Journal of
Medical Genetics



# Mutations specific to the Rac-GEF domain of TRIO cause intellectual disability and microcephaly

| | |
|---|---|
| Journal: | *Journal of Medical Genetics* |
| Manuscript ID | Draft |
| Article Type: | Original Article |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Pengelly, Reuben; University of Southampton, Genomic Informatics Group<br>Greville-Haygate, Stephanie; Princess Anne Hospital<br>Schmidt, Susanne; Centre de Recherche de Biochimie Macromoleculaire<br>Seaby, Eleanor; University of Southampton, Genomic Informatics Group; University of Southampton,<br>Fagotto-Kaufman, Christine; Centre de Recherche de Biochimie Macromoleculaire<br>Jabal-Ameli-Forooshani, Reza; University of Southampton<br>Mehta, Sarju; East Anglian Medical Genetics Service, Addenbrooke's Hospital, Department of Clinical Genetics<br>Parker, Michael; Sheffield Children's NHS Foundation Trust, Department of Clinical Genetics<br>Goudie, David; Ninewells Hospital<br>Mercer, Catherine; Wessex Clinical Genetics Service,<br>DDD study, the<br>Debant, Anne; Centre de Recherche de Biochimie Macromoleculaire<br>Ennis, Sarah; University of Southampton, Genetic Epidemiology<br>Baralle, Diana; University of Southampton, Human Genetic and Genomics |
| Keywords: | microcephaly, Rac, TRIO, Db1, Rho GTPase |
| | |

## INTRODUCTION

Neurodevelopmental delay is genetically heterogenous and affects between 1% and 3% of the general population.[1] The utilisation of next generation genomic techniques such as Whole Exome Sequencing (WES) has facilitated the identification of numerous novel pathogenic variants implicated in the evolution of neurodevelopmental pathology.[2] One such gene on chromosome 5, *TRIO*, has recently been identified as a candidate for human neurodevelopmental delay.[3]

Neurodevelopment is a complex process requiring coordinated axonal guidance and neuronal migration to facilitate the delivery of neurons from the neuroepithelium to their synaptic target.[4,5] This process is driven by specific molecular cues which regulate the neuronal growth cone, residing at the tip of the axon.[5,6] Motility of the growth cone is, in part, regulated through remodelling of the actin cytoskeleton.[7,8]

Members of the Rho GTPase family are key modulators of cytoskeletal dynamics.[9,10] They play an essential role in neurodevelopment through the control of actin cytoskeleton dynamics, including motility of the growth cone.[11] Rho GTPases are activated by guanine nucleotide exchange factors (GEFs). *TRIO* encodes a guanine nucleotide exchange factor expressed ubiquitously in many tissues, including the central nervous system (CNS).[4,12] It is highly expressed in many areas of the developing brain including the cerebellum, cortex, hippocampi and thalami.[9,12] Interestingly, *TRIO* can be alternatively spliced, and as a result, can encode several isoforms whose expression is nervous-system specific.[9,13] Trio is thought to be one of the major regulators of neuronal development by controlling actin cytoskeleton remodelling through the activation of the GTPase Rac1.[14] The importance of Trio in neuronal development is further emphasised by the fact that total knock-out or specific deletion of *TRIO* in the mouse nervous system is embryonically lethal; embryos display defects in brain organisation and reduced brain size.[4,15] Deletion of *TRIO,* specifically in the hippocampus and in the cortex during early embryogenesis, results in aberrant organisation of these structures, impairing the learning ability of these mice.[12]

Our findings consolidate and extend the human phenotype recently reported by Ba et al.[3] Through the application of parent/offspring WES analysis, we report three children from across the UK with microcephaly and neurodevelopmental delay who harbour *de novo* missense mutations in *TRIO*. We also report a family with *TRIO* mutations that segregate autosomal dominantly. We show for the first time that *TRIO* mutations identified in patients affect Trio function and consequently could be responsible for the phenotype observed.

## METHODS

Patients 1, 2 and 3 were assessed at the Wessex Clinical Genetics Service (Figure 1). Exome sequencing of patients 2 and 3 was performed on DNA extracted from whole blood. Sequencing and

analysis were undertaken as previously described;[16] all variant positions reported are defined on GRCh37 (hg19) and *TRIO* transcript NM_007118. To validate the sample provenance, a parallel SNP panel was applied to confirm data identity.[17]

Patients 4, 5 and 6 were referred to regional Clinical Genetics services across the UK, where they were recruited to the Deciphering Developmental Disorders (DDD) study (http://www.ddduk.org). DDD has so far investigated over 4000 children with severe, undiagnosed developmental delay, and their parents, using a combination of genome-wide assays to detect all major classes of genetic variation in the protein-coding portion of the genome. Clinical information and phenotypes have been recorded using the Human Phenotype Ontology via a secure web portal within the DECIPHER database.[18,19] DNA samples from patients and their parents were analysed by the Wellcome Trust Sanger Institute using high-resolution microarray analysis (array-comparative genomic hybridisation (CGH) and SNP-genotyping) to investigate copy number variants (CNVs) in the affected child, and exome sequencing to investigate single nucleotide variants (SNVs) and small insertions/deletions (indels). Putative *de novo* sequence variants were validated using targeted Sanger sequencing. The population prevalence (minor allele frequency) of each variant in nearly 15 000 samples from diverse populations was recorded, and the effect of each genomic variant was predicted using the Ensembl Variant Effect Predictor.[20] Likely diagnostic variants in known developmental disorder genes were fed back to the referring clinical geneticists for external Sanger validation and discussion with the family via the patient's record in DECIPHER, where they can be viewed in an interactive genome browser. Full genomic datasets were also deposited in the European Genome–Phenome Archive (http://www.ebi.ac.uk/ega).

Functional analysis to assess input of variants was undertaken. *TRIO* point mutants were generated by introducing a point mutant in the wild-type (wt) form of GFP-tagged Trio using the Quick change site-directed mutagenesis kit (Stratagene). The Rac1-GTP pull-down assay was performed using the Cdc42/Rac1-interactive binding (CRIB) domain of PAK1 as described.[6] Total HEK293T lysates and corresponding pull-downs retained on GST-Sepharose beads were processed for Western blotting using the Rac1 (BD) and GFP (Clinisciences) antibodies.

## RESULTS

### Patients 1, 2 and 3

Patient 1 (see Figure 1) was whole exome sequenced and a frameshift deletion (p.Gln1489Argfs*11) in *TRIO* was identified, which was paternally inherited**.** This variant is predicted to cause nonsense-mediated decay of the transcript, and thus prevent expression of this allele.[21] She was last assessed at the age of 17 months and was displaying signs of mild developmental delay; she sat unsupported at 9 months and walked unaided at 17 months. First words were spoken at 17 months and there was

evidence of mild delay in expression and comprehension. She is a fussy eater and has dietetic input to increase calorific intake. There were some subtle behavioural signs including poor attention. She is microcephalic with a head circumference five standard deviations below the mean. Dysmorphic features included a short nose, long philtrum, thin upper lip, epicanthic folds, 2/3 toe syndactyly and an almost absent 5$^{th}$ toe nail. She has right radial aplasia, a rudimentary thumb and absent first metacarpal. She was found to have multiple cardiac septal defects at birth; at the age of three she has two small apical muscular ventricular septal defects and a patent foramen ovale, none of which are of concern clinically. The pregnancy was complicated by pre-existing maternal type II diabetes mellitus, which required insulin therapy. She also has a 15q11.2 microdeletion.

Patient 2 is the father of patient 1 and was genotyped using traditional Sanger methods. He harbours the same deletion in *TRIO* in addition to the same 15q11.2 microdeletion. He displayed mild learning difficulties in childhood and attended a specialist school. He is microcephalic with an occipital frontal circumference (OFC) 3 standard deviations below the mean. Other dysmorphic features include a straight nose, small jaw, pointed features, low anterior hair line, flattened thenar eminence, tapering digits, fifth finger clinodactyly and dental delay including absence or failed eruption of the lower central incisors and all four lateral molars.

Patient 3 is the uncle of patient 1 and brother of patient 2, who underwent whole exome sequencing. He harbours the same deletion in *TRIO* in addition to a pathogenic variant in *KCNJ2*. He has learning difficulties and is microcephalic with a head circumference 5 standard deviations below the mean. He has similar dysmorphic features to his brother including a straight nose, small jaw, low anterior hair line, pointed features and short and tapering fingers. He has a structurally normal heart but experiences ventricular ectopic beats which may be attributable to his co-existing variant in *KCNJ2*.[22] Similarly to his brother, he too has dental abnormalities with absence of any upper or lower lateral incisors.

**Patient 4**

Patient 4 has a *de novo* missense mutation in *TRIO* (p.Arg1428Gln). She was last assessed at the age of 16 years, when she had evident global developmental delay. From a gross motor perspective, she sat unsupported at 10 months and walked at 22 months. It was not possible to elucidate the age of her first words, however at the age of 16 years, although talkative, she remains unable to read or write. No feeding difficulties have been reported. This individual has numerous behavioural difficulties including stereotypies and obsessive compulsive traits. There was previous concern about aggressive and disruptive behaviour which has now resolved. She is microcephalic with an OFC 5.41 standard deviations below the mean. Some dysmorphism was noted including congenital ptosis, upslanting palpebral fissures, large fleshy ears and 5$^{th}$ finger clinodactyly. Neurological features include insensitivity to pain and urinary incontinence.

**Patient 5**

Patient 5 was last assessed at the age of 8 years. She has a *de novo* missense mutation in *TRIO* (p.Pro1461Thr). She has global developmental delay and is microcephalic with an OFC 5 standard deviations below the mean. She sat unsupported at 11 months and walked unaided at 2½ -3 years. Her first words were spoken between 4 and 5 years. Early feeding difficulties were reported including a poor suck, impaired bottle feeding and failure to thrive. Behavioural features include attention deficit hyperactivity disorder and poor sleep.

**Patient 6**

Patient 6 has a *de novo* missense mutation in *TRIO* (p.Asn1080Ile) and she was last assessed at the age of 9 years. She has global developmental delay, most notably affecting gross motor and language development. She sat unsupported at 11 months and walked unaided at 4-5 years. She has no verbal communication and utilises Makaton. She is still in nappies. There were some early feeding difficulties requiring gastrostomy; these have now resolved, although she remains with a thin body habitus. She has behavioural features that include hand stereotypies and aggressive episodes; she bites herself and can attack her younger brother. Head circumference is within the normal range. She has facial features that resemble Angelman-like facies with plagiocephaly. Neurological features include nocturnal tonic clonic seizures, and a wide-based and ataxic gait.

***TRIO* mutations**

The six patients presented in this study have mutations in *TRIO*, a gene containing 57 exons that encodes a distinct member of the Dbl family of GEFs, displaying strong conservation across evolution.[12] Alternative splicing of *TRIO* results in isoforms that harbour either one or both of the highly conserved DH-PH domains. Out of the six Trio isoforms, five contain the first DH-PH domain. Following stimuli from growth factor or cytokine receptors, each PH domain downstream of a DH domain uniquely targets a specific intracellular Rho-GDP complex and facilitates catalytic exchange of GDP for GTP. DH1-PH1 domains specifically activate RhoG and Rac1.

We set out to test whether the mutations identified in Trio could impact on its function and consequently contribute to the phenotypes observed in the six patients. The RhoGEF Trio is a major regulator of neuronal development, mainly through its activation of Rac1. Patients 1, 2 and 3 share a frameshift mutation (p.Q1489fs*) resulting in a truncated Trio protein (Figure 3); it would be expected that this truncated transcript would be degraded by nonsense-mediated decay, resulting in the production of negligible protein product. Patients 4, 5 and 6 harbour *de novo* missense mutations. Interestingly, among the four mutations identified, three lie within the GEFD1 domain, responsible for Rac1 activation (p.R1428Q, p.P1461T and p.Q1489fs*) (Figure 3C). Furthermore, the mutations affect highly conserved residues, since both R1428 and P1461 are present in Trio from invertebrates

to mammals (Figure 4B). They lie respectively within the alpha-5 and alpha-6 helices of the DH domain, which have been shown to interact with the target GTPase and occur at the protein-protein interface (Figure 4A). The truncated mutant of Trio, p.Q1489fs*, is missing the PH1 domain, which is shown to be required for efficient GDP/GTP exchange.[23] In contrast to the other 5 patients, individual 6 has a missense mutation (p.N1080I) within a spectrin repeat (Figure 3C).

We tested whether the mutations identified in our 6 patients affected Trio-mediated Rac1 activation (Figures 3D and 3E). We introduced four mutations individually into the Trio cDNA and expressed wt or mutant GFP-tagged Trio proteins in HEK293T cells. Rac1-GTP pull down experiments were performed using the Cdc42/Rac1-interactive binding (CRIB) domain of PAK1. As expected, Rac1-GTP was efficiently pulled down from cells expressing wt Trio. In contrast, the pull down of active Rac1 from cells expressing the Trio p.R1428Q, p.P1461T or the truncated mutant p.Q1489fs* was strongly reduced, albeit to a lesser extent than the fully dead GEF mutant used as a negative control (Q1427A/L1435E).[24]. The results obtained with the two point mutants in the GEFD1 domain are therefore consistent with a crucial function of these two residues in Rac1 activation. The p.N1080I mutant (in individual 6) did not affect Trio-mediated Rac1 activation. Taken together, our results reveal that in five out of six patients harbouring a mutation in the *TRIO* gene, Rac1 activation is strongly reduced, demonstrating that Trio function is affected in these patients.

**DISCUSSION**

*TRIO* is highly expressed in the developing brain; rat models show transcripts present in all major brain areas postnatally.[9,25] *TRIO* and its orthologues play a fundamental role in mammalian neuronal development, with mutations implicated in impaired neuronal migration, whereby abnormal function results in global brain reduction and aberrant structural developmental of the hippocampus, cerebellum and cortex.[4,15] Complete knockout mice are embryonically lethal; they have smaller brains and aberrant organisation in the hippocampus and hindbrain.[4,12,15] A mouse model recently reported with a *Trio* knockout specific to the hippocampus and brain cortex addressed the role of Trio in postnatal brain development, concluding that *Trio* regulates the neuronal development of the hippocampus and affects the learning ability and intelligence of adult mice.[12]

Five of the six patients we report, share a core phenotype comprising intellectual disability (ID), behavioural difficulties, subtle dysmorphism and microcephaly. This presentation aligns with the phenotype recently presented by Ba et al.,[3] who screened 2 300 ID cases and identified four truncating mutations in *TRIO*. Similarly, they described skeletal anomalies, short stature, feeding difficulties and facial asymmetry. Perhaps most strikingly however, are the similarities of the digital dysmorpology, most notably phalyngeal hypoplasia, swollen proximal interphalangeal joints (PIP) joints and tapering fingers shared between their study and our findings. The microcephalic presentation is likely to be primary in nature, in which the skull is small because the underlying brain

is small. Noteworthy, the microcephaly we describe is more severe than in the individuals reported by Ba et al.[3], whom all harboured loss of function mutations. Of interest, we showed that microcephaly was on present in individuals who harboured mutations in the GEFD1 domain affecting Rac1 activation by Trio. Since Trio function in the nervous system is mainly mediated by Rac1 activation, we can propose from these data that alteration of Rac1 activation by Trio variants is associated with microcephaly. Furthermore, we have recently shown that Trio is implicated in cell division by counteracting the effect of MgcRacGAP on Rac1 during cytokinesis.[26] Interestingly, many genes associated with cell cycle regulation have been found to be mutated in different forms of microcephaly.[27,28] Therefore, the microcephaly phenotype observed in patients with *TRIO* mutations could also originate from a cell cycle defect.

In contrast to patients 1 - 5, one individual in our study (individual 6) is somewhat of a phenotypic outlier; although she has ID, behavioural difficulties and dysmorphism, she is the only patient we report without microcephaly and whom has seizures, complete speech failure and plagiocephaly. Unlike individuals 1 – 5 whom have mutations affecting the PH-DH1 domain, individual 6 has a missense mutation which affects a spectrin repeat and was functionally shown not to affect Trio-mediated Rac1 activation (Figure 3). Spectrin repeats are important for Trio function as they bind many Trio regulators, such as NAV1, DISC1 and KidIns220.[14,29] Therefore, we can hypothesise that the mutant Trio, p.N1080I, is mediated by a pathway independent of Rac1 activation, and could impair the binding of Trio to its interactors, potentially leading to protein mislocalisation.

*TRIO* is expressed in many tissue types, however its role outside of the central nervous system remains to be fully elucidated. Interpretation of a truly specific *TRIO* phenotype is confounded by additional findings; notably a 15q11.2 microdeletion in patients 1 and 2 and a pathogenic *KCNJ2* variant in patient 3. Mutations in *KCNJ2* have been associated with Anderson-Tawil syndrome, short QT and familial atrial fibrillation,[22,30] likely explaining the ventricular ectopic beats seen in individual 3. It is also of note that individual 1 has right radial aplasia and ventricular septal defects, which may be secondary to an *in utero* environment complicated by maternal type II diabetes.[31]

Two of our probands displayed additional neurological signs including seizures, ataxia and pain insensitivity, and three of the probands had feeding difficulties. Furthermore, the intellectual disability reported was variable. The phenotypic variability could be related to the differing variant types (loss of function versus missense) and the importance of mutations affecting different exon positions within the DH-PH1 domain and their consequent effect on protein function. Of particular interest, there was little difference in phenotypic severity between individuals harbouring loss of function mutations versus missense mutations that did not significantly alter protein length, nor in the biological disruption of Trio mediated Rac1 activity.

**CONCLUSIONS**

| Phenotype | Patient 1 (Proband) | Patient 2 (Father) | Patient 3 (Paternal Uncle) | Patient 4 | Patient 5 | Patient 6 |
|---|---|---|---|---|---|---|



We are the first study to describe pathogenic missense mutations in *TRIO* and a *TRIO* syndrome that segregates in an autosomal dominant pattern. Our findings corroborate and extrapolate previous literature; we show that mutations in *TRIO* affecting the RhoGEF1 domain cause a phenotype comprising microcephaly, intellectual disability, behavioural difficulties and dysmorphism with specific digital features. We functionally prove that genotypic, patient-specific *TRIO* mutants cause protein dysfunction modulated through reduced Rac1 activity, and that microcephaly was only present in individuals who harboured mutations in the GEFD1 domain. It now remains to be established whether the phenotypic contribution of *TRIO* variants in patients are attributable to the function of Trio in cell division and/or neuronal differentiation.

Table 1. Clinical Phenotype

*(+) present; (-) absent; AD – autosomal dominant; ADHD – attention deficit hyperactivity disorder; DM – diabetes mellitus; F – female; M – male; NR – recorded but absent; PFO – patent foramen ovale; PIP – proximal interphalyngeal; RBBB – right bundle branch block; sd – standard deviations; VSD – ventricle septal defect*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Background** | *TRIO* Mutation | Frameshift | Frameshift | Frameshift | Missense | Missense | Missense |
| | Coding Change | c.4466delA | c.4466delA | c.4466delA | c.4283 G>A | c.4381 C>A | c.3239 A>T |
| | Protein Change | pGln1489Argfs*11 | pGln1489Argfs*11 | pGln1489Argfs*11 | p.Arg1428Gln | p.Pro1461Thr | p.Asn1080Ile |
| | Exon | Exon 30 | Exon 30 | Exon 30 | Exon 28 | Exon 29 | Exon 19 |
| | Domain affected | DH1 | DH1 | DH1 | DH1 | DH1 | Spectrin repeat |
| | MAF | Novel | Novel | Novel | Novel | Novel | Novel |
| | Inheritance | Inherited | Inherited | Inherited | AD *de novo* | AD *de novo* | AD *de novo* |
| | Sex | F | M | M | F | F | F |
| | Gestational Age (weeks) | 38 | - | - | 41 | 38 | 40 |
| **Development** | Age at Last Assessment | 17 months | 36 years | 10 years | 16 years | 8 years | 9 years |
| **Early Milestones** | 1st Smile | NR | NR | NR | 8 weeks | 36 weeks | NR |
| | Sitting Unsupported | 9 months | NR | NR | 10 months | 11 months | 11 months |
| | Walking Unaided | 17 months | NR | NR | 22 months | 2.5-3 years | 4-5 years |
| | First Words | 17 months | NR | NR | Unknown | 4-5 years | Unknown |
| **Current Developmental Level** | General | Mild developmental delay | Mild learning difficulties attended special school | Mild developmental delay | Global developmental delay | Global developmental delay | Global developmental delay |
| | Language | Mild delay in expression and comprehension | | Learning difficulties | Unable to read or write but very talkative | | Non-verbal (Makaton) |
| **Growth** | Height | 70cm (<0.4th) | 167cm (9th) | 126cm (2nd) | 159.4cm (25th) | 121.2cm (9th) | 121.6cm (2nd) |
| | Weight | 7.9kg (<0.4th) | 69.9kg (50th-75th) | 20.5kg (<0.4th) | 41.1kg (0.4th) | 21.2kg (9th) | 20.2kg (2nd) |
| | OFC | 42cm (-5sd) | 52cm (-3sd) | 48cm (-5sd) | 48cm (-5sd) | 47cm (-5sd) | 54.3cm (75th) |
| **Neurological** | Stereotypies | - | NR | NR | + | - | + |
| | Aggression | - | | | + | - | + |
| | Poor Attention | + | | | - | + ADHD | - |
| | Other | - | | | obsessive compulsive traits | disrupted sleep | - |
| | Other | - | - | - | Pain insensitivity, urinary incontinence | - | Seizures (nocturnal tonic clonic), gait ataxia |
| **Gastrointestinal** | Infantile feeding difficulties | + | NR | NR | - | + | + |
| | Other | Dietician input | | | Constipation | | Previous gastrostomy |
| **Skeletal** | Digits | Short tapering fingers with swelling of PIP joints5th finger cleinodactyly | Short tapering fingers with swelling of PIP joints and shortening of the metacarpals and phalanges | Short tapering fingers with swelling of PIP joints, 5th finger cleinodactyly, 2/3 left-sided toe syndactyly | Short tapering fingers with swelling of PIP joints, 5th finger cleinodactyly, 2/3 left-sided toe syndactyly | 2/3 toe syndactyly and almost absent 5th toe nail | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Spine | Rotational scoliosis, right radial aplasia and rudimentary thumb and absent metacarpal, no radial synestosis | Minor bony abnormalities of the low dorsal vertebrae with rotational scoliosis of the thoracolumbar spine | - | - | - | Thoracolumbar scoliosis |
| **Additional** | Facial | Short nose, long philtrum, thin upper lip, epicanthic folds, sonophyrs | Straight nose, small jaw, pointed features, low anterior hair line, asymmetry | Straight nose, small jaw, low anterior hair line with frontal upsweep of hair, pointed features, asymmetry | Congenital ptosis, upslanting palpebral fissures, large fleshy ears, sonophrys | flat nasal bridge, sonophrys | Plagiocephaly, Angelman-like facies, asymmetry |
| | Dental | NR | Dental delay | Dental delay | Dental overcrowding | NR | NR |
| | Other | 2 VSDs and PFO, Maternal Type II DM | NR | Asymptomatic ventricular ectopics with RBBB | Good musical memory | Strabismus, perforated ear drum | NR |
| | Additional Variants | 15q11.2 microdeletion | 15q11.2 microdeletion | KCNJ2 (p.T75M) | - | - | - |

## ACKNOWLEDGMENTS AND AFFILIATIONS

Thank you to all the patients and their families for participating in this study. We further extend our gratitude to the DDD study. The DDD study presents independent research commissioned by the Health Innovation Challenge Fund [grant number HICF-1009-003], a parallel funding partnership between the Wellcome Trust and the Department of Health, and the Wellcome Trust Sanger Institute [grant number WT098051]. The views expressed in this publication are those of the author(s) and not necessarily those of the Wellcome Trust or the Department of Health. The study has UK Research Ethics Committee approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC). The research team acknowledges the support of the National Institute for Health Research, through the Comprehensive Clinical Research Network.

## Funding

RJP is supported by the University of Southampton.

## COMPETING INTERESTS

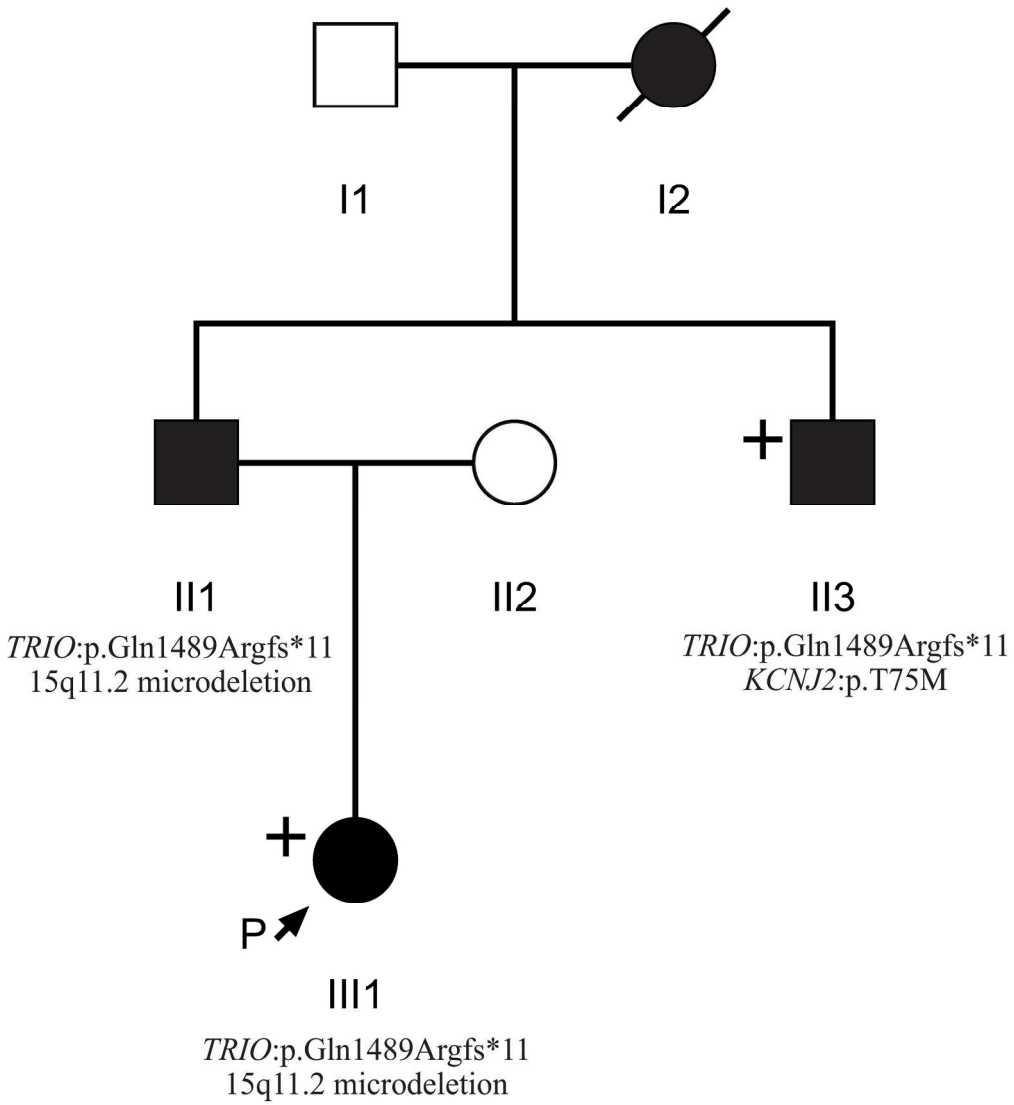
None to declare.

## REFERENCES

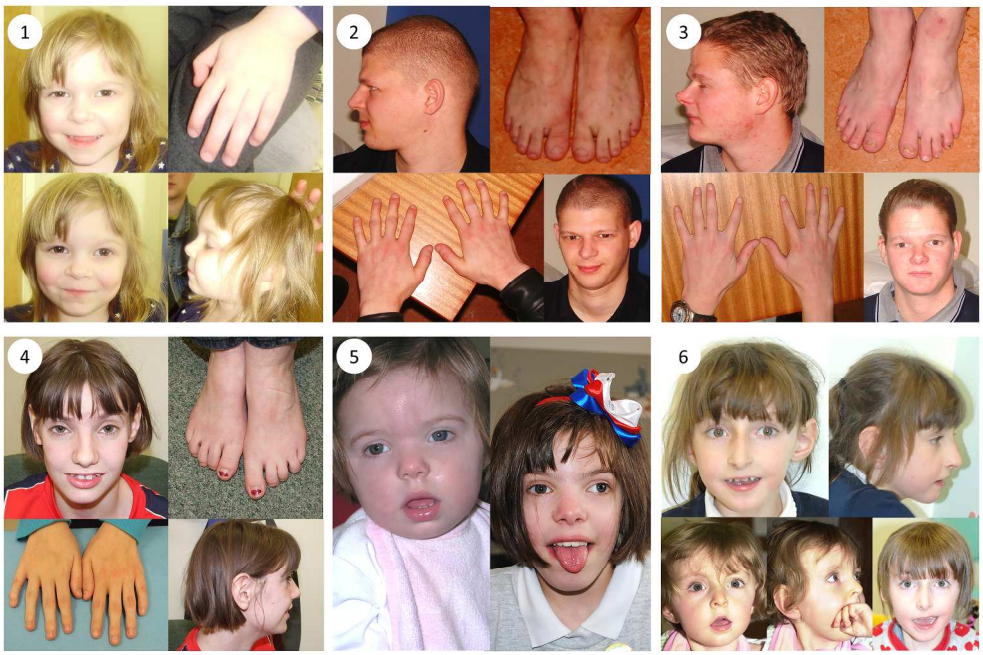
1. van Bokhoven H. Genetic and epigenetic networks in intellectual disabilities. Annu Rev Genet 2011;**45**:81-104

2. de Ligt J, Willemsen MH, van Bon BWM, Kleefstra T, Yntema HG, Kroes T, Vulto-van Silfhout AT, Koolen DA, de Vries P, Gilissen C, del Rosario M, Hoischen A, Scheffer H, de Vries BBA, Brunner HG, Veltman JA, Vissers LELM. Diagnostic Exome Sequencing in Persons with Severe Intellectual Disability. N Engl J Med 2012;**367**(20):1921-29 doi: doi:10.1056/NEJMoa1206524[published Online First: Epub Date]|.
3. Ba W, Yan Y, Reijnders MR, Schuurs-Hoeijmakers JH, Feenstra I, Bongers EM, Bosch DG, De Leeuw N, Pfundt R, Gilissen C. TRIO loss of function is associated with mild intellectual disability and affects dendritic branching and synapse function. Hum Mol Genet 2015:ddv618
4. Peng Y-J, He W-Q, Tang J, Tao T, Chen C, Gao Y-Q, Zhang W-C, He X-Y, Dai Y-Y, Zhu N-C. Trio is a key guanine nucleotide exchange factor coordinating regulation of the migration and morphogenesis of granule cells in the developing cerebellum. J Biol Chem 2010;**285**(32):24834-44
5. Huber AB, Kolodkin AL, Ginty DD, Cloutier J-F. Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. Annu Rev Neurosci 2003;**26**(1):509-63
6. Briançon-Marjollet A, Ghogha A, Nawabi H, Triki I, Auziol C, Fromont S, Piché C, Enslen H, Chebli K, Cloutier J-F. Trio mediates netrin-1-induced Rac1 activation in axon outgrowth and guidance. Mol Cell Biol 2008;**28**(7):2314-23
7. Dent EW, Gupton SL, Gertler FB. The growth cone cytoskeleton in axon outgrowth and guidance. Cold Spring Harbor perspectives in biology 2011;**3**(3):a001800
8. Dent EW, Gertler FB. Cytoskeletal dynamics and transport in growth cone motility and axon guidance. Neuron 2003;**40**(2):209-27
9. Ma XM, Huang JP, Eipper BA, Mains RE. Expression of Trio, a member of the Dbl family of Rho GEFs in the developing rat brain. J Comp Neurol 2005;**482**(4):333-48
10. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature 2002;**420**(6916):629-35
11. Govek E-E, Newey SE, Van Aelst L. The role of the Rho GTPases in neuronal development. Genes Dev 2005;**19**(1):1-49
12. Zong W, Liu S, Wang X, Zhang J, Zhang T, Liu Z, Wang D, Zhang A, Zhu M, Gao J. Trio gene is required for mouse learning ability. Brain Res 2015;**1608**:82-90
13. Portales-Casamar E, Briançon-Marjollet A, Fromont S, Triboulet R, Debant A. Identification of novel neuronal isoforms of the Rho-GEF Trio. Biol Cell 2006;**98**(3):183-93
14. Schmidt S, Debant A. Function and regulation of the Rho guanine nucleotide exchange factor Trio. Small GTPases 2014;**5**(4):e983880
15. O'Brien SP, Seipel K, Medley QG, Bronson R, Segal R, Streuli M. Skeletal muscle deformity and neuronal disorder in Trio exchange factor-deficient mouse embryos. Proceedings of the National Academy of Sciences 2000;**97**(22):12074-78
16. Pengelly RJ, Upstill-Goddard R, Arias L, Martinez J, Gibson J, Knut M, Collins AL, Ennis S, Collins A, Briceno I. Resolving clinical diagnoses for syndromic cleft lip and/or palate phenotypes using whole-exome sequencing. Clin Genet 2014:n/a-n/a doi: 10.1111/cge.12547[published Online First: Epub Date]|.
17. Pengelly RJ, Gibson J, Andreoletti G, Collins A, Mattocks CJ, Ennis S. A SNP profiling panel for sample tracking in whole-exome sequencing studies. Genome medicine 2013;**5**(9):89
18. Robinson PN, Mundlos S. The human phenotype ontology. Clin Genet 2010;**77**(6):525-34
19. Bragin E, Chatzimichali EA, Wright CF, Hurles ME, Firth HV, Bevan AP, Swaminathan GJ. DECIPHER: database for the interpretation of phenotype-linked plausibly pathogenic sequence and copy-number variation. Nucleic Acids Res 2013:gkt937
20. McLaren W, Pritchard B, Rios D, Chen Y, Flicek P, Cunningham F. Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. Bioinformatics 2010;**26**(16):2069-70
21. Hu J, Ng PC. Predicting the effects of frameshifting indels. Genome Biol 2012;**13**(2):R9
22. Statland JM, Tawil R, Venance SL. Andersen-Tawil Syndrome. 2013
23. Bellanger JM, Estrach S, Schmidt S, Briançon-Marjollet A, Zugasti O, Fromont S, Debant A. Different regulation of the Trio Dbl-Homology domains by their associated PH domains. Biol Cell 2003;**95**(9):625-34

24. Estrach S, Schmidt S, Diriong S, Penna A, Blangy A, Fort P, Debant A. The Human Rho-GEF trio and its target GTPase RhoG are involved in the NGF pathway, leading to neurite outgrowth. Curr Biol 2002;**12**(4):307-12
25. McPherson CE, Eipper BA, Mains RE. Multiple novel isoforms of Trio are expressed in the developing rat brain. Gene 2005;**347**(1):125-35 doi: http://dx.doi.org/10.1016/j.gene.2004.12.028[published Online First: Epub Date]|.
26. Cannet A, Schmidt S, Delaval B, Debant A. Identification of a mitotic Rac-GEF, Trio, that counteracts MgcRacGAP function during cytokinesis. Mol Biol Cell 2014;**25**(25):4063-71
27. Congenital microcephaly. American Journal of Medical Genetics Part C: Seminars in Medical Genetics; 2014. Wiley Online Library.
28. Barbelanne M, Tsang WY. Molecular and cellular basis of autosomal recessive primary microcephaly. BioMed research international 2014;**2014**
29. van Haren J, Boudeau J, Schmidt S, Basu S, Liu Z, Lammers D, Demmers J, Benhari J, Grosveld F, Debant A. Dynamic microtubules catalyze formation of navigator-TRIO complexes to regulate neurite extension. Curr Biol 2014;**24**(15):1778-85
30. Deo M, Ruan Y, Pandit SV, Shah K, Berenfeld O, Blaufox A, Cerrone M, Noujaim SF, Denegri M, Jalife J. KCNJ2 mutation in short QT syndrome 3 results in atrial fibrillation and ventricular proarrhythmia. Proceedings of the National Academy of Sciences 2013;**110**(11):4291-96
31. Grix A, Opitz JM. Malformations in infants of diabetic mothers. Am J Med Genet 1982;**13**(2):131-37
32. Mercer CL, Keeton B, Dennis NR. Familial multiple ventricular extrasystoles, short stature, craniofacial abnormalities and digital hypoplasia: a further case of Stoll syndrome? Clin Dysmorphol 2008;**17**(2):91-93
33. Skowronek KR, Guo F, Zheng Y, Nassar N. The C-terminal basic tail of RhoG assists the guanine nucleotide exchange factor trio in binding to phospholipids. J Biol Chem 2004;**279**(36):37895-907
34. Chhatriwala MK, Betts L, Worthylake DK, Sondek J. The DH and PH domains of Trio coordinately engage Rho GTPases for their efficient activation. J Mol Biol 2007;**368**(5):1307-20
35. DeLano WL. The PyMOL molecular graphics system. 2002

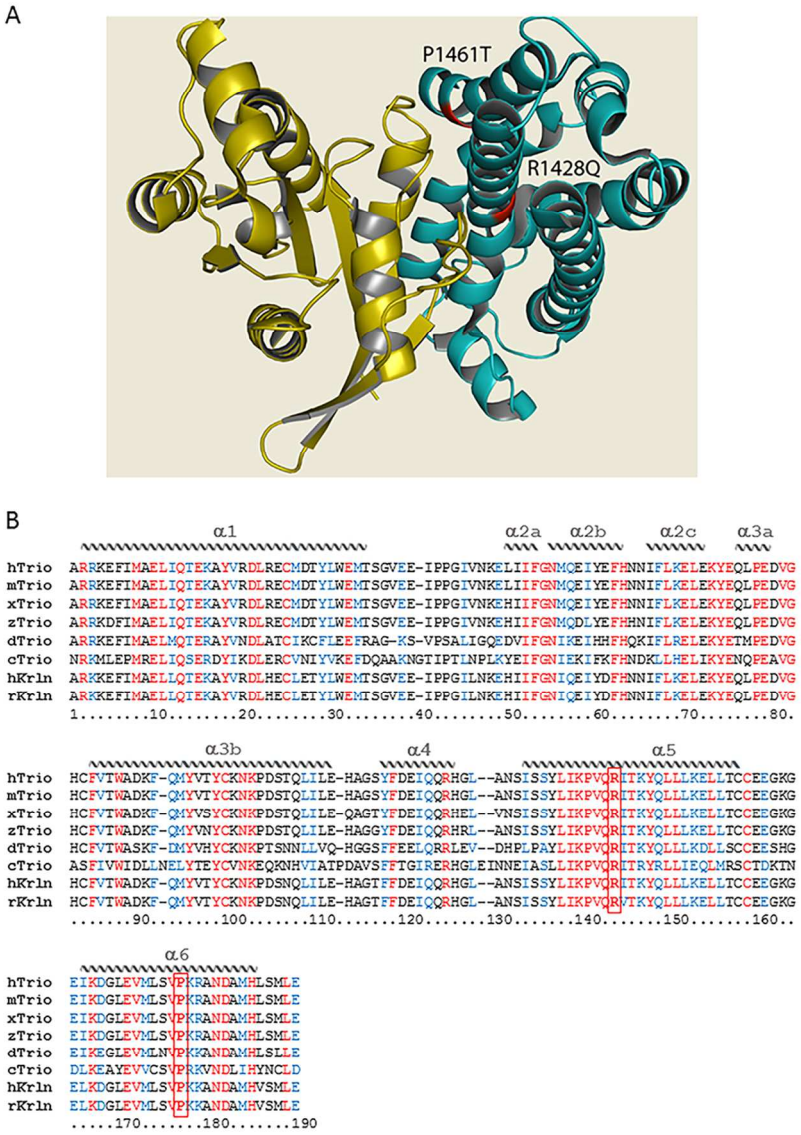



Pedigree of Individuals 1, 2 and 3 (III1, II1 and II2 respectively) obtained through the Wessex Clinical Genetics Service. Affected individuals are shaded in black. Individuals who underwent whole exome sequencing are labelled with a "+". Genotypic information confirmed by Sanger sequencing is displayed where known.
196x215mm (300 x 300 DPI)




Clinical Photographs

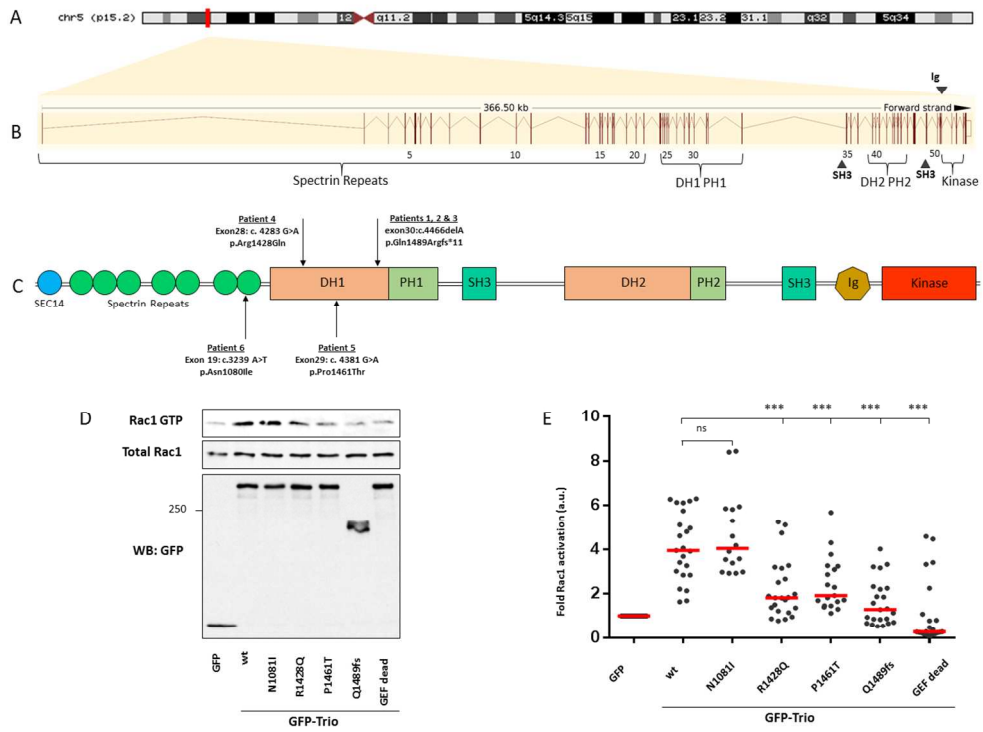
Photographs of individuals 1 to 6, taken at ages 3, 25 years, 22 years, 18, (2 & 11), and (18 months, 7 & 10) respectively. All individuals harbour TRIO mutations. Individual 1 is the daughter of individual 2. Individuals 2 and 3 are brothers and have been previously described.[32] Individuals 4, 5 and 6 are unrelated. Common features amongst studied individuals include microcephaly, mild dysmorphic facies, tapering fingers with prominent proximal interphalyngeal joints, 5th finger clinodactyly and 2/3 toe syndactyly.
254x169mm (300 x 300 DPI)




TRIO and its functional domains

(A) Genomic location of TRIO on the short arm of chromosome 5p15.2. (B) Graphical representation of the TRIO gene, comprising its 57 exons (vertical lines) spanning 366.5 Kb. Exons are numbered alongside their relative position to coding domains. (C) Schematic overview of the Trio protein and its domains alongside the four mutations identified in the 6 patients from this study. Trio displays three enzymatic domains. Each GEF module contains a catalytic domain, called Dbl Homology (DH) domain (in reference to Dbl, the first RhoGEF identified as an oncogene in mammalian cells), and a Pleckstrin-Homology (PH) domain that plays a role in GEF activation and localisation. The first GEF domain, GEFD1, activates Rac1 and RhoG. The second GEF domain, GEFD2, acts on RhoA. In addition, Trio harbours numerous accessory domains. Listed from the N-terminus to the C-terminus, these include: a CRAL-Trio/Sec14 motif; several spectrin-like repeats; two Src-Homology 3 (SH3) motifs; and an Immunoglobulin (Ig)-like domain. (D) Rac1-GTP pull down assay. HEK293T cells (transfected as indicated) were lysed and active GTP-Rac1 was affinity purified using the

Cdc42/Rac1-interactive binding (CRIB) domain of PAK1, immobilised on Glutathione-Sepharose beads. Purified GTP-bound and total Rac1 were detected by Western blot, using an anti-Rac1 antibody. Protein expression in the cell lysates was verified by immunoblotting with an anti-GFP antibody. One representative experiment is shown. (E) Quantification of the Rac1-GTP pull down assay shown in (D). Rac1 activation mediated by wt Trio was arbitrarily set to 100%, in order to be able to compare the individual experiments. The % of Rac activation was calculated from at least six independent experiments (mean ±SEM). *p < 0.015, ***p < 0.0001. Of note, all three mutations lying in the DH1 domain strongly affect Trio-mediated Rac1 activation.
84x122mm (300 x 300 DPI)




The Structure of Trio

(A) Quaternary structure of the DH domain of Trio (teal) in complex with the small GTPase substrate (yellow). Mutations within the DH seen in patients 4 and 5 are indicated in red; both mutations can be seen to occur at the protein-substrate interface. PDB IDs: DH domain – 1NTY; GTPase substrate – 1KZ7.[33,34] Figure generated using PyMOL.[35] (B) Sequence alignment of the DH1 domain of Trio (and Kalirin) across evolution.Sequences were obtained from NCBI databases and aligned with Clustal Omega. Identical residues are labelled in red, similar residues in blue. The structural features of the protein domain (alpha- helices) are depicted schematically and labelled on top of the sequence. The position of the mutations R1428Q and P1461T are indicated in bold and boxed in red. Note that R1428Q and P1461T each affect a highly conserved residue within a very conserved region of the DH domain, in helices alpha-5 and alpha-6. Helices alpha-1, alpha-3b, alpha-5 and alpha-6 make contact with the target GTPase. Represented species are: homo sapiens (h); mus musculus (m); rattus norvegicus (r); xenopus laevis (x); danio rerio (z); drosophila melanogaster (d); caenorhabditis elegans (c).
355x266mm (96 x 96 DPI)