateral cystic lesions in the cerebral white matter: possibly small lacunar infarcts, multiple lesions in the deep white matter frontoparietal, mild dilatation of the posterior horn of the lateral ventricles. He has mild insufficiency of all four cardiac valves and non-synchronic left ventricular contractions. Previous investigation included normal metabolic screening in urine and blood, 250k SNP array and targeted sequencing for *TCF4*, *PTPN11* and *ANKRD11*. Whole exome sequencing (WES) revealed a heterozygous *de novo* mutation in *RAC1*: Chr7(GRCh37):g.6426860G>A; NM_018890.3:c.53G>A; p.(Cys18Tyr).

Individual 2 (male, 9 years) – He is the only child of non-consanguineous parents of Dutch ancestry. There was no family history of developmental delay. Pregnancy was complicated by pregnancy induced hypertension. He was born at 32 weeks and 4 days of gestation via a Caesarian section for fetal distress and intra-uterine growth retardation. He had frequent pneumonias and hyperbilirubinemia during infancy. Both motor and language development were delayed. Psychological assessment at the age of 7 years showed mild to moderate intellectual disability with an IQ of 51. He is hyperactive with poor attention and has a high pain threshold. He has a friendly personality. He has a history of constipation and frequent dental caries. At the age of 9 years his height was 128 cm (-1.5SD), weight was 24 kg (-0.5 SD) and OFC was 47.7 cm (-3 SD). He has low set, posteriorly rotated and dysplastic ears, full eyebrows, long palpebral fissures, broad nasal tip, flat philtrum, broad mouth and pointed chin. Brain MRI at the age of 2.5 years showed mildly enlarged lateral ventricles, hypoplastic pons , hypoplastic lower lobe of the cerebellar vermis, mega cisterna

magna, enlarged fourth ventricle, thin splenium and rostrum of the corpus callosum. Previous genetic investigations included normal karyotyping, analysis of subtelomeric regions and 250k SNP array. WES revealed a heterozygous *de novo* mutation in *RAC1*: Chr7(GRCh37):g.6431563A>G; NM_018890.3:c.116A>G p.(Asn39Ser).

Individual 3 (male, 15 years, DDD 260739) – He is born to non-consanguineous parents. Increased nuchal thickness was noted prenatally. He was born at 40 weeks of gestation. He was noted to have a poor suck and had neonatal feeding problems. His developmental milestones are not available. Currently, he has a few single words. At 15 years of age his OFC was 47 cm (-5 SD). Trio WES sequencing revealed a heterozygous *de novo* mutation in the *RAC1* gene Chr7(GRCh37):g.6431665C>T; NM_018890.3: c.218C>T p.(Pro73Leu).

Individual 4 (male, 6 months) – He is the first child of healthy non-consanguineous couple of French Canadian-Polish and Armenian descent. There was a family history of postaxial polydactyly of the feet in the maternal aunt and grandmother. Prenatal scans revealed multiple fetal abnormalities, including cerebellar hypoplasia, shortened corpus callosum, prominent cisterna magna, an echogenic intra-cardiac focus, hypertelorism, postaxial polydactyly on all four limbs, single umbilical artery, and near term onset of growth restriction. At birth, the baby was also found to have a large anterior fontanelle, flat nasal bridge, broad upturned nose, hypoplastic alae nasi, full lips, umbilical hernia, cryptorchidism and hypospadias. The baby was microcephalic at birth (with a head circumference at 2 SD below the mean); birth weight and length were within the normal range (both at the 30th centile). The boy required several days of respiratory support initially, and had recurrent apneic episodes in the first few months of life; consequent investigations revealed tracheobronchomalacia, central apnea and subclinical seizures. On assessment at 2 and 4 months of age, he had global developmental delay, central hypotonia, microcephaly (-2.5 SD) and poor weight gain (-3 SD). His length remained in the normal range (20th centile). Brain MRI showed abnormal cerebellar foliation, lower vermis hypoplasia, large supravermian cyst, mega cisterna magna, abnormal brainstem with loss of normal pontomedullary sulcation, and short corpus callosum with tapering of the posterior body and splenium. Echocardiogram revealed bicuspid aortic valve, patent foramen ovale and patent ductus arteriosus. Karyotype and 250k SNP-array were normal. WES revealed a heterozygous d*e novo* novel mutation in exon 7 of the *RAC1* gene: Chr7(GRCh37):g. 6441968G>A; NM_018890.3: c.470G>A p.(Cys157Tyr) and a maternally inherited novel variant of uncertain significance was also found in the *KIAA2022* gene (c.4228C>T; p.Pro1410Ser). While *KIAA2022* mutations are a known cause of X-linked mental retardation, the dysmorphic features and multiple anomalies found in this case do not fit the phenotype associated with mutations.[1; 2]

Individual 5 (male, 12y, DDD 265249) - is one of non-identical twins born to non-consanguineous parents. He was born at 38 weeks of gestation with a weight of 2.2 Kg (-2.17 SD). He was noted to be mildly hypotonic soon after birth. There is no history of neonatal feeding difficulties. He achieved social smile at 12 weeks of age. He has severe global developmental delay. He could sit without support from 19 months and can walk short distances with support from the age of 4 years. He does not have any expressive speech and his understanding is severely impaired. He has bilateral sensorineural hearing loss and mild visual impairment. He used to have head stereotypies as a young child but they have resolved. He has never had seizures but possibility of absences has been raised, although not proven. He was found to have a ventricular septal defect that was closed surgically at the age of 2 years. He has a small penis. His OFC at 8 years was 53 cm (0.07SD) and 56.5 cm (+1 SD) at 12 years of age. At 8 years his height was 134 cm (+1.03 SD). He has prominent metopic suture, wave-shaped palpebral fissures, a high palate, a prominent nasal bridge with low columella and dysplastic ears. A brain MRI at 2 years of age showed polymicrogyria. Previous testing included normal karyotype, Fragile X studies, urine metabolic screen, very long chain fatty acid levels, plasma lactate and SNP6 microarray. WES sequencing revealed a heterozygous *de novo* mutation in the *RAC1* gene Chr7(GRCh37):g.643163T>G; NM_018890.3: c.190T>G p.(Tyr64Asp).

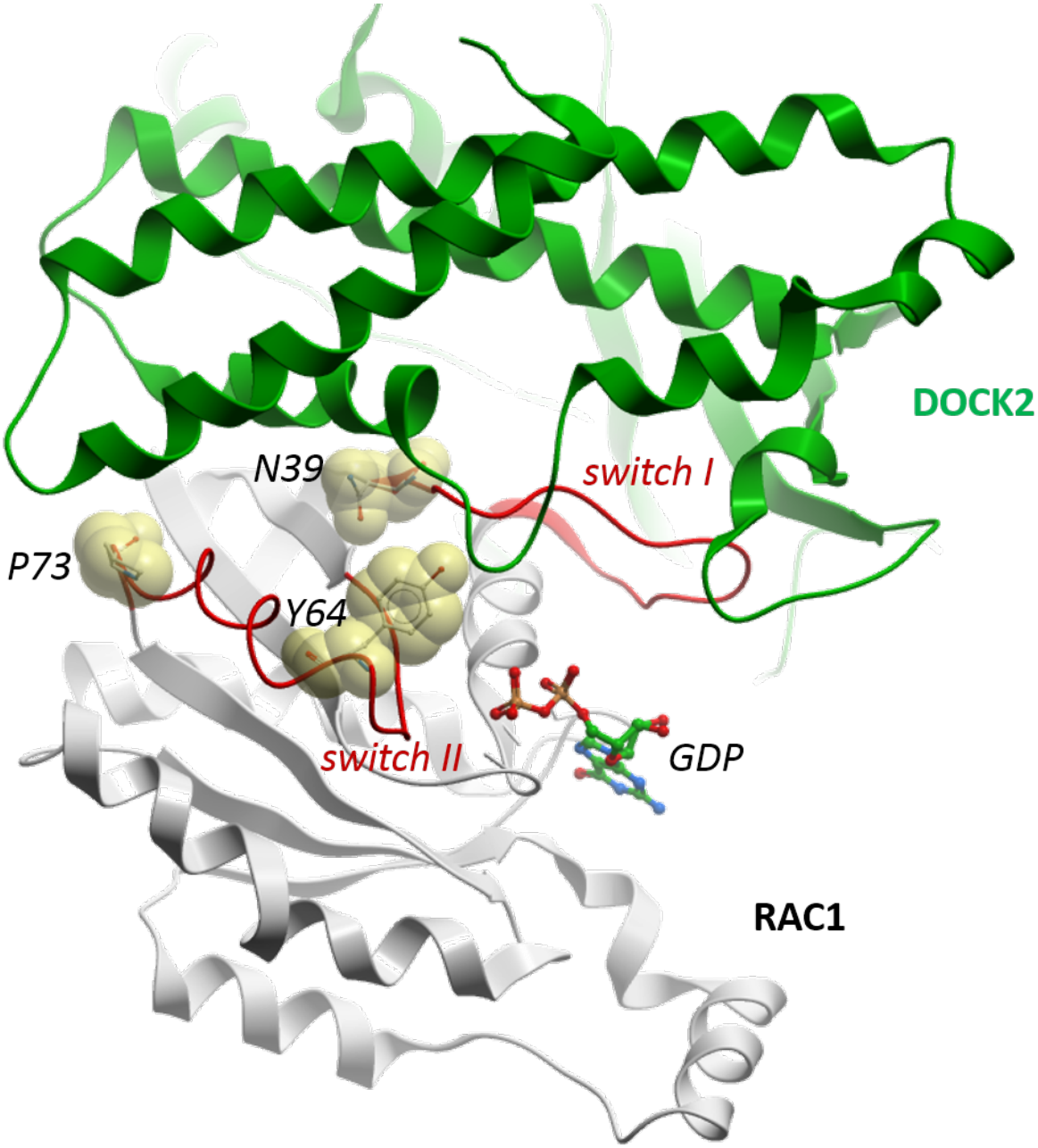
Individual 6 (male, 33 months, DDD 270776) – He is the first child of non-consanguineous parents, who both have sensorineural hearing loss. The pregnancy was uneventful. The child was born at 39 weeks of gestation with a birth weight of 3.54 Kgs (0.42 SD). He has normal hearing. The birth OFC is not available but he was referred to Paediatrics at 4 months of age for macrophaly and his OFC at 11 months was 52cm (+1 SD). He was able to sit without support from 12 months and started walking independently between the ages of 2 and 2.5 years. At the age of 33 months his OFC was 56 cm (+4.16 SD). He has a prominent broad forehead, slightly up-slanted palpebral fissures, an open mouth and a 'scooped out' appearance on lateral view. A MRI brain scan at 12 months of age showed non-specific white matter change in the right frontal and parietal lobes in the peri-ventricular distribution. Previous genetic investigations included normal *PTEN* sequencing, array CGH, urine organic acids, white cell enzymes and Fragile X. WES revealed a heterozygous *de novo* mutation in *RAC1*: Chr7(GRCh37):g.6431598G>A; NM_018890.3: c.151G>A p.(Val51Met).

Individual 7 (male, 4 years and 5 months) – He is eldest of two children born to non-consanguineous parents of African American ancestry. Family history was negative for developmental delay and intellectual disability. The pregnancy was unremarkable, and the patient was born at term by spontaneous vaginal delivery. He had no perinatal problems. He was noted to have developmental

delay at 15 months of age when he was not crawling or walking and did not have any words at that time. He started having seizures at 1 year of age which were well controlled with Depakote. His EEG showed generalized cortical dysfunction and multifocal induced seizures. By 2 years of age he was walking but only had between 3 and 5 words which he used inconsistently. He is on the autistic spectrum. He has mild truncal hypotonia and symmetric hyporeflexia. He has a history of recurrent ear infections. He has significant eczema with associated hypopigmentation. At the age of 4 years 5 months his height was 109 cm (0 SD), weight was 23 kg (+2.5 SD) and OFC was 59.5 cm (+4.5 SD). He has a prominent broad forehead, up-slanted palpebral fissures, upturned ear lobes, an open mouth and a 'scooped out' appearance on lateral view. A brain MRI showed tiny punctate white matter lesions in the periventricular area. Previous testing included normal high resolution chromosomes, CytoScan microarray, plasma amino acids, urine organic acids, fragile X analysis, myotonic dystrophy analysis, *PTEN* sequencing, and a NGS lysosomal gene panel. Trio WES sequencing revealed a heterozygous *de novo* mutation in the *RAC1* gene Chr7(GRCh37):g.6431598G>C; NM_018890.3:c.151G>C p.(Val51Leu).

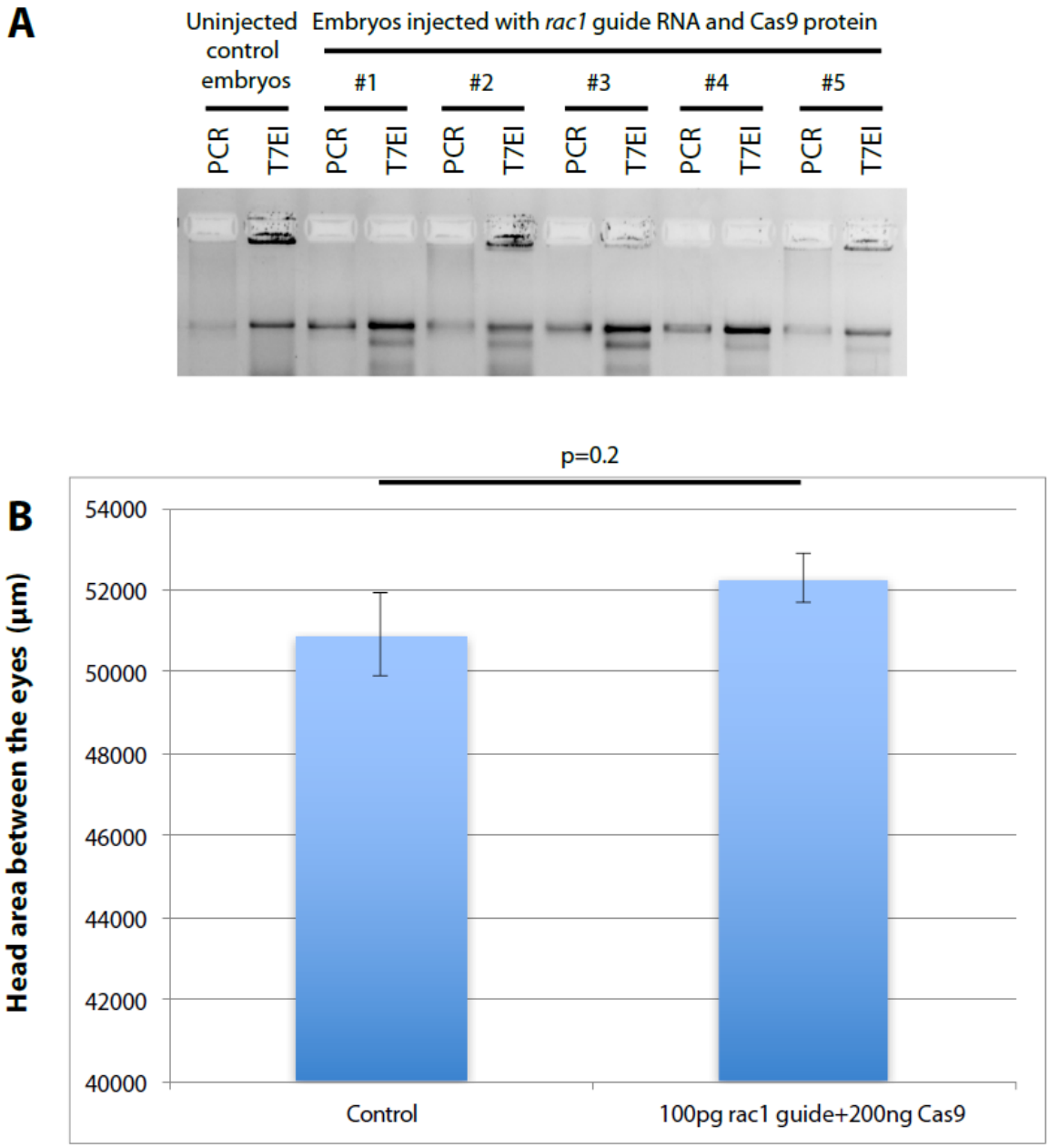
Supplemental figures and legends

Figure S1. Crystal structure of the RAC1-DOCK2 complex (PDB code 2YIN).



This figure highlights the use of Switch I and Switch II motifs (red) of RAC1 (grey cartoon) to interact with the guanine exchange factor DOCK2 (green cartoon). The three mutations predicted to affect this interaction are mapped onto RAC1 as spheres.

Figure S2. Evidence for the efficiency of the CRISPR reagent used to suppress the endogenous expression of *rac1* in developing zebrafish embryos.

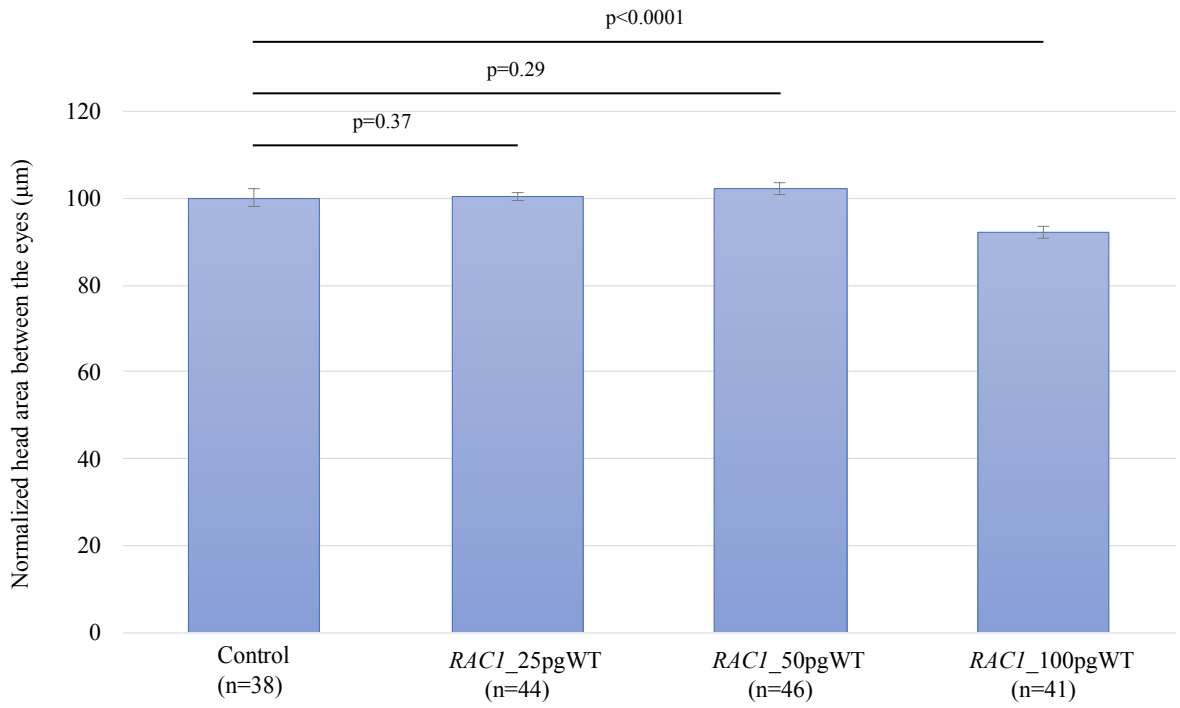


(A) For the cloning of the *rac1* guide template RNA was annealed the two oligonucleotides rac1_guide2_F: 5'-TAGGACCAGTAAACCTGGGATTGT-3' and rac1_guide2_R: 5'-AAACACAATCCCAGGTTTACTGGT-3'. The *rac1* guide oligonucleotide sequences were ligated into the pT7Cas9sgRNA vector (Addgene) using the *Bsm* BI sites. For the generation of the guide RNA, the template DNA was linearized with *Bam* HI, purified by phenol/chloroform extraction and *in vitro* transcribed using the MEGAshortscript T7 kit (Invitrogen). To generate F0 CRISPR mutants we injected 1nl containing 100pg *rac1* guide RNA and 200ng Cas9 protein (PNA bio, CP01) to 1-cell stage embryos. To determine the efficiency of the guide RNA, embryos were allowed to grow to 5dpf, at

which time they were euthanized and subjected to digestion with proteinase K (Life Technologies, AM2548) to extract genomic DNA. The targeted locus was PCR amplified using the drrac1_g2test_1F 5'-TCCCCAATTACATTTGTCATCA-3' and drrac1_g2test_1R 5'-ACTCATGGATATCGGCAAGC-3' primer pair. The PCR amplicons were subject to digestion using T7 endonuclease I (New England Biolabs, M0302L) at 37 °C for 1 hr and were visualized on a 2% agarose gel. For Sanger sequencing of individual products from the *rac1* locus, PCR fragments from four embryos with a positive T7assay were cloned into the pCR4/TOPO TA cloning vector (Life technologies, 450030) and 40 colonies from each cloned embryo were Sanger sequenced. Gel image showing the efficiency of the *rac1* guide RNA following T7 endonuclease assay evaluation. The first two lanes show control amplicons from the locus flanking the targeted sequence, with no aberrations observed. In the embryos injected with *rac1* guide RNA and Cas9 protein, aberrations are evident for embryos #1 - #5, showing that the guide efficiently introduced sequence aberrations in all injected embryos.

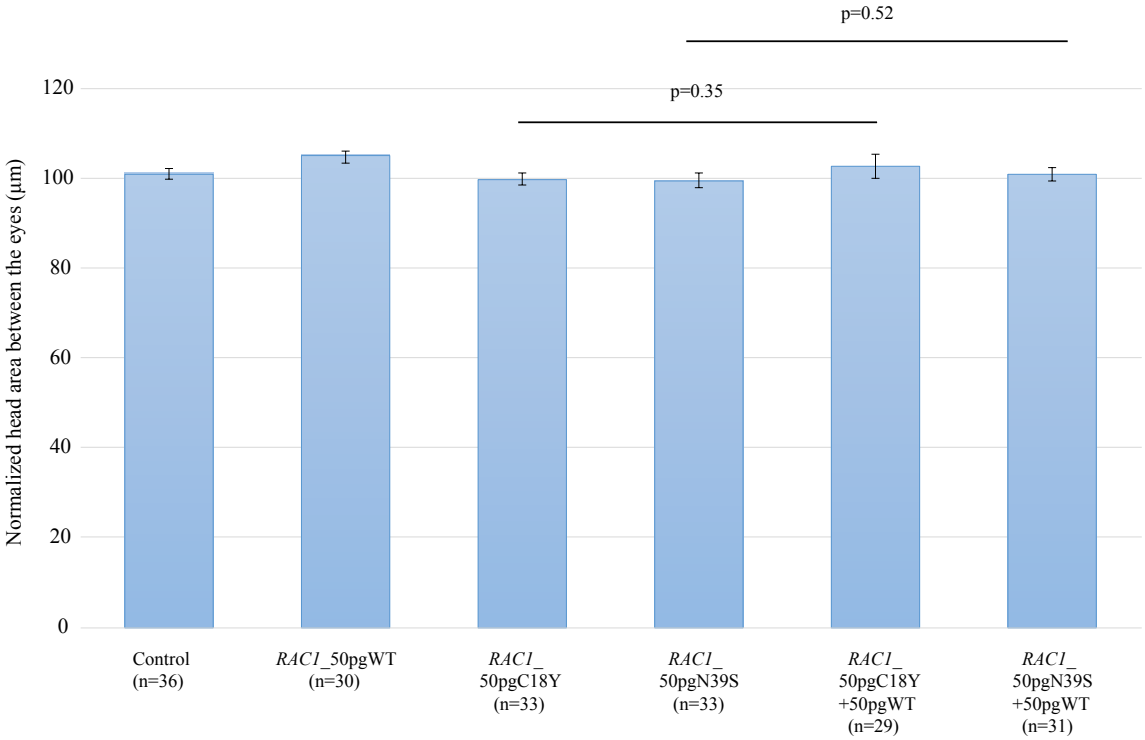
(B) Bar graph showing the quantification of the head size phenotype in control embryos and larvae injected with *rac1* guide RNA and Cas9. Statistical analyses were performed by student`s t-test.

Figure S3. Dose curve showing the effect of progressively increasing doses of WT *RAC1* in 5dpf zebrafish larvae.



Bar graph showing the quantification of the head size phenotype in control embryos and embryos injected with progressively increasing doses of WT *RAC1* message. The bars represent cumulative normalized plotted experiments across two biological replicas. Statistical analyses were performed by student`s t-test.

Figure S4. The headsize phenotype caused by overexpression of d*e novo* mutations p.Cys18Tyr and p.Asn39Ser in *RAC1* cannot be antagonized by concomitant overexpression of WT *RAC1* message.



Bar graph showing the quantification of the head size phenotype in control embryos and embryos injected with WT, mutant or mutant + WT *RAC1* message. The bars represent normalized plotted experiments. Statistical analyses were performed by student`s t-test.

Supplemental references

1. Cantagrel, V., Lossi, A.M., Boulanger, S., Depetris, D., Mattei, M.G., Gecz, J., Schwartz, C.E., Van Maldergem, L., and Villard, L. (2004). Disruption of a new X linked gene highly expressed in brain in a family with two mentally retarded males. Journal of medical genetics 41, 736-742.
2. Van Maldergem, L., Hou, Q., Kalscheuer, V.M., Rio, M., Doco-Fenzy, M., Medeira, A., de Brouwer, A.P., Cabrol, C., Haas, S.A., Cacciagli, P., et al. (2013). Loss of function of KIAA2022 causes mild to severe intellectual disability with an autism spectrum disorder and impairs neurite outgrowth. Human molecular genetics 22, 3306-3314.