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Truncated Cables1 causes agenesis of the corpus callosum in mice

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Agenesis of the corpus callosum (ACC) is a congenital abnormality of the brain structure. More than 60 genes are known to be involved in corpus callosum development. However, the molecular mechanisms underlying ACC are not fully understood. Previously, we produced a novel transgenic mouse strain, TAS, carrying genes of the tetracycline-inducible expression system that are not involved in brain development, and inherited ACC was observed in the brains of all homozygous TAS mice. Although ACC was probably induced by transgene insertion mutation, the causative gene and the molecular mechanism of its pathogenesis remain unclear. Here, we first performed interphase three-color fluorescence *in situ* hybridization (FISH) analysis to determine the genomic insertion site. Transgenes were inserted into chromosome 18 ~12.0 Mb from the centromere. Gene expression analysis and genomic PCR walking showed that the genomic region containing exon 4 of *Cables1* was deleted by transgene insertion and the other exons of *Cables1* were intact. The mutant allele was designated as *Cables1*^{TAS}. Interestingly, *Cables1*^{TAS} mRNA consisted of exons 1–3 of *Cables1* and part of the transgene that encoded a novel truncated Cables1 protein. Homozygous TAS mice exhibited mRNA expression of *Cables1*^{TAS} in the fetal cerebrum, but not that of wild-type *Cables1*. To investigate whether a dominant negative effect of Cables1^{TAS} or complete loss of function of Cables1 gives rise to ACC, we produced *Cables1*-null mutant mice. ACC was not observed in *Cables1*-null mutant mice, suggesting that a dominant negative effect of Cables1^{TAS} impairs callosal formation. Moreover, ACC frequency in *Cables1*$^{+/TAS}$ mice was significantly lower than that in *Cables1*$^{-/TAS}$ mice, indicating that wild-type *Cables1* interfered with the dominant negative effect of *Cables1*^{TAS}. This study indicated that truncated Cables1 causes ACC and wild-type Cables1 contributes to callosal formation.
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The corpus callosum is the largest fiber tract in the brain, consisting of 200–350 million and 7 million fibers in humans and mice, respectively,[1,2] and serves as a bridge to transfer information between the right and left cerebral hemispheres. Agenesis of the corpus callosum (ACC), which is characterized by absence of the corpus callosum,[3] is a congenital brain abnormality that may occur in isolation or in association with other central nervous system or systemic malformations in humans. Neonatal and prenatal imaging studies have suggested that ACC occurs in at least 1:4000 live births in the human population.[4,5] In other studies, the estimated incidence rates of ACC were reported as 3–5% of patients with neurodevelopmental disorders and 2–3% of the population with mental impairment.[6–8]

Reverse genetic analyses of mice identified more than 60 causative genes of ACC, and these genes are involved in several processes in the formation of corpus callous.[9] The *Sp8*, *Pax6*, and *Otx2* genes are essential for patterning of the developing forebrain. Analyses of mouse strains with mutations in these genes indicated their involvement in ACC with multiple brain malformations and strong craniofacial abnormalities.[10–12] As both brain midline fusion defects and ACC were observed in several inbred mouse strains (eg, BALBc/Wah1 and 129/J),[13] telencephalic

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midline fusion appears to be necessary for corpus callosum development. Correct development of the cerebral cortex is also important for formation of the corpus callosum because callosal neurons, which extend axons across the corpus callosum, are located in the cerebral cortex. Indeed, *N-cadherin* gene-mutant mice showed ACC because of abnormal development of the cerebral cortex.[14] Furthermore, the projection of callosal axons from the cortex is regulated by various axon guidance molecules, including Slit/Robo, Netrin/DCC, Ephs/Ephrins, and Semaphorins/Neuropilins. Indeed, abnormal corpus callosum development has been reported in mice with deficiencies in these genes.[15–22]

Occasionally, random integration of transgene DNA into the host genome disrupts an endogenous gene and causes a class of mutations called transgene insertion mutations.[23] Approximately 5–15% of random DNA insertion events in transgenic mice are associated with an abnormal phenotype by transgene insertion mutation.[24] The integrated transgene may affect endogenous loci close to the transgene and result in inactivation of the endogenous gene or creation of a fusion with an endogenous gene. When transgene insertion mutation results in unexpected phenotypic alterations in mice, knowledge regarding the precise location of transgene insertion within the genome could be useful to discover genes responsible for defects.

Previously, we developed a transgenic mouse strain, TAS (reverse *t*etracycline-controlled trans*a*ctivator and tetracycline-controlled transcriptional *s*ilencer), that showed ACC accompanied with formation of Probst bundles, as seen in human patients.[25] The complete penetrance of ACC was observed in homozygous TAS mice. Furthermore, axons of the corpus callosum were not repelled by the midline structures. TAS mice carry both the reverse tetracycline-controlled transactivator (rtTA) gene and the tetracycline-controlled transcriptional silencer gene (tTS) in the BALB/c genetic background. As these transgene products do not affect brain development and another founder line carrying both rtTA and tTS did not exhibit ACC, we hypothesized that ACC in TAS was caused by transgene insertion mutation. In addition, we reported previously that rtTA and tTS were located in the region between 9.3 and 16.9 Mb from the centromere on chromosome 18 in TAS, and there is no critical gene known to be involved in ACC in the candidate genomic region.

Although ACC in TAS mice is probably induced by transgene insertion mutation, the causative gene and its pathological mechanism have not yet been clarified. Here, we identified the genomic insertion site of the transgene on chromosome 18 at ~12 Mb from the centromere by multicolor fluorescence *in situ* hybridization (FISH) analysis. Furthermore, the insertional mutation was shown to generate a novel mutant *Cables1* allele. Analysis of *Cables1*-null mutant mice indicated that complete loss of function of Cables1 did not contribute to callosal malformation. Finally, we demonstrated that the frequency of ACC in *Cables1*$^{+/TAS}$ mice was significantly lower than that in *Cables1*$^{-/TAS}$ mice. These data suggest that ACC in TAS mice is caused by the N-terminal half of Cables1, a molecule responsible for the complex organization of the corpus callosum.

MATERIALS AND METHODS

Animals

The TAS mice used in this study have been described previously.[25] BALB/cAJ, C57BL/6J, and ICR mice were purchased from CLEA Japan. Animals were kept as described previously.[25] Animal experiments were carried out humanely in accordance with the Regulations for Animal Experiments of the University of Tsukuba and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and with approval from the Institutional Animal Experiment Committee of our university.

KB Staining

The brains of adult mice were rapidly immersed in 10% neutral buffered formalin for at least 1 week before embedding. Subsequently, brains were dehydrated by passage through an ascending series of alcohol concentrations, cleared in xylene, and embedded in paraffin. Paraffin blocks were cut into serial sections 6 μm thick on a microtome and mounted on glass slides. Sections were stained with Klüver–Barrera (KB) stain according to standard protocols.

Immunofluorescence

The neonatal brains were fixed in 4% paraformaldehyde in PBS. After overnight fixation at 4 °C, the brains were cryoprotected in 30% sucrose and frozen in OCT compound (Sakura Finetek). Cryostat sections 30 μm thick were blocked with 0.2% Triton X-100 in SuperBlock® Blocking Buffer in TBS (Pierce). The sections were incubated with rat anti-L1CAM antibody (1:100; Millipore). Subsequently, the sections were washed in PBS and incubated with mouse Cy3-GFAP antibody (1:200; Sigma) and Alexa Fluor 350 goat anti-rat IgG (1:200; Life Technologies).

FISH

R-banded chromosome preparations were made from the spleen lymphocytes of heterozygous TAS mice as previously described.[25] After hardening at 65 °C, the chromosome spreads were denatured in 70% formamide/2 × SSC for 2 min, then dehydrated in ethanol (70 and 100%) at 4 °C for 15 min. RP23-232N9 and RP23-101N14 bacterial artificial chromosome (BAC) DNAs were purified by NucleoBond BAC 100 (Macherey-Nagel). These BAC and tTS DNA probes were labeled by nick translation (Roche) with biotin-dUTP (Roche), Cy3-dUTP (GE Healthcare), and Cy-5-dUTP (GE Healthcare), respectively. Repeat sequences in BAC DNA probes were blocked with 10 μg Cot1 DNA (Life Technologies). Aliquots of 1 μg of each BAC probe and

500 ng of tTS DNA probe in formamide were denatured at 75 °C for 10 min and applied to slides with hybridization buffer containing 200 μg/μl dextran sulfate sodium salt (Sigma), 200 μg/μl bovine serum albumin (Roche), and 2 × SSC. After overnight hybridization at 37 °C, the slides were washed in 1 × , 2 × , and 4 × SSC and incubated with avidin-FITC (Roche) at 37 °C for 1 h.

RT-PCR

Total RNA was prepared from the brains of embryos using ISOGEN (Nippon Gene). cDNA was synthesized as described previously.[25] PCR was performed with AmpliTaq Gold® PCR Master Mix (Life Technologies) and various primers (Supplementary Table 1).

Sequencing

Genomic and cDNA sequencing analyses were performed with a BigDye® Terminator v3.1 Cycle Sequencing Kit and Applied Biosystems 3130 Genetic Analyzer (Life Technologies).

Northern Blotting

Aliquots of 10 μg of total mouse embryonic cerebral RNA (E17.5 and E19.5) were denatured, fractionated, and transferred onto Hybond N^+ (GE Healthcare) according to standard protocols. The 5′- and 3′-cDNA probes were derived from mouse brain cDNA with specific primers (Supplementary Table 2). Hybridization and detection were performed using a DIG Northern Starter Kit (Roche).

Transfection and Western Blotting

cDNA fragments of *Cables1* and *Cables1*^{TAS} CDS, which were derived from wild-type and TAS mice, were introduced into the pc3 mammalian expression vector. These constructs were transfected into HEK293T cells by LipofectAMINE LTX (Life Technologies). Cables1 and Cables1^{TAS} proteins from transfected cells were detected with rabbit anti-N-terminal Cables1 antibody (M280; 1:100; Santa Cruz) and HRP-conjugated donkey anti-rabbit IgG secondary antibody (1:4000; GE Healthcare).

Gene Targeting

The *Cables1*^{null} targeting vector contained the *Neo*^{r} cassette (Figure 5a). The 3.8-kb 5′-homology arm and 4.6-kb 3′-homology arm were amplified from BALB/c mouse genomic DNA with Cables1^{null} specific primers (Supplementary Table 3). The *Cables1*^{null} targeting vector was introduced into BALB/c embryonic stem cells by electroporation.[26] Genomic DNA from G418-resistant colonies were screened for homologous recombination by Southern blotting.

RESULTS

Identification of Transgene Insertion Site in TAS Mice

We recently developed a novel transgenic mouse strain carrying rtTA and tTS, the components of the Tet-on/off gene expression system. Unexpectedly, only one line of transgenic mice showed ACC (Figure 1b), designated as TAS.[25] As rtTA and tTS have not been reported to affect cerebral development, we hypothesized that ACC in TAS mice was due to disruption of an endogenous gene by transgene insertion. A recent study showed that both rtTA and tTS transgenes are closely linked and inserted into the region between 9.3 and 16.9 Mb from the centromere on chromosome 18 in TAS mice.

To narrow down the insertion site, we performed three-color FISH analyses. The DNA probes from BAC clones RP23-232N9 (derived from chr.18: 11 108 363 to 11 290 545) and RP23-101N14 (derived from chr.18: 12 743 636 to 12 933 531) were labeled with Cy3 and Cy5, respectively. A transgenic DNA probe was labeled with FITC. Although these three-color signals were detected on chromosome 18, the signals were not separated on metaphase chromosome spreads (Figure 2a). In contrast, the FITC signal was detected between Cy3 and Cy5 signals on interphase chromosome spreads (Figure 2b). These observations indicated transgene insertion in the region between 11.3 and 12.7 Mb on chromosome 18 (Figure 2c). We then examined the expression of 14 genes located around the region of transgene insertion (Figure 2c). RT-PCR analyses indicated that only the *Cables1* gene expression pattern was different between TAS and wild-type mice (Figure 2d).

Exon 4 of *Cables1* Is Disrupted by Transgene Insertion

The *Cables1* gene is located 12 Mb from the centromere on mouse chromosome 18. *Cables1* consists of 10 exons, and alternative splicing results in two transcripts. Exon 4 of *Cables1* isoform I is not included in isoform II (Figure 4c). We performed genomic PCR walking in the *Cables1* locus to determine the transgene insertion site. Consistent with the results of RT-PCR analyses, long-PCR (data not shown) and sequence analyses (Figure 3a) showed that the genomic region from 12 058 849 to 12 070 825 bp on chromosome 18, including exon 4 of *Cables1*, was deleted by transgenes insertion (Figure 3b). The novel mutant allele of *Cables1* was designated as *Cables1*^{TAS}. As multiple copies of the transgenes (rtTA and tTS) were inserted into the genome,[25] no amplification product was obtained from *Cables1*^{TAS} by genomic PCR analysis with a primer set derived from the 5′ and 3′ flanking regions of the deletion (data not shown).

De Novo Truncating Mutation in *Cables1*

No amplification of *Cables1* cDNA was observed in TAS homozygous mutant mice by RT-PCR analysis using primers derived from exons 2 and 7 (Figure 2d). In addition, genomic analysis revealed that only exon 4 of *Cables1* was deleted (Figure 3b). These results suggested that truncated *Cables1* mRNA, including exons 1–3, may be expressed in TAS mice. Next, we investigated *Cables1* expression in the fetal cerebrum at E17.5 and 19.5, because the corpus callosum dramatically develops late in gestation. Northern blotting

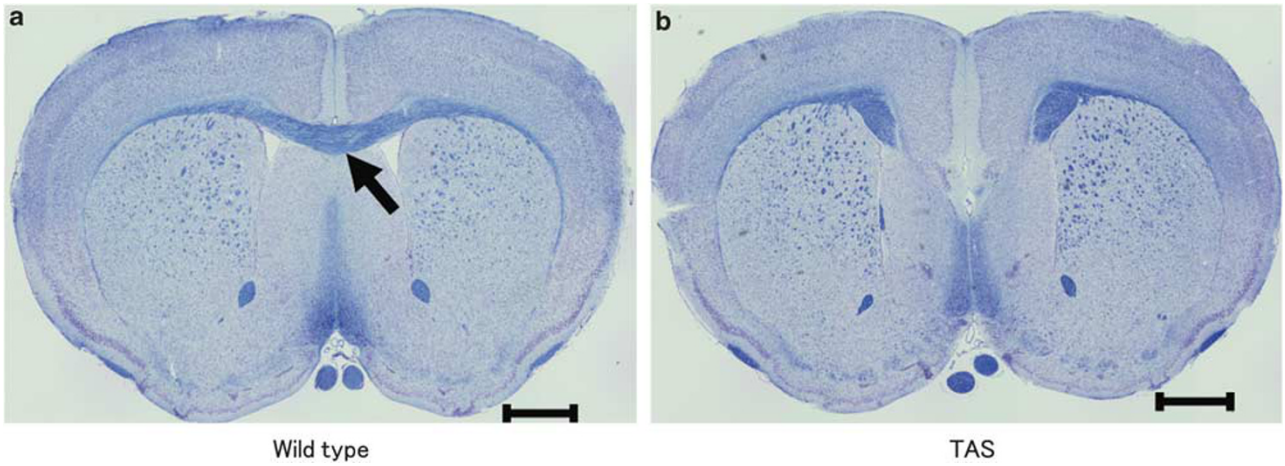

Figure 1 Agenesis of the corpus callosum in TAS. Coronal sections through the rostral levels of the cerebrum in adult wild-type (**a**) and adult TAS (**b**) mice were stained with Klüver–Barrera (KB). Arrow indicates the corpus callosum; scale bar = 1 mm (**a**, **b**).

analysis with 5'-cDNA probe (derived from part of exons 1–3) revealed that *Cables1* mRNA was shorter in TAS fetuses (*Cables1*$^{TAS/TAS}$) than in wild-type fetuses. In contrast, no band was detected in TAS fetuses (*Cables1*$^{TAS/TAS}$) with 3′-cDNA probe derived from exons 7–9 (Figure 4a). These results suggested that exons 1–3 of *Cables1* are included in *Cables1*^{TAS} mRNA and the other exons are not transcribed in TAS (*Cables1*$^{TAS/TAS}$). In addition, *Cables1*^{TAS} mRNA more than 2 kb in length was detected with 5′-cDNA probe, even though a 1425-bp fragment comprising exons 1–3 of *Cables1*. Sequence analysis was then performed to investigate *Cables1*^{TAS} mRNA in detail. Interestingly, exons 1–3 of *Cables1* mRNA were linked to the transgene in *Cables1*^{TAS} (Figure 4b). Furthermore, a frameshift mutation was detected in the transgene following exon 3 of the *Cables1*^{TAS} transcript, resulting in a stop codon (TAG) 69 bp downstream from the exon 1–3 boundary (Figure 4c).

To confirm whether a mutant small protein is translated from *Cables1*^{TAS}, HEK293T cells were transfected with *Cables1*^{TAS} or wild-type *Cables1* expression vector. As expected, western blotting analysis with an antibody to the N-terminus of Cables1 (M-280) indicated that mutant protein derived from *Cables1*^{TAS} was smaller than the wild-type protein (Figure 4d). Furthermore, the molecular weight of the mutant protein product from *Cables1*^{TAS} was similar to the predicted size (33.5 kDa). These results suggested that the abnormal truncated (N-terminal half) Cables1 protein could be expressed in TAS mice. Western blotting analysis was then performed to detect truncated Cables1 in the brain of these mice. However, anti-Cables1 antibody (M-280) did not specifically detect wild-type or truncated Cables1 protein in brain lysate (data not shown).

Development of Normal Corpus Callosum in *Cables1*-Null Mutant Mice

Although reverse genetics analyses indicated various abnormalities in *Cables1*-deficient mice (eg, development of endometrial hyperplasia, progression of colon cancer, and poor oocyte quality),[27–29] the effect of *Cables1*-null mutation on the corpus callosum has not been reported. To determine whether null or TAS mutation gives rise to ACC, it is necessary to establish Cables1-deficient mice in the BALB/c genetic background, because ACC in TAS was seen with the BALB/c (not C57BL/6) genetic background.[25] We successfully produced *Cables1*-null mutant mice in the BALB/c genetic background (Figures 5a–c). The targeting vector was introduced into BALB/c embryonic stem cells by electroporation.[26] Five distinct clones were obtained, one of which (clone 10) was used to generate germline chimeras by aggregation with tetraploid ICR mouse embryos. *Cables1*$^{-/-}$ mice were obtained from intercrosses of *Cables1*$^{-/+}$ mice at Mendelian ratios. These mice were genotyped by genomic Southern blotting (Figure 5b), and RT-PCR analysis with primers derived from exons 2 and 7 revealed that *Cables1* was not expressed in *Cables1*$^{-/-}$ mice (Figure 5c). We then confirmed that a normal corpus callosum was formed in the brains of all *Cables1*$^{-/-}$ adult mice examined ($n = 7$; Figure 5d). These data indicated that lack of *Cables1* function is not responsible for ACC in TAS mice.

Relationship between *Cables1* Genotype and ACC

To understand the relationship between the *Cables1* genotype and ACC phenotype in more detail, *Cables1*$^{+/TAS}$ mice were crossed with *Cables1*$^{-/-}$ mice. As a result, we obtained eight *Cables1*$^{+/TAS}$ and 6 *Cables1*$^{-/TAS}$ mice and examined gross callosum structures at 0 days of age by immunofluorescence with L1-CAM and GFAP antibodies. Similar to *Cables1*$^{TAS/TAS}$, all of the *Cables1*$^{-/TAS}$ mice showed ACC (Figure 6b). In our previous study, all *Cables1*$^{TAS/TAS}$ ($n = 12$) and 62% of *Cables1*$^{+/TAS}$($n = 37$) mice developed ACC.[25] The *Cables1* genotypes and frequency of ACC are summarized in Table 1. Taken together, these results indicated that ACC occurred only in mice bearing the *Cables1*^{TAS} allele. These data indicated that ACC is caused not

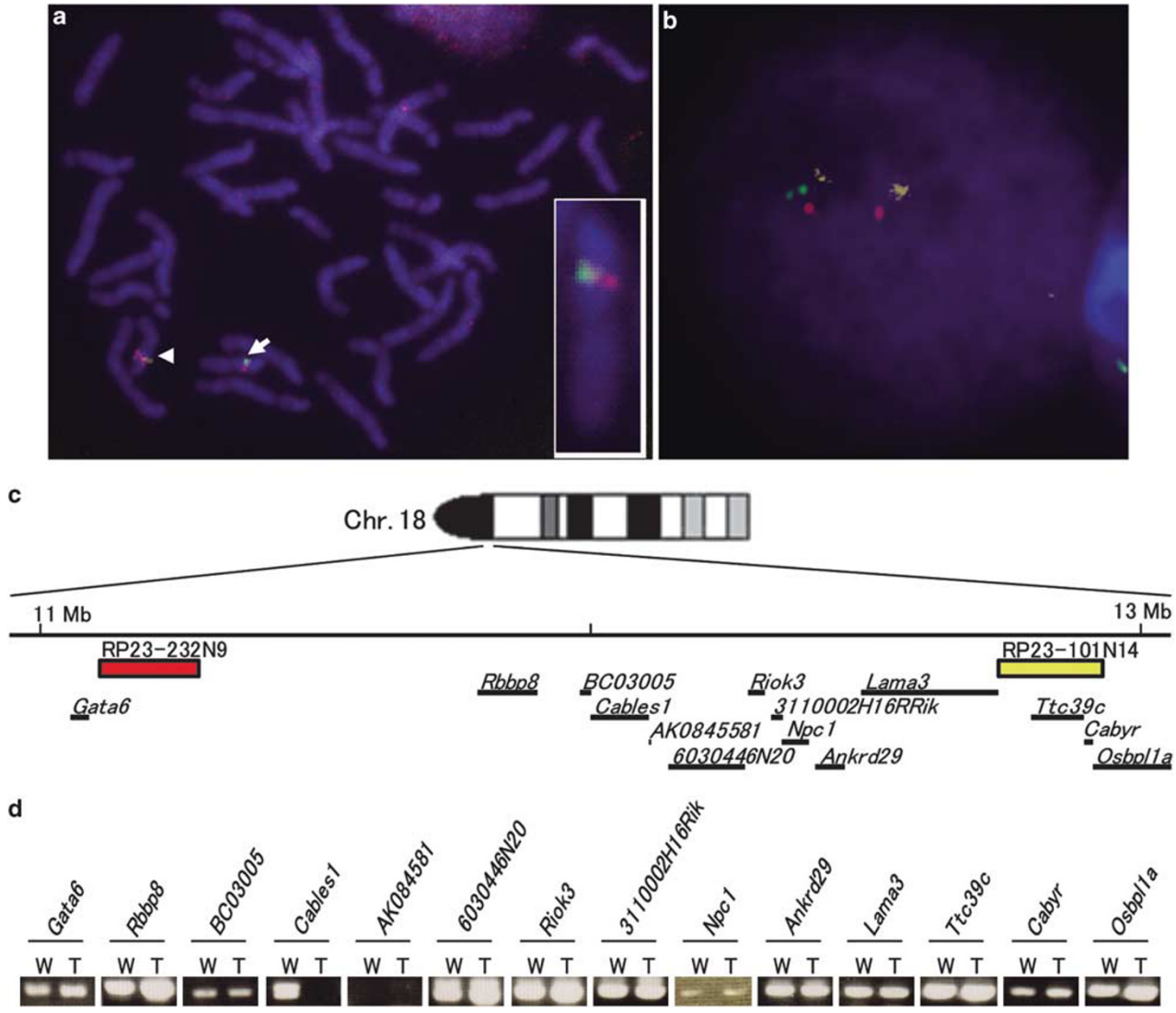

Figure 2 Identification of transgene insertion site. (**a**) FISH to metaphase heterozygous TAS chromosome spreads. The arrow indicates transgene (green) signals colocalized with RP23-232N9 (red) and RP23-101N14 (yellow). The white box shows an enlarged view of this chromosome and indicates that the three-color signals are not separated. The arrowhead indicates red and yellow signals on the nontransgenic chromosome. (**b**) FISH to interphase heterozygous TAS chromosome spreads. Transgene (green) signals were observed between RP23-232N9 (red) and RP23-101N14 (yellow) signals. (**c**) The genomic region between RP23-232N9 (red box) and RP32-101N14 (yellow box) contains 14 known and predicted genes. (**d**) RT-PCR analysis of the 14 genes showed that only the *Cables1* gene expression pattern was different between TAS and wild-type mice. W, wild-type mouse; T, TAS mouse.

by loss of *Cables1* function but by $Cables1^{TAS}$ mutation. Interestingly, as ACC frequency in $Cables1^{+/TAS}$ mice was lower than that in $Cables1^{-/TAS}$ mice, wild-type *Cables1* interfered with the dominant negative effect of $Cables1^{TAS}$, suggesting that wild-type Cables1 may be involved in corpus callosum development.

DISCUSSION

TAS mice are a useful model in which to investigate genes involved in regulation of corpus callosum development and to gain new insights into the molecular genetics of ACC.[25] Here we showed that exon 4 of *Cables1* was deleted by transgene insertion mutation in TAS mice. We also showed that the abnormal *Cables1* mRNA, which is composed of exons 1–3 of *Cables1* and a short part of the transgene, was expressed in the TAS mouse fetal brain. In addition, truncated Cables1 protein was shown to be generated from $Cables^{TAS}$ transcript. The callosal appearance in *Cables1*-null mutant mice supported the conclusion that ACC in TAS mice is because of the dominant negative effect of $Cables^{TAS}$ mutation on callosal organization. Moreover, we showed the relationship between *Cables1* genotype and ACC, demonstrating the contribution of wild-type Cables1 to callosal formation.

Three-color interphase FISH analyses revealed transgene insertion in the region between 11.3 and 12.7 Mb from the centromere of chromosome 18 in TAC mice (Figures 2b

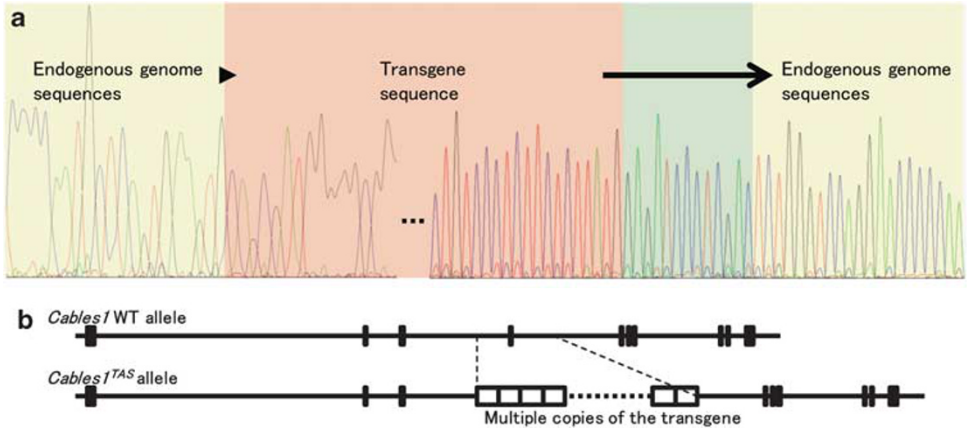

Figure 3 Disruption of *Cables1* gene caused by transgene insertion. (**a**) Results of genomic sequencing analysis. The transgenic sequence (red background) was directly connected (arrowhead) to 5585 bp upstream of exon 4 of *Cables1* (yellow background). The 3′-end of the transgene connected (arrow) to neither the genomic nor transgenic sequence (green background) and subsequently connected to 6314 bp downstream of exon 4 (red background). Dotted line indicates multiple copies of the transgene. (**b**) The schematic diagram shows a comparison of *Cables1* genomic structure between wild-type and *TAS* alleles.

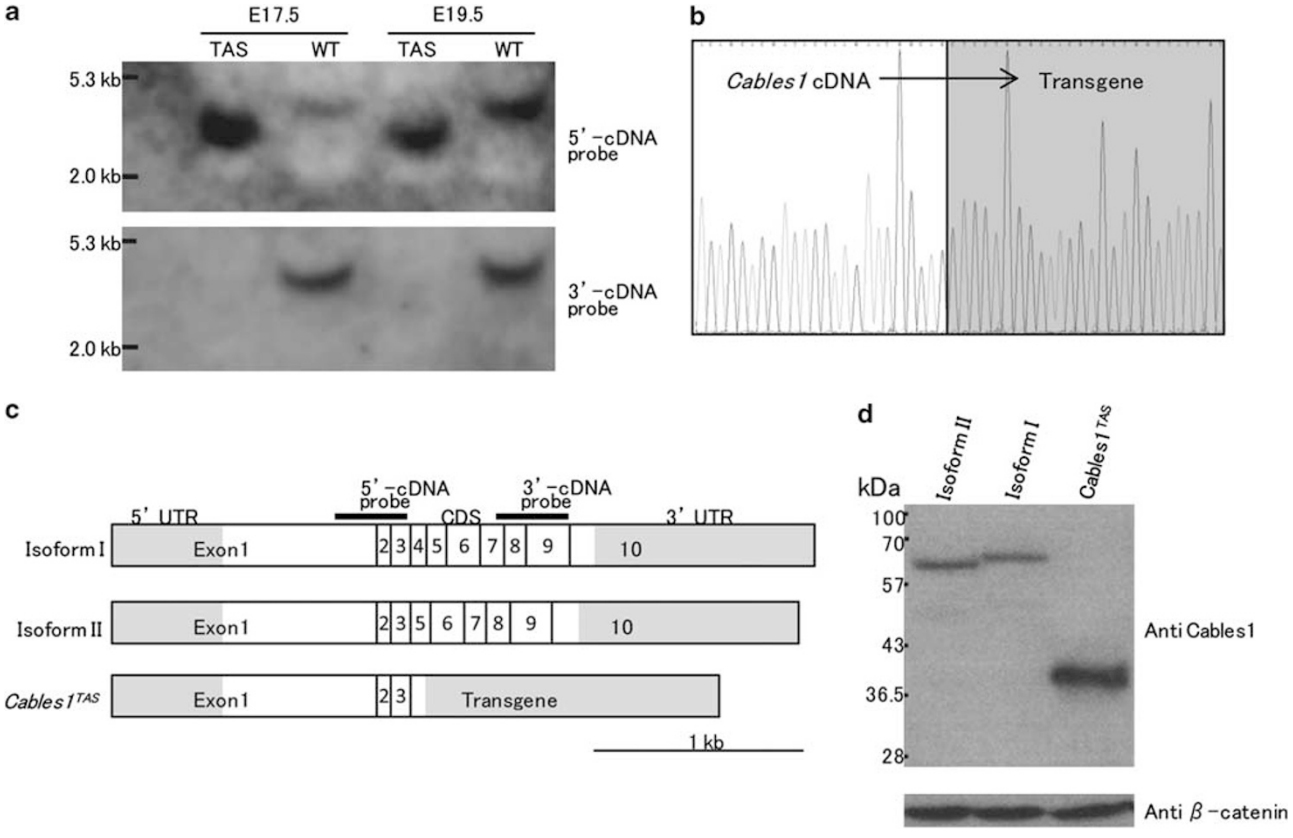

Figure 4 Mutated *Cables1* mRNA in TAS. (**a**) Northern blot analysis of *Cables1* in the fetal cerebrum of wild-type and TAS mice. Shorter mRNA derived from TAS was detected only with the 5′-cDNA probe. (**b**) cDNA sequencing analysis. The transgenic sequence (gray background) was directly connected (arrow) to the 3′-end of exon 3 (white background). (**c**) Three schematic diagrams indicating isoform I, isoform II, and TAS mutant *Cables1* mRNA. Gray and white boxes indicate untranslated region and coding sequence, respectively. (**d**) Three variants of Cables1 protein were expressed in HEK293T cells. These proteins were analyzed by western blotting. Cables1^{TAS} protein was smaller than the wild type. UTR, untranslated region; CDS, coding sequence.

and c). A search in the UCSC genomic database (http://genome.ucsc.edu/) indicated that there are 14 genes in this genomic region. Figure 2d shows that there were no transcriptional differences between wild-type and homozygous TAS mice for any of these genes except *Cables1*. Furthermore, we confirmed that there were no differences in the

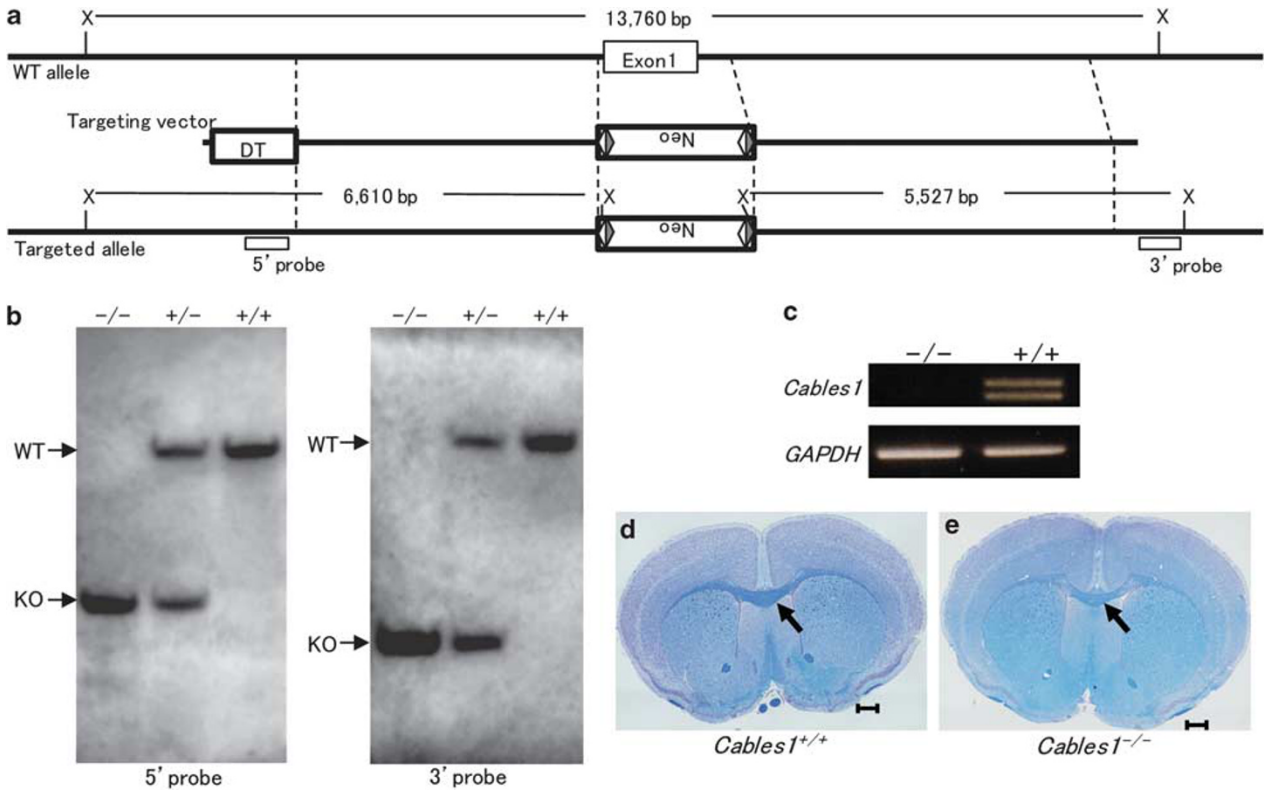

Figure 5 *Cables1*-null mutant mouse. (**a**) Schematic of the null allele in which exon 1 was deleted by homologous recombination in BALB/c ES cells. X, *Xba*I site; Neo, neomycin resistance cassette; DT, diphtheria toxin cassette; white triangle, LoxP; gray triangle, FRT. (**b**) Southern blotting of genomic DNA derived from *Cables1*$^{+/+}$, *Cables1*$^{-/+}$, and *Cables1*$^{-/-}$ mice. (**c**) RT-PCR of *Cables1* from exon 2 through exon 7 demonstrated the absence of both isoform I and isoform II *Cables1* products in *Cables1*$^{-/-}$ mice. Coronal sections through the rostral levels of the cerebrum in adult *Cables1*$^{+/+}$ (**d**) and *Cables1*$^{-/-}$ (**e**) mice were stained with Klüver–Barrera (KB). Arrows indicate the corpus callosum; scale bar = 1 mm.

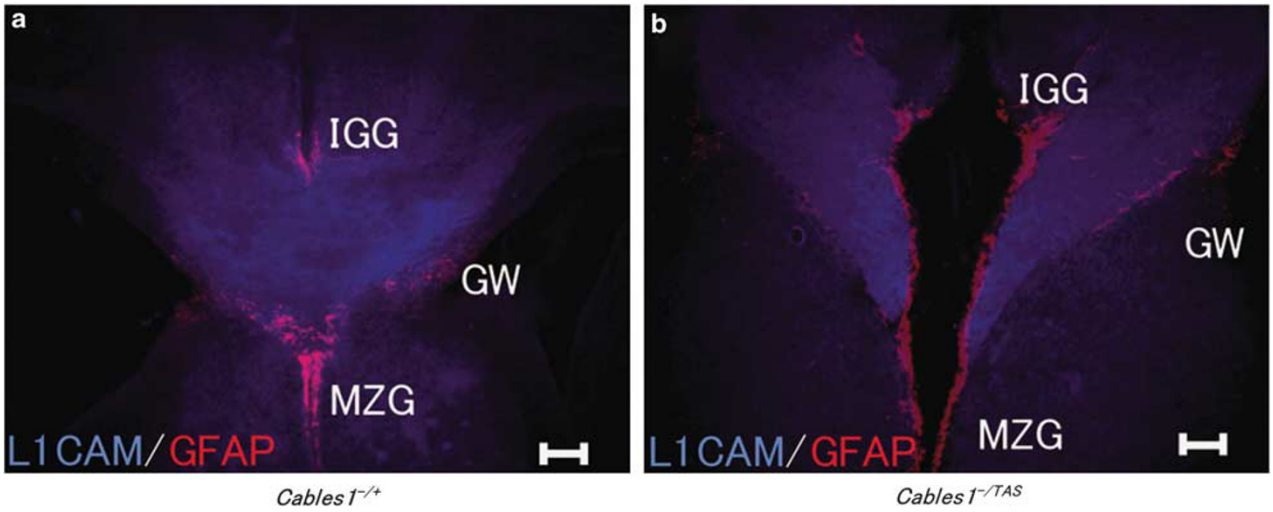

Figure 6 ACC in *Cables1*$^{-/TAS}$. Coronal sections in P0 *Cables1*$^{-/+}$ (**a**) and *Cables1*$^{-/TAS}$ (**b**). Staining with antibodies against L1CAM (blue) and GFAP (red). Callosal axons did not cross the midline, and GFAP-expressing IGG and MZG were indistinguishable from *Cables1*$^{-/TAS}$ (**b**). GW, glial wedge; IGG, indusium griseum glia; MZG, midline zipper glia; scale bar = 100 μm.

expression of 32 additional genes located in the vicinity of the candidate region between wild-type mice and homozygous TAS mice (data not shown). These results indicate that only *Cables1* is disrupted by transgene insertion, suggesting that ACC is caused by *Cables1* gene mutation in TAS mice.

A complete *CABLES1* is also present in the human genome and is mapped to the long arm of chromosome 18 at

20.71–20.84 Mb from the centromere (http://genome.ucsc.edu). *CABLES1*, originally called *iK3-1*, has been isolated using the yeast two-hybrid system with cyclin-dependent kinase 3 (CDK3) cDNA as bait to evaluate cdk3 function.[30] In addition to the interaction with CDK3, CABLES1 is functionally connected to p53 and p73 in cell death, suggesting that it is a molecule involved in harmonizing cell cycle progression and cell death.[31] Previous studies showed that *Cables1*-null mutant mice displayed increased cellular proliferation (eg, endometrial hyperplasia, colon cancer, and oocyte development).[27–29] In contrast to *Cables1* loss-of-function mutation, overexpression of *Cables1* in a colon cancer cell line showed tumor suppressor activity, including inhibition of colony formation and cell growth.[32] However, there have been no reports that aberrant expression of *Cable1* is involved in neural defects *in vivo*.

Zukerberg *et al*[33] showed that Cables1 is expressed in the brain, where it is present in postmitotic neurons of the cerebral cortex, and interacts directly with Cdk5 and Abl1 (Abelson murine leukemia viral oncogene homolog 1) in the brain lysate. Furthermore, Cables1 and Cdk5 were localized at the tips of growing axons. They suggested that the association of Cables1 and Cdk5 is part of the signaling pathway that operates during brain development. Moreover, Rhee *et al*[34] demonstrated that Cables1 is the essential molecule connecting Robo-associated Abl and N-cadherin-associated β-catenin. Slit/Robo signaling is known to play a crucial role in cortical axon guidance across the corpus callosum by the midline glial population.[35] This is the first report regarding a *Cables1* allele associated with an acallosal phenotype. We also clearly showed that a novel *Cables1* mutation in TAS mice generates a truncated Cables1 product. These observations strongly suggest that Cables1 may be involved in development of the carpus callosum.

We produced a unique *Cables1*-null mutant strain derived from BALB/c ES cells to evaluate Cables1 function in BALB/c mice, because it is known that the genetic background has a strong influence on the development of ACC in mice.[12] Unexpectedly, the complete loss-of-function Cables1 mutant showed no alterations in callosal formation in mice, even with the same genetic background as TAS mice. Although we cannot as yet provide direct evidence for the lack of phenotype in the loss-of-function Cables1 mutant, the discrepancy in ACC frequency between Cables$^{+/TAS}$ mice (62%) and Cables1$^{-/TAS}$ mice (100%) is suggestive of the possible mechanism. In mice, expression of truncated Cables1 resulted in acallosal brain development and *Cables1*^{TAS} gene dosage was associated with the incidence of ACC, indicating a dominant negative function of Cables^{TAS} on callosal formation. In contrast, the absence of Cables1 had no negative influence on brain development, suggesting the presence of a molecule(s) functionally redundant to Cables1. Furthermore, the significantly lower frequency of ACC in *Cables1*$^{+/TAS}$ mice compared with *Cables1*$^{-/TAS}$ mice indicated that the wild-type Cables1 serves to form the corpus callosum. Therefore, one copy of *Cable1*^{TAS} may be sufficient to impair the function of the homozygous redundant gene. This speculation is summarized in Figure 7.

Table 1 Frequency of ACC in *Cables1*-mutant mice

Genotype	No. of mice examined	No. of mice with ACC (%)[a]
Cables1$^{+/+}$	59	0 (0%)[b]
Cables1$^{+/TAS}$	37	23 (62%)[b]
Cables1$^{TAS/TAS}$	12	12 (100%)[b]
Cables1$^{-/-}$	7	0 (0%)
Cables1$^{-/TAS}$	6	6 (100%)

[a]No. of mice with ACC/no. of mice examined.
[b]These data were reported in our previous study.[25]

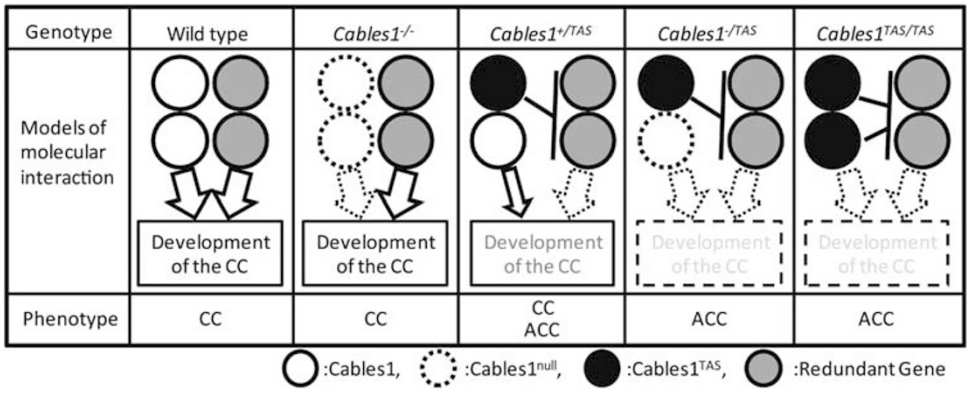

Figure 7 Model for gene interaction between *Cables1*, *Cables1*^{TAS}, and redundant gene. *Cables1* and its redundant gene play a role in the development of corpus callosum, and *Cables1*^{TAS} may impair redundant gene function. As the redundant gene functions in development of the corpus callosum, ACC was not observed in *Cables1*-null mutant mice. In heterozygous TAS mice (*Cables1*$^{+/TAS}$), even though redundant gene function was impaired by *Cables1*^{TAS}, one copy of the *Cables1* gene served to form the corpus callosum. In contrast, all *Cables1*$^{TAS/TAS}$ and *Cables1*$^{-/TAS}$ mice showed ACC because *Cables1* and its redundant gene were not expressed or were impaired by *Cables1*^{TAS}, respectively.

The Cables family has two members in mammals, designated as Cables1 and Cables2, and the latter is a possible candidate with functional redundancy. *Cables2* was cloned by cross-hybridization with Cables1 and RT-PCR.[36] It has been reported that Cdk5 and Abl1 associate with Cables2, similar to Cables1. Furthermore, Cables2 is expressed in a variety of tissues, including the brain. Although no data have been reported regarding colocalization of Cables1 and Cables2 expression in the brain, the effect of Cables1 dysfunction may be masked by a redundant function of Cables2 in callosal development.

Integration of adhesive interactions with directional information from attractive and repulsive cues is essential for the correct progression of callosal axons. Cables1 connects Robo-associated Abl and N-cadherin-associated β-catenin. Truncated Cable1 includes the N-terminal half (298 amino acids) of the wild-type protein (568 amino acids). Cables1 lacking the N-terminal one-third of the protein shows markedly decreased association with Abl1 compared with the wild type.[33] Although the precise localization of Abl1 interaction site(s) of Cables1 is unknown, five of the six SH3 domain-binding motifs (PXXP) in Cables1 are located in the N-terminal region. The SH3 domain regulates the kinase activity by interacting with the kinase domain.[37] Cables1^{TAS} also contains PXXP domains. The function of Abl1 may be affected by binding to the truncated Cables1. The Abl family of nonreceptor tyrosine kinases consists of Abl1 and its paralog Abl2. Although Abl1-deficient mice show normal brain formation, mice lacking both Abl1 and Abl2 kinases develop neurulation defects at 11 dpc, suggesting that both kinases play fundamental roles in central nervous system development and function.[38] Therefore, truncated Cables1 may target both Abl1 and Abl2. Further studies are required to determine whether both Abl kinases contribute to the corpus callosum organization in cerebral cells expressing Cables1 after 15 dpc and whether truncated Cable1 impairs the functions of both Abl kinases.

In conclusion, the results presented here clearly indicated that ACC in TAS mice is caused by a novel mutant gene *Cables1*^{TAS}. Although the molecular mechanisms underlying the observed effects of truncated Cables1 and its related proteins are unclear, these findings will contribute to our understanding of corpus callosum development.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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