

1 ***De novo* mutations in the GTP/GDP-binding region of RALA, a RAS-like small GTPase,**
2 **cause intellectual disability and developmental delay**

3

4 **Short/Running Title:**

5 *RALA* mutations and neurodevelopmental disorders

6

7 **Authors and affiliations**

8 Susan M. Hiatt^{1¶}, Matthew B. Neu^{1,2¶}, Ryne C. Ramaker^{1,2}, Andrew A. Hardigan^{1,2},
9 Jeremy W. Prokop³, Miroslava Hancarova⁴, Darina Prchalova⁴, Marketa Havlovicova⁴, Jan
10 Prchal⁵, Viktor Stranecky⁶, Dwight K.C. Yim⁷, Zöe Powis⁸, Boris Keren⁹, Caroline Nava⁹,
11 Cyril Mignot^{9, 10, 11}, Marlene Rio^{10, 12}, Anya Revah-Politi¹³, Parisa Hemati¹³, Nicholas
12 Stong¹³, Alejandro D. Iglesias¹⁴, Sharon F. Suchy¹⁵, Rebecca Willaert¹⁵, Ingrid M.
13 Wentzensen¹⁵, Patricia G. Wheeler¹⁶, Lauren Brick¹⁷, Mariya Kozenko¹⁷, Anna C.E. Hurst²,
14 James W. Wheless^{18, 19}, Yves Lacassie^{20, 21}, Richard M. Myers¹, Gregory S. Barsh¹, Zdenek
15 Sedlacek⁴, Gregory M. Cooper^{1*}

16

17 ¹HudsonAlpha Institute for Biotechnology, Huntsville, AL, USA

18 ² Department of Genetics, University of Alabama at Birmingham, Birmingham, AL, USA

19 ³Department of Pediatrics and Human Development, Michigan State University, East

20 Lansing, MI, USA

21 ⁴Department of Biology and Medical Genetics, Charles University 2nd Faculty of

22 Medicine and University Hospital Motol, Prague, Czech Republic

- 23 ⁵Laboratory of NMR Spectroscopy, University of Chemistry and Technology, Prague,
24 Czech Republic
- 25 ⁶Department of Pediatrics and Adolescent Medicine, Diagnostic and Research Unit for
26 Rare Diseases, Charles University 1st Faculty of Medicine and General University
27 Hospital, Prague, Czech Republic
- 28 ⁷Kaiser Permanente-Hawaii, Honolulu, HI, USA
- 29 ⁸Department of Emerging Genetic Medicine, Ambry Genetics, Aliso Viejo, CA, USA
- 30 ⁹Department of Genetics, La Pitié-Salpêtrière Hospital, Assistance Publique-Hôpitaux de
31 Paris, Paris, France
- 32 ¹⁰Centre de Référence Déficiences Intellectuelles de Causes Rares, Paris, France
- 33 ¹¹Groupe de Recherche Clinique UPMC "Déficiência Intellectuelle et Autisme", Paris,
34 France
- 35 ¹²Assistance Publique-Hôpitaux de Paris, service de Génétique, Hôpital Necker-Enfants-
36 Malades, Paris, France
- 37 ¹³Institute for Genomic Medicine, Columbia University Medical Center, New York, NY,
38 USA
- 39 ¹⁴Division of Clinical Genetics, Department of Pediatrics, Columbia University Medical
40 Center, New York, NY, USA
- 41 ¹⁵GeneDx, Gaithersburg, MD, USA
- 42 ¹⁶Arnold Palmer Hospital, Division of Genetics, Orlando, FL, USA
- 43 ¹⁷Department of Genetics, McMaster Children's Hospital, Hamilton, Ontario, Canada

44 ¹⁸Division of Pediatric Neurology, University of Tennessee Health Science Center,
45 Neuroscience Institute & Le Bonheur Comprehensive Epilepsy Program, Memphis, TN,
46 USA

47 ¹⁹Le Bonheur Children's Hospital, Memphis, TN, USA

48 ²⁰Division of Clinical Genetics, Louisiana State University Health Sciences Center, New
49 Orleans, LA, USA

50 ²¹Department of Genetics, Children's Hospital, New Orleans, LA, USA

51

52 *Corresponding Author

53 E-mail: gcooper@hudsonalpha.org (GMC)

54

55 ¶These authors contributed equally to this work.

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60 **Abstract**

61 Mutations that alter signaling of RAS/MAPK-family proteins give rise to a group
62 of Mendelian diseases known as RASopathies, but the matrix of genotype-phenotype
63 relationships is still incomplete, in part because there are many RAS-related proteins,
64 and in part because the phenotypic consequences may be variable and/or pleiotropic.
65 Here, we describe a cohort of ten cases, drawn from six clinical sites and over 16,000
66 sequenced probands, with *de novo* protein-altering variation in *RALA*, a RAS-like small
67 GTPase. All probands present with speech and motor delays, and most have intellectual
68 disability, low weight, short stature, and facial dysmorphism. The observed rate of *de*
69 *novo* *RALA* variants in affected probands is significantly higher ($p=4.93 \times 10^{-11}$) than
70 expected from the estimated mutation rate. Further, all *de novo* variants described
71 here affect conserved residues within the GTP/GDP-binding region of *RALA*; in fact, six
72 alleles arose at only two codons, Val25 and Lys128. We directly assayed GTP hydrolysis
73 and *RALA* effector-protein binding, and all but one tested variant significantly reduced
74 both activities. The one exception, S157A, reduced GTP hydrolysis but significantly
75 increased *RALA*-effector binding, an observation similar to that seen for oncogenic RAS
76 variants. These results show the power of data sharing for the interpretation and
77 analysis of rare variation, expand the spectrum of molecular causes of developmental
78 disability to include *RALA*, and provide additional insight into the pathogenesis of
79 human disease caused by mutations in small GTPases.

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83 **Author Summary**

84 While many causes of developmental disabilities have been identified, a large number of
85 affected children cannot be diagnosed despite extensive medical testing. Previously
86 unknown genetic factors are likely to be the culprits in many of these cases. Using DNA
87 sequencing, and by sharing information among many doctors and researchers, we have
88 identified a set of individuals with developmental problems who all have changes to the
89 same gene, *RALA*. The affected individuals all have similar symptoms, including
90 intellectual disability, speech delay (or no speech), and problems with motor skills like
91 walking. In nearly all of these cases (10 of 11), the genetic change found in the child was
92 not inherited from either parent. The locations and biological properties of these
93 changes suggest that they are likely to disrupt the normal functions of *RALA* and cause
94 significant health problems. We also performed experiments to show that the genetic
95 changes found in these individuals alter two key functions of *RALA*. Together, we have
96 provided evidence that genetic changes in *RALA* can cause DD/ID. These results will
97 allow doctors and researchers to identify additional children with the same condition,
98 providing a clinical diagnosis to these families and leading to new research
99 opportunities.

100

101 Introduction

102 Developmental delay and intellectual disability (DD/ID) affect about 1-2% of individuals
103 worldwide [1]. Many highly penetrant genetic variants underlying DD/ID have been
104 identified, but a large fraction of disease risk remains unexplained [2, 3]. While some
105 DD/ID-cases may result from environmental factors and small-effect common variants
106 [4] it is likely that many probands harbor pathogenic, highly penetrant variation in as-
107 yet-unknown disease-associated genes.

108 The RASopathies are a group of genetic conditions often associated with
109 developmental disorders [5], having in common mutational disruption of genes in the
110 RAS/MAPK pathway that alter patterns of signal transduction. RASopathies are
111 individually rare and pleiotropic but are collectively one of the most common causes of
112 developmental disorders. Associated features include neurocognitive impairment,
113 craniofacial dysmorphology, anomalies of the cardiovascular and musculoskeletal
114 systems, cutaneous lesions, and increased risk of tumor formation [6]. For example,
115 variation in *HRAS* is associated with Costello Syndrome (MIM:218040) and Noonan
116 Syndrome (MIM:609942), variation in *KRAS* is associated with Cardiofaciocutaneous
117 syndromes (MIM:615278), and variation in *NRAS* has been observed in probands with
118 RASopathy-associated phenotypes [7].

119 Given the genetic and phenotypic heterogeneity among DD/ID in general and
120 RASopathies in particular, collaboration and data sharing among clinicians, researchers,
121 and sequencing centers is necessary to enable, or accelerate, discoveries of new forms

122 of disease. One tool to facilitate such collaborations is GeneMatcher, launched in 2013
123 as a way to connect researchers and clinicians with interests in specific genes [8].

124 Here, we present details of a cohort, assembled via GeneMatcher, of eleven total
125 probands (including one set of monozygotic twins) with protein-altering variation in
126 *RALA*, which encodes a RAS-like small GTPase; the variants arose *de novo* in ten of these
127 probands. All probands present with developmental delay. Detailed phenotyping,
128 computational analyses of observed variation, and functional studies lead to the
129 conclusion that variation affecting the GTPase activity and downstream signaling of
130 *RALA* underlies a new neurodevelopmental RASopathy-like disorder.

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132

133 **Results**

134 This study originated as a collaboration facilitated by GeneMatcher through shared
135 interests in *RALA* as a result of observations from exome sequencing (ES) or genome
136 sequencing (GS) as part of DD/ID-related clinical or research testing. In the Methods
137 and Appendix S1, we describe the research sites that identified one or more affected
138 probands reported in this study, the methods used for sequencing and analysis, and
139 related details. In total, we identified *RALA* mutations in eleven affected probands from
140 ten unrelated families. These variants were identified from a combined cohort of over
141 16,000 probands sequenced by six groups who independently submitted *RALA* to
142 GeneMatcher (Table 1, Appendix S1).

143

Table 1. Genotypes and phenotypes of individuals with variation in *RALA*.

145

	Proband 1	Proband 2	Proband 3	Proband 4*	Proband 5*	Proband 6	Proband 7	Proband 8	Proband 9	Proband 10	Proband 11
Sequencing Site	Site A	Site B	Site C	Site D	Site D	Site E	Site F	Site F	Site F	Site F	Site A
Variant (NM_005402.3)	c.73G>A	c.73G>A	c.73G>A	c.73G>T	c.73G>T	c.383A>G	c.383A>G	c.389A>G	c.469T>G	c.472_474delGCT	c.526C>T
Variant (NP_005393.2)	p.(V25M)	p.(V25M)	p.(V25M)	p.(V25L)	p.(V25L)	p.(K128R)	p.(K128R)	p.(D130G)	p.(S157A)	p.(A158del)	p.(R176X)
CADD v1.3	33	33	33	33	33	26.6	26.6	29.6	31	22.1	41
Inheritance	de novo	de novo	de novo	de novo	de novo	de novo	de novo	de novo	de novo	de novo	unknown
Age at last examination	11y	1y 8m	7y 5m	15y	15y	13y	2y 8m	3y 6m	3y 9m	2y 3m	16m
Gender	female	male	male	male	male	male	female	male	male	male	male
Growth Parameters											
Length at birth <10%ile	-	-	-	+	+	NR	-	NR	-	-	+
Weight at birth <10%ile	-	-	-	+	-	NR	-	-	-	-	+
Height at last examination <10%ile	+	-	+	+	+	NR	-	+	-	-	+
Weight at last examination <10%ile	+	+	+	+	+	+	+	+	-	-	-
OFC at last evaluation (%ile)	NR	90-97	75 (at 5 y)	53	53	90	56	75	75-80	>98	<3
Cognitive abilities	moderate ID	severe ID	ID/global developmental delay	profound ID	profound ID	ID/severe global developmental delay	ID/developmental delay	moderate to marked ID	global developmental delay	global developmental delay	profound global developmental delay
Verbal abilities	speech delay	absent speech	speech delay	absent speech	absent speech	absent speech	absent speech	absent speech	speech delay	speech delay	absent speech (tracheostomy in)

												place)
Autism Spectrum Disorder	+	+	+	NR	NR	NR	NR	NR	NR	NR	NR	NR
Hypotonia	-	+	+	+	+	+	+	+	+	+	+	+
Able to walk?	+	-	+	-	-	-	-	-	+	-	-	-
Facial dysmorphism	+	+	-	+	+	+	+	-	+	+	+	+
Seizures	+	-	-	+	+	+	-	+	-	-	-	+
Skeletal Anomalies	mid-fifth finger clinodactyly	fifth finger clinodactyly , 2/3 toe syndactyly	NR	long ,thin fingers with hyperexten sible joints	long ,thin fingers with hyperexten sible joints	NR	fifth toe clinodactyly , 2/3 toe syndactyly	left mild clubfoot	NR	NR	NR	NR
Brain MRI**	normal	abnormal	normal	abnormal	abnormal	abnormal	abnormal	abnormal	abnormal	abnormal	abnormal	abnormal
Other variants of interest**	-	+	-	-	-	-	+	+	-	+	+	+

146

147 *Probands 4 and 5 are monozygotic twins.

148 **See clinical summaries in Appendix S2 for further description of MRI findings, other variants of interest, and additional phenotype information.

149 CADD, Combined Annotation-Dependent Depletion [9]; y, years; m, months; NR, not reported; OFC, occipitofrontal circumference; ID, intellectual disability.

150

151

152

153 *Phenotypic details*

154 All eleven probands presented with speech problems, including absent speech in seven
155 and speech delay in the remaining four. Ten of the eleven probands are reported to have
156 hypotonia, with eight unable to walk. Intellectual disability was specifically noted for 8 of 11,
157 (but not ruled out for the remaining three, see Table 1). Birth measurements were available for
158 nine probands and three (33%) reported either length or weight (or both) at less than the tenth
159 percentile. Height and weight measurements at last examination were available for all
160 probands (except for height in one). Six of ten probands (60%) were reported to have heights
161 less than the 10th percentile at last examination, while eight of eleven (73%) were reported to
162 have weights less than the 10th percentile. Three probands had head circumference
163 measurements greater than or equal to the 90th percentile at last evaluation. Nine of eleven
164 probands were reported to have dysmorphic facial features. Several consistent features were
165 observed, including a broad, prominent forehead, horizontal eyebrows, epicanthus, mild ptosis,
166 slightly anteverted nares, wide nasal bridge, short philtrum, thin upper lip vermilion with an
167 exaggerated Cupid's bow, pointed chin, and low-set ears with increased posterior angulation
168 (Figure 1).

169 Additional common but variable features were observed: seizures were present in most
170 probands (6/11), as were structural brain abnormalities detected by MRI (9/11). Six of eleven
171 probands were reported to have skeletal anomalies such as clinodactyly (3 of 6) and/or 2/3 toe
172 syndactyly (2 of 6). None of the probands are reported to have had cancer. Clinical summaries
173 with additional details are available in Appendix S2.

174

175 *Molecular characterization of variation*

176 Genetic variation within this cohort includes eight *de novo* heterozygous missense
177 variants (in nine probands, including the monozygotic twin pair), one *de novo* heterozygous in-
178 frame deletion of one amino acid, and one heterozygous premature stop of unknown
179 inheritance (Table 1, Figure 2A). Except for R176X (see below), all observed variants are absent
180 from gnomAD [10] and TopMed genomes (“Bravo”) [11]. These variants have CADD scores
181 ranging from 22.1 to 41 suggesting they are highly deleterious, similar to the majority of
182 mutations previously reported to cause Mendelian diseases [9].

183 Seven probands (1-7), including the monozygotic twin pair, harbor recurrent *de novo*
184 variants affecting one of only two codons, those encoding residues Val25 and Lys128, while the
185 remaining three *de novo* variants affect Asp130, Ser157, and Ala158. All of these residues are
186 computationally annotated as one of 24 residues, within a total protein length of 206 amino
187 acids, that form the GTP/GDP-binding region of the RALA protein (Figure 2, Methods). While
188 Val25 does not directly interact with GTP/GDP, variation observed at this position (Val25Met
189 and Val25Leu) would likely result in distortion of the structure of the GTP/GDP-binding pocket
190 (Figure 2B, 2C, Supplemental Figure S1). Lys128, Asp130 and Ser157 all form hydrogen bonds
191 with GTP/GDP in the wild type protein (Figure 2B, 2C, Supplemental Figures S2-S4). Although
192 Lys128Arg would retain the positive charge of the side chain, steric hindrance resulting from
193 the larger size of the Arg side chain would likely result in disruption of this binding pocket
194 (Supplemental Figure S2). Both Asp130Gly and Ser157Ala are predicted to result in loss of
195 hydrogen bond formation (Figure 2B, 2C, Supplemental Figures S3, S4). The remaining *de novo*
196 variant, an in-frame deletion of Ala158, results in a shift of Lys159 into the GTP/GDP binding

197 region of RALA, which likely hinders GTP/GDP binding (Supplemental Figure S5). Variation at all
198 five of these residues is thus predicted to alter GTP/GDP binding. This conclusion is consistent
199 with the high degree of conservation at these residues throughout evolution in RALA
200 (Supplemental Figure S6) as well as in other related genes including HRAS, KRAS, and NRAS
201 (Supplemental Figure S7) and RAP1A/B and RHOA[12].

202 The predicted nonsense variant Arg176X in proband 11 lies within the last exon of *RALA*,
203 and thus may not result in nonsense-mediated decay (NMD) of the transcript. This would yield
204 a protein that lacks the 29 C-terminal residues (Supplemental Figure S8), which are known to
205 contain at least two critical regulatory regions. Phosphorylation of Ser194 by Aurora kinase A
206 (AURKA) activates RALA, affects its localization, and results in activation of downstream
207 effectors like RALBP1 [13, 14]. Additionally, the C-terminal CAAX motif (CCIL in the case of
208 RALA) is essential for proper localization and activation of RALA via prenylation of Cys203 [15,
209 16].

210

211 *Enrichment and clustering of missense variation*

212 We next assessed whether the *de novo* variants in our cohort were enriched compared
213 to that which would be expected in the absence of a disease association. Eight unrelated
214 individuals were drawn from cohorts of at least 400 proband-parent trios, collectively spanning
215 16,086 probands (Appendix S1). When comparing the frequency of observed *de novo* variation
216 to the expected background frequency of *de novo* missense or loss-of-function variation in
217 *RALA* (6.16×10^{-6} per chromosome) [17], we find a highly significant enrichment for *de novo*
218 variants in affected probands (8 observed *de novo* variants in 32172 screened alleles vs. 0.198

219 expected, Exact Binomial test $p=4.93 \times 10^{-11}$). We note that this p-value is likely conservative, as
220 it results from comparison of the observed rate to the expected frequency of *de novo* variation
221 over the entire gene. However, six of the nine *de novo* alleles affect only two codons, and all
222 observed *de novo* variants are within the GTP-interacting space of 24 residues (11.7% of the
223 206-aa protein, Figure 2A). This clustering likely reflects a mechanism of disease that depends
224 specifically on alterations to GTP/GDP binding and, subsequently, RALA signaling.

225 Population genetic data also support pathogenicity of these variants. *RALA* has a pLI
226 score of 0.95 in ExAC [10], suggesting that it is intolerant to loss-of-function variation. While
227 *RALA* has an RVIS score rank [18] of 50.45%, it also has an observed/expected ratio percentile of
228 0.92%, a score that has been suggested to be more accurate for small proteins wherein
229 observed and expected allele counts are relatively small [19]. Furthermore, population genetic
230 data also support the likely special relevance of mutations in the GTP/GDP-binding pocket. No
231 high-quality (“PASS” only) missense variants are observed at any frequency at any of the 24
232 GTP/GDP-coordinating residues in either gnomAD [10] or BRAVO[11]; in contrast, there are
233 missense variants observed at 34 of the 182 *RALA* residues outside the GTP/GDP-interaction
234 region (Supplemental Table S1). This distribution across *RALA* is likely non-random (Fisher’s
235 exact test $p=0.017$) and suggestive of especially high variation intolerance in this region of
236 *RALA*.

237

238 *Comparison to disease associated with RAS-family GTPases*

239 *RALA* and other RAS-family GTPases have a high degree of similarity, and germline
240 variation in other RAS-family GTPases is known to be associated with developmental disorders

241 [5]. Comparisons of phenotypes observed here to those reported in these RASopathies
242 suggest considerable overlap, including DD/ID, growth retardation, macrocephaly, high broad
243 forehead and mildly dysplastic dorsally rotated ears. Further, we compared the specific
244 variants observed here to variants in HRAS, KRAS, or NRAS, reported as pathogenic for
245 RASopathies (Supplemental Table S2, Supplemental Figure S7). *De novo* heterozygous missense
246 variation at Val14 of KRAS, the homologous equivalent of Val25 in RALA, was previously
247 reported in four unrelated individuals with Noonan syndrome [20, 21]. Functional studies
248 showed that this variant may alter intrinsic and stimulated GTPase activity and may increase
249 the rate of GDP release [20, 21]. A *de novo* variant in HRAS at Lys117, the homologous
250 equivalent of Lys128 in RALA, was found in two unrelated probands with Costello Syndrome
251 [22]. Lastly, a *de novo* HRAS variant at Ala146, the homologous equivalent of Ala158 in RALA,
252 was reported in at least three patients with Costello Syndrome [23]. Variation at this residue
253 has also been reported as a recurrent somatic variant in colorectal cancers [24].

254

255 *Functional analysis*

256 We investigated the functional consequences of the variants described above by
257 expressing and purifying recombinant RALA proteins, and then measuring their abilities to
258 hydrolyze GTP and to interact with an immobilized RALA effector protein (see Methods). While
259 wild-type RALA showed robust GTPase activity under these experimental conditions, all
260 mutants tested here exhibited a dramatic reduction in GTPase activity, including a mutant RALA
261 that was not observed in probands but which carries a missense substitution, G23D,
262 homologous to the G12D KRAS or HRAS variant commonly observed in tumor tissue (Figure 3A).

263 As GTPase activity of mutant RAS family proteins alone is not always a clear indication of
264 downstream effects [20, 21], we also assessed binding of these mutants to a RALA effector
265 protein using an ELISA-based method (see Methods). In this assay, recombinant G23D RALA
266 protein exhibited approximately two-fold increased binding ($p < 0.0001$, Figure 3B), as
267 anticipated for a constitutively active gain-of-function alteration [20, 25]. V25L, V25M, D130G
268 and R176X each showed a roughly 2-5-fold reduction in effector binding compared to wild-type
269 (each $p < 0.0001$, Figure 3B). In contrast, the S157A mutant exhibited increased binding
270 compared to wild-type, suggesting that it may act in a constitutively-active manner similar to
271 G23D ($p < 0.0001$, Figure 3B). We note that while there is some variation among mutants in the
272 efficiency of protein production and purification (Methods, Supplemental Figure S9), whether
273 or not one normalizes to relative band intensity from Western blots of purified protein does not
274 qualitatively affect these conclusions (Supplemental Figure S10).

275

276 *Other candidate variants in probands with RALA variants*

277 In this and other cases of rare disease sequencing, it is important to consider other
278 variation in any given patient that may be pathogenic. In six of the eleven cases presented here,
279 the *RALA* variant was found to be the only plausible candidate. In five cases, other variants
280 were discovered that were also initially considered as potential disease-causing mutations.
281 (Table 1, Appendix S2). Proband 2 has a hemizygous variant in *FLNA* (p.V606L), inherited from
282 his unaffected heterozygous mother. Phenotype comparison, consultation with a filaminopathy
283 disease expert, and application of the ACMG variant interpretation guidelines [26] resulted in
284 the scoring of this variant as a VUS. Interestingly, *FLNA* is an interaction partner of *RALA* [27],

285 but the relevance of this variant is unclear. Proband 7 has a *de novo* variant in *SHANK2*
286 (p.A1101T); however, this allele is present in gnomAD three times and thus is not likely to be a
287 highly penetrant allele resulting in DD/ID. Proband 8 has a variant in *SCN1A* p.R187Q; however,
288 this variant was inherited from an unaffected father, is present in gnomAD in one heterozygote,
289 and, according to the referring clinician, the phenotype observed in the proband is not
290 consistent with Dravet syndrome. Finally, proband 10 carries a paternally-inherited 1.349 Mb
291 duplication of 1q21.1-q21.2. This duplication has been reported to be associated with mild to
292 moderate DD/ID, autism spectrum disorders, ADHD and behavioral problems, and other
293 variable features [28]. While the patient may have some phenotypic features of this
294 duplication, the patient's MRI findings and severity of delays are not likely explained by this
295 inherited duplication.

296 Proband 11 carries a nonsense variant, R176X, which is unusual given the apparent
297 specificity for the GTP/GDP-binding region of RALA observed in the other cases in our cohort.
298 Clinically, we consider the R176X to be a variant of uncertain significance for several reasons.
299 The R176X allele has been observed twice in the Bravo genome database, and parental DNA for
300 this proband was not available, so we do not know whether the variant is *de novo* or inherited.
301 In addition, the proband has microcephaly and more profound delays than others in the cohort,
302 and also has large regions of homozygosity consistent with parental consanguinity. These
303 regions of homozygosity suggests an additional and/or more complex molecular pathogenesis.
304

305 Discussion

306 The rapidly expanding application of genome sequencing to clinical settings is rapidly
307 expanding our knowledge of mutations that cause rare disease, and has engendered new
308 strategies for analysis, new rubrics for molecular pathology, and new platforms for
309 collaboration. Here we apply these advances to show that mutations in the GTP/GDP-binding
310 region of *RALA* cause developmental and speech delay, together with minor dysmorphic
311 features. Mutations in RAS family members and RAS signaling pathways are well-recognized
312 causes of several dysmorphic syndromes and cancer, but germline mutations in *RALA* have not
313 been previously associated with disease. Our results add to basic knowledge about the biology
314 and function of RAS family members, raise new questions about the molecular pathogenesis of
315 mutations that affect small GTPases, and have important implications for clinical genomics.

316 Among the small GTPases, *RALA* and *RALB* are the most closely related to the RAS
317 subfamily (~50% amino acid similarity), and function as a third arm of the RAS effector pathway
318 in addition to RAF and PI3K activation [5]. *RALA* and *RALB* have different expression patterns—
319 *RALA* is broadly expressed whereas expression of *RALB* is enriched in endocrine tissues [29]—
320 but also exhibit some degree of genetic redundancy: in gene-targeted mice, loss of function for
321 *RALA* causes a severe neural tube defect that is exacerbated by simultaneous loss of *RALB* [30].
322 In neuronal culture systems, *RALA* has been implicated in the development, plasticity,
323 polarization, migration, branching, and spine growth of neurons [31-35], as well as the renewal
324 of synaptic vesicles and trafficking of NMDA, AMPA, and dopamine receptors to the
325 postsynaptic membrane [27, 34, 36]. These studies evaluated the effects of *RALA* in multiple
326 ways, including through loss of function studies (e.g., using RNA interference), and designed

327 mutational alterations to GTP/GDP hydrolysis, suggesting that multiple types of RALA
328 perturbation have molecular and cellular consequences. Several aspects of our results suggest
329 that developmental delay in humans is not caused by a simple loss-of-function for RALA. Our
330 patients are all heterozygous, whereas in mice, heterozygosity for loss of function does not
331 obviously affect development or viability [30]. In our functional assay, all of the proband alleles
332 exhibited reduced GTPase activity, similar to most oncogenic RAS alleles. However, they
333 exhibited variability in their ability to bind RALA effector protein, with one showing increased
334 effector binding and the others all reducing effector binding. Simillar variability of *in vitro*
335 functional effects were reported for *KRAS* GTP/GDP-binding domain mutations observed in
336 patients with developmental disorders [21]. Importantly, all of the proband alleles assessed
337 here to be pathogenic are *de novo* missense variants in the GDP/GTP-binding domain, including
338 six recurring at only two codons. This observation is in contrast to the variation in large human
339 population datasets which is only observed outside of this domain. Taken together, our data
340 suggest that the molecular pathogenesis of developmental delay in the patients described here
341 is brought about by a genetic mechanism that specifically depends on perturbations to the
342 normal GTP-GDP cycling of RALA.

343 In summary, we show that *de novo* variation affecting *RALA* in individuals with DD/ID is
344 highly enriched compared to background mutational models, exhibits clear spatial clustering
345 in/near to the GTP/GDP-binding region, tends to affect positions whose homologous
346 equivalents in other small GTPases are reported to harbor disease-associated variation, and
347 significantly alters GTPase activity and RALA effector binding in *in vitro* functional assays. These

348 observations add to the diverse and pleiotropic group of Mendelian disorders caused by
349 variation in RAS-family GTPases and related RAS pathways.
350

351 **Materials and Methods**

352 *Informed consent*

353 Informed consent to publish de-identified data was obtained from all participating families, and
354 informed consent to publish clinical photographs was also obtained when applicable. Collection
355 and analysis of sequencing data from all participants was conducted with the approval of
356 appropriate human subjects research governing bodies.

357

358 *Exome/Genome sequencing*

359 Exome sequencing (ES) or genome sequencing (GS) was performed at each of the following
360 sites in either a research or clinical setting. Additional details, including cohort sizes used in p-
361 value calculations, are provided in Supplemental Materials and Methods, Appendix S1.

Proband	Site	Site Name	Experiment Type, Research/Clinical Subjects
1	A	HudsonAlpha Institute for Biotechnology	GS, Trio Research
2	B	Charles University	ES, Trio Research
3	C	Ambry Genetics	ES, Trio Clinical
4	D	La Pitié-Salpêtrière Hospital	ES, Trio Clinical
5	D	La Pitié-Salpêtrière Hospital	Sanger only, monozygotic twin of proband 4 Clinical
6	E	Institute for Genomic Medicine	ES, Trio Research

at Columbia University Medical
Center

7, 8, 9, 10	F	GeneDx	ES, Trio	Clinical
11	A	HudsonAlpha Institute for Biotechnology	GS, Proband Only	Research

362

363

364 *Three dimensional modeling*

365 The protein structure determined by Holbourn et al. [37] was used for the assessment of the
366 potential effect of the mutations on RALA activity (PDB ID: 2BOV). The structure was visualized
367 using PyMOL 0.99rc6[38]. Additional protein modeling was performed as previously described
368 [39]. The GTP/GDP-binding residues of RALA were defined as those in which any atom of a
369 residue (side chain or backbone) lies within 1.5 angstroms of an atom of the ligand.

370

371 *Cloning, protein expression, and purification*

372 RALA cDNA was synthesized (Integrated DNA Technologies, Skokie, IL, USA) based on the coding
373 sequence of NM_005402.3, with substitutions identified in patients described here (probands
374 1-9, 11; see Note below) used to represent variation. Following PCR amplification, coding
375 sequences were cloned into Champion™ pET302/NT-His (ThermoFisher Scientific, Waltham,
376 MA, USA, # K630203) using Gibson Assembly Master Mix (New England BioLabs, Ipswich, MA,
377 USA, #E2611). All *RALA* coding sequences were Sanger sequenced and compared to
378 NM_005402.3. The only differences within the coding regions of *RALA* were those observed in

379 the probands. Single Step (KRX) Competent Cells (#L3002, Promega Corporation, Madison WI,
380 USA) were transformed with plasmids, and bacteria were grown overnight at 37°C in LB plus
381 ampicillin. Bacteria were diluted 1:100 in fresh LB plus 0.05% glucose and 0.1% rhamnose to
382 induce a 6-His-tagged recombinant RALA protein. Bacteria were collected after 8 h incubation
383 at 25°C, and snap-frozen on dry ice. 6-His-tagged proteins were purified using Dynabeads™ His-
384 Tag Isolation and Pulldown (#10103D, ThermoFisher Scientific, Waltham, MA, USA) according to
385 the manufacturer's protocol. Protein purity was assessed using standard SDS-PAGE and
386 Coomassie Blue staining. Protein concentration was quantified using a Take3 microplate reader
387 (BioTek, Winooski, VT, USA) by assessing absorbance at 280 nm. Protein amounts were
388 normalized among samples in Dynabead elution buffer prior to use in assays.

389

390 *GTPase activity*

391 GTPase activity of 0.95 µg of purified, recombinant proteins was assessed using the GTPase-
392 Glo™ Assay (#V7681, Promega Corporation, Madison WI, USA). Luminescence was quantified
393 using an LMax II 384 Microplate Reader (Molecular Devices, San Jose, CA, USA).

394

395 *G-LISA™*

396 Binding of purified, recombinant proteins to a proprietary Ral effector protein was assessed
397 using the RalA G-LISA™ Activation Assay Kit (#BK129, Cytoskeleton, Inc. Denver, CO), as per the
398 manufacturer's protocol. Briefly, purified RALA protein was incubated in the presence or
399 absence of 15 µM GTP (#P115A, Promega) for 1.5 h at 25°C, then 23.75 ng of purified RALA/GTP

400 mixture was applied to the Ral-BP binding plate. A Take3 microplate reader was used for
401 quantification of this colorimetric assay.

402

403 *Western Blot*

404 Purified proteins were detected using a polyclonal RALA Antibody (#3526S, Cell Signaling
405 Technology, Danvers, MA, USA) at a dilution of 1:1000, and an anti-rabbit IgG secondary
406 antibody (#926-32211, IRDye® 800CW Goat anti-Rabbit IgG, Li-cor, Lincoln, NB, USA) at a
407 dilution of 1:20,000. An Odyssey CLx Imaging System (Li-cor, Lincoln, NB, USA) was used to
408 visualize the Western. Relative quantification of the image was performed using Image J
409 (<https://imagej.net/>).

410

411 We note that while we attempted to study the effects of all variation observed here, Proband
412 10 was identified after functional validation began, and the recombinant protein with the
413 K128R variant (observed in probands 6 and 7) was not able to be expressed and purified
414 consistently. Thus GTPase and G-LISA™ results are not shown for K128R or A158del.

415

416

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424

425 **Figure Legends**

426 **Figure 1. Facial features of individuals with variation in *RALA*.** Overlapping features include a
427 broad, prominent forehead, horizontal eyebrows, epicanthus, mild ptosis, slightly anteverted
428 nares, wide nasal bridge, short philtrum, thin upper lip vermillion with an exaggerated Cupid's
429 bow, pointed chin, and low-set ears with increased posterior angulation.

430

431 **Figure 2. Variation observed in *RALA* clusters in GTP/GDP-binding regions.** A. Linear model of
432 *RALA*, including GTP/GDP-binding regions (depicted in yellow, as defined by molecular modeling
433 data) and the CAAX motif (CCIL in the case of *RALA*; depicted in green). Positions of amino acid
434 residues that form the GTP/GDP-binding region are listed below the model, and residues within
435 those regions are listed above the model. Residues affected by variation observed here are
436 shown in red. The predicted protein changes for described variation are shown above the
437 affected amino acid residues. B. Positions of *RALA* amino acid residues affected by variation
438 relative to the GDP molecule. C. A zoomed in view of the variation observed within the
439 GTP/GDP-binding region. GDP is shown in a licorice representation in orange. The *RALA* protein
440 is shown in a cartoon representation in green, with the mutated residues in licorice
441 representation. V25 is in yellow, K128 in blue, D130 in red, S157 in magenta, and A158 in black.
442 Hydrogen bonds between the side chains of these amino acids and GDP are shown as black
443 dashed lines. See Supplemental Figures S1-S5, S8 for consequences of individual variants on the
444 protein structure.

445

446 **Figure 3. Missense variation in RALA affects GTPase activity and RALA effector binding. A.**
447 GTPase activity of purified recombinant RALA proteins was assessed using a luminescence
448 assay. Raw luminescence values (measuring remaining free GTP) were subtracted from 100 to
449 calculate activity, and were then normalized to a no template control (NTC). WT, wild-type
450 RALA. G23D, predicted constitutively active mutant (not from a proband). ** indicates p-value
451 = 0.0015 compared to WT, *** indicates p-value = 0.0003, and **** indicates p-value < 0.0001
452 compared to WT. Mean values of one experiment performed in triplicate are shown. B. Binding
453 of purified recombinant RALA proteins to an effector molecule was assessed using an ELISA-
454 based assay. Absorbances were normalized to a no template control (NTC). Mean values of one
455 experiment performed in triplicate are shown. WT, wild-type RALA. **** indicates p-value <
456 0.0001 compared to WT. ##### indicates p-value < 0.0001 compared to NTC. ### indicates p-
457 value = 0.0001 compared to NTC.
458
459

460 **Conflicts of Interest**

461 ZP is an employee of Ambry Genetics, which provides exome sequencing as a commercially available test.

462 IMW, RW, SFS are employees of GeneDx, Inc., a wholly owned subsidiary of OPKO Health, Inc. that

463 also offers commercial exome sequencing. The remaining authors declare no conflicts of interest

464

465

466

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469 preparation of the manuscript.

470

471 **Supplemental Files**

472 **Appendix S1.** Supplemental Materials and Methods.

473 **Appendix S2.** Clinical summaries.

474 **Appendix S3.** Supplemental Figures and Tables.

475

476

477

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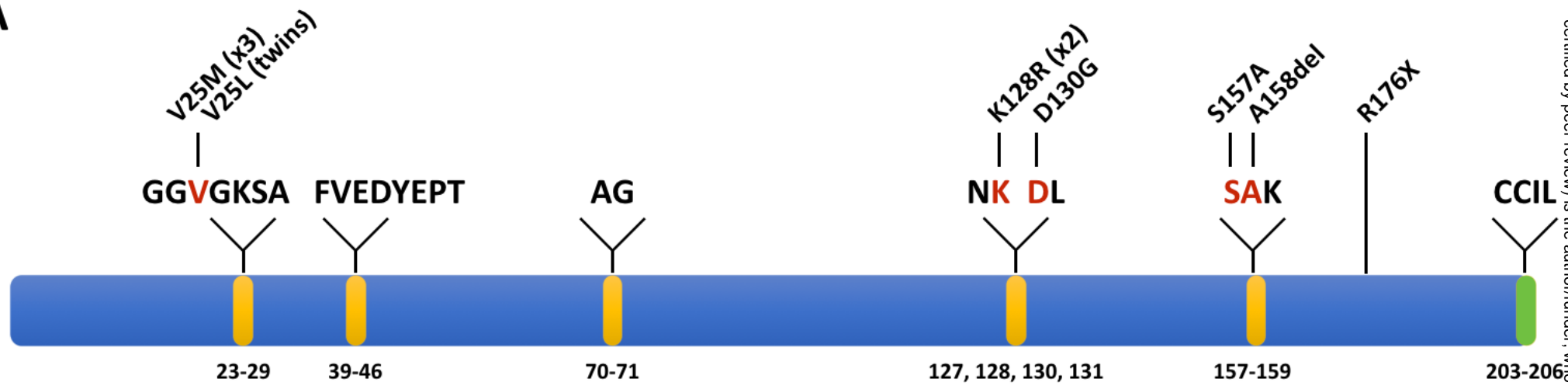
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Figure 1 was removed because it contains identifiable images. See Page 11 for descriptions of overlapping dysmorphic features, in addition to clinical summaries in Appendix S2.

Figure 2

A



B



C

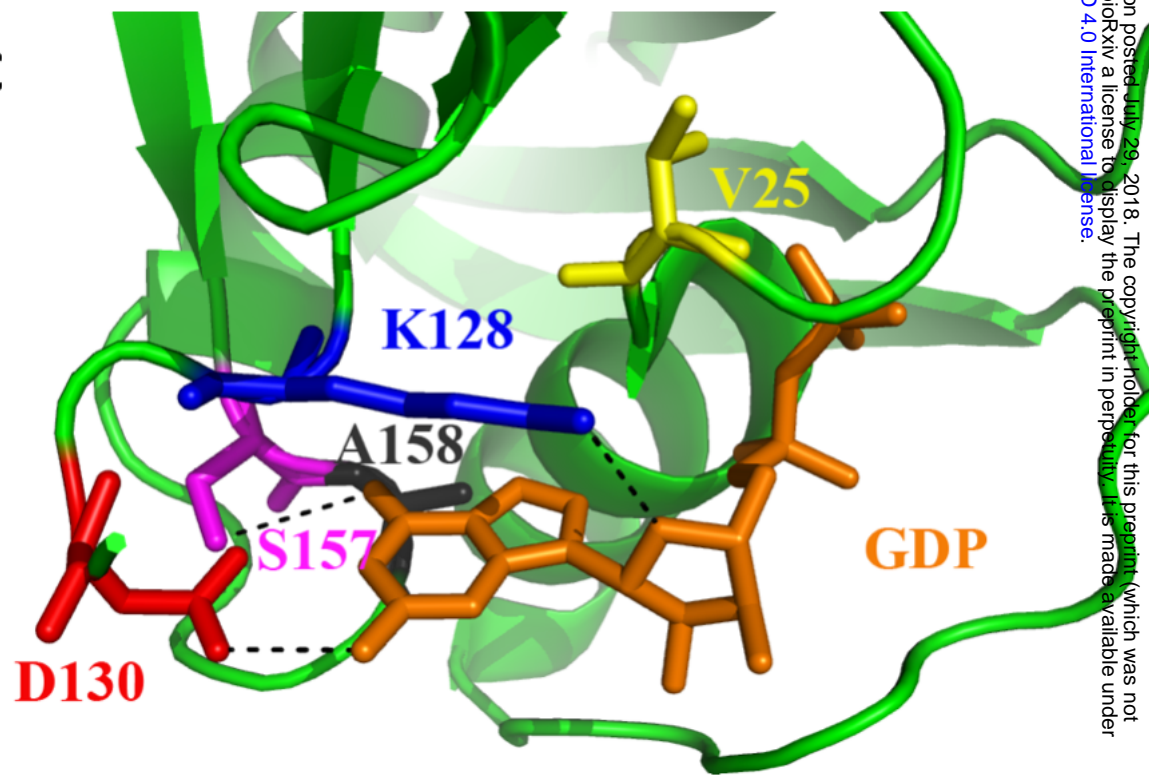


Figure 3

