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Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis

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Atopic disease, including atopic dermatitis (eczema), allergy and asthma, has increased in frequency in recent decades[1] and now affects ~20% of the population in the developed world. Twin and family studies have shown that predisposition to atopic disease is highly heritable[2]. Although most genetic studies have focused on immunological mechanisms, a primary epithelial barrier defect has been anticipated[3]. Filaggrin is a key protein that facilitates terminal differentiation of the epidermis and formation of the skin barrier. Here we show that two independent loss-of-function genetic variants (R510X and 2282del4) in the gene encoding filaggrin (*FLG*) are very strong predisposing factors for atopic dermatitis. These variants are carried by ~9% of people of European origin. These variants also show highly significant association with asthma occurring in the context of atopic dermatitis. This work establishes a key role for impaired skin barrier function in the development of atopic disease.

The prominent keratohyalin granules seen by light microscopy in the granular layers of the outer epidermis are predominantly composed of the 400-kDa polyprotein profilaggrin[4,5]. Upon terminal differentiation of these keratinocytes to form the squames, profilaggrin is cleaved into 10–12 copies of the 37-kDa filaggrin protein[6]. These polypeptides aggregate the keratin cytoskeleton system to form a dense protein-lipid matrix that is cross-linked by transglutaminases to form the cornified cell envelope. This structure prevents epidermal water loss and also impedes the entry of allergens, toxic chemicals and infectious organisms[4]. Filaggrin (encoded by *FLG*) is also expressed in oral and nasal mucosa, where it is also assumed to contribute to epithelial barrier function[7]. Extending previous work that established the genetic linkage of the filaggrin locus to 1q21 (ref. 8), we recently identified two filaggrin mutations, R501X and 2282del4, both of which lead to complete loss of processed filaggrin product[9]. These null mutations were shown to be the cause of ichthyosis vulgaris in 15 families and isolated cases (maximum two-point lod score, Z_{max}, = 8.11 at $\theta = 0$)[9]. Ichthyosis vulgaris is semidominant: that is, heterozygotes had either no discernible phenotype or milder ichthyosis, whereas homozygotes or compound heterozygotes had marked ichthyosis and an overt histological skin barrier defect[9].

In our families with ichthyosis vulgaris, many individuals null or heterozygous for filaggrin also had atopic dermatitis ('eczema') and, in a few cases, also had asthma (**Fig. 1**). Specifically, atopic dermatitis was prevalent in the individuals with mild ichthyosis vulgaris, all of whom were heterozygous for a *FLG* null allele (13/29; 44%). Atopic dermatitis was particularly common in individuals with severe ichthyosis vulgaris, all of whom were homozygous or compound heterozygous for *FLG* null alleles (16/21; 76%). None of the individuals in these families who lacked a *FLG* null allele had atopic dermatitis ($n = 13$).

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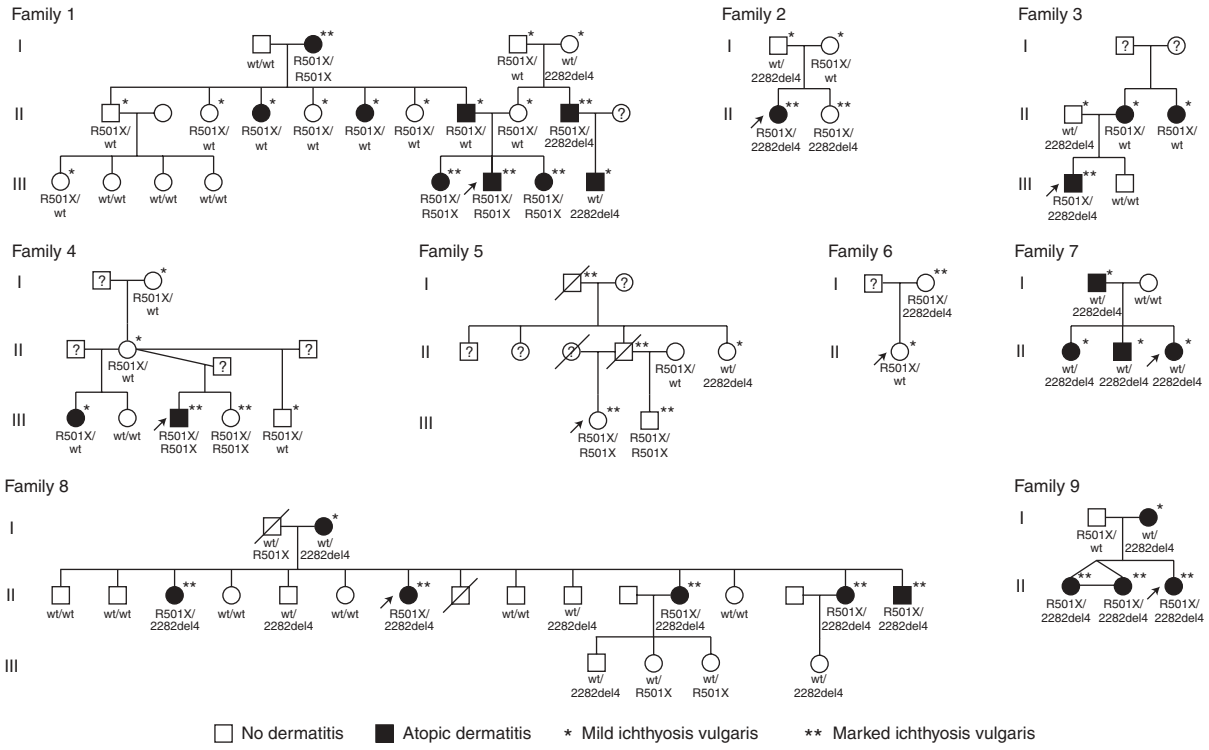

Figure 1 Pedigrees of the nine families studied with semidominant inheritance of ichthyosis vulgaris and atopic dermatitis ('eczema'). Open symbols denote absence of atopic dermatitis; filled symbols denote physician-diagnosed atopic dermatitis; * denotes physician-diagnosed mild presentation of ichthyosis vulgaris and ** denotes physician-diagnosed marked ichthyosis vulgaris phenotype. Genotypes for the two *FLG* null alleles R501X and 2282del4 are shown, where known. Some genotypes in family 8 and in generation II of family 1 were inferred from the previously reported haplotype data for the *FLG* locus[8]. All phenotypes shown were determined through direct examination by experienced dermatologists. Families 1–6 and family 8 have been reported previously[9]. Here, using a semidominant model with penetrance reduced to 45%, on the basis of our asthma/atopic dermatitis cohort study (**Table 2**), linkage between the *FLG* null alleles and atopic dermatitis gives a statistically significant two-point lod score, demonstrating that, in these families, atopic dermatitis behaves as a low-penetrance transmissible trait that cosegregates with *FLG* null alleles. wt, wild-type for the R501X and 2282del4 variants.

Thus, atopic dermatitis is inherited as a semidominant trait in these families with high penetrance in *FLG*-null homozygotes or compound heterozygotes and reduced penetrance in heterozygotes. Only six individuals from these families had asthma (two R501X heterozygotes, two R501X homozygotes and two R501X/2282del4 compound heterozygotes), and the lod score for asthma was not significant. In contrast, genetic linkage between atopic dermatitis and *FLG* null alleles in these families gave a significant two-point lod score ($Z_{max} = 3.27$) with no recombination ($\theta = 0$) when penetrance in heterozygotes and homozygotes was set at 45% (on the basis of our atopic dermatitis/asthma cohort study, described below). Recalculation with 30% penetrance still gave a significant lod score ($Z_{max} = 3.08$) at $\theta = 0$. Notably, we did not observe any recombination between *FLG* and atopic dermatitis in these families. These data strongly imply that *FLG* null alleles are a frequent transmissible predisposing factor in common atopic dermatitis.

To investigate this further, we obtained a small cohort consisting of 52 Irish pediatric patients who presented with dermatologist-diagnosed atopic dermatitis. The clinical characteristics of the atopic dermatitis cohort are summarized in **Supplementary Table 1** online. The combined allele frequency for *FLG* R501X and 2282del4 variants in an anonymous unselected Irish control population ($n = 189$) was 0.042. The variants were greatly overrepresented in the cohort with atopic dermatitis, with a combined allele frequency of 0.330, demonstrating a highly significant dominant risk for atopic dermatitis (**Table 1**; odds ratio (OR) = 13.4; 95% confidence interval (c.i.) = 6.2–27.5, $P < 0.0001$). Both homozygotes and compound heterozygotes were identified in this patient group ($n = 6$). Nearly half of the individuals with atopic dermatitis also had documented asthma, and this was similar for those with different *FLG* genotypes. Specifically, 48% of *FLG* null carriers had asthma; 41% of individuals with the wild-type *FLG* variants had asthma.

Table 1 Frequency of *FLG* null alleles in the Irish AD cohort

	R501X		2282del4		Combined genotype		
Genotype	Population controls	AD	Population controls	AD	Population controls	AD	AD + asthma
AA	177	32	182	38	170	23	9
Aa	12	20	4	13	16	23	10
aa	0	0	0	1	0	6	2
	189	52	186	52	186	52	21
	$P = 6 \times 10^{-9}$		$P = 8 \times 10^{-10}$		$P = 3 \times 10^{-17}$		$P = 6 \times 10^{-12}$

FLG null alleles are overrepresented in the Irish children attending hospital dermatology clinics with atopic dermatitis (AD). The association seen here was stronger with atopic dermatitis than with the combined atopic dermatitis plus asthma phenotype. 'AA' refers to wild-type/wild-type *FLG* genotype for R501X and 2282del4 variants; 'Aa' refers to heterozygous genotype for either R501X or 2282del4 and 'aa' refers to homozygous R501X or 2282del4 genotype or compound heterozygous genotype.

Table 2 Frequency of *FLG* null alleles in the Scottish childhood asthma cohort

	R501X		2282del4		Combined genotype		
Genotype	Population controls	Asthma	Population controls	Asthma	Population controls	Asthma	AD + asthma
AA	950	551	970	559	914	509	215
Aa	55	51	38	43	89	88	57
aa	3	2	0	2	5	7	7
	1008	604	1008	604	1008	604	279
	$P = 0.024$		$P = 0.00089$		$P = 6 \times 10^{-5}$		$P = 4.8 \times 10^{-11}$

Both R510X and 2282del4 are overrepresented in a Scottish childhood asthma cohort, and the strongest association was with the combined atopic dermatitis plus asthma phenotype. 'AA' refers to wild-type/wild-type *FLG* genotype for R501X and 2282del4 variants; 'Aa' refers to heterozygous genotype for either R501X or 2282del4 and 'aa' refers to homozygous R501X or 2282del4 genotype or compound heterozygous genotype.

We proceeded to replicate this association in a larger cohort ascertained on the basis of asthma, but, notably, ~50% of this patient cohort also had a history of atopic dermatitis in association with asthma. Both *FLG* variants were genotyped in 1,008 Scottish schoolchildren with unknown disease status (the population cohort) and in 604 Scottish schoolchildren and adolescents with physician-diagnosed asthma from the BREATHE study (**Table 2**). Both groups were recruited from the Tayside area of Northeast Scotland. The clinical characteristics of the asthma cohort are summarized in **Supplementary Table 1**. The frequency of carriers of R501X was 5.8%, and the 2282del4 variant was present in 3.8% of the schoolchildren, giving a combined carrier frequency of 9.6%. Both filaggrin variants were overrepresented in the asthmatic cohort (R501X: 9.2%, 2282del4: 7.5%, combined carrier frequency: 15.7%), with carriers of either allele demonstrating a dominant risk for the asthma phenotype alone (R501X dominant: OR = 1.6, 95% c.i. = 1.1–2.3, $P = 0.025$; 2282del4 dominant: OR = 2.1, 95% c.i. = 1.3–3.2, $P = 0.002$; combined genotype dominant: OR = 1.8, 95% c.i. = 1.3–2.5, $P = 0.0002$). We found homozygotes for both variants in the asthma cohort, as well as two compound heterozygotes. In contrast, we did not observe any homozygotes of 2282del4 in the population controls.

Atopic dermatitis is frequently associated with asthma[3], and because filaggrin is a major epidermal protein, *FLG* null alleles should associate more strongly with the combined atopic dermatitis/asthma phenotype than with asthma alone. Consistent with this, 72% of all the children in the asthma cohort carrying a *FLG* null allele had atopic dermatitis, in contrast to only 46% of those without these filaggrin variants. The *FLG* null allele carrier frequency in this asthma cohort was 23% (**Table 2** and **Fig. 2a**; OR = 3.3, 95% c.i. = 2.0–5.6, $P = 0.000004$). A codominant model comparing individuals with asthma and atopic dermatitis to the population controls provided the best fit (**Table 2**, $P = 4.8 \times 10^{-11}$). This model performed particularly well, as every individual in the homozygous null group had atopic dermatitis ($n = 7$). Notably, the exclusion of the *FLG* null individuals still produced a substantial association between heterozygotes and atopic dermatitis (OR = 3.1, 95% c.i. = 1.8–5.3, $P = 0.000013$), confirming the risk conferred by heterozygous *FLG* variants. The association with asthma was solely in individuals who also had atopic dermatitis, as there was no association with asthma in the absence of atopic dermatitis (**Fig. 2b**; OR = 0.8, 95% c.i. = 0.5–1.3). Thus, *FLG* variants are only a predisposing factor for the clinical subtype of asthma that occurs in the context of existing atopic dermatitis.

To provide further replication of this disease association and to gain longitudinal information into the population risks of the *FLG* variants, we genotyped the two *FLG* null variants in 372 Danish children who had been followed prospectively since pregnancy in a cohort study that recruited Danish mothers with asthma (the COPSAC study)[10]. The frequency of carriers of R501X was 5%, and the 2282del4 variant was present in 7% of the children, with a combined carrier frequency of ~11%. Homozygotes for both variants were observed in three children in the COPSAC cohort, two with atopic dermatitis. The combined filaggrin variants were overrepresented in the children with atopic dermatitis compared with children without this diagnosis (**Table 3**; hazard ratio = 2.8; 95% c.i. = 1.7–4.5, $P < 0.0001$). The majority of individuals carrying the *FLG* null variants had atopic dermatitis (63%), whereas only 40% of the individuals with wild-type *FLG* had atopic dermatitis. In this prospective study, 17.5% of all individuals with atopic dermatitis were carriers of *FLG* null alleles. The number of individuals in this cohort with asthma was small, as expected for this young age (median age of asthma onset = 3 years); however, we detected a significant increase in atopic dermatitis plus asthma in carriers of the *FLG* null alleles when compared with the non-atopic individuals (**Table 3**). This was not significant when compared with individuals without asthma but with atopic dermatitis, thus confirming the previous observations that the increased asthma accompanies the increase in atopic dermatitis but is not an independent association. In addition, the longitudinal

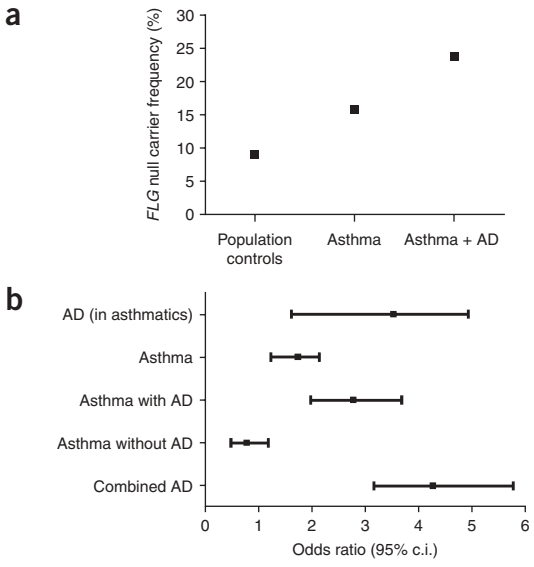

Figure 2 Filaggrin variants represent a substantial fraction of individuals with asthma and atopic dermatitis (AD). (**a**) Carrier frequencies of the combined filaggrin null mutations in the Scottish population, the asthmatic population and the asthmatic population with atopic dermatitis. (**b**) Filaggrin variants are selectively associated with individuals with both asthma and atopic dermatitis, and no association is seen with asthma in the absence of atopic dermatitis. Odds ratios are shown with the population frequency as the reference, except for the comparison, atopic dermatitis (in asthmatics), in which the referent category is asthmatics without atopic dermatitis. The Scottish and Irish atopic dermatitis cases and population controls were combined, and the odds ratio is shown ('Combined AD').

Table 3 Frequency of *FLG* null variants in the Danish birth cohort

Genotype	Combined genotype: Individuals without AD	Combined genotype: Individuals with AD	AD + asthma
AA	175	117	21
Aa	14	23	5
aa	1	2	0
	190	142	25
		$P = 0.006$[a]	$P = 0.027$[a]

FLG null variants are overrepresented in the children with atopic dermatitis at 3 years of age in a Danish birth cohort study (COPSAC). Here again, the association with atopic dermatitis (AD) is more significant than with atopic dermatitis with asthma, although it should be borne in mind that the children in this cohort are well below the median age of onset of asthma. 'AA' refers to wild-type/wild-type *FLG* genotype for R501X and 2282del4 variants; 'Aa' refers to heterozygous genotype for either R501X or 2282del4; 'aa' refers to homozygous R501X or 2282del4 genotype or compound heterozygous genotype. [a]Compared with individuals without atopic dermatitis.

analysis demonstrated a substantial increased risk for total asthma that did not quite reach statistical significance (hazard ratio = 2.0; 95% c.i. = 0.9–4.1; $P = 0.07$).

Atopic disease seems to be increasing in incidence in Western nations, with Scotland and Ireland having the highest incidence of asthma worldwide[11]. We therefore examined the frequency of the *FLG* variants in the Centre d'Etude du Polymorphisme Humain (CEPH) Human Genome Diversity panel plus additional samples (**Supplementary Table 2** online). This showed that both variants were absent in non-European populations such as those of African and Asian origin. We observed only two heterozygotes (one each for R510X and 2282del4) in the CEPH biodiversity panel, in individuals of Mexican and Basque origin, respectively. Similarly, we identified three 2282del4 heterozygotes in the Indian population. European ancestry is possible in all these cases.

In children, atopic dermatitis is often the earliest sign of atopic disease, and in many cases may be followed by food allergy, asthma and allergic rhinitis[12]. Individuals with atopic dermatitis patients have impaired barrier function and an inherently abnormal stratum corneum in both lesional and nonlesional skin[13]. There are several lines of evidence implicating reduced filaggrin expression in atopic dermatitis. Immunohistochemistry and ELISA assays have demonstrated filaggrin deficiency in both lesional and nonlesional skin of atopic dermatitis patients[14,15]. Recently, microarray analysis of active atopic skin demonstrated decreased filaggrin mRNA expression[16]. Genome-wide analysis has shown several linkage peaks for atopic dermatitis, including the region of 1q21 encompassing the *FLG* locus[17]. Finally, we recently identified two common semidominant mutations in *FLG* in several familial cases of ichthyosis vulgaris, many of whom have atopic dermatitis[9]. Ultrastructural analysis of known *FLG* null homozygotes with ichthyosis vulgaris demonstrated a poorly formed stratum corneum[9]. The association of ichthyosis vulgaris with the atopic diathesis is well established: 37–50% of people with ichthyosis vulgaris have atopic diseases[18,19], and ~8% of atopic dermatitis patients have classical features of ichthyosis vulgaris[18,20].

Over the past several years, the mechanisms by which allergen exposure through the skin could initiate systemic allergy and predispose to asthma have become clearer. Epicutaneous sensitization with a protein allergen has been shown to induce a Th2-type immune response with concomitant high IgE production in mice[21]. In mice sensitized with ovalbumin, cutaneous exposure of this allergen induced a T cell– and eosinophil-rich cellular infiltrate along with a cytokine response that closely mirrored those seen in atopic dermatitis. In addition to generating allergen-specific IgE, these mice developed airway hyperresponsiveness on intravenous challenge with methacholine after a single inhalation challenge with ovalbumin[22]. Although these studies have not been repeated in human subjects, these observations in mice imply that the presence of a heritable defect in the skin barrier may lead to increased susceptibility to transepidermal allergen transfer, therefore amplifying the epidermal-driven Th2 response and provoking the onset of atopic disease.

Although we have identified two common mutations, it is likely that other *FLG* mutations will be identified in Western European populations. It is also probable that other populations will have specific mutation profiles, some of which may allow expression of small numbers of filaggrin repeats compared with R501X and 2282del4, that lead to the complete loss of filaggrin peptide production[9]. The penetrance of the *FLG* null alleles may be further modulated by the known allelic variation in repeat number (10–12 repeats; ref. 6), which may in itself predispose to dry skin phenotypes[23]. Furthermore, as the epidermal differentiation gene cluster on 1q21 contains many other genes involved in skin barrier function[4], it is possible that variants in neighboring genes are also involved in the pathogenesis of atopic dermatitis and asthma. The presence of two common *FLG* null alleles plus potential effects from nearby genes (*FLG* is one of seven 'fused S100 protein' genes in a tightly linked gene cluster) may have combined to lessen the observed genetic linkage to this locus[17]. Thus, heritable skin barrier defects involving 1q21-encoded epithelial barrier proteins may be highly prevalent or even a prerequisite for atopic dermatitis.

Our data provide robust evidence of a heritable genetic defect common to atopic dermatitis and associated asthma. We have initially shown transmission of the *FLG* variants in families with ichthyosis vulgaris and atopic dermatitis, which we then confirmed in two population-based case-control studies and in a prospective birth cohort study. We have thus replicated this strong association in four complementary data sets. In all of these studies, each of the two independent *FLG* null alleles showed highly significant association with atopic dermatitis and a corresponding increase in asthma, providing further within-study replication. Our data suggest that these variants should be studied in different contexts related to atopy. In our families with ichthyosis vulgaris, asthma was much less common than atopic dermatitis: of 59 people carrying one or two *FLG* null alleles, 10% had asthma, whereas 49% had atopic dermatitis (**Fig. 1**). The exact contribution to the overall prevalence of atopic dermatitis and asthma is complicated, with temporal and disease severity issues, in addition to environmental effects. The highest frequency of the *FLG* null alleles was observed in a cohort ascertained in an atopic dermatitis clinic and therefore represents the greater severity and widest age range of children, whereas the lowest prevalence (17%) was observed in the careful survey of a birth cohort from Denmark born of asthmatic mothers[10], which would include all degrees of severity of atopic dermatitis and is an accumulated risk to 3 years of age. Notably, although the prevalence was lower in the birth cohort, the observed penetrance of the *FLG* null alleles was very high, with 63% of the *FLG* null allele carriers demonstrating atopic dermatitis by the age of 3. The precise relationship of filaggrin status to the general atopic state and to specific intermediate phenotypes such as food allergy, bronchial hyperreactivity, house dust mite allergy[24] and the suggested distinctions between intrinsic and extrinsic atopic dermatitis[25] and asthma will be further delineated by analysis of additional cohorts.

In conclusion, this study firmly establishes inherited reduction or loss of filaggrin expression as a major predisposing factor in atopic dermatitis and provides a molecular mechanism to define the coexistence of a clinical subtype of asthma with atopic dermatitis. Two common mutations, 2282del4 and R501X, seem to be the major filaggrin variants in populations of European origin. Further longitudinal studies of individuals carrying these *FLG* null alleles will help define the lifetime health risks associated with this specific barrier function deficit.

METHODS

Study populations. All DNA samples were collected with informed consent, in compliance with all principles of the Helsinki Accord. Families with ichthyosis vulgaris were from dermatology clinics in Ireland, Scotland and the USA, as previously described[9]. For the Irish atopic dermatitis cohort, all patients met both the Hannifin and Rajka and UK Working Party definition of atopic dermatitis[26,27] as assessed by an experienced dermatologist (A.D.I., G.M.O'R. or R.M.W.). Severity of atopic dermatitis was assessed using the validated Nottingham Eczema Severity Score (NESS)[28]. All asthma patients were recruited through the children's asthma clinics in primary or secondary care under the Tayside BREATHE study and asthma diagnosed by the physician according to the Scottish Intercollegiate Guidelines Network/British Thoracic Society Diagnostic Guidelines. In addition, parents were asked, "Has your child ever had eczema or an itchy rash?" Local (Tayside) allele frequencies were obtained from a group of children (ages 4–10) recruited from primary schools as previously described[29]. For the birth cohort analysis (**Table 3**), we used 372 Danish children followed prospectively since pregnancy in a cohort study recruiting Danish mothers with asthma (COPSAC study)[10]. The development of atopic dermatitis and asthma during the first 3 years of life was monitored prospectively by close clinical follow-up and diary cards with complete follow-up data for 332 of the children. Atopic dermatitis was defined at regular clinic visits using the criteria of Hannifin and Rajka and asthmatic symptoms were monitored by daily diaries[30]. Asian and North African population samples (obtained by K.McE.; see **Supplementary Table 2**) were used in addition to the CEPH Human genome diversity panel.

Filaggrin genotyping. All primers and probe sequences are shown in **Supplementary Table 3** online. Mutation R501X creates a new *Nla*III restriction enzyme site, and 2282del4 creates a new *Dra*III site, which were used to screen short, highly specific PCR fragments for these variants, as described previously[9]. Genotyping for R501X was also performed using a TaqMan-based allelic discrimination assay (Applied Biosystems). Standard procedures were used based on Applied Biosystems reagents and 10-μl reaction volumes. Allelic discrimination was assessed using an Applied Biosystems 7700 sequence detection system. Mutation 2282del4 was also genotyped by sizing a fluorescently labeled PCR fragment on an Applied Biosystems 3100 or 3730 DNA sequencer. Ten-microliter PCR reactions were carried out using primers DEL4.F2 and DEL4.R1 in AmpliTaq Gold buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems), 10 nmol of each dNTP and 1 unit AmpliTaq Gold DNA polymerase. Reactions were amplified as follows: 94 °C (12 min), one cycle; 94 °C (15 s), 58 °C (30 s) and 72 °C (45 s), 30 cycles; and 72 °C (5 min), one cycle. Fragments were diluted 1:60 and sized against ROX-500 size markers according to the manufacturer's recommended protocol (Applied Biosystems). The wild-type allele was 199 bp, and the 2282del4 allele was 195 bp.

Statistical analysis. All statistical analysis in the association study was performed using SPSS for Windows, v.11.5 or Instat 3 for Macintosh (Graphpad Software). Allele frequencies were compared using Pearson χ^2 tests of the three genotype frequencies (AA, Aa, aa). Odds ratios for dominant models (AA versus aX) were determined using Fisher exact tests and binary logistic regression. All *P* values shown are not corrected for multiple testing, as all the tests of the primary hypotheses firmly rejected the null hypothesis. All variants were in Hardy-Weinberg Equilibrium (HWE), with the exception of R501X in the Tayside population control sample ($\chi^2 = 4.97$, $P = 0.026$). This deviation was not significant after correction for multiple testing, or after combining with the other European samples (**Table 2**), and there was no *a priori* reason for this one sample to deviate from HWE. Lod scores were calculated with MLINK algorithm of LINKAGE version 5.1, using a semi-dominant model of the disease with a range of low penetrance values (30, 45 and 70%). The penetrance of 45% was estimated from the asthma cohort and therefore represents a realistic approximation. The combined European mutant allele frequency was assumed to be 0.045 (**Supplementary Table 2**). For longitudinal survival analysis of the Danish COPSAC cohort, Cox's regression was used to determine the association of genotype with atopic dermatitis from birth until 3 years of age.

URLs. Scottish Intercollegiate Guidelines Network/British Thoracic Society Diagnostic Guidelines: http://www.sign.ac.uk/guidelines/; CEPH Human genome diversity panel: http://www.cephb.fr/HGDP-CEPH-Panel/.

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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