

Supplemental Data

Germline-Activating *RRAS2* Mutations

Cause Noonan Syndrome

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Supplemental Note: a case report

HU1 was a boy and the third child of healthy non-consanguineous parents. Polyhydramnios and enlargement of cerebral ventricles were pointed out in his gestational period. He was born at 36 weeks of gestational age by vaginal delivery. His body weight, body length, and head circumference at birth were 2,644 g ($-0.31SD$), 49.0 cm ($+0.83SD$), and 37.7 cm ($+3.8SD$), respectively. He had respiratory distress due to hypotonia and respiratory care was initiated by nasal directional positive airway pressure. Oxygenation was continued by 31 days of age. He had macrocephaly, low set ears, micrognathia, unilateral cryptorchidism, hydrocele testicle, and micropenis. On the other side, no obvious pulmonary artery stenosis and lymphatic malformation were pointed out. Trans-fontanel ultrasonography showed an enlargement of ventricles. His chromosomal G-banding and microarray analysis were normal. At the age of 5 months, analyses of organic acids, acylcarnitine, and very long chain fatty acids were normal. At the same age, he started tube feeding because he had a gastroesophageal reflux leading to a respiratory disease. A head magnetic resonance imaging at age 6 months showed enlargement of his lateral, third, and fourth ventricles but no Chiari malformations. His brain myelination was equivalent to that expected for a 4-month-old infant (Fig. 1K). He had a gastrostomy at the age of 13 months and a ventriculoperitoneal shunt surgery at age 14 months. At the

age of 14 months, eosinophilia and eosinophils in stool were found, suggesting the presence of a milk allergy. After the age of 2 years and 2 months, vomiting persisted and he had a ketotic hypoglycemia (blood glucose 29 mg/dL, acetoacetic acid 196 μ mol/L; normal < 55 μ mol/L, 3-hydroxybutyric acid 108 μ mol/L; normal < 85, NH₃ 40 μ g/dL; normal 30–86 μ g/dL) at age 2 years and 3 months. The laboratory test data on admission showed elevated brain natriuretic peptide (BNP, 798 pg/mL; normal < 18.4 pg/mL) and decreased insulin-like growth factor 1 (< 4 ng/mL; normal 24–164 ng/mL). Echocardiography revealed cardiac hypofunction without the findings of hypertrophic or dilated cardiomyopathy. Adenovirus 41 was detected by virus isolation from his urine and stool. The cardiac hypofunction persisted after this infectious episode. At the age of 3 years 0 month, he had clinical signs suggesting infection such as hard breathing, cough, and rhinorrhea. His white blood cell count and C-reactive protein were slightly elevated. His respiratory disease got worse during 4 days, and he was hospitalized. BNP on admission was 586 pg/mL. Ultrasound imaging showed marked dilation of left ventricle and contractile dysfunction. After three days of admission, he died of cardiac failure in spite of mechanical ventilation and administration of catecholamines in an intensive care unit. He had severe failure to thrive and developmental delay throughout his life. At age 3 years, his body weight, body length, and head circumference were 6,145

g (-5.0 SD), 72.5 cm (-5.9 SD), and 51.0 cm (-0.1 SD), respectively. He was able to smile, pursue something visually, and shake his head, but not to control his head.

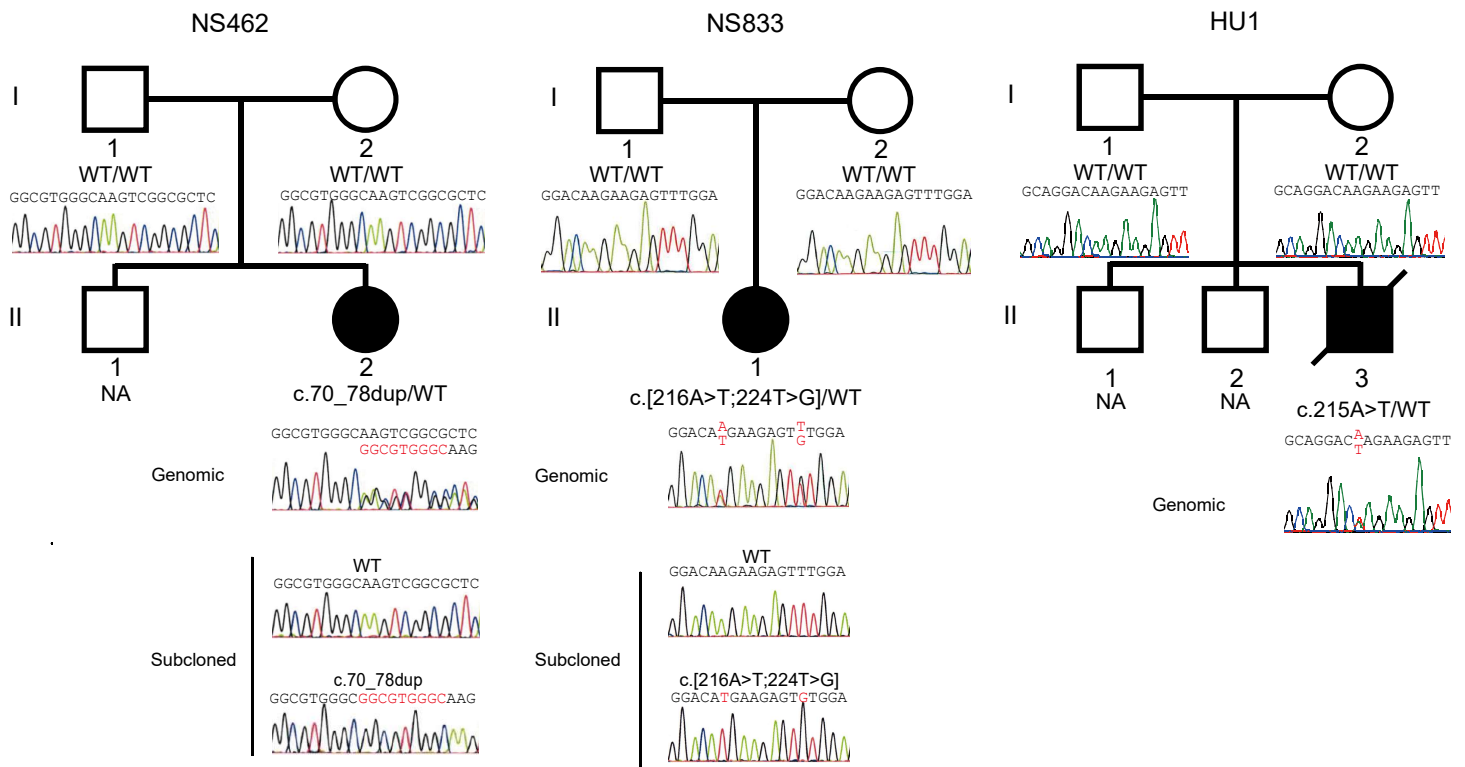


Figure S1. Sanger sequencing of *de novo* variants in *RRAS2* identified in individuals with Noonan syndrome (NS)

Four *de novo* variants in *RRAS2* were identified in three individuals with NS. PCR products amplified using genomic DNA from peripheral blood of individuals NS462 (II-2) and NS833 (II-1) were subcloned into pCR4 TOPO vector and sequenced. Subcloned sequence of NS833 shows that the two variants were on the same chromosome. Abbreviations: WT, wide-type; and NA, not available

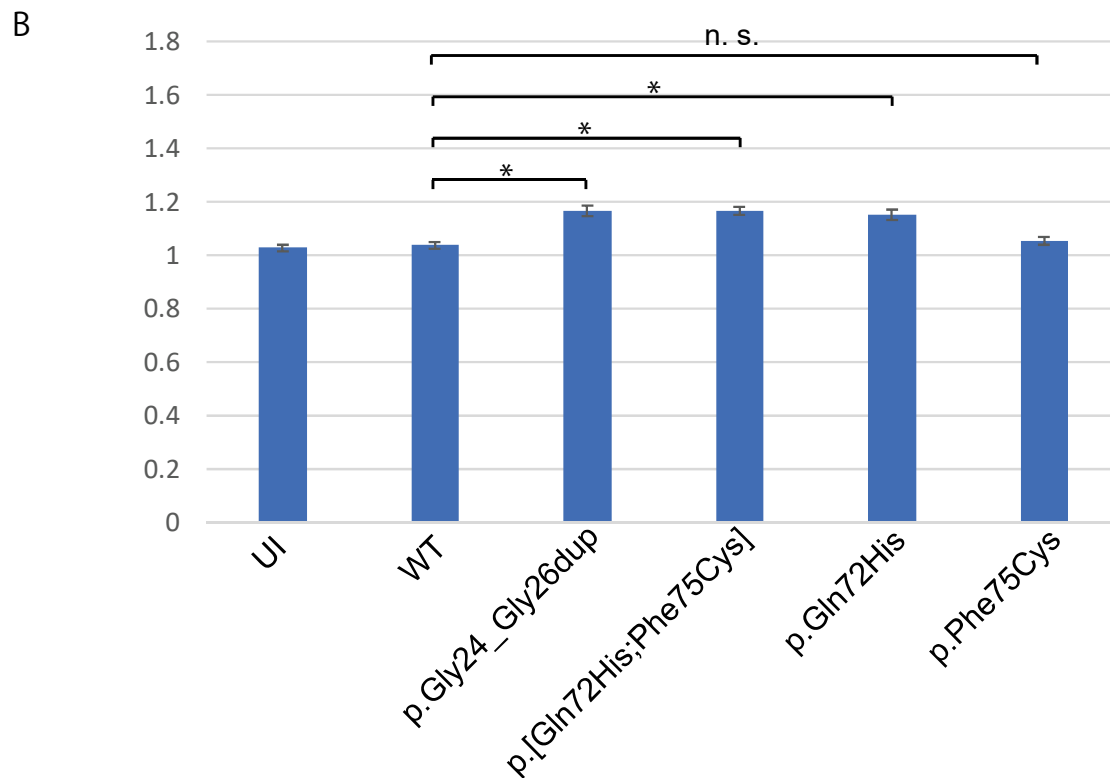
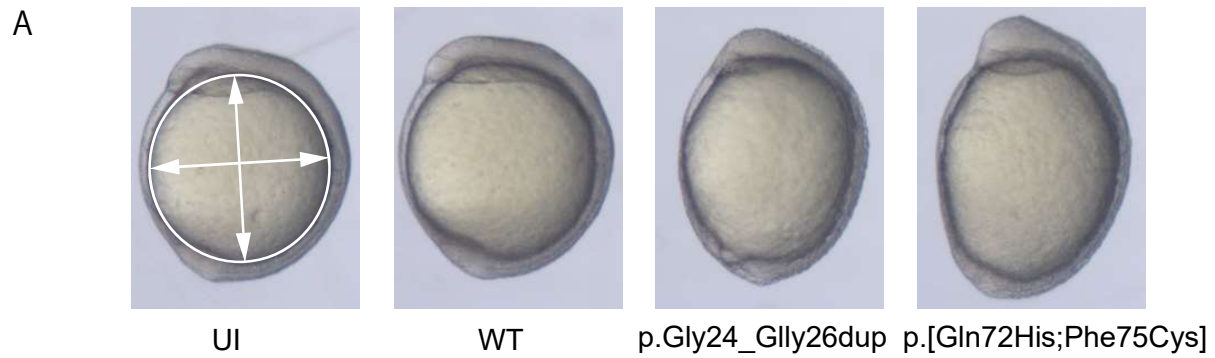
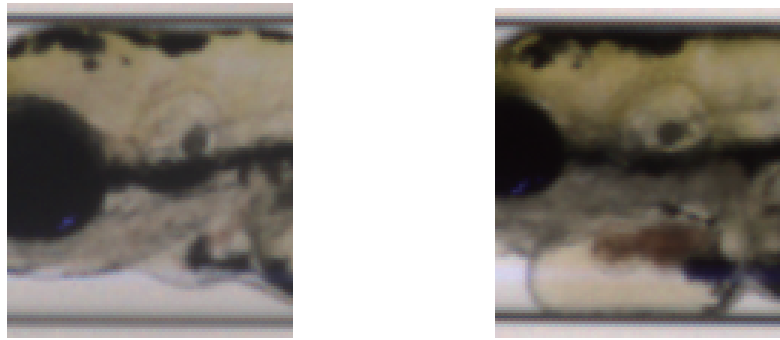


Figure S2. Morphology of zebrafish embryos injected with wild-type (WT) or mutant *RRAS2* mRNA at 11 hpf

(A) Embryos were injected at the 1–4 cell stages with synthetic RNA (25 pg) encoding *RRAS2* (wild-type or mutant) and morphology was assessed at 11 hpf. The major and minor axes (pointed by axes) of the yolk were measured using Image J. Representative batches of embryos are depicted. (B) Quantification of the ratio of the major and minor axes upon injection with synthetic RNAs encoding WT or mutant *RRAS2* as indicated. $n = 76–85$ embryos per batch. Error bars represent standard errors of the mean. n.s., not significant;

*: $P < 0.001$

A



B

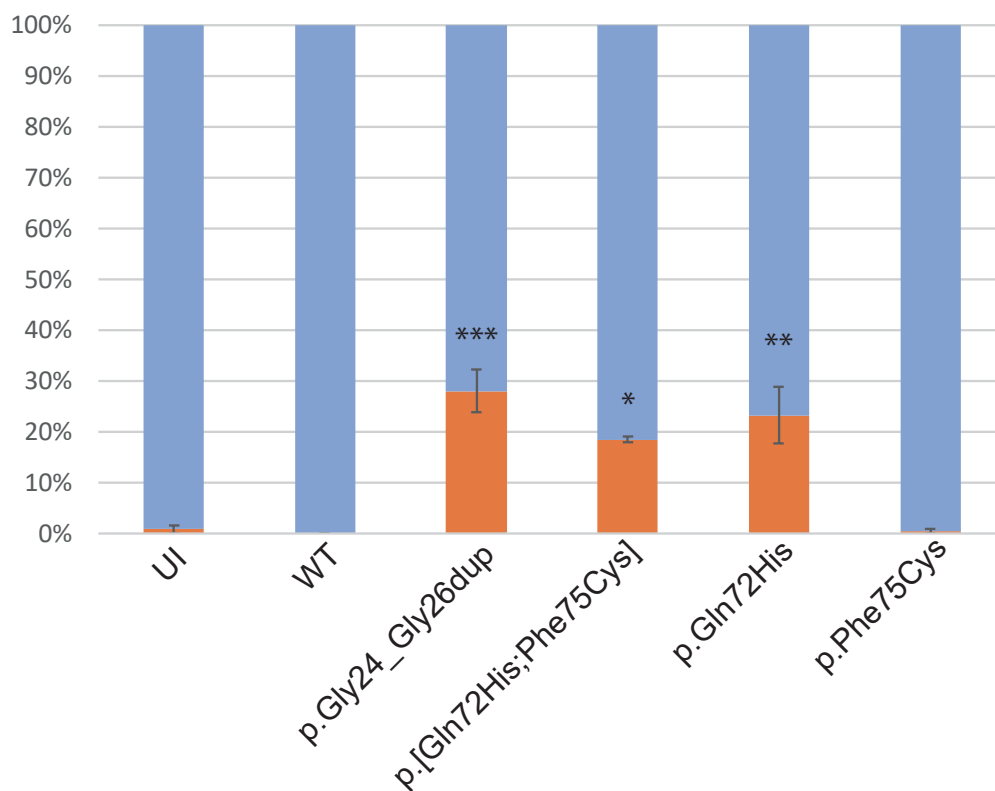


Figure S3. Pericardial edema of zebrafish embryos injected with wild-type (WT) or mutant *RRAS2* mRNA at 3 days post fertilization (dpf)

(A) Representative image of larvae at 3 dpf. Left panel: normal morphology of pericardium. Right panel:

pericardial edema. (B) Ratio of larvae with pericardial edema. Experiments were repeated three times and the mean percentages of larvae with pericardial edema were shown. $n = 32-86$ larvae per batch.

Error bars show standard errors of the mean. ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$, compared with WT.

Table S2. *In Silico* predictions of *RRAS2* variants in individuals with NS.

Chr	Position	Ref	Alt	Nucleotide change (NM_012250.5)	Amino acid change (NP_036382.2)	PROVEAN	Mutation Taster	Polyphen-2 (Humdiv)	CADD >30
11	14,380,338	-	GCCCACGCC	c.70_78dup	p.Gly24_Gly26dup	Deleterious	polymorphism	-	No (17.69)
11	14,316,389	T	A	c.216A>T	p.Gln72His	Deleterious	disease causing	Probably damaging	No (27.1)
11	14,316,390	T	A	c.215A>T	p.Gln72Leu	Deleterious	disease causing	Possibly damaging	No (28.4)
11	14,316,381	A	C	c.224T>G	p.Phe75Cys	Deleterious	disease causing	Probably damaging	Yes (31)

Table S3. Primer pairs used to amplify coding exons and their flanking introns in *RRAS2*

Flagment	Exon	Forward	Reverse
1	1 ^a	5'-F-gtttcattctctgccagcca	5'-R-tagagtggagaggggatgc
2	1 ^b	5'-F-tcatgcatatgcagcacctca	5'-R-gaccacattcctgagaagc
3	2 ^{a,b}	5'-F-gcctcaagtgatccttctc	5'-R-catgggctaattccagatc
4	3 and 4 ^{a, b, c}	5'-F-gctctctagaggactcaca	5'-R-cacttaagtggcatggagc
5	5 ^{a,b, c}	5'-F-aaacaacttggccttcttggc	5'-R-ccctagaaaggaatcacttcc
6	6 ^{a,b, c}	5'-F-ggaaagagagaaattccctcg	5'-R-ggctagaaaggtaccaacaag

a, in NM_001177314.1; b, in NM_012250.5; c, in NM_001177315.1 and NM_001102669.2; F, 5'- gtaaacgacggccagt; R, 5'- aggaaacagctatgacc. Exons 1 and 2 of NM_001177315.1 and NM_001102669.2 were non-coding exons.

Supplemental Materials and Methods

Genetic analysis

This study was approved by the Ethics Committee of the Tohoku University School of Medicine. Written informed consent was obtained from all subjects involved in the study or from their parents. This study included 27 individuals for WES and 191 individuals for Sanger sequencing with suspected NS or related disorders who were not found to harbor a mutation based on Sanger sequence screening, which included *PTPN11* (exons 1–15), *SHOC2* (exon 1), *KRAS* (exons 1–5), *RAF1* (exons 7, 14, and 17), *HRAS* (exons 1–5), *BRAF* (exons 6 and 11–16), *MAP2K1* (exons 2 and 3), *MAP2K2* (exons 2 and 3), *SOS1* (exons 1–23), and *RIT1* (exons 1–6). We extracted DNA from peripheral blood according to standard procedures, from hair and fingernails using IsoHair (Nippon Gene, Tokyo, Japan), and/or from saliva using Oragene (DNA Genotek, Ottawa, Ontario, Canada). Libraries for WES were prepared using the SureSelect Human All Exon Kit Ver 4 (NS462) or Ver 6 (NS833 and others) (Agilent technologies, Santa Clara, CA, USA). Libraries were sequenced for 101 (NS462) or 126 (NS833 and others) bases with paired-ends on Hiseq 2500 (Illumina, San Diego, CA, USA). Mapping, variant calls, and annotations were performed as described¹. WES on DNAs from HU1 and his parents were performed as described previously². Microarray

analysis of DNA from the peripheral blood samples of NS462 was performed using CytoScan HD array (Affymetrix, Santa Clara, CA, USA). Sanger sequencing was performed as described³ using primers listed in Supplemental Table 3.

Plasmid construction and site-direct mutagenesis

We generated human *RRAS2* cDNA by RT-PCR using human leukocyte cDNA as a template. The human *RRAS2* ORF (NM_012250.5) was amplified by PCR using a forward primer containing a *Bam*HI site and a reverse primer containing an *Eco*RI site. The amplified fragment was digested with *Bam*HI and *Eco*RI and cloned into pCS2+. Mutagenesis was performed using a QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). All vectors were confirmed by Sanger sequencing.

Active RAS pull-down assay and immunoblotting

To evaluate binding between RBD and *RRAS2*, we performed a RAS pull-down assay. The RAS Assay Reagent (RAF-1 RBD agarose, #14-278) was purchased from Millipore (Burlington, MA, USA). HEK293 cells were plated in 6-cm dishes at 5×10^5 cells per well and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Twenty-four

hours later, cells were transfected using Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) with 5 µg WT or mutant *RRAS2* construct. The next day, medium was replaced by serum-free DMEM. Cells were harvested after serum starvation for 24 hours. The pull-down assay was performed according to the manufacturer's protocol of the RAS Activation Assay kit (#17-218, Millipore) using lysis/wash buffer (25 mM Tris/HCl pH8.0, 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 5% glycerol) instead of Mg²⁺ Lysis/Wash Buffer (# 20-168, Millipore). RAF-1 RBD agarose beads after pull-down reaction and cell lysates were mixed with 4x Laemmli sample buffer (Biorad, Hercules, CA, USA) and boiled at 95 °C for 5 minutes. Electrophoresis was performed on 5-20% gradient gels (E-R520L, ATTO, Tokyo, Japan). Antibodies against RRAS2 (PA5-22123, Thermo Fisher Scientific, Waltham, MA, USA), p44/42 MAPK (#9102, Cell Signaling Technology (CST), Danvers, MA, USA), phospho-p44/42 MAPK (#9101, CST), MEK1/2 (#8727, CST), phospho-MEK1/2 (#9154, CST), AKT(#9272, CST), phospho-AKT (#9272 for Ser473 and #2965 for Thr308, CST) and β-actin (A5316, Sigma, St. Louis, MO, USA) were used for immunoblotting. All experiments were performed in triplicates. Intensities of suggested molecules were quantified with Image J. Statistical analyses were performed by Dunnett's test using the multcomp package of R_3.5.1.

Luciferase assay

We performed a reporter assay to determine the effect on downstream of RAS/MAPK signaling pathway. HEK293 cells were plated in 24-well plates at 5×10^4 cells per well. After 24 hours, cells were transfected transiently with 300 ng pFR-luc, 15 ng pFA2-ELK1, 10 ng phRLnull-luc, and 50 ng WT or mutant *RRAS2* construct using Lipofectamine 3000 (Invitrogen). After 18 hours, cells were serum starved in DMEM for 24 hours. Cells were then harvested in passive lysis buffer, and luciferase activity was assayed using the Promega Dual-Luciferase Assay Kit (Promega, Madison, WI, USA). Renilla luciferase expressed by phRLnull-luc was used to normalize transfection efficiency. The experiments were performed using three wells in each condition. Statistical analyses were performed by Dunnett's test using the multcomp package of R_3.5.1.

Zebrafish embryos

All zebrafish-related experiments were performed in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee.

Zebrafish embryos were obtained by natural mating of transgenic (*-1.4coll1a1:egfp⁴*) adults on AB background and were maintained at 28°C on a 14 h/10 h light/dark cycle.

RNA injections in zebrafish

pCS2+ constructs of WT and variant *RRAS2* were linearized with *NotI*, and capped mRNA was transcribed with the mMessage mMachine SP6 Kit (Thermo Fisher) according to the manufacturer's instructions. Approximately 1 nL cocktail of 5 or 25 ng/ μ L RNA and 0.2% phenol red were injected into yolks of embryos at the 1–4 cell stage. At 11 hpf, we obtained images of embryos to determine yolk elongation. At 3 dpf, larval batches were anesthetized with 0.2 mg/mL Tricaine and imaged using the Vertebrate Automated Screening Technology Bioimager (VAST, Union Biometrica, Holliston, MA, USA). We obtained fluorescent images of GFP-positive cells on ventrally positioned larvae and dorsal and lateral brightfield images of whole larvae using VAST. Phenotypic analyses (measuring length of the major and minor axes of embryos at 11 hpf and length of body and head at 3 dpf) were performed using Image J. Statistical analyses were performed by Dunnett's test using the multcomp package of R_3.5.1. or Student's t test using Excel.

References

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