RAC1 missense mutations in developmental disorders with diverse phenotypes

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Abstract

RAC1 is a widely studied Rho GTPase, a class of molecules that modulate numerous cellular functions essential for normal development. RAC1 is highly conserved across species and is under strict mutational constraint. We report seven individuals with distinct *de novo* missense *RAC1* mutations and varying degrees of developmental delay, brain malformations and additional phenotypes. Four individuals each carrying one of c.53G>A;p.(Cys18Tyr), c.116A>G;p.(Asn39Ser), c.218C>Y;p.(Pro73Leu) and c.470G>A;(p.Cys157Tyr) variants were microcephalic, with head circumferences between -2.5 to -5 SD. In contrast, two individuals carrying the c.151G>A;p.(Val51Met) and c.151G>C;p.(Val51Leu) alleles were macrocephalic with head circumferences of +4.16 and +4.5 SD. One individual carrying the c.190T>G;p.(Tyr64Asp) allele had head circumference in the normal range. Collectively, we observed an extraordinary spread of ~10 SD of head circumferences orchestrated by distinct mutations in the same gene. *In silico* modeling, mouse fibroblasts spreading assays and *in vivo* overexpression assays using zebrafish as a surrogate model demonstrated that the p.(Cys18Tyr) and p.(Asn39Ser) *RAC1* mutations function as dominant negative alleles and result in microcephaly, reduced neuronal proliferation and cerebellar abnormalities *in vivo*. Conversely, the p.Tyr64Asp mutation is constitutively active. The effects of the remaining mutations is probably context dependent. These findings highlight the importance of *RAC1* in neuronal development. Along with TRIO and HACE1, a sub-category of rare developmental disorders is emerging with RAC1 as the central player. We show that ultra-rare disorders caused by private, non-recurrent missense mutations that result in varying phenotypes, are challenging to dissect, but can be delineated through focused international collaboration.

Main text

Developmental disorders (DDs) are etiologically extremely heterogeneous and affect 2–5% of individuals.[1; 2] *De novo* mutations account for a substantial proportion of DDs and are thought to underlie approximately 400,000 new DD cases world-wide annually.[3] Recently, large-scale next generation sequencing studies have led to the identification of several DD-associated genes that lead to clinical manifestations through protein truncating or recurrent missense variants.[4-6] However, rare disorders caused by private, non-recurrent missense mutations that result in varying phenotypes, remain challenging to dissect.[7]

Several human DDs are known to result from mutations in members of the RAS superfamily of small GTPases.[8] The RAS superfamily is further divided into smaller families, one of which is the 22-member Rho family. Rho GTPases cycle between active GTP-bound, and inactive GDP-bound states. Their activity is regulated by guanine nucleotide exchange factors (GEFs), GTPases activating proteins (GAPs) and guanine nucleotide dissociated inhibitors (GDIs).[9] Rho GTPases modulate essential cellular functions, including cell polarity, migration, vesicle trafficking and cytokinesis, and play crucial roles in neuronal development, neuronal survival and neurodegeneration.[10-12] However, no human DDs caused by mutations in genes encoding Rho GTPases are known.

One of the most widely studied Rho GTPases is the RAS-related C3 Botulinum Toxin Substrate 1 (RAC1 [MIM602048]).[13] RAC1 is part of the RAC Rho GTPases subfamily that also includes RAC2 [MIM602049], RAC3 [MIM602050] and RhoG [MIM179505].[14] RAC1 is an important modulator of the cytoskeleton, with a critical function in phagocytosis, mesenchymal-like migration, neuronal polarization, axonal growth, adhesion and differentiation of multiple cell types.[10; 15; 16]Additionally, it is involved in cellular growth and cell-cycle regulation via mTOR signaling.[17] In mouse studies, Rac1 is required for the formation of three germ layers during gastrulation with *Rac1*-knockout mice being embryonic lethal.[18] Conditional forebrain-specific *Rac1*-knockout mice display impaired neuronal

migration, abnormal dendritic growth and remodelling, disruption of lamellipodia formation, reduced neuronal proliferation, premature differentiation and microcephaly.[19-21] Here, we report *de novo* missense *RAC1* mutations in individuals with DD with divergent phenotypes.

All procedures followed were in accordance with the ethical standards of the institutional and national responsible committees on human experimentation and that proper informed consent was obtained. Review of data from 4,293 families, who underwent trio whole exome sequencing (WES) as part of the Deciphering Developmental Disorders study[3] led to identification of three individuals with *de novo RAC1* (NM_006908) missense mutations - individual 3 with c.218C>T, p.(Pro73Leu); individual 5 with c.190T>G, p.(Tyr64Asp); and individual 6 with c.151G>A, p.(Val51Met) (Table 1; Figures 1 and 2). Two additional individuals were independently ascertained through family-based diagnostic WES - individual 1 with c.53G>A, p.(Cys18Tyr) in and individual 2 with c.116A>G, p.(Asn39Ser) (Table 1; Figures 1 and 2). While functional studies were ongoing for the five individuals, two additional individuals were identified via the GeneMatcher tool[22] or through international collaboration: individual 4 with c.470G>A, p.(Cys157Tyr); and individual 7 with c.151G>C, p.(Val51Leu); (Table 1; Figures 1 and 2).

Human *RAC1* (ENSG00000136238) encodes six transcripts, of which two are protein coding: RAC1 and RAC1B (Figure 1A). Of the two protein-coding transcripts, NM_006908 (RAC1, ENST00000348035.8) lacks exon 4 and encodes the shorter RAC1 isoform of 192 amino acid, which is ubiquitously expressed in all tissues.[23] *RAC1* is under strict mutational constraint with only 15 missense variants observed versus 75.9 expected in ~60,000 exomes catalogued in the ExAC database (z-score = 3.42).[24] Moreover, 7 of these 15 observed missense variants are located in exon 4, which is included only in RAC1B (ENST00000356142.4) that encodes the longer isoform and is mainly expressed in gastro-intestinal and epithelial tissues.[25] Of particular note, there are no missense variants in exons 1, 2, 3 and 5 in the ExAC database (Figure 1B). Six out of seven mutations

described here are in exons 2 and 3. The p.Cys157Tyr change (p.Cys176Tyr in the longer RAC1B transcript) lies in a sub-region of exon 7 with no known germline human missense variants (Figure 1B). None of the identified mutations were present in any of the in-house variant databases of the four centers participating in this study. All six amino acids affected by the seven mutations are highly conserved among different species (Figure 1C). All reported *RAC1* mutations are within or in proximity to G box residues and/or conserved residues present in 90% of the RAS superfamily members (Figure 1C).[26] Collectively, the genetic data were strongly supportive of deleteriousness for each of the seven mutations.

Informed consent for publication of photographs was obtained from legal guardians. Detailed clinical information was collected on all affected individuals (Table 1; Figure 2) (See Case reports in Supplementary information). All seven individuals (age range 4.5 months – 15 years) had moderate to severe intellectual disability (ID) and variable degrees of neurological involvement including hypotonia (4/7), epilepsy (3/7), behavioral problems (3/7) and stereotypic movements (2/7). However, their occipital frontal circumferences (OFC) were remarkably different: individuals 1, 2 and 3 and 4 were microcephalic (OFCs of -2.5, -3, -5 and -2.5 SD respectively); individual 5 with p.Tyr64Asp mutation had a normal OFC (+1 SD); and individuals 6 and 7, both with mutations affecting p.Val51, were macrocephalic (OFCs of +4.16 and +4.5 SD respectively) (Table 1; Figure 2). Hypoplasia of the corpus callosum and the cerebellar vermis were the commonest features observed on available Magnetic resonance imaging (MRI) studies of individuals with microcephaly. However, they all had additional abnormalities (Case reports in Supplementary information; Figure 2). Individual 5 (with normal OFC) was reported to have polymicrogyria and hypoplastic corpus callosum (images not available). The two individuals with macrocephaly (individuals 6 and 7) showed periventricular white matter lesions (Table 1; Figure 2). Arched eyebrows, dysplastic ears, prominent nasal bridges and overhanging columellae were shared between a majority of individuals without macrocephaly (Case reports in Supplementary information; Figure 2), but their facial dysmorphism

does not overlap sufficiently to make this condition recognizable via their gestalt. Both individuals with macrocephaly displayed prominent broad foreheads, open mouth appearance and scooped out appearance on lateral view (Figure 2). Collectively, the clinical data suggested a remarkable phenotypic variability in our cohort.

We mapped each of the seven identified mutations onto the available crystal structure of human RAC1 (Protein Data Bank code 3TH5) (Figure 1D) to gain insights into their effect on the protein structure and function. The mutation-induced putative effects could be categorized into three groups: (i) p.Cys18 and p.Cys157 are respectively located in and adjacent to the guanine nucleotide binding site that binds GTP/GDP. These two cysteine thiols can be oxidized by glutathionylation, a post-translational modification that alters GTP binding and exchange activities of RAC1.[27] The p.Cys18Tyr and p.Cys157Tyr substitutions introduce a bulky aromatic residue in place of the thiol group and are expected to impact GTPase activity either by directly interfering with GTP binding, or indirectly by abolishing the post-translational modification; (ii) p.Asn39 is part of the Switch I motif, while p.Tyr64 and p.Pro73 are respectively within and adjacent to the Switch II motif. Both Switch motifs are highly conserved regions involved in the interactions with various GEFs and GAPs (e.g. Rex1, DOCK)[28], mediating the conformational changes for guanine nucleotide exchange (Figure S1), and downstream effectors. Missense mutations affecting these residues are likely to impact the protein-protein interactions directly; (iii) p.Val51 has no obvious involvement in GTP binding or interactions with GEF/GAPs. Free energy calculations by FoldX (in the SNPeffect 4.0 server)[29] revealed a reduction of protein stability for these two substitutions (ΔΔG of 2.13 and 1.94 kcal/mol for p.Val51Met and p.Val51Leu respectively).

RAC1 is known to regulate the spreading of fibroblasts plated onto fibronectin.[30] The genetic data and *in silico* modeling suggested that the phenotypes are unlikely to result from haploinsufficiency. We reasoned that the *RAC1* mutations identified in this studycould be dominant negative or acting

as constitutively active. If so, these mutations should result in changes in fibroblast spreading. To test this hypothesis, we introduced selected *RAC1* mutations in NIH3T3 fibroblasts. Mammalian expression plasmids encoding GFP-Rac1, GFP-Rac1-T17N and GFP-Rac1-Q61L were obtained from Prof Viki Allan (Manchester). A Quikchange Lightning kit (Agilent Technologies) was used to introduce point mutations in the GFP-Rac1 expression plasmid. We generated 6/7 mutations identified in this study (p.Cys18Tyr, p.Asn39Ser, p.Val51Met, p.Tyr64Asp, p.Pro73Leu and p.Cys157Tyr) along with p.Thr17Asn that is known to have a dominant negative effect[31] and p.Gln61Leu which is known to result in constitutive protein activation.[32] NIH3T3 fibroblasts were cultured in a 24 well plate and were transfected with the Rac expression plasmids 24hr after plating using Fugene 6 reagent (Promega). 48 h after transfection, the fibroblasts were trypsinised then replated onto fibronectin coated coverslips. 30 min after re-plating, the coverslips were rinsed with PBS and fixed with 4% paraformaldehyde (PFA). The fixed cells were then permeabilised with 0.1% Triton-X100 in PBS followed by blocking with 1% BSA in PBS. The cells were stained with a rabbit anti-GFP (Invitrogen) antibody (1:500), followed by an Alexa-488 anti-rabbit secondary antibody (Invitrogen) and Alexa568-phalloidin (Invitrogen) before mounting in Prolong Gold (Invitrogen). Cells were imaged on a Nikon A1R confocal microscope, using a 60x 1.4NA oil objective.

Cell circularity values were obtained using ImageJ software. Cell perimeters were identified using the ImageJ 'threshold' function and then circularity index ($4\pi \times \text{area}/\text{perimeter}^2$) calculated using the 'analyze particles' function. Circularity datasets were statistically analyzed using one-way ANOVA with Dunnett's correction for multiple comparisons. At least 50 cells were analyzed for each dataset except Asn39Ser, Tyr64Asp, Cys157Tyr (>40 cells) and Gln61Leu (25 cells). Data was pooled from three independent experiments and highly-expressing cells were excluded. Morphology was also assessed qualitatively by classifying cells according to their predominant actin protrusion type. Three categories were used: (i) >50% of cell perimeter occupied of filopodia; (ii) >50% of perimeter

occupied by lamellipodia/ruffles; and (iii) mixed protrusions, with neither type occupying 50% of the perimeter.

The majority (51%) of cells transfected with wild-type Rac1 exhibited mixed protrusions, with the remaining cells split almost equally between those in which >50% the perimeter was occupied by filopodia or lamellipodia/ruffles (Figure 3A). As observed previously, lamellipodia formation was inhibited by transfecting the known dominant negative p.Thr17Asn variant, resulting in a filopodia-rich cell perimeter and a significantly reduced circularity index (Figure 3A-C).[30] By contrast, expression of the known constitutively active p.Gln61Leu variant resulted in virtually all cells exhibiting large lamellipodia or membrane ruffles, and a significantly increased circularity index.[33] Expression of the p.Cys18Tyr and and p.Asn39Ser, mutations (seen in individuals 1 and 2 with microcephaly) resulted in a phenotype reminiscent of dominant negative Rac1, with an increase in the proportion of cells rich in filopodia and a reduction in cells rich in lamellipodia/ruffles (Figure 3A,C). Consistently, these mutations exhibited a significantly decreased circularity index (Figure 3B). By contrast, cells transfected with the p.Tyr64Asp mutation resulted in a phenotype more reminiscent of constitutive active Rac1, with significantly increased circularity index and a greater proportion of cells exhibiting lamellipodia or ruffles (Figure 3A-C). Cells expressing p.Val51Met, p.Pro73Leu and p.Cys157Tyr all showed a tendency towards increased filopodia and reduced lamellipodia, but did not result in a significant change in circularity index relative to cells expressing wild-type Rac, suggesting at most a modest impact on Rac function in this assay (Fig. 3A-C). All the *Rac1* mutation constructs exhibited similar cellular localization to wild-type Rac1 with the exception of p.Tyr64Asp, which appeared to localize strongly to the leading edge of ruffles and lamellipodia (Fig. 3C).

Next, we sought to explore the *in vivo* effects of *RAC1* mutations in zebrafish embryos. Towards this, we identified *rac1a* as the sole zebrafish ortholog with highest degree of homology to the human

RAC1 (90% similarity, 90% identity) and used the CRISPR/Cas9 system to introduce deletions, as a way to explore the phenotypic effects induced by loss of function of *RAC1*. Guide RNAs targeting the *Danio rerio* coding region of *rac1* were generated as previously described.[34; 35] We observed sequence aberrations in 30% of the evaluated *rac1* clones (Figure S2A). Assessment of mosaic F0 embryos injected with a guide against exon 2 did not result in statistically different head size counts between F0 CRISPR and control embryos (Figure S2B). No overt morphological changes were observed in the genetically edited embryos. We next considered a dominant effect of the *RAC1* alleles. To test this hypothesis, we cloned the human wild-type *RAC1* mRNA (NM_018890) into the pCS2+ vector and transcribed *in vitro* using the SP6 Message Machine kit (Ambion). The mouse fibroblasts spreading assays had indicated that dominant negative effects could be common mechanism for *RAC1* mutations. We, therefore, introduced the two mutations (p.Cys18Tyr and p.Asn39Ser) that were shown to be the strongest phenocopies of the known dominant negative mutation using Phusion high-fidelity DNA polymerase (New England Biolabs) and custom-designed primers. Additionally, for sake of comparison, we also introduced the known dominant negative (p.Thr17Asn) and constitutively active (p.Gln61Leu) mutations. Based on the dose-curve for the titration of the effect that WT *RAC1* had on the headsize phenotype (Figure S3) we injected 50pg of WT or mutant RNA into wild-type zebrafish embryos at the 1-to 4-cell stage. The injected larvae were grown to 5 dpf and imaged live on dorsal view. The area of the head was traced excluding the eyes from the measurements and statistical significance was calculated using student`s t-test. All experiments were repeated three times and scored blind to injection cocktail. Injection of p.Thr17Asn, p.Cys18Tyr or p.Asn39Ser in zebrafish embryos induced a significant decrease in head-size ($p<0.0005$) compared to controls (Figures 4A and 4B). Injection of p.Gln61Leu induced a significant increase in head-size ($p<0.0001$) compared to controls (Figures 4A and 4B). Importantly, the head-size could not be rescued by co-injection of mutant *RAC1* with WT message (Figure S4) arguing further in favor of these *RAC1* mutations acting as dominant alleles in vivo, consistent with our results of mouse fibroblasts spreading assay.

Driven by the fact that RAC1 is involved in neuronal proliferation[20], we next assessed neuronal proliferation in the brain of embryos injected with WT or a subset of mutant *RAC1* (p.Cys18Tyr or p.Asn39Ser). To do this, the injected embryos were fixed overnight at 48 hours post fertilization (hpf) in Dent's fixative (80% methanol, 20% DMSO) at 4°C. The embryos were first rehydrated in progressively decreasing methanol solutions, bleached with 10% H_2O_2, 0.5% KOH and 0.1% Triton-X. After two washes in PBS, the tissue was permeabilized with proteinase K followed by post-fixation with 4% PFA. PFA-fixed embryos were washed first in PBS and subsequently in IF buffer (0.1% Tween-20, 1% BSA in PBS) for 10 minutes at room temperature. The embryos were incubated in the blocking buffer (10% FBS, 1% BSA in PBS) for 1 hour at room temperature. After two washes in IF Buffer for 10 minutes each, embryos were incubated overnight at 4°C with 1:500 phospho histone 3 (PH3, a marker for proliferating cells) primary antibody (ser10)-R (sc-8656-R, rabbit, Santa Cruz) in blocking solution. After two additional washes in IF Buffer for 10 minutes each, embryos were incubated in the secondary antibody solution [Alexa Fluor goat anti-mouse IgG (A21207, Invitrogen) and donkey anti-rabbit (A21206, Invitrogen), 1:1000] in blocking solution, for 1 hour at room temperature. Proliferating cells were quantified by counting all positive cells on a dorsal view of a 48hpf embryos, excluding the eyes from the scored area, using the ITCN ImageJ plugin that counts cells with 10 pixel width and 5 pixel minimum distance between them in order to be considered as separate cells. Statistical significance for this assay was established using student`s t-test. This assay showed significantly reduced cellular proliferation for both mutants ($p=0.0012$ for p.Cys18Tyr and $p=0.0018$ for p.Asn39Ser when compared to WT *RAC1*; Figure 4C, 4D).

Finally, armed with prior knowledge that RAC1 has a known role in cerebellar development[36] together with the observation that cerebellar abnormalities were reported in 3/7 individuals in our cohort, we sought to study the effect of the p.Cys18Tyr and p.Asn39Ser alleles on cerebellar development of our zebrafish model. For the assessment of the cerebellum, embryos were fixed at

72hpf in Dent's fixative and subsequently whole-mount stained following the same staining protocol as for neuronal proliferation and using a primary antibody against acetylated tubulin (1:1000; Thr7451, mouse, Sigma-Aldrich). The embryos were then scored qualitatively assaying the integrity of the cerebellum by scoring for the presence and organization of axons along the midline of the structure (highlighted with a green dashed box; Figure 4E) and statistical significance was determined using a χ^2-test. Structural defects in the integrity of the cerebellum consisted of depletion of the axons that cross the midline were observed only upon overexpression of *RAC1*p.Cys18Tyr (p=0.01) but not *RAC1*p.Asn39Ser (p=0.2) (Figure 4E, 4F). The latter is likely consistent with the more pronounced cerebellar defect observed in individual 1 with p.Cys18Tyr mutation (Figure 2B).

In summary, we report seven individuals with *de novo* missense *RAC1* mutations and variable developmental delay with additional features. Remarkably, the OCFs observed in this study ranged from -5SD to +4.5SD. Previously, some chromosomal regions have been associated with both microcephaly (deletions) and macrocephaly (duplications).[37-39] However, it is extremely rare that point mutations within the same gene can cause differences of such magnitude (~10 SD) in head size of affected individuals. It is interesting to note that RAC1 is involved in mTOR signaling and other disorders in this pathway also result in significant alterations in head size.[17; 40; 41] The variability of RAC1-phenotypes appear to be dependent on specific mutations, although the contribution of genetic background cannot be ruled out. *In silico* modeling, mouse fibroblasts spreading assays and zebrafish experiments demonstrate that some *RAC1* mutations (p.Cys18Tyr, and p.Asn39Ser) phenocopy known dominant negative allele (p.Thr17Asn) and result in reduced neuronal proliferation, microcephaly and cerebellar abnormalities *in vivo*. On the other hand, the *in vitro* effects of p.Tyr64Asp, seen in affected individual 5 with OFC within the normal range, are similar to the known constitutively active *RAC1* mutation. Of note, *in vitro* expression of dominant negative and constitutively active *Rac1* have been previously shown to cause opposite effects on dendritic

growth and morphology.[18] However, the link between the human mutations and the resultant phenotypes is likely to be complex depending on the balance between interactors, regulators and effectors of RAC1 signaling.[42] This is likely to be especially true for other *RAC1* mutations identified in this study that could not be clearly classed as being either dominant negative or constitutively active (p.Val51Met, p.Pro73Leu and p.Cys157Tyr). Further studies will be required in the future to uncover the precise underlying mechanisms of phenotypic variability of *RAC1* mutations.

Our findings show that mutations in genes encoding members of the RhoGTPases-family can cause DDs. Of note, mutations in *TRIO* [MIM601893], a gene that encodes a RAC1 GEF, have been shown to result in mild intellectual disability.[43; 44] Interestingly, missense mutations in its RAC-GEF domain result in a more severe phenotype with global developmental delay, microcephaly and reduced RAC1 activity.[43] Furthermore, bi-allelic mutations in *HACE1* [MIM610876], a known interactor of RAC1, have been recently shown to result in an autosomal recessive syndrome with macrocephaly.[45] Notably, overexpression of a WAVE mutant has been demonstrated to partially rescue axon growth in *Rac1*-knock-out neurons[19] suggesting some of this emerging group of conditions could be potentially treatable. Overall, a potentially treatable sub-category of rare developmental disorders appears to be emerging with RAC1 as the central player.

Finally, our results show that ultra-rare disorders caused by private, non-recurrent missense mutations, resulting in varying phenotypic effects severity and degrees of severity, are challenging to dissect but can be delineated through focused international collaborations.

Supplemental Data description:

1. Supplemental Note: Case Reports

2. Figure S1: Crystal structure of the RAC1-DOCK2 complex

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

4. Figure S3: Phenotype caused by d*e novo* mutations Cys18Tyr and Asn39Ser in *RAC1* cannot be antagonized by concomitant overexpression of WT *RAC1* message.

Web resources

OMIM: https://www.ncbi.nlm.nih.gov/omim

ExAC: http://exac.broadinstitute.org/gene/ENSG00000136238

ImageJ Software: https://imagej.net/Welcome

Molsoft: http://www.molsoft.com/icm_pro.html

Protein Data Bank: http://www.rcsb.org/pdb/home/home.do

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References

1. Sheridan, E., Wright, J., Small, N., Corry, P.C., Oddie, S., Whibley, C., Petherick, E.S., Malik, T., Pawson, N., McKinney, P.A., et al. (2013). Risk factors for congenital anomaly in a multiethnic birth cohort: an analysis of the Born in Bradford study. Lancet 382, 1350-1359.
2. Ropers, H.H. (2010). Genetics of early onset cognitive impairment. Annual review of genomics and human genetics 11, 161-187.
3. Deciphering Developmental Disorders, S. (2017). Prevalence and architecture of de novo mutations in developmental disorders. Nature 542, 433-438.
4. de Ligt, J., Willemsen, M.H., van Bon, B.W., Kleefstra, T., Yntema, H.G., Kroes, T., Vulto-van Silfhout, A.T., Koolen, D.A., de Vries, P., Gilissen, C., et al. (2012). Diagnostic exome sequencing in persons with severe intellectual disability. The New England journal of medicine 367, 1921-1929.
5. Iossifov, I., O'Roak, B.J., Sanders, S.J., Ronemus, M., Krumm, N., Levy, D., Stessman, H.A., Witherspoon, K.T., Vives, L., Patterson, K.E., et al. (2014). The contribution of de novo coding mutations to autism spectrum disorder. Nature 515, 216-221.
6. Deciphering Developmental Disorders, S. (2015). Large-scale discovery of novel genetic causes of developmental disorders. Nature 519, 223-228.
7. MacArthur, D.G., Manolio, T.A., Dimmock, D.P., Rehm, H.L., Shendure, J., Abecasis, G.R., Adams, D.R., Altman, R.B., Antonarakis, S.E., Ashley, E.A., et al. (2014). Guidelines for investigating causality of sequence variants in human disease. Nature 508, 469-476.
8. Rauen, K.A. (2013). The RASopathies. Annual review of genomics and human genetics 14, 355-369.
9. Van Aelst, L., and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. Genes & development 11, 2295-2322.
10. Heasman, S.J., and Ridley, A.J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nature reviews Molecular cell biology 9, 690-701.
11. Duquette, P.M., and Lamarche-Vane, N. (2014). Rho GTPases in embryonic development. Small GTPases 5, 8.
12. Stankiewicz, T.R., and Linseman, D.A. (2014). Rho family GTPases: key players in neuronal development, neuronal survival, and neurodegeneration. Frontiers in cellular neuroscience 8, 314.
13. Aspenstrom, P., Fransson, A., and Saras, J. (2004). Rho GTPases have diverse effects on the organization of the actin filament system. The Biochemical journal 377, 327-337.
14. Boureux, A., Vignal, E., Faure, S., and Fort, P. (2007). Evolution of the Rho family of ras-like GTPases in eukaryotes. Molecular biology and evolution 24, 203-216.
15. Tahirovic, S., Hellal, F., Neukirchen, D., Hindges, R., Garvalov, B.K., Flynn, K.C., Stradal, T.E., Chrostek-Grashoff, A., Brakebusch, C., and Bradke, F. (2010). Rac1 regulates neuronal polarization through the WAVE complex. The Journal of neuroscience : the official journal of the Society for Neuroscience 30, 6930-6943.
16. Wojnacki, J., Quassollo, G., Marzolo, M.P., and Caceres, A. (2014). Rho GTPases at the crossroad of signaling networks in mammals: impact of Rho-GTPases on microtubule organization and dynamics. Small GTPases 5, e28430.
17. Saci, A., Cantley, L.C., and Carpenter, C.L. (2011). Rac1 regulates the activity of mTORC1 and mTORC2 and controls cellular size. Molecular cell 42, 50-61.
18. Sugihara, K., Nakatsuji, N., Nakamura, K., Nakao, K., Hashimoto, R., Otani, H., Sakagami, H., Kondo, H., Nozawa, S., Aiba, A., et al. (1998). Rac1 is required for the formation of three germ layers during gastrulation. Oncogene 17, 3427-3433.
19. Threadgill, R., Bobb, K., and Ghosh, A. (1997). Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42. Neuron 19, 625-634.

20. Leone, D.P., Srinivasan, K., Brakebusch, C., and McConnell, S.K. (2010). The rho GTPase Rac1 is required for proliferation and survival of progenitors in the developing forebrain. Developmental neurobiology 70, 659-678.
21. Chen, L., Melendez, J., Campbell, K., Kuan, C.Y., and Zheng, Y. (2009). Rac1 deficiency in the forebrain results in neural progenitor reduction and microcephaly. Developmental biology 325, 162-170.
22. Sobreira, N., Schiettecatte, F., Valle, D., and Hamosh, A. (2015). GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Human mutation 36, 928-930.
23. Matos, P., Skaug, J., Marques, B., Beck, S., Verissimo, F., Gespach, C., Boavida, M.G., Scherer, S.W., and Jordan, P. (2000). Small GTPase Rac1: structure, localization, and expression of the human gene. Biochemical and biophysical research communications 277, 741-751.
24. Lek, M., Karczewski, K.J., Minikel, E.V., Samocha, K.E., Banks, E., Fennell, T., O'Donnell-Luria, A.H., Ware, J.S., Hill, A.J., Cummings, B.B., et al. (2016). Analysis of protein-coding genetic variation in 60,706 humans. Nature 536, 285-291.
25. Jordan, P., Brazao, R., Boavida, M.G., Gespach, C., and Chastre, E. (1999). Cloning of a novel human Rac1b splice variant with increased expression in colorectal tumors. Oncogene 18, 6835-6839.
26. Colicelli, J. (2004). Human RAS superfamily proteins and related GTPases. Science's STKE : signal transduction knowledge environment 2004, RE13.
27. Hobbs, G.A., Mitchell, L.E., Arrington, M.E., Gunawardena, H.P., DeCristo, M.J., Loeser, R.F., Chen, X., Cox, A.D., and Campbell, S.L. (2015). Redox regulation of Rac1 by thiol oxidation. Free radical biology & medicine 79, 237-250.
28. Gao, Y., Xing, J., Streuli, M., Leto, T.L., and Zheng, Y. (2001). Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors. The Journal of biological chemistry 276, 47530-47541.
29. De Baets, G., Van Durme, J., Reumers, J., Maurer-Stroh, S., Vanhee, P., Dopazo, J., Schymkowitz, J., and Rousseau, F. (2012). SNPeffect 4.0: on-line prediction of molecular and structural effects of protein-coding variants. Nucleic acids research 40, D935-939.
30. Price, L.S., Leng, J., Schwartz, M.A., and Bokoch, G.M. (1998). Activation of Rac and Cdc42 by integrins mediates cell spreading. Molecular biology of the cell 9, 1863-1871.
31. Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70, 401-410.
32. Machesky, L.M., and Hall, A. (1997). Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization. The Journal of cell biology 138, 913-926.
33. Michiels, F., Habets, G.G., Stam, J.C., van der Kammen, R.A., and Collard, J.G. (1995). A role for Rac in Tiam1-induced membrane ruffling and invasion. Nature 375, 338-340.
34. Jao, L.E., Wente, S.R., and Chen, W. (2013). Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proceedings of the National Academy of Sciences of the United States of America 110, 13904-13909.
35. Perles, Z., Moon, S., Ta-Shma, A., Yaacov, B., Francescatto, L., Edvardson, S., Rein, A.J., Elpeleg, O., and Katsanis, N. (2015). A human laterality disorder caused by a homozygous deleterious mutation in MMP21. Journal of medical genetics 52, 840-847.
36. Mulherkar, S., Uddin, M.D., Couvillon, A.D., Sillitoe, R.V., and Tolias, K.F. (2014). The small GTPases RhoA and Rac1 regulate cerebellar development by controlling cell morphogenesis, migration and foliation. Developmental biology 394, 39-53.
37. Nevado, J., Rosenfeld, J.A., Mena, R., Palomares-Bralo, M., Vallespin, E., Angeles Mori, M., Tenorio, J.A., Gripp, K.W., Denenberg, E., Del Campo, M., et al. (2015). PIAS4 is associated with macro/microcephaly in the novel interstitial 19p13.3 microdeletion/microduplication syndrome. European journal of human genetics : EJHG 23, 1615-1626.

38. Van Dijck, A., van der Werf, I.M., Reyniers, E., Scheers, S., Azage, M., Siefkas, K., Van der Aa, N., Lacroix, A., Rosenfeld, J., Argiropoulos, B., et al. (2015). Five patients with a chromosome 1q21.1 triplication show macrocephaly, increased weight and facial similarities. European journal of medical genetics 58, 503-508.
39. Shinawi, M., Liu, P., Kang, S.H., Shen, J., Belmont, J.W., Scott, D.A., Probst, F.J., Craigen, W.J., Graham, B.H., Pursley, A., et al. (2010). Recurrent reciprocal 16p11.2 rearrangements associated with global developmental delay, behavioural problems, dysmorphism, epilepsy, and abnormal head size. Journal of medical genetics 47, 332-341.
40. Riviere, J.B., Mirzaa, G.M., O'Roak, B.J., Beddaoui, M., Alcantara, D., Conway, R.L., St-Onge, J., Schwartzentruber, J.A., Gripp, K.W., Nikkel, S.M., et al. (2012). De novo germline and postzygotic mutations in AKT3, PIK3R2 and PIK3CA cause a spectrum of related megalencephaly syndromes. Nature genetics 44, 934-940.
41. Rosner, M., Hanneder, M., Siegel, N., Valli, A., Fuchs, C., and Hengstschlager, M. (2008). The mTOR pathway and its role in human genetic diseases. Mutation research 659, 284-292.
42. Hetmanski, J.H., Schwartz, J.M., and Caswell, P.T. (2016). Rationalizing Rac1 and RhoA GTPase signaling: A mathematical approach. Small GTPases, 1-6.
43. Pengelly, R.J., Greville-Heygate, S., Schmidt, S., Seaby, E.G., Jabalameli, M.R., Mehta, S.G., Parker, M.J., Goudie, D., Fagotto-Kaufmann, C., Mercer, C., et al. (2016). Mutations specific to the Rac-GEF domain of TRIO cause intellectual disability and microcephaly. Journal of medical genetics.
44. Ba, W., Yan, Y., Reijnders, M.R., Schuurs-Hoeijmakers, J.H., Feenstra, I., Bongers, E.M., Bosch, D.G., De Leeuw, N., Pfundt, R., Gilissen, C., et al. (2016). TRIO loss of function is associated with mild intellectual disability and affects dendritic branching and synapse function. Human molecular genetics 25, 892-902.
45. Hollstein, R., Parry, D.A., Nalbach, L., Logan, C.V., Strom, T.M., Hartill, V.L., Carr, I.M., Korenke, G.C., Uppal, S., Ahmed, M., et al. (2015). HACE1 deficiency causes an autosomal recessive neurodevelopmental syndrome. Journal of medical genetics 52, 797-803.

Figure Legends

Figure 1: Properties of germline *RAC1* variants.

(A) *RAC1* is located on human chromosome 7p22.1. Exon-intron structure of the two protein-coding *RAC1* transcripts is shown. . The ubiquitously expressed *RAC1* transcript is composed of six exons (blue vertical lines). *RAC1B* (mainly expressed in gastro-intestinal and epithelial cells) contains an additional exon (exon 4; grey vertical line).

(B) Schematic of *RAC1* gene, with the coding exons shown as blue rectangles and the introns as blue lines. Exon 4 that is only expressed in the longer *RAC1B* transcript is shown as a grey rectangle. The red triangles represent the positions of the mutations identified in this study. The red oval within exon 4 shows the two splice donor variants detected in the EXAC database. The purple ovals represent the missense variants in the ExAC database.

(C) Amino acid sequence of the RAC1 protein across positions p.1-p.192. Red amino acids are highly conserved in >90% of the RAS family members and blue amino acids represent RAS protein G box consensus residues. Positions of the identified missense mutations (in red) are marked with dotted lines and the high conservation among different species of these amino acids, is shown in the rectangle below.

(D) Crystal structure of human RAC1 protein showing the position of the identified missense mutations presented in this study. The sites of mutation are indicated by sticks in spheres. The mutation sites are color-coded according to their putative impact on guanine nucleotide binding (cyan), protein-protein interactions (yellow) and structural stability (red). For reference, the non-hydrolyzable GTP analogue GMPPNP bound to the structure is shown in sticks. The structure was analysed and this figure was generated using the program ICM-Pro v3.8 (Molsoft, LLC).

Figure 2: Facial dysmorphism and brain abnormalities observed in individuals with *RAC1* mutations

(A) Frontal and lateral photographs of the faces of individuals 1-3 and 5-7. Individuals 1, 2 and 3 were microcephalic, individual 5 had a normal occipital-frontal circumference and individuals 6 and 7

had macrocephaly. Variable degrees of arched eyebrows, dysplastic ears, prominent nasal bridge and overhanging columella were noted. Individuals with macrocephaly have prominent broad foreheads, slightly up-slanted palpebral fissures, open mouths and 'scooped out' appearance on lateral view.

(B) Left upper panel (1) – brain MRI images of individual 1: Axial T2 images showing dysgenetic vermis and right cerebellar hemisphere (left), cystis lesions in the right frontal deep white matter (middle, arrow) and sagittal T1 images (right) showing enlarged cisterna magna (arrow). Left lower panel (2) – brain MRI images of individual 2: Axial T2 images showing hypoplastic cerebellar vermis (left), slightly enlarged lateral ventricles (middle) and sagittal T1 images (right) showing hypoplastic corpus callosum, pons (arrow head), hypoplastic lower vermis, enlarged 4th ventricle and cisterna magna (arrow). Right upper panel (6)– brain MRI image of individual 6: Axial T2 image showing non-specific white matter changes in the frontal and parietal lobes. No hydrocephalus or extra-axial fluid was observed.

Figure 3: Distinct *RAC1* mutations phenocopy dominant negative or constitutively active variants

(A) NIH3T3 fibroblasts expressing the indicated Rac1 construct were fixed 30 min following plating onto fibronectin and stained with Alexa-568 phalloidin to reveal the actin cytoskeleton and anti-GFP to reveal the expressed construct. Cells were imaged by confocal microscopy and divided into the three indicated categories . At least 50 cells were analysed for each dataset except Asn39Ser, Tyr64Asp, Cys157Tyr (40 cells) and Gln61Leu (25 cells). Data was pooled from three independent experiments. **(B)** Graph showing mean circularity index of cells expressing indicated Rac1 constructs. Error bars indicate S.E.M. Datasets statistically analysed using ANOVA test with Dunnett's correction for multiple comparisons. Asterisks indicate datasets significantly different to WT (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). (C) Images of cells expressing the indicated RAC1 mutation. In each case the cell is a representative example of the most common morphological category for that mutation (see Panel A). Top panels show Alexa-568 phalloidin staining to reveal F-actin distribution, while the panels immediately below show a magnified view of the boxed region from the top panels to

highlight the morphology of actin protrusions at the cell periphery. The third row of panels show the GFP channel of the same cells to reveal the distribution of the expressed RAC1 mutation and the bottom panels show a merge of the F-actin (red) and GFP (green) channels. All scale bars indicate 10 μm.

Figure 4: Overexpression of human *RAC1* mutations p.Cys18Tyr and p.Asn39Ser cause microcephaly, while p.Q61L causes the mirror phenotype in zebrafish embryos

Functional assessment of *de novo* variants in *RAC1* by *in vivo* complementation in zebrafish larvae. **(A)** Dorsal view of 5 days post fertilization (dpf) control and overexpressant larvae. For each experiment, embryos were injected with either WT or mutant *RAC1* human mRNA message. The embryos were allowed to grow to 5 dpf and imaged live for head size. The midbrain area between the eyes highlighted with the dashed red line in panel A was measured for every imaged embryo, to produce a quantitative score. **(B)** Bar graph of normalized values showing the quantification of the head size phenotype in control embryos and embryos injected with either WT or mutant human *RAC1* message, from two plotted experiments. Statistical analyses were performed by student`s t-test. **(C)** Dorsal view of 2dpf control and overexpressant larvae, fixed in Dent's fixative and whole-mount immuno-stained with an anti-phospho histone 3 (PH3) antibody that marks proliferating cells. For each experiment, embryos were injected with either WT or mutant *RAC1* human mRNA message. The embryos were allowed to grow to 2dpf and subsequently were fixed, stained and imaged. Embryos were imaged dorsally and z-stacks were acquired every 100nm. The z-stacks were collapsed to produce an extended depth of focus (EDF) image that was then processed for scoring. The PH3-positive cells were counted for every embryo imaged using the ITCN plugin from ImageJ, to produce a quantitative score. **(D)** Bar graph of normalized values showing the quantification of the proliferating cell count phenotype, across three biological replicas. Statistical analyses for the neuronal proliferation experiments were performed by student`s t-test. **(E)** Dorsal view of 3 dpf control and overexpressant larvae for *RAC1* WT and mutant conditions. The area of the cerebellum consisting of the neuronal axons that cross the midline is highlighted with a green dashed box. At

least 50 embryos per condition were imaged live and evaluated at 3dpf for the depletion of axons within the area highlighted. **(F)** Bar graph of cumulative plotted experiments across three biological replicas showing percentages of embryos with cerebellar defects. Statistical analyses for the cerebellar integrity assay using a χ^2-test.

Tables

Table 1: RAC1 mutations and phenotype

	Individual 1 ***Microcephaly***	**Individual 2** ***Microcephaly***	**Individual 3** ***Microcephaly***	**Individual 4** ***Microcephaly***	**Individual 5** ***Normal OFC***	**Individual 6** ***Macrocephaly***	**Individual 7** ***Macrocephaly***
Gender	Male	Male	Male	Male	Male	Male	Male
Ethnicity	Caucasian/Egypt	Caucasian	Caucasian	Caucasian/Armenian	Caucasian/Asian	Caucasian	African American
Age of examination	13 years	9 years	15 years	4.5 months	12 years	33 months	4 years 5 months
Mutation (NM_006908)							
Chromosome position (Hg19)	Chr7:6426860G>A	Chr7:6431563A>G	Chr7:6431665C>T	chr7:6441968G>A	Chr7:643163T>G	Chr7:6431598G>A	Chr7:6431598G>C
cDNA change	c.53G>A	c.116A>G	c.218C>T	c.470G>A	c.190T>G	c.151G>A	c.151G>C
Amino acid change	p.(Cys18Tyr)	p.(Asn39Ser)	p.(Pro73Leu)	p.(Cys157Tyr)	p.(Tyr64Asp)	p.(Val51Met)	p.(Val51Leu)
Likely effect of the mutation	Dominant negative	Dominant negative	Unknown	Unknown	Constitutively active	Unknown	Unkown
Growth							
Height	127 cm (-2.5 SD)	128 cm (-2.5 SD)	Unknown	62 cm (-1 SD)	134 cm (+1.03 SD) (8 yrs)	Unknown	109 cm (0 SD)
Weight	30 kg (0 SD)	24 kg (-0.5 SD)	Unknown	4.8 kg (-3 SD)	Unknown	Unknown	23 kg (+2.5 SD)
Head circumference	50 cm (-2.5 SD)	47.7 cm (-3 SD)	47 cm (-5 SD)	39 cm (-2.5 SD)	56.5 cm (+1 SD)	57 cm (+4.16 SD)	59.5 cm (+4.5 SD)
Development							
Intellectual disability	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Mild/Moderate/Severe	Moderate	Mild-Moderate	Severe	Unknown	Severe	Moderate	Unknown
Neurological							
Epilepsy	Yes	No	Unknown	Yes	No	Unknown	Yes
Hypotonia	Yes	No	Unknown	Yes	Yes	Unknown	Yes
Behavioural problems	No	Yes - Hyperactive	Unknown	Unknown	Yes - Sleep disturbances	Unknown	Yes - Autism
Stereotypic movements	No	No	Unknown	No	Yes	Unknown	Yes
Brain MRI abnormalities							
Cerebellar abnormalities	Yes	Yes	Unknown	Yes	No	No	No
Hypoplasia corpus callosum	Yes	Yes	Unknown	Yes	Yes	No	No
Enlarged lateral ventricles	Yes	Yes	Unknown	No	No	No	No
Enlarged fourth ventricle	No	Yes	Unknown	No	No	No	No
Thin pons brain stem	No	Yes	Unknown	Yes	No	No	No
Mega cisterna magna	Yes	Yes	Unknown	Yes	No	No	No
Polymicrogyria	No	No	Unknown	No	Yes	No	No
White matter lesions	Yes	No	Unknown	No	No	Yes	Yes
Congenital abnormalities							
Cardiac abnormalities	Yes – NS LVC; IV	No	Unknown	Yes – PDA, PFO, BAV	Yes - VSD	Unknown	No
Hypospadia	No	No	Unknown	Yes	Yes	Unknown	No
Other							
Neonatal feeding difficulties	Yes	Yes	Yes	No	No	Unknown	No
Other	Plagiocephaly; Scoliosis; Small hands and feet; hyperlaxity; brachydactyly 5th digit; SC bilateral	Recurrent pneumonias; Eczema	Diabetes Mellitus	umbilical hernia, tracheobronchomalacia; cryptorchidism	Mild visual impairment; congenital sensorineural hearing impairment; Abnormal creases hand	None	Non-verbal; Recurrent otitis media; Eczema

Abbreviations: BAV = bicuspid aortic valve; IV = insufficiency all valves; NS LVC = Non-synchronic left ventricle contractions; PDA = patent ductus arteriosus; PFO = patent foramen ovale; SC = simian crease; VSD = ventricular septum defect