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Variation in a range of mTOR-related genes associates with intracranial volume and intellectual disability


De novo mutations in specific mTOR pathway genes cause brain overgrowth in the context of intellectual disability (ID). By analyzing 101 mMTOR-related genes in a large ID patient cohort and two independent population cohorts, we show that these genes modulate brain growth in health and disease. We report the mTOR activator gene \( RHEB \) as an ID gene that is associated with megalencephaly when mutated. Functional testing of mutant \( RHEB \) in vertebrate animal models indicates pathway hyperactivation with a concomitant increase in cell and head size, aberrant neuronal migration, and induction of seizures, concordant with the human phenotype. This study reveals that tight control of brain volume is exerted through a large community of mTOR-related genes. Human brain volume can be altered, by either rare disruptive events causing hyperactivation of the pathway, or through the collective effects of common alleles.

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Many aspects of brain homeostasis, among which are measures of total brain volume, are highly heritable. Genome-wide association studies (GWAS) of brain volume have shown a polygenic architecture in the general population, with individual common genetic variants explaining <1% of phenotypic variance. Neurodevelopmental disorders, such as intellectual disability (ID) and autism spectrum disorder (ASD), have been associated with significant brain overgrowth. In ID, up to 6% of the patients are macrocephalic. One of the key regulators of normal brain development is the evolutionarily conserved Ser/Thr protein kinase Mammalian Target Of Rapamycin (MTOR). The role of the mTOR pathway in brain development and function has been intensively studied both in vitro and in vivo using different mouse models. In these models, mutations in either the downstream effectors of mTOR, or the most important upstream regulators of mTOR, such as Ras homolog enriched in brain (Rheb), tuberous sclerosis 1 (Tsc1), and Tsc2, have been tested. Collectively, all studies provide strong evidence that proper mTOR signaling is involved in key aspects of brain development, such as neuronal progenitor maintenance and differentiation (including regulation of neuronal polarity, soma size and neurite outgrowth) and neuronal migration. In the mature brain, mTOR is an important regulator of synapse formation and synaptic function, in particular through its role in regulating protein translation and elongation. Not surprisingly, hyperactivity of the mTOR pathway in mice can lead to a myriad of phenotypes such as macrocephaly, seizures, and behavioral abnormalities.

Fig. 1 Schematic overview of selected mTOR-related genes. Schematic representation of the genes (n = 101) included in our mTOR-related gene-set based on three different authoritative publications. Both proteins acting upstream of mTOR and proteins acting downstream of mTOR were included. Additionally, we subdivided the total set in two subsets: the RAS-MAPK-mTOR subset and the PI3K-AKT-mTOR subset. In both subsets, downstream genes are included as well. Genes in which we identified de novo mutations in this study were marked with a yellow star, and genes previously reported to cause ID and (relative) macrocephaly are shown in bolt and dark green.
In contrast, sustained downregulation of the mTOR pathway appears to have little effect on neuronal function and behavior. Findings that the epilepsy and behavioral deficits in mice can be rescued by mTOR inhibitors, offers a broad therapeutic window in which patients can potentially be treated. Indeed, recent studies indicated that mTOR inhibition is a promising treatment for epilepsy in tuberous sclerosis complex (TSC) patients.

Given the large body of evidence implying mTOR function in key aspects of brain development, it is not surprising that hyperactivating, somatic, and germline mutations in components of the PI3K-AKT3-mTOR pathway have been linked with rare ID syndromes associated with (hemi)megalencephaly, focal cortical dysplasia, and epilepsy. We were struck by the apparent recurrence of mTOR-related mutations in ID, the persistent co-morbid megalencephaly and the absence of studies investigating the overall contribution of the mTOR pathway to ID and brain growth. Considering this knowledge gap, we sought to identify deleterious germline mutations in mTOR-related genes, and assess their contribution to the development of ID and pathology of syndromic ID patients represents the extreme end of a more continuous contribution of the mTOR pathway to human brain development and neuroanatomical variance in the population. Our data indeed indicate that mTOR variation significantly contributes to megalencephaly in a large ID cohort and brain size in the population. Furthermore, we present that de novo mutations in a key regulator of mTOR, RHEB, causes severe ID, epilepsy and megalencephaly in humans. By functionally testing the RHEB mutations in vertebrate animal models, we show that the specific mutations cause hyperactivation of mTOR, with a concomitant increase in cell and head size, aberrant neuronal migration and induction of seizures, concordant with the human phenotype. The extent of mTOR activation likely affects brain volume in humans. In extreme cases, highly deleterious mutations can lead to profound pathology. For such patients, functional restoration of the pathway through treatment with selective mTOR inhibitors might be of direct clinical utility.

**Results**

**mTOR-related mutations are associated with macrocephaly.** To assess the overall burden of mTOR defects to ID, we performed whole-exome sequencing (WES) in a cohort of 826 patients with ID cataloguing de novo mutations (Supplementary Data 1) in a set of 101 mTOR-related genes (Supplementary Data 2, Fig. 1). We identified 17 de novo mutations affecting 10 different mTOR-related genes, providing a possible genetic diagnosis in 2.1% of our cohort. Five of the identified genes were known ID genes (PIK3R1, PIK3R2, RAF1, PPP2R5D, MTOC) and five (RHEB, RAC1, PPP2R5E, PPP2CA, ERK1) were not associated with ID previously (Fig. 1, Table 1, Supplementary Table 1). Of the five novel genes (RHEB, RAC1, and PPP2CA) showed a significant enrichment for de novo mutations in our patient cohort (Table 1, Supplementary Table 1). Combining the gene-specific mutation rates of all individual mTOR-related genes, we found a significant enrichment for de novo mutations in mTOR-related genes (p = 3.50e-04 (Supplementary Table 1). Additionally, we found significant spatial clustering of de novo missense variants for a single gene (PPP2R5D: RHEB).

**Table 1 Identified mutations with bonferroni corrected p-value and occipital frontal circumference (OFC) of patients with de novo mutations in mTOR-related genes**

<table>
<thead>
<tr>
<th>Patient key</th>
<th>Gene</th>
<th>c.DNA</th>
<th>Protein change</th>
<th>Known ID gene</th>
<th>OFC</th>
<th>Bonferroni-corrected p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RHEB</td>
<td>c.202 T&gt;C</td>
<td>p.(S68P)</td>
<td>No</td>
<td>+2.5 SD</td>
<td>4.514e–03*</td>
</tr>
<tr>
<td>2</td>
<td>RHEB</td>
<td>c.110 C&gt;T</td>
<td>p.(P37L)</td>
<td>No</td>
<td>+2.5 SD</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>RHEB</td>
<td>c.110 C&gt;T</td>
<td>p.(P37L)</td>
<td>No</td>
<td>+2.5 SD</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>RAC1</td>
<td>c.53 G&gt;A</td>
<td>p.(C18Y)</td>
<td>No</td>
<td>&lt;−2.5 SD</td>
<td>7.657e–03</td>
</tr>
<tr>
<td>5</td>
<td>RAC1</td>
<td>c.116 A&gt;G</td>
<td>p.(N395S)</td>
<td>No</td>
<td>&lt;−2.5 SD</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>PPP2RSE</td>
<td>c.605 T&gt;G</td>
<td>p.(V202G)</td>
<td>No</td>
<td>No data</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>PPP2CA</td>
<td>c.882dup</td>
<td>p.(R295*)</td>
<td>No</td>
<td>No data</td>
<td>1.696e–02</td>
</tr>
<tr>
<td>8</td>
<td>PPP2CA</td>
<td>c.572 A&gt;G</td>
<td>p.(H191R)</td>
<td>No</td>
<td>+2.5 SD</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>ERK1</td>
<td>c.569 T&gt;C</td>
<td>p.(I90T)</td>
<td>No</td>
<td>&lt;−2.5 SD</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>PIK3R1</td>
<td>c.1559 C&gt;G</td>
<td>p.(N453K)</td>
<td>Yes</td>
<td>Normal</td>
<td>7.662e–02</td>
</tr>
<tr>
<td>11</td>
<td>PIK3R1</td>
<td>c.1692 C&gt;G</td>
<td>p.(N564K)</td>
<td>Yes</td>
<td>+2.5 SD</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>PIK3R2</td>
<td>c.1117 G&gt;A</td>
<td>p.(G373R)</td>
<td>Yes</td>
<td>+2.5 SD</td>
<td>1</td>
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<tr>
<td>13</td>
<td>RAF1</td>
<td>c.1082 G&gt;C</td>
<td>p.(G361A)</td>
<td>Yes</td>
<td>Normal</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>PPP2RSD</td>
<td>c.1258 G&gt;A</td>
<td>p.(E420K)</td>
<td>Yes</td>
<td>No data</td>
<td>7.832e–04</td>
</tr>
<tr>
<td>15</td>
<td>PPP2RSD</td>
<td>c.598 G&gt;A</td>
<td>p.(E200K)</td>
<td>Yes</td>
<td>+2.5 SD</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>PPP2RSD</td>
<td>c.592 G&gt;A</td>
<td>p.(E198K)</td>
<td>Yes</td>
<td>+2.5 SD</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>MTOC</td>
<td>c.4555 G&gt;A</td>
<td>p.(A1519T)</td>
<td>Yes</td>
<td>+2.5 SD</td>
<td>1</td>
</tr>
</tbody>
</table>

OFC: occipital frontal circumference; SD: standard deviation.

*In the statistical enrichment analysis, the RHEB p.(P37L) variant was considered as a single event.

**Table 2 Number of patients with macrocephaly, normal OFC, and microcephaly**

<table>
<thead>
<tr>
<th></th>
<th>Macrocephaly</th>
<th>Normal OFC</th>
<th>Microcephaly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with clinical data (n = 732)</td>
<td>47</td>
<td>580</td>
<td>105</td>
</tr>
<tr>
<td>Patients with de novo mutation(s) (n = 553)</td>
<td>35</td>
<td>442</td>
<td>76</td>
</tr>
<tr>
<td>Patients with de novo mutation in mTOR-related gene (n = 14)</td>
<td>9</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Patients with de novo mutation in gene not related to mTOR (n = 539)</td>
<td>26</td>
<td>440</td>
<td>73</td>
</tr>
</tbody>
</table>
De novo mutations in RHEB cause an ID syndrome associated with megalencephaly. 

**Fig. 2** De novo mutations in RHEB cause an ID syndrome associated with megalencephaly. 

- **a** Pedigree structure, disease status and genotype information for patients with changes in RHEB. 
- **b** Schematic of the RHEB protein and the RAS domain. 
- **c** Schematic of the N-terminal portion of the human RHEB protein. 

**mTOR pathway contributes to intracranial volume.** Driven by the high frequency of brain overgrowth described in the literature and the strong enrichment of macrocephaly in patients with mutations in mTOR-related genes in our cohort, we tested our set of 101 mTOR pathway genes for an association with intracranial volume (ICV) in the general population (Fig. 1). 

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3. **www.nature.com/naturecommunications
of PI3K-AKT-mTOR than RAS-MAPK-mTOR with ICV (PI3K-AKT-mTOR: \( p_{\text{self-contained}} = 0.00092471, p_{\text{competitive}} = 0.0079133 \); RAS-MAPK-mTOR: \( p_{\text{self-contained}} = 2.2885e^{-07}, p_{\text{competitive}} = 0.068983 \)). The role of the PI3K-AKT-mTOR pathway in volumetric variation of the brain was further strengthened by testing the previously described Reactome PI3K_AKT_activation gene-set \(^{42} \) (35 genes; \( p_{\text{self-contained}} = 3.8649e^{-13}, p_{\text{competitive}} = 0.00028957 \); Supplementary Table 5). Not all 96 genes in the gene-set analysis showed significant association with ICV individually. The most strongly associated individual gene was AKT3 \((P = 2.22E^{-05})\) and in total, 18 genes of the mTOR gene set including \( APC (P = 0.00042) \), and the new ID gene RHEB \((P = 0.0041) \), showed nominally significant association with ICV (Supplementary Fig. 1; Supplementary Table 6).

### RHEB mutations cause increased neuronal cell and head size.

Two of the three individuals with de novo RHEB mutations were siblings and carried the same heterozygous p.(Pro37Leu) mutation, while a sporadic individual carried the p.(Ser68Pro) allele. The p.(Pro37Leu) mutation was not identified in either parent, suggesting parental gonadal mosaicism (Fig. 2a). The RHEB mutations are located in the RAS domain (Fig. 2b, c) and are absent from ExAC, EVS, or our internal clinical exome databases. All three individuals (Fig. 2d) with de novo RHEB mutations had short stature \((-2 \text{ to } -3 \text{ SD})\) and early brain overgrowth with pronounced macrocephaly during childhood \((+2.5/-3 \text{ SD})\). They had severe to profound ID with hypotonia, as well as autism spectrum disorder. Two of three individuals were reported to have epilepsy. No epileptic episodes were noted for the third patient, but EEG recordings were suggestive of epileptic discharges (Supplementary Note, Supplementary Table 7). Brain magnetic resonance imaging (MRI) evaluation of the patient with the p.(Ser68Pro) allele, confirmed megalencephaly with broad frontal lobes and mild dilatation of the lateral ventricles. The MRI scan further showed a thickened rostrum of the corpus callosum and small splenium, and mild hypoplasia of the lower cerebellar vermis (Fig. 2e).

We selected the RHEB mutations to obtain experimental evidence for our hypothesis that de novo changes in mTOR-related genes are likely due to a gain-of-function mechanism, resulting in hyperactivation of mTOR, as previously shown for other syndromic neurodevelopmental cases associated with macrocephaly. We first tested in vitro whether the RHEB de
novel changes have an impact on overall mTOR activity levels. Given that mTORC1 regulates cell size, we used primary hippocampal neuron soma size as a readout to assess differences between RHEB-WT overexpressing vs. RHEBp.P37L and RHEBp.S68P overexpressing neurons. A significant increase in soma size was detected already in RHEB-WT transfected neurons, suggesting that RHEB is a highly dosage sensitive gene, likely causing hyperactivation of the mTOR pathway.

Overexpression of the RHEB mutant proteins caused an increase in soma size, confirming that these mutations do not cause a loss of function (Fig. 3a, b, one-way ANOVA, $p < 0.0001$, $F(3,260) = 50.35$; control vector vs. RHEB-WT: $p < 0.0001$; control vector vs. RHEBp.P37L: $p < 0.0001$; control vector vs. RHEBp.S68P: $p < 0.0001$ by Tukey’s multiple comparisons test). Notably, overexpression of RHEBp.P37L had the strongest effect inducing a significantly pronounced increase in soma size compared to RHEB-WT ($p < 0.05$) and RHEBp.S68P ($p < 0.05$).

We next sought to evaluate the relevance of these variants in the development of neuroanatomical phenotypes in a developing zebrafish in vivo model. Toward this, we identified the sole zebrafish $rheb$ ortholog (96% similarity, 91% identity). First, we corroborated that the variants identified are not acting through a loss of function mechanism by generating a CRISPR-Cas9 system to introduce deletions. Assessment of head size in mosaic F0 embryos injected with a guideRNA against exon 3 showed microcephaly in two biological replicates, which was opposite to the phenotype observed in the patients (Supplementary Fig. 2).

We next evaluated the effect of the $rheb$ alleles on head size under a gain of function and mTOR hyperactivating paradigm, as suggested through our in vitro studies. To test this hypothesis, we injected human WT or mutant $rheb$ mRNA into 1- to 4-cell stage zebrafish embryos. Expression of WT human $rheb$ induced a significant increase in the headsize area of 5 dpf larvae ($p = 0.0013$). Overexpression of either RHEBp.P37L or p.S68P, also resulted in significantly increased headsize, reminiscent of the megalencephaly seen in our patients ($p < 0.0001$ for either mutant allele when compared to WT RHEB; Fig. 3d, e). This finding was reproducible across three independent biological replicates.

**Fig. 4** Overexpression of RHEB mutants in vivo causes deficits in neuronal migration and seizures in mouse. **a, b** Representative images of E14.5 in utero electroporated P0 brains (a) or P7 brains (b), with an enlargement showing the migratory pathway of the transfected cells (tdTomato+) from the intermediate zone (IZ) and subplate (SP) to the more superficial layers of the cortex (CP = cortical plate and MZ = marginal zone). **c** Quantification of the neuronal migration pattern observed in different conditions. Data are presented as mean ± SEM. Statistical significance was assessed by two-way repeated measure ANOVA followed by Bonferroni’s post hoc test (for bins 2–4: * indicates significant difference between control vector and the different RHEB conditions ($p < 0.0001$); † indicates significant difference between the RHEB-WT and all other conditions ($p < 0.0001$); for bins 9 and 10: * indicates significant difference between control vector and RHEBp.P37L and RHEBp.S68P; ‡ indicates significant difference between RHEB-WT and RHEBp.P37L and RHEBp.S68P ($p < 0.0001$); \* indicates significant difference between RHEB-WT and RHEBp.P37L and RHEBp.S68P ($p < 0.0001$); ‡ indicates significant difference between RHEBp.P37L and RHEBp.S68P ($p < 0.0001$). **d** Kaplan–Meier graph representing onset of tonic-clonic seizures in successfully targeted mice. The insert legends of the graph show $N_{seizures}/N_{mice}$ (c) or $N_{seizures}/N_{total}$ (d).
Rapamycin rescues neuronal soma and head size defects. Antagonists of the mTOR pathway, such as rapamycin, can ameliorate some of neurological deficits associated with mTOR hyperactivity33–35, 36, 37. To evaluate whether this is true for the RHEB activating mutations described here, we treated the neurons with 20 nM rapamycin or vehicle 1 day after transfection for 3 days and assessed neuronal soma size. We found that while the soma size of RHEB-WT overexpressing neurons nominally decreased, a statistically significant reduction of neuronal soma size was observed for both RHEBp.P37L and RHEBp.S68P and a trend in the same direction was seen for RHEB-WT (Fig. 3c, two-way ANOVA, effect of treatment \( p < 0.0001 \), F(1,89) = 16.29; RHEBp.P37L vehicle vs. RHEBp.P37L Rapamycin \( p < 0.01 \); RHEBp.S68P vehicle vs. RHEBp.S68P Rapamycin: \( p < 0.05 \); RHEB-WT vehicle vs. RHEB-WT Rapamycin: \( p = 0.1 \); by Bonferroni’s multiple comparisons test). Taken together, these data show that overexpression of either wild-type or mutant RHEB induces an mTOR-dependent increase in soma size and that this phenotype can be rescued through the administration of the known mTOR antagonist rapamycin. Grounded on the in vitro observations, we next explored the possibility of rapamycin serving as a putative therapeutic agent in vivo. Toward this, we coinjected rapamycin together with WT or mutant RHEB mRNA in zebrafish embryos and we compared the embryos’ head size at 5 dpf. Rapamycin alone did not induce any appreciable neuroanatomical pathologies, or indeed any overt morphological phenotype(s) (Fig. 3e). In contrast, rapamycin sufficiently and reproducibly rescued the macrocephalic phenotype induced by both RHEBp.P37L and RHEBp.S68P, suggesting that suppression of mTOR hyperactivity might present a therapeutic target for disease amelioration (Fig. 3e).

RHEB mutations affect neuronal migration and induce seizures. Previous studies have shown that mTOR signaling is not only involved in cell morphology and growth, but also plays a role in neuronal migration. Increased mTOR activity in vivo, induced either by overexpression of a constitutively active RHEB or by inactivating mutations in the Tsc1 or Tsc2 genes, two negative regulators of RHEB, causes neuronal migration defects12–17. We performed in utero electroporation at E14.5 to induce the in vivo overexpression of RHEB-WT, RHEBp.P37L, and RHEBp.S68P, and tested the effect of the RHEB mutations on neuronal migration in the still developing somatosensory cortex of P0 pups18. Although in neuronal cultures overexpression of RHEB-WT and RHEB mutants increased soma size equally, the results obtained in vivo showed notable differences between these proteins. We observed that while cells transfected with the control vector efficiently migrated to the cortical plate (CP), cells transfected with RHEB-WT could be found in all the layers of the cortex (Fig. 4a). Strikingly, the majority of cells transfected with either RHEBp.P37L or RHEBp.S68P remained in the subplate (SP), indicating more severe migration deficits compared to RHEB-WT overexpression (Fig. 4a). At P7, when the cortical layers are more defined, the difference between RHEB-WT and RHEBp.P37L or RHEBp.S68P was even more striking (Fig. 4b). Analysis of the number of tdTomato-positive cells present in the different cortical layers showed a significant difference between the four different conditions (two-way repeated measure ANOVA, effect of interaction: \( F(27,180) = 13.73, p < 0.0001 \)), consistent with our previous in vitro and in vivo studies that the mutations in RHEB are gain-of-function hyperactivating mutations. Consistent with our findings in primary neuronal cultures, post hoc analysis revealed that the RHEBp.P37L mutation yielded the strongest effects among evaluated conditions (Fig. 4c).

Neuronal migration deficits are often linked with seizures and ID45. Additionally, the link between an epileptogenic phenotype and hyperactivity of the mTOR pathway has been established from studies in both human and mice26, 28, 30, 48. Interestingly, knockdown of the TSC1 gene, a negative regulator of the mTOR pathway, in just a subset of cortical neurons reduces the threshold for seizure induction34. Careful monitoring of the in utero electroporated mice, revealed that overexpression of RHEB-WT, RHEBp.P37L, and RHEBp.S68P resulted in spontaneous tonic–clonic seizures starting at P20 (Supplementary Movie 1). Seizures were particularly common in mice expressing mutant RHEB, whereas 20% (2/10) of mice expressing RHEB1-WT developed epilepsy, all (7/7) mice expressing RHEBp.P37L and 83% (5/6) of mice expressing RHEBp.S68P developed spontaneous seizures (Fig. 4d). Consistent with our findings from primary neuronal cultures and neuronal migration following in utero electroporation, the RHEBp.P37L allele was shown to have the strongest effect, as the mice expressing this allele also showed a significantly earlier onset of seizures (log-rank (Mantel–Cox) \( p < 0.01 \); compared to RHEBp.S68P; Fig. 4d). Taken together, our in vivo results further corroborate the conclusion that the missense mutations in RHEB act as dominant activating mutations.

Discussion

Here we studied the contribution of mTOR-related genes to ID and brain overgrowth in 826 ID patients unscreened for any other phenotypic features and found 17 germline de novo mutations in genes related to mTOR, providing a possible genetic diagnosis for 2.1% of our cohort. We show that genes encoding components of the mTOR pathway, contribute to rare and common alleles that impact brain volume and provide insight into neurodevelopmental processes mediated through mTOR hyperactivity and outlook to potential treatment options for a subset of patients with ID.

A significant fraction of patients harboring a de novo mutation in mTOR-related genes was observed to be macrocephalic. The link between mTOR mutations and ID and/or head size differences has already been established through numerous studies that identified genes such as AKT3, PIK3CA, PPP2R5D, and recently MTOR itself38–40, 49. In fact, from the 23 genes previously reported to cause ID among our gene-set of 101 mTOR-related genes, most (18/23, 78%) have been associated in the literature with macrocephaly or relative macrocephaly. Our study significantly extends these findings: of the 35 patients with macrocephaly in the complete cohort, 9 patients (26%) harbored a de novo mutation in mTOR-related genes. As such, genes in this pathway should be carefully evaluated in patients with ID and macrocephaly.

Motivated by the high frequency of brain overgrowth in previous reports of mTOR-related syndromes, and in patients with mutations in mTOR-related genes in our cohort, we reasoned that the highly penetrant activating alleles that we identified de novo might represent only a fraction of alleles associated with severe neurocognitive disorders, and that more common and less penetrant alleles might be associated with head and brain growth in the general population. Indeed, a combined analysis of common variants of all 96 autosomal genes in the mTOR-related gene-set showed significant association with ICV in two large imaging genetics samples from the CHARGE and ENIGMA consortia, confirming our initial hypothesis. Interestingly, the PI3K-AKT-activation pathway (35 genes from the reactome gene-set) was recently shown to be among the most strongly associated pathways for ICV in an enrichment analysis testing 671 Reactome gene-sets using the same cohorts42. Our
analyses support and expand this conclusion by testing a different, carefully selected gene-set (only 15 out of 96 genes overlapping). Taken together, our data support a model by which mTOR-related genes, including the newly discovered ID gene RHEB, contribute to variation in brain growth, through common and rare genetic variants, in health and disease. Our observations corroborate, how rare disorders can inform biological mechanisms underlying common traits in the general population.

There is ongoing debate on the precise genetic composition of gene-sets. Gene-set databases, such as KEGG, Ingenuity, and others, all differ in their coverage of specific biological pathways and their functional annotations. In line with this observation, the number of genes mapped to pathways may also vary greatly across the databases[30]. For mTOR-related gene-sets, inclusion in databases is incomplete, with key proteins and protein complexes such as RAC1, RAG, MEK, and PP2A missing. For this reason, we used three authoritative reviews on mTOR signaling describing both upstream and downstream interactors of mTOR and then used additional evidence from the literature to subdivide various protein complexes into their constituent proteins and genes. Therefore, our selection of 101 mTOR-related genes might be incomplete and additional genes are likely to be involved in mTOR signaling. For this reason and because of limitation of our methods to detect reliably somatic mosaicism, a mechanism thought to be a significant contributor to mutation burden in this pathway[38–40], we postulate that the diagnostic rate within our cohort (2.1%) might represent the lower bound of the estimate.

In this study, we identified de novo mutations in both known ID genes (PIK3R1, PIK3R2, RAF1, PPP2R5D, MTOR) and novel candidate ID genes (RHEB, RAC1, PPP2R5E, PPP2CA, ERK1). For the most frequently mutated gene, RHEB, we show that hyperactivating mutations cause an ID syndrome with brain overgrowth and epilepsy. The finding that these mutations are hyperactivating, is in line with the observation that loss of RHEB activity does not result in overt neurological phenotypes in Rheb mutant mice[32]. Several mechanisms, such as increased proliferation, increased soma size and reduced apoptosis are known to have a role in the development of megalencephaly[51]. We observed a significant increase in soma size upon over-expressing WT and mutant RHEB alleles in vitro. Since RHEB is the canonical activator of mTOR, this finding is consistent with other reports that have highlighted mTOR as a main regulator of cell size[5, 11, 15, 52]. In vivo, we postulate that the increased soma size might represent one of the mechanisms through which macrocephaly occurs, as the zebrafish embryos injected with mutant RHEB were phenotypically concordant with the human patients. Further dissecting the pathomechanism(s) underlying RHEB-associated ID, we showed severe neuronal migration defects in mouse embryos electroporated with mutant RHEB and an increased incidence of epileptogenic activity postnatally. These findings are reminiscent to what has been observed for mutations of MTOR itself. Constitutive activation of mTORC1 causes enlarged neuronal somata in rodent neurons, and focal cortical expression of MTOR mutations has been reported to disrupt neuronal migration and to cause spontaneous seizures by in utero electroporation in mice[40, 41, 53]. This observation shows that activating mutations in different genes of the mTORC1 branch of the mTOR pathway act through convergent mechanisms and have similar phenotypic outcomes.

Based on these observations, we reasoned that patients with activating RHEB changes might be able to benefit from therapies that result in a reduction of mTOR activity, such as rapamycin. Indeed, we here showed that suppression of mTOR levels through the administration of the mTOR antagonist rapamycin can significantly and reproducibly prevent both the neuronal soma size phenotype in vitro and the macrocephaly phenotype in vivo. Recent studies have reported successful implementation of mTOR inhibitor treatment in individuals with TSC-associated epilepsy and brain tumors[35–37]. In a conceptually similar paradigm, fibroblasts from a patient with an mTOR activating PIKCA change, were treated successfully with the PI3K inhibitors wortmannin or LY294002, which abrogated the overactivation of the pathway[54]. It is premature to advocate the use of rapamycin in patients with ID and mutations in all mTOR-related genes, not least because of the potentially adverse effects induced by prolonged exposure to this agent[55]. However, we speculate that targeted administration of mTOR inhibitors (rapamycin, wortmannin, everolimus as well as currently emerging second-generation drugs), perhaps during critical postnatal neurodevelopmental windows, might be of significant benefit to patients. In that context, rapid molecular diagnosis in both known ID genes and candidate ID genes, would be a critical component of the treatment decision process.

In conclusion, our data show that a large number of mTOR-related genes together modulate human brain volume in the population. Severe disruption of such mTOR-related genes can cause intellectual disability and brain overgrowth, most likely through mTOR hyperactivation.

Methods

Subjects and mutation analysis. We evaluated a cohort of 826 patients with ID, who had undergone diagnostic trio WES at Radboud University Medical Center (Radboudumc). We included 820 simplex patients described previously, as well as three sib pairs excluded from the earlier study[30]. Diagnostic WES was approved by the medical ethics committee of the Radboud University Medical Center (Commissie Mensegebonden Onderzoek, Nijmegen, The Netherlands (registration number 2011-118)). Written informed consent was obtained from all individuals or their legal guardians. We collected all available clinical information and performed deep phenotyping of individuals with a de novo mutation in RHEB. Consent for publication of photographs was obtained. Brain images were re-evaluated where available.

Selection of mTOR-related genes. We focused on the two well-described, convergent pathways in which mTOR acts upstream of AKT-mTOR pathway and the RAS-MAPK-mTOR pathway. We defined a list of 101 mTOR-related genes based on three authoritative reviews on the mTOR regulators[37–39]. Protein complexes were mapped to single proteins and genes based on information available in the literature. The final list contains 101 mTOR-related genes: 96 map on autosomes and five map on the X-chromosome.

Identification of mutations and collection of OFC data. From our cohort of 826 patients with ID, we selected all de novo mutations that affect mTOR-related genes. All mutations were confirmed by Sanger sequencing. mTOR-related genes were considered to be known ID genes, if present in our recently published list containing over 1500 known ID genes[26]. We performed a literature search by querying Pubmed to investigate which of the known mTOR-related ID genes have been associated with large or small head size. Within our cohort of 826 patients, individuals were classified as macrocephalic (OFC > +2.5 SD), normocephalic (OFC between −2.5 SD and +2.5 SD) or unknown. We used Fisher’s Exact test to calculate enrichment of macrocephaly in mTOR-related mutation carriers. The significance threshold was set at p < 0.05.

Gene-based enrichment. To assess whether mTOR-related genes were significantly enriched for functional de novo mutations in our cohort, we tested each of the 101 genes using a statistical model as described previously[26]. For this statistical enrichment analysis, the RHEB p.(P37L) variant was considered as one single event. Multiple testing correction was performed by the Bonferroni procedure based on 101 tested genes. Additionally, we tested whether the mTOR pathway as a whole was enriched for functional de novo mutations in our cohort by combining the gene-specific mutation rates of all individual genes in the pathway.

Clustering analysis. Clustering analysis was performed by generating the full cDNA for the respective RefSeq genes. To increase the statistical power of the spatial clustering of the recurrently mutated genes, we added de novo missense variants from the denovo-db[60] annotated by our in-house pipeline (Supplementary Data 5). The locations of observed de novo missense mutations were randomly sampled 100,000 times over the cDNA of the gene after which the distances (in base pairs) between the mutations were normalized for the total coding size of the respective gene. The geometric mean (the nth root of the product of n
ENIGMA and CHARGE study populations and data description. This study reports data on 25,974 subjects of Caucasian ancestry from 46 study sites that are part of the Enhancing Neuroimaging Genetics through Meta-Analysis (ENIGMA)69 consortium (13,171 subjects) and Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE; 12,803 subjects)70. Briefly, the ENIGMA consortium brings together numerous studies, mainly with case-control design, which performed neuroimaging in a range of neuropsychiatric or neurodegenerative diseases, as well as healthy normative populations. The CHARGE consortium is a collaboration of predominantly population-based cohort studies that investigate the genetic and molecular underpinnings of age-related complex diseases and traits. ENIGMA and CHARGE data were downloaded from the Reactome_PI3K_AKT_activation gene-set, consisting of 38 genes, was tested whether the regression coefficient of this expression was larger than 0, tests whether the gene-set analyses within the individual cohorts, we meta-analyzed data of both cohorts on the gene-level followed by gene-set analysis. Post hoc, the potential influence of sample size on the strength of the association of each gene with the phenotype (ICV), with higher numbers of autosomal SNPs, separately. Since data from the genome-wide association analyses only included the genome-wide SNP data from the different studies (ENIGMA2 and CHARGE), the data set, using self-contained and competitive testing65. For the gene-set analysis, the genome-wide summary statistics of the CHARGE consortium has been requested by the CHARGE consortium. 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1.5 mL NB medium supplemented with 2% B27, 1% penicillin/streptomycin and 1% glutamax (Invitrogen), and dissociated using a 5 mL pipette. Following dissociation, neurons were plated at a density of 2.5 X 10^4 cells per well on 35 mm glass coverslips at a density of 3 X 10^4 or 5 X 10^4 cells per coverslip for the axon length measurements and 1 X 10^4 cells per coverslip for all the other experiments. The plates were stored at 37°C/5% CO₂ until the day of transfection. Neurons were transfected at 3 days in vitro (DIV3, DIV7, and DIV14) with the following DNA constructs: control vector (1.8 µg per coverslip), RHEB-WT, RHEBp.P37L, and RHEBp.S68P (all 2.5 µg per coverslip). Plasmids were transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). For the rescue experiments, 20 nM Rapamycin (dissolved in 0.1% Ethanol) was applied to the cultures post transfection for 4 days. Neurons were fixed (4 days after Rapamycin experiment) or 5 days (for soma size) post transfection with 4% paraformaldehyde (PFA)/4% sucrose and incubated overnight at 4°C with primary antibodies in GDB buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton X-100, 30 mM phosphate buffer, pH 7.4). The following primary antibodies were used: guinea-pig anti MAP2 (1:500, catalogue number: 188004, Synaptic System) to stain for dendrites, and rabbit anti-RHEB (1:100, catalogue number: 4935, Cell Signaling). Donkey anti-guinea-pig Alexa647- and donkey anti-rabbit-Alexa488-conjugated were used as secondary antibodies (all 1:200, catalogue numbers: 706-605-148 and 711-545-152, respectively, Jackson Immunol. Research). Slides were mounted using mowiol-DABCO mounting medium. Confocal images were acquired using a Leica SP5 microscope with a 63× oil objective (×20 objective, 0.5 zoom, 1024 × 1024 pixels) were taken from 2 to 3 non-consecutive sections from 2 and 3 electroporated animals per control and mutant groups. Images were rotated to correctly position the cortical layers, and the number of cells in each selected layer was counted using the analyze particles plugin. The results were exported to a spreadsheet for further analysis. Cortical areas from the pia to the ventricle were divided in 10 equal-sized bins and the percentage of tdTomato-positive cells per bin was calculated. For immunofluorescence, mice were deeply anesthetized with an overdose of Nembutal and transverse section perfused with 4% PFA. Brains were extracted and post-fixed in 4% PFA. Brains were then embedded in gelatin and cryoprotected in 30% sucrose in 0.1 M phosphate buffer (PB), frozen on dry ice, and sectioned using a freezing microtome (40/50 µm thick). Free-floating coronal sections were washed in 0.1 M PB and a few selected sections were counterstained with 4’6-diamidino-2-phenylindole solution (DAPI, 1:10,000, Invitrogen) before being mounted with mowiol® (Sigma-Aldrich) on glass. Overview images of the coronal sections were acquired by tile scan imaging using a LSM780 confocal microscope (Zeiss) with a x10 objective. Zoom-in images of the targeted area were taken using a x20 objective. For seizure observations, mice obtained after in utero electroporation were observed daily starting at P18. General behavior and postnatal seizure observation were assessed following abnormal behaviors such as hyperactivity, the presence of stereotypical behaviors and the presence of tonic–clonic seizures, either spontaneous or induced upon mild handling. Weaned mice were video-monitored for 24 h per day in the Phenotyper (Noldus), to assess seizure onset. Abnormal behaviors and onset of seizures were scored and analyzed for each mouse by an expert experimentalist who had been blinded to the identity of samples (i.e., which plasmid had been transfected).

Statistical analysis used for the mouse studies. Statistical difference in soma size between the RHEB WT and mutants was determined using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons. The effect of Rapamycin treatment on soma size was determined using two-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons. For the analysis of the in utero electroporation data, a two-way ANOVA repeated measure was performed, followed by the Bonferroni’s multiple comparisons test. For the analysis of epilepsy onset, the log-rank Mantel–Cox test was used. The significance threshold was set at p < 0.05. Data are presented as mean ± standard error of the mean (SEM).


Data availability. Material requests for zebrafish experiments should be addressed to N.K. (Nicholas.katsanis@duke.edu). Material requests for neuronal culture and mouse studies should be addressed to G.M.v.W. (g.vanwoerden@erasmusmc.nl) or Y.E. (y.e.sgersem@erasmusmc.nl). Material requests for gene set analysis should be addressed to M.K.I (Marijke.klein@radboudumc.nl) or B.E. (Barbara.Franke@radboudumc.nl). All other material requests should be addressed to M.R. (margot.reijnders@radboudumc.nl) or H.B. (han.brunner@radboudumc.nl).

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findings to this project. The original publication of those findings as well as the list of contributing samples and people can be found on the ENIGMA website: http://enigma.ini.usc.edu/. The Neurology Working Group of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium also contributed with an independent set of summary statistics of consortium findings. The contributing cohorts and persons can be found on previous publications of the CHARGE consortium as listed on the website: http://www.chargeconsortium.com/. Part of this work was carried out on the Dutch national e-infrastructure with the support of SURF Foundation. Barbara Franke is supported by funding from a personal Vici grant of the Netherlands Organisation for Scientific Research (NWO; grant 016-130-669), from the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreements no. 602805 (Aggressotype) and no. 602450 (IMAGEMEND), and from the European Community’s Horizon 2020 Programme (H2020/2014–2020) under grant agreement no. 643051 (MiND). In addition, her work is supported by a grant for the ENIGMA Consortium (grant number U54 EB020403) from the BD2K Initiative of a cross-NIH partnership.

Author contributions

Additional information
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