Desmoplakin and periplakin genetically and functionally contribute to eosinophilic esophagitis

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Eosinophilic esophagitis (EoE) is a chronic allergic inflammatory disease with a complex underlying genetic etiology. Herein, we conduct whole-exome sequencing of a multi-generation EoE pedigree (discovery set) and 61 additional multiplex families with EoE (replication set). A series of rare, heterozygous, missense variants are identified in the genes encoding the desmosome-associated proteins DSP and PPL in 21% of the multiplex families. Esophageal biopsies from patients with these variants retain dilated intercellular spaces and decrease DSP and PPL expression even during disease remission. These variants affect barrier integrity, cell motility and RhoGTPase activity in esophageal epithelial cells and have increased susceptibility to calpain-14-mediated degradation. An acquired loss of esophageal DSP and PPL is present in non-familial EoE. Taken together, herein, we uncover a pathogenic role for desmosomal dysfunction in EoE, providing a deeper mechanistic understanding of tissue-specific allergic responses.

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Eosinophilic esophagitis (EoE) is an emerging chronic allergic inflammatory esophageal disease clinically characterized by esophageal dysfunction; histologically by esophageal eosinophilia, epithelial hyperplasia, and dilated intercellular spaces (DIS) associated with impaired barrier function; and a high degree of heritability. We have previously described high proband concordance in monozygotic twins (58%), substantiating a genetic etiology. Most genetic studies have focused on analyzing common genetic variants by genome-wide association studies (GWAS), with evidence implicating the epithelial gene products calpain 14 (CAPN14) and thymic stromal lymphopoietin (TSLP), respectively. Mechanistic studies have substantiated that CAPN14 contributes to impaired epithelial barrier function, mediated in part by loss of expression of desmoglein 1 (DSG1), and that TSLP promotes adaptive type 2 T cell immunity associated with IL-5 and IL-13 overproduction. Despite these advances, the causal gene variants and/or genomic networks for EoE pathogenesis remain unclear.

Common genetic variants in identified pathways only partially explain heritability of EoE. Odds ratios (OR) for previously associated single-nucleotide polymorphisms (SNPs) range from 0.76–2.57, with the less frequent variants having larger effect sizes. These results suggest that rare variants are more likely to be disease-causing with moderate-to-large effect sizes. A challenge for GWAS, especially for rare conditions like EoE, is that rare variants are excluded due to power concerns. In this study, we identify rare genetic variants conferring substantial risk for EoE by using a multiplex family-based study design and whole-exome sequencing (WES). We show the functionality of sequence variants by bioinformatics and experimental support at gene and variant levels following the guidelines of the National Human Genome Research Institute.

**Results**

**Candidate variants in EoE.** The schematic workflow for the WES analysis is depicted in Fig. 1a. We initially conducted WES on the DNA from an extended pedigree (discovery set), which included five subjects who had histologically confirmed EoE and were across multiple generations (Fig. 1b and Supplementary Fig. 1). WES identified two coincident, heterozygous, missense variants in desmoplakin (DSP) and periplakin (PPL) [DSP: p.G46D (c.137 G → A), PPL: p.V1377E (c.4130 T → A)] (Fig. 1a–b) only in affected family members. Both DSP and PPL are members of the plakin protein family that localize to desmosomes and are highly expressed in the esophagus (Supplementary Table 1 and Supplementary Fig. 2). DSP and PPL are critical components of desmosomal structures in epithelial cells, which are responsible for maintaining the structural integrity of cell contacts and cell migration.

Notably, DSP and PPL were expressed primarily in the squamous epithelium with strong relative expression in the esophageal mucosa (Supplementary Fig. 2). Subsequent Sanger sequencing of all 32 family members confirmed co-segregation of the two variants with each of the five affected members (Supplementary Fig. 1). Two additional members (#12 and #28) had both variants; #12 had a diagnosis of esophageal stricture, and #28 had reflux symptoms but had never undergone an esophagogastroduodenoscopy (EGD) (Fig. 1b). Potentially, both might have EoE. Additional family members had one variant, DSP or PPL. Co-segregation was found only in the variants in DSP and PPL, but not other candidate genes. The strong co-segregation of these variants supports a joint role of these variants in EoE etiology.

**Frequency of DSP and PPL mutations in EoE.** We sequenced additional multiplex families (replication set), which included 61 index patients of European ancestry. In addition to the identified discovery variants (1 DSP, 1 PPL), we observed 6 DSP variants in six unique multiplex families (one variant per family) and 5 PPL variants in six other multiplex families (one variant shared by two families), which we confirmed by Sanger sequencing of all family members (genotype–phenotype concordance 78.3%, P = 0.005) (Fig. 1c). From the discovery and replication set, we identified a total of seven variants in DSP and six variants in PPL. Only the discovery family had identified coincident DSP and PPL variants. These 13 variants of DSP and PPL are exceedingly rare based on the Exome Aggregation Consortium (ExAC), predicted to have a deleterious effect on protein function and evolutionarily conserved (Supplementary Tables 2–5). For DSP variants, the mutant amino acids are within the plakoglobin binding site, plakin domain and homodimerization site (Fig. 1d). For PPL variants, the mutant amino acids are within the plakin domain and the rod domain that forms complexes with envoplakin (Fig. 1d).

To evaluate whether these rare variants in DSP and PPL were enriched in EoE compared to controls, we examined rare genetic burden tests by case-control association studies with the use of EXAC. At the variant level, three of the 13 variants were not in the ExAC database, and eight reached statistical significance (Table 6). At the gene level, the frequency of DSP or PPL variants in the index patients from the 62 EoE multiplex families were higher than that of a control cohort of European ancestry from the ExAC database (P = 0.0021, Fisher’s exact test) (Table 2). This result was replicated by an independent control cohort from the UK Biobank (P = 0.023, Fisher’s exact test) (Table 2).

Although we found enrichment of rare variants in these genes, we did not see the joint segregation of those two variants in the replication set. Given the fact that DSP and PPL were seen in the control cohorts, we speculate that isolated DSP and PPL variants are not sufficient for the disease but cooperate with secondary hits in the desmosomal genes. In the case of the discovery family, the second hit is the co-occurrence of damaging variants in both DSP and PPL, whereas, in the replication families, the co-occurrence of the DSP or PPL variants with additional rare variants in other genes in the desmosome were enriched (P = 0.0052, Fisher’s exact test) (Table 2 and S7), providing evidence that rare genetic variants in the desmosomal genes associate with EoE.

**Esophageal DSP and PPL expression and associated features in EoE.** To obtain further in vivo evidence of the relevance of EoE-associated mutations to disease manifestations, esophageal biopsy specimens obtained from familial EoE (62 multiplex families), non-familial EoE (511 non-multiplex families), and controls (93 individuals) (Table S8) were analyzed using approaches including quantifying peak esophageal eosinophil counts, EoE histology scoring system (HSS), EoE diagnostic panel (EDP), single-cell RNA sequencing and immunofluorescence staining. Esophageal DSP and PPL expression were markedly lower in patients with active EoE than inactive EoE and controls (Fig. 2a), as was the expression of genes encoding other desmosomal proteins, such as DSG1 (Supplementary Fig. 5a). Among patients with active EoE having similar esophageal eosinophil counts, EoE histology scoring system, EoE diagnostic panel, single-cell RNA sequencing and immunofluorescence staining, esophageal DSP and PPL expression were markedly lower in patients with active EoE than inactive EoE and controls (Fig. 2a).
impaired barrier function, which is an upstream event in the induction of TSLP\(^1\). TSLP is overexpressed in EoE and genetically and functionally linked with EoE\(^5,9\). In this regard, it is also notable that patients with familial EoE harboring DSP or PPL variants expressed higher TSLP mRNA levels than did patients with non-familial EoE (2.1 fold, \(P = 0.001\)) (Fig. 2b); the difference in TSLP expression was independent of peak eosinophil levels in the esophagus. These data support a possible relationship between desmosome variants and the type 2 skewing and allergic responses observed in EoE.

With regard to EoE histologic features, DSP and PPL expression inversely correlated with structural features and eosinophilic features (Fig. 2c, Supplementary Fig. 6a for non-familial EoE). Of note, non-familial EoE showed histologic changes that varied with the severity of disease activity, whereas familial EoE demonstrated retained structural features, mainly driven by DIS, regardless of disease activity (Table S9). This differential histology between non-familial and familial EoE was independent of esophageal eosinophil levels—peak esophageal eosinophil counts were not significantly different between...
Table 1: Families with EoE and pathogenic variants in DSP and/or PPL.

| Patient | Gender | Variant | Gene(s) | Protein | cDNA | Age at onset (years) | Atopic status | Peak Eos/hpf | Endoscopic phenotype | Symptoms |
|---------|--------|---------|---------|---------|------|----------------------|--------------|-------------|----------------------|----------|
| 1       | Male   | DSP     | p.G46D  | c.G137A | 4.3  | BA, AR, AnFA         | 177          | Inflammatory | Dysphagia, GERD, N/V, Pain |
| 2       | Female | PPL     | p.V1377E| c.T4130A| 2.5  | BA                   | 77           | Normal-appearance | Dysphagia, GERD, N/V, Pain |
| 3       | Male   | DSP     | p.R808C | c.C2422T| 5.7  | BA, AR               | 145          | Inflammatory | Dysphagia, GERD, N/V, Pain |
| 4       | Female | DSP     | p.Y895C | c.A2684G| 4.1  | BA, AR, AnFA         | 100          | Normal-appearance | Dysphagia, GERD, N/V, Pain |
| 5       | Male   | PPL     | p.R108C | c.C322T | 4.2  | BA                   | 147          | Inflammatory | Dysphagia, GERD, N/V, Pain |
| 6       | Female | PPL     | p.R108C | c.C322T | 4.2  | BA                   | 147          | Inflammatory | Dysphagia, GERD, N/V, Pain |
| 7       | Female | PPL     | p.E1163K| c.G3487A| 3.8  | BA, AR, AnFA         | 33           | Inflammatory | Dysphagia, GERD, N/V, Pain |
| 8       | Male   | PPL     | p.E632K | c.G1894A| 14.3 | BA                   | 50           | Inflammatory | Dysphagia, GERD, N/V, Pain |
| 9       | Female | PPL     | p.L1154V| c.C3460G| 3.5  | BA, AR, AnFA         | 52           | Inflammatory | Dysphagia, GERD, N/V, Pain |
| 10      | Female | PPL     | p.L1154V| c.C3460G| 3.5  | BA, AR, AnFA         | 52           | Inflammatory | Dysphagia, GERD, N/V, Pain |
| 11      | Female | PPL     | p.E1163K| c.G3487A| 3.8  | BA, AR, AnFA         | 33           | Inflammatory | Dysphagia, GERD, N/V, Pain |

a Discovery set DSP desmoplakin, PPL periplakin, BA bronchial asthma, AR allergic rhinitis, AnFA anaphylaxis by food, GERD gastroesophageal reflux, N/V nausea and/or vomiting, eos/hpf eosinophils per high-power microscopic field.
uncover the role of and DSP co-expression of sequencing analysis of human esophageal biopsies also revealed and/or DSP homeostasis, we generated complementary Fig. 8b.

Moreover, knocking out DSP and/or PPL in the suprabasal and differentiated epithelial cells (Supplementary Fig. 8d). Single-cell RNA sequencing analysis of human esophageal biopsies also revealed co-expression of DSP and PPL at the single epithelial cell level (Supplementary Fig. 7) in some of the epithelial cells.

Table 2 DSP or PPL rare variant burden analysis in eosinophilic esophagitis (EoE).

| Exome Aggregation Consortium (ExAC) | Multiplex EoE cases (n = 62) | Controls (n = 33,370) | P-value (Fisher’s exact test) |
|------------------------------------|-----------------------------|----------------------|-----------------------------|
| DSP or PPL                         | Minor allele | Major allele | Minor allele | Major allele |                          |
| DSP or PPL                         | 14           | 234         | 3079        | 13,040      | 0.0021                   |

**UK Biobank**

| Multiplex EoE cases (n = 62) | Controls (n = 4,822) | P-value (Fisher’s exact test) |
|-------------------------------|----------------------|-----------------------------|
| DSP or PPL                    | Subjects with variants | Subjects without variants | Subjects with variants | Subjects without variants |                          |
| DSP or PPL                    | 13                    | 49                     | 532                   | 4,290                    | 0.023                    |
| DSP or PPL with other rare variants in desmosome (GO:0030057) | 8                     | 54                     | 206                   | 4,616                    | 0.0052                   |

EoE eosinophilic esophagitis, ExAC Exome Aggregation Consortium, UK United Kingdom, GO gene ontology. Two-tailed P-values were determined by the Fisher’s exact test.

Individuals of European ancestry from ExAC Database (https://gnomad.broadinstitute.org/). Observed allele counts were based on two alleles of each gene.

Non-EoE, individuals of European ancestry from the UK Biobank (https://www.ukbiobank.ac.uk/).

non-familial and familial inactive EoE nor between non-familial and familial active EoE (Supplementary Fig. 6b–c). These findings suggest that decreased DSP and/or PPL expression, via the presence of DSP and/or PPL variants in familial EoE, impairs the epithelial barrier and causes fixed histologic features independent of esophageal eosinophilia. Immunofluorescence staining revealed that DSP is highly expressed in the basal and differentiating epithelial cells, whereas PPL is highly expressed in the suprabasal and differentiated epithelial cells (Supplementary Fig. 6d–g). Notably, DSP and PPL protein expression were reduced in esophageal biopsies obtained from patients with DSP and/or PPL variants, respectively, regardless of disease activity (Fig. 2d, e and Supplementary Fig. 6d–g). Single-cell RNA sequencing analysis of human esophageal biopsies also revealed co-expression of DSP and PPL at the single epithelial cell level (Supplementary Fig. 7) in some of the epithelial cells.

DSP and PPL deficiency in esophageal epithelial cells. To uncover the role of DSP and PPL in esophageal epithelial homeostasis, we generated DSP- and PPL-deficient human esophageal cell lines (EPC2 cells) via clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated genome editing. We examined the effect of DSP and PPL deletion on the barrier integrity following air–liquid interface (ALI) culture (Supplementary Fig. 8a). DSP and PPL knockout (KO) cells demonstrated acantholysis and significantly decreased barrier function, the latter determined by reduced transepithelial electrical resistance (TEER) and increased permeability to fluorescein isothiocyanate (FITC)-dextran compared to control cells (Supplementary Fig. 8b–d). Moreover, DSP KO cells and PPL KO cells showed increased migratory ability following a physical cellular barrier injury (Supplementary Fig. 8e). Taken together, DSP or PPL loss in esophageal epithelial cells modifies cell motility and barrier integrity.

Consequences of EoE-associated DSP and PPL variants. To further investigate the consequences of the EoE-associated missense variants in DSP and PPL, we transduced EPC2 cells with constructs encoding non-variant and mutated variant DSP and PPL. Immunoblotting of the cell extracts showed that expression of all mutant DSP and PPL constructs were equivalent to that of the wild-type construct (Supplementary Fig. 9). We examined the effect of DSP and PPL variants on the barrier integrity following epithelial culture at the ALI. Impaired epithelial barrier integrity and reduced barrier function were observed in cells transduced with the mutated variants compared with the wild-type DSP and PPL (Fig. 3a–c).

We assessed epithelial cell reconstitution using a wound healing assay and found that cells transduced with the mutated variants closed the wound faster than did control cells transduced with wild-type DSP and PPL (Fig. 3d and Supplementary Fig. 10). Notably, similar to mutated variants in this study, partial gene silencing and some of the known DSP mutations [p.R451G from arrhythmogenic cardiomyopathy and p.H586P from severe dermatitis, multiple allergies, and metabolic wasting (SAM) syndrome] also showed increased cell motility (Supplementary Fig. 10b and d). In non-esophageal keratinocytes (HaCaT cells), there were similar mutational effects, although variability was observed (Supplementary Fig. 10d, e). Regarding a potential mechanism, the observed effects on cell motility in the wound healing assay were suggestive of aberrant RhoGTPase signaling, especially because it was reported that DSP knockout cells have lower RhoA activity. To investigate whether desmosomal variants affect RhoGTPase signaling, we measured levels of GTP-bound RhoA (active RhoA). Of note, RhoA activity was reduced in most of the cells transfected with variant DSP or PPL compared to cells transfected with wild-type versions of these genes (Fig. 4a). Additionally, restoration of Rho activity (using the Rho activator calpeptin) rescued the enhanced migration. Furthermore, blocking Rho signaling (via the Rho kinase inhibitor Y27632) further increased migration (Fig. 4b). Taken together, the RhoA pathway is likely associated with the DSP and PPL mutational effects. Indeed, we also demonstrated decreased active RhoA in biopsies from active EoE compared to that of inactive EoE and normal controls (Fig. 4c). Taken together, these data suggest that loss of function in epithelial homeostasis (by functional haploinsufficiency or dominant-negative effects of overexpressed mutant protein) is likely a relevant pathogenic mechanism.

Calpain 14–mediated desmosomal protein degradation. The identified DSP and PPL variants associated with EoE are distinct from the pathogenic variants associated with other phenotypes such as arrhythmias. We aimed to understand the reason for the non-redundant phenotypes, i.e., esophageal but not other tissue involvement, in the EoE multiplex families having DSP and PPL variants. Of note, although we primarily observed missense
mutations associated with EoE, more severe truncating mutations of DSP and PPL are associated with skin and heart diseases\textsuperscript{25-27}. Thus, we hypothesized that the identified genetic variants might interact with tissue-specific pathways through a post-translational mechanism. We have previously identified CAPN14 as an esophageal-specific functional protease that induces esophageal epithelial barrier impairment and loss of DSG1 expression, likely by a degradative mechanism\textsuperscript{6,6}. In this context, we hypothesized that cells carrying the DSP or PPL genetic variants might be more susceptible to CAPN14 proteolysis than cells carrying wild-type DSP or PPL. In order to test this hypothesis, we co-transfected expression vectors encoding CAPN14 with DSP (wild-type and mutant) or with PPL (wild-type and mutant). Cellular lysates were subsequently incubated with calcium to activate endogenous CAPN14, but the degradation was less with the enzymatically active CAPN14-C101A, supporting that CAPN14 proteolysis contributes to this degradation (Fig. 5a). Of note, DSP and PPL mutants showed increased degradation compared to wild type (Fig. 5b). Considering the preferential expression of CAPN14 in

![Fig. 2 Expression of DSP and PPL in patients with EoE.](Image)

(a) DSP (left) and PPL (middle) mRNA expression in esophageal biopsies from controls and patients with non-familial EoE (inactive and active). Each point represents an individual subject ([Control, n = 48; Inactive EoE, n = 51; Active EoE, n = 147]; red data points represent patients with DSP or PPL variants (not included in the statistics)). Statistics: DSP (Control vs. Inactive EoE, P = 0.9999; Control vs. Active EoE, P = 0.0007; Inactive EoE vs. Active EoE, P = 0.0009); PPL (Control vs. Inactive EoE, P = 0.9999; Control vs. Active EoE, P = 0.0001; Inactive EoE vs. Active EoE, P < 0.0001). Correlation plot of DSP and PPL mRNA expression is also shown (right) (n = 246). Statistics: P < 0.0001. **Peak esophageal eosinophilic counts (left) and TSLP mRNA expression (right) are plotted by groups for non-familial and familial esophageal EoE with DSP or PPL variants (non-familial EoE, n = 115; familial EoE with variants, n = 15). All samples were from the biopsies during the active disease state. The dashed line indicates the diagnostic threshold of EoE (15 eosinophils/hpf). Statistics: peak esophageal eosinophil count, P = 0.0927; TSLP, P = 0.0010. ***Correlation plots of gene expressions (left: DSP, right: PPL) and histologic scores (red: eosinophilic features, blue: structural features) (n = 68). Statistics: left (DSP with eosinophilic features, P = 0.0022; DSP with structural features, P = 0.0001), right (PPL with eosinophilic features, P = 0.0014; PPL with structural features, P < 0.0001). (d) Representative western blot analysis of DSP and PPL among control individuals (n = 4), patients with non-familial EoE (n = 4), and patients with familial EoE with DSP or PPL variants (n = 6). GAPDH serves as a loading control. For patients with EoE, all samples were from the biopsies during the inactive disease state. Statistics: DSP (control vs. non-familial EoE, P = 0.7833; control vs. familial EoE, P = 0.0022; non-familial EoE vs. familial EoE, P = 0.0007); PPL (control vs. non-familial EoE, P = 0.9061; control vs. familial EoE, P = 0.0142). For panels a, b, d, and e, data are presented as mean ± SEM. For panels a-e, two-tailed P-values were determined by the following tests: a (left and middle), Kruskal-Wallis test followed by a Dunn multiple-comparison test; b, the unpaired t-test; c, Spearman’s rank correlation coefficient (multiple comparisons were not applied); and d, e, one-way ANOVA test followed by a Tukey’s multiple comparisons test. *P < 0.05, **P < 0.01 and ***P < 0.001. EoE eosinophilic esophagitis, DSP desmoplakin, PPL periplakin, GAPDH Glyceraldehyde 3-phosphate dehydrogenase, hpf high-power microscopic field, IQR interquartile range, DAPI 4',6-diamidino-2-phenylindole, MW molecular weight, NS not significant, SEM standard error of the mean.

![Fig. 5](Image)

(b) Peak tissue eosinophil count and histologic scores (red: eosinophilic features, blue: structural features) (n = 68). Statistics: peak esophageal eosinophil count, P = 0.0927; TSLP, P = 0.0010. ***Correlation plots of gene expressions (left: DSP, right: PPL) and histologic scores (red: eosinophilic features, blue: structural features) (n = 68). Statistics: left (DSP with eosinophilic features, P = 0.0022; DSP with structural features, P = 0.0001), right (PPL with eosinophilic features, P = 0.0014; PPL with structural features, P < 0.0001). (d) Representative western blot analysis of DSP and PPL among control individuals (n = 4), patients with non-familial EoE (n = 4), and patients with familial EoE with DSP or PPL variants (n = 6). GAPDH serves as a loading control. For patients with EoE, all samples were from the biopsies during the inactive disease state. Statistics: DSP (control vs. non-familial EoE, P = 0.7833; control vs. familial EoE, P = 0.0022; non-familial EoE vs. familial EoE, P = 0.0007); PPL (control vs. non-familial EoE, P = 0.9061; control vs. familial EoE, P = 0.0142). For panels a, b, d, and e, data are presented as mean ± SEM. For panels a-e, two-tailed P-values were determined by the following tests: a (left and middle), Kruskal-Wallis test followed by a Dunn multiple-comparison test; b, the unpaired t-test; c, Spearman’s rank correlation coefficient (multiple comparisons were not applied); and d, e, one-way ANOVA test followed by a Tukey’s multiple comparisons test. *P < 0.05, **P < 0.01 and ***P < 0.001. EoE eosinophilic esophagitis, DSP desmoplakin, PPL periplakin, GAPDH Glyceraldehyde 3-phosphate dehydrogenase, hpf high-power microscopic field, IQR interquartile range, DAPI 4',6-diamidino-2-phenylindole, MW molecular weight, NS not significant, SEM standard error of the mean.
Discussion

Our study advances the field by identifying a pathogenic role for desmosomal dysfunction in EoE and the likely intersection of this dysfunction with calpain-14 and RhoGTPase-mediated pathways. More specifically, we identified DSP and PPL variants as likely being functionally involved in disease pathogenesis, as substantiated by multiple lines of evidence. First, DSP and PPL variants, encoding desmosomal proteins, strongly segregated in a multigenerational EoE pedigree (discovery) and were enriched in 21% of EoE multiplex families. Second, a series of functional analyses using an organotypic-like ALI culture system demonstrated that modulating wild-type DSP and PPL expression in vitro was functionally sufficient to induce changes in epithelial integrity (e.g., acantholysis) and barrier impairment, processes that are dysregulated in EoE30,31. Third, we determined that the identified DSP and PPL variants encode for proteins that have enhanced susceptibility to degradation by calpain-14, an esophageal specific protease that is involved in disease pathogenesis (based on GWAS, epigenetic and functional studies)6,8,32, thereby providing a pathogenic mechanism for the previously unexplained tissue-specific nature of this condition. Fourth,

esophageal mucosa (Fig. 5c) and its strong association with EoE28,30, these results provide a potential mechanistic framework to understand the functional significance of the DSP and PPL mutants, as well as a potential mechanism that may link the observed esophageus-specific phenotype (compared to skin or heart disease phenotypes). Notably, CAPN14 mRNA expression in esophageal biopsies showed a trend of being lower in patients with familial EoE than non-familial EoE (P = 0.23) (Supplementary Fig. 11a). However, there was no difference in the allele frequency of a CAPN14-tagging SNP between families with or without DSP and/or PPL rare variants (Supplementary Fig. 11b). Finally, we hypothesized that inhibiting calpain activity would ameliorate the increased CAPN14-mediated degradation. We focused our attention on a pharmacologic drug with broad calpain inhibitory activity, using SNJ-1945, an agent designed to have high water solubility and bioavailability29. Indeed, SNJ-1945 rescued cleavage by CAPN14 at least in part (Fig. 5d). Taken together, these findings provide a deeper molecular understanding of EoE pathogenesis and substantiate the potential therapeutic value of calpain inhibitors in modifying the identified genetic pathway.

Fig. 3 Effects of EoE-associated DSP and PPL variants. a Representative hematoxylin and eosin (H&E)-stained sections of EPC2 cells stably transduced with constructs encoding non-variant and mutated DSP or PPL after air–liquid interface (ALI) differentiation (day 14). Arrows point to the non-cellular areas that were formed. Scale bar: 50 μM. Data are representative of three experiments performed in duplicate. b The transepithelial electrical resistance (TEER) and c FITC-dextran flux measurements are shown for EPC2 cells grown at the ALI. d Wound healing assays performed in EPC2 cells transduced with constructs encoding non-variant and mutated DSP or PPL. Quantification of the wound closure after 8 h was shown. For panels a–d data are representative of three experiments performed in duplicate and are presented as mean ± SEM. Statistics (versus non-variants): b (p.G46D, P = 0.0324; p.R808C, P = 0.0059; p.Y895C, P = 0.0042; p.1067_1068del, P = 0.0006; p.N1215S, P = 0.0005; p.R1340C, P = 0.0003; p.E1723Q, P = 0.0012; p.R108C, P = 0.0041; p.E632K, P = 0.0131; p.K1051V, P = 0.0189; p.L1154V, P = 0.0004; p.E1163K, P = 0.0024; p.V1377E, P = 0.0291), c (p.G46D, P = 0.1542; p.R808C, P = 0.0274; p.Y895C, P = 0.0151; p.1067_1068del, P = 0.0062; p.N1215S, P = 0.1189; p.R1340C, P = 0.0244; p.E1723Q, P = 0.0385; p.R108C, P = 0.0187; p.E632K, P = 0.066; p.K1051V, P = 0.0536; p.L1154V, P = 0.0022; p.E1163K, P = 0.0304; p.V1377E, P = 0.1601) and d (p.G46D, P = 0.9917; p.R808C, P = 0.0103; p.Y895C, P = 0.005; p.1067_1068del, P = 0.0031; p.N1215S, P = 0.0944; p.R1340C, P = 0.0187; p.E1723Q, P = 0.4656; p.R108C, P = 0.0249; p.E632K, P = 0.0748; p.K1051V, P = 0.9471; p.L1154V, P = 0.0016; p.E1163K, P = 0.0194; p.V1377E, P = 0.9261). For panels b–d, two-tailed P-values were determined by the one-way ANOVA test followed by a Dunnett’s multiple-comparison test. *P < 0.05, **P < 0.01, and ***P < 0.001. EoE eosinophilic esophagitis, DSP desmoplakin, PPL periplakin, FITC fluorescein isothiocyanate, SEM standard error of the mean.
expression of the identified DSP and PPL variants modified epithelial motility in a wound model and mechanistically affected RhoGTPase signaling. Fifth, we observed that DSP and PPL loss occurs in non-familial EoE, substantiating that the pathway identified initially by rare familial EoE cases is broadly applicable to familial and non-familial EoE. We present evidence that non-familial EoE likely has an acquired dysregulation (differential DSP and PPL expression on the basis of disease activity), whereas the defect in multiplex families appears to be fixed, as demonstrated by the retained DIS and decreased DSP and PPL expression even during disease remission. Sixth, we have found that familial EoE harboring DSP or PPL variants express higher TSLP mRNA levels than does non-familial EoE, providing a mechanistic link between desmosomal variants and the observed type 2 immune skewing and allergic responses in EoE. This is particularly interesting as common genetic variants in TSLP confer susceptibility to EoE5,9.

Our results mechanistically explain the previous findings of abnormal desmosome structure and barrier protein expression and impaired barrier function in biopsy specimens of patients with EoE16,33,34. Previous observations demonstrated that loss of

Fig. 4 Effect of DSP and PPL variants on RhoA activity. a Active RhoA assays performed in EPC2 cells transduced with constructs encoding non-variant and mutated DSP or PPL. Statistics (versus non-variants): p.G46D, P = 0.132; p.R808C, P = 0.0691; p.Y895C, P = 0.0453; p.1067_1068del, P = 0.1013; p.N1215S, P = 0.2131; p.R1340C, P = 0.048; p.E1723Q, P = 0.0619; p.R108C, P = 0.1484; p.E632K, P = 0.1443; p.K1051V, P = 0.1589; p.L1154V, P = 0.1449; p.E1163K, P = 0.0335; p.V1377E, P = 0.8738. b Wound healing assays performed in EPC2 cells transduced with constructs encoding non-variant and mutated DSP (p.Y895C) treated with the Rho activator CN01 or the Rho kinase inhibitor Y27632. Quantification of the wound closure after 12 h was shown. Scale bar: 500 μM. Statistics: Non-variant vs. Variant, P = 0.0001; Non-variant vs. Variant + CN01, P = 0.7658; Non-variant vs. Variant + Y27632, P < 0.0001; Variant vs. Variant + CN01, P = 0.0002; Variant vs. Variant + Y27632, P = 0.0394; Variant + CN01 vs. Variant + Y27632, P < 0.0001. c Enzyme-linked immunosorbent assay of the level of active RhoA (RhoA-GTP) in protein lysates of biopsies from control individuals, non-variant DSP PPL and familial patients with inactive EoE and non-familial patients with active EoE (Control, Variant, Active EoE, Inactive EoE). aP = 0.0443; bP = 0.7658; cP = 0.2131; dP = 0.0035; eP = 0.0398; fP = 0.0419; gP = 0.0035; hP = 0.048; iP = 0.0479. For panels a, b, c, data are presented as mean ± SEM, with markers representing biologically independent subjects. For panels a-c, two-tailed P-values were determined by the following tests: the one-way ANOVA test followed by a Dunnett’s multiple-comparison test (a) or Tukey’s multiple comparisons test (b, c). *P < 0.05, **P < 0.01, and ***P < 0.001. DSP desmoplakin, PPL periplakin, MW molecular weight, SEM standard error of the mean.
desmosomal proteins results in a spectrum of epithelial integrity defects depending on the severity of disruption. This is most evident in humans, as a large number of mutations covering most desmosomal genes have been described. For instance, homozygous mutations in the epithelial adhesion molecule DSG1 cause SAM syndrome and are associated with EoE. Multiple studies have emphasized that the inherited and acquired defects of plakins (i.e., DSP or PPL) in humans and animal models potentially lead to dramatic manifestations in the epithelium, which is consistent with our mechanistic in vitro findings and clinical findings of EoE. Thus, the decreased DSP and PPL expression identified herein may exert a deleterious effect on epithelial homeostasis, which underlies the disease phenotype seen in EoE.

Besides the impact of activated eosinophils as a functional endpoint, a growing body of evidence supports a role for altered barrier function in EoE. The histopathologic hallmark of EoE is the presence of eosinophils in a hyperplastic esophageal epithelium. Dysregulation of intercellular junctions suggests a mechanism whereby inflamed and hyperproliferative esophageal epithelial tissue can be penetrated by allergens, thereby amplifying allergic inflammation, although whether these alterations in the epithelia represent a primary causative feature or a reactive secondary event following eosinophilic inflammation is uncertain due to disease heterogeneity in EoE. The genetic evidence presented herein supports a primary role for desmosomal dysfunction in EoE, at least in the identified familial cases.

It is notable that individuals with familial EoE with DSP/PPL variants had low peak eosinophil counts even during disease remission, which is similar to individuals with non-familial EoE. However, inactive EoE was defined by the eosinophil counts (<15 eosinophils/HPF), which may be insufficient to fully characterize the disease state or differentiate subtle mechanisms or outcomes of familial EoE during disease remission. For instance, although anti-inflammatory therapy (e.g., steroids) and elimination of causal food allergens can decrease eosinophil counts, subjects with DSP/PPL variants may maintain a basal level of “leakiness”. Thus, more intense therapy may be required in patients harboring the identified genetic variants. Notably, there were stronger relationships between DSP and PPL expression and structural features than eosinophil features in non-familial EoE. In familial EoE, we demonstrated a specific disruption of structural features in the esophageal epithelium of patients with DSP and PPL variants regardless of disease activity status, suggesting that barrier dysfunction occurred as a primary fixed defect. As we did not have detailed clinical data, it is unclear whether DSP and PPL variants may contribute to disease severity and/or treatment responses; a further characterization of clinical features will be a valuable future endeavor.

A challenge in the field has been explaining how a generalized impaired barrier function can result in the tissue-specific nature of EoE. Though DSP and PPL are highly expressed in the esophagus, they are also abundant in some tissues that experience...
and the likely intersection of this with CAPN14- and RhoGTPase-mediated pathways. As therapeutic mainstays for EoE currently target inflammation (e.g., corticosteroids) or indicate allergen avoidance (e.g., dietary therapy), these findings prompt the development of therapeutics to normalize epithelial barrier function focused on desmosome function and provide a means for improved diagnostics based on targeted genome sequencing. These findings also highlight the value of WES in deciphering complex traits, providing a paradigm for assessing complex traits by starting with familial cases and extending the findings to the more common presentation.

**Methods**

**Study participants and design.** We recruited patients with eosinophilic esophagitis (EoE) and family members in Cincinnati Children’s Hospital Medical Center (CCHMC), Cincinnati, USA, from 2005 through 2017. Diagnosis of EoE was defined as symptoms consistent with esophageal inflammation and/or dysfunction and ≥15 eosinophils per high-power field (eos/hpf) in distal esophageal biopsies regardless of concurrent proton pump inhibitor (PPI) use, consistent with inter- national consensus diagnostic criteria. Subjects with histologically confirmed EoE were defined as “affected”, whereas those with no histologic evidence of EoE were defined as “unaffected”. All families were ascertained through a proband with EoE. In addition to familial EoE (62 multiplex families), controls and non-familial EoE (non-multiplex families) were included in the study for histologic and molecular analysis.

Whole-exome sequencing (WES) was performed on 62 unrelated families with European ancestry who have multiple family members with EoE across the US (21 states). The cohort of patients comprised two sets: Family F430 (as discovery set) and 61 families (as replication set). The first step was to identify a variant that segregated with affected family members of a large pedigree with EoE (Family F430) and sequenced the exomes of five affected family members (Patients 1, 2, 4, 11, and 19). After observing the presence of a variant in each of the five affected members, we then performed Sanger sequencing of samples obtained from all 32 Family F430 members, including spouses, to determine whether the same variant was segregated with affected status. The second step involved screening for additional variants of the candidate EoE genes in the 61 families with EoE. We sequenced the exome of one affected family member (index patient) per family, followed by Sanger sequencing for all first-degree relatives of the index patients. The third step was, if a gene were identified as a strong candidate, to obtain evidence for its role in disease pathogenesis using functional biologic assays. The identification of variants that were functionally shown to be pathogenic in a gene known to encode a protein that has a critical role in epithelial homeostasis would provide evidence of its function in EoE pathogenesis.

This study involving human subjects was conducted under the approved IRB (Cincinnati Children’s Hospital Medical Center) protocol number IRB00002720. An informed consent/assent form for consent to participate in research was received from the subjects and/or their legal guardians per institutional guidelines prior to inclusion in the study. Study participants were also made aware that their involvement in the study was voluntary, and their declination to participate did not interfere with their standard of care.

**Whole-exome sequencing (WES).** Genomic DNA was extracted with the use of kits that were designed to obtain saliva samples (OG-500, DNA Genotek) or peripheral blood (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany), according to the manufacturer’s instructions. WES was performed in the CCHMC Genetic Variation and Gene Discovery Core, the Oklahoma Medical Research Foundation (OMRF) Genomics facility, Perkin Elmer, and Broad Institute. Libraries were prepared using the Illumina TruSeq capture kit and sequenced on an Illumina HiSeq2000 or HiSeq4000 to generate 100–150× base, paired-end reads. Sequencing reads were aligned using Burrows-Wheeler Aligner (BWA) and GRRch37 human reference genome, and variant calls were made simultaneously following the Genome Analysis Toolkit (GATK) Best Practices by using the GATK Unified Genotyper software.

**WES variant filtering.** Variant filtering was performed using SNP and Variation Suite (SVS) software (Golden Helix Inc.) and the Cincinnati Analytical Suite for Sequencing Informatics (CASSI). Filters included minimum read depths >15 and genotype quality scores >20. Genotype calls were filtered using alternate allele ratios of <0.15 for homozygotes for the reference allele, 0.30–0.70 for heterozygotes, and >0.85 for homozygotes for the alternate allele.

**Sanger sequencing.** All variants identified by WES were validated by Sanger sequencing of PCR amplified DNA. Of all families with the proband having variants, Sanger sequencing was performed on all affected and unaffected members whose DNA samples were available. Primer pairs were designed with the software program (primer 3) to amplify 100–300 bp from the variants. The primers are...
Minor allele frequencies. Rare variants were annotated by Annotate Variation (ANNOVAR)17, identified and filtered using public data from the NCBI RefSeq genome build 105, the 1,000 Genomes Project (1kG Phase1 - Variant Frequencies)25, and the Exome Aggregation Consortium (ExAC) (ExAC Variant Frequencies 0.3 v2, BROAD)14. Rare variants were filtered using a minor allele frequency (MAF) cutoff of <0.01 (0.1%) in the European ancestry populations. In addition to the public databases, variant frequencies were filtered by using a MAF cutoff of <0.01 (1%) against an internal CCHMC database of WES data from 357 patients with various diseases, including macrophage-activating syndrome, systemic lupus erythematosus and juvenile idiopathic arthritis46.

Algorithms predicting conservation and pathogenicity. Functional predictions (dbNSFP)50–52 were filtered using a MAF cutoff of <0.01 (1%) against an internal CCHMC database of WES data from 357 patients with various diseases, including macrophage-activating syndrome, systemic lupus erythematosus and juvenile idiopathic arthritis46.

Molecular expression and histologic analysis by esophageal biopsies. Esophageal biopsy specimens taken from familial EoE (62 multiplex families), 93 controls, and non-familial EoE (511 non-multiplex families) were analyzed using several approaches, including EoE Diagnostic Panel (EDP)17, single-cell RNA sequencing18, quantification of peak esophageal eosinophils, EoE Histology Scoring System (EoEHSS)25 and expression of DSP and PPL by western blot and immunofluorescence staining49. For non-familial EoE, children and adults with EoE but not from multiplex families and who were presenting for standard of care were independently enrolled in the study for histologic and molecular analyses. Active EoE was defined as esophageal biopsy specimens that showed ≥15 eosinophils/hpf, and inactive EoE was defined as less than 15 eosinophils/hpf in patients with a previous history of EoE15. Patients with the condition of these patients may not be completely normal, they serve as a relevant control group for comparison with EoE15.

Endoscopic sample collection from patients. Subjects undergoing diagnostic endoscopy for ongoing clinical care consented/assented to provide additional esophageal biopsy specimen for research in addition to the standard clinical practice of obtaining esophageal endoscopic biopsy specimens. The research biopsy specimens were obtained after specimens had been obtained for clinical purposes. Distal esophageal biopsy was used throughout the study because this is typically obtained during endoscopy, represents the conventional location of biopsies, and allows ready comparison to the previous transcriptomic studies17,18, which are generally limited to this region. The tissues obtained for research were then placed in RNAlater buffer (Qiagen) and later processed for RNA extraction.

PCR amplification of DSP, PPL, and representative EoE gene analysis. Fresh biopsy specimens were stored in RNAlater until they were subjected to RNA isolation using the miRNeasy kit (Qiagen) per the manufacturer's instructions. Esophageal biopsy RNA (N = 246; 147 active EoE, 51 inactive EoE, and 48 unaffected controls) were isolated (Table S8 in the Supplementary information). After RNA quantity and quality analyses with a NanoDrop spectrometer, an aliquot of 500 ng of RNA was acquired for reverse transcription by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories), according to the manufacturer’s protocol. In addition to DSP and PPL transcripts, the transcriptomic signature of esophageal biopsy samples was obtained using an EoE Diagnostic Panel (EDP) comprising a set of 96 esophageal transcripts (which includes housekeeping proteins)17. DSP (Hs00189942_m1), PPL (Hs00160312_m1) and representative EoE genes in EDP were amplified from cDNA stock generated by the methods described above. The primers are reported in Supplementary Table Table 11. Using TaqMan Universal Master Mix II (Applied Biosystems), TaqMan real-time PCR amplification was performed on the Quant Studio 7 (Life Technologies). After the qPCR reactions had completed, raw cycle thresholds (Ct) values for each gene were generated and calculated using GeneQuantik GX 12.6 (Agilent Technologies) for statistical analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

SNP genotyping. DNA from patients was obtained from saliva or peripheral blood samples. rs10192210 in CAPN14 was genotyped in 62 unrelated families with European ancestry who have multiple family members with EoE by using TaqMan assay (Hs01037120_c1; Hs01457450_c2).
expression control for all analyzed genes. Samples with a GAPDH value of <30 CT value were considered acceptable for analysis. The expression CT value of the housekeeping gene GAPDH was subtracted from each gene of interest (GOI) CT value to acquire the ΔCT calculated12,67.

Single-cell RNA sequencing. Single-cell suspensions were prepared from biopsies. Bulk population cells were directly subjected to the 10X mass genomics chip (10X Genomics, Inc) targeting 10,000 simultaneously captured live events for next-generation sequencing. Each cell was uniquely barcoded during the cDNA library generation stage, followed by Illumina sequencing at CCHMC’s Genetic Variation and Gene Discovery Core, which allocated to a total read of ~320 million (two lanes/flow cell). Sparse data matrices, provided by 10X Genomics, were used as input into Seurat for further analysis31. For analysis of all sequenced esophageal samples, cells were filtered on the basis of unique feature counts (.001) or <200. Genes with ≤20% mitochondrial counts were filtered out. Only genes expressed in at least three cells were retained. The total number of cells passing the filters and captured across all patients was 39,562 cells, and 20,208 genes passed the quality control check. Of these cells, 30,967 cells were classified as epithelial cells. Principal component analysis (PCA) was performed over the list of the variable genes. Data were subjected to Uniform Manifold Approximation and Projection (UMAP) and shared nearest neighbor (SNN) modularity optimization-based clustering. Using PCA and SNN modularity optimization-based clustering algorithm with a resolution of 0.5 and UMAP, we identified eight distinct clusters, including three epithelial clusters. Top marker genes with high specificity were used to classify each cell type on the basis of existing biological knowledge. To calculate relative expression, data were processed by the first log normalizing the expression and then scaling the data so that it was centered at zero.

Histologic features. Histologic features were assessed by peak eosinophil counts and the EoE histology scoring system (HSS)16. Eight features of esophageal biopsies were defined and evaluated. Eosinophil infiltration (Ei) was graded using peak eosinophil counts obtained by counting eosinophils in the most densely infamed hpt. Additional features were basal zone hyperplasia (BZH), >15% of the total epithelial thickness; eosinophil abscess (EA), solid mass of intraepithelial eosinophils; eosinophil surface layering (SL), linear alignment of eosinophils parallel to the epithelial surface; dilated intercellular spaces (DIS), spaces around squamous epithelial cells that exhibit intercellular bridges; surface epithelial alteration (SEA), surface epithelial cells that exhibit altered tinctorial properties, manifesting as dark red staining, with or without intraepithelial eosinophils; dyskeratotic epithelial cells (DEC), individual cells with deeply eosinophilic cytoplasm and hyperchromatic nuclei; and lamina propria fibrosis (LPF), thickened connective tissue fibers in the lamina propria. Each feature was scored separately for grade (severity) or stage (extent) of the abnormality using a 4-point scale (0 = normal; 3 = most severe or extensive). The total score for each feature was defined as the sum of the scores for grade and stage. Therefore, the total score for each feature ranges from 0–6 because each score ranges from 0–3. HSS features were also grouped into those that relate to inflammation (i.e., a score of peak eosinophil count, eosinophil abscesses, eosinophil surface layering, and surface epithelial alteration) and those that relate to architectural aspects (basal zone hyperplasia, dilated intercellular spaces, dyskeratotic epithelial cells, and lamina propria fibrosis). For each group, the ratio of the sum of the assigned total scores for each evaluated feature divided by the maximum possible score for that biopsy (range, 0–1).

Immunofluorescence. For immunofluorescence of biopsies, DSP (sc-390975, Santa Cruz) (1:50 dilution) and PLL (ab131269, Abcam) (1:100 dilution) antibodies were used on at least 4 distal esophageal biopsies from control individuals and from patients with EoE with or without variants. Isotype antibodies were used as negative controls. The nuclei were stained with DAPI. The slides were blocked with PBS with 10% donkey serum. The secondary antibodies (1:625 dilution) were used: donkey anti-mouse Alexa Fluor 488 or donkey anti-rabbit Alexa Fluor 647 (Invitrogen). Imaging was performed with a Nikon A1 inverted confocal microscope (Nikon). The fluorescence intensity of each biopsy was calculated using the ImageJ calculation for corrected total fluorescence = integrated density—(area of background readings)23. For each gene, fluorescence intensity was determined with a ratio of control individuals to patients with EoE with or without variants for ease of interpretation.

Cell culture. Various cell types were used in the study: EPC2 (iHERT-derived immortalized human esophageal epithelial cell line), HaCat (immortalized human skin keratinocyte line), and HEK293T (human embryonic kidney cell line 293T). The EPC2 was a gift from Anil Rustgi (University of Pennsylvania, Philadelphia, PA) who was a member of the In-Fusion method (Takara). The EPC2 cells were grown and transduced with high-calcium (1.8 mM) media for 5 days, after which the media from the upper culture-Insert 4 Well was replaced with low calcium (0.3 mM) media supplemented with 10% FBS. EPC2 cells were transduced with pX459M2, pX459M2-DSPg2, or pX459M2-PLLg1 using Vioner Red reagent (Origene) according to the manufacturer’s protocol. Transduced cells were selected using puromycin, expanded, dispersed, and plated by limiting dilution in 96-well plates in the presence of irradiated NIH 3T3 feeder cells. The resulting EPC2 cell clones were expanded, and genomic DNA was extracted using the Quick gDNA micro prep kit (Zymo Research) according to the manufacturer’s protocol. For each clone, genomic DNA in the vicinity of the predicted editing region was amplified with the following primers (a) DSP nested PCR, we first amplified the gDNA with RB6848 (5'-CAACACCAACACC CAGCTC-3') and RB6853 (5'-GTACCGGGCGTGTGGTCAC-3'), and then gel extracted the 662-bp product and amplified with RB6852 (5'-CCGAGTAGTGAC TGGCAGG-3') and RB6851 (5'-AGTTCTTCTCGGCACCCG-3') and (b) PLL, we amplified with RB8710 (5'-GGACGCGACCGCAAGCTCTG-3') and RB8711 (5'-CGAGTTCTCCCTCACAGGAC-3'). The PCR products were sequenced with (a) RB8652 or (b) RB8711 to assess whether editing to produce a frameshift occurred. Knockout of protein expression was confirmed by western blot analysis.

DSP and PLL overexpression. For overexpression in EPC2 cells, the wild-type and identified mutants of DSP or PLL were synthesized into a pLVX-IRES-puro lentivector according to the In-Fusion method (Takara). The EPC2 cells were grown and transduced in KSF. The viruses, obtained from a 60 mm dish of HEK293T cells, were precipitated using Lenti-X concentrated solution and resuspended in 300 μl of KSF. KSF (150 μl) was mixed with polybrein to a final concentration of 5 μg/ml and used to transduce EPC2 cells grown to 60–70% confluency on a 6-well plate by centrifugation at 2000 g at 4 °C for 1 h. All cells underwent puromycin selection (1 μg/ml) for 1–2 weeks and were later kept in puromycin at 0.5 μg/ml. The puromycin was removed 24 h prior to the beginning of the experiment. Expression levels were validated by quantitative PCR and western blot analyses.

Transsparticular electrical resistance (TEER) and fluorescein isothiocyanate (FITC)-dextran flux measurements. EPC2 cells were subjected to the culture at the 60% confluence well filter insert (0.45 μm pore size; Millicell-CM, Millipore, Billerica, MA). The Culture-Insert 4 Well allows for wound healing assays with four cell-free gaps with a 0.5 mm gap width and one center area with a 1 mm diagonal distance. After removal of the insert, cells were washed three times, incubated at 37 °C and 5% CO2, and observed at 8 h or 12 h post wounding. Each wound was imaged at the time of wounding (0 h) and 8 h or 12 h post wounding using an Evsos inverted microscope (Thermo Fisher Scientific). The gap area was quantified using the ImageJ plugin MRR Wound Healing Tool (http://dev.mrr.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool). Percent change in wound area was defined as [100 × (W0-W8)/W0] (W0: wound width at 0 h; W8: wound width at 8 h).

Active RhoA assay. Active RhoA was measured using the RhoA G-LISA Activation Assay Kit, according to the manufacturer’s recommendations (Cytoskeleton). Briefly, cells were washed and lysed by the ice-cold lysis buffer with a protease inhibitor cocktail. As for the esophageal tissue biopsies, the frozen tissue samples were homogenized in ice-cold lysis buffer with a protease inhibitor cocktail. Lysates were clarified by a 1 cm mini centrifugation, and supernatants were snap-frozen in liquid nitrogen. Total protein contents were assayed by bicomichronic acid (BCA) (Thermo Fisher Scientific). The lysates were diluted into the buffer into the pre-coated plates provided with the RhoA G-LISA kit. The plate was placed on an orbital microplate shaker at 400 rpm for 30 min at 4 °C and then incubated with anti-RhoA primary antibody (1:250), followed by a secondary antibody (1:62.5), on an orbital microplate shaker at 400 rpm for 45 min each at room temperature. The plate was washed 3 times with PBS and incubated with the HRP-conjugated antibody (1:1000) for 15 min. Absorbance was read at 490 nm using a microplate reader (BioTek). Total RhoA levels of each lysate were quantified by western blot analysis.
**Calpain-14-mediated degradation assay.** For in vitro calpain-14 degradation assays, HEK293T cells were co-transfected with DSP or PPL (non-variant or mutant) and CAPN14-FLAG or enzymatically inactive CAPN14-C101A-FLAG using TransIT-LT (Mirus Bio), as indicated. After 48 h, transfected cells were lysed without a protease inhibitor cocktail. To equalize each cell lysate, total protein concentrations were measured by BCA (Thermo Fisher). Cell lysates were incubated for 15 min at 37 °C in an equal volume of assay buffer (40 mM Tris HCl, 50 mM NaCl, 2 mM DTT) with and without 1 mM CaCl2. For calpain inhibitor assay, samples were pre-incubated with SNJ-1945 (250 μM) for 2 min at room temperature before addition of assay buffer. Reactions were terminated by the addition of 4 × protein sample buffer followed by boiling for 5 min. The resulting products were separated on SDS-PAGE gel and processed by western blot analysis. The protein remaining after activation of CAPN14 was determined by the difference in band intensity [e.g., (100 × after activation)/before activation].

**Statistical analysis.** Statistical analyses were performed using the JMP v13.1 (SAS Institute, Cary, NC), R statistical computing environment (version 3.1.2). Genespring GX 12.6 (Agilent Technologies, Santa Clara, CA) and GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA). Descriptive studies were not tested for statistical significance. Data are presented as n (%) or mean ± standard error of the mean (SEM) unless otherwise stated. Missing data were excluded from all analytical statistics. For categorical data, the chi-square test or Fisher’s exact test was used to ascertain differences. For continuous data, some variables exhibited normal distributions, whereas others exhibited deviations; thus, we employed both parametric and non-parametric tests to compare groups as appropriate. For the comparison of two groups, either t-tests or Mann–Whitney U test were used. For the comparison of three or more groups, we used one-way analysis of variance (ANOVA) followed by a Dunnett’s multiple-comparison test or the Kruskal–Wallis test followed by a Dunn multiple-comparison test. Correlations were calculated using Spearman correlations. Benjamini–Hochberg correction was applied for multiple testing. A significant P-value was defined as <0.05.

**Data availability**
The patients’ genome data obtained by whole-exome sequencing during the current study are not fully available in a public or closed repository because study participants did not give full consent for releasing data; a subset of the sequences is deposited in the dbGAP accession phs010112.v1.p2. These data can be accessed under the condition that a joint research plan is made by the researchers and approved by the ethics committees. The exophagomal molecular expression data by EDP and single-cell RNA sequencing have been deposited in EDPExpress (https://edpexpress.research.cchmc.org/data/). All other data supporting the findings of this study are included within the article or the Supplementary information and are available from the corresponding author upon request. Source data are provided with this publication.

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Author contributions
T.S. and M.R. conceived the study and wrote the manuscript. K.M.K. and L.I.M. contributed the genetic analysis, statistical consultation, and support. N.A.B., N.Z., M.H.C., and G.A.O. contributed the genetic analysis, statistical consultation, and support. N.Z., M.H.C., and N.A.B. obtained funding and led the study. M.E.R. contributed the genetic analysis, statistical consultation, and support. N.Z., M.H.C., and G.A.O. also grateful to their colleagues and clinical support staff for procuring biopsies, saliva and blood samples, and clinical data. We also thank S. Hottinger for editorial assistance. This work was supported by grants [R37 AD45898, RO1 AI124855, U19 AI070235, and P30 DK078392 (Gene and Protein Expression Core); the Campaign Urging Research for Eosinophilic Esophagitis (CURED); the Sunshine Charitable Foundation and its supporters, Dennis A. Bunning and David G. Bunning] (to M.R.) and grant K99/R00 AI158660 (to T.S.).

Competing interests
The authors declare competing financial interests. M.E.R. is a consultant for Palm One, Spoon Guru, Celgene, Shire, Astra Zeneca, GlaxoSmithKline, Allakos, Adare, Regeneron, and Novartis and has an equity interest in Palm One, Clostridia, and Spoon Guru, and royalties foruszumab (Teva Pharmaceuticals). M.E.R. is an inventor of patents owned by Cincinnati Children’s Hospital Medical Center. M.E.R. is a consultant for Shire, Regeneron, Receptos/Celgene, GlaxoSmithKline, Escocap, Astra Zeneca, Arena, and Allakos and has received research funding from Shire, Regeneron and Receptos/Celgene. V.A.M. is a consultant for Shire and has received research funding from Shire. T.S. has received research support from JSIS Overseas Research Fellowships and is a co-inventor of patents owned by Cincinnati Children’s Hospital Medical Center. All other authors declare no competing interests.
