Recurrent NFIA K125E substitution represents a loss-of-function allele: Sensitive in vitro and in vivo assays for nontruncating alleles

Tomoko Uehara1 | Rikako Sanuki2 | Yurie Ogura3 | Atsushi Yokoyama4 | Takeshi Yoshida4 | Hiroshi Futagawa5 | Hiroshi Yoshihashi5 | Mamiko Yamada1 | Hisato Suzuki1 | Toshiki Takenouchi6 | Kohei Matsubara2 | Hiromi Hirata3 | Kenjiro Kosaki1 | Toshiyuki Takano-Shimizu2

1Center for Medical Genetics, Keio University School of Medicine, Tokyo, Japan
2Advanced Insect Research Promotion Center, Kyoto Institute of Technology, Kyoto, Japan
3Department of Chemistry and Biological Science, College of Science and Engineering, Aoyama Gakuin University, Sagamihara, Kanagawa, Japan
4Department of Pediatrics, Kyoto University Graduate School of Medicine, Tokyo, Japan
5Department of Genetics, Tokyo Metropolitan Children’s Medical Center, Tokyo, Japan
6Department of Pediatrics, Keio University Hospital, Tokyo, Japan

Correspondence
Toshiyuki Takano-Shimizu, Advanced Insect Research Promotion Center, Kyoto Institute of Technology, Saga Ippongi-cho, Ukyo-ku, Kyoto 616-8354, Japan.
Email: fruitfly@kit.ac.jp

Kenjiro Kosaki, Center for Medical Genetics, Keio University School of Medicine, 35 Shinanomachi Shinjuku-ku, Tokyo 160-8582, Japan.
Email: kkosaki@keio.jp

Funding information
Japan Agency for Medical Research and Development, Grant/Award Numbers: 18ek0109288h0002, 18ek0109301, 19ek0109288h0003, 19gk0110038h, 20ek0109484h0001

Abstract
Nuclear factor I A (NFIA) is a transcription factor that belongs to the NFI family. Truncating variants or intragenic deletion of the NFIA gene are known to cause the human neurodevelopmental disorder known as NFIA-related disorder, but no patient heterozygous for a missense mutation has been reported. Here, we document two unrelated patients with typical phenotypic features of the NFIA-related disorder who shared a missense variant p.Lys125Glu (K125E) in the NFIA gene. Patient 1 was a 6-year-old female with global developmental delay, corpus callosum anomaly, macrocephaly, and dysmorphic facial features. Patient 2 was a 14-month-old male with corpus callosum anomaly and macrocephaly. By using Drosophila and zebrafish models, we functionally evaluated the effect of the K125E substitution. Ectopic expression of wild-type human NFIA in Drosophila caused developmental defects such as eye malformation and premature death, while that of human NFIA K125E variant allele did not. nfia-deficient zebrafish embryos showed defects of midline-crossing axons in the midbrain/hindbrain boundary. This impairment of commissural neurons was rescued by expression of wild-type human NFIA, but not by that of mutant variant harboring K125E substitution. In accordance with these in vivo functional analyses, we showed that the K125E mutation impaired the transcriptional regulation of HES1 promoter in cultured cells. Taken together, we concluded that the K125E variant in the NFIA gene is a loss-of-function mutation.

KEYWORDS
corpus callosum anomaly, loss-of-function, model organisms, NFIA

Tomoko Uehara, Rikako Sanuki, and Yurie Ogura contributed equally to this study.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2021 The Authors. American Journal of Medical Genetics Part A published by Wiley Periodicals LLC.
1 | INTRODUCTION

The prevalence of intragenic deletions in patients with aplasia or hypoplasia of the corpus callosum and developmental delay suggests that haploinsufficiency of the *Nuclear factor I A* (*NFIA*) gene is a primary cause of chromosome 1p32-p31 deletion syndrome or brain malformations with or without urinary tract defects (MIM 613735) (Bayat et al., 2017; Hollenbeck et al., 2017; Mikhail et al., 2011; Nyboe et al., 2015; Rao et al., 2014). Identification of frameshift and nonsense mutations in *NFIA* further supports this notion (Negishi et al., 2015; Revah-Politi et al., 2017; Zhang et al., 2020). Indeed, the mouse ortholog of this conserved transcription factor, *Nfia*, is required for differentiation and maturation of astrocyte and oligodendrocyte and its loss results in the aplasia/hypoplasia of corpus callosum and urinary tract defects (das Neves et al., 1999; Lu et al., 2007).

Despite growing recognition of the impact of *NFIA* haploinsufficiency on the neurodevelopmental disorder, there is no patient heterozygous for a pathogenic missense variant to date (but see also Zenker et al., 2019 for three candidate pathogenic variants). Here, we report the same de novo missense mutation K125E in the *NFIA* gene in two unrelated patients. By using *Drosophila* and *zebrafish* models (Suzuki et al., 2019; Uehara et al., 2020) as well as cell culture system, we unambiguously demonstrated that this K125E missense variant represents a loss-of-function pathogenic allele. Presently reported in vivo assays will be useful for functional evaluation of other missense variants of *NFIA*.

2 | CLINICAL REPORT

Patient 1 was a 6-year-old female who was the first child of healthy and nonconsanguineous Japanese parents. She was born after an uncomplicated pregnancy at 35 weeks and 4 days of gestation. Her weight at birth was 2142 g (−0.5 SD), length was 43 cm (−1.2 SD), and head circumference was 33 cm (−0.8 SD). After birth, she showed tachycardia. Head ultrasound showed ventricular enlargement and intraventricular hemorrhage. She had been in neonatal intensive care unit since 34-day-old. After discharge, she attended her hospital regularly due to cerebral palsy and global developmental delay. At 2 years of age, she underwent an operation for exotropia. She had congenital hearing loss and wore hearing aids at 5 years of age. Head magnetic resonance imaging (MRI) at 5 years of age showed thin corpus callosum, cyst of septi pellucidi, ventricular wall irregularity, and periventricular leukomalacia (Figure 1a). She showed distinctive dysmorphic features with high hairline, small eyes, anteverted nares, a depressed nasal bridge, a broad columella, and a thin upper-lip (Figure 1b). Her developmental milestones were delayed. She started to walk at the age of 3 years. She also stated to

![FIGURE 1](https://wileyonlinelibrary.com)

**Clinical characteristics of two patients with the same NFIA variant.** (a) Results of head MRI of Patient 1 at 5 years of age. The picture above shows sagittal T1-weighted image. The picture below shows axial T2-weighted fluid-attenuated inversion recovery image. Note thin corpus callosum, cyst of septi pellucidi, ventricular wall irregularity, and decreased white matter volume. (b) Pictures of Patient 1 at 6 years of age. Note the high hairline, small eyes, anteverted nares, a depressed nasal bridge, a broad columella, and a thin upper-lip. (c) Results of head MRI of Patient 2 at 1 month of age. The picture above shows sagittal-T2-weighted image. The picture below shows axial diffusion-weighted image. Note polycerebral gyrus at parasylvius fissures, cortical dysplasia of bilateral cerebral hemisphere, partial myelination delay, and hypoplasia of corpus callosum. [Color figure can be viewed at wileyonlinelibrary.com]
speak her words at the age of 3 years and spoke only few words at the age of 6 years. Her developmental quotient as assessed using the WISC-IV test was 23. Her physical growth was also delayed. At 6 years of age, her weight was 15.9 kg (~1.3 SD), height was 102.4 cm (~2.4 SD), and head circumference was 54.5 cm (~2.4 SD). She had no urogenital anomalies. Patient 2 was a 14-month-old boy who was born at 34 weeks gestation. He had been diagnosed at 34 weeks gestation with a head enlargement. His weight at birth was 2635 g (~2.1 SD), length was 47.5 cm (~1.6 SD), and head circumference was 35.3 cm (~3.0 SD). After birth, a head MRI showed polycylic gyri at parasympatines fissures, cortical dysplasia of bilateral cerebral hemisphere, partial myelination delay, and hypoplasia of corpus callosum (Figure 1c). He had mild congenital hearing anomalies. He showed distinctive dysmorphic features with high hairline, thick eyebrow, short nose, anteverted nares, long philtrum, thin upper-lip vermilion, and a retrognathia. His developmental milestones were delayed; he gained head control and rolling over at 8 months of age, sat, and walked independently at 1 year of age. He was able to stand with support at 1 year of age. At 1 year and 1 month of age, his weight was 10.42 kg (~0.3 SD), height was 81.0 cm (~1.9 SD), and head circumference was 52.0 cm (~3.9 SD).

3 | METHODS

3.1 | Mutation analysis

Approval from the local institutional review board and informed consent from the patients' parents were obtained prior to the molecular studies. Whole exome sequencing using a SureSelect XT Human All exon V6 Panel (Agilent Technology, Santa Clara, California) on a HiSeq platform (Illumina, San Diego, California) was performed for Patient 1 and her parents. Medical exome sequencing using the TruSight One Sequencing Panel (Illumina) on a MiSeq platform (Illumina) was performed for Patient 2 and his parents. We confirmed their results by performing Sanger sequencing with the following primers: NFIA sense, 5’-AAA ACC AGA GGT CAA GCA GAA G-3’, and NFIA antisense, 5’-ATT CTC ACC ATC GCA CTT ACC T-3’.

3.2 | Functional assays in Drosophila

We PCR-amplified the NFIA open reading frame sequence from a human cDNA clone (Kazusa DNA Research Institute, Chiba, Japan, ORK00836) and subcloned it into a modified pENTR221 vector. The K125E mutation was introduced into the subclone by site-directed mutagenesis. Both subcloned fragments were verified by sequencing, and transferred into a destination vector pUASg-attB (Bischof et al., 2013) via the LR reaction (Invitrogen). The plasmid DNA was injected into embryos carrying the attP40 landing site for phiC31 integrase-mediated transformation (y¹ v¹ P[y¹+t7.7]=nos-phiC31)int. NLS[X]. P[y¹+t7.7]=CaryP] attP40, Bloomington Drosophila Stock Center, #25709). The wildtype and mutant NFIA transgenes were expressed via the GAL4/UAS system. The GAL4 drivers used in this study were nSyb-GAL4 [y¹ w[1118]; P[y¹+t7.7] w[+mC]=nSyb-GAL4. P{attP2, Bloomington Drosophila Stock Center, BDSC# 51941] for pan-neuronal expression, GMR-GAL4 [w; P[w[+mC]=GAL4-ninaE.GMR] 12, KYOTO Stock Center, DGRC# 106207] for expression in the retina, and ey-GAL4 [w; P[w[+mC]=GAL4-ey.HJ3-8, KYOTO Stock Center, DGRC# 108283] for expression in the eye-antenna disc.

3.3 | Functional assays in zebrafish

Zebrafish (Danio rerio) were reared and maintained under a 14 h light and 10 h dark photoperiod according to the standard protocol. Zebrafish carrying the nfia Q232X nonsense mutant allele (nfia<sup>Q232X</sup>) was obtained from Zebrafish International Resource Center and was used for rescue experiments with wild-type and K125E mRNA. nfia<sup>Q232X</sup> was previously generated by a targeting induced local lesions in genomes project (Kettleborough et al., 2013). For rescue experiments, wild-type human NFIA coding sequence was generated by a DNA synthesis service (Fasmac, Japan) and subcloned into an expression vector pCS2+. The K125E point mutation was introduced into human NFIA by the QuickChange method (Agilent Technologies) using following two primers: 5’-GCTGCAACACCTTTAAGCATTTC TTGGGGATGTTCTAATG-3’ and 5’-CATTAGATACCATCCCAAG AAATGCTTAAAGAGTTTGTGCAGC-3’. These constructs were used to generate wild-type and mutant NFIA mRNA using the mMESSAGE mMACHINE SP6 Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. The human NFIA mRNAs (100 pg) were injected into 1–2-cell stage zebrafish embryos produced by crossing nfia heterozygous mutant carrier fish. Embryos were fixed at 72 h postfertilization (hpf) and subjected to immunolabeling using anti-acetylated α-tubulin (clone 6-11B-1; Sigma), HRP-conjugated anti-mouse IgG (Invitrogen), and ImmPACT DAB Substrate (Vector Laboratories). For genotyping of immunolabeled embryos, the region surrounding the Q232X mutation site was amplified by genomic PCR using following two primers; 5’-CTGTATTCGTCATGCTCATTACAGTAACGC-3’ and 5’-GCTCAATGTATGCTCCCAAGAAAG-3’. PCR products were digested with Fai I restriction enzyme (SibEnzyme, Russia) and separated by 15% polyacrylamide gel electrophoresis. This zebrafish study was approved by Animal Care and Use Committee of Aoyama Gakuin University (A9/2020) and carried out according to the Aoyama Gakuin University Animal Care and Use Guideline.

3.4 | Cell culture

293T cells (RCB2202, provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan) were grown in Dulbecco's modified Eagle's medium (044–29765, Fujifilm Wako Pure Chemical, Osaka, Japan), supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France) and a penicillin-streptomycin solution (Nacalaï Tesque, Kyoto, Japan), and cultured at 37°C and 5% CO2 condition.
3.5 | NanoLuc reporter assay

The human 0.6-kb (~602 to +44) GFAP and 1.2-kb (~1039 to +135) HES1 promoter regions were amplified by PCR from human genome and were subcloned into the reporter vector pNL2.2 (Promega Corp., Madison, Wisconsin). The pNL2.2 reporter constructs were cotransfected with pCAGGS-Luc2, and pCAGGS-NFIA-3xFlag plasmids into 293T cells using the calcium phosphate-mediated method. Two days later, the cells were lysed in passive lysis buffer (Promega Corp.), and the luciferase and NanoLuc activities were measured using the Nano-Glo Dual-Luciferase reporter assay system (Promega Corp.) according to the manufacturer’s instructions.

4 | RESULTS

4.1 | Variants identified in two patients

Trio exome analysis showed that both patients had the same de novo nonsynonymous mutation in the NFIA gene (NM_001134673.4), c.373A>G (p.Lys125Glu). The DNA-binding domains of NFIA and its paralogs are highly conserved and the K125 residue is identical across vertebrates and invertebrates studied (Figure 2). Indeed, the combined annotation-dependent depletion scores (Kircher et al., 2014) for the K125E variant was 26.8. This variant was absent in the database of 3552 normal Japanese individuals (Japanese Multi Omics Reference Panel) (https://jmorp.megabank.tohoku.ac.jp/202001/variants) (Tadaka et al., 2019) and also absent in the Genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org/).

4.2 | Functional assays of K125E variant in Drosophila

To assess the functional significance of the K125E mutation in vivo, we introduced the wild-type and mutant human NFIA transgenes into Drosophila and expressed them in the nervous system and imaginal discs using the GAL4/UAS system. We found that ectopic expression of wild-type NFIA allele (NFIAWT) in the Drosophila retina by the GMR-GAL4 driver caused defects in eye development (Figure 3a). However, the K125E allele (NFIAK125E) did not cause any change in the morphology (Figure 3b). Likewise, when driven by the pan-neuronal nSyb-GAL4 driver, NFIAWT, but not NFIAK125E, caused embryonic or first larval lethality (data not shown). What is more, NFIA expression by the

**FIGURE 2** Amino acid sequence alignment of the DNA-binding domains of human NFIA with its orthologs and paralogs. Sequences and domain definitions were obtained from UniProt (The UniProt Consortium, 2019). HsPA: human NFIA (Q12857, residues 1–194); HsPB: human NFIB (Q00712, 1–194); HsPC: human NFIC (P08651, 1–194); HsPD: human NFIX (Q14938, 1–194); Mmu: mouse Nfia (Q02780, 24–217); Xtr: frog nfia (A0A618RQ42, 1–194); Dre: zebrafish nfia (F1R2R8, 1–193); Dme: fly NfI (Q86P06, 1–192); and Cel: nematode nfi-1 (Q9631, 44–237). The K125E mutation site is indicated by an arrow; 6th and 7th mutagenized sites in Armentero et al. (1994) are underlined.
ey-GAL4 caused an antenna-to-leg transformation (Figure 3c,d). The well-known antenna-to-leg transformation by ectopic expression of the HOM-C genes (e.g., Antennapedia) is due to suppression of homothorax (hth) gene transcription and subsequent failure of nuclear localization of Extradenticle homeodomain protein (Yao et al., 1999). We then searched the sequence of the fly hth gene (Drosophila melanogaster R6.37; Larkin et al., 2021) and identified a consensus binding site for NFIA (5′-TTGGCNNNNNGCCAA-3′) approximately 450 bp upstream of the transcription start site. It is likely that hth gene transcription was suppressed by ectopic expression of NFIA in the first instar larvae when the eye and antenna fates are not yet segregated (Wang & Sun, 2012). By contrast, expression of NFIAK125E by ey-GAL4 did not have any effect either (Figure 3e,f). In sum, all of the dominant effects of NFIA expression were completely abolished in the mutant allele.

4.3 | Functional assays of K125E variant in zebrafish

To further address the functional consequences of the K125E mutation in a vertebrate model, zebrafish, we employed the nfia nonsense mutant (nfia<sup>w16768</sup>) as an NFIA-deficient animal model and performed an mRNA rescue assay. As described previously (Chitnis & Kuwada, 1990; Wilson et al., 1990), commissural axons crossed the midline in the midbrain/hindbrain boundary region in wild-type and nfia heterozygous mutant embryos at 3 dpf (Figure 4a,b), but the axons did not cross the midline in nfia homozygous mutant (Figure 4c). The lack of commissural fibers in mutants was rescued by injection of wild-type human NFIA<sup>WT</sup> mRNA (Figure 4d). On the other hand, the injection of NFIAK125E mRNA failed to rescue the phenotype (Figure 4e). Collectively, these zebrafish assays indicate that nfia is essential for neural development and suggest that NFIAK125E is a loss-of-function mutation of human NFIA.

4.4 | K125E mutation impaired the transcriptional regulation ability of NFIA

Because NFIA is known to repress transcription of Hes1 and to activate that of Gfap by direct binding to the promoter regions in mouse (Miura et al., 1990; Piper et al., 2010), we performed luciferase reporter assay using the human GFAP and the HES1 promoters to determine whether NFIAK125E has the ability to regulate transcription (Figure 5a). Induction of exogenous NFIA<sup>WT</sup> expression repressed HES1 promoter activity in a dose-dependent manner; however, NFIAK125E showed no repressive activity at all in HEK293T cells (Figure 5b). Furthermore, dose-dependent GFAP promoter activation by NFIA<sup>WT</sup> was, although not completely, attenuated in NFIAK125E (Figure 5c). These results indicate that the K125E mutation severely impaired the NFIA transcriptional activity.

5 | DISCUSSION

In this article, we report the recurrent heterozygous missense mutation K125E in the NFIA gene in two unrelated patients with an intellectual disability, corpus callosum anomaly, and macrocephaly. Our in vitro and in vivo analyses consistently indicate that this variant represents a loss-of-function allele. The previous studies proposing haploinsufficiency of the NFIA gene as a primary cause of the NFIA-related disorder have relied on deletions or truncating mutations (i.e., frameshift mutation or nonsense mutation) (Bayat et al., 2017;
A few missense mutations have also been reported to be pathogenic or likely pathogenic (Zenker et al., 2019), but their pathogenicity has not been experimentally verified yet. Therefore, this is
The first case report of NFIA missense variant associated with the neurodevelopmental disorder.

Nuclear factor I family proteins are found to bind the palindromic consensus sequence as homo- or heterodimers (Gronostajski, 2000). Although dimerization is essential for DNA binding, these two activities can be separated by mutations (Armentero et al., 1994). K125 residue is sandwiched between two mutants, 6th and 7th mutations in Armentero et al. (1994); Figure 2), that disrupt the DNA-binding activity, but the former does not impair the dimerization activity. If the K125E mutant protein can still dimerize, but cannot bind the target sequences, its detrimental effect may be even stronger than that of truncating variants.

As in Figure 2, the K125 residue is conserved in all four human NFI family genes and the missense mutations in NFIB and NFIX are also classified as pathogenic or probably pathogenic in the ClinVar database (Landrum et al., 2018). In particular, NFIB K126E mutation (at the site corresponding to K125 residue in NFIA) causes a severe loss of transcriptional activity and is one of the variants associated with intellectual disability and macrocephaly (Schanze et al., 2018). In addition, two mutations at the same K125 residue have been reported in NFIX. One is K125E in a patient with Malan syndrome (Gurrieri et al., 2015) and another is K125N in a patient with developmental disabilities (Lu et al., 2017); both patients had macrocephaly. Taken together, all these findings underscore the importance of the K125 residue for NFI function.

The hypoplasia of corpus callosum and macrocephaly may represent diagnostic clues to the NFIA-related disorder. Indeed, macrocephaly was shown in 14 of 14 reported patients with truncating variants or intragenic deletions in the NFIA gene (Bayat et al., 2017; Mikhail et al., 2011; Negishi et al., 2015; Nyboe et al., 2015; Rao et al., 2014; Revah-Politi et al., 2017; Zhang et al., 2020) (Table 1). Consistent with this shared feature, knockout mice for Nfia, Nfib, and Nfix all exhibit severe brain malformations including megalencephaly (Campbell et al., 2008; Chang et al., 2013; das Neves et al., 1999). This megalencephaly is hypothesized to be due to delayed radial glia differentiation, which promotes extended self-renewal and results in an increased number of

| TABLE 1 | Summary of the patients with neurodevelopmental disorder and heterozygous variants in NFIA |
|---------|---------------------------------|
| Patient | Patient 2 | Patients with truncating variants or intragenic deletion of the NFIA gene |
| Variants in NFIA | p.Lys125Glu | p.Lys125Glu | p.Gln54ProfsTer49 |
| | | | p.Arg69Ter x3 (two patients were a same family) |
| | | | p.Pro365HisTer32 |
| | | | p.Arg74Ter |
| | | | intragenic deletion x8 (each two patients and four patients were same families) |
| Sex | Female | Male | 8 males and 6 females | 9 males and 7 females |
| Developmental delay/intellectual disability | Severe (DQ23) | Mild – moderate | 12/14 | 14/16 |
| Macrocephaly | Present | Present | 14/14 | 16/16 |
| Head MRI | Thin corpus callosum, cyst of septi pellucidi, ventricular wall irregularity, decreased white matter volume | Polycerebral gyrus at parasyvulus fissures, cortical dysplasia of bilateral cerebral hemisphere, partial myelination delay, hypoplasia of corpus callosum | Abnormality of corpus callosum: 13/14 | Abnormality of corpus callosum: 15/16 |
| Facial dysmorphism | High forehead, small eyes, anteverted nares, depressed nasal bridge, broad columella, thin upper-lip vermilion, high-arched palate | High forehead, thick eyebrow, short nose, anteverted nares, long philtrum, thin upper-lip vermilion, retrognathia | High forehead (8/13), eye anomalies (2/13) | High forehead (10/15) eye anomalies (3/15) (one was not available) |
| Hearing impairment | Present | Present | 2/12 | 4/14 (two were not available) |
| Urogenital problems | Absent | Absent | 4/13 | 4/15 (one was not available) |
neural progenitors (Zenker et al., 2019). Corpus callosum hypoplasia was also shown in 13 of these 14 patients (Table 1) and is indeed an important feature of the NFIA-related disorder. In mouse, formation of the corpus callosum requires astroglial-mediated remodeling of the interhemispheric midline (das Neves et al., 1999; Gobius et al., 2016). Knockout of Nfia and Nfib delays differentiation of midline zipper glia cells from radial glia, which prevents normal interhemispheric remodeling and affects subsequent callosal tract formation (Gobius et al., 2016). Our in vitro experiments clearly show that the repressive effect of NFIA on the HES1 promoter was severely impaired by the K125E mutation (Figure 5b). Therefore, it is possible that cellular differentiation from radial glia was delayed and overgrowth of progenitor cells caused the subsequent macrocephaly in the present patients (Piper et al., 2010). The observation of commissural defects in our zebrafish model deficient for nfia also substantiates the association of NFIA disruption with hypoplasia of corpus callosum.

Finally, we anticipate that other NFIA missense variants may also be associated with the neurodevelopmental disease. If so, our in vitro and in vivo assays would be a valuable tool for diagnosis, especially for evaluating whether a missense mutation is a loss-of-function.

ACKNOWLEDGMENTS
We thank Mrs. Chika Kanoe, Mrs. Keiko Tsukue, and Mrs. Yumi Obayashi for their technical assistance in the preparation of this article. This work was supported by the Japan Agency for Medical Research and Development under Grant Numbers 18ek0109301, 18ek0109288h0002, 19gk0110038h, 19ek0109288h0003, and 20ek0109484h0001.

CONFLICT OF INTEREST
The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
U.T., R.S., Y.O., H.H., K.K., and T.T-S. wrote the main manuscript text. R.S., Y.O., H.H., K.M., and T.T-S. designed and performed all the experiments. U.T., A.Y., T.Y., T.F., H.Y., M.Y., H.S., T.T., and K.K. have contributed to data collection and interpretation, and critically reviewed the manuscript. All authors contributed to analysis and interpretation of data. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available in Database of Pathogenic Variants at https://dpv.cmg.med.keio.ac.jp/dpv-pub/variants, reference number [DPVS:13139.1].

ORCID
Tomoko Uehara https://orcid.org/0000-0002-1497-7686
Mamiko Yamada https://orcid.org/0000-0002-4039-8899

REFERENCES
Armentero, M. T., Horvitz, M., & Mermod, N. (1994). Targeting of DNA polymerase to the adenovirus origin of DNA replication by interaction with nuclear factor I. Proceedings of the National Academy of Sciences United States of America, 91(24), 11537–11541. https://doi.org/10.1073/pnas.91.24.11537
Bayat, A., Kirchhoff, M., Madsen, C. G., Roos, L., & Kreiborg, S. (2017). Familial craniofacial abnormality and polymicrogyria associated with a microdeletion affecting the NFIA gene. Clinical Dysmorphology, 26(3), 148–153. https://doi.org/10.1097/MCD.0000000000001812
Bischof, J., Björklund, M., Furger, E., Schertel, C., Taiapale, J., & Basler, K. (2013). A versatile platform for creating a comprehensive UAS-ORFeome library in drosophila. Development, 140(11), 2434–2442. https://doi.org/10.1242/dev.088757
Campbell, C. E., Piper, M., Plachez, C., Yeh, Y.-T., Baizer, J. S., Osiński, J. M., Litwack, E. D., Richards, L. J., & Gronostajski, R. M. (2008). The transcription factor Nfix is essential for normal brain development. BMC Developmental Biology, 8, 52. https://doi.org/10.1186/1471-213X-8-52
Chang, C.-Y., Pasolli, H. A., Giannopoulou, E. G., Guasch, G., Gronostajski, R. M., Elemento, O., & Fuchs, E. (2013). Nfip1 is a governor of epithelial-melanocyte stem cell behaviour in a shared niche. Nature, 495, 98–102. https://doi.org/10.1038/nature11847
Chen, C. P., Su, Y. N., Chen, Y. Y., Chern, S. R., Liu, Y. P., Wu, P. C., Lee, C. C., Chen, Y. T., & Wang, W. (2011). Chromosome 1p32-p31 deletion syndrome: Prenatal diagnosis by array comparative genomic hybridization using uncultured amniocytes and association with NFIA haploinsufficiency, ventriculomegaly, corpus callosum hypogenesis, abnormal external genitalia, and intrauterine growth restriction. Taiwanese Journal of Obstetrics and Gynecology, 50(3), 345–352. https://doi.org/10.1016/j.tjog.2011.07.014
Chitnis, A. B., & Kuwada, J. Y. (1990). Axonogenesis in the brain of zebrafish embryos. Journal of Neuroscience, 10(6), 1892–1905. https://doi.org/10.1523/JNEUROSCI.10-06-01892.1990
das Neves, L., Duchała, C. S., Tolentino-Silva, F., Haixiu, M. A., Colmenares, C., Macklin, W. B., Campbell, C. E., Butz, K. G., & Gronostajski, R. M. (1999). Disruption of the murine nuclear factor I-A gene (Nfia) results in perinatal lethality, hydrocephalus, and agenesis of the corpus callosum. Proceedings of the National Academy of Sciences United States of America, 96(21), 11946–11951. https://doi.org/10.1073/pnas.96.21.11946
Gobius, I., Morcom, L., Suárez, R., Bunt, J., Bukshpun, P., Reardon, W., Dobyns, W. B., Rubenstein, J. L., Barkovich, A. J., Sherr, E. H., & Richards, L. J. (2016). Astrogial-mediated remodeling of the interhemispheric midline is required for the formation of the corpus callosum. Cell Reports, 17(3), 735–747. https://doi.org/10.1016/j.celrep.2016.09.033
Gronostajski, R. M. (2000). Roles of the NFI/CTF gene family in transcription and development. Gene, 249(1-2), 31–45. https://doi.org/10.1016/S0378-1119(00)00140-2
Gurrieri, F., Cavaliere, M. L., Wischnjejer, A., Mammì, C., Nerì, G., Pisanti, M. A., Rodella, G., Laganà, C., & Priolo, M. (2015). NFIX mutations affecting the DNA-binding domain cause a peculiar overgrowth syndrome (Malan syndrome): A new patients series. European Journal of Medical Genetics, 58(9), 488–491. https://doi.org/10.1016/j.ejmg.2015.06.009
Hollenbeck, D., Williams, C. L., Drazba, K., Descartes, M., Korf, B. R., Rutledge, S. L., Lose, E. J., Robin, N. H., Carroll, A. J., & Médail, F. M. (2017). Clinical relevance of small copy-number variants in
chromosomal microarray clinical testing. Genetics in Medicine, 19(4), 377–385. https://doi.org/10.1038/gim.2016.132

Kettleborough, R. N. W., Busch-Nentwich, E. M., Harvey, S. A., Dooley, C. M., de Bruijn, E., van Eeden, F., Sealy, I., White, R. J., Herd, C., Nijman, J. I., Fénes, Y., Mehrotra, S., Schallig, C., Gibbons, R., Wall, N., Carruthers, S., Hall, A., Yen, J., Cuppen, E., & Stemple, D. L. (2013). A systematic genome-wide analysis of zebrafish protein-coding gene function. Nature, 494(7446), 494–497. https://doi.org/10.1038/nature11992

Kircher, M., Witten, D. M., Jain, P., O’Roak, B. J., Cooper, G. M., & Shendure, J. (2014). A general framework for estimating the relative pathogenicity of human genetic variants. Nature Genetics, 46(3), 310–315. https://doi.org/10.1038/ng.2892

Koehler, U., Holinski-Feder, E., Ertl-Wagner, B., Kunz, J., von Moers, A., von Voss, H., & Schell-Apalick, C. (2010). A novel 1p31.3p32.2 deletion involving the NFIA gene detected by array CGH in a patient with macrocephaly and hypoplasia of the corpus callosum. European Journal of Pediatrics, 169(4), 463–468. https://doi.org/10.1007/s00431-009-1057-2

Landrum, M. J., Lee, J. M., Benson, M., Brown, G. R., Chao, C., Chitipirilla, S., Gu, B., Hart, J., Hoffman, D., Jang, W., Karapetyan, K., Katz, K., Liu, C., Maddipatla, Z., Malheiro, A., McDaniel, K., Ovetsky, M., Riley, G., Zhou, G., Holmes, J. B., ... Ertl-Wagner, B., Kunz, J., von Moers, A., Kircher, M., Witten, D. M., Jain, P., O’Roak, B. J., Cooper, G. M., & Nyboe, D., Kreiborg, S., Kirchhoff, M., & Hove, H. B. (2015). Familial craniosynostosis is associated with a CNS malformation syndrome with clinical features of Sotos syndrome and Malan syndrome. Yamamoto, T. (2017). Mutations in NSD1 are a critical gene in 1p32-p31 deletion syndrome: A four patient series. American Journal of Medical Genetics Part A, 173A(12), 3158–3164. https://doi.org/10.1002/ajmg.a.34177

Piper, M., Barry, G., Hawkins, J., Mason, S., Lindwall, C., Little, E., Sarkar, A., Smith, A. G., Moldrich, R. X., Boyle, G. M., Tole, S., Gronostajski, R. M., Bailey, T. L., & Richards, L. J. (2010). NFIA controls telencephalic progenitor cell differentiation through repression of the notch effector Hes1. The Journal of Neuroscience, 30(27), 9127–9139. https://doi.org/10.1523/JNEUROSCI.6167-09.2010

Quiring, R., Walldorf, U., Kloet, U., & Gehring, W. J. (1994). Homology of the eyeless gene of drosophila to the small eye gene in mice and Aniridia in humans. Science, 265(5173), 785–789. https://doi.org/10.1126/science.7914031

Rao, A., Donnell, S., Bain, N., Meldrum, C., Shorter, D., & Goel, H. (2014). An intragenic deletion of the NFIA gene in a patient with a hypoplastic corpus callosum, craniofacial abnormalities and urinary tract defects. European Journal of Medical Genetics, 57(2–3), 65–70. https://doi.org/10.1016/ejmg.2013.12.011

Revah-Politi, A., Ganapathi, M., Bier, L., Cho, M. T., Goldstein, D. B., Hemati, P., Iglesias, A., Juusola, J., Pappas, P., Petrovski, S., Wilson, A. L., Aggarwal, V. S., & Anyane-Yeboa, K. (2017). Loss-of-function variants in NFIB provide further support that NFIA is a critical gene in 3p23-p31 deletion syndrome: Four patient series. American Journal of Medical Genetics Part A, 173A(12), 3158–3164. https://doi.org/10.1002/ajmg.a.34177

Schanze, I., Bunt, J., Lim, J. W. C., Schanze, D., Dean, R. J., Alders, M., Blanchet, P., Attié-Bitach, T., Berland, S., Boogert, J., Boppudi, S., Bridges, C. J., Cho, M. T., Dobyns, W. B., Donnai, D., Douglas, J., Earl, D. L., Edwards, T. J., Faivre, L., Fregeau, B., ... Richards, L. J. (2018). NFIB haploinsufficiency is associated with intellectual disability and macrocephaly. American Journal of Human Genetics, 103(5), 752–768. https://doi.org/10.1016/j.ajhg.2018.10.006

Suzuki, H., Yoshida, T., Morisada, N., Uehara, T., Kosaki, K., Sato, K., Matsubara, K., Takano-Shimizu, T., & Takenouchi, T. (2019). De novo NSF mutations cause early infantile encephalopathy. Annals of Clinical and Translational Neurology, 6(11), 2334–2339. https://doi.org/10.1002/acne.20917

Tadaka, S., Katsuoka, F., Ueki, M., Kojima, K., Makino, S., Sato, S., Otsuki, A., Gocho, C., Sakurai-Yageta, M., Danjoh, I., Motoike, I. N., Yamaguchi-Kabata, Y., Shiroma, M., Koshiba, S., Nagasaki, M., Minegishi, N., Hozawa, A., Kuriyama, S., Shimizu, A., Yasuda, J., ... Kinoshita, K. (2019). 3.5KJPNv2: An allele frequency panel of 3552 Japanese individuals including the X chromosome. Human Genome Variation, 6, 28. https://doi.org/10.1038/s41444-019-0059-5

_ts, T., & Takagi, T. (1999). Estimating transcription factor binding affinity on DNA. Bioinformatics, 15(78), 622–630. https://doi.org/10.1093/bioinformatics/15.7.622

Uehara, T., Abe, K., Oginuma, M., Ishitani, S., Yoshihashi, H., Okamoto, N., Takenouchi, T., Kosaki, K., & Ishitani, T. (2020). Pathogenesis of CDK8-associated disorder: Two patients with novel CDK8 variants and in vitro and in vivo functional analyses of the variants. Scientific Reports, 10, 1745. https://doi.org/10.1038/s41598-020-74642-4

Urbach, R., & Technau, G. M. (2003). Molecular markers for identified neuronal progenitor cell differentiation through repression of the notch effector Hes1. Developmental Neurobiology, 130(16), 3621–3637. https://doi.org/10.1242/dev.00553

The UniProt Consortium. (2019). UniProt: A worldwide hub of protein knowledge. Nucleic Acids Research, 47(D1), D566–D515. https://doi.org/10.1093/nar/gky1049

Wang, C.-W., & Sun, Y. H. (2012). Segregation of eye and antenna fates maintained by mutual antagonism in Drosophila. Development, 139(18), 3413–3421. https://doi.org/10.1242/dev.078857

Wilson, S. W., Ross, L. S., Parrett, T., & Easter, S. S., Jr. (1990). The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, Brachydanio rerio. Development, 108(1), 121–145. https://dev.biologists.org/content/108/1/121

Yao, L.-C., Liaw, G.-J., Pai, C.-Y., & Sun, Y. H. (1999). A common mechanism for antenna-to-leg transformation in Drosophila: Suppression of
homothorax transcription by four HOM-C genes. Developmental Biology, 211, 268–276.

Zenker, M., Bunt, J., Schanze, I., Schanze, D., Piper, M., Priolo, M., Gerkes, E. H., Gronostajski, R. M., Richards, L. J., Vogt, J., Wessels, M. W., & Hennekam, R. C. (2019). Variants in nuclear factor I genes influence growth and development. American Journal of Medical Genetics. Part C, 181(4), 611–626. https://doi.org/10.1002/ajmg.c.31747

Zhang, Y., Lin, C. M., Zheng, X. L., & Abuduxikuer, K. (2020). A novel NFIA gene nonsense mutation in a Chinese patient with macrocephaly, corpus callosum hypoplasia, developmental delay, and dysmorphic features. Molecular Genetics & Genomic Medicine, 8(11), e1492. https://doi.org/10.1002/mgg3.1492

How to cite this article: Uehara, T., Sanuki, R., Ogura, Y., Yokoyama, A., Yoshida, T., Futagawa, H., Yoshihashi, H., Yamada, M., Suzuki, H., Takenouchi, T., Matsubara, K., Hirata, H., Kosaki, K., & Takano-Shimizu, T. (2021). Recurrent NFIA K125E substitution represents a loss-of-function allele: Sensitive in vitro and in vivo assays for nontruncating alleles. American Journal of Medical Genetics Part A Part A, 185A: 2084–2093. https://doi.org/10.1002/ajmg.a.62226