

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

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Abstract

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

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Introduction

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

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

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

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

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

Results

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

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

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	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
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
Brain MRI**	normal	abnormal	normal	abnormal	abnormal	abnormal	abnormal	abnormal	abnormal	abnormal	abnormal
Other variants of interest**	-	+	-	-	-	-	+	+	-	+	+

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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.

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Phenotypic details

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

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

Molecular characterization of variation

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

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

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

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

Enrichment and clustering of missense variation

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

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

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

Comparison to disease associated with RAS-family GTPases

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

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

Functional analysis

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

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

Other candidate variants in probands with RALA variants

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

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

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

Discussion

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

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

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

In summary, we show that *de novo* variation affecting *RALA* in individuals with DD/ID is highly enriched compared to background mutational models, exhibits clear spatial clustering in/near to the GTP/GDP-binding region, tends to affect positions whose homologous equivalents in other small GTPases are reported to harbor disease-associated variation, and 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.
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Materials and Methods

Informed consent

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

Exome/Genome sequencing

Exome sequencing (ES) or genome sequencing (GS) was performed at each of the following sites in either a research or clinical setting. Additional details, including cohort sizes used in p-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	GS, Proband Only	Research
		Biotechnology		

Three dimensional modeling

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

Cloning, protein expression, and purification

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

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

GTPase activity

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

G-LISA™

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

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

Western Blot

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

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

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424

Figure Legends

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

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

Figure 3. Missense variation in RALA affects GTPase activity and RALA effector binding. A.

GTPase activity of purified recombinant RALA proteins was assessed using a luminescence assay. Raw luminescence values (measuring remaining free GTP) were subtracted from 100 to calculate activity, and were then normalized to a no template control (NTC). WT, wild-type RALA. G23D, predicted constitutively active mutant (not from a proband). ** indicates p-value = 0.0015 compared to WT, *** indicates p-value = 0.0003, and **** indicates p-value < 0.0001 compared to WT. Mean values of one experiment performed in triplicate are shown. B. Binding of purified recombinant RALA proteins to an effector molecule was assessed using an ELISA-based assay. Absorbances were normalized to a no template control (NTC). Mean values of one experiment performed in triplicate are shown. WT, wild-type RALA. **** indicates p-value < 0.0001 compared to WT. ##### indicates p-value < 0.0001 compared to NTC. ### indicates p-value = 0.0001 compared to NTC.

Conflicts of Interest

ZP is an employee of Ambry Genetics, which provides exome sequencing as a commercially available test. IMW, RW, SFS are employees of GeneDx, Inc., a wholly owned subsidiary of OPKO Health, Inc. that also offers commercial exome sequencing. The remaining authors declare no conflicts of interest

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Supplemental Files

Appendix S1. Supplemental Materials and Methods.

Appendix S2. Clinical summaries.

Appendix S3. Supplemental Figures and Tables.

References

1. Ropers HH. Genetics of intellectual disability. *Curr Opin Genet Dev.* 2008;18(3):241-50.
2. Yang Y, Muzny DM, Xia F, Niu Z, Person R, Ding Y, et al. Molecular findings among patients referred for clinical whole-exome sequencing. *JAMA.* 2014;312(18):1870-9.
3. Taylor JC, Martin HC, Lise S, Broxholme J, Cazier JB, Rimmer A, et al. Factors influencing success of clinical genome sequencing across a broad spectrum of disorders. *Nat Genet.* 2015;47(7):717-26.
4. Niemi M, Martin HC, Rice DL, Gallone G, Gordon S, Kelemen M. Common genetic variants contribute to risk of rare severe neurodevelopmental disorders; 2018. Preprint. Available from bioRxiv: <https://doi.org/10.1101/309070>.
5. Simanshu DK, Nissley DV, McCormick F. RAS Proteins and Their Regulators in Human Disease. *Cell.* 2017;170(1):17-33.
6. Cao H, Alrejaye N, Klein OD, Goodwin AF, Oberoi S. A review of craniofacial and dental findings of the RASopathies. *Orthod Craniofac Res.* 2017;20 Suppl 1:32-8.
7. Altmuller F, Lissewski C, Bertola D, Flex E, Stark Z, Spranger S, et al. Genotype and phenotype spectrum of NRAS germline variants. *Eur J Hum Genet.* 2017;25(7):823-31.
8. Sobreira N, Schiettecatte F, Valle D, Hamosh A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. *Hum Mutat.* 2015;36(10):928-30.
9. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet.* 2014;46(3):310-5.

- 499 10. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of
500 protein-coding genetic variation in 60,706 humans. *Nature*. 2016;536(7616):285-91.
- 501 11. The NHLBI Trans-Omics for Precision Medicine (TOPMed) Whole Genome Sequencing
502 Program. BRAVO variant browser. University of Michigan and NHLBI. 2018 [cited 20 June 2018].
503 Available from: <https://bravo.sph.umich.edu/freeze5/hg38/>.
- 504 12. Wilson JM, Prokop JW, Lorimer E, Ntantie E, Williams CL. Differences in the
505 Phosphorylation-Dependent Regulation of Prenylation of Rap1A and Rap1B. *J Mol Biol*.
506 2016;428(24 Pt B):4929-45.
- 507 13. Wu JC, Chen TY, Yu CT, Tsai SJ, Hsu JM, Tang MJ, et al. Identification of V23RAlA-Ser194
508 as a critical mediator for Aurora-A-induced cellular motility and transformation by small pool
509 expression screening. *J Biol Chem*. 2005;280(10):9013-22.
- 510 14. Lim KH, Brady DC, Kashatus DF, Ancrile BB, Der CJ, Cox AD, et al. Aurora-A
511 phosphorylates, activates, and relocalizes the small GTPase RalA. *Mol Cell Biol*. 2010;30(2):508-
512 23.
- 513 15. Gentry LR, Nishimura A, Cox AD, Martin TD, Tsygankov D, Nishida M, et al. Divergent
514 roles of CAAX motif-signaled posttranslational modifications in the regulation and subcellular
515 localization of Ral GTPases. *J Biol Chem*. 2015;290(37):22851-61.
- 516 16. Kinsella BT, Erdman RA, Maltese WA. Carboxyl-terminal isoprenylation of ras-related
517 GTP-binding proteins encoded by rac1, rac2, and ralA. *J Biol Chem*. 1991;266(15):9786-94.
- 518 17. Samocha KE, Robinson EB, Sanders SJ, Stevens C, Sabo A, McGrath LM, et al. A
519 framework for the interpretation of de novo mutation in human disease. *Nat Genet*.
520 2014;46(9):944-50.

18. Petrovski S, Wang Q, Heinzen EL, Allen AS, Goldstein DB. Genic intolerance to functional variation and the interpretation of personal genomes. *PLoS Genet.* 2013;9(8):e1003709.
19. Petrovski S, Ren N, Goldstein D. Genic Intolerance. Institute for Genomic Medicine. 2018 [cited 20 June 2018]. Available from: <http://genic-intolerance.org/about.jsp>.
20. Schubbert S, Zenker M, Rowe SL, Boll S, Klein C, Bollag G, et al. Germline KRAS mutations cause Noonan syndrome. *Nat Genet.* 2006;38(3):331-6.
21. Gremer L, Merbitz-Zahradnik T, Dvorsky R, Cirstea IC, Kratz CP, Zenker M, et al. Germline KRAS mutations cause aberrant biochemical and physical properties leading to developmental disorders. *Hum Mutat.* 2011;32(1):33-43.
22. Kerr B, Delrue MA, Sigaudy S, Perveen R, Marche M, Burgelin I, et al. Genotype-phenotype correlation in Costello syndrome: HRAS mutation analysis in 43 cases. *J Med Genet.* 2006;43(5):401-5.
23. Chiu AT, Leung GK, Chu YW, Gripp KW, Chung BH. A novel patient with an attenuated Costello syndrome phenotype due to an HRAS mutation affecting codon 146-Literature review and update. *Am J Med Genet A.* 2017;173(4):1109-14.
24. Edkins S, O'Meara S, Parker A, Stevens C, Reis M, Jones S, et al. Recurrent KRAS codon 146 mutations in human colorectal cancer. *Cancer Biol Ther.* 2006;5(8):928-32.
25. Chen XW, Leto D, Chiang SH, Wang Q, Saltiel AR. Activation of RalA is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c. *Dev Cell.* 2007;13(3):391-404.
26. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American

543 College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet
544 Med. 2015;17(5):405-24.

545 27. Zheng M, Zhang X, Sun N, Min C, Zhang X, Kim KM. RalA employs GRK2 and beta-
546 arrestins for the filamin A-mediated regulation of trafficking and signaling of dopamine D2 and
547 D3 receptor. Biochim Biophys Acta. 2016;1863(8):2072-83.

548 28. National Library of Medicine (US). 1q21.1 microduplication: Genetics Home Reference
549 [Internet]. Bethesda, MD. 2018 [cited 26 June 2018]. Available from:
550 <https://ghr.nlm.nih.gov/condition/1q211-microduplication>.

551 29. Thul PJ, Akesson L, Wiking M, Mahdessian D, Geladaki A, Ait Blal H, et al. A subcellular
552 map of the human proteome. Science. 2017;356(6340). Available from: v18.proteinatlas.org,
553 <https://www.proteinatlas.org/ENSG00000006451-RALA/tissue>,
554 <https://www.proteinatlas.org/ENSG00000144118-RALB/tissue>). Cited 27 June 2018.

555 30. Peschard P, McCarthy A, Leblanc-Dominguez V, Yeo M, Guichard S, Stamp G, et al.
556 Genetic deletion of RALA and RALB small GTPases reveals redundant functions in development
557 and tumorigenesis. Curr Biol. 2012;22(21):2063-8.

558 31. Carmena A, Makarova A, Speicher S. The Rap1-Rgl-Ral signaling network regulates
559 neuroblast cortical polarity and spindle orientation. J Cell Biol. 2011;195(4):553-62.

560 32. Jossin Y, Cooper JA. Reelin, Rap1 and N-cadherin orient the migration of multipolar
561 neurons in the developing neocortex. Nat Neurosci. 2011;14(6):697-703.

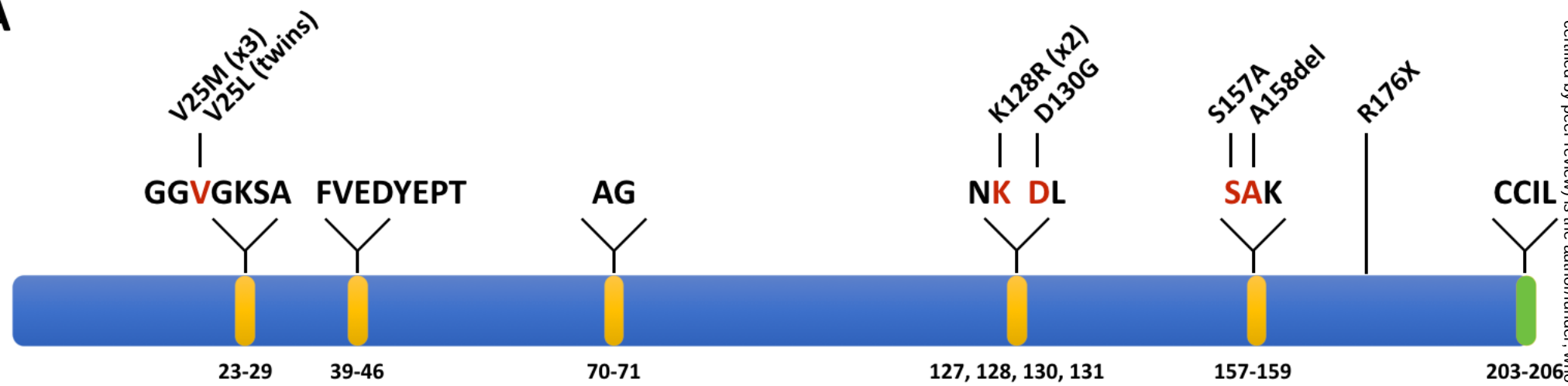
562 33. Lalli G, Hall A. Ral GTPases regulate neurite branching through GAP-43 and the exocyst
563 complex. J Cell Biol. 2005;171(5):857-69.

34. Teodoro RO, Pekkurnaz G, Nasser A, Higashi-Kovtun ME, Balakireva M, McLachlan IG, et al. Ral mediates activity-dependent growth of postsynaptic membranes via recruitment of the exocyst. *Embo J.* 2013;32(14):2039-55.
35. Lalli G. RalA and the exocyst complex influence neuronal polarity through PAR-3 and aPKC. *J Cell Sci.* 2009;122(Pt 10):1499-506.
36. Polzin A, Shipitsin M, Goi T, Feig LA, Turner TJ. Ral-GTPase influences the regulation of the readily releasable pool of synaptic vesicles. *Mol Cell Biol.* 2002;22(6):1714-22.
37. Holbourn KP, Sutton JM, Evans HR, Shone CC, Acharya KR. Molecular recognition of an ADP-ribosylating *Clostridium botulinum* C3 exoenzyme by RalA GTPase. *Proc Natl Acad Sci U S A.* 2005;102(15):5357-62.
38. DeLano WL. PyMOL. DeLano Scientific, San Carlos, CA, USA. 2002.
39. Prokop JW, Lazar J, Crapitto G, Smith DC, Worthey EA, Jacob HJ. Molecular modeling in the age of clinical genomics, the enterprise of the next generation. *J Mol Model.* 2017;23(3):75.

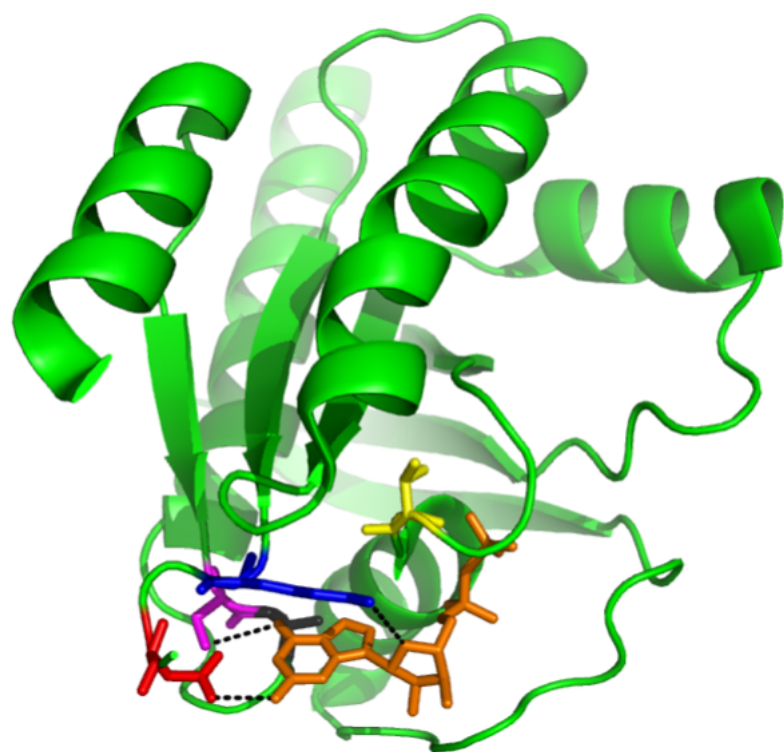
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See Page 11 for descriptions of overlapping dysmorphic
features, in addition to clinical summaries in Appendix S2.**

Figure 2

A



B



C

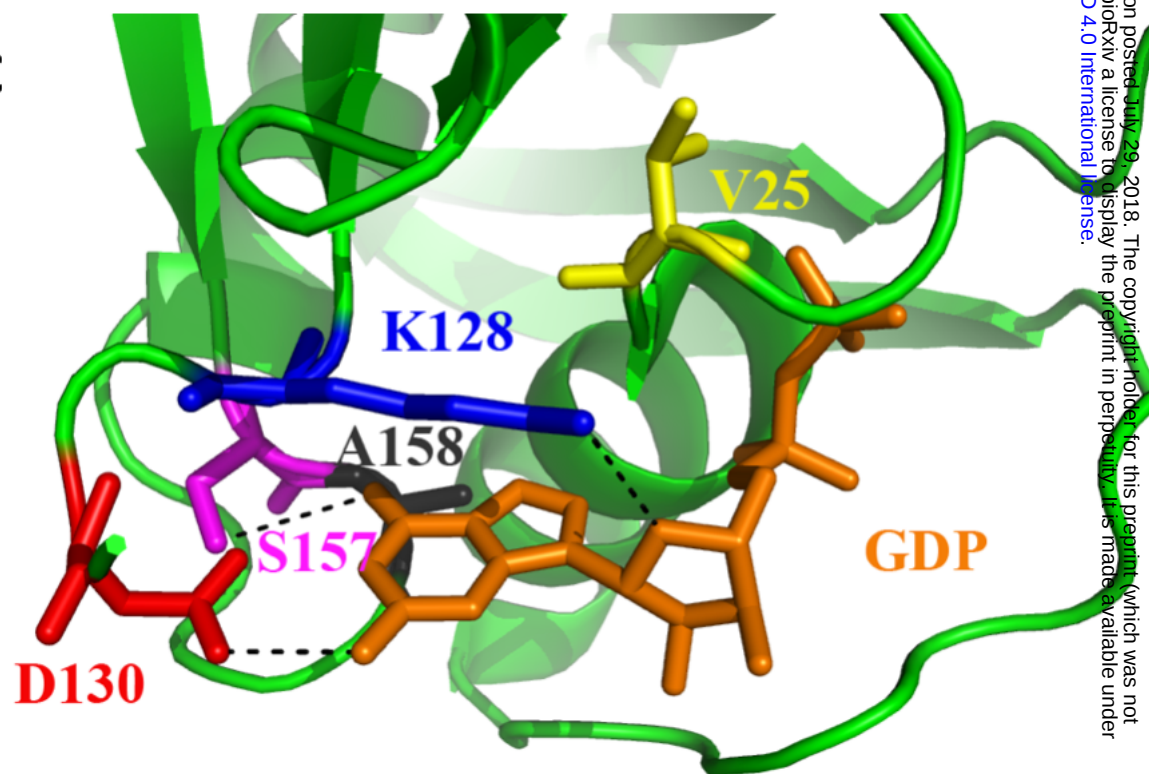


Figure 3

