Human Mutation

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Brief Report

Loss of interaction between plectin and type XVII collagen results in epidermolysis bullosa simplex

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Abstract

Plectin is a linker protein that interacts with intermediate filaments and β4 integrin in hemidesmosomes of the epidermal basement membrane zone (BMZ). Type XVII collagen (COL17) has been suggested as another candidate plectin binding partner in hemidesmosomes. Here, we demonstrate that plectin-COL17 binding helps to maintain epidermal BMZ organization. We identified an epidermolysis bullosa (EB) simplex patient as having markedly diminished expression of plectin and COL17 in skin. The patient is compound heterozygous for sequence variants in the plectin gene (*PLEC*); one is a truncation and the other is a small in-frame deletion sequence variant. The in-frame deletion is located in the putative COL17-binding domain of plectin and abolishes the plectin-COL17 interaction in vitro. These results imply that disrupted interaction between plectin and COL17 is involved in the development of epidermolysis bullosa. Our study suggests that protein-protein binding defects may underlie EB in patients with unidentified disease-causing sequence variants.

Key words: plectin, *PLEC*, type XVII collagen, epidermolysis bullosa simplex

Plectin, encoded by *PLEC* (*PLEC*; MIM# 601282), serves as a linker protein between intermediate filaments and hemidesmosomes in the epidermal basement membrane zone (BMZ) (Castanon, et al., 2013; Natsuga, 2015; Winter and Wiche, 2013). β4 integrin has been well characterized as a binding partner of plectin in hemidesmosomes (Koster, et al., 2004; Rezniczek, et al., 1998). In addition to β4 integrin, the transmembrane protein type XVII collagen (COL17, also known as BP180 or BPAG2) has been implicated as interacting with plectin in yeast two-hybrid assays (Koster, et al., 2003). The Y domain (Arg618 to Val819), a rich α-helical region, was proposed as a putative COL17-binding domain of plectin (Koster, et al., 2003). The crystal structure of the plectin spectrin repeats around the Y domain was recently described (Ortega, et al., 2011). However, the physiological and pathological roles of the plectin-COL17 interaction *in vivo* in mammals have not yet been elucidated.

Epidermolysis bullosa simplex, basal type (basal EBS) is a group of congenital blistering diseases that are characterized by skin detachment within basal keratinocytes observable under electron microscopy (Fine, et al., 2014). Plectin deficiency leads to subtypes of autosomal recessive basal EBS that include EBS with muscular dystrophy (EBS-MD), EBS with pyloric atresia (EBS-

PA) and skin-only EBS (Gostynska, et al., 2015; Natsuga, 2015; Winter and Wiche, 2013).

Here, we present an EBS patient with a plectin disease-causing sequence variant in the COL17-binding domain and demonstrate that the sequence variant disturbs the interaction between plectin and COL17 *in vitro*.

The proband was born to non-consanguineous Japanese parents. He had suffered from skin fragility since birth. In the first week of life, erosions and small blisters were seen on his trunk and extremities (**Supp. Fig. S1a-b**). His developmental status was otherwise normal, and no muscle weakness or gastrointestinal complications were observed in the first 3 years of life. Electron microscopy of skin specimens from the proband revealed skin detachment within basal keratinocytes accompanied by hypoplastic hemidesmosomes at the bottom of the blisters (**Supp. Fig. S1c-e**). Keratin aggregates were not present in the cytoplasm of the keratinocytes. The clinical manifestations and ultrastructural findings led to the diagnosis of EBS, basal subtype (Fine, et al., 2014).

To identify the targeted protein in this EBS case, we evaluated the expression of various BMZ proteins in the proband's skin (**Supp. Fig. S2a-h**) (Natsuga, et al., 2010a). We found that plectin staining in the epidermal BMZ was

diminished compared with that of normal control skin (**Supp. Fig. S2a-c**) as determined using several antibodies directed to different portions of the plectin molecule. In addition, COL17 labeling in skin samples from the proband was also reduced relative to that of skin samples from the control subject. In contrast, β4 integrin labeling in proband tissue was comparable to control tissue (**Supp. Fig. S2d-h**). The expression patterns of other BMZ proteins, including BP230, α6 integrin, type IV collagen, laminin-332 and type VII collagen, were not affected (data not shown). To confirm the results obtained from the skin specimens, we evaluated cultured epidermal keratinocytes collected from the proband and the control patient. Immunoblot analysis revealed that expression of plectin and COL17 was reduced in the proband's keratinocytes compared with the control, whereas expression of β4 integrin was unaffected (**Fig. 1a-d**). These results suggest that among hemidesmosomal components, plectin and COL17 are selectively reduced in the EBS proband's keratinocytes.

Ultrastructure, immunofluorescence and Western blot analysis have indicated that genetic abnormalities may be present in *PLEC* (encoding plectin), *COL17A1* (encoding COL17) or other EBS causative genes. Direct sequencing of the *COL17A1*, *KRT5*, *KRT14*, *DST*, *EXPH5* and *ITGB4* genes revealed no

disease-causing sequence variants in the proband's genomic DNA. Further cytogenic DNA array analysis failed to detect gross deletions or insertions in *COL17A1*. *PLEC* sequence variant analysis revealed the proband to be compound heterozygous for c.2264_2266del (p.Phe755del) in exon 19 (Winter, et al., 2016) (**Fig. 1e**) and previously undescribed c.9194dup (p.Ser3066GlufsTer55) in exon 32 (**Fig. 1f**). The proband's mother and father were carriers for a heterozygous allele of c.2264_2266del or c.9194dup, respectively (data not shown). These sequence variants were submitted to the public Leiden Open Variation Database 3.0 (https://databases.lovd.nl/shared/individuals/00119121). Generally, truncation sequence variants that lead to premature termination codons in the last exon of the gene have been regarded as being exempt from nonsense-mediated mRNA decay. However, previous studies suggested that truncation sequence variants in exon 32 might be subject to nonsense-mediated mRNA decay (Natsuga, et al., 2010b), possibly through microRNA-regulated mRNA qualification (Zhao, et al., 2014). Therefore, c.9194dup in exon 32 is expected to lead to null expression of the allele (**Fig. 1g**). In contrast, c.2264_2266del (p.Phe755del) is an in-frame deletion that is not expected to affect gene expression (**Fig. 1g**). p.Phe755del is

located in the Y domain of plectin (Arg618 to Val819), which was predicted to be a COL17-binding domain by the yeast two-hybrid assay (Koster, et al., 2003) (**Fig. 1g**). Although c.2264_2266del is adjacent to the splice acceptor site, PCR products derived from the cDNA of proband and control keratinocytes spanning the exon18-exon19 junction exhibited a single band (**Supp. Fig. S3a**). Direct sequencing of the PCR products revealed a wild type and a c.2264_2266del allele (**Supp. Fig. S3b**), suggesting that *PLEC* splicing is not altered by c.2264_2266del. Along with the null expression of one allele of *PLEC*, these findings indicate that an in-frame deletion in the putative COL17-binding domain of plectin is disease-causing for the EBS phenotype.

Quantitative RT-PCR revealed that *PLEC* gene expression was reduced in keratinocytes cultured from the proband (**Supp. Fig. S4a**), which is consistent with the sequence variant data and protein expression studies. Expression of a major transcript variant of *PLEC* expressed in the hemidesmosome (encoding plectin 1a isoform) was reduced in keratinocytes from the proband (**Supp. Fig. S4b**). Unexpectedly, *COL17A1* gene expression was also diminished in the proband's cells compared with control cells (**Supp. Fig. S4c**).

In light of the results of genetic analyses, we hypothesized that the in-frame

deletion of plectin in the putative COL17-binding domain specifically hinders plectin-COL17 binding. To test this hypothesis, we prepared three V5-tagged vectors with *PLEC* cDNA fragments: 1) PLEC1-819, 2) PLEC1-617 and 3) PLEC1-819ΔPhe755 (**Fig. 2a**). PLEC1-819 spans Met1 to Val819 of plectin. PLEC1-617 does not harbor the Y domain (Arg618 to Val819), the putative COL17-binding domain. PLEC1-819ΔPhe755 lacks Phe755, which was deleted in the proband's allele. We transfected the three types of *PLEC* gene constructs into HEK293 cells that stably expressed FLAG-COL17 and performed immunoprecipitation-immunoblot analysis. After immunoprecipitation with anti-V5 antibody (plectin), immunoblot using an anti-FLAG antibody (COL17) revealed FLAG-tagged COL17 in the lysates of PLEC1-819-transfected cells but not in the PLEC1-617 cell extracts. These results suggest that plectin-COL17 binding in mammalian cells occurs between Ala618 and Val819 in plectin, as predicted by the yeast two-hybrid assay (Koster, et al., 2003) (**Fig. 2b**). PLEC1-819ΔPhe755 failed to bind to COL17 in the transfected cells (**Fig. 2b**). To further investigate the interaction between plectin and COL17, GST-tagged recombinant partial plectin and the full-length COL17 (FLAG-COL17) recombinant proteins were subjected to a protein-protein binding assay (**Fig. 2c**). The full-length COL17

(FLAG-COL17, input) was detected at approximately 180 kDa with CBB staining (**Fig. 2d**). GST (~25 kDa) and GST-tagged partial plectin proteins (PLEC618-819 and PLEC618-819Δ755Phe, both ~50 kDa) were also detected with CBB. PLEC618-819 was bound to FLAG-COL17, but the binding of PLEC618-819Δ755Phe to FLAG-COL17 was significantly diminished (**Fig. 2d, 2e**). These results indicate that in-frame deletion of Phe755 in the COL17-binding domain greatly reduces plectin-COL17 binding.

In addition to the p.Phe755del detected in the proband and in the recent case series (Winter, et al., 2016), several other EBS cases with *PLEC* in-frame deletion/insertion sequence variants have been described: 1) c.954_956dup (p.Leu319dup) in exon 9 (Bauer, et al., 2001), 2) c.1530_1531insGTCTGCGTGTACCGTCTGCTCCGACGCCTGGTAGGC (p.Ala510_Ile511insValCysValTyrArgLeuLeuArgArgLeuValGly) in exon 14 (the detailed information kindly provided by Prof. Jouni Uitto) (Pfendner, et al., 2005), and 3) c.2677_2685del (p.Gln893_Ala895del) in exon 21 (Pulkkinen, et al., 1996; Yiu, et al., 2011). p.Leu319dup is near the actin-binding domain (ABD), to which β4 integrin primarily binds (Geerts, et al., 1999; Rezniczek, et al., 1998); this sequence variant has been shown to lead to protein self-aggregation and

impaired binding between β4 integrin and plectin (Bauer, et al., 2001). c.1530_1531insGTCTGCGTGTACCGTCTGCTCCGACGCCTGGTAGGC (p.Ala510_Ile511insValCysValTyrArgLeuLeuArgArgLeuValGly) (Pfendner, et al., 2005) is localized in the plakin domain but outside the putative COL17-binding domain (Arg618 to Val819). p.Gln893_Ala895del was previously described as being located within the Y domain of plectin (Koster, et al., 2003), but the sequence variant is apparently outside the Arg618 to Val819 region. This is in line with immunofluorescence data showing normal COL17 labeling on the BMZ of an EBS patient with p.Gln893_Ala895del (Pulkkinen, et al., 1996). Thus, to our knowledge, the p.Phe755del detected in the proband in the current study is the first disease-causing sequence variant to be identified within the putative COL17-binding domain of plectin.

The intracytoplasmic in-frame deletion of COL17 has also been reported to contribute to an EBS phenotype (Fontao, et al., 2004). This deletion abolishes the association of COL17 with plectin together with β4 integrin and BP230 (Fontao, et al., 2004). In addition, COL17-null cultured keratinocytes fail to incorporate plectin into hemidesmosome-like structures (Koster, et al., 2003). These studies also support the importance of the plectin-COL17 interaction in

hemidesmosome formation *in vitro*. Our study highlighted the significance of the plectin-COL17 interaction *in vivo*.

As flawed protein-protein interactions affect protein stability, the reduced protein levels of plectin and COL17 together with the maintenance of β4 integrin are consistent with a defective plectin-COL17 interaction due to a *PLEC* sequence variant in the COL17-binding domain. However, gene expression levels of plectin and COL17 were also diminished in cells cultured from the proband. Given that plectin knockdown in normal human epidermal keratinocytes does not alter COL17 gene expression (data not shown), the reason for the reduction in COL17 gene expression in the proband's cells remains unclear, although it might be associated with other sequence variants in proband cells.

In addition to binding to the ABD (Geerts, et al., 1999; Rezniczek, et al., 1998), β4 integrin may also bind to other domains of plectin (Koster, et al., 2004; Rezniczek, et al., 1998). Although the plakin domain of plectin was reported to bind to β4 integrin in a blot overlay assay (Rezniczek, et al., 1998), the binding was not confirmed in a yeast two-hybrid assay (Koster, et al., 2004). We cannot exclude the possibility that p.Phe755del in plectin somehow affects β4 integrin-plectin binding. However, because the amount and expression pattern of β4

integrin were not altered in the proband, we believe that p.Phe755del specifically disturbs the plectin-COL17 interaction.

Although recent technical advances in pyrosequencing have contributed greatly to the field of genodermatoses, including EB (Takeichi, et al., 2015), the conventional sequence variant search strategy starting from immunohistochemical antigen mapping and electron microscopy to targeted Sanger sequencing is still a powerful tool for detecting genetic abnormalities in EB patients. Most of the recessive inherited EB cases show null or profoundly diminished expression of one BMZ component. However, one limitation of this strategy is that in-frame deletion or insertion sequence variants might not always reduce protein expression levels. This limitation is exemplified by the fact that targeted genes are not found in up to 15% of EB cases (Takeichi, et al., 2015). Therefore, the defective protein-protein binding seen in our study may contribute to the pathology in some undiagnosed EBS cases.

In conclusion, our study is the first to detect a plectin disease-causing sequence variant within the COL17-binding domain in EBS. Abnormalities in the protein-binding domain may disturb the formation of cell structures, leading to devastating diseases such as EB.

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Disclosure statement

The authors have no conflict of interest to declare.

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Figure legends

Figure 1. Immunoblot analysis of cultured keratinocyte lysates and *PLEC* sequence variant analysis

(a-d) Lysates from cultured epidermal keratinocytes of the proband and normal control were examined. The amounts of plectin (a, HD1-121) and COL17 (b, 09040) are reduced in the proband compared with the normal control, while β4 integrin (H101) in the proband is comparable with that of the normal control (c). Tubulin (ab6046) was used as a loading control. (d) The relative ratio of plectin/COL17/β4 integrin to tubulin in the proband was normalized to the expression level in normal control samples. Data were analyzed with Student's t-test. ** $0.001<p<0.01$. (e, f) The sequencing data for TA cloning products from the proband's genomic DNA. In the wild-type allele derived from the proband's father, exon 19 begins with two TTCs, whereas the mutant allele from the mother has only one TTC, resulting in the deletion of one phenylalanine at the protein level (e). Because the HGVS guideline (http://varnomen.hgvs.org/) (den Dunnen, et al., 2016) recommends that the most 3' position possible be arbitrarily assigned to have changed for all descriptions, this in-frame deletion sequence variant was designated as c.2264_2266del/p.Phe755del (e). c.9194dup in exon 32 was also

detected in the paternal allele (but not in the maternal allele), leading to the replacement of serine at the 3066 position with glutamate, followed by frameshift leading to a premature termination codon (PTC) 54 codons downstream (p.Ser3066GlufsTer55) (f). (g) A schematic of the plectin gene and the protein structure. Note that c.2264_2266del/p.Phe755del is located in the putative COL17-binding domain of plectin (Arg618 to Val819). The sequence variants were numbered based on the reference sequence NM_000445.4 encoding plectin isoform 1c. IF, intermediate filaments.

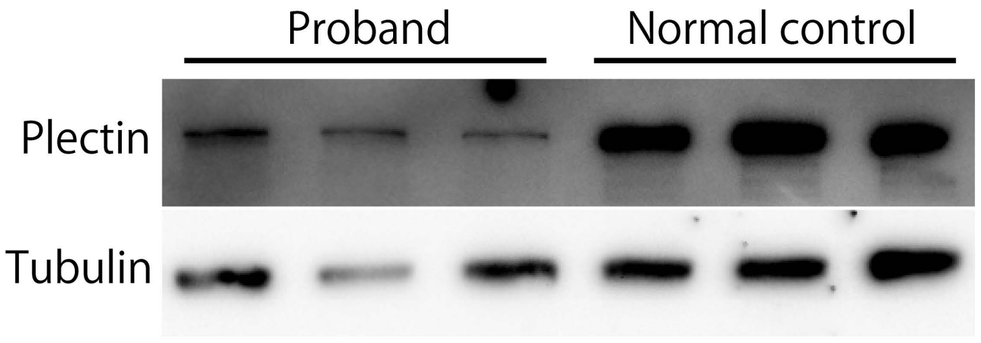
Figure 2. Immunoprecipitation-immunoblot assay

(a) Three plectin fragments with V5-tag were prepared. PLEC1-819 harbors the putative COL17-binding domain (Arg618 to Val819), whereas PLEC1-617 does not. PLEC1-819ΔPhe755 does not contain Phe755, which is deleted in the maternal allele of the proband. Each construct was transiently transfected into HEK293 cells with stable COL17 expression. COL17 was tagged with FLAG peptides. (b) Transfection of each construct was confirmed by immunoblot of cell lysates using an anti-V5 antibody. Stable COL17 expression is evident from anti-FLAG antibody detection by immunoblot. β-actin was used as the loading control

(left column). Immunoprecipitation using the anti-V5 antibody for each cell lysate reveals the bands of each plectin fragment through immunoblot. COL17, detected by the anti-FLAG antibody, is observed in PLEC1-819-transfected cells but not in PLEC1-617-transfected or PLEC1-819ΔPhe755-transfected cells after immunoprecipitation (right column). (c) GST and GST-tagged plectin fragments were prepared and used for the protein-protein binding assay with the recombinant full-length COL17 (FLAG-COL17). PLEC618-819 consists of the putative COL17-binding domain (Arg618 to Val819) and GST at the N-terminus. PLEC618-819ΔPhe755 is a recombinant protein in which Phe755 was deleted from PLEC618-819. (d) Protein-protein binding assay. FLAG-COL17 (input) reveals a band between 250 kDa and 150 kDa with CBB staining. GST (25 kDa) and GST-tagged plectin fragments (~50 kDa) are observed with CBB staining. Immunoblotting using the anti-FLAG antibody reveals a band of the supernatants from PLEC618-819-Sepharose incubated with FLAG-COL17. In contrast, GST does not bind to FLAG-COL17. PLEC618-819ΔPhe755 shows much weaker binding to FLAG-COL17 compared with PLEC618-819. M = molecular weight marker. (e) The relative ratio of COL17 to bait protein in PLEC618-819ΔPhe755 was normalized to PLEC618-819. Data were obtained from three independent

experiments and analyzed with Student’s t-test. * $0.01<p<0.05$.

a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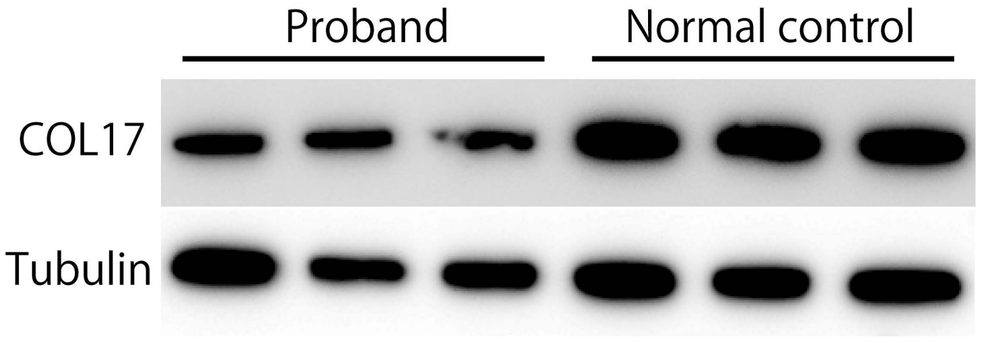
Normal control
Plectin
Tubulin

b


Proband
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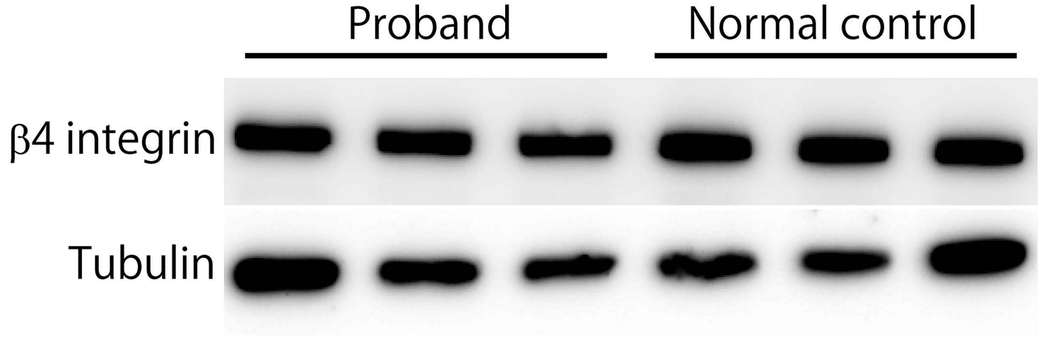
COL17
Tubulin

c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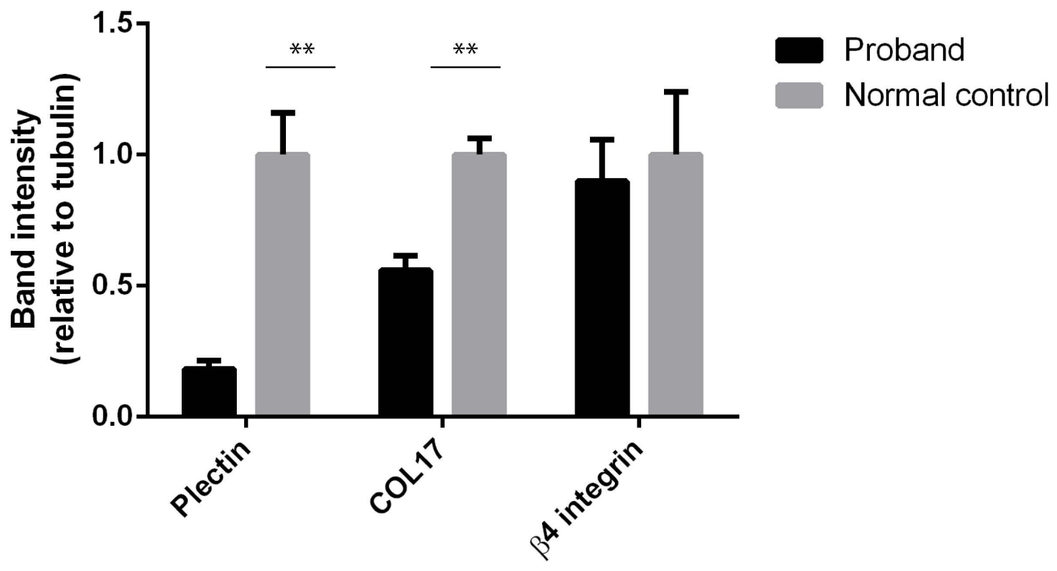
d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1.0
0.5
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Plectin
COL17
β4 integrin
Proband
Normal control

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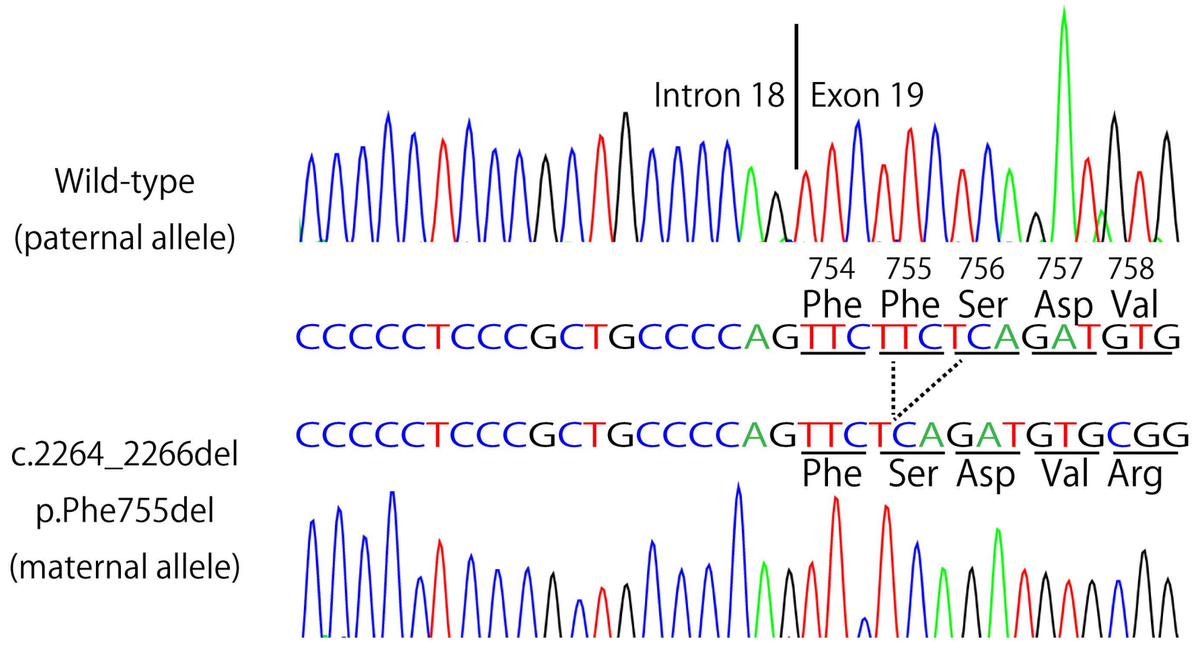
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Exon 19
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Phe Phe Ser Asp Val
CCCCCTCCCGCTGCCCCAGTTCTTCTCAGATGTG
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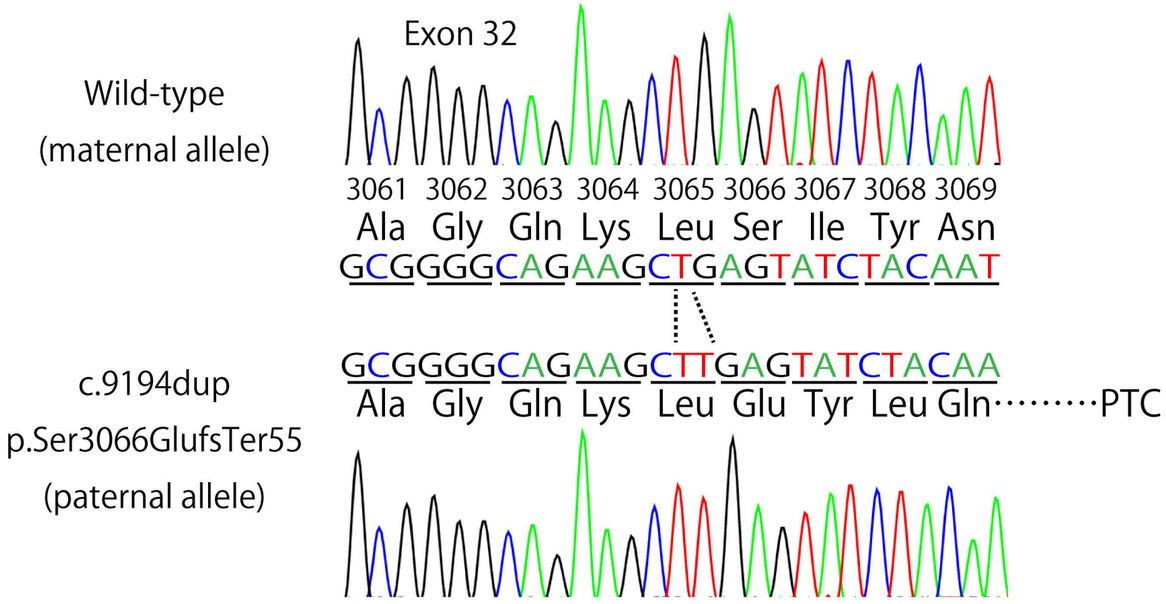
f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(maternal allele)
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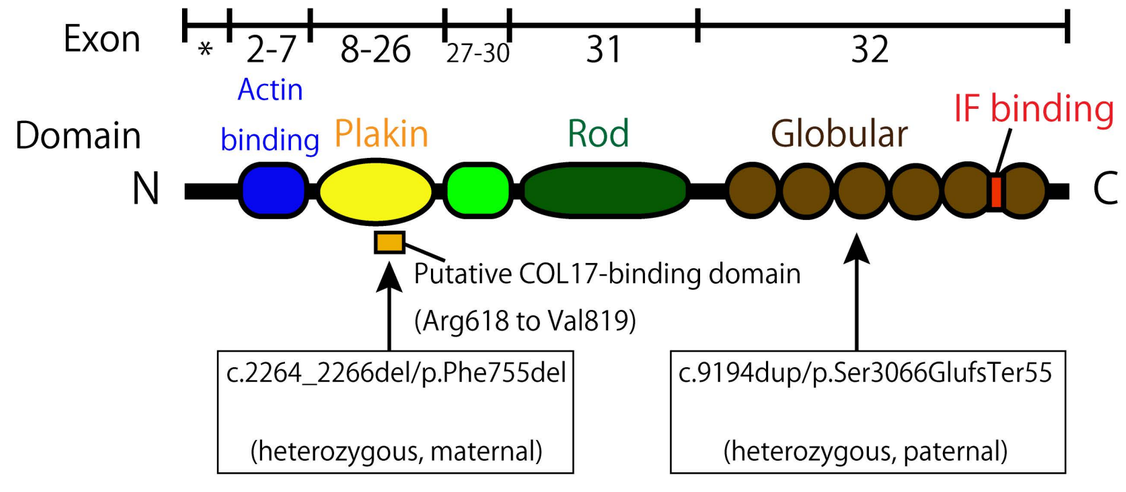
g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Actin binding
Domain
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Rod
Globular
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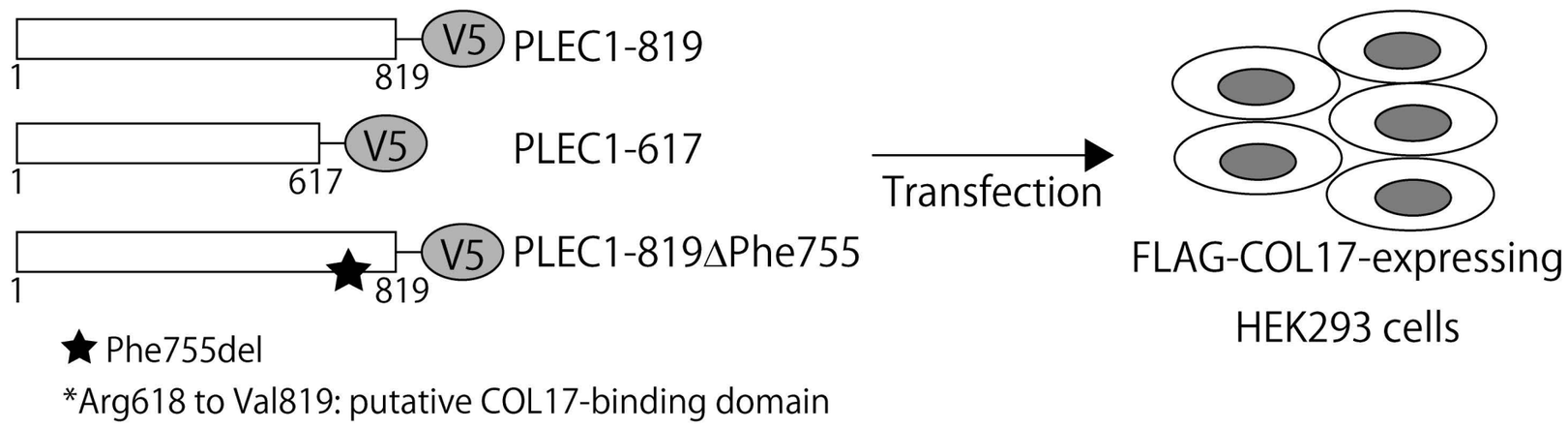
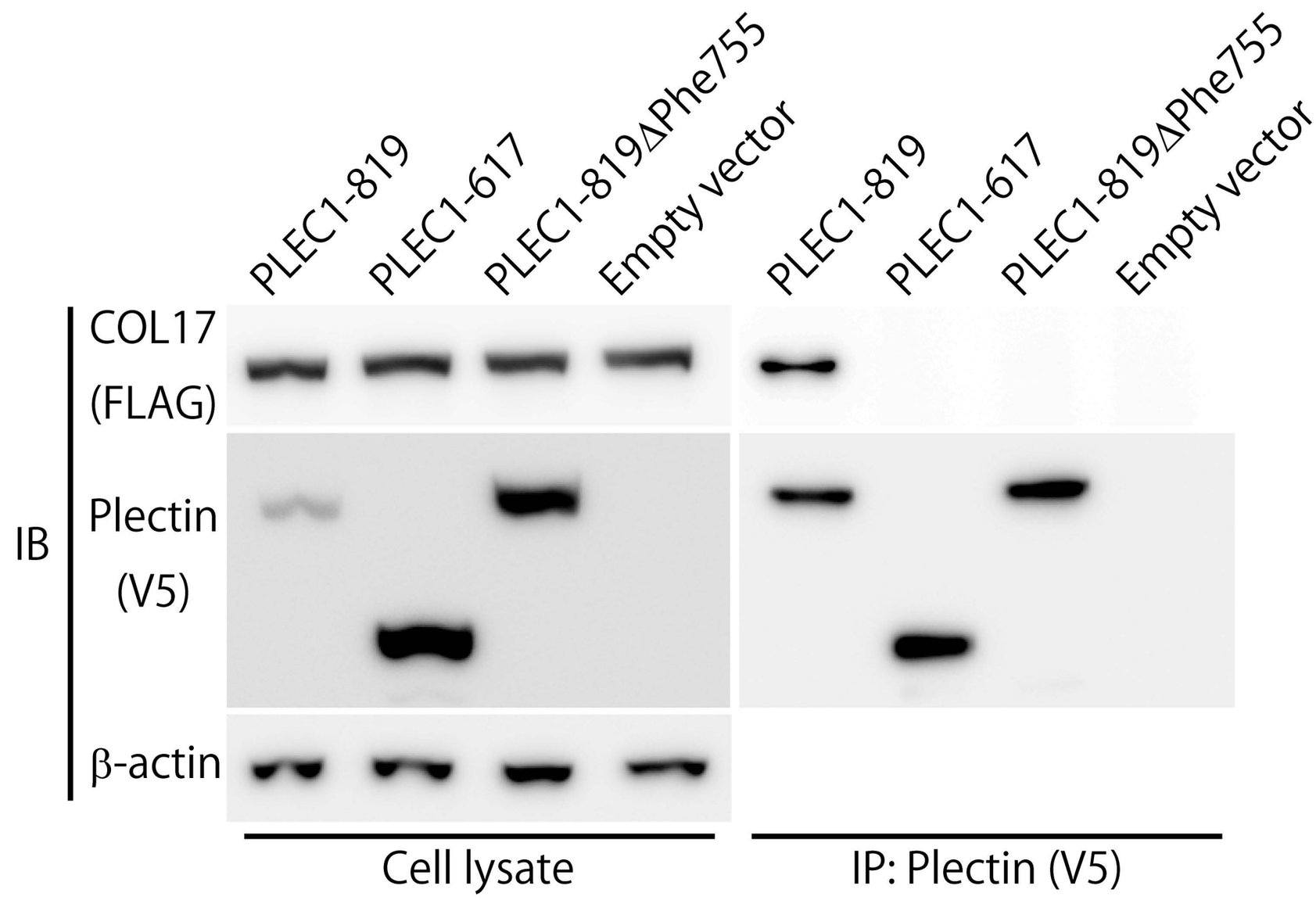
(Arg618 to Val819)
c.2264_2266del/p.Phe755del
(heterozygous, maternal)
c.9194dup/p.Ser3066GlufsTer55
(heterozygous, paternal)

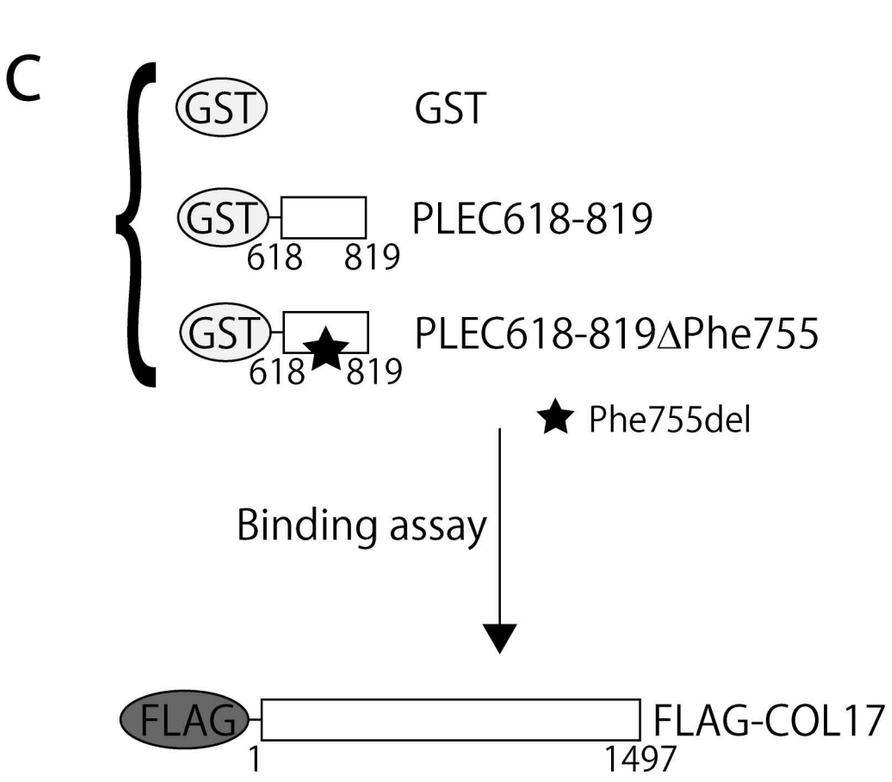
a


V5
PLEC1-819
1
819
V5
PLEC1-617
1
617
V5
PLEC1-819ΔPhe755
1
819
Phe755del
*Arg618 to Val819: putative COL17-binding domain
Transfection
FLAG-COL17-expressing
HEK293 cells

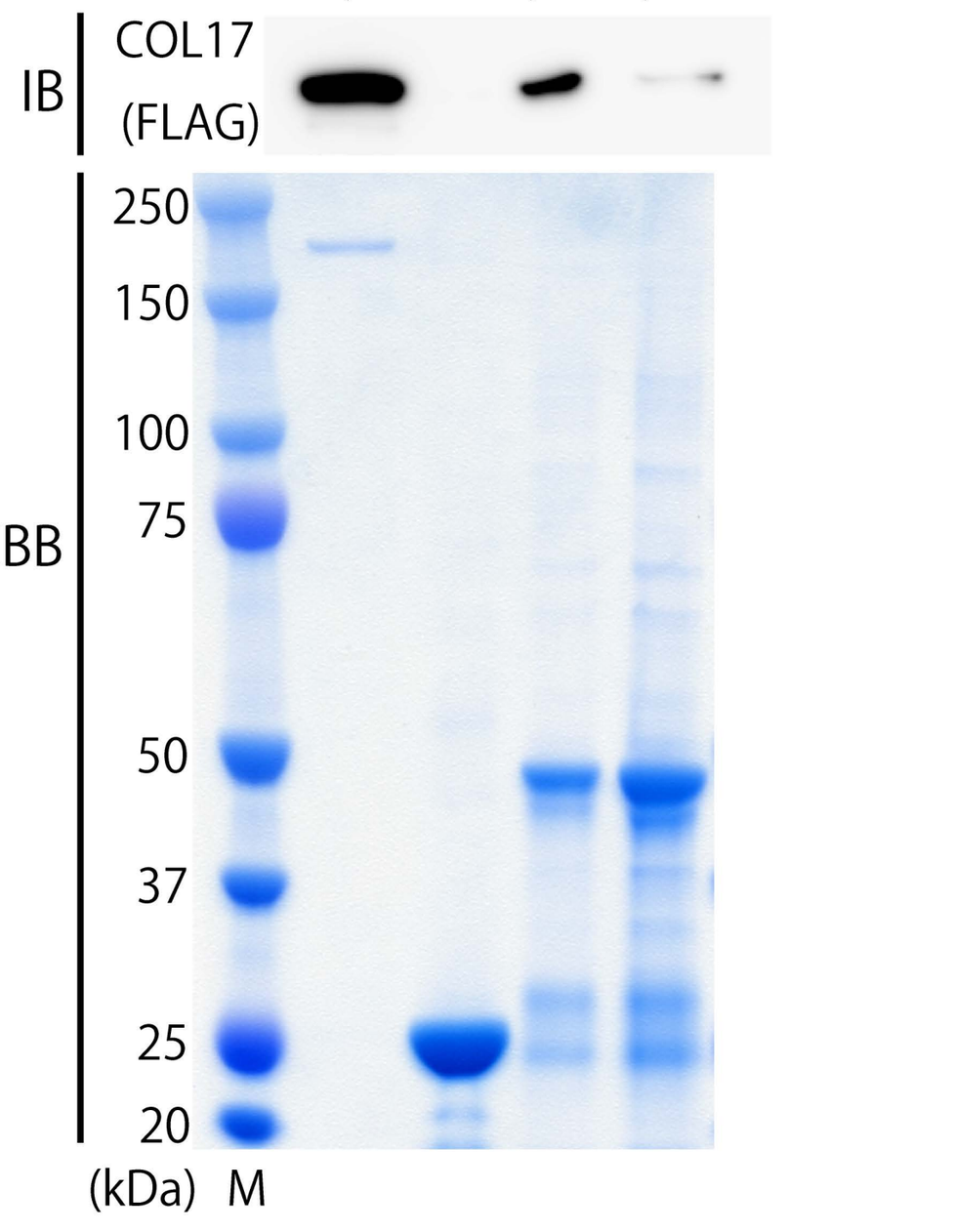
b


PLEC1-819
PLEC1-617
PLEC1-819ΔPhe755
Empty vector
PLEC1-819
PLEC1-617
PLEC1-819ΔPhe755
Empty vector
IB
COL17
(FLAG)
Plectin
(V5)
β-actin
Cell lysate
IP: Plectin (V5)

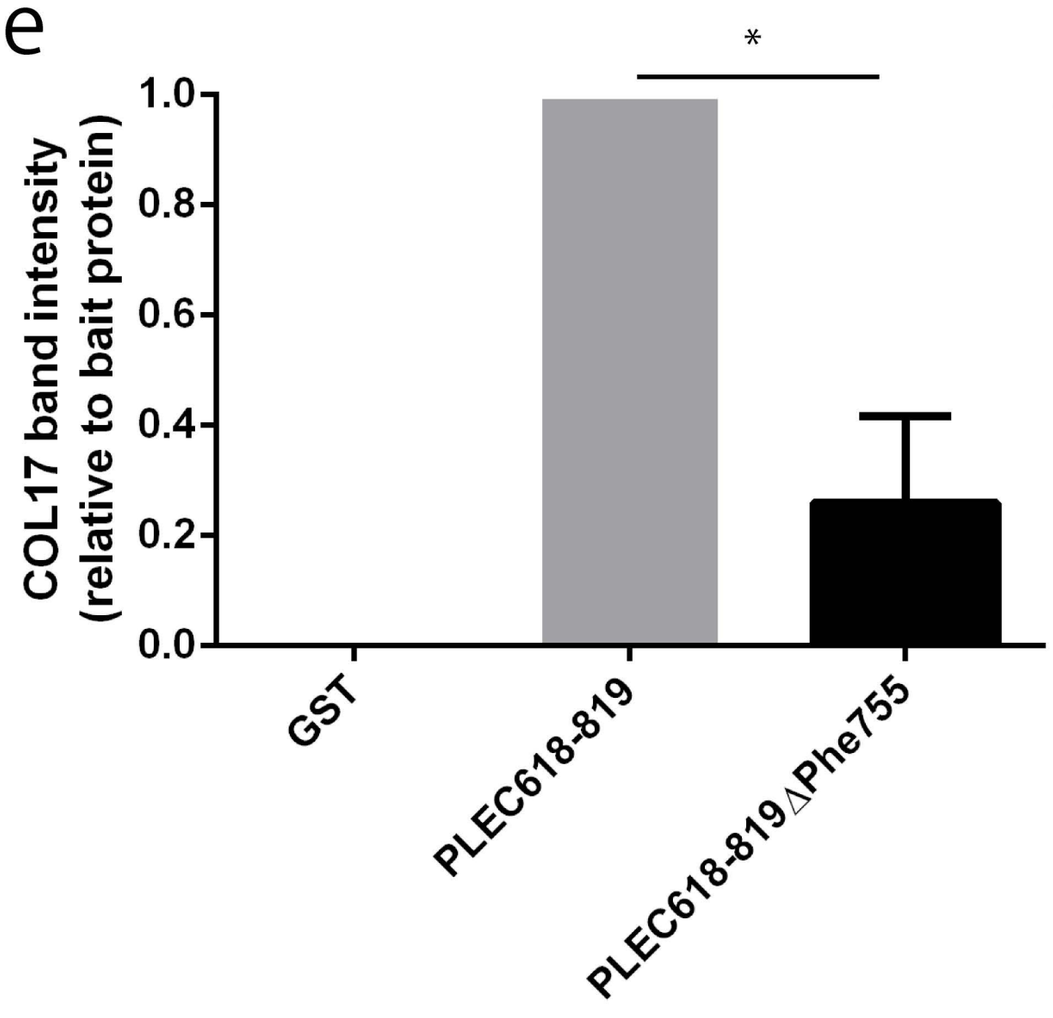
c

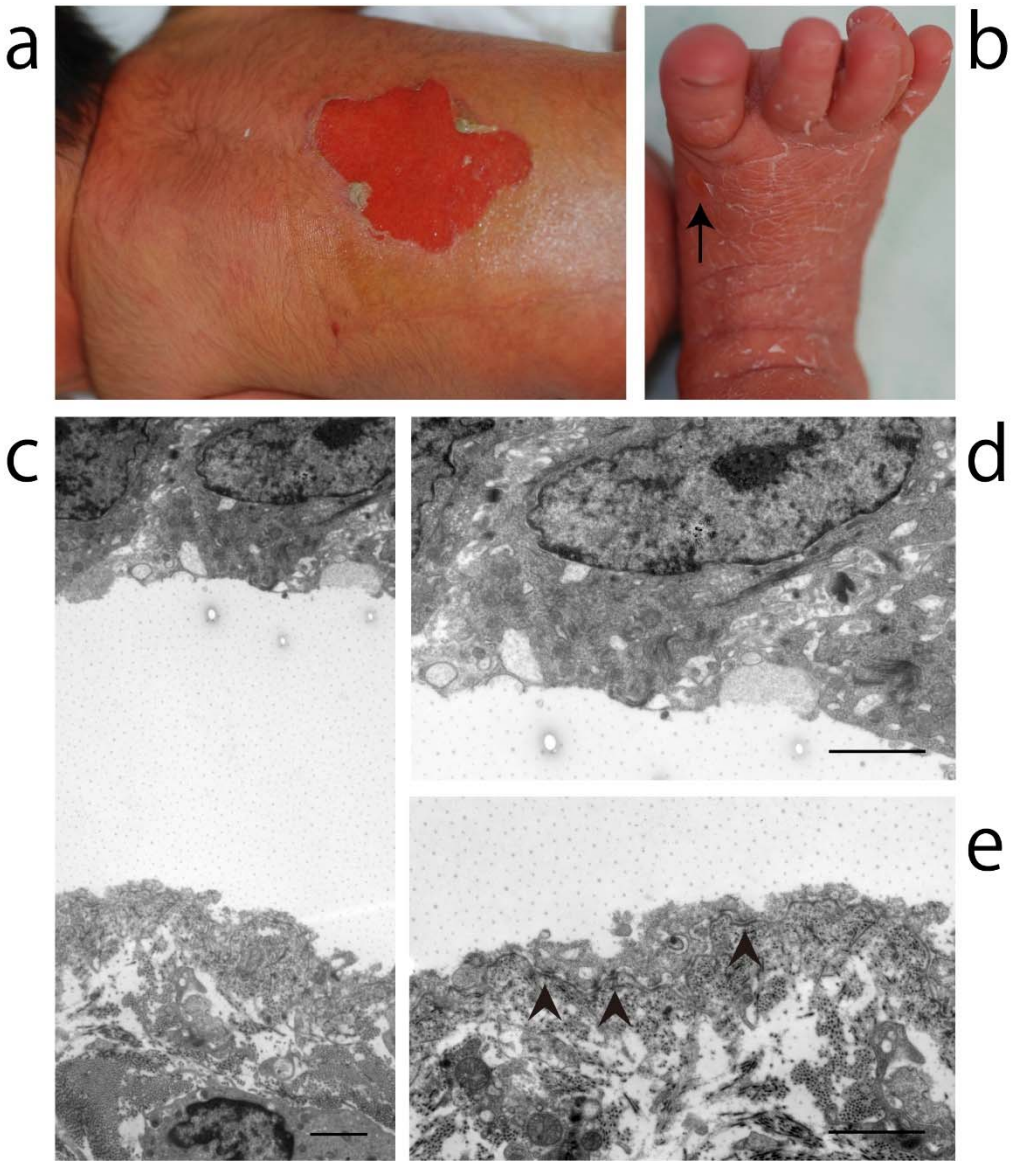

GST
GST
PLEC618-819
618
819
PLEC618-819ΔPhe755
618
819
Phe755del
Binding assay
FLAG
FLAG-COL17
1
1497

d


Input (FLAG-COL17)
GST
PLEC618-819
PLEC618-819ΔPhe755
IB
COL17
(FLAG)
CBB
250
150
100
75
50
37
25
20
(kDa)
M

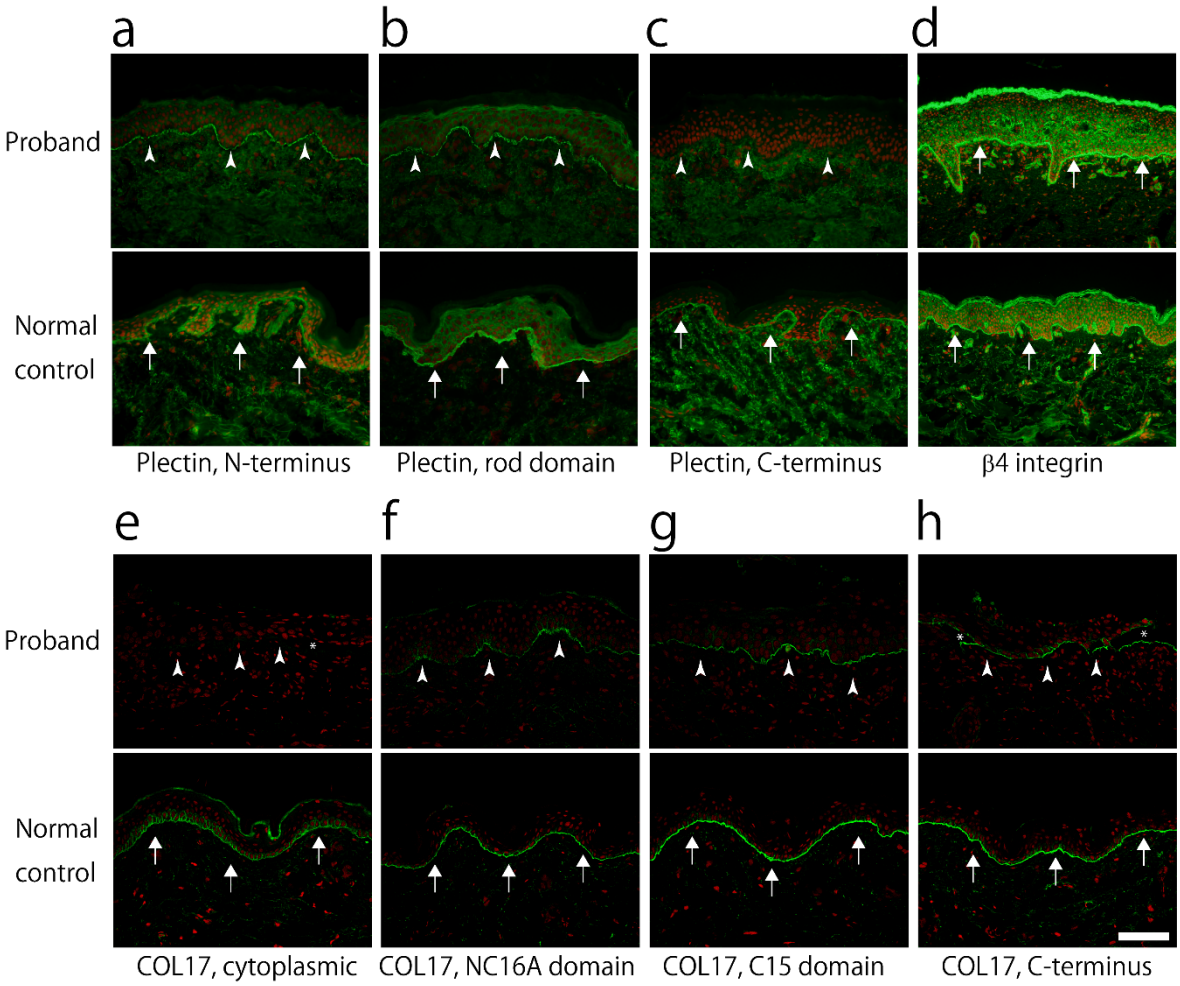
e


*
COL17 band intensity
(relative to bait protein)
1.0
0.8
0.6
0.4
0.2
0.0
GST
PLEC618-819
PLEC618-819ΔPhe755



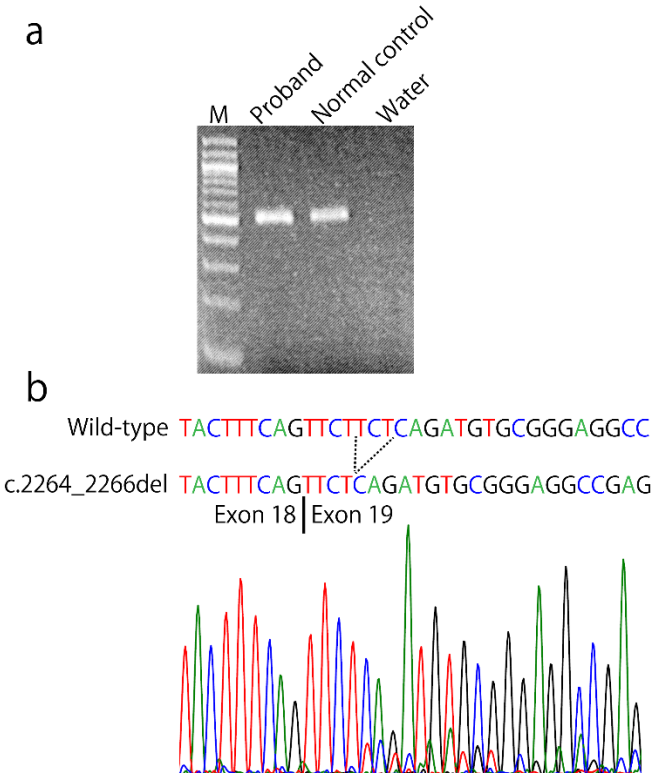
Supplementary Figure 1. Clinical manifestations and electron microscopy

(a) An erosion of 5 cm in diameter is visible on the proband's back in the first week of life. (b) A vesicle on the right foot (arrow). (c-e) Electron micrographs of skin split within basal keratinocytes. Higher-magnification images of the epidermal side (d) and the dermal side (e). Hypoplastic hemidesmosomes can be observed at the base of the blister (arrowheads) (e). Bar = 2 µm.



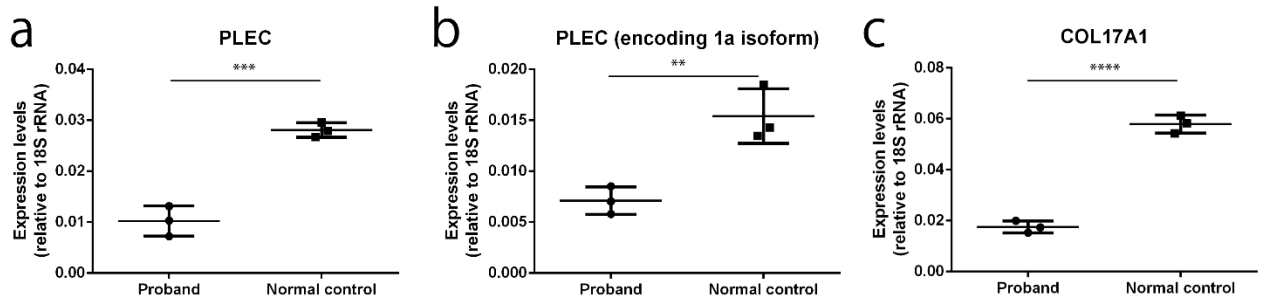
Supplementary Figure 2. Immunofluorescence studies of skin specimens

The upper images are from skin specimens of the proband, and the lower ones are from those of a normal control. All of the antibodies against plectin (a-c), β4 integrin (d) and type XVII collagen (COL17) (e-h) are bound to the dermal-epidermal junction. Labeling of plectin and COL17 is diminished in the proband (arrowheads) compared with the normal control (arrows), while β4 integrin staining is comparable (arrows). PI was used for nuclear staining. Bar = 50 μm.



Supplementary Figure 3. Direct sequencing of exon-exon boundaries in the proband's cDNA

(a) Agarose gel electrophoresis of PCR products around the boundary of *PLEC* exon 18 and exon 19 from cDNA of the proband and a normal control. cDNA was obtained from the cultured epidermal keratinocytes. The forward primer was in exon 17, and the reverse one was in exon 21. A single band of 512 base pairs (bp) is seen in both samples. M: 100-bp marker. (b) Direct sequencing of the PCR products from the proband's cDNA shows both wild-type and c.2264_2266del alleles as seen in the analysis of genomic DNA. No other splicing variant is observed.



Supplementary Figure 4. Quantitative RT-PCR (qRT-PCR) on cultured keratinocytes

Gene expression of *PLEC* (a), *PLEC* transcript variant 11, encoding plectin 1a (b) and *COL17A1* (c) is significantly reduced in the proband's keratinocytes compared with those of the normal control.

Materials and Methods

Electron microscopy

Ultrastructural observation was performed as described previously (Natsuga, et al., 2011). Briefly, skin biopsy samples were fixed in 5% glutaraldehyde solution, followed by post-fixation in 1% OsO4. After dehydration, samples were embedded in Epon 812 (TAAB, UK) and sectioned for electron microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined via JEM1400 transmission electron microscopy (Tokyo, Japan).

Antibodies

The following antibodies were used: monoclonal antibody (mAb) PN643 against the N-terminus of plectin; mAb HD1-121 against the rod domain of plectin; mAb PC815 against the C-terminus of plectin; mAb 3E1 (Chemicon International, Temecula, CA) and polyclonal antibody (polyAb) H101 (Santa Cruz Biotechnology, Santa Cruz, CA) against β4 integrin; mAb 1A8c against the cytoplasmic portion of COL17; polyAb 11-NC16A-R1 against the NC16A domain of COL17; mAb D20 against the C15 domain of COL17; mAb 233 and polyAb 09040 against the C-terminus of COL17; mAb GoH3 (Chemicon International,

CA) against α6 integrin; mAb GB3 (Sera-lab, Cambridge, UK) against laminin 332; mAb LH7.2 (Sigma, St. Louis, MO) against type VII collagen; mAb PHM-12+CIV22 against type IV collagen (NeoMarkers, Fremont, CA); polyAb S1193 against BP230; mAb AC15 against β-actin (Sigma, St. Louis, MO); polyAb ab6046 (abcam, Cambridge, UK) against tubulin; anti-V5 antibody (Invitrogen); and mAb M2 against FLAG (Sigma). The details of mAbs PN643, HD1-121, PC815, 1A8c, D20 and 233 were described in previous reports (Gache, et al., 1996; Hirako, et al., 1998; Natsuga, et al., 2010). 11-NC16A-R1 and 09040 were prepared as described previously (Natsuga, et al., 2012; Ujiie, et al., 2014). S1193 was donated by Prof. J. R. Stanley of the University of Pennsylvania.

Cell culture and immunoblot analysis

Cultured epidermal keratinocytes were obtained from skin biopsy samples and maintained with Cnt-Prime (CELLnTEC, Bern, Switzerland). Cultured keratinocytes were lysed in NP-40-containing buffer (1% NP-40, 25 mM Tris-HCl, 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail (Sigma)) on ice for 30 min. Cell debris was removed by

centrifugation, and supernatant was collected and mixed with Laemmli's sample buffer. After boiling, samples were separated by gel electrophoresis on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and transferred to a PVDF membrane. The membrane was incubated with primary antibodies (HD1-121, 09040, H101 and ab6046) overnight at 4°C followed by incubation with secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 hr at room temperature. The blots were detected using Clarity Western ECL Substrate (Bio-rad). The band intensity was calculated using ImageJ.

Immunofluorescence

For staining of skin samples, skin biopsy specimens were embedded in optimal cutting temperature (OCT) compound and quickly frozen with dry ice. Five-micron cryostat sections were incubated with primary antibodies (1A8c, 11-NC16AR1, D20, 233, PN643, HD121, PC815 and H101) overnight at 4°C. After washing in PBS, sections were incubated with secondary antibodies conjugated with fluorescein-isothiocyanate (FITC) or Alexa488 for 1 hr at room temperature. All stained samples were imaged with a confocal laser scanning microscope (Olympus Fluoview FV1000).

Sequence variant analysis

Genomic DNA extracted from peripheral blood was used as a template for polymerase chain reaction (PCR) amplification. All coding exons including exon-intron boundaries of *PLEC* (Nakamura, et al., 2005), *COL17A1* (Gatalica, et al., 1997), *ITGB4* (Takizawa, et al., 1997), *KRT5/KRT14* (Hut *et al.*, 2000; Stephens et al., 1997), *DST* (Groves, et al., 2010), and *EXPH5* (Pigors, et al., 2014) were amplified according to previously described methods. DNA sequencing of the PCR products was performed with an ABI 3100 sequencer (PerkinElmer Life Sciences–ABI, Foster City, CA). The *PLEC* sequence variant nomenclature follows the Human Genome Variation Society (HGVS) guidelines (http://varnomen.hgvs.org/) (den Dunnen, et al., 2016) according to the reference sequence NM_000445.4 encoding plectin isoform 1c with +1 as the A of the ATG initiation codon. Cytogenic DNA array analysis on the proband's genomic DNA was performed using the CytoScan HD Array (Affymetrix) according to the manufacturer's instructions. PCR on *PLEC* cDNA spanning the boundary of exon 18 and exon 19 was performed using the following primers; GAGCTCCAAAATGCTGGGGA (forward, on exon 17),

CTGCTGAGCACCTTCCAGTG (reverse, on exon 21).

Quantitative RT-PCR (qRT-PCR)

RNA was extracted from cultured cells with an RNeasy kit (Qiagen), and cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System (Life Technologies). qRT-PCR was carried out using specific primers and SYBR Select Master Mix (Life Technologies) and was performed on the ABI 7000 sequence detection system. All samples were compared with human 18S rRNA (NR_003286.4) as a housekeeping control. The following primers were used: *PLEC*: CACTGGAGATCCAGCGACAG (forward, on exon 31) and CACCGTCTGCATCTCCTCAG (reverse, on exon 32); *PLEC* transcript variant 11 (NM_201384.2, encoding the plectin 1a isoform): AGAACCAGCTCGGAGGACAA (forward, on exon 1a) and GGTCACTGATGTGCCTCTGG (reverse, on exon 3); *COL17A1* (NM_000494.3): TCAACCAGAGGACGGAGTCA (forward) and TCGACTCCCCTTGAGCAAAC (reverse); 18S rRNA: GGCGCCCCCTCGATGCTCTTAG (forward) and GCTCGGGCCTGCTTTGAACACTCT (reverse).

Plasmid construction

cDNAs containing the *PLEC* coding region encoding Met1 to Val819 or Met1 to Tyr617 (PLEC1-819 and PLEC1-617, respectively) were subcloned into the pcDNA3.1V5-His vector (Invitrogen). In addition, cDNA encoding Arg618 to Val819 was subcloned into pGEX6P-1 (GE Healthcare) (PLEC618-819). Constructs with *PLEC* cDNA harboring p.Phe755del (PLEC1-819ΔPhe755 or PLEC618-819ΔPhe755) were generated using the GeneTailor Site-Directed Mutagenesis System (Invitrogen) on PLEC1-819 or PLEC618-819.

Immunoprecipitation-immunoblot assay

HEK293 cells stably expressing COL17-FLAG were prepared as described previously (Li, et al., 2010). Plasmids were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). Cell lysates were obtained using NP-40-containing buffer 48 hours after transfection. For immunoprecipitation, protein-G Sepharose (GE Healthcare) was coupled with anti-V5 antibody, followed by incubation with cell lysates at 4°C overnight. After washing of the beads with PBS containing 0.1% NP-40, bound proteins were eluted by boiling

for 5 min with Laemmli's sample buffer. Immunoblotting using anti-V5 antibody or anti-FLAG antibody was performed on the immunoprecipitated samples or the cell lysates mixed with sample buffer as described above. mAb AC15 against β-actin was used to confirm equal loading. The band intensity was calculated using ImageJ.

Protein-protein binding assay

The recombinant full-length COL17 with a FLAG-tag on the N-terminus (FLAG-COL17) was prepared and purified from HEK293 cells stably expressing the protein (K Izumi et al., manuscript in preparation). The original cDNA was a kind gift from Professor Kim B. Yancey. PLEC constructs (PLEC618-819 or PLEC618-819ΔPhe755 or pGEX6P-1 only) were transformed into BL21 cells (GE Healthcare). Transformed cells were grown in LB broth at 25°C with the Overnight Express Autoinduction system (Novagen). The pellets were lysed with B-PER II (Pierce) containing lysozyme (Sigma-Aldrich). After centrifugation, supernatants were added to glutathione Sepharose 4B (GE Healthcare) and washed with binding buffer (Tris 50 mM, NaCl 150 mM, Triton-X 1%). The Sepharose was incubated with the recombinant full-length COL17 in the binding

buffer at 4°C overnight. After washing, the Sepharose was boiled with Laemmli's sample buffer and centrifuged. The supernatants were separated by gel electrophoresis as described above, and the gels were stained with CBB or used for subsequent immunoblotting using anti-FLAG antibody. The band intensity was calculated using ImageJ (https://imagej.nih.gov/ij/) (Schneider, et al., 2012).

Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software, CA). P values were determined with Student's t-test. P-values are indicated as *0.01<p<0.05, **0.001<p<0.01.

Ethics

The institutional review board of Hokkaido University Graduate School of Medicine approved all procedures. The study was conducted according to the Declaration of Helsinki Principles. Participants or their legal guardians gave written informed consent.

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