

A homozygous frameshift mutation in the mouse *Flg* gene facilitates enhanced percutaneous allergen priming

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Loss-of-function mutations in the *FLG* (filaggrin) gene cause the semidominant keratinizing disorder ichthyosis vulgaris¹ and convey major genetic risk for atopic dermatitis (eczema)^{2–4}, eczema-associated asthma^{2,3} and other allergic phenotypes⁵. Several low-frequency *FLG* null alleles occur in Europeans and Asians, with a cumulative frequency of ~9% in Europe⁴. Here we report a 1-bp deletion mutation, 5303delA, analogous to common human *FLG* mutations, within the murine *Flg* gene in the spontaneous mouse mutant flaky tail (ft). We demonstrate that topical application of allergen to mice homozygous for this mutation results in cutaneous inflammatory infiltrates and enhanced cutaneous allergen priming with development of allergen-specific antibody responses. These data validate flaky tail as a useful model of filaggrin deficiency and provide experimental evidence for the hypothesis that antigen transfer through a defective epidermal barrier is a key mechanism underlying elevated IgE sensitization and initiation of cutaneous inflammation in humans with filaggrin-related atopic disease.

Eczema is a common disease affecting 15–30% of children and 2–10% of adults in developed countries⁶. There is a strong genetic association with atopic disorders including asthma, hay fever and food allergy⁷. Elevated total IgE is observed in 60–80% of individuals with eczema attending secondary care. Although IgE sensitization increases in the first few years of life in children with eczema⁸, the timing and pathogenic role of IgE sensitization in the development of atopic dermatitis remains uncertain⁹. Recent evidence strongly suggests that children with early-onset (<3 months) and more severe eczema have the greatest risk of IgE sensitization. Although debate remains as to the relative contributions of inherited barrier defects versus

immunological aberrations, the major genetic risk identified to date is associated with loss-of-function mutations in *FLG*, encoding the skin barrier protein profilaggrin/filaggrin^{2,4}.

The spontaneous recessive mouse mutant flaky tail (ft) arose in 1958 on the background of an existing recessive hair phenotype, matted (ma), and since then has been maintained on a mixed strain¹⁰. Flaky tail mice have dry, flaky skin and annular tail and paw constrictions in the neonatal period¹¹. Protein blot analysis shows that ft/ft mice express a truncated profilaggrin (~215 kDa) instead of the normal high-molecular-weight profilaggrin (>500 kDa)¹¹. Although processed filaggrin in these mice has been shown to be virtually absent at the protein level¹¹, the underlying genetic mechanism is unknown. Given the likely genetic similarity between flaky tail mice and human ichthyosis vulgaris and atopic eczema, we sought to identify the genetic mechanism underlying this mutant and study in detail the function of the skin barrier in ft/ft mice with respect to ease of allergen priming.

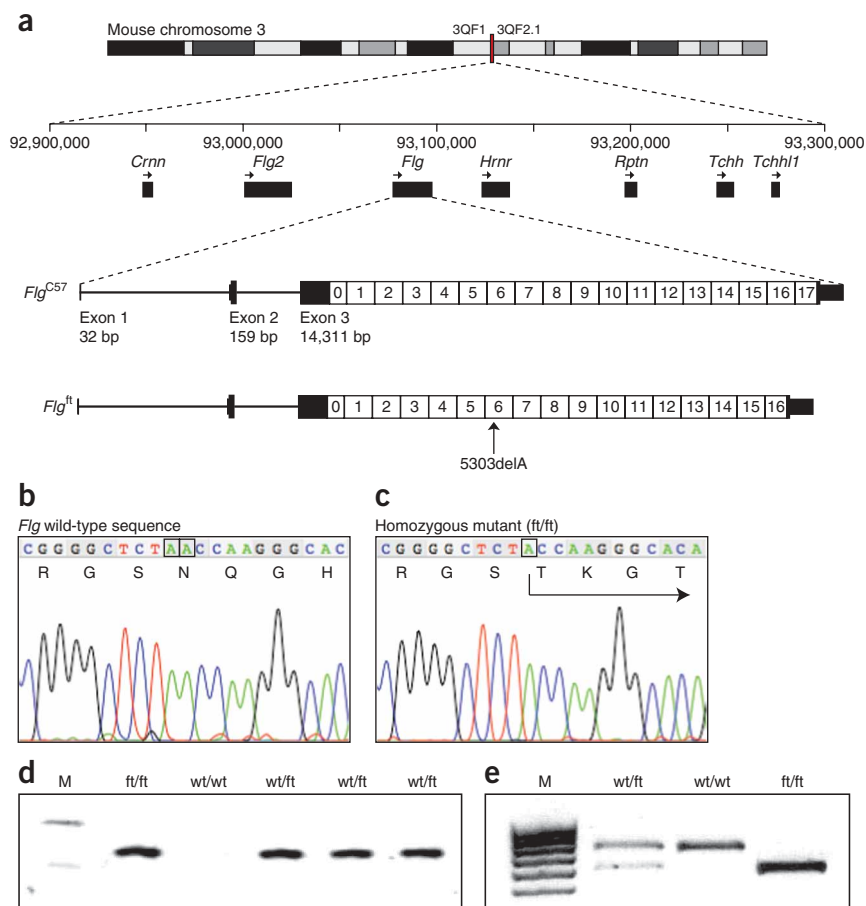
The mouse filaggrin gene, *Flg*, is located on chromosome band 3QF2.1 within a cluster of seven genes encoding the fused S100 family of filaggrin-like proteins (Fig. 1). The organization of this cluster is identical to the orthologous human cluster. We manually annotated the intron-exon organization of the 20,150-bp *Flg* gene from the current build of the C57BL/6 mouse genome (Fig. 1a and Supplementary Table 1 online). The genomic sequence derived from BAC clone RP23-227A12 contains small assembly errors owing to incomplete resolution of the tandem repeats within *Flg*. We resolved this issue by aligning individual filaggrin repeats and correcting the five detected assembly errors (Supplementary Table 2 online) so that the complete ORF was in-frame with the reported short partial 5' and 3' cDNA sequences for mouse *Flg*^{12,13}. The predicted 496-kDa mouse profilaggrin consists of 4,654 amino acids, including 16 full

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Figure 1 *Flg* gene structure and mutation identification. (a) *Flg* resides within a cluster of seven genes encoding closely related fused-S100 proteins, spanning ~350 kb on chromosomal band 3QF21. Like human *FLG*, mouse *Flg* has three exons. Exon 1 consists of 5'-UTR sequences only; exon 2 contains the ATG and encodes part of the N-terminal S100 calcium-binding domain of profilaggrin; and exon 3 encodes the remainder of the S100 domain, the B-domain and the filaggrin polyprotein repeat domain. In the July 2007 mouse genome sequence annotated here (*Flg*^{C57}), the latter consists of 16 near-perfect repeats encoding a 250-amino-acid filaggrin protein (1–16) flanked by two imperfect repeats (0 and 17). Repeat 17 is followed by a short tail domain. In *ft* mice, the exon 3 sequence (*Flg*^{ft}) lacks one repeat owing to a 750-bp in-frame deletion, as well as the frameshift mutation 5303delA. There are also several additional SNPs and small in-frame insertions/deletions in *Flg* that differ between the two strains; their DNA sequences overall are 99.3% identical, excluding the repeat copy number variation. (b) Wild-type *Flg*, corresponding to codons 1765–1771, with amino-acid translation. The A dinucleotide involved in the mutation is boxed. (c) Corresponding *Flg* sequence derived from a homozygous *ft/ft* mouse, showing the homozygous frameshift mutation designated 5303delA, predicting truncation sequence Asn1768ThrfsX154. The single A nucleotide remaining at the site of the mutation is boxed. (d) Allele-specific genotyping of the parents and F₁ offspring of a cross between an *ft/ft* homozygote and a wild-type mouse. M, molecular weight markers, upper band = 118 bp; lower band = 72 bp. The allele-specific band is only amplified from mice carrying the *ft* mutation. (e) Genotyping for an *Accl* restriction site polymorphism in filaggrin repeat 1, approximately 4 kb upstream of the 5303delA mutation. The 678-bp C57BL/6 allele does not digest; the *ft* allele digests to yield fragments of 559 bp and 134 bp. M, molecular weight markers.



near-identical repeats of a 250-amino-acid filaggrin consensus sequence (repeats 1–16), flanked by two incomplete repeats (repeats 0 and 17; **Fig. 1a**). Most repeats are less than 1% different in protein sequence from each another. The amino terminus consists of a conserved S100 calcium-binding domain¹⁴ and a B-domain postulated to have a signaling function^{12,15}. The domain organization is identical to that of human profilaggrin⁴, except that the mouse protein has 16 repeats of a smaller 250-amino-acid sequence versus 10–12 repeats of 324 amino acids in humans⁴. The number of repeats has been shown to vary among mouse strains¹⁶, as it does in the human population^{4,17}. Downstream of repeat 17 is a unique C-terminal

domain. A polyclonal antibody raised against a synthetic peptide derived from the C-terminal tail domain recognized full-length ~500-kDa profilaggrin in wild-type mice, as well as intermediates from profilaggrin-to-filaggrin processing (**Fig. 2**), validating the predicted amino-acid sequence. Although traces of truncated profilaggrin (~215 kDa) can be seen in *ft/ft* homozygotes by total protein staining (**Fig. 2a**) and in protein blots using antibodies against a filaggrin repeat epitope, as previously reported¹¹, they are not recognized by the C-terminal antibody, predicting a truncating mutation (**Fig. 2b**).

Long-range PCR methods used to identify human *FLG* mutations^{1,4} proved unable to amplify the larger exon 3 of mouse *Flg*. We therefore

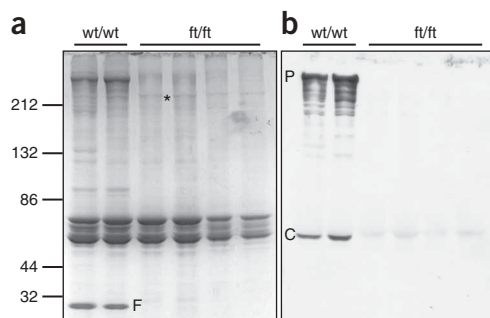


Figure 2 A truncated profilaggrin lacking the C terminus is expressed in flaky tail mouse skin. Urea/Tris protein extracts from wild-type and *ft/ft* mice were fractionated by SDS-PAGE and immunoblotted with a peptide antibody developed to the C terminus of mouse profilaggrin. (a) Coomassie brilliant blue-stained protein gel to control for loading. (b) Corresponding immunoblot probed with the C-terminal antibody. The antibody detects both full-length profilaggrin (P) and a putative C-terminal processing product (C) in wild-type mouse extracts; however, these immunoreactive products are not detected in *ft/ft* mice (b). Note the lack of filaggrin (F) in *ft/ft* mice (a), as observed previously¹¹. The mutant profilaggrin protein in *ft/ft* mice is faintly visible in a at an apparent molecular weight of ~215 kDa (*). This is readily detectable with a mouse filaggrin antibody as reported previously¹¹. Molecular weight marker sizes are shown at left.

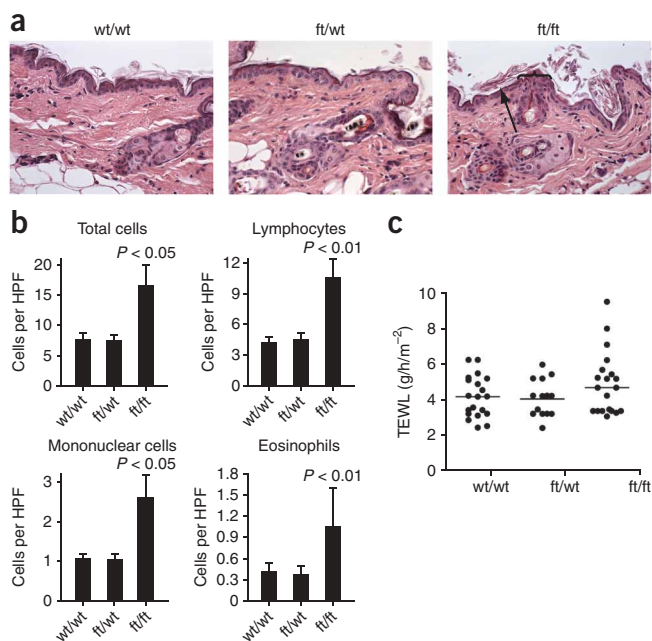


Figure 3 Cutaneous inflammation in untreated ft/ft mice but not wt/wt or wt/ft animals. **(a)** Representative photomicrographs of skin sections from age- and sex-matched wt/wt, wt/ft and ft/ft mice. Ft/ft mice had orthokeratotic hyperkeratosis (arrow) with occasional foci of acanthosis (bracket) compared to wt/wt and wt/ft mice (original magnification $\times 40$). **(b)** Numbers of skin infiltrating cells, lymphocytes, eosinophils and mononuclear cells were detected per high-power field (HPF). Cells were counted on 15–20 HPF ($\times 1,000$) on hematoxylin and eosin-stained sections of 5–6 wt/wt, wt/ft and ft/ft mice. Data represent the mean; error bars represent s.e.m. Student's *t*-test, or corrected Welch corrected *t*-test, was used to determine statistical differences between groups. **(c)** TEWL analysis of untreated wt/wt, wt/ft and ft/ft mice. Values are individual mice, and mean bars are shown.

used a shotgun sequencing method whereby six species of short PCR fragments were generated and cloned using primers designed to amplify any of the repeat units, as recently used to detect human *FLG* mutations¹⁸. By sequencing >900 clones, we detected a 1-bp deletion in clones resulting from four different PCR fragments (Fig. 1b,c). Allele-specific PCR genotyping showed that this deletion completely cosegregated with the ft phenotype in many mouse crosses (Fig. 1d). We also detected an *AccI* restriction fragment length polymorphism in filaggrin repeat 1 in tight linkage with the mutation. This marker proved to be convenient for genotyping ft mice, as heterozygotes are more easily scored than by allele-specific PCR (Fig. 1e). The ft mutation was subsequently backcrossed and maintained in our laboratories on the C57BL/6 background. Early in the course of backcrossing, we were able to remove the matted hair allele (ma) originally present on this mixed strain.

Given the nature of the shotgun method initially used, it was not possible to assemble the complete ft sequence for exon 3. However, we constructed a BAC library from an ft/ft homozygote and isolated a clone containing the entire *Flg* locus. We then subcloned exon 3 by recombineering and oversequenced by $\sim$ ninefold the resultant plasmid by random transposon insertion methodology. We sequenced exons 1 and 2 from ft/ft mice by standard PCR methods, allowing full assembly of the *Flg* sequence from the mutant strain. By this means, the mutation was unambiguously designated as 5303delA in filaggrin repeat 6, leading to premature termination 154 codons downstream (Asn1768ThrfsX154). The *Flg* sequence from ft mice was one filaggrin repeat shorter than the C57BL/6 sequence, consistent with earlier studies reporting variation in filaggrin repeat copy number both in mice and humans^{4,17}. Otherwise, the coding sequence showed 99.3% identity between C57BL/6 and ft, with no other loss-of-function variants detected. Partial sequencing of *Flg* in different mouse strains suggested that the *Flg* locus in ft was originally derived from the C3H strain (data not shown).

We took skin biopsies for histopathological analysis from 6- to 8-week-old age- and sex-matched wt/wt (wild-type), wt/ft (heterozygous) and ft/ft (homozygous) mice. No overt differences were noted between wt/wt and wt/ft (Fig. 3a). In contrast, ft/ft mice had a mild

diffuse orthokeratotic hyperkeratosis (a hypertrophy of the stratum corneum of the skin characterized by the presence of non-nucleated cells). We also observed occasional foci of acanthosis (abnormal thickening of the stratum spinosum layer of the skin), which varied in magnitude among individual ft/ft mice (Fig. 3a). In our hands, ft/ft mice have a phenotype consistent with the original description¹¹ but do not have significant acanthosis or alterations to the stratum granulosum as previously reported¹¹ possibly because histopathological analysis was done at a younger age. We observed a broad spectrum of severity of skin pathologies among individual ft/ft mice, including some with sporadic superficial dermal cellular infiltrates. We further characterized the cutaneous cellular infiltrate and, according to the number of cells detected per field ($\times 1,000$), we observed significantly more total cells ($P < 0.05$), lymphocytes ($P < 0.01$), eosinophils ($P < 0.05$) and mononuclear cells ($P < 0.01$) in ft/ft relative to wt/wt and wt/ft mice (Fig. 3b). To address the baseline integrity of the skin barrier of ft animals, we measured transepidermal water loss (TEWL) on the skin of adult age-matched wt/wt, wt/ft and ft/ft mice. Although there were higher individual TEWL readings in ft/ft mice, possibly reflecting the spectrum of severity in skin inflammation within this group, there was no statistically significant difference in baseline TEWL among the three groups (Fig. 3c).

To test allergen priming of the skin in these mice, we used the widely studied and clinically relevant allergen ovalbumin (OVA). We applied OVA or PBS (as a vehicle control) to the shaved abdominal skin of wt/wt, wt/ft and ft/ft mice (for full protocol, see **Supplementary Fig. 1a** online). Cutaneous exposure to OVA did not lead to gross skin lesions in any mice (data not shown). Skin removed from the site of allergen challenge showed evidence of some edema and a non-significant increase in cellular infiltrates but no gross damage or inflammation in wt/wt or ft/ft mice (Fig. 4a,b). In contrast, exposure of OVA to the skin of ft/ft mice induced diffuse mild acanthosis, with significant infiltrates of mixed, predominantly lymphocytic inflammatory cells, in addition to eosinophils and mononuclear cells (Fig. 4a,b). Measurement of TEWL at the site of allergen challenge 24 h after OVA application revealed a significant ($P < 0.0001$) elevation in TEWL of OVA-treated ft/ft mice relative to PBS-treated ft/ft and wt/wt and wt/ft mice (Fig. 4c). Application of OVA to wt/wt or wt/ft mice caused no alteration in TEWL (Fig. 4c).

We then analyzed the systemic immune response to OVA. Following cutaneous allergen exposure, wt/wt mice did not have elevated levels of total serum IgE, nor did they generate an antibody (IgG or IgE) response to OVA (Fig. 5a and **Supplementary Fig. 2** online), and spleen cells from wt/wt mice did not proliferate or produce cytokines when exposed to OVA *in vitro* (Fig. 5b,c). In contrast, percutaneous allergen exposure in ft/ft mice generated OVA-specific IgG and OVA-specific IgE that was significantly ($P > 0.005$) elevated relative to other PBS- or OVA-treated groups (Fig. 5a). The absence of allergen-specific responses in wt/wt mice upon allergen-challenge to

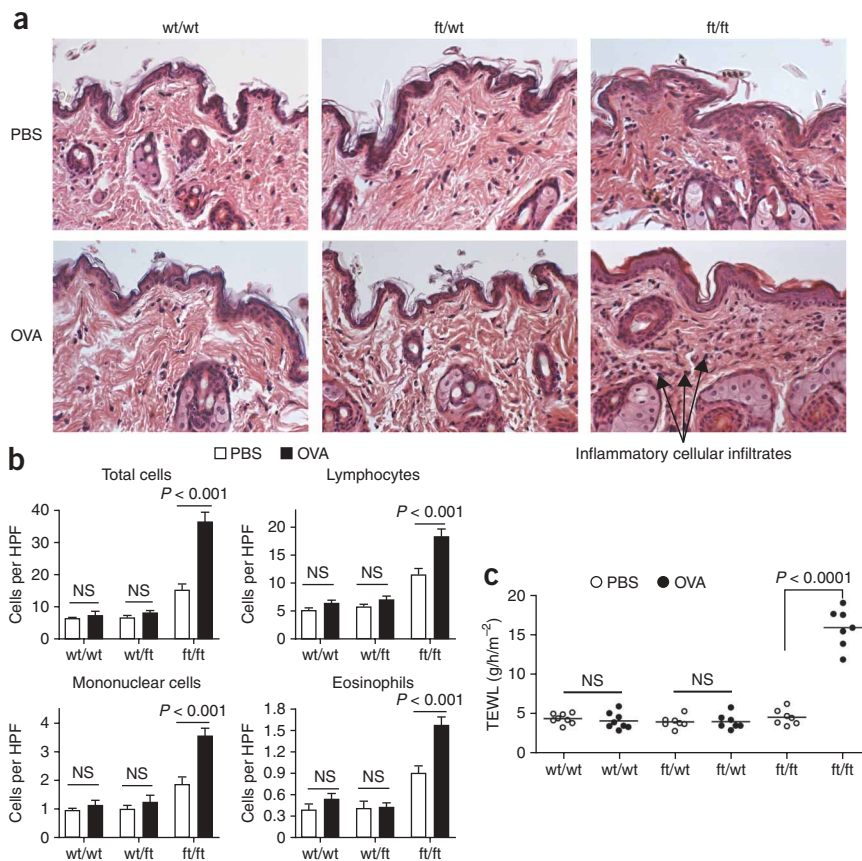


Figure 4 Allergen exposure exacerbates skin inflammation in ft/ft mice but not wt/wt or wt/ft animals. **(a)** Representative photomicrographs of skin sections from age- and sex-matched wt/wt, wt/ft and ft/ft mice exposed to OVA or PBS as a vehicle control. Ft/ft mice had diffuse mild acanthosis and marked increases in dermal cell infiltration when cutaneously challenged with OVA, compared to wt/wt and wt/ft mice (original magnification $\times 40$). **(b)** Quantification of numbers of skin infiltrating cells, lymphocytes, eosinophils and mononuclear cells detected per high-power field (HPF). Cells were counted at on 15–20 HPF ($\times 1,000$) on hematoxylin and eosin-stained sections of 11–14 wt/wt, wt/ft and ft/ft mice. Data represent the mean; error bars represent s.e.m. Student's *t*-test, or corrected Welch corrected *t*-test, was used to determine statistical differences between groups. NS; nonsignificant. **(c)** TEWL analysis of skin of OVA-exposed wt/wt, wt/ft and ft/ft mice. Values relate to individual mice, and mean bars are shown.

PBS-exposed mice (**Supplementary Figs. 3 and 4** online). No airway inflammation was seen on lung histology sections from wt/wt or ft/ft mice exposed to OVA via the skin (**Supplementary Fig. 5a** online), which was subsequently confirmed and quantified by scoring histology sections (data not shown). Furthermore, there was no significant elevation in the numbers of total cells and eosinophils recovered in bronchoalveolar lavage

(BAL) fluid between OVA-exposed or PBS-treated ft/ft and wt/wt mice (**Supplementary Fig. 5c,d**). OVA application to barrier-disrupted skin of wt/wt mice, as used to induce AHR on the more Th2 immunoreactive BALB/c strain previously²⁵, also failed to induce AHR in tape-stripped C57BL/6 mice (**Supplementary Fig. 4a**). The inability to induce AHR after cutaneous OVA challenge may thus reflect the use of C57BL/6 mice, which are less susceptible than BALB/c mice to allergen-induced Th2 inflammation.

To confirm that the OVA sensitization shown here in ft/ft is due to a specific defect in the skin barrier rather than a generalized altered immunity, we gave intraperitoneal injections of OVA and adjuvant (Alum) to ft/ft and wt/wt mice. We then evaluated allergen-specific responses (**Supplementary Fig. 3b** online). ft/ft mice intraperitoneally immunized with OVA and adjuvant developed allergen-specific immune responses comparable to wt/wt animals, with both groups developing a Th2-biased OVA-specific cytokine and antibody isotype response (**Supplementary Fig. 6** online).

To address whether the lack of pulmonary inflammation in ft/ft mice following allergen challenge was due to altered lung immunity and development of AHR in ft/ft mice, we challenged these animals and wt/wt mice intraperitoneally with OVA and adjuvant followed by pulmonary allergen sensitization (**Supplementary Fig. 3a**). There was comparable induction of AHR, determined as lung resistance (R_L), in wt/wt and ft/ft mice (**Supplementary Fig. 7** online). Furthermore, intraperitoneal challenge with OVA and adjuvant resulted in airway inflammation in both wt/wt and ft/ft mice (**Supplementary Fig. 5b**), with no differences between groups in the magnitude of lung inflammation detected (data not shown). Therefore ft/ft mice develop allergic lung inflammation after intraperitoneal priming with OVA and adjuvant but not when sensitized via the skin to OVA in the

intact skin has been reported previously in the background strain of the ft/ft mice used here (C57BL/6)¹⁹; however, an allergic response can be generated when OVA is applied after the skin barrier has been disrupted by tape-stripping²⁰. Spleen cells from OVA-exposed ft/ft mice proliferated in a dose-dependent manner in response to OVA, whereas cells from other test groups did not proliferate (**Fig. 5b**). None of the PBS-treated groups developed OVA-specific cytokine responses in splenic cells (data not shown). Spleen cells from OVA-treated wt/wt and wt/ft mice did not secrete cytokines in response to OVA (**Fig. 5c**). In contrast, ft/ft mice produced OVA-specific Th2 (IL-4, IL-5, IL-13), Th1 (IFN- γ), regulatory (IL-10) and Th17 (IL-17) cytokines (**Fig. 5c**), indicating a generalized allergen-specific cytokine response that was not solely Th2 skewed. Consistent with a mixed T helper cytokine response in ft/ft mice treated with OVA cutaneously, there was elevation of serum levels of both OVA-specific IgG2a and IgG1, antibody isotypes that are induced by Th1 and Th2 cytokines, respectively²¹ (**Supplementary Fig. 2b**). In these studies, wt/ft animals were comparable to wt/wt mice with respect to all parameters analyzed (**Figs. 3–5**).

These data show that ft/ft mice generate allergen-specific IgE and cytokine responses following cutaneous allergen challenge to intact skin. Several large studies have shown that *FLG* loss-of-function mutations predispose to asthma in the context of atopic dermatitis^{5,22–24}. To experimentally address this association using the mouse model, we treated ft/ft and wt/wt animals that had been percutaneously treated with OVA with OVA aerosol to evoke pulmonary sensitization and airway hyper-responsiveness (AHR), and analyzed pulmonary responses (**Supplementary Fig. 1b**). AHR, determined as airway resistance (R_L), was not induced in OVA-exposed ft/ft or wt/wt mice, with both OVA-exposed groups having similar R_L values as

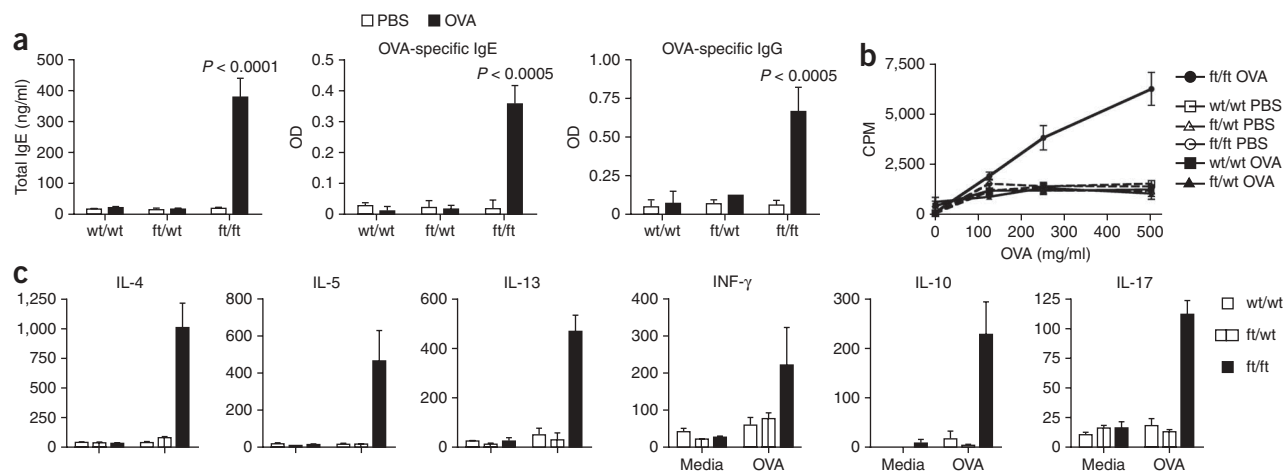


Figure 5 Elevated OVA-specific immune response in allergen exposed ft/ft mice but not wt/wt or wt/ft animals. **(a)** ELISA detection of levels of total IgE, OVA-specific IgE and IgG in serum from wt/wt, wt/ft and ft/ft mice treated with PBS or OVA on skin. Data are mean plus s.e.m. (from 7–12 mice). **(b)** Cell proliferation to OVA of spleen cells from wt/wt, wt/ft and ft/ft mice treated with PBS or OVA on skin. Data represent the mean of spleens from 3–4 individual mice per group; error bars represent s.e.m. **(c)** Cytokine production by spleen cells from OVA-exposed wt/wt, wt/ft and ft/ft mice treated with PBS or OVA. Cells were cultured in media or OVA (500 μ g/ml) and cytokines detected by ELISA. Data represent the mean of spleens from 3–4 individual mice per group; error bars represent s.e.m. Student's *t*-test, or corrected Welch corrected *t*-test, was used to determine statistical differences between groups.

absence of adjuvant. Further work is needed to dissect whether this is due to the type of OVA-specific response generated in the absence of Alum, a Th2-inducing adjuvant, as opposed to the response generated following skin exposure to allergen in the absence of adjuvant.

Here we have identified the flaky tail mutation as a frameshift mutation 5303delA in repeat 6 of *Flg* (Fig. 1). Several truncating mutations have been reported in human *FLG*⁴, including three in repeat 6. Notably, the truncated profilaggrin species resulting from mutations in repeats 7 and 10 of *FLG* have been shown to be highly unstable and incapable of being processed to functional filaggrin⁴, as shown in the ft mouse here (Fig. 2) and previously¹¹. Thus, the 5303delA mutation is a null allele, analogous to many of the human *FLG* mutations now emerging, and might be a better model for the human mutations than a knockout allele, which has proven difficult to generate owing to the repetitive nature of this locus (R.B.P. and W.H.I.M., unpublished data). Availability of genotyping assays for the mutant allele will allow generation of congenic strains and double mutant mice to further dissect the immunological and biological effects of *Flg* mutations.

Although ft/ft mice closely model the phenotypic, histological and ultrastructural characteristics of ichthyosis vulgaris in humans¹¹, we show here that these filaggrin-deficient mice are also predisposed to develop sensitization following percutaneous exposure to a model allergen, and develop cutaneous inflammatory infiltrates and allergen-specific immune responses following allergen sensitization (Fig. 5). Following sensitization, a further skin barrier defect, as measured by elevated TEWL, is seen, suggesting that the mild initial heritable barrier defect is exacerbated by allergic sensitization. This suggests that sensitization might also be an early event in filaggrin-deficient humans. Several known modifiers (for example, Th2 cytokines, including IL-13 and IL-4) have been shown to downregulate expression of filaggrin²⁶ and other key epidermal structural proteins²⁷, and unknown genetic factors may also influence the phenotype of ft/ft mice; these areas require further experimentation. It is noteworthy that PBS-treated ft/ft mice showed no elevation of total IgE up to 12–13 weeks of age (Fig. 5). Indeed, in 8-month-old untreated ft/ft mice, we have observed no increase in nonspecific IgE or elevated Th2

cytokine (IL-4, IL-5 and IL-13) production, and there are no previous reports of spontaneous atopy or eczema in ft/ft mice^{10,11}. Thus, ft/ft mice maintained under specific pathogen-free conditions do not appear to develop spontaneous allergic responses, in contrast to NC/Nga mice²⁸.

As noted above, the role of elevated IgE in human atopic dermatitis remains unresolved. Given a recent report of a mouse model of intrinsic atopic eczema²⁹, which highlights the potential for eczema in mice in the absence of elevated IgE, caution is needed in assigning an obligate pathogenic role for elevated IgE in mouse atopic dermatitis. Although clarifying this relationship will require more experimentation on mouse models in addition to human studies, we have shown enhanced percutaneous priming in a mouse model of filaggrin deficiency that hints at possible mechanisms in the human disease. These findings set the scene for the use and further refinement of this animal model to further dissect the role of filaggrin in skin barrier function and the genesis of atopic eczema.

METHODS

Sequence analysis. We annotated the genomic sequence of the mouse *Flg* locus derived from BAC clone RP23-227A12 using the ClustalW tools within MacVector version 9.0. To identify the causative mutation in ft/ft mice, we applied the shotgun method recently developed to identify *FLG* mutations in humans with atopic dermatitis¹⁸. We used six pairs of PCR primers based on *Flg* exon 3 sequence on BAC clone RP23-227A12, designed to amplify each filaggrin homologous sequence unit (Supplementary Table 3 online). The PCR products were cloned into the TA-cloning vector pGEM-tEasy (Promega). Plasmid DNA template was generated using the TempliPhi system (GE Healthcare Biosciences) and directly sequenced. We identified an identical 1-bp deletion of A in shotgun clones derived from four independent PCR fragments. To confirm the mutation and determine the full sequence of *Flg* from the mutant strain, we constructed a BAC library from homozygous ft/ft mice, which was screened by hybridization, and the resultant hits were confirmed by PCR (Amplicon Express). From a BAC containing the entire locus, we subcloned exon 3 into a plasmid vector by recombining (Red/ET system, Gene Bridges). The subclone was subjected to random transposon insertion using the EZ-Tn5 <KAN-2> insertion kit (EPICENTRE Biotechnologies), and 96 transposon insertion clones were sequenced bidirectionally from the Tn5 insertion site, allowing full assembly of exon 3 with ~ninefold oversequencing.

Genotyping. For allele-specific PCR detection of the ft mutation, we amplified genomic DNA (2 ng) with primers mFlgdelA-F and mFlgdelA-R (**Supplementary Table 3**) using $\times 2$ KOD FX PCR buffer containing 1.5 nmol $MgCl_2$, 0.2 U of KOD FX DNA polymerase (Toyobo Biochemicals). PCR amplification conditions were as follows: (94 °C, 2 min) $\times 1$; (94 °C, 15 s; 70 °C, 30 s; 72 °C, 2 min) $\times 35$; (72 °C, 5 min) $\times 1$. PCR products were analyzed on 12% polyacrylamide gels. This PCR primer set is positive for ft/ft and wt/ft but negative for wt/wt (**Fig. 2d**). To more conveniently genotype animals, we developed a PCR assay to detect a 15-bp insertion polymorphism at the 5' end of exon 3 in linkage disequilibrium with the ft mutation. This insertion creates an *AccI* restriction site. We used primers FTins15.F and FTins15.R (**Supplementary Table 3**) in standard PCR reactions using the following amplification conditions: (94 °C, 5 min) $\times 1$; (94 °C, 30 s; 58 °C, 30 s; 72 °C, 1 min) $\times 35$; (72 °C, 5 min) $\times 1$. Five microliters of PCR product were digested overnight with 2.5 U *AccI* and resolved on 1.5% agarose gels. The 678-bp C57BL/6 allele does not digest; the ft allele digests to yield fragments of 559 bp and 134 bp (**Fig. 2e**).

C-terminal antibody. The C-terminal antibody specific for mouse profilaggrin was generated against the amino-acid sequence ESIFTAKHLDFNQSHS. The peptide was conjugated to KLH via an N-terminal cysteine residue before injection into New Zealand white rabbits (Genemed Synthesis). The serum was affinity purified before use. Urea/Tris extracts were prepared from newborn homozygous ft/ft mice and control C57BL/6 mice as described¹¹. Protein samples were separated on 5–12% SDS/polyacrylamide gels and transferred to PVDF membrane (Millipore). Blots were probed with C-terminal antibody and immunoreactive proteins visualized with ECL (Amersham/GE Healthcare). A duplicate gel was stained with Coomassie Brilliant Blue R-250.

Mice. Breeding pairs of flaky tail (ft/ft) mice (original strain from Jackson Laboratory Stock a/a ma ft/ma ft/JSun; JR#9078; provided by J.P.S.) were backcrossed four generations onto a C57BL/6J strain background; C57BL/6J mice were used as wild-type (wt/wt) controls. Female mice were used in all experiments unless otherwise indicated. Flaky tail (ft/ft) mice were crossed with wild-type C57BL/6J mice to generate flaky tail heterozygous (wt/ft) mice. The OVA cutaneous sensitization experiments were repeated with F7 backcrossed mice, with littermates from wt/ft to wt/ft matings genotyped as wt/wt, wt/ft and ft/ft and used in experiments, giving identical results as the F4 backcross (data not shown). The mice were housed in SPF conditions, with irradiated diet and bedding provided. All animal experiments were done in compliance with Irish Department of Health and Children regulations and approved by the Trinity College Dublin's BioResources ethical review board.

Cutaneous challenge with OVA. We challenged the intact skin of wt/wt, wt/ft and ft/ft mice with OVA (Sigma Chemicals) by cutaneous exposure. We applied OVA in a regimen involving three cycles of daily exposure to OVA for 5 consecutive days (see **Supplementary Fig. 1a**). Abdominal hair was carefully shaved 24 h before application of OVA; mice with any visual damage to the skin were not used. OVA (fraction V; Sigma) was prepared in PBS (Dulbecco's PBS; Sigma) at 1 mg/ml. Mice were restrained and 50 μ l of OVA solution, or 50 μ l of PBS, was applied to the shaved skin and allowed to air dry. To address systemic allergen treatment, we challenged wt/wt and ft/ft mice with OVA in alum as an adjuvant by intraperitoneal injection as described³⁰ (see **Supplementary Fig. 3b**).

Measurement of trans-epidermal water loss (TEWL). We used a Courage and Khazaka Tewameter TM210 (Enviroderm) to measure TEWL. TEWL analysis of the abdomen of male and female adult (6–10 weeks of age) wt/wt and ft/ft mice occurred 24 h after shaving. Any mice with visual damage to the skin following shaving were not included in the analysis. TEWL was recorded at ambient temperature 19–21 °C and humidity 50% $\pm$ 5. In mice sensitized to OVA, TEWL was measured in PBS- and OVA-treated mice after OVA application.

Spleen cell preparation. Single-cell suspensions were prepared from spleens. Spleen cells were cultured in RPMI 1640 (Gibco-Invitrogen) supplemented with 10% (v/v) heat-inactivated FCS (FCS; Labtech), 2 mM L-glutamine (Gibco), 50 U/ml penicillin and 50 μ g/ml streptomycin (Gibco). Spleen cells were plated at 5×10^6 cells/ml. OVA was subjected to endotoxin removal for use in cell assays as described³⁰. Cells were unstimulated (media) or stimulated with OVA (250 and 500 μ g/ml) in a 24-well plate (Greiner Bio-One) at 37 °C

for 72 h. Supernatants were harvested after 72 h and cytokine levels (IL-4, IL-5, IL-13, IFN- γ , IL-10 and IL-17) were measured by ELISA. For cell proliferation analysis, cells were exposed to a range of concentrations of OVA in triplicate wells on 96-well plates. Cultures were pulsed with 1 μ Ci per well [³H]thymidine (Amersham) for the last 14 h of culture. Cells were harvested with a cell harvester (Tomec) and [³H]thymidine incorporation was measured by a Wallac β -counter (Perkin-Elmer).

Antibody and cytokine ELISA. Serum OVA-specific IgG, IgG1 and IgG2a levels were detected by direct ELISA using serial dilutions of sera. OVA-specific IgE was measured using biotinylated-OVA with 1:10 sera dilutions. Total serum IgE was measured using PharMingen antibodies (BD Biosciences), according to the manufacturer's instructions. We detected total serum IgG using commercial capture antibodies and IgG standard (Zymed Laboratories; Sigma). Sandwich ELISAs were done to quantify levels of specific cytokines in the supernatants cell cultures (IL-4, IL-5 and IL-13, assays supplied by PharMingen; IL-10, IL-17 and IFN- γ , assays supplied by R&D Systems).

Histology. Abdominal skin of age- and sex-matched mice (wt/wt, wt/ft and ft/ft) that were untreated or exposed to OVA was removed and fixed in 10% formaldehyde saline. Paraffin sections were stained with hematoxylin and eosin. Tissue sections were analyzed with slides blinded to genotype and exposure to allergen. To quantify the individual inflammatory cells infiltrating the skin, we used a previous described scoring system²⁰. We counted the total number of cells, lymphocytes, eosinophils and mononuclear cells per $\times 1,000$ high-power fields (HPF) of view, with 15–20 HPFs scored per mouse. Lungs were processed for histopathological analysis as described³⁰.

OVA challenge regimen for airway hyper-responsiveness (AHR). wt/wt and ft/ft mice were exposed to OVA cutaneously or intraperitoneally and then exposed to 20 min of continuous aerosol of 1% OVA each day for five consecutive days (as shown in **Supplementary Figs. 1b** and **3a**, respectively). Epicutaneous exposure to OVA was achieved by repeated tape-stripping of shaved skin²⁵ before OVA application using the regimen in **Supplementary Figure 1b**. AHR was analyzed the day following the final aerosol challenge. Mice were anesthetized, tracheostomized and ventilated. AHR was measured using a whole-body plethysmograph with a pneumotachograph linked to a transducer (EMMS). The average lung resistance (R_L) and the area under the resistance curve (R_L AUC) parameters were quantified for 3-min periods at baseline and after exposure to nebulized endotoxin-free PBS (Sigma) and acetyl- β -methylcholine chloride (Sigma) at 10, 30, 60 and 120 mg/ml. Broncho-alveolar lavage (BAL) fluids were collected by cannulating the trachea and lavaging the lungs with a 1.0 ml of ice-cold PBS. BAL cells were pelleted, washed and counted. The numbers of eosinophils in BAL were analyzed by flow cytometry as described³⁰. Lungs were removed from mice for histology.

Statistics. All animal experiments were repeated at least twice. GraphPad Prism and GraphPad Instat software were used for data analysis. Difference between groups was determined by Student's *t*-test or, if nonparametric, Welch corrected *t*-test. Differences were considered significant when $P < 0.05$.

Accession codes. BAC clone RP23-227A12, AC158361; 5' *Flg*, AF510860; 3' *Flg*, J03458; *Flg* complete sequence from ft mouse strain, FJ824603.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

The study was designed by W.H.I.M., P.G.F., A.D.I. and M.A. Molecular biology was performed by T.S., A. Sandilands, L.E.C., H.K., A. Shiohama, A.K., N.S. and J.K. Bioinformatics was performed by W.H.I.M. C-terminal profilaggrin antibody and immunoblot data was generated by R.B.P. and P.F. The initial mixed strain ft mice were provided by J.P.S. Mouse backcrossing, immunology and histological experiments were performed by P.G.F., S.P.S., N.E.M. and J.J.C. The manuscript was written by P.G.F., T.S., J.K., M.A., R.B.P., A.D.I. and W.H.I.M.

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SUPPLEMENTARY DATA

A homozygous frameshift mutation in the murine filaggrin gene facilitates enhanced percutaneous allergen priming

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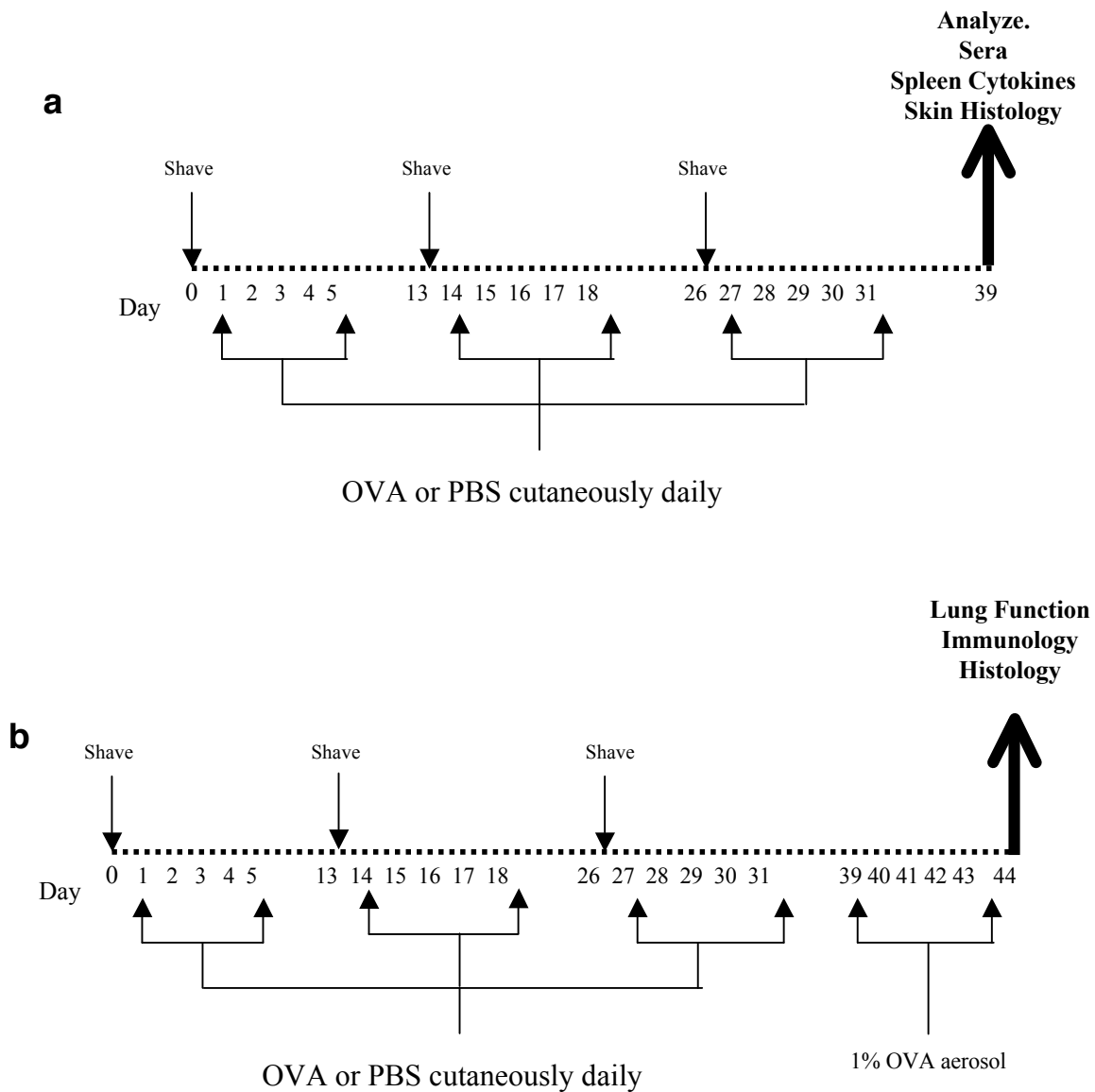
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* Equal contributions; ** Equal contributions

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Fig S1.

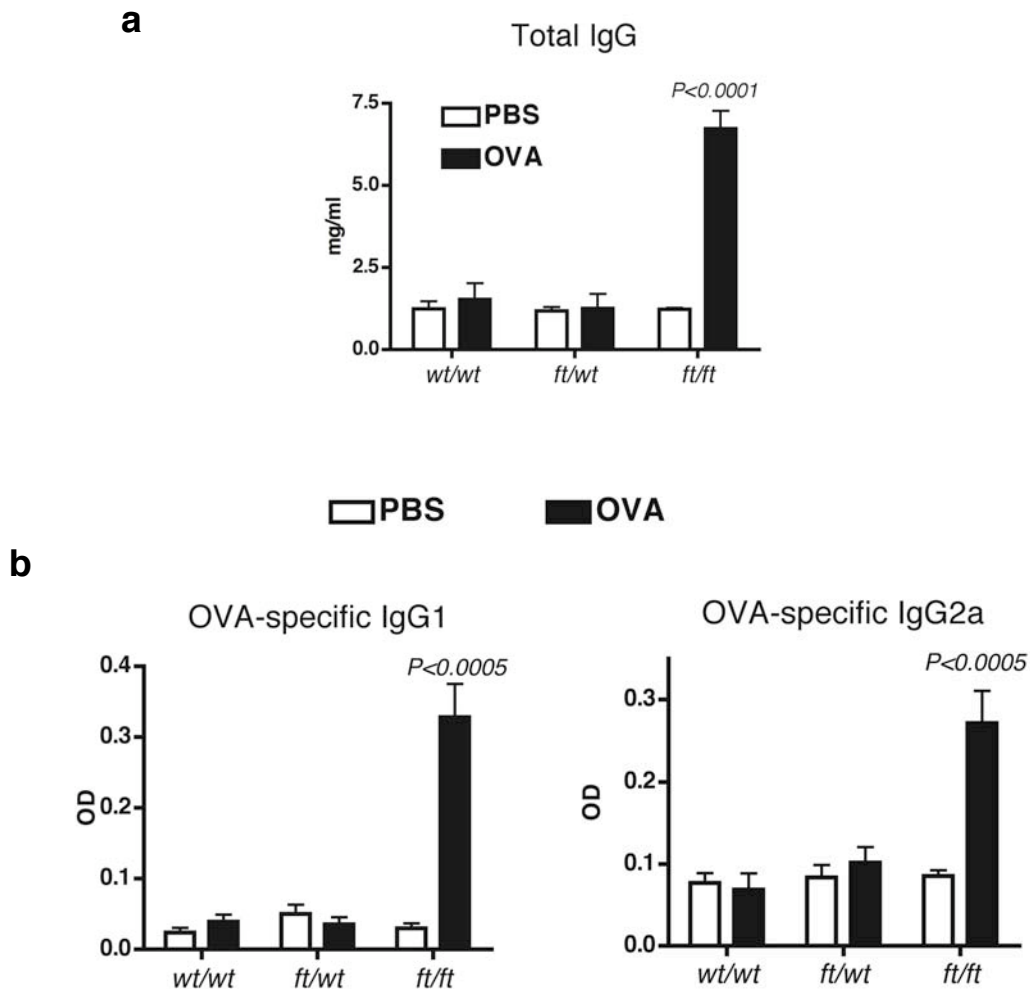


Supplementary Figure 1.

(a) Protocol for cutaneous application of Ovalbumin (OVA) to the intact skin of *wt/wt*, *ft/wt* and *ft/ft* mice. The abdomen was shaved on indicated days. A suspension of OVA (50 μ g in 50 μ l PBS) or 50 μ l PBS was applied to abdomen as shown.

To investigate pulmonary responses **(b)**, *wt/wt* and *ft/ft* mice were challenged with OVA as described **(a)**, prior to subsequent pulmonary OVA challenge. Mice were subjected to 20 mins of 1% OVA aerosol challenge for 5 days as indicated. Lung function was analysed by measurement of lung resistance in a dose response to methacholine challenge.

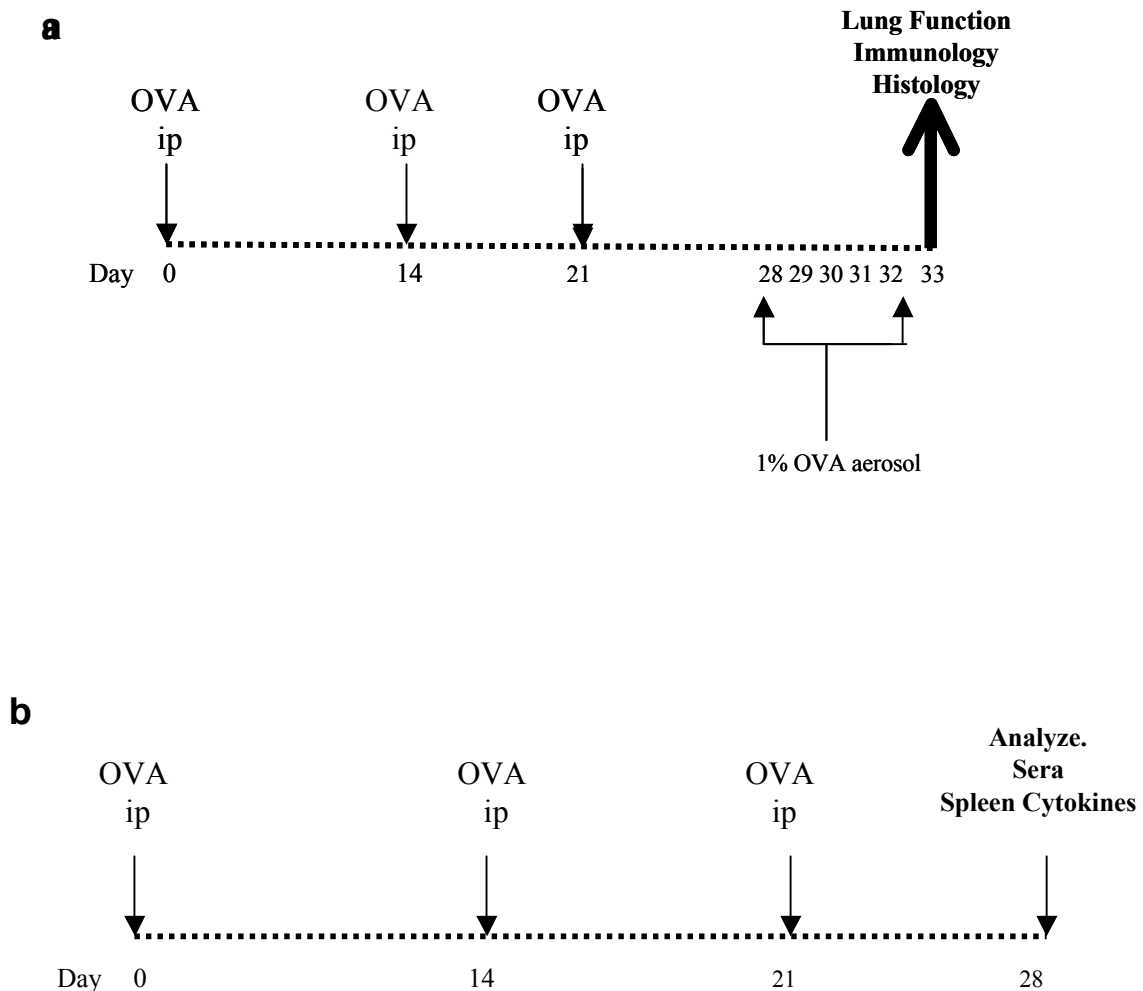
Fig S2.



Supplementary Figure 2.

ELISA detection of serum levels of **(a)** Total IgG and **(b)** OVA-specific IgG1 and IgG2a isotypes in *wt/wt*, *ft/wt* and *ft/ft* mice, exposed to Ovalbumin (OVA) or PBS by cutaneous application according to the sensitization protocol as described (**Fig S1a**). Student's or Welch's corrected t-test was used to test for statistical differences between PBS and OVA-treated groups. Data are Mean $\pm$ SEM from 4-7 mice. Data representative of two separate experiments.

Fig. S3

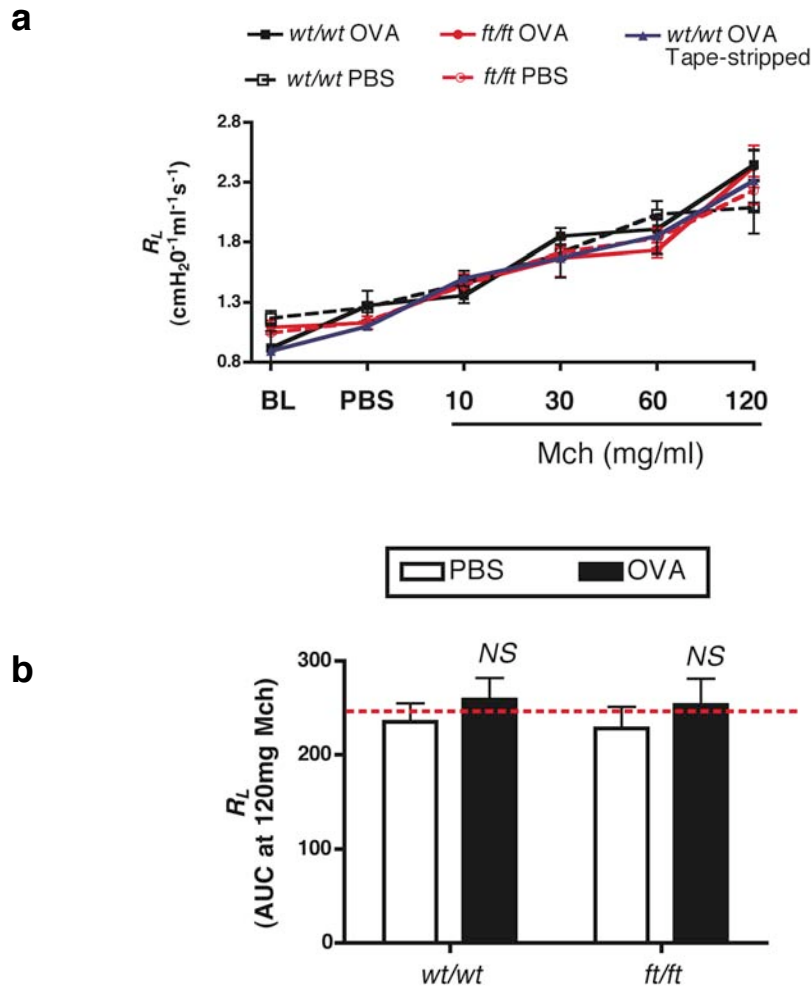


Supplementary Figure 3.

(a) Protocol for intraperitoneal (ip) challenge of *wt/wt* and *ft/ft* mice with Ovalbumin (OVA) and subsequent pulmonary OVA challenge. Mice were subjected to 20 mins of 1% OVA aerosol challenge for 5 days as indicated. Lung Functions was analysed by measurement of lung resistance in a dose response to methacholine challenge.

(b) Protocol for systemic immunization of *wt/wt* and *ft/ft* mice with OVA and adjuvant. On days 0, 14 and 21 mice received an ip injection of 100 μ l of a sensitization solution containing 20 μ g OVA and 2 mg ImjectTM Alum (Pierce) prepared in PBS, as described Mangan *et al.* 2006. Mice received 100 μ l PBS ip as a vehicle control.

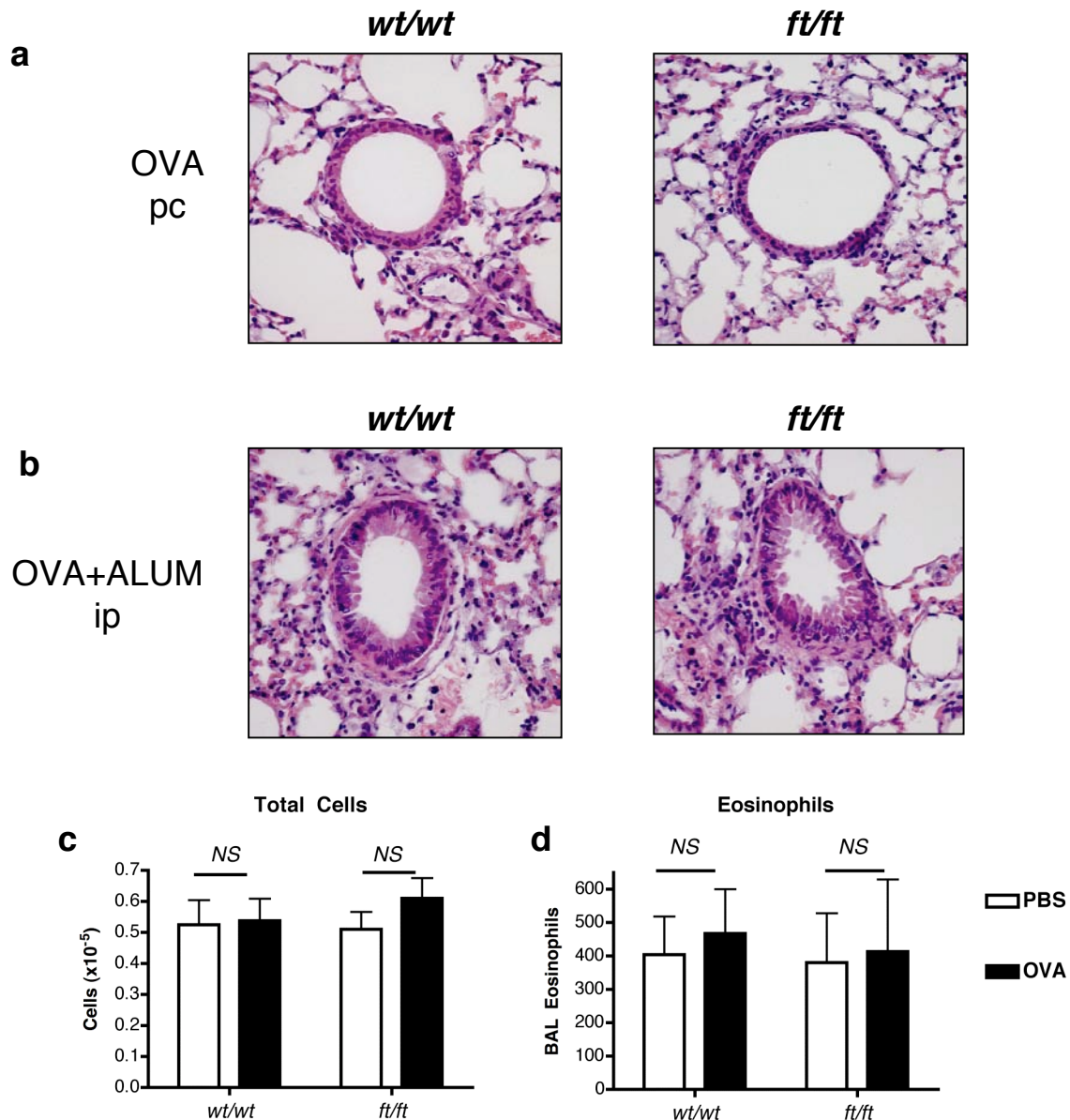
Fig. S4



Supplementary Figure 4.

No alterations in airway hyper responsiveness determined as lung resistance (R_L), in *wt/wt* and *ft/ft* mice cutaneously challenged with Ovalbumin (OVA), or in *wt/wt* mice exposed epicutaneously to OVA after tape-stripping. Mice were cutaneously challenged with OVA as described (**Fig. S1b**). For epicutaneous challenge, *wt/wt* mice were tape-stripped before application of OVA to skin. **(a)** Dose-response curve to Methacholine (Mch) challenge. BL = Baseline R_L . **(b)** Area Under Curve (AUC) of R_L values at 120 mg/ml Mch. The hatched red line is AUC for *wt/wt* mice exposed epicutaneously (tape-stripped) to OVA. Student's t-test was used to test for statistical differences between groups (NS = Non Significant). Data are Mean $\pm$ SEM from 5-7 mice. Data representative of two separate experiments.

Fig. S5

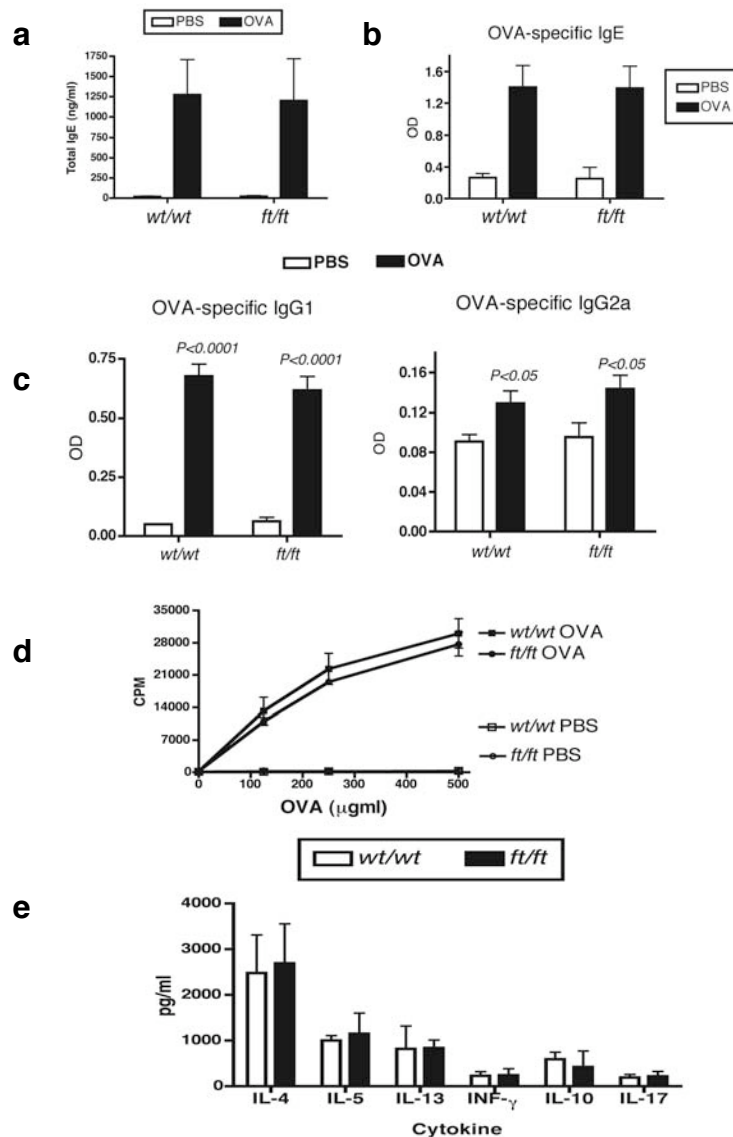


Supplementary Figure 5.

Representative photomicrographs of hematoxylin and eosin-stained lung sections (**a&b**), (original magnification $\times 40$). (**a**) Absence of pulmonary inflammation in *wt/wt* and *ft/ft* mice following cutaneous (pc) treatment with Ovalbumin (OVA) and subsequent exposure to OVA via aerosol as described (**Fig. S1b**). (**b**) Bronchial hyperplasia and cellular infiltrates in both *wt/wt* and *ft/ft* mice intraperitoneally (ip) immunized with OVA and adjuvant (Alum) and subsequent challenge with OVA via aerosol as described (**Fig. S3a**).

Comparable numbers of total cells (**c**) and eosinophils (**d**) were recovered in bronchoalveolar lavage (BAL) of *wt/wt* and *ft/ft* mice cutaneously treated with OVA or PBS prior to aerosol challenge as described (**Fig. S1b**). (**c**) Total number of cells recovered in BAL fluid. (**d**) Absolute numbers of eosinophils recovered in BAL. BAL eosinophils were detected by flow cytometry as CD4⁺CD8⁻CD19⁻CCR3⁺ (see Ref 31). Student's t-test was used to test for statistical differences between groups (NS = Non Significant). Data are Mean $\pm$ SEM from 4-7 mice. Data representative of three separate experiments.

Fig. S6

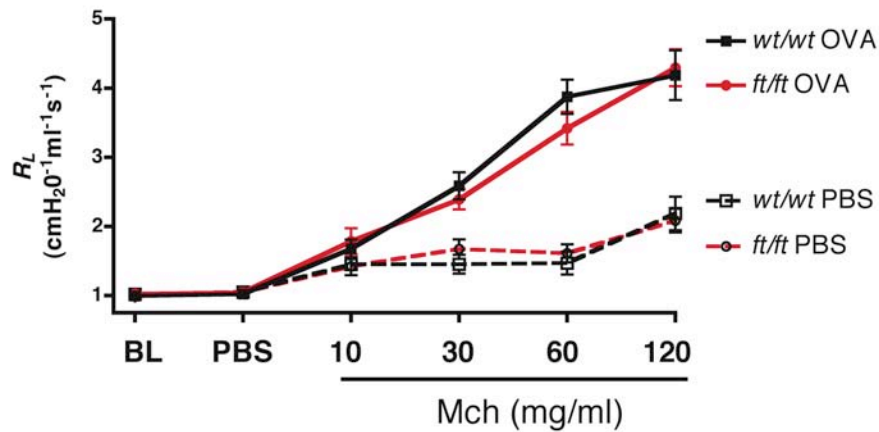


Supplementary Figure 6.

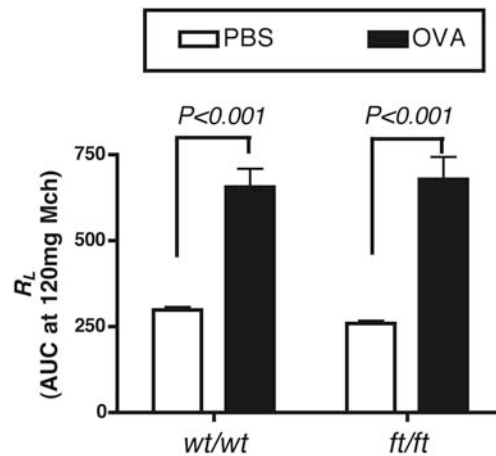
No differences in immunological responses between *wt/wt* and *ft/ft* mice injected intraperitoneally with Ovalbumin (OVA). Immunization protocol is as described (**Fig. S3b**). (**a**) Total Serum IgE and (**b**) OVA-specific IgE in PBS- or OVA-immunized mice. (**c**). Greater induction of OVA-specific IgG1 than IgG2a ($P < 0.0001$ and $P < 0.05$, respectively, relative to PBS-injected mice) in *wt/wt* and *ft/ft* mice injected with OVA and adjuvant. (**d**). Proliferation of spleen cells from PBS- or OVA-immunized *wt/wt* and *ft/ft* mice in response to OVA. (**e**). ELISA detection of OVA-induced cytokines from spleen cells from OVA-immunized *wt/wt* and *ft/ft* mice. Data are from 4-6 mice and representative of two separate experiments.

Fig. S7

a



b



Supplementary Figure 7.

Comparable induction of airway hyper responsiveness determined as lung resistance (R_L), in *wt/wt* and *ft/ft* mice. Mice were intraperitoneally challenged with Ovalbumin (OVA) as described (**Fig. S3a**). (**a**) Dose-response curve to Methacholine (Mch) challenge. BL = Baseline R_L . (**b**). Area Under Curve (AUC) of R_L values at 120 mg/ml Mch. Student's t-test was used to test for statistical differences between PBS and OVA immunized groups. There was no statistical differences in R_L between *wt/wt* and *ft/ft* mice. Data are Mean $\pm$ SEM from 4-6 mice. Data representative of two separate experiments.

Supplementary Table S1

Intron-exon organization of the murine filaggrin gene (*flg*), chromosomal band 3qF1-3qF2.1 (base numbering in base pairs from mm9, July 2007 assembly of the mouse genome).

Feature	Start	End	Size
Exon 1	93077463	93077494	32
Intron 1	93077495	93081473	3979
Exon 2	93081474	93081632	159
Intron 2	93081633	93083302	1670
Exon 3	93083303	93097613	14311

Supplementary Table S2

Resolution of assembly errors in *flg* ORF within filaggrin tandem repeat region.

<i>mRNA position</i>	<i>Genomic DNA position (mm9, July 2007)</i>
3186T>G	93,086,297T>G
3195T>G	93,086,306T>G
3312delGC	93,086,423delGC
3343insG	93,086,454insG
5946delG	93,089,057delG

Supplementary Table S3

Primers used for shotgun sequencing and genotyping.

<i>Designation</i>	<i>Purpose</i>	<i>Sequence 5'-3'</i>
mFlgdelA-F	Mutation delA genotyping	CGC CAG GTC CCG GGG CTC TAC
mFlgdelA-R	Mutation delA genotyping	TCT TGA GCC TGA TCT TGC GCT GA
FTins15.F	<i>Ft</i> 15 bp in/del marker	CAT CTC CAG TCA GGG CTG ACC
FTins15.R	<i>Ft</i> 15 bp in/del marker	GCT GCC TGT GGC CGG ACT CG
FTshotgun.F1	PCR of shotgun fragment 1	AAG ATC AGG CTC AGG AGG AAG
FTshotgun.R1	PCR of shotgun fragment 1	GAG CTG TGC CCT TGG TTA GAG
FTshotgun.F2	PCR of shotgun fragment 2	CCA GCG ACA GCG AGG GCC ACT C
FTshotgun.R2	PCR of shotgun fragment 2	CTC GCT GAC CCC TCG ACG CCC T
FTshotgun.F3	PCR of shotgun fragment 3	GAG GGC CGG ATC CAG CTC CGG CTC A
FTshotgun.R3	PCR of shotgun fragment 3	CTT CGA CCT GGA CTC CAG AAT GGA C
FTshotgun.F4	PCR of shotgun fragment 4	GCT GTT CGT GCT CAT GCT GGT G
FTshotgun.R4	PCR of shotgun fragment 4	GGG CTC CCT GGT GTC CTC TC
FTshotgun.F5	PCR of shotgun fragment 5	CAC GGC TCC GGA TAC TAC TAT G
FTshotgun.R5	PCR of shotgun fragment 5	GAC CGC TGC TCG TGC CTC TGG C
FTshotgun.F6	PCR of shotgun fragment 6	TCT CGC CAC CAG GCC ACT CTC
FTshotgun.R6	PCR of shotgun fragment 6	GAG AGA GGA GCT GTG CCC TTG