

ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

Dominant atopy risk mutations identified by mouse forward genetic analysis

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Abstract

Background: Atopy, the overall tendency to become sensitized to an allergen, is heritable but seldom ascribed to mutations within specific genes. Atopic individuals develop abnormally elevated IgE responses to immunization with potential allergens. To gain insight into the genetic causes of atopy, we carried out a forward genetic screen for atopy in mice.

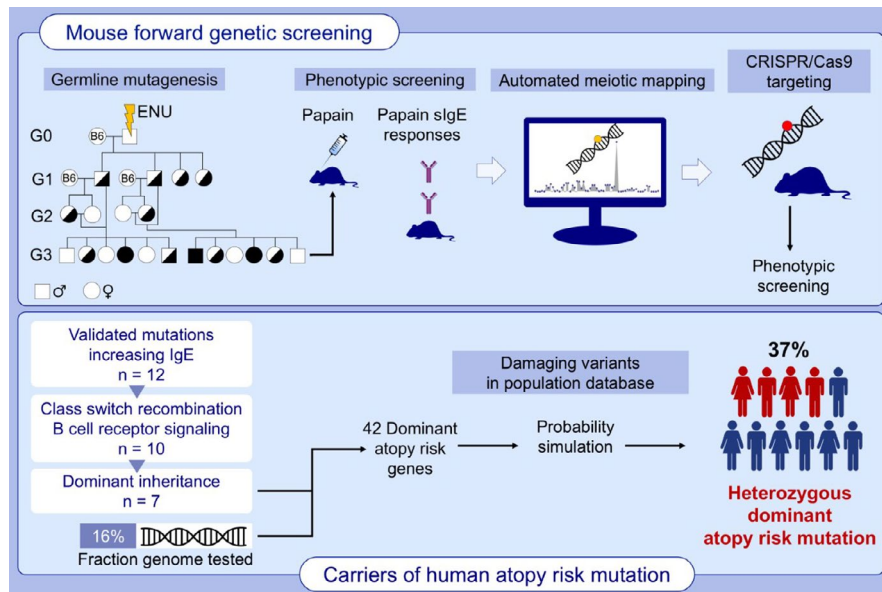
Methods: We screened mice carrying homozygous and heterozygous *N*-ethyl-*N*-nitrosourea (ENU)-induced germline mutations for aberrant antigen-specific IgE and IgG1 production in response to immunization with the model allergen papain. Candidate genes were validated by independent gene mutation.

Results: Of 31 candidate genes selected for investigation, the effects of mutations in 23 genes on papain-specific IgE or IgG1 were verified. Among the 20 verified genes influencing the IgE response, eight were necessary for the response, while 12 repressed IgE. Nine genes were not previously implicated in the IgE response. Fifteen genes encoded proteins contributing to IgE class switch recombination or B-cell receptor signaling. The precise roles of the five remaining genes (*Flcn*, *Map1lc3b*, *Me2*, *Prkd2*, and *Scarb2*) remain to be determined. Loss-of-function mutations in nine of the 12 genes limiting the IgE response were dominant or semi-dominant for the IgE phenotype but did not cause immunodeficiency in the heterozygous state. Using damaging allele frequencies for the corresponding human genes and in silico simulations (Monte Carlo) of undiscovered atopy mutations, we estimated the percentage of humans with heterozygous atopy risk mutations.

Conclusions: Up to 37% of individuals may be heterozygous carriers for at least one dominant atopy risk mutation.

KEYWORDS

allergy, atopy, IgE, IgG1, papain



GRAPHICAL ABSTRACT

This study uses a classical genetic screen employing automated meiotic mapping of pregenotyped mice and CRISPR/Cas9-based verification to identify numerous mutations causing altered IgE responses to papain immunization. Three-quarters of mutations that increased allergen-specific IgE are dominant or semidominant, without causing immunodeficiency in the heterozygous state. Up to 37% of immunocompetent humans may carry a heterozygous, dominant atopy risk mutation. Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; ENU, *N*-ethyl-*N*-nitrosourea.

1 | INTRODUCTION

Immunoglobulin E (IgE) antibodies contribute to protective immunity, particularly against parasites, but in atopic individuals, high levels of IgE mediate allergic reactions to otherwise innocuous antigens. Concentrations of IgE antibodies in circulation are much lower than those of other immunoglobulin classes, but become elevated in individuals with allergic disease. Several mechanisms limit circulating IgE concentration and the proportion of IgE-expressing B lymphoid cells. These include negative regulation of IgE class switch recombination (CSR),^{1–3} the short intrinsic half-life of IgE,⁴ and capture or clearance of IgE from the serum by mouse FcεRII and human FcεRI, respectively.^{5,6} Class switched IgE-expressing B cells are themselves tightly controlled by a strong propensity to differentiate into short-lived plasma cells rather than germinal center cells; they may also be programmed to undergo apoptosis.^{7–12} These effects are mediated by membrane IgE (mIgE) in an antigen-independent manner via constitutive signaling through CD19, Syk, Btk, PI3K, Akt, IRF4, and BLNK, leading to the generation of short-lived plasma cells and cell death, BCR downregulation, and/or delayed cell cycle progression.^{12,13} mIgE and CD19 signaling may suppress the generation of IgE-expressing long-lived plasma cells.

Atopy is known to be heritable^{14–17} but the relative contributions of variants of mIgE, its signaling pathway, and other pathways, to the overall incidence of atopy in the human population, are unknown. In addition, whether *de novo* mutations arising in the population might be causal factors for the increasing commonness of reported allergic

diseases is an open question. We present a mouse forward genetic screen to identify genes with nonredundant function in the regulation of the IgE response to allergens. Intact pedigrees of third-generation (G3) C57BL/6J mice with mixed zygosity for *N*-ethyl-*N*-nitrosourea (ENU)-induced mutations were immunized by intraperitoneal (i.p.) injection with the model allergen papain. Like many allergens, papain is a cysteine protease that stimulates Th2 responses, IgE production, and eosinophilia.^{18–20} Papain immunization elicits papain-specific antibodies in the absence of alum or other adjuvants; thus, papain acts as both adjuvant and antigen. Two weeks after immunization, serum papain-specific IgE was measured, along with papain-specific IgG1 in some instances. Mutations causing either an increase or decrease in the papain-specific IgE or IgG1 responses were identified by automated genetic mapping.²¹ We extrapolate from the number of validated mutations and the genome saturation achieved to estimate a genomic footprint for atopy under the specific conditions of our screen. We discuss the implications of our findings in understanding the etiology of allergic disease in humans.

2 | MATERIALS AND METHODS

Detailed descriptions of CRISPR/Cas9 gene targeting in mice, measurement of eosinophils, ELISA analysis, flow cytometry, T cell-dependent and T cell-independent antigen immunization and measurement of antibody responses, and measurement of TNF production by macrophages are provided in the online supplement.

2.1 | Mice

Male C57BL/6J mice (designated G0) were mutagenized with ENU as previously described.²² Three generations (G1, G2, and G3) of mice were bred from the G0 mice; G3 mice were subjected to phenotypic screening.²¹ The sources of previously reported mouse strains are provided in the online supplement. Male and female mice were used in all experiments and data for males and females were combined for analysis. Mice were maintained at the University of Texas Southwestern Medical Center. All studies were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 | Immunizations

For dose-response studies, C57BL/6J mice were injected i.p. on day 0 with the indicated dose of papain (EMD Millipore, 5125 Carica papaya) in 0.5 mL PBS. For screening, G3 mice were injected i.p. on day 0 with 0.5 mg papain in 0.5 mL PBS; subcutaneous immunization (0.5 mg papain in 0.2 mL PBS injected over the shoulders) was performed on a small subset of G3 mice ($n = 1709$) with equivalent effects and screening data were combined with those from i.p. immunizations. On day 15 after immunization, blood was collected in microcentrifuge tubes (Fisher Scientific) for ELISA analysis. G3 mice were injected with ovalbumin (OVA, Sigma-Aldrich, A5503) adsorbed to Alum (InvivoGen, vac-alu-250) in a 1:1 ratio and administered by intramuscular injection; blood was collected 14 days after immunization for ELISA analysis. Intraperitoneal NP-Ficoll immunizations were performed as previously described.²³

2.3 | ELISA

ELISA analysis to detect serum IgE or IgG1 was performed using standard techniques detailed in the supporting information.

2.4 | Automated genetic mapping

Automated genetic mapping was performed as previously described.²¹ Briefly, genotypes at all mutation sites present in the exomes of G3 mice were determined prior to phenotypic screening: tail DNA from G1 males was subjected to whole exome sequencing using an Illumina HiSeq2500 instrument; G2 and G3 mice were then genotyped at the identified mutation sites using an Ion PGM

(Life Technologies). Following phenotypic screening, linkage analysis using recessive, additive, and dominant models of inheritance was performed for every mutation in the pedigree using the program Linkage Analyzer; phenotypic data scatter plots and Manhattan plots were displayed using the program Linkage Explorer.

2.5 | Comparison of frequency of dominant and semi-dominant phenotypes in other screens

We compared the frequency of dominant and semi-dominant mutations encountered in the papain screen to that identified in all other screens performed in our laboratory. First, we calculated the ratio of validated dominant or semi-dominant phenotypes among all papain-specific IgE phenotypes. Then, we counted all phenotypes from all screens that had a dominant, semi-dominant, or recessive mode of inheritance and met the following criteria: (a) probably damaging or probably null causative mutation, (b) total mouse number in pedigree ≥ 20 , (c) at least three mice of every genotype (wild type, heterozygous and homozygous for the variant allele), and (d) $P < .001$ with Bonferroni's correction. The proportion of dominant and semi-dominant mutations was then calculated. The Fisher exact test was used to compare the ratios for the papain screen vs all other screens.

2.6 | Calculating prevalence of atopy risk genes with heterozygous mutations

Monitoring genomic saturation for genes with mutations screened once in the homozygous state in the papain screen was performed as previously described²¹ with adjustments using correction factors for PolyPhen-2 classifications [CorrectionFactorPPN2] to account for the overestimation of damage by PolyPhen-2 as previously described.²⁴ Dividing the number of validated dominant and semi-dominant CSR/BCR gene mutations (seven) by the percent genome saturation (16.5%) equals the estimated total number of dominant/semi-dominant CSR/BCR genes expected to increase allergen-specific IgE (42 genes).

To estimate the frequency of persons carrying a mutation in the heterozygous state that would increase atopy, we started by calculating the average probability of genetic damage for each gene ($P_{\text{GeneDamaged}}$) based on the variant alleles and their frequencies in the ExAC (F_{ExAC}) database (alleles from TCGA database excluded).²⁵ The damaging effect of an allele was predicted using PolyPhen-2 and then F_{ExAC} for that allele was corrected by multiplying with CorrectionFactorPPN2 to reflect the "true" probability²⁴ of damage. For each gene, corrected frequencies for all alleles were summed to give $P_{\text{GeneDamaged}}$:

$$P_{\text{GeneDamaged}} = [(F_{\text{ExAC-Null1}} + F_{\text{ExAC-Null2}} + \dots + F_{\text{ExAC-NullX}}) \times \text{CorrectionFactorPPN2}_{\text{Null}} (0.61)] + [(F_{\text{ExAC-ProbDamg1}} + F_{\text{ExAC-ProbDamg2}} + \dots + F_{\text{ExAC-ProbDamgX}}) \times \text{CorrectionFactorPPN2}_{\text{ProbDamg}} (0.17)]$$

Next, we performed a Monte Carlo simulation to randomly select a set of genes equal to the total number of dominant atopy genes ($n = 42$) with the seven validated genes set as constants in the simulation. We used $P_{\text{GeneDamaged}}$ to determine the probability that a person has no damaging mutations in any of the 42 randomly selected genes. Then, we subtracted that value from 1 to calculate the probability that an individual is heterozygous for at least 1 damaging mutation in a dominant atopy risk gene ($P_{\text{AtopicMutation}}$):

$$P_{\text{AtopicMutation}} = 1 - \prod_{i=1}^{42} (1 - P_{\text{GeneDamaged}})$$

We performed 1000 unique Monte Carlo simulations and averaged the resulting $P_{\text{AtopicMutation}}$ across all 1000 simulations to obtain 37.5%.

2.7 | Candidate explorer

Candidate explorer (CE) is a software tool to assess the likelihood that a putative mutation is causative for an observed phenotype. CE utilizes a supervised machine learning algorithm to classify putative mutations into four categories: excellent candidate, good candidate, potential candidate, and not good candidate. The training set consisted of 1378 verified and 2120 excluded mutation-phenotype associations. We used the CRISPR/Cas9 system to generate germline mutant alleles and assayed them for phenotypic effect. The input vector is comprised of over 60 features describing the properties of the mutations and the characteristics of the phenotypic data, including mutation damage probability by PolyPhen-2 and SIFT, gene lethality, clustering of observations within one genotype group, overlaps between different genotype groups, and consistency among multiple pedigrees. The prediction model is built using a random forest algorithm implemented in the R caret package. Using potential candidate as a cutoff, the current version of CE achieves 73% accuracy (90% sensitivity and 62% specificity). The program had worse performance for the atopy screen ($n = 19$) with an overall accuracy of 42% (recall/sensitivity 67% and specificity 20%). The atopy screen made up a minority of cases in the training set for CE ($n = 25$), so the weighting of certain characteristics is not optimized for this screen.

2.8 | Statistical analysis

Data represent mean $\pm$ SD in all graphs depicting error bars. The statistical significance of differences between experimental groups in Figure S2 was determined by Student's *t* test using GraphPad Prism 6 (* $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$; **** $P \leq .0001$; ns, not significant with $P > .05$). The *P* values of association between genotype and phenotype were calculated using a likelihood ratio test from a generalized linear model or generalized linear mixed effect model and Bonferroni's correction applied.²¹

3 | RESULTS

3.1 | Genetic screen for mutations altering antigen-specific IgE and IgG responses to papain

Wild-type mice upregulated eosinophils and IgE in response to intraperitoneal administration of papain (Figure S1A,B), from which both elevated and diminished IgE responses could be distinguished (Figure S1A). Conducted in this manner, the screen detects aberrant primary IgE responses during the sensitization phase, ranging from complete failure to elevated responses characteristic of atopy (Figure S1C).

Initial studies to validate the screen included examination of T-cell deficient *Lck*^{-/-} mice (Figure S2A) and *Nfkb1*- or *Sharpin*-deficient mice (Figure S2B,C). We found that papain-induced IgE and IgG1 depended on T cells and NF- κ B signaling, as previously reported.^{26,27} IgE and IgG1 responses to papain were unaffected by deficiencies of MyD88, NLRP3, or CARD9 (Figure S2D-F), consistent with previous reports.^{20,28} However, we detected slightly elevated antigen-specific IgG1 production by *Tlr4*^{lps3/lps3} mice (Figure S2G), in contrast to reduced amounts produced by *Tlr4*^{-/-} mice reported previously.²⁸ This difference may reflect the different inocula (papain vs papain + ovalbumin) and immunization routes (i.p. vs s.c.) in the two experiments. We show for the first time the independence of IgE production from the intracellular nucleic acid sensors cGAS or MAVS (Figure S2H).

We screened 16 177 G3 mice from 704 pedigrees for IgE responses. These mice carried 38 710 nonsynonymous heterozygous or homozygous mutations in the coding regions or splice junctions of 14 654 genes. Computational assessment of the number of genes modified by damaging and/or null alleles²⁴ screened once or more in the homozygous state indicated 16.5% genome saturation among all annotated protein-encoding genes. For IgG1 responses, the corresponding quantities were: 3886 G3 mice from 180 pedigrees representing 12 218 mutations in 7956 genes (5.81% genome saturation).

3.2 | Selection and validation of candidate genes

We restricted our search for mutations affecting IgE or IgG1 responses to those (a) found in pedigrees with 10 or more G3 mice, (b) with zero or more instances of homozygosity (to allow for detection of dominant or semi-dominant homozygous lethal mutations), and (c) with at least two mice homozygous for the reference allele of the affected gene. *P* values for linkage of mutations to altered IgE or IgG1 phenotypes were calculated using recessive, dominant, and semi-dominant models of inheritance.²¹ A criterion of $P < .05$ with Bonferroni's correction for the number of mutations in each pedigree was used to flag mutations as candidates, which were then evaluated with an in-house machine learning tool, Candidate Explorer (CE), for the likelihood of being causative for the phenotype, that is, recapitulating the phenotype in mice with an independently generated mutation in the same gene (Figure 1A). CE evaluates gene-phenotype

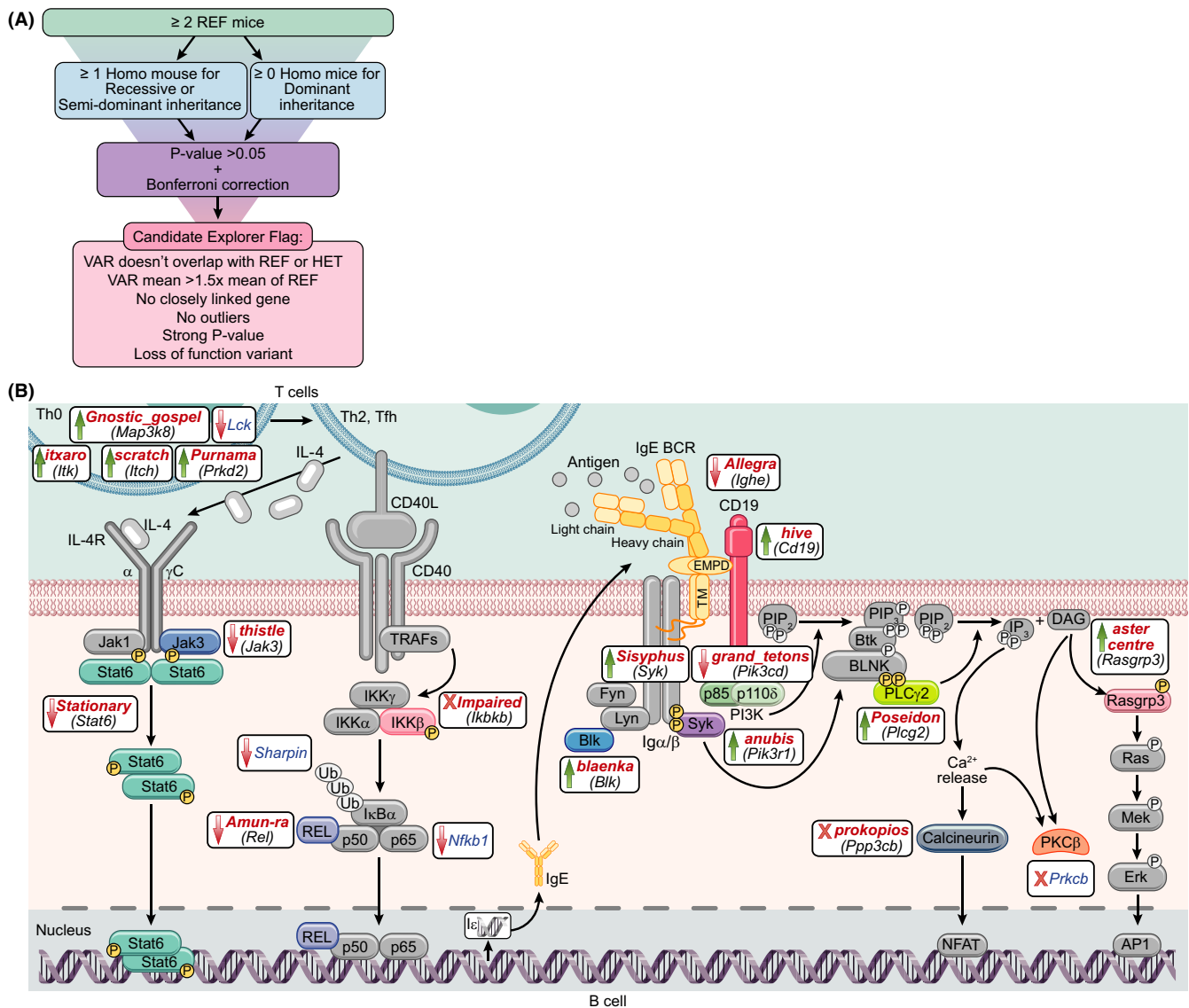


FIGURE 1 A, Scheme showing candidate mutation selection criteria and several of the factors positively correlated with stronger Candidate Explorer ratings. B, Pathways implicated by the atopy screen, see Supporting Information text for details. Signals from Th2 and Tfh cells induce B-cell class switch recombination to IgE, which is secreted or expressed on the membrane of B cells as the B-cell receptor (BCR). Signaling pathways downstream of the BCR and CD19 are shown. The phenotype name is shown in red font with the name of the mutated gene in italics and parenthesis. Gene names in blue font indicate a gene tested based on hypothesis outside of G3 screening. An arrow indicates the direction of IgE change in the atopy screen as a result of the gene mutation (green up = increased IgE, red down = decreased IgE). A red "X" indicates a mutation that failed to be validated

associations based on approximately 60 variables describing properties of the mutations and phenotypic data (Figure 1A); the program is regularly trained and updated as new data are collected. For example, mutation damage probability, gauged by PolyPhen-2 and SIFT and gene essentiality, makes a minor contribution to the CE score. More highly weighted in CE scoring are properties of the phenotypic data, such as attributing multiple phenotypes to one genotype, different alleles of the same gene producing the same phenotype, and having no phenotypic overlap between REF/HET and VAR mice. In addition, the CE score will be higher for mutations that result in large differences between REF/HET and VAR phenotypes compared to mutations that result in smaller differences.

Among the group of 219 genes affected by 221 mutations with a CE rating of "potential," "good," or "excellent candidate" (Data S1; predicted validation frequency of 73% for this group at the time of writing), we selected 22 genes for validation testing (Table 1 and Figure 1B). These genes generally fell into three biological categories based on published data: class switch recombination (CSR) (Figure 2), B-cell receptor (BCR) signaling (Figures 3 and 4), and others (neither CSR nor BCR signaling, Figure 5 and Figure S3). All phenotypes ascribed to CSR genes were concordant with previous reports. A substantial number of genes encoding BCR signaling components scored in the screen, suggestive of biological relevance, and we therefore selected them for validation testing.

TABLE 1 Summary of atopy screen genes

	High IgE	Figure	Low IgE	Figure	High IgG1	Figure	Low IgG1	Figure
Targeted: validated	<i>Blk</i> ^{b,d,e}	3D						
	<i>Cd19</i> ^{a,d,e}	4A						
	<i>Itch</i> ^{a,d}	2A						
	<i>Map1lc3b</i> ^{a,d,e}	5E						
	<i>Pik3r1</i> ^{a,c,e}	4E	<i>Flcn</i> ^{a,c}	5M	-		<i>Nr4a3</i> ^{a,c}	S3C
	<i>Plcg2</i> ^{a,c,e}	3L	<i>Me2</i> ^{b,c}	5I				
	<i>Prkd2</i> ^{a,c,e}	5A						
	<i>Rasgrp3</i> ^{a,c,e}	3P						
	<i>Scarb2</i> ^{b,d}	5Q						
	<i>Syk</i> ^{a,d,e}	3H						
Not targeted, literature verified	<i>Itk</i> ^a	2E	<i>Ighe</i> ^a	3A			<i>Jak3</i> ^{a,b}	2H
	<i>Map3k8</i> ^{a,e}	S3E	<i>Rel</i> ^a	2N	-		<i>Pik3cd</i> ^a	4I
			<i>Stat6</i> ^a	2K			<i>Rel</i>	2N
Hypothesis: validated			<i>Lck</i> ^c	S2A				
	-		<i>Nfkb1</i>	S2B	-		-	
			<i>Sharpin</i>	S2C				
Hypothesis: failed validation	<i>Prkcb</i> ^b	S7A	-		-		-	
Targeted: failed validation	<i>Arid4a</i> ^{b,c}	S6D					<i>Ikbkb</i> ^{a,c}	S4A
	<i>Ppp3cb</i> ^{a,c}	S5E	<i>Ikbkb</i> ^{a,c}	S5A	<i>Tnfaip2</i> ^{a,c}	S5O	<i>Snx14</i> ^{a,c}	S5I
	<i>Zfyve26</i> ^{b,d}	S6E					<i>Stx17</i> ^{a,c}	S5N

^a"Potential," "Good," or "Excellent" candidate" by Candidate Explorer (22 genes).

^b"Not good candidate" by Candidate Explorer (57% -predicted validation frequency) (5 genes).

^cKnockout or replacement allele generated by CRISPR/Cas9 targeting (14 genes; see Table S1).

^dPublished knockout obtained (7 genes).

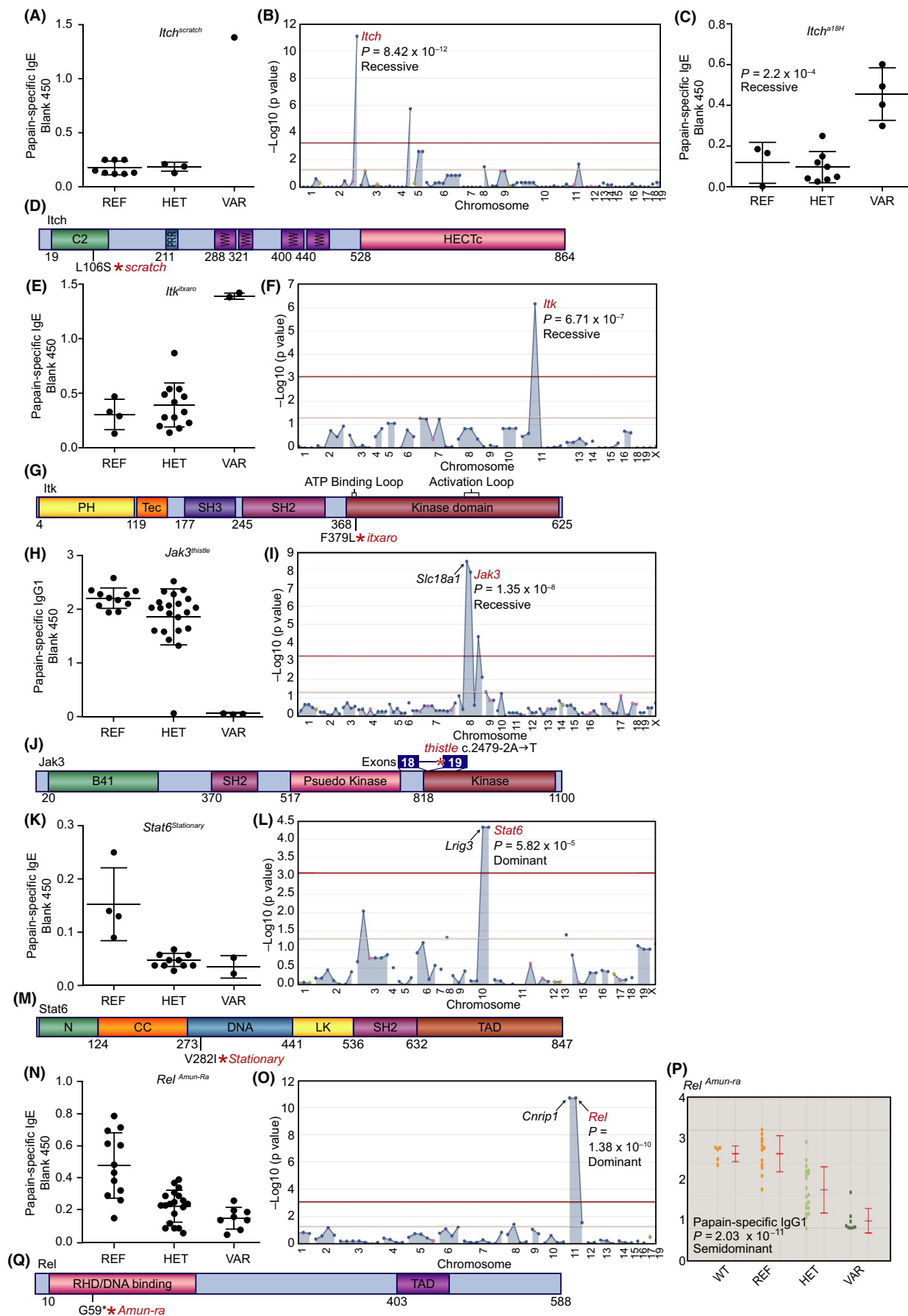
^eDominant or semi-dominant inheritance in the screen or validation allele.

Validation of the candidate genes was pursued by generating CRISPR-targeted mutations in wild-type genomes. CRISPR-induced knockout strains were created when the candidate phenotype was consistent with a loss-of-function mutation and evidence existed that homozygous null alleles were not lethal (10 genes). Replacement alleles (replicating the original ENU-induced DNA change) by CRISPR targeting are relatively more challenging to obtain; these were produced when evidence of lethality existed for homozygous null mutants or when a gain-of-function mutation was suspected (four genes). Mutations that phenocopied the original ENU-induced mutation were considered validated. Some

candidate variants were in genes where loss-of-function variants have a well-established impact on IgE production (*Itk*, *Jak3*, *Stat6*, *Rel* [Figure 2]; *Pik3cd* [Figure 4]; and *Ighe* [Figure 3]). When these candidates phenocopied the published phenotype, we considered them "literature verified" and did not pursue experimental validation (seven genes).

Ten of the 22 selected genes (Table 1) were validated by testing mice for their IgE or IgG1 responses (*Cd19*, *Flcn*, *Itch*, *Map1lc3b*, *Nr4a3*, *Pik3r1*, *Plcg2*, *Prkd2*, *Rasgrp3*, and *Syk*). Five genes were similarly tested but failed to be validated (*Ikbkb*, *Ppp3cb*, *Snx14*, *Stx17*, and *Tnfaip2*). Lastly, for seven genes (*Ighe*, *Itk*, *Jak3*, *Map3k8*, *Pik3cd*,

FIGURE 2 Screening results of *Itch*, *Itk*, *Jak3*, *Stat6*, and *Rel* alleles for effects on papain-specific IgE or IgG1 responses to papain immunization. For each gene, a scatter plot of the phenotypic data for mice carrying the original ENU-induced allele, Manhattan plot of linkage analysis data, scatter plot of phenotypic data for mice carrying an independently generated allele (created in-house or obtained from another source for validation testing), and protein domain diagram depicting ENU-mutated residue and allele name are shown. Manhattan plots display the $-\log_{10}$ P values (Y axis) plotted vs the chromosomal positions (X axis) of all mutations identified in the G1 founder of the pedigree. Linkage peak is labeled with gene, P value, and inheritance mode. Horizontal red and beige lines represent thresholds of $P = .05$ with or without Bonferroni's correction, respectively. (A-D) *Itch*, (E-G) *Itk*, (H-J) *Jak3*, (K-M) *Stat6*, and (N-Q) *Rel*. REF, homozygous for the reference allele; HET, heterozygous for the reference and variant allele; VAR, homozygous for the variant allele; C2, calcium-binding domain; PRR, proline-rich region; WW, domain with 2 conserved Trp (W) residues; HECTc, domain homologous to E6-AP carboxyl terminus; PH, pleckstrin homology domain; Tec, transient erythroblastopenia of childhood; SH3, Src homology 3 domain; SH2, Src homology 2 domain; B41, bank 4.1 homologues; CC, coiled coil; DNA, DNA-binding domain; LK, linker domain; RHD, Rel homology domain; TAD, transactivation domain. Data points represent individual mice (A, C, E, H, K, N, and P). Data are representative of 1-2 independent experiments. Central line indicates mean, and error bars indicate SD



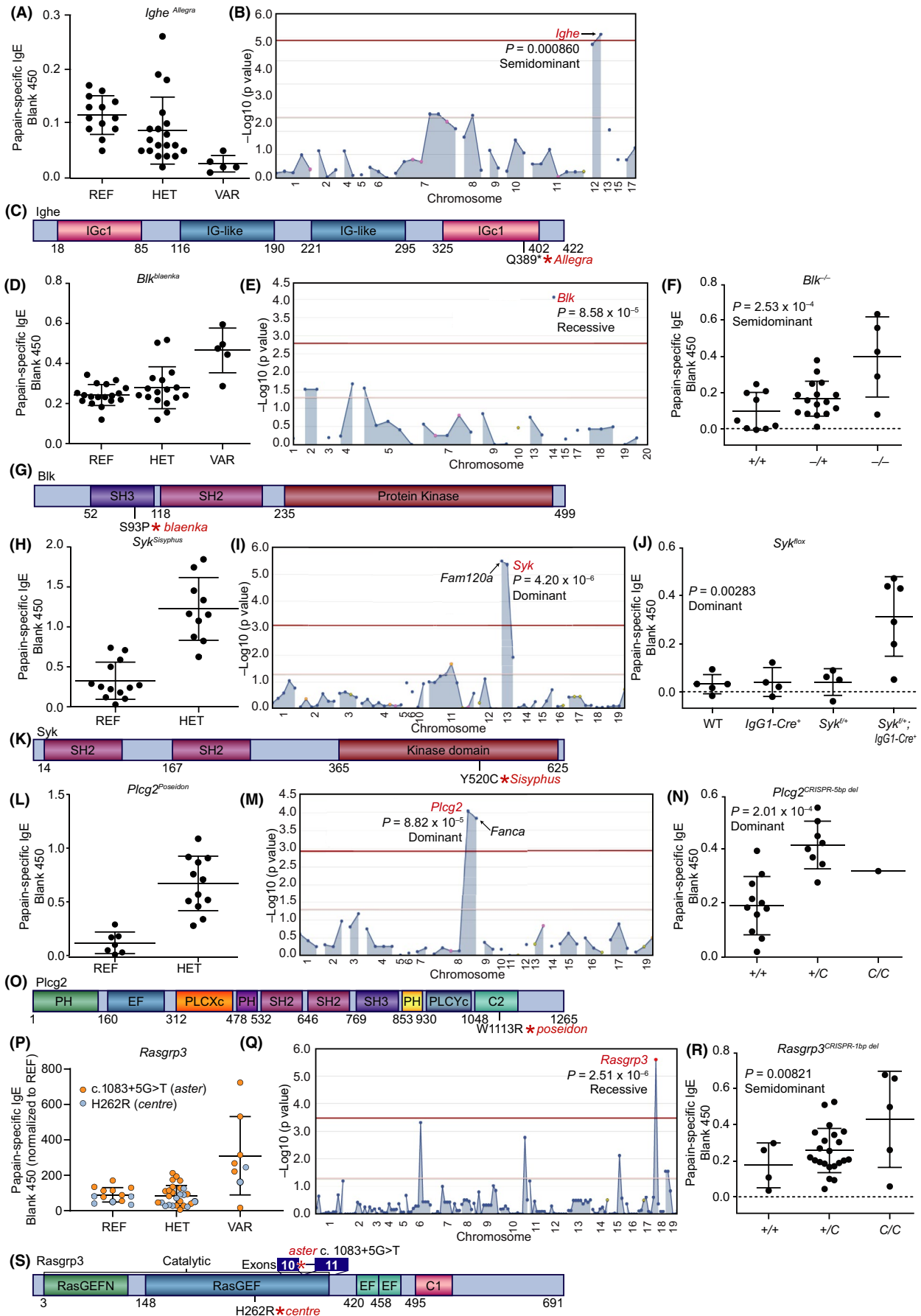


FIGURE 3 Screening and validation testing of *Ighe*, *Blk*, *Syk*, *Plcg2*, and *Rasgrp3* alleles for effects on papain-specific IgE responses to papain immunization. Data are displayed as in Figure 2. (A-C) *Ighe*, (D-G) *Blk*, (H-K) *Syk*, (L-O) *Plcg2*, and (P-S) *Rasgrp3*. The *Rasgrp3*^{aster} and *Rasgrp3*^{centre} pedigrees were combined to a scatter plot of normalized serum papain-specific IgE after papain immunization (P) and corresponding Manhattan plot of combined linkage analysis data (Q). Manhattan plots display the $-\log_{10} P$ values (Y axis) plotted vs the chromosomal positions (X axis) of all mutations identified in the G1 founder(s) of the pedigree(s). REF, homozygous for the reference allele; HET, heterozygous for the reference and variant allele; VAR, homozygous for the variant allele; C, CRISPR/Cas9-targeted allele; Cre, engineered Cre recombinase allele; -/-, engineered null allele; IGc1, immunoglobulin C-type; IG-like, immunoglobulin-like; SH2, Src homology 2 domain; SH3, Src homology 3 domain; PH, pleckstrin homology domain; EF, EF hand domain; PLCXc, phospholipase C, catalytic domain X; PLCYc, phospholipase C, catalytic domain Y; C2, calcium-binding domain; RasGEFN, guanine nucleotide exchange factor for Ras-like GTPases, N-terminal motif; RasGEF, guanine nucleotide exchange factor for Ras-like small GTPases; C1, diacylglycerol (DAG)-binding domain. Data points represent individual mice (A, D, F, H, J, L, N, P, R). Data are representative of 2-3 independent experiments. Central line indicates mean, and error bars indicate SD

Rel, and *Stat6*) mutant mice were not subjected to phenotypic testing but the genes were regarded as validated based on published reports ("Not Targeted, Literature Verified" in Table 1).

Additionally, five genes (*Blk*, *Me2*, *Scarb2*, and the linked pair *Zfyve26/Arid4a*) containing mutations ranked "not good candidate" (lower CE-predicted validation frequency of 39%), but which had $P < .05$ for linkage to their respective IgE or IgG1 phenotypes, appeared otherwise robust and were selected for validation testing (Table S1). Three of the five genes (*Blk*, *Me2*, and *Scarb2*) were validated; two genes (*Zfyve26* and *Arid4a*) representing one phenotype failed to be validated.

3.3 | Summary of implicated genes and their effects

Mutations in 17 genes—either validated by robust LOF alleles or strongly supported by previous experimental observations—influenced IgE responses and were detected in screening (Table 1). For 12 of these genes, the effect of mutations was to augment the IgE response (*Blk*, *Cd19*, *Itch*, *Itk*, *Map1lc3b*, *Map3k8*, *Pik3r1*, *Plcg2*, *Prkd2*, *Rasgrp3*, *Scarb2*, and *Syk*). We noted that a substantial fraction of these mutations (9 out of 12) displayed dominant or semi-dominant phenotypic effects (Table 1, marked with superscripted "e"). For the remaining five validated genes, mutations diminished or abolished IgE responses (*Fln*, *Ighe*, *Me2*, *Rel*, and *Stat6*). Null alleles of *Ppp3cb*, *Zfyve26*, and *Arid4a*, and a replacement allele of *Ikbkb* failed to validate as affecting IgE (Table 1). Finally, one gene, *Prkcb*, was not implicated in screening, but its well-described function in BCR signaling led us to test an existing *Prkcb* allele, *Untied*,²⁹ for an effect on the papain-induced IgE response. No effect was observed (Figure S7A,B).

Diminished IgG1 responses were attributed to mutations in four genes (*Jak3*, *Nr4a3*, *Pik3cd*, and *Rel*; Table 1); mutations in *Ikbkb*, *Snx14*, and *Stx17* failed to be validated (Figure S5). No validated ENU-induced mutations were found to increase the IgG1 response (*Tnfrsf2* failed to validate; Figure S5Q).

4 | DISCUSSION

The forward genetic analysis of mice with a defined genetic background permitted unbiased and unambiguous assignment of

causality to single gene variants responsible for aberrant IgE or IgG1 phenotypes. Because mice and humans generate similar adaptive immune responses, our findings likely have implications for atopy in humans. To our knowledge, no other group has approached the genetic basis of atopy in this way.

Including *Lck*, *Prkcb*, *Sharpin*, and *Nfkb1*, we investigated 32 mutations in 31 genes for their effects on the quantity of serum papain-specific IgE or IgG1 produced in response to immunization. We suggest that measurement of allergen-specific IgE more accurately reflects atopy than total IgE or basal IgE, which have not consistently been associated with anaphylaxis or food allergy.³⁰ Using CRISPR-targeted knockout/replacement alleles or published, available knockout alleles, we validated the effects of mutations in 23 (74%) of the 31 genes; the effects of eight mutations (26%) were not validated. Many of the validated genes aligned to signaling pathways important for the production of IgE. Among 20 validated gene candidates identified in screening or hypothesized to affect the IgE response, 15 were in either the CSR or BCR signaling pathways (Figure 1B). We discuss putative mechanisms by which specific mutations alter IgE responses to papain in the Supporting Information; we describe relevant pathways beginning with Th2 stimulation leading to IgE CSR, then BCR signaling, and ending with a summary of genes with unknown function in the IgE response.

Many validated genes were kinases (*Itk*, *Jak3*, *Map3k8*, *Blk*, *Syk*, *Pik3cd*, and *Prkd2*) or GTPases (*Rasgrp3* and *Pik3r1*) affected by ENU-induced mutations in the catalytic domain. It may be that catalytic domain mutations generally have a more significant impact on protein function than mutations in noncatalytic domains. For these kinase domain mutations, we recapitulated the original phenotypes with null alleles, confirming that the ENU-induced mutations conferred a loss of function. However, in several instances the complete knockout revealed a heterozygous increase in IgE not observed for the ENU-induced mutation, which was recessive (*Blk*, *Pik3r1*, and *Rasgrp3*; also *Cd19*, not a kinase). These findings suggest that the ENU-induced mutations in *Blk*, *Cd19*, *Pik3r1*, and *Rasgrp3* were relatively mild and heterozygosity resulted in retention of >50% of wild-type protein function, which was sufficient to confer the wild-type phenotype. In contrast, 50% of wild-type protein function caused by heterozygosity for a null allele was insufficient for normal IgE production. Our genetic findings support previous

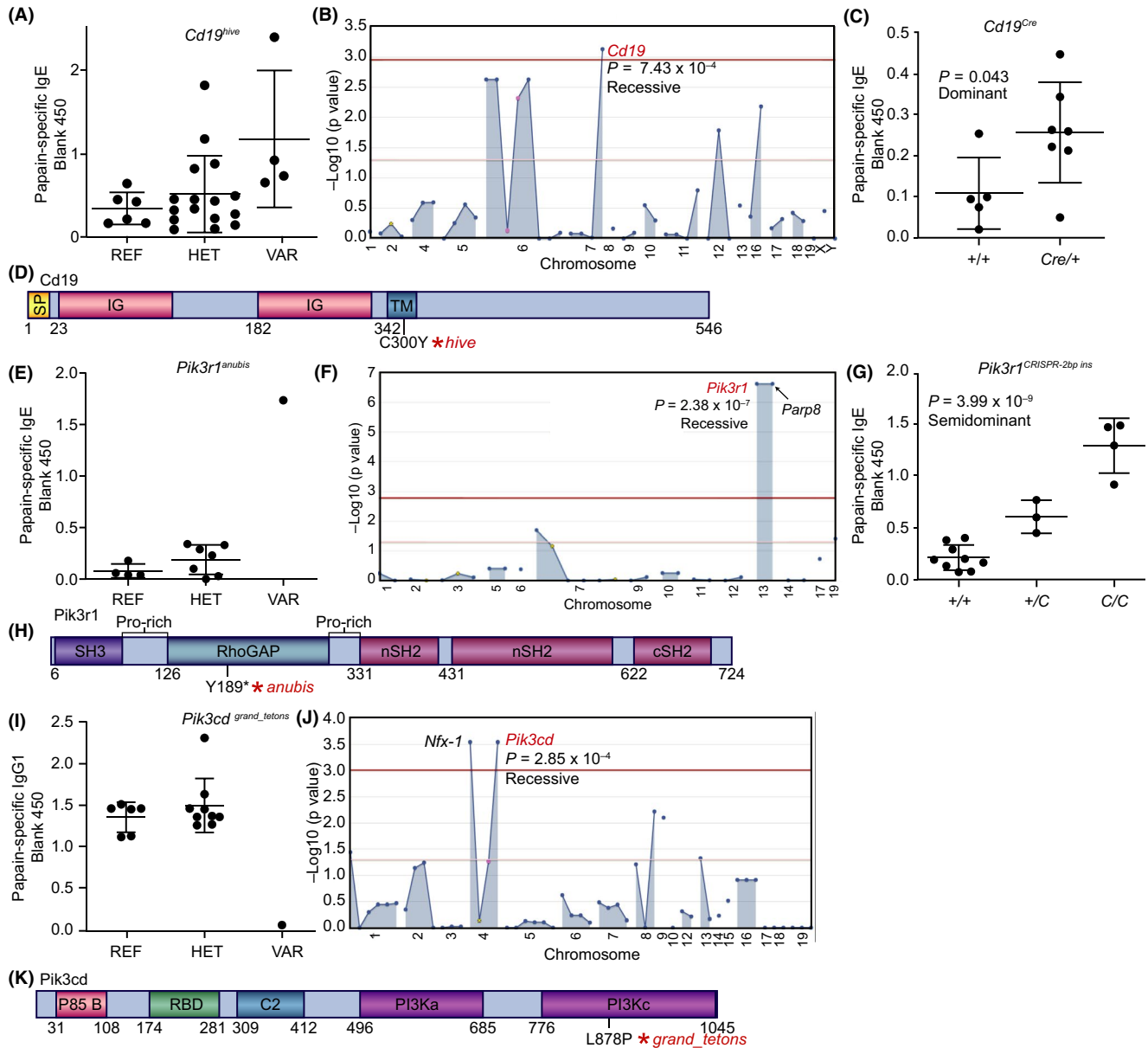
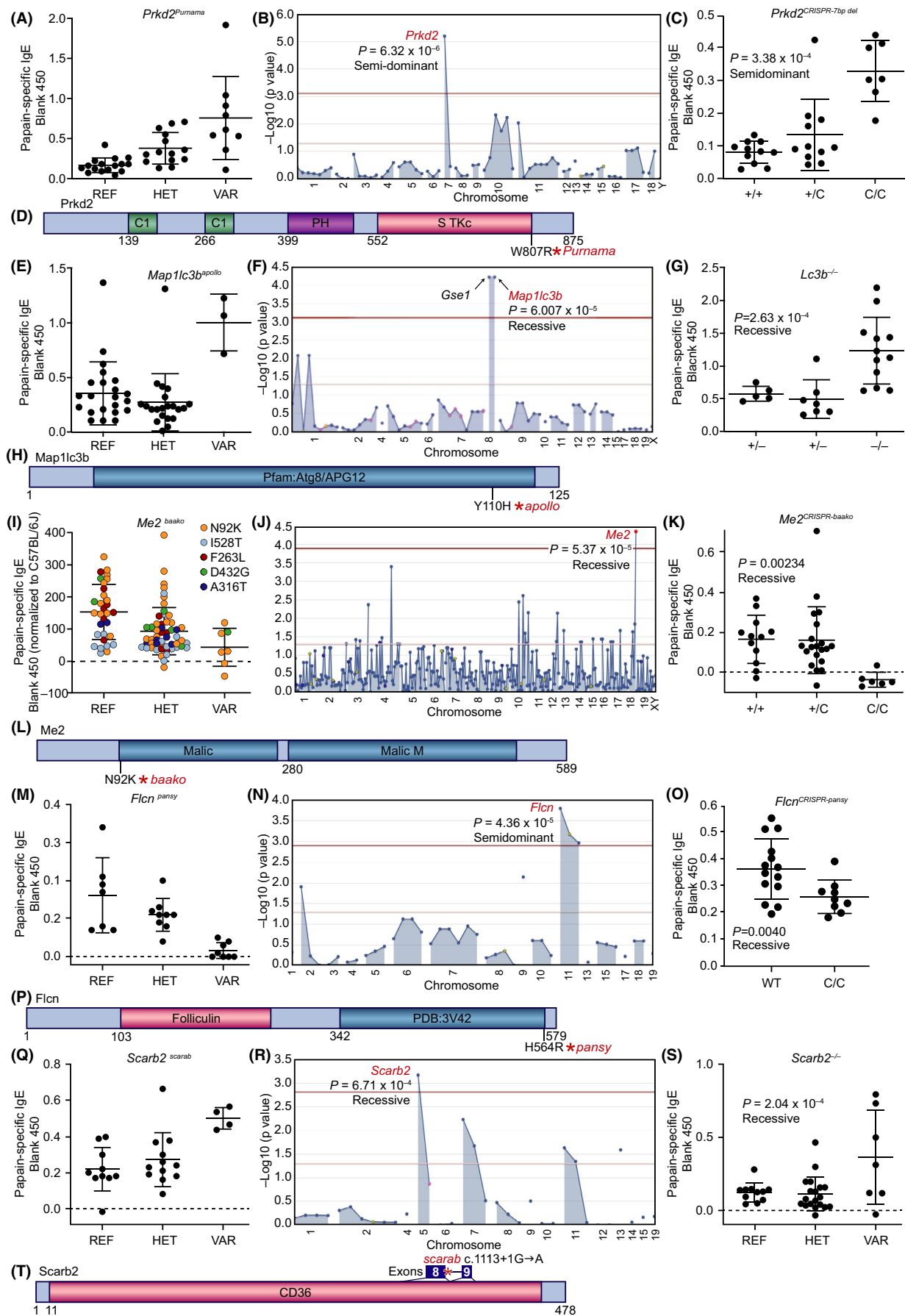


FIGURE 4 Screening data for *Cd19*, *Pik3r1*, and *Pik3cd* alleles on papain-specific IgE or IgG1 responses to papain immunization. Data are displayed as in Figure 2. (A–D) *Cd19*, (E–H) *Pik3r1*, (G–I) and *Pik3cd*, (I–K). REF, homozygous for the reference allele; HET, heterozygous for the reference and variant allele; VAR, homozygous for the variant allele; C, CRISPR/Cas9-targeted allele; Cre, engineered Cre recombinase allele; SP, signal peptide; IG, immunoglobulin; TM, transmembrane; SH3, Src homology 3 domain; RhoGAP, GTPase-activator protein for Rho-like GTPases; SH2, Src homology 2 domain; nSH2, Src homology 2 domain; cSH2, Src homology 2 domain; p85B, p85-binding domain; RBD, Ras-binding domain; C2, calcium-binding domain; PI3Ka, phosphoinositide 3-kinase, accessory domain; PI3Kc, phosphoinositide 3-kinase, catalytic domain. Data points represent individual mice (A, C, E, G, I). Data are from 1 to 2 independent experiments. Central line indicates mean, and error bars indicate SD

FIGURE 5 Screening and validation testing of *Prkd2*, *Map1lc3b*, *Me2*, *Fln*, and *Scarb2* alleles for effects on papain-specific IgE responses to papain immunization. Data are displayed as in Figure 2. (A–D) *Prkd2*, (E–H) *Map1lc3b*, (I–L) *Me2*, (M–P) *Fln*, and *Scarb2* (Q–T). (I and J) Multiple pedigrees containing distinct mutations in *Me2* were combined for linkage analysis. Mutations, each from a single pedigree, are color coded in the phenotypic data scatter plot (I) (papain-specific IgE normalized to B6 controls, not shown). REF, homozygous for the reference allele; HET, heterozygous for the reference and variant allele; VAR, homozygous for the variant allele; C, CRISPR/Cas9-targeted allele; –/–, engineered null allele; C1, diacylglycerol (DAG)-binding domain; PH, pleckstrin homology domain; S TKc, serine/threonine protein kinases, catalytic domain; SH3, Src homology 3 domain; SH2, Src homology 2 domain; Malic, Malic enzyme, N-terminal domain; Malic M, Malic enzyme, NAD-binding domain; CD36, scavenger receptor domain. Data points represent individual mice (A, C, E, G, I, K, M, O, Q, S). Data are representative of 1–3 independent experiments. Central line indicates mean, and error bars indicate SD



reports that constitutive signaling from the IgE BCR initiates gene expression changes that affect cell fate and lifespan to limit IgE production. These specific events are still unclear but could be elucidated by transcriptomic analysis using bulk RNA-Seq and single-cell RNA-Seq of IgE B cells.^{31,32} Recurrent haploinsufficient phenotypes caused by mutations of BCR signaling components suggest moderate decreases in signaling curtail the events that limit IgE production. Other mutations causing a dominant decrease in IgE were in nuclear transcription factors that dimerize to exert their activity (*Rel* and *Stat6*).

Considering the number of validated genes in the CSR and BCR immune pathways (10 increasing IgE) and the genome saturation (16.5%) for the atopy screen to date, we estimated that a total of 60 CSR/BCR-related genes might be expected to increase papain-specific IgE should saturation reach 100%. However, this conservative calculation likely underestimates the number of genes that affect antigen-specific IgE responses since only a single allergen, papain, was used and genes not known to participate in these pathways were found to increase antigen-specific IgE (*Map1lc3b*, *Prkd2*, and *Scarb2*). Alternative atopic sensitization methods or allergens may find additional genes or pathways. Further, the immunization method of intraperitoneal injection was artificial but more practical for large scale screening compared to a labor intensive but more physiologic method such as exposure by skin, inhalation or ingestion. This could prevent detection of genes such as *Flg* (encoding filaggrin) that lead to atopy by disturbing skin integrity.^{33–35}

Determining a model system that would resemble the human situation should be disease-specific. For example, several of the BCR signaling mutants may appropriately model human food allergy and allergic rhinitis, which involve increased allergen-specific IgE in the absence of immunodeficiency. Some mutants may be unsuitable for modeling non-IgE mediated disease such as eosinophilic esophagitis, but the *Stat6* mutant may be an interesting model to study the effect of blocking IL-4 signaling to treat allergies.³⁶ The *Plcg2* mutant displayed increased allergen-specific IgE and may be a good model to study the pathology and mechanisms underlying the increased IgE and atopy associated with mutations in human *PLCG2*.³⁷

A significant finding from our screen was the high incidence of dominant or semi-dominant inheritance patterns among elevated IgE phenotypes. Of the 12 validated gene candidates for increased IgE, nine were dominant or semi-dominant allelic variants, a frequency significantly higher than the frequency of dominant or semi-dominant alleles causative of all other CRISPR-validated phenotypes screened in our group ($P = 3.33 \times 10^{-5}$, Fisher's exact test, $n = 9$ Atopy^{Dom}, 3 Atopy^{Recessive}, 190 Other^{Dom}, 854 Other^{Recessive}, Table S3). These dominant or semi-dominant mutations were apparently innocuous in the heterozygous state with respect to immune competence (as indicated by phenotypic testing in other immunologic screens; Table S2), suggesting that they are not gain-of-function variants, and importantly, reflecting the clinical presentation of most allergic patients. We speculate that

heterozygous mutations with dominant effects on atopy, together with the large number of genes estimated to comprise the genomic footprint of the high IgE-response phenotype (~60 genes from CSR/BCR signaling alone), may explain the high incidence of heritable atopic disease in humans (as in normally nonatopic mice modified by a random mutagen).

Indeed, among the seven validated, dominant CSR/BCR mutations described here, 1.3% of human subjects in the ExAC database are heterozygous for null or probably damaging alleles ($n = 780$ alleles; Data S2) of the genes; on a per gene basis, this amounts to an allele frequency that is on average less than the 1% allele frequency needed for identification by genome-wide association studies. Overall, we estimate that 37.5% of the population carries a dominant CSR/BCR signaling atopy risk mutation in the heterozygous state (Materials and Methods). An atopic mutation will predispose an individual to being sensitized to an allergen and they may not manifest allergic symptoms. An additive effect of heterozygous atopic mutations at multiple loci may increase the risk of allergic disease. Thus, rare, damaging mutations in the heterozygous state may underlie allergy and atopy in otherwise healthy children and adults.

5 | CONCLUSIONS

We present the first forward genetic screen for mutations that influence atopy, which identified 31 candidates of which 74% (23/31) were validated. Of 20 genes that affected IgE in our screen, 55% (11/20) were not previously known to influence atopy. Although further work will be necessary to confirm similar associations in humans, a forward genetic approach in mice will yield important contributions to resolving genetic determinants of allergy. These discoveries can guide genome-wide association studies, which so far have revealed only weak associations with allergy pathophysiology. Given the current genomic saturation rate, we expect that many more novel gene-atopy associations remain to be discovered. We estimate that a heterozygous mutation in at least one of these genes may be present in 37% of the human population.

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CONFLICTS OF INTEREST

Dr SoRelle reports a grant from Howard Hughes Medical Institute during the conduct of the study. Dr Chen, Dr Jianhui, Dr Yue, Dr Jin Huk Choi, Dr Kuan-Wen Wang, Dr Zhong, Ms Hildebrand, Dr Russell, Dr Russell, Dr Xu, Dr Xiaowei Zhan, Dr Bu, Dr Tao Wang, Dr

Mihwa Choi, Dr Tang, Dr Ludwig, Dr Xiaoming Zhan, Dr Li, and Dr Moresco have nothing to disclose. Dr Beutler reports grants from National Institutes of Health and from the Lyda Hill Foundation during the conduct of the study.

AUTHOR CONTRIBUTIONS

JAS and BB conceived the study and acquired funding. ZC, JW, TY, JHC, KW, X.Zhong, DX, Xiaowei Zhan, CHB, TW, MC, MT, SL, Xiaoming Zhan, and XL designed the methodology. JAS, ZC, JW, TY, JHC, KW, X.Zhong, SH, JR, LS, DX, Xiaowei Zhan, CHB, TW, MC, MT, SL, Xiaoming Zhan, and XL performed experiments. JAS, TW, and BB performed formal analysis. JR, SL, Xiaoming Zhan, XL, and BB provided resources. JAS, JW, TY, JHC, KW, X.Zhong, and SL validated the data. BB supervised the study. JAS wrote the original draft. JAS, EMYM, and BB reviewed and edited the manuscript.

DATA AVAILABILITY STATEMENT

All data are provided in the main text and supplementary materials.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supporting Information

Dominant atopy risk mutations identified by mouse forward genetic analysis

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Materials and Methods

Mice

C57BL/6J, *Blk*^{-/-} (*Blk*^{tm1Tara}; #017624), *Cd19*^{Cre} (*Cd19*^{tm1(cre)Cgn}; #006785), *Ighg1*^{cre} (*Ighg1*^{tm1(cre)Cgn}; #010611), *Map1lc3b*^{KO} (*Map1lc3b*^{tm1Mrab}; #009336), *Sharpin*^{cpdm/cpdm} (#007599), *Syk-flox* (*Syk*^{tm1.2Tara}; #017309), and *Zfyve26*^{em1J} (#26273) mice were purchased from The Jackson Laboratory. *Mavs*^{-/-} (1), *cGas*^{-/-} (*Mb21d1*^{-/-}) (2), *Myd88*^{-/-} (3), and *Prckb*^{Untied/Untied} (4) mice have been previously described. *Nfkb1*^{Finlay/Finlay}, *Tlr4*^{lps3/lps3}, and *Nlrp3*^{Park6/Park6} mice were generated by ENU mutagenesis (5) and are described at <http://mutagenetix.utsouthwestern.edu>; they are available from the Mutant Mouse Regional Resource Center. Mice with mutations in *Arid4a*, *Flcn*, *Ikbkb*, *Lck*, *Me2*, *Nr4a3*, *Pik3r1*, *Plcg2*, *Ppp3cb*, *Prkd2*, *Rasgrp3*, *Snx14*, *Stx17*, and *Tnfaip2* were generated by gene targeting using the CRISPR/Cas9 system. *Card9*^{-/-} mice were from Dr. Xin Lin; *Itch*^{a18H/a18H} mice were a kind gift from Dr. Lydia Matesic; *Scarb2*^{-/-} mice were a kind gift from Dr. Paul Saftig (6).

Generation of alternate alleles by CRISPR/Cas9 gene targeting

Female C57BL/6J mice were superovulated by injecting them with 6.5 units of pregnant mare's serum gonadotropin (PMSG; Millipore: 367222), then 6.5 units of human chorionic gonadotropin (hCG; Sigma-Aldrich: C1063) 48 h later. The superovulated females were subsequently mated with C57BL/6Jcl male mice (The Jackson Laboratory) overnight. The following day, fertilized eggs were collected from the oviducts of the female mice and *in vitro* transcribed Cas9 mRNA (50 ng/μl) was injected into the pronucleus or cytoplasm of the fertilized eggs along with small base-pairing guide RNA for *Arid4a* (50 ng/μl 5'- GCATTCTCGTCATCACTGC-3'), *Flcn* (50 ng/μl, 5'- TTCGGACGGTGGACATGAGA-3'), *Ikbkb* (50 ng/μl, 5'- GGAGCTCTACCGAAGACTCA-3'), *Lck* (50 ng/μl, 5'- AACTGTGCAGGGCGATAACC -3'), *Me2* (50 ng/μl, 5'-GACTTTCAATATCATCCTGC-3'), *Nr4a3* (50 ng/μl, 5'- ACCAAAGAGCCCACTACAAC-3'), *Pik3r1* (50 ng/μl 5'- AGTCGTACAGTGCTCTGTAC -3'), *Plcg2* (50 ng/μl 5'- CCCTGTCTGGGCTCCAAC -3'), *Ppp3cb* (50 ng/μl, 5'- CTTAGTACAGCAGCTGCAA -3'), *Prkd2* (50 ng/μl, 5'- TCTCAGCCACCCATGGTTAC -3'), *Rasgrp3* (50 ng/μl, 5'- CGTACCGATTGGAATTCT -3'), *Snx14* (50 ng/μl, 5'- ACCATATTAAGAGGGGAATG -3'), *Stx17* (50 ng/μl, 5'- CCACTTTGAAGCTGCGAGG -3'), or *Tnfaip2* (50 ng/μl, 5'- ATGGGGCTCTGATACCTTC -3'). Replacement alleles required template mRNA for *Me2* (5'- gcttaatttcagatacatttatataatgggcatccaagaaagaaaAgagaaactgttctatagaatTctgcaggatgatattgaaagtctgatgccgattgtgtacacgccaacagttggtcttgcctgctgtcaatatg -3') and *Nr4a3* (5'- gcctattccaagtgtgatctttaaacagtggtttttccccctctgtatctcatcagttgtgAgtacagacagtctgaaagggaggagaggtctgtgcc

ttccaaaccaaagagtccactacaacaAgagccctcgagccctcccg -3') where the first capitalized letter is the replacement mutation and the second capitalized letter is a silent mutation to improve replacement efficiency. The injected embryos were cultured in M16 medium (Sigma-Aldrich: M7292) at 37°C and 95% air/5% CO₂. For the production of mutant mice, 2-cell stage embryos were transferred into the ampulla of the oviduct (10-20 embryos per oviduct) of pseudopregnant Hsd:ICR (CD-1) females (Harlan Laboratories). The resulting mutations transmitted to the germline and bred to homozygosity are included in Table S1.

Measurement of eosinophils

A DREW Hemavet complete blood count instrument gave a count and percentage of eosinophils using EDTA anticoagulated blood.

ELISA

Blood was centrifuged at 4000 rpm for 5 min to separate serum. Polystyrene high binding 96-well microplates (Corning) were coated with 10 µg/ml papain (EMD Millipore, 5125 Carica papaya) in PBS overnight at 4°C and then blocked with 2% (wt/vol) BSA in PBS for 1-4 h at room temperature. Serum samples were diluted 1:10 (for IgE) or 1:100 (for IgG1) in 1% (wt/vol) BSA in PBS, added to the ELISA plates, and incubated overnight at 4°C. Plates were washed and then incubated with HRP-conjugated goat anti-mouse IgE or IgG1 (Southern Biotechnology), developed with SureBlue TMB Microwell Peroxidase Substrate (KPL) and 2 M sulfuric acid, and read at 450 nm on a microplate reader. Raw data (blank 450) represent absorbance at 450 nm after subtracting absorbance for a blank well (1% BSA in PBS) unless otherwise indicated in the figures. OVA-specific IgE was assayed by coating the plates with 10 µg/ml OVA then performing the same steps as for papain-specific IgE (serum dilution 1:10), except an OVA-specific IgE standard was used for quantitation. Total IgE was measured according to the manufacturer's instructions (eBioscience, 88-90460-76) using serum at a dilution of 1:50. Serum NP-specific IgM was measured as described previously (7).

Flow cytometry

Peripheral blood cells were isolated, and red blood cell (RBC) lysis buffer was added to remove RBCs. Cells were stained in the presence of mouse CD16/32 antibody for 1 hour at 4°C with 15 mouse fluorochrome-conjugated monoclonal antibodies specific for the following murine cell surface markers encompassing the major immune lineages: B220, CD3ε, CD4, CD5, CD8α, CD11b, CD11c, CD19, CD43, CD44, CD62L, F4/80, IgD, IgM, and NK1.1 (1:200-300 dilution for all antibodies). After staining, cells were washed twice in phosphate-buffered saline (PBS) and analyzed by flow cytometry as previously described (8).

T cell-dependent and T cell-independent antibody responses

G3 mice were immunized i.m. with the T-dependent antigen aluminum hydroxide-precipitated ovalbumin (OVA/alum, 200 µg; Invivogen) on day 0. Fourteen days after OVA/alum immunization, blood was collected in MiniCollect tubes (Mercedes Medical) and centrifuged at 1500 × g to separate the serum for enzyme-linked immunosorbent assay (ELISA) analysis of OVA-specific IgG and total IgE. Three days after bleeding (day 17), mice were immunized with another T-dependent antigen, recombinant

Semliki Forest virus-encoded β -galactosidase (rSFV- β -Gal, 2×10^6 I.U.) (9), and the T-independent antigen NP-Ficoll (50 μ g i.p.; Biosearch Technologies, day 25) as previously described (7). Six days after NP-Ficoll immunization, blood was collected for ELISA analysis of NP-specific IgM or β -Gal-specific IgG as previously described (10).

Stimulation of peritoneal macrophages with innate immune receptor agonists

Macrophages were elicited by i.p. injection of 2 ml BBL thioglycollate medium, brewer modified (4%; BD Biosciences) and recovered 4 d later by peritoneal lavage with 5 ml PBS. The peritoneal macrophages were cultured at 37 °C in 95% air and 5% CO₂ in DMEM cell culture medium (DMEM containing 10% FBS (Gemini Bio Products), 1% penicillin and streptomycin (Life Technologies)). Cytokines were measured in the culture medium after 16 hours of incubation with Toll-like receptor agonists (LPS, CpG, poly(I:C), R848, and Pam₃CSK₄) as described previously (11). Nigericin was added to macrophages for 1 hour after overnight LPS stimulation and supernatant was collected to measure IL-1 β as previously described (11). Ultra-pure LPS and R848 were obtained from Enzo Life Sciences; Pam₃CSK₄ was obtained from EMC Microcollections (Tübingen, Germany); nigericin was obtained from Sigma (St. Louis, MO), poly(I:C) was obtained from Enzo Life Sciences (San Diego, CA), and CpG DNA was obtained from IDT (San Diego, CA). TNF, IL-1 β and type I IFN concentrations were measured with ELISA kits (eBioscience).

Discussion

Pathways implicated in the atopy screen (Fig. 1B)

Class-switch recombination

IgE CSR depends on the interaction of B cell-expressed CD40 with CD40 ligand (CD40L) expressed by cognate T helper cells, and on the cytokine IL-4 produced by T helper type 2 (Th2) cells (12). Abrogation of the papain-specific IgE response caused by T cell deficiency in *Lck*^{-/-} mice (Fig. S2A) is consistent with the established requirement for T cell help in CSR.

Regulation in the T cell compartment. A non-polar to polar missense mutation (*scratch*, p.L106S) at a highly conserved position of *Itch* resulted in high IgE levels (Fig. 2A-D). *Itch* encodes an E3 ubiquitin ligase that negatively regulates Th2 differentiation. Mice lacking *Itch* were reported to develop severe skin and pulmonary inflammation, and T cells from these mice displayed an activated phenotype and produced elevated Th2 cytokines leading to increased serum IgG1 and IgE (13, 14). We confirmed the increased production of papain-specific IgE in immunized α^{18H} mice, which harbor a partial inversion of *Itch*. Increased papain-specific IgE was also detected in mice with a homozygous missense mutation in *Itk* (*itxaro*, p.F379L, Fig 2E-G), affecting the highly conserved ATP-binding loop of the kinase domain. *Itk*, as a component of the proximal TCR signaling complex, is required for activation of phospholipase C γ 1 (PLC γ 1) (15), explaining the effect of the *itxaro* mutation on TCR signaling, T cell development, and hence, T cell numbers (Fig. S4B). A null mutation of *Itk* was reported to increase the numbers of $\gamma\delta$ NKT cells producing Th2 cytokines when stimulated, and expressing costimulatory molecules that provide B cell help, resulting in elevated levels of serum IgE (16-18), consistent with the phenotype of homozygous

Itk^{itxaro} mice. Mutations of *ITCH* or *ITK* cause similar phenotypes in humans and mice, characterized by multisystem autoimmune disease and lymphoproliferative syndrome, respectively (19-21).

We identified a physiochemically disparate missense mutation in a highly conserved position of the tyrosine kinase domain of *Map3k8* (*Gnostic_gospel*, p.R376C) that resulted in increased papain-specific IgE in homozygous and heterozygous mice (Fig. S3E-G). TLR signaling defects were also detected in these animals (Table S2), as previously reported, supporting the deleterious effect of the observed variant (22, 23). Our data are consistent with a report that elevated levels of IgE were detected in *Map3k8* deficient mice after ovalbumin immunization due to increased IL-4 production by T cells in these animals (24). Interestingly, the overproduction of IgE occurred despite decreased IgE production by *Map3k8* deficient B cells in culture, attributed to impaired MKK1/2 and ERK activation downstream from CD40 (24).

A canonical splice acceptor site mutation in intron 18 of *Jak3* (*thistle*, c.2479-2A>T) was predicted to cause skipping of exon 19, a frameshift, and premature termination in the kinase domain. This presumed loss of function variant was linked to a decrease in papain-specific IgG1 (Fig. 2H-J). These data are consistent with the known role of JAK3 in mediating signaling from γ_c -containing cytokine receptors, including the IL-7 receptor necessary for B and T cell development, the IL-15 receptor necessary for NK cell development, and the IL-4 receptor, which signals STAT6-dependent transcription of the C ϵ GLT in B cells (25, 26).

A missense mutation of *Stat6* (*Stationary*, p.V263I) was linked to a dominant decrease in papain-specific IgE (Fig. 2K-M). The mutation affects a highly conserved amino acid at the N-terminus of the DNA binding domain (Fig. 2M), and there is a small physicochemical difference between valine and isoleucine (27). Based on the crystal structure of human STAT6 bound to DNA (PDB 5D39), V282 (identical in the human protein) appears to be adjacent to the DNA binding interface. The *Stationary* mutation may therefore impair DNA binding of mutant STAT6 dimers, affecting STAT6 homodimerization, which mediates IgE class switching.

Although homozygous null mutations of *Ikkkb* are embryonic lethal (28), a missense mutation (*Impaired*, p.L576P) in the conserved dimerization domain led to viable mice that had a semidominant reduction of papain-specific IgE and IgG1 responses (Fig. S4A and S5A-D). Proline missense mutations can affect protein structure, but this mutant appears to retain some function as the mice were viable. Defective function of the IKK complex leading to a failure of NF- κ B activation likely underlies the *Impaired* phenotype. We hypothesize this *Ikkkb* mutation failed to be validated either because of redundancy between IKK β and IKK α in canonical NF- κ B activation (29) or due to insufficient impairment to IKK β function.

A nonsense mutation in the REL homology/DNA binding domain of *Rel* (*Amun_ra*, p.G59*, Fig. 2N-Q) would be anticipated to cause a loss of protein function, and was linked to decreased papain-specific IgG1 and IgE. Rel dimerizes with other NF- κ B family members to mediate class switching, and the dominant decrease in IgE but semi-dominant decrease in IgG1 suggests Rel is more important for IgE class switching while residual function permits class switching to IgG1.

Finally, the inhibitory effect on papain-specific IgE of the semidominant *Ighe* mutation *Allegra* (p. Q389*, Fig. 3A-C) may not be due to impaired IgE CSR per se, but rather due to lack of IgE expression as a result of nonsense mediated decay of the transcript.

B cell receptor signaling

Four B cell-specific proteins (Blk, Syk, PLC γ 2, Rasgrp3), the BCR coreceptor CD19, and its signaling mediator PI3K (composed of regulatory subunit p85 α encoded by *Pik3r1* and catalytic subunit p110 δ encoded by *Pik3cd*) were implicated as suppressors of the papain-specific IgE response. We hypothesized the alleles to be loss-of-function, as gain-of-function alleles are much rarer; therefore, we used knockout alleles to confirm the role of each gene. A missense mutation in *Blk* (*blaenka*, p.S93P, Fig. 3D-G) caused a large physicochemical change at a conserved residue in the Src homology 3 (SH3) domain, which is essential for kinase activity (30). A missense mutation in the Syk kinase domain (*Sisyphus*, p.Y520C, Fig. 3H-K) affecting Tyr520, which is normally autophosphorylated following Syk binding to phosphorylated ITAMs, caused a dominant increase in papain-specific IgE. Phosphorylation of Tyr519 and Tyr520 are essential for signal transduction, but not absolutely required for kinase activity (31, 32). A conditional knockout of *Syk* by an IgG1-Cre recombinase demonstrated the B-cell intrinsic nature of the phenotype.

Syk recruits the Blnk scaffold subsequently activating Plcg2, which was linked to a dominant increase in papain-specific IgE (*Poseidon*, p.W1113R, Fig. 3L-O) and validated by CRISPR targeted knockout allele. Plcg2 activates Rasgrp3 indirectly through inositol-triphosphate (IP3) mediated calcium release and diacylglycerol (DAG) signaling. Mutations in human PLCG2 were associated with increased atopy and allergies (33), consistent with our data. Interestingly, two high papain-specific IgE phenotypes, *centre* and *Aster*, mapped to *Rasgrp3* (p.H262R and c.11083+5G>T, Fig. 3P-S). The *centre* allele was a missense mutation causing a small physicochemical change but at a conserved site in the Ras guanine exchange factor (RasGEF) catalytic domain of the gene. The *Aster* splice site mutation was predicted to cause exon 11 skipping with an in-frame deletion of 26 amino acids from the end of the RasGEF domain. Although Candidate Explorer ranked *Aster* a “not good candidate,” *centre* was ranked an “excellent candidate,” and by combining the phenotypic data from both pedigrees into a “superpedigree” (34) we found strong linkage of the elevated IgE phenotype to *Rasgrp3*, which was verified by a CRISPR targeted knockout allele and implicates downstream Mek/Erk signaling in IgE regulation.

A *Cd19* promoter-driven Cre recombinase was not used with the *Syk*-flox allele, because this haploinsufficient *Cd19-Cre* allele was found to increase IgE when validating the recessive *hive* phenotype linked to a missense change of cysteine to tyrosine in the transmembrane domain of *Cd19* (p.C300Y, Fig. 4A-D). CD19 signals through the PI3K pathway; PI3K itself was hit in this screen: *Pik3r1* and *Pik3cd*. Papain-specific IgE was increased by a nonsense mutation in *Pik3r1* (*anubis*, p.Y189*, Fig. 4E-H), which prematurely truncated the major regulatory isoform (p85 α); p85 α deficiency is known to be viable but immunodeficient in the homozygous state. The validation of this phenotype by a CRISPR-targeted knockout allele showed a semi-dominant mode of inheritance with increased IgE in the heterozygous state, in contrast to the ENU-induced allele that was recessive.

Our findings are consistent with work showing that CD19 haploinsufficiency or BLNK/p85 α /p110 δ deficiency resulted in elevated concentrations of antigen-specific IgE in mice immunized with NP-CGG, attributed to increased GC IgE⁺ B cells (35-38). However, the role of BCR and PI3K in the regulation of IgE⁺ B cells may not be straightforward, since other reports indicate that genetically increasing PI3K signaling by PTEN deletion in B cells confers the ability to generate IgE⁺ memory B cells and augments the IgE response(39); these reports propose reduced surface BCR expression causes weak BCR signaling limiting IgE⁺ memory B cell survival (39, 40). Our findings of elevated IgE responses caused by hypomorphic mutations in multiple components of the BCR and PI3K pathways align with the former reports.

However, we found that mutations in two proteins implicated in BCR signaling, calcineurin A β , encoded by *Ppp3cb* (Fig. S5E-H), and PKC β (Fig. S7) were not validated to have an effect on papain-specific IgE compared to wild-type mice. The lack of effect of the *Ppp3cb* mutation is probably a result of redundancy with another catalytic subunit of the calcineurin phosphatase (41). PKC β is required for NF- κ B activation downstream from the BCR, and mice deficient in PKC β had reduced numbers of mature peripheral B cells and defective T cell-dependent and -independent antibody responses (4, 42). In addition, PKC β -deficient B cells displayed impaired germinal center formation and plasma cell differentiation following T cell-dependent antigen challenge (43). The molecular effects of the *Prkcb*^{Untied} mutation are not known, and interpretation of the normal phenotype of mice with the *Untied* mutation is difficult considering that PKC β influences many of the signaling molecules and cell fate decisions that regulate IgE-expressing cells, sometimes in opposing directions.

Other proteins that affect the IgE response

Mutations in *Me2*, *Scarb2*, *Fln*, *Prkd2*, and *Map1lc3b* affected IgE responses by uncertain mechanisms. A combination of pedigrees with *Me2* variants was used to perform a superpedigree analysis to map the p.N95K variant named *baako* to a decrease in IgE (Fig. 5I-L). The amino acid change of a small, polar asparagine to a large, positively charged lysine at a highly conserved site of the malate oxidoreductase domain was replicated by CRISPR and validated the original phenotype (Fig. 5K).

A decreased IgE phenotype named *pansy* was linked to an A to G transition at c.1691 resulted in a missense mutation of p.H564R at the C-terminus of the *Fln* gene (Fig. 5M-P). This new amino acid is a stronger base of similar size and charge in a highly conserved position that does not lie in a known functional domain. Homozygous null mutants of *Fln* are known to be lethal, so the ENU-allele was reproduced by CRISPR and validated the low IgE phenotype (Fig. 5O).

Increased papain-specific IgE in the recessive *apollo* phenotype was linked to a missense mutation in *Map1lc3b* at a conserved amino acid site (p.Y110H) in the Atg8 autophagy domain (Fig. 5E-H). A previously described knockout allele (44) confirmed this phenotype (Fig. 5G) resulted from loss of function of this ubiquitin like modifier, which is important for autophagosome formation, but not essential for canonical autophagy.

A missense mutation in the serine/threonine kinase catalytic domain of *Prkd2* (*Purnama*, p.W807R) was linked to a semi-dominant increase in papain-specific IgE (Fig. 5A, B). This change from a large, non-polar aromatic tryptophan to a charged arginine at a highly conserved site was suspected to decrease protein function (Fig. 5D), which was confirmed when the phenotype was validated with a CRISPR-targeted frameshift allele (Fig. 5C).

An increase in papain-specific IgE was linked to a splice site mutation of *Scarb2* (*scarab*, c.1113G>A, Fig. 5Q-T). Aberrant splicing was predicted to use a cryptic splice site in intron 8 leading to a 55 nucleotide insertion, which causes a frameshifted product after amino acid 371 that terminates after the inclusion of 36 aberrant amino acids. The protein contains a single CD36 domain, which occupies almost its entire linear sequence. *Scarb2* is an integral membrane protein on the lysosome and functions as a scavenger receptor for oxidized LDL. The *scarab* phenotype was validated by a previously described knockout allele (Fig. 5S) (6).

Phenotypes that failed to be validated

Given the validated *apollo* phenotype, we were interested to test whether other autophagy related candidate genes (*Snx14*, *Stx17*, and *Zfyve26*) would be confirmed. Decreased papain-specific IgG1 was linked to missense mutations of *Snx14* (*santorini*, p.Y180C, Fig. S5I, J) and *Stx17* (*cronus*, p.V241M, Fig. S3A, B), encoding components of the SNARE complex that fuses lysosomes to autophagosomes. However, neither homozygotes with CRISPR induced replacement alleles nor compound heterozygotes with a knockout allele recreated the original phenotype (Fig. S5K, N). A missense mutation at a poorly conserved site in an unknown domain of *Zfyve26* (*eros*, p.M313K, Fig. S6A-C) was linked to elevated papains-specific IgE. However, *Zfyve26* and the closely linked *Arid4a* failed validation since homozygous KO mice displayed normal rather than elevated IgE responses to papain (Fig. S6D-F). Lastly, missense mutations in *Tnfaip2* and *Akt1* were linked to increased papain-specific IgG1, but a CRISPR induced replacement allele of the *Tnfaip2* mutation did not validate the original finding (Fig. S5O-R).

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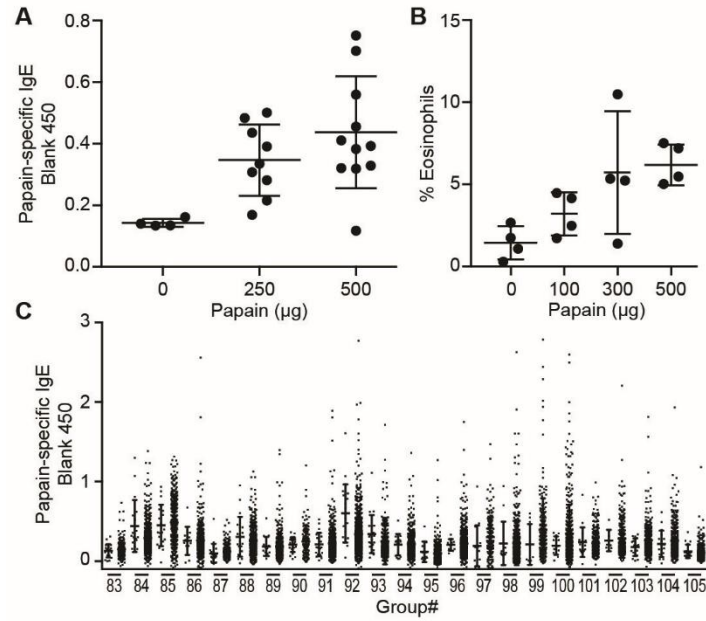


Fig. S1. IgE and eosinophils induced by papain immunization in mice. C57BL/6J or G3 mice carrying ENU-induced mutations were immunized i.p. with papain on day 0. Papain-specific IgE on day 15 (A) or frequency of eosinophils on day 6 (B) in serum from C57BL/6J mice. (C) Example of screening results showing papain-specific IgE in serum for 23 groups of G3 mice on day 15 after immunization with 0.5 mg papain/mouse. Data points represent individual mice. Data are representative of one (C) or two (A, B) independent experiments. Central line indicates mean, error bars indicate S.D.

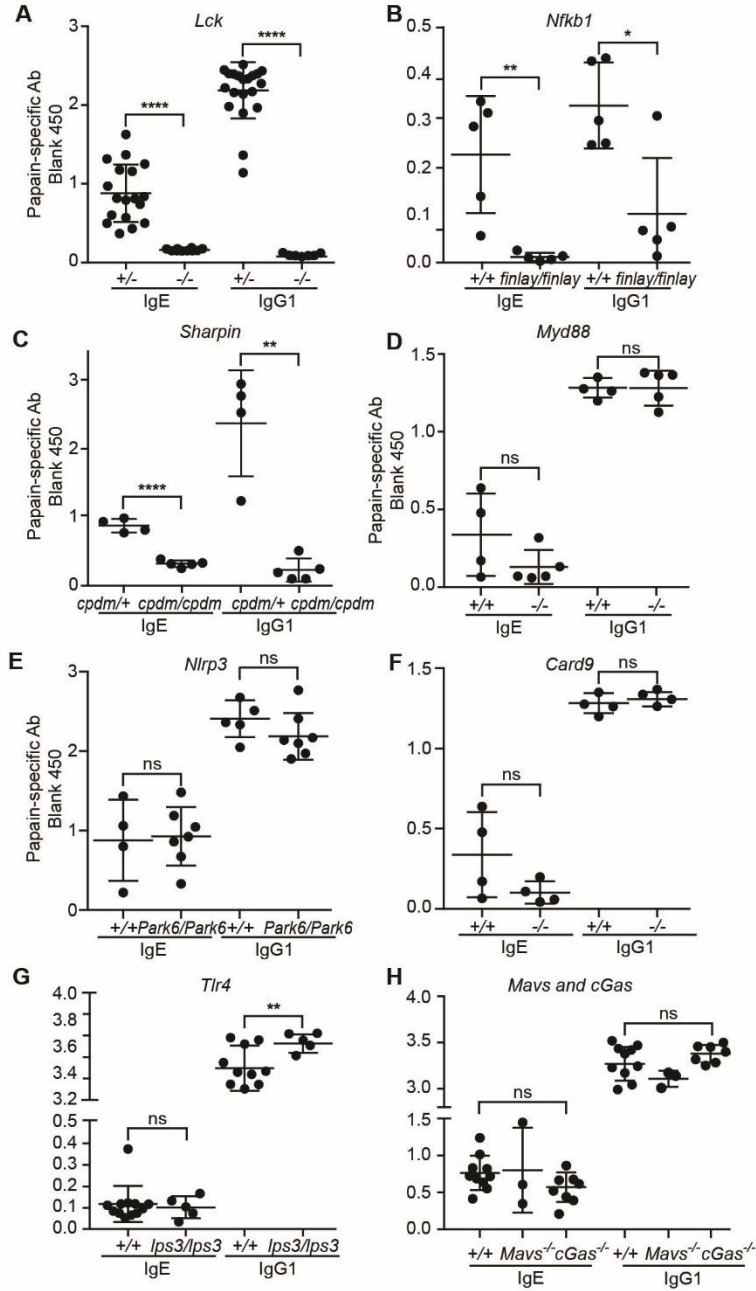


Fig. S2. Validation of the screening protocol using mice deficient in NF-κB signaling or innate immune signaling. Serum papain-specific IgE and IgG1 on day 14 after papain immunization in (A) *Lck*^{-/-}, (B) *Nfkb1*^{finlay/finlay}, (C) *Sharpin*^{cpdm/cpdm}, (D) *Myd88*^{-/-}, (E) *Nlrp3*^{Park6/Park6}, (F) *Card9*^{-/-}, (G), *Tlr4*^{lps3/lps3} (H), and *Mavs*^{-/-}, *cGas*^{-/-} mice. “-/-” represents an engineered null allele. Data points represent individual mice. Data are representative of 1-2 independent experiments. Central line indicates mean, error bars indicate S.D.

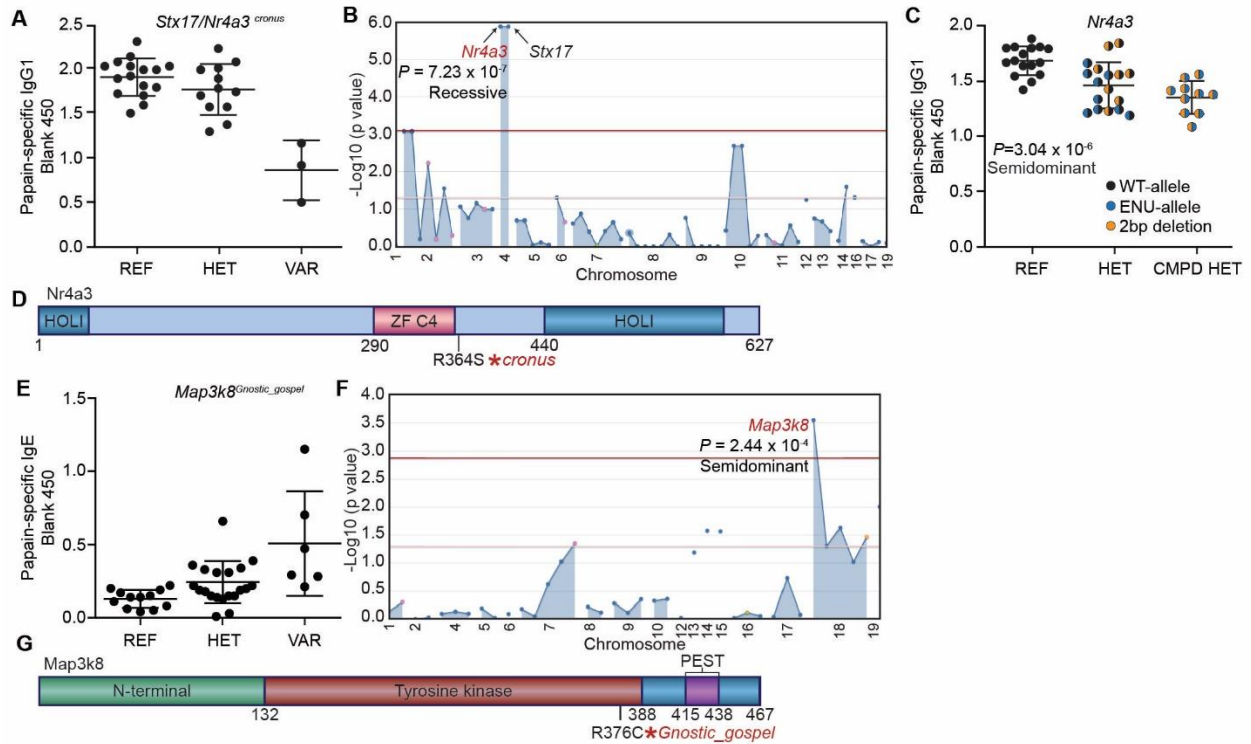


Fig. S3. Screening and validation testing of *Nr4a3* and *Map3k8* alleles for effects on papain-specific IgE or IgG1 responses to papain immunization. Data are displayed as in Fig. 2. (A-D) *Nr4a3* (E-G) *Map3k8*. *Stx17* is linked to *Nr4a3* and phenotypic data scatter plot for the *Stx17* CRISPR mutation is in Fig. S5N. REF, homozygous for the reference allele; HET, heterozygous for the reference and variant allele; VAR, homozygous for the variant allele; CPMD HET, compound heterozygous for two variant alleles; C, CRISPR/Cas9-targeted allele; HOLI, Ligand binding domain of hormone receptors; ZF C4, c4 zinc finger in nuclear hormone receptors; PEST, domain rich in proline (P), glutamic acid (E), serine (S) and threonine (T). Data points represent individual mice (A, C, E). Data are representative of 1-2 independent experiments. Central line indicates mean, error bars indicate S.D.

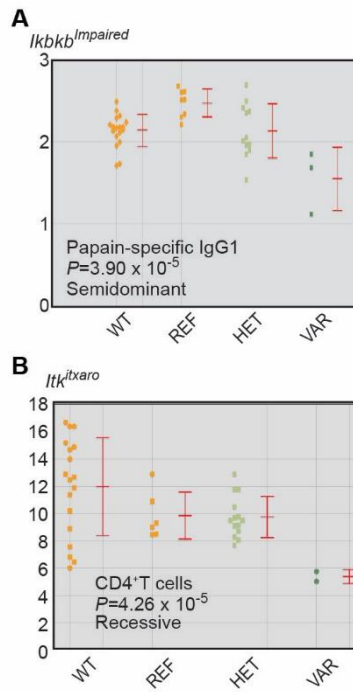


Fig. S4. Phenotypic screening data for *Ikbkb* and *Itk* alleles. (A) Serum papain-specific IgG1 after papain immunization of mice with the *Ikbkb^{impaired}* allele. (B) Frequency of CD4⁺ T cells in the blood of mice with the *Itk^{itxaro}* allele. WT, C57BL/6J; REF, homozygous for the reference allele; HET, heterozygous for the reference and variant allele; VAR, homozygous for the variant allele. Data points represent individual mice. Data are from one experiment. Central line indicates mean, error bars indicate S.D.

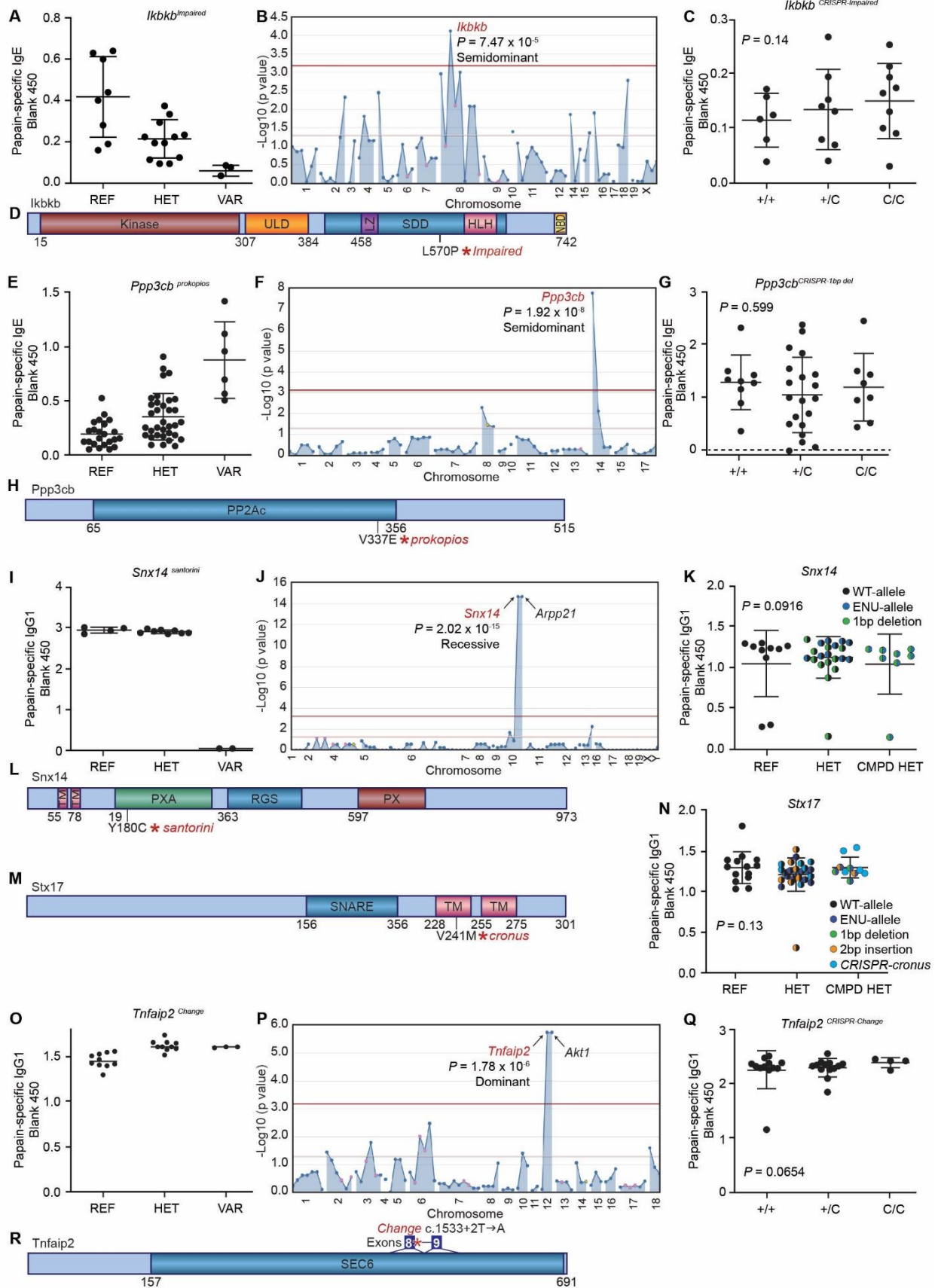


Fig. S5. Screening data for *Ikbkb*, *Ppp3cb*, *Snx14*, *Stx17*, and *Tnfaip2* alleles on papain-specific IgE or IgG1 responses to papain immunization. These alleles were considered not validated based on CRISPR targeted (C, G, K, N, Q) alleles. Data are displayed as in Fig. 2. (A-D) *Ikbkb*, (E-H) *Ppp3cb*, (I-L) *Snx14*, (M-N) *Stx17*, and (O-R) *Tnfaip2*. REF, homozygous for the reference allele; HET, heterozygous for the reference and variant allele; VAR, homozygous for the variant allele; C, CRISPR/Cas9-targeted allele; CPMD HET, compound heterozygous for two variant alleles; ULD, Ubiquitin-like domain; LZ, leucine zipper; HLH, helix-loop-helix; NBD, nucleotide binding domain; PP2Ac, Protein phosphatase 2A, catalytic domain; TM, transmembrane; SNARE, SNAP-Receptor; PX, Phox homology domain; PXA, PX-associated; RGS, Regulation of G protein Signaling; Sec6, Exocyst complex component 6. Data points represent individual mice (A, C, E, G, I, K, N, O, Q). Data are from one experiment. Central line indicates mean, error bars indicate S.D.

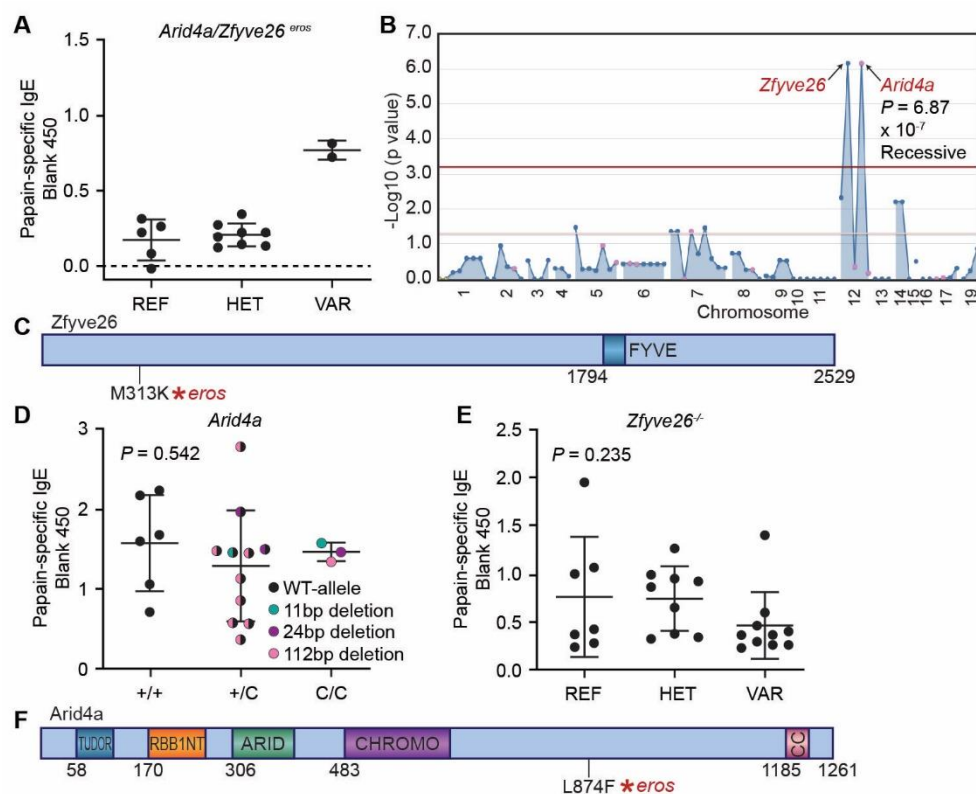


Figure S6. Screening data for *Arid4a/Zfyve26* alleles on papain-specific IgE or IgG1 responses to papain immunization. *Arid4a* is linked to *Zfyve26*. (A) Phenotypic data scatter plot and (B) Manhattan plot are presented for the linked mutations. These alleles were considered not validated based on CRISPR targeted (D) or an existing null (E) allele. REF, homozygous for the reference allele; HET, heterozygous for the reference and variant allele; VAR, homozygous for the variant allele; C, CRISPR/Cas9-targeted allele; FYVE, present in Fab1, YOTB, Vac1, and EEA1; RBB1NT, Retinoblastoma-binding protein 1; ARID, AT-rich interaction domain; CHROMO, chromatin organization modifier domain; CC, coiled coil domain. Data points represent individual mice (A, D, E). Data are from one experiment. Central line indicates mean, error bars indicate S.D.

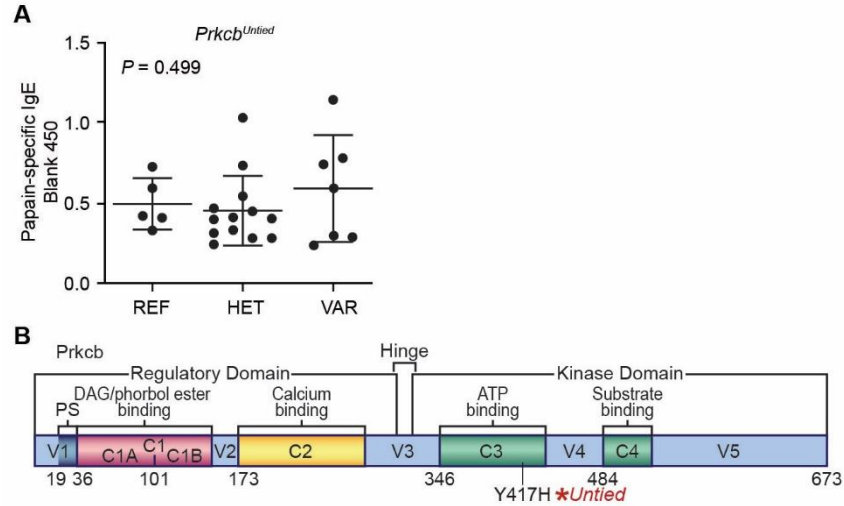


Fig. S7. Screening data for the *Prkcb* allele on papain-specific IgE response to papain immunization. (A) Scatter plot of serum papain-specific IgE after papain immunization of mice. (B) The protein domain diagram of *Prkcb* showing the location of the *Untied* mutation. REF, homozygous for the reference allele; HET, heterozygous for the reference and variant allele; VAR, homozygous for the variant allele; C1, phorbol 12-myristate 13-acetate (PMA)/ diacylglycerol (DAG) binding domain; C2, calcium dependent phospholipid binding domain; C3, ATP binding domain of protein kinase; C4, substrate binding domain of protein kinase; PS, pseudosubstrate domain; V1-5, variable domain 1-5. Data points represent individual mice. Data are from one experiment. Error bars indicate S.D.

Table S1. Genes, alleles, and results of validation testing

Gene	Mutation	Coding change	Protein change	P	N (REF, HET, VAR)	Phenotype	Inheritance
Arid4a	11bp deletion, 24bp deletion, 112bp deletion	c.827_837 del CAGTGATGACG	p.S276R fs*5	0.54	(6, 12, 3)	Papain-specific IgG1	Not validated
		c.828_854 del AGTGATGACGAA GAATGCCAGCT GAGGAACA insTGAGAACT	p.277*				
		c.821_906+27 del CCTCCAGCAGTG ATGACGAAGAAT GCCAGCTGAGG AACATGAAGAGG AGAAGGAAAAA GAGGCCAAAAA GGAGGAAGAAG AGCTGGTAGGTC AACGCTGTGGTT TGTTTGT	p.S272A fs*1				
Blk	Blk ^{tm1Tara}			4.57 x 10 ⁻⁴	(8, 14, 5)	↑ Papain-specific IgE	Semi-dominant
Cd19	Cd19 ^{tm1(cre)Cgn}			0.013	(5, 7, 0)	↑ Papain-specific IgE	Dominant
Flcn	H564R (Replacement)	c.1691AT>GG	p.H564R	0.0040	(13, 0, 9)	↓ Papain-specific IgE	Recessive
Ikbbk	L570P (Replacement)	c.1709T>C	p.L570P	0.14	(6, 8, 9)	Papain-specific IgE	Not validated
Itch	Itch ^{a18H}			0.00274	(3, 8, 4)	↑ Papain-specific IgE	Recessive
Lck	1bp deletion	c.198delA	p.V66Sfs*36	8.75 x 10 ⁻¹⁷	(0, 18, 10)	↓ Papain-specific IgE	Recessive
Map1lc3b	Map1lc3b ^{tm1Mrab}			2.63 x 10 ⁻⁴	(5, 7, 12)	↑ Papain-specific IgE	Recessive
Me2	N92K (Replacement)	c.276A>T	p.N92K	0.00234	(12, 22, 6)	↓ Papain-specific IgE	Recessive

Nfkb1	Nfkb1 ^{Finlay}	c.1751T>A	p.L584*	0.0089	(5, 0, 5)	↓ Papain-specific IgE	Recessive
Nr4a3	R364S (ENU) x 2bp deletion	c.1150_1151 delCA	p.Q384T fs*31	1.23 x 10 ⁻⁴	(16, 24, 12)	↓ Papain-specific IgE	Semi-dominant
				3.04 x 10 ⁻⁶	(15, 17, 10)	↓ Papain-specific IgG1	Semi-dominant
Pik3r1	2bp insertion	c.25 insCA	p.R9Sfs*22	3.99 x 10 ⁻⁹	(9, 3, 4)	↑ Papain-specific IgE	Semi-dominant
Plcg2	5bp deletion	c.3346_3351 delCTCAGGinsA	p.T1116K fs*5	2.01 x 10 ⁻⁴	(11, 8, 1)	↑ Papain-specific IgE	Dominant
Ppp3cb	1bp deletion	c.988 delG	p.V330Y fs*1	0.599	(8,20,8)	Papain-specific IgE	Not validated
Prkcb	Prkcb ^{Untied}	c.1249T>C	p. Y417H	0.50	(5, 14, 7)	Papain-specific IgE	Not validated
Prkd2	7bp deletion	c.2423_2427+2d elTACAGGT	p.L808*	3.38 x 10 ⁻⁴	(5, 11, 7)	↑ Papain-specific IgE	Semi-dominant
Rasgrp3	1bp deletion	c.1151 delA	p.N384I fs*37	0.00821	(4, 22, 5)	↑ Papain-specific IgE	Semi-dominant
Scarb2	Scarb2 ^{tm1Psa}			2.04 x 10 ⁻⁴	(9, 16, 5)	↑ Papain-specific IgE	Recessive
Sharpin	Sharpin ^{cpdm}			0.00414	(4, 0, 5)	↓ Papain-specific IgE	Recessive
Snx14	(ENU) x 1bp deletion	c.542+1 delG	Critical splice site	0.09	(9, 22,8)	Papain-specific IgG1	Not validated
Stx17	V241M (ENU) x 1bp deletion, 2bp insertion, V241M (Replacement)	c.743 delT c.744 insTC c. 721G>A	p.L249P fs*17 p.L250S fs*17 p.V241M	0.13	(14, 28, 10)	Papain-specific IgG1	Not validated
Syk	Syk ^{flox/+} x Ighg1 ^{Cre}			0.00283	(9, 6, 0)	↑ Papain-specific IgE	Dominant
Tnfaip2	c.1459+2T>A (Replacement)	c.1459+2T>A	Critical splice site	0.424	(13, 12, 4)	Papain-specific IgG1	Not validated
Zfyve26	Zfyve26 ^{em1J}			0.23	(8, 17, 6)	Papain-specific IgE	Not validated

In some cases the original ENU allele was retrieved and crossed to a KO allele (designated “ENU,” refer to main figures for the mutation), or the original single base pair mutation was recreated by CRISPR/Cas9 targeting (designated “Replacement,” described in this table).

Table S2. Significant changes to immunity in the heterozygous state of dominant IgE genes.

% change P value N= REF,HET,VAR	TD	TI	CD4	CD8	B	NK	NLRP3	LPS	CpG	PolyI:C	R848	Pam ₃ CSK ₄
Gene												
<i>Blk</i>^{blaenka}	NS 21,23,5	NS 21,23,5	NS 22,23,5	NS 22,23,5	NS 22,23,5	NS 22,23,5	NS 19,18,1	NS 20,21,3	NS 3,8,0	NS 3,8,0	NS 20,21,3	NS 20,21,3
<i>Cd19</i>^{hive}	NS 5,18,4	NS 5,18,4	NS 6,18,4	NS 6,18,4	NS 6,18,4	NS 6,18,4	NS 5,15,4	NS 5,17,4	NS 5,15,4	NS 5,15,4	NS 5,17,4	NS 5,17,4
<i>Map1lc3b</i>^{apollo}	NS 16,17,3	NS 16,17,3	NS 18,20,3	NS 18,20,3	NS 18,20,3	NS 18,20,3	NS 16,18,3	NS 16,18,3	NS 14,14,3	NS 14,14,3	NS 16,18,3	NS 16,18,3
<i>Map3k8</i>^{Gnostic} _{_gospel}	NS 12,20,6	NS 12,20,6	NS 12,20,6	NS 12,20,6	NS 12,20,6	NS 12,20,6	-24% 3.5x10 ⁻⁴ 12,21,6	-41% 3.8x10 ⁻⁵ 8,17,5	-39% 5.8x10 ⁻⁵ 7,16,6	-32% 6.8x10 ⁻⁴ 7,17,5	NS 8,17,5	-35% 1.2x10 ⁻³ 8,17,5
<i>Pik3r1</i>^{anubis}	NS 4,7,1	NS 4,7,1	NS 4,7,1	NS 4,7,1	NS 4,7,1	NS 4,7,1	NS 4,7,1	NS 4,7,1	NS 4,7,1	NS 3,7,1	NS 4,7,1	NS 4,7,1
<i>Plcg2</i>^{Poseidon}	NS 7,14,0	-33% 6.9x10 ⁻⁴ 7,14,0	NS 7,14,0	NS 7,14,0	NS 7,14,0	NS 7,14,0	NS 6,10,0	NS 7,11,0	NS 5,10,0	NS 5,10,0	-56% 3.2x10 ⁻⁴ 7,11,0	NS 7,11,0
<i>Prkd2</i>^{Purnama}	NS 16,14,9	NS 16,14,8	NS 16,14,9	NS 16,14,9	NS 16,14,9	NS 16,14,9	NS 12,9,7	NS 13,9,7	NS 11,9,7	NS 13,9,7	NS 13,9,7	NS 13,9,7
<i>Rasgrp3</i>^{aster}	NS 9,16,6	-24% ^{SP} 2.9x10 ⁻⁶ 44,61,19	NS 9,17,6	NS 9,17,6	NS 9,17,6	NS 9,17,6	NS 5,16,4	NS 6,17,4	NS 5,16,4	NS 5,16,4	NS 6,17,4	NS 6,17,4
<i>Syk</i>^{Sisyphus}	NS 14,9,0	NS 14,9,0	NS 14,9,0	NS 14,9,0	NS 14,9,0	+46% 4.1x10 ⁻⁷ 14,9,0	+82% 3.4x10 ⁻⁴ 8,8,0	NS 9,9,0	NS 8,8,0	NS 8,8,0	NS 9,9,0	NS 9,9,0

TD: T-dependent IgG response to rSFV-βGal, TI: T-independent IgM response to NP-Ficoll, CD4: percent of CD4⁺ T cells in the peripheral blood, CD8: percent of CD8⁺ T cells in the peripheral blood, B: percent of B cells in the peripheral blood, NK: percent of NK cells in the peripheral blood, NLRP3: IL-1β secretion from peritoneal macrophages in response to LPS/nigericin stimulation; TLR screens measured TNF secretion from peritoneal macrophages in response to LPS (lipopolysaccharide), CpG (single stranded DNA with CpG dinucleotides), Poly(I:C) (double stranded RNA consisting of polyriboinosinic:polyribocytidylic acid), R848 (resiquimod), and Pam₃CSK₄. If a heterozygous mutation resulted in a response significantly different from that of C57BL/6J controls (dominant or semi-dominant model of inheritance), then the percent change with respect to controls is shown along with the p-value and the number of mice of each genotype (REF, HET, VAR).

^{SP} Denotes linkage analysis was performed after combining multiple pedigrees with the same allele into a superpedigree for analysis.

Table S3. Contingency tables for the number of semi-dominant/dominant and recessive alleles found to increase papain-specific IgE compared to semi-dominant/dominant and recessive alleles from all other verified phenotypes performed by our lab.

Observed	Papain-IgE	Others
Dom + Semi-dom	9	190
Recessive	3	854
Expected	Papain-IgE	Others
Dom + Semi-dom	2	197
Recessive	10	847