



Molecular Editing of Cellular Responses by the High-Affinity Receptor for IgE
Ryo Suzuki *et al.*
Science **343**, 1021 (2014);
DOI: 10.1126/science.1246976

This copy is for your personal, non-commercial use only.

If you wish to distribute this article to others, you can order high-quality copies for your colleagues, clients, or customers by [clicking here](#).

Permission to republish or repurpose articles or portions of articles can be obtained by following the guidelines [here](#).

The following resources related to this article are available online at www.sciencemag.org (this information is current as of November 19, 2014):

Updated information and services, including high-resolution figures, can be found in the online version of this article at:

<http://www.sciencemag.org/content/343/6174/1021.full.html>

Supporting Online Material can be found at:

<http://www.sciencemag.org/content/suppl/2014/02/06/science.1246976.DC1.html>

A list of selected additional articles on the Science Web sites **related to this article** can be found at:

<http://www.sciencemag.org/content/343/6174/1021.full.html#related>

This article **cites 34 articles**, 13 of which can be accessed free:

<http://www.sciencemag.org/content/343/6174/1021.full.html#ref-list-1>

This article has been **cited by 2 articles** hosted by HighWire Press; see:

<http://www.sciencemag.org/content/343/6174/1021.full.html#related-urls>

This article appears in the following **subject collections**:

Cell Biology

http://www.sciencemag.org/cgi/collection/cell_biol

N_e^{EUR}/N_e^{ASN} is 1.29 [95% confidence interval (CI) of 1.15 to 1.57] and that East Asians received 20.2% (95% CI of 13.4 to 27.1%) more Neandertal sequence in the second pulse (10). We note that additional unexplored models may provide a better fit to the data, and refining demographic models of hominin evolution is an important area for future work.

The collection of surviving Neandertal lineages that we identified allows us to search for signatures of adaptive introgression (15, 16). First, we used introgressed variants that exhibit large allele frequency differences between Europeans and East Asians ($F_{ST} > 0.40$, $P < 0.001$ by simulation) (10) to identify four significantly differentiated regions (Fig. 4 and table S10) (10). Introgressed haplotypes in two of these regions span genes that play important roles in the integumentary system: *BNC2* on chromosome 9 and *POU2F3* on chromosome 11. *BNC2* encodes a zinc finger protein expressed in keratinocytes and other tissues (17) and has been associated with skin pigmentation levels in Europeans (18). The adaptive haplotype has a frequency of ~70% in Europeans and is completely absent in East Asians (Fig. 2B). *POU2F3* is a homeobox transcription factor expressed in the epidermis and mediates keratinocyte proliferation and differentiation (19, 20). The adaptive haplotype in East Asians has a frequency of ~66% and is found at less than 1% frequency in Europeans (Fig. 2B). No coding introgressed variants were found in *BNC2* or *POU2F3*, although several highly differentiated introgressed variants were located in functional noncoding elements (21) (Fig. 2B), suggesting that modern humans acquired adaptive regulatory sequences at these loci. We also searched for shared signatures of adaptive introgression between East Asians and Europeans,

identifying six distinct regions that have introgressed haplotype frequencies greater than 40% in both populations (Fig. 4 and table S11) ($P < 10^{-4}$ by simulation) (10). One of these regions lies in the type II cluster of keratin genes on 12q13 (table S11), further suggesting that Neandertals provided modern humans with adaptive variation for skin phenotypes. In total, 8 of the 10 candidate introgressed regions overlap protein-coding genes (Fig. 4).

This study shows that the fragmented remnants of the Neandertal genome carried in the DNA of modern humans can be robustly identified, allowing, in aggregate, substantial amounts of Neandertal sequence to be recovered. In principle, our approach can be used in the absence of an archaic reference sequence, potentially allowing the discovery and characterization of previously unknown hominins that interbred with modern humans (22–24). This fossil-free paradigm of sequencing archaic genomes holds considerable promise for revealing insights into hominin evolution, the population genetics characteristics of archaic hominins, how introgression has influenced extant patterns of human genomic diversity, and narrowing the search for genetic changes that endow distinctly human phenotypes.

References and Notes

1. A. D. Twyford, R. A. Ennos, *Heredity* **108**, 179–189 (2012).
2. D. Zinner, M. L. Arnold, C. Roos, *Evol. Anthropol.* **20**, 96–103 (2011).
3. R. E. Green *et al.*, *Science* **328**, 710–722 (2010).
4. D. Reich *et al.*, *Nature* **468**, 1053–1060 (2010).
5. M. Meyer *et al.*, *Science* **338**, 222–226 (2012).
6. A. Eriksson, A. Manica, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 13956–13960 (2012).
7. M. A. Yang, A. S. Malaspina, E. Y. Durand, M. Slatkin, *Mol. Biol. Evol.* **29**, 2987–2995 (2012).

8. S. Sankararaman, N. Patterson, H. Li, S. Pääbo, D. Reich, *PLOS Genet.* **8**, e1002947 (2012).
9. J. D. Wall *et al.*, *Genetics* **194**, 199–209 (2013).
10. Supplementary materials are available on Science Online.
11. V. Plagnol, J. D. Wall, *PLOS Genet.* **2**, e105 (2006).
12. G. R. Abecasis *et al.*, *Nature* **491**, 56–65 (2012).
13. W. Enard *et al.*, *Nature* **418**, 869–872 (2002).
14. M. Currat, L. Excoffier, *Proc. Natl. Acad. Sci. U.S.A.* **108**, 15129–15134 (2011).
15. F. L. Mendez, J. C. Watkins, M. F. Hammer, *Am. J. Hum. Genet.* **91**, 265–274 (2012).
16. L. Abi-Rached *et al.*, *Science* **334**, 89–94 (2011).
17. A. Vanhoutteghem, P. Djian, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 12423–12428 (2006).
18. L. C. Jacobs *et al.*, *Hum. Genet.* **132**, 147–158 (2013).
19. A. Cabral, D. F. Fischer, W. P. Vermeij, C. Backendorf, *J. Biol. Chem.* **278**, 17792–17799 (2003).
20. H. Takemoto *et al.*, *J. Dermatol. Sci.* **60**, 203–205 (2010).
21. B. E. Bernstein *et al.*, *Nature* **489**, 57–74 (2012).
22. J. D. Wall, K. E. Lohmueller, V. Plagnol, *Mol. Biol. Evol.* **26**, 1823–1827 (2009).
23. M. F. Hammer, A. E. Woerner, F. L. Mendez, J. C. Watkins, J. D. Wall, *Proc. Natl. Acad. Sci. U.S.A.* **108**, 15123–15128 (2011).
24. J. Lachance *et al.*, *Cell* **150**, 457–469 (2012).

Acknowledgments: We thank members of the Akey laboratory, S. Browning, B. Browning, and J. Duffy for critical feedback related to this work; S. Pääbo for providing access to high-coverage Neandertal sequence data; and L. Jäuregui for help in figure preparation. A description of where sequence data used in our analyses can be found in the supplementary materials. Introgressed regions and variants can be downloaded from <http://akeylab.gs.washington.edu/downloads.shtml>. J.M.A. is a paid consultant of Glenview Capital.

Supplementary Materials

www.sciencemag.org/content/343/6174/1017/suppl/DC1
Materials and Methods
Figs. S1 to S15
Tables S1 to S11
References (25–45)

12 September 2013; accepted 6 December 2013
Published online 29 January 2014;
10.1126/science.1245938

Molecular Editing of Cellular Responses by the High-Affinity Receptor for IgE

Ryo Suzuki,¹ Sarah Leach,¹ Wenhua Liu,² Evelyn Ralston,² Jörg Scheffel,¹ Weiguo Zhang,³ Clifford A. Lowell,⁴ Juan Rivera^{1*}

Cellular responses elicited by cell surface receptors differ according to stimulus strength. We investigated how the high-affinity receptor for immunoglobulin E (IgE) modulates the response of mast cells to a high- or low-affinity stimulus. Both high- and low-affinity stimuli elicited similar receptor phosphorylation; however, differences were observed in receptor cluster size, mobility, distribution, and the cells' effector responses. Low-affinity stimulation increased receptor association with the Src family kinase Fgr and shifted signals from the adapter LAT1 to the related adapter LAT2. LAT1-dependent calcium signals required for mast cell degranulation were dampened, but the role of LAT2 in chemokine production was enhanced, altering immune cell recruitment at the site of inflammation. These findings uncover how receptor discrimination of stimulus strength can be interpreted as distinct *in vivo* outcomes.

It has long been recognized that there are many subtleties in how receptors function to determine a cell's response. For example,

vegetative growth of the yeast *Saccharomyces cerevisiae* is elicited by low pheromone concentrations recognized by the pheromone receptor

Ste2, whereas intermediate and high pheromone concentrations sensed by this receptor lead to chemotrophic growth or mating, respectively (1). Mathematical modeling suggests that yeast translate pheromone concentration as the duration of the transmitted signal (2).

We explored how the high-affinity immunoglobulin E (IgE) receptor FcεRI discriminates high- from low-affinity stimulation to modulate the mast cells' effector responses. Engagement of FcεRI on mast cells and basophils is central to allergic responses (3, 4). Allergic individuals may produce IgE antibodies to offending allergens (a term used for allergy-inducing antigens). These IgE antibodies bind [via their crystallizable

¹Laboratory of Molecular Immunogenetics, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD 20892, USA. ²Light Imaging Section, Office of Science and Technology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD 20892, USA. ³Department of Immunology, Duke University School of Medicine, Durham, NC 27710, USA. ⁴Department of Laboratory Medicine, University of California, San Francisco, CA 94143, USA.

*Corresponding author. E-mail: juan_rivera@nih.gov

Fig. 1. Mast cell responses differ following DNP or 2NP stimulation of FcεRI. (A) Degranulation (as measured by β-hexosaminidase release) of wild-type (WT) BMMCs after stimulation with indicated concentrations of DNP or 2NP. ****P* < 0.001; two-way analysis of variance (ANOVA). (B) Leukotriene B₄ (LTB₄) secretion from BMMCs induced by treatment with 2NP (3000 ng/ml) is less than that with DNP (30 ng/ml). ***P* < 0.01, ****P* < 0.001; one-way ANOVA. (C) Tumor necrosis factor-α (TNFα), interleukin-6 (IL6), and IL13 are similarly affected at the concentrations of DNP and 2NP indicated in (B). **P* < 0.05, ***P* < 0.01, ****P* < 0.001; one-way ANOVA. (D) Release of CCL2, CCL3, and CCL4 is increased after stimulation with 2NP relative to DNP [conditions as in (B)]. ***P* < 0.01, ****P* < 0.001; one-way ANOVA. Data were collected from four to eight individual experiments.

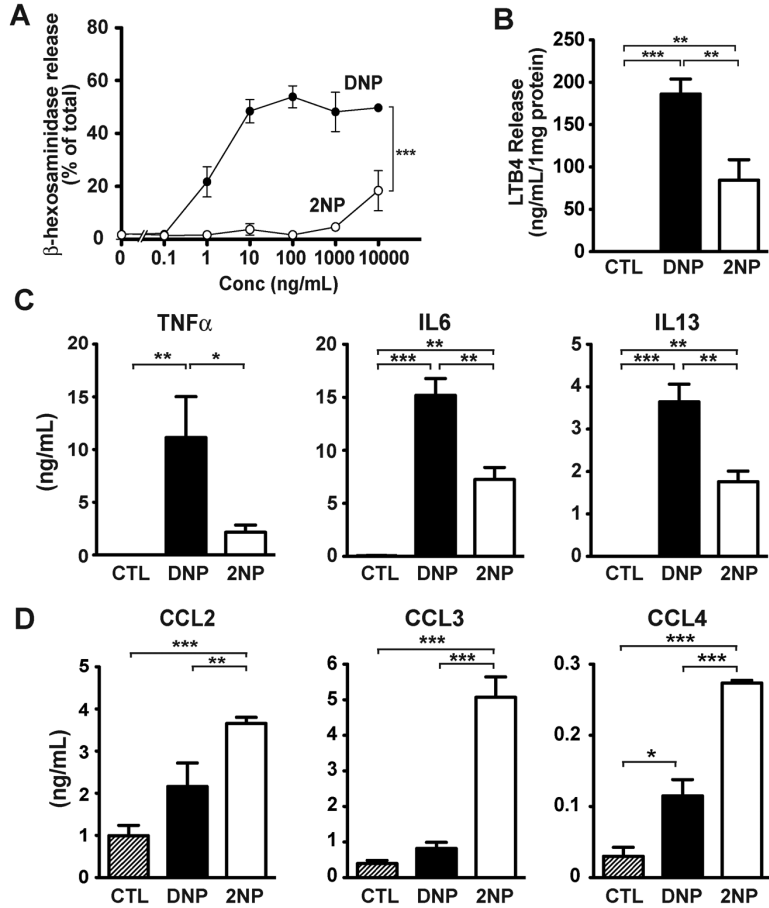
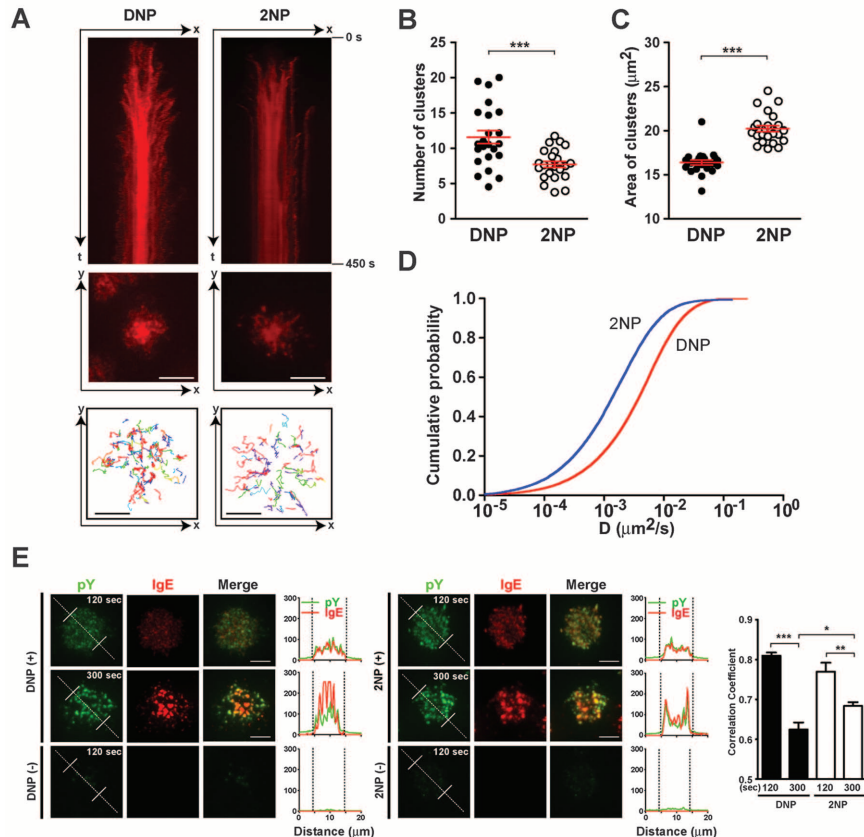


Fig. 2. Characteristics of FcεRI clusters differ with 2NP or DNP stimulation of BMMCs. (A) Kymographs of a mast cell labeled with Alexa Fluor 568-labeled IgE (as an FcεRI marker) as captured by TIRF microscopy. Static view of IgE-FcεRI microcluster dynamics (trajectory) captured upon cell contact with DNP or 2NP imbedded in a planar supported lipid bilayer (see supplementary materials) is shown in two dimensions (x and y) of movement. Movement of receptors clusters with time (t) is also shown. Sequential image sections for 0.5 to 450 s are shown in chronological order. Scale bars, 5 μm. (B) The average number of IgE-FcεRI clusters on DNP- or 2NP-imbedded planar supported lipid bilayer is different. Red bars are means ± SE from 24 individual cells. ****P* < 0.001, Student's *t* test. (C) Average area of IgE-FcεRI clusters on DNP- or 2NP-imbedded planar supported lipid bilayers differs. Red bars are means ± SE from 24 individual cells. ****P* < 0.001, Student's *t* test. (D) Cumulative probability distribution of the diffusion coefficient of individual FcεRI clusters at any given time from analysis of 7835 to 11,198 FcεRI clusters on 24 different cells for DNP and 2NP. (E) Tyrosine phosphorylation with IgE-FcεRI microclusters in DNP- or 2NP-treated cells as determined by intracellular staining with antibodies to phosphotyrosine. Fluorescence intensity in cross section (white dotted line) is shown. Localization was analyzed for more than 40 cells in each condition. Scale bars, 5 μm. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; one-way ANOVA. Data are representative of at least three independent experiments.



fragment (Fc)] to FcεRI with high affinity, with the half-life of IgE bound to FcεRI measured in days (5). Interaction of antigen with FcεRI-bound

antigen-specific IgE clusters the individual receptors (6, 7); this step is required for generation of intracellular signals that cause mast cells and

basophils to release allergic mediators (3, 8). The antigen-binding (Fab) portion of FcεRI-bound IgE antibodies may differ in their affinity for the antigen [as seen in allergic individuals (9)], presumably affecting the duration of the transmitted signal and subsequent outcome. Whether FcεRI functionally distinguishes differences in the affinity of IgE antibody and antigen interactions is not clear. To investigate this, we used two previously described antigens (10): dinitrophenyl-caproate-Fab (DNP, high affinity) and 2-nitrophenyl-caproate-Fab (2NP, low affinity). These differ in their relative affinities for binding to FcεRI-bound DNP-specific IgE by approximately three orders of magnitude. In bone marrow–derived mouse mast cells (BMMCs) (11), FcεRI phosphorylation was similar with approximately 100 times as much 2NP (3000 ng/ml) as DNP (30 ng/ml) (fig. S1A); the kinetics of FcεRI phosphorylation were unaltered at these concentrations (fig. S1B). However, cellular responses differed as 2NP elicited less than 20% of the DNP-induced degranulation response (Fig. 1A) at 3000 ng/ml and 30 ng/ml, respectively, and showed reduced leukotriene B4 (Fig. 1B) and cytokine production (Fig. 1C) but enhanced chemokine production (Fig. 1D). DNP- and 2NP-induced responses required the presence of DNP-specific IgE (fig. S2, A and B), and 2NP treatment had no effect on responses initiated through ovalbumin (OVA)–specific IgE (fig. S2, C and D).

To explore the differences in DNP- and 2NP-induced FcεRI clustering, we used total internal reflection fluorescence (TIRF) microscopy to study DNP-specific IgE-bearing mast cells after their contact with either a DNP- or 2NP-embedded planar supported lipid bilayer, while maintaining equal receptor phosphorylation and differences in mast cell degranulation (fig. S3, A and B). Exposure to DNP resulted in highly mobile receptor clusters that moved from the cell periphery toward the cell center to form a synapse-like localization as described for the T cell receptor (12, 13) (movie S1). In contrast, treatment with 2NP revealed slower movement of receptor clusters and a diffuse distribution with a loosely organized synapse-like structure at the cell center (movie S2). Analysis of receptor cluster movement with time (Fig. 2A) revealed greater numbers of receptor clusters at the periphery in 2NP-treated cells. The total number of clusters formed at any given time was greater upon DNP treatment (Fig. 2B), but the area occupied by these clusters was larger after 2NP treatment (Fig. 2C). The relative mobility of receptor clusters in cells treated with 2NP was on average one-third that of clusters formed by DNP treatment (Fig. 2D). Phosphotyrosine (a hallmark of intracellular signaling) was localized with both DNP- and 2NP-formed receptor clusters, although stronger colocalization was evident after 2NP treatment (Fig. 2E). These findings demonstrate that the FcεRI clusters induced by DNP or 2NP differ spatiotemporally.

We explored whether differences in the spatiotemporal behavior of receptor clustering by DNP

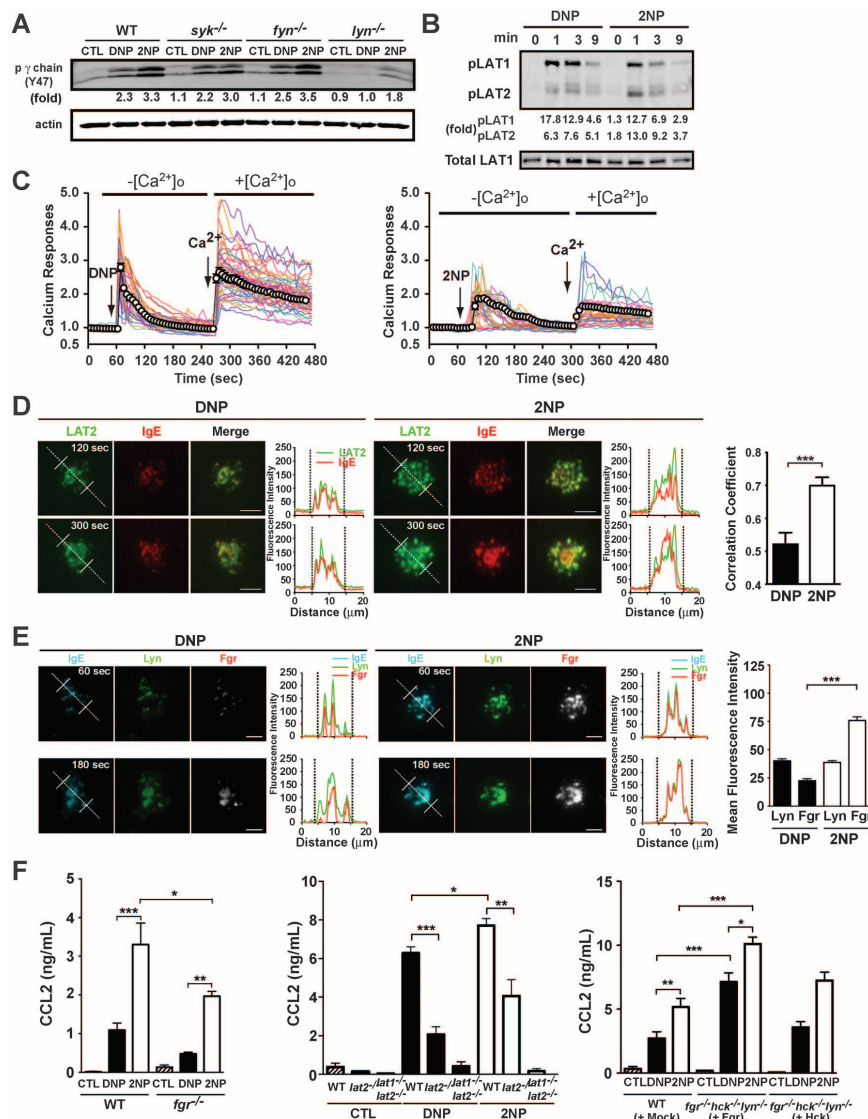


Fig. 3. Distinct molecular signals are induced in 2NP-treated BMMCs: Enhanced role for Fgr and LAT2. (A) Phosphorylation of the FcεRI (ITAM γ Y47) in WT, *syk*^{-/-}, *fyn*^{-/-}, and *lyn*^{-/-} BMMCs after DNP (30 ng/ml) or 2NP (3000 ng/ml) treatment. Relative increase is normalized to the control (CTL) for WT. (B) Kinetics of LAT1 and LAT2 phosphorylation detected by Western blot differ in cells treated with DNP or 2NP. Relative increase is normalized to DNP (0 min). (C) Single cell analysis of BMMC calcium responses in the absence or presence of extracellular Ca²⁺ (–[Ca²⁺]_o; +[Ca²⁺]_o) as indicated. Fluorescence intensity of a single cell with time (more than 300 cells were monitored in total) was measured and shown as the “calcium response” normalized to the baseline fluorescence. Single cells, solid colored lines; averaged responses, black line with open circles. (D) Localization of the LAT2-RFP (green) in *lat2*^{-/-} BMMCs with Alexa Fluor 647–conjugated IgE (red, marking FcεRI) after DNP or 2NP treatment. Images were captured by TIRF microscopy at 120 or 300 s. Fluorescence intensities of IgE-FcεRI microclusters and LAT2-RFP are shown in the graphs. Colocalization was analyzed in 15 to 30 cells for each condition. ****P* < 0.001, Student’s *t* test. Scale bar, 5 μ m. (E) Fluorescence intensities of Lyn-YFP (green), Fgr-RFP (white), IgE-FcεRI microclusters (cyan) in BMMCs derived from *fgr*^{-/-} *hck*^{-/-} *lyn*^{-/-} mice treated with DNP or 2NP for 60 or 120 s. Mean fluorescence intensity was measured from at least 50 cells for each condition. ****P* < 0.001, Student’s *t* test. Scale bar, 5 μ m. (F) CCL2 secretion by BMMCs from the indicated mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; one-way ANOVA. Data are means $\pm$ SE from at least four individual experiments.

or 2NP were translated into changes in FcεRI-generated intracellular signals. Analysis of the phosphorylation of FcεRIγ at Tyr⁴⁷ (Y47) showed increased phosphorylation at this site (Fig. 3A) after cell treatment with 2NP, relative to DNP, and the increased phosphorylation was not dependent on Syk, Fyn, or Lyn; however, Lyn deficiency reduced the overall phosphorylation of FcεRI for both stimuli (14). In contrast, phosphorylation of Syk on the activation loop tyrosines (Y519 and Y520), a marker of its activity, was reduced with 2NP treatment (fig. S4A). The adapter molecule LAT1 (linker for activation of T cells-1) is a target of Syk and scaffolds multiple signaling molecules at the plasma membrane, including the phospholipases PLCγ1 and PLCγ2, whose activity is essential for initiating release of calcium from intracellular stores. Phosphorylation of LAT1 (Fig. 3B) and of PLCγ1 and PLCγ2 (fig. S4B) was greater in DNP-treated cells than in 2NP-treated cells. Calcium mobilization, a key regulator of degranulation (15), from intracellular or extracellular sources was also greater upon DNP treatment (Fig. 3C), consistent with the better degranulation of DNP-treated cells relative to 2NP-treated cells. Phosphorylation of the LAT1-related adapter LAT2 was increased (Fig. 3B) in 2NP-treated mast cells. Greater colocalization of LAT2 with FcεRI was observed in 2NP-treated cells relative to DNP-treated cells (movies S3 and S4), and these differences (as determined

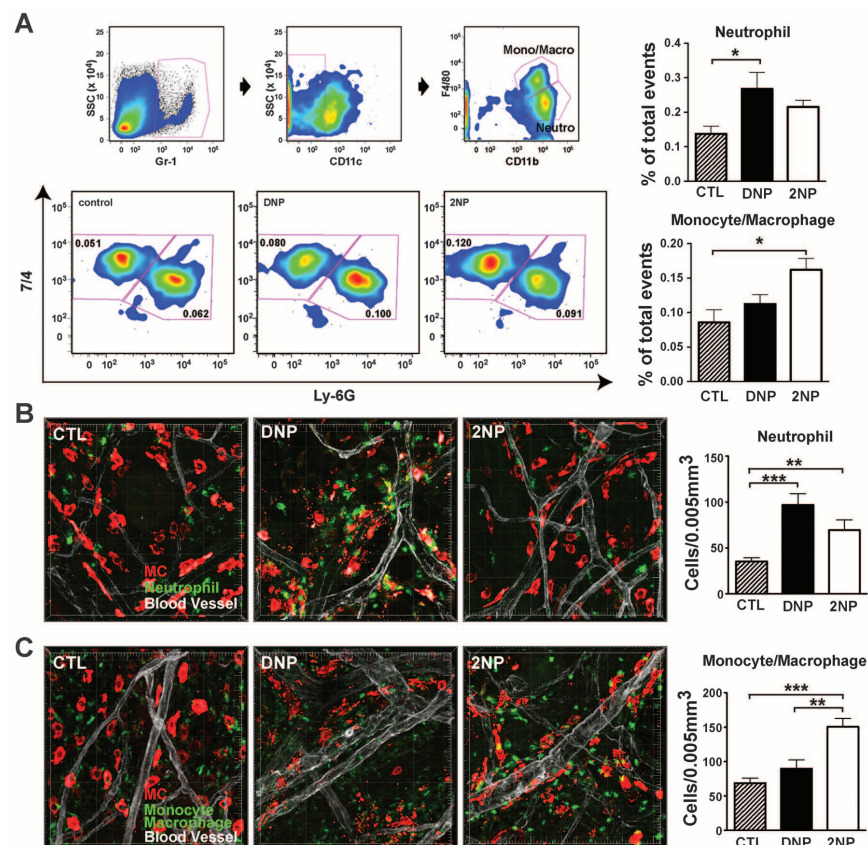
by fluorescence intensity) were quantifiable (Fig. 3D). Thus, the differences in FcεRI clustering by DNP or 2NP alter the balance of molecular signaling, dampening some (i.e., LAT1 and PLCγ phosphorylation) but enhancing others (i.e., LAT2 phosphorylation).

Although the absence of Lyn in mast cells caused decreased phosphorylation of FcεRIγ on Y47 (Fig. 3A), phosphorylation at this site was greater in 2NP-treated cells relative to DNP-treated cells, which suggested the possible phosphorylation of FcεRI by another tyrosine kinase after 2NP treatment. FcεRI phosphorylation was completely inhibited by the Src family kinase selective inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (also known as AG1879 or PP2) (fig. S5A). Multiple Src family kinases are expressed in mast cells including Fgr, Fyn, Hck, Lyn, and Yes, although Yes is seemingly restricted to transformed mast cell lines (16). Hck deficiency in mast cells results in enhanced FcεRI phosphorylation, and Fyn does not contribute to FcεRI phosphorylation (17, 18), but recent work has suggested that Fgr can associate with FcεRI (19) (fig. S5B) and contribute to its function. In mast cells derived from *fgr*^{-/-} *hck*^{-/-} *lyn*^{-/-} mice, we found no phosphorylation of FcεRI, LAT1, or LAT2 (fig. S5C). Reconstitution of these cells with a retrovirus encoding Hck also failed to restore phosphorylation of the aforementioned molecules. How-

ever, retroviral reconstitution with Fgr restored the phosphorylation of FcεRI, LAT1, and LAT2, even in the absence of Lyn. Phosphorylation of LAT2 was increased relative to LAT1 (fig. S5C). Localization of FcεRI with Fgr or Lyn in cells reconstituted with both proteins revealed increased colocalization of Fgr in 2NP-treated cells relative to DNP-treated cells but no significant change in Lyn colocalization with FcεRI (movies S5 and S6). The amount of Fgr associated with FcεRI (as determined by fluorescence intensity) in 2NP-treated cells was more than twice that in DNP-treated cells (Fig. 3E). Thus, differences in FcεRI clustering result in changes in the balance of receptor-associated kinases and subsequent downstream signaling. To determine whether Fgr or LAT2 contributed to the enhanced chemokine response observed after 2NP stimulation, we analyzed the chemokine (C-C motif) ligand 2 (CCL2) response of Fgr- or LAT2-deficient mast cells after treatment with DNP or 2NP. Fgr or LAT2 deficiency impaired CCL2 secretion in response to both DNP and 2NP (Fig. 3F). Conversely, overexpression of Fgr enhanced production of CCL2 in response to both DNP and 2NP (Fig. 3F). Fgr or LAT2 deficiency enhanced 2NP-induced mast cell degranulation (fig. S5D), demonstrating negative regulatory control of this response.

Subtle changes in cell signaling may have consequences in vivo. To test this, we used a passive cutaneous anaphylaxis (PCA) model, which

Fig. 4. Immune cell recruitment at the site of inflammation differs in mice treated with DNP or 2NP after passive cutaneous anaphylaxis (PCA). (A) Fluorescence-activated cell sorter identification of cell types recruited to the site of inflammation. Monocytes or macrophages and neutrophils were identified as Gr-1⁺ (hi) CD11c⁻ CD11b⁺ F4/80⁺ (intermediate) neutrophil [7/4⁺ Ly-6G⁻ and Gr-1⁺ (hi) CD11c⁻ CD11b⁺ F4/80⁺ (intermediate) neutrophil [7/4⁺ Ly-6G⁺, respectively. Data are means ± SE from at least six individual experiments. **P* < 0.05; one-way ANOVA. (B and C) Whole-mount ear tissue immunostaining after PCA reaction reveals differential immune cell recruitment after DNP or 2NP treatment. Mast cells (red) are located along blood vessel (white); degranulation was observed under DNP challenge (middle panel). Total number of neutrophils [green in (B)] or monocyte or macrophages [green in (C)] in random images from six to eight ears per group were counted and normalized per 0.005 mm². Data are means ± SE. ***P* < 0.01, ****P* < 0.001; one-way ANOVA. One representative image of at least three independent experiments is shown for (B) and (C).



provides a localized assessment of mast cell responses, maintaining the factor of 100 times the 2NP concentration relative to that of DNP. At 30 min after exposure to DNP or 2NP, vascular permeability as measured by extravasation of Evans blue dye (see supplementary materials) was significantly higher in DNP- than 2NP-treated mice (fig. S6A). Consistent with this result, more mast cells were degranulated in animals treated with DNP. Ear swelling was significantly different 30 min after DNP or 2NP treatment but narrowed with time (fig. S6B), and the increase in the thickness of the dermis was similar at 3 hours after stimulation (fig. S6C). Immune cell infiltration was similarly increased in animals exposed to DNP or 2NP (fig. S6D). By 12 hours after stimulation, the thickness of the dermis returned to that of control mice (fig. S6E) but immune cell infiltrates were still elevated relative to control mice (fig. S6F). Given that an inflammatory response was initiated by either DNP or 2NP, we explored the cell types involved. Gr-1⁺ CD11c⁺ CD11b⁺ cells were distinguished on the basis of the myeloid marker 7/4 [Ly-6B.2, which is somewhat more highly expressed by recently generated inflammatory macrophages (20)] from neutrophils as marked by Ly-6G (Fig. 4A). Exposure to DNP caused increased numbers of neutrophils relative to inflammatory macrophages, whereas this ratio was reversed in animals treated with 2NP, consistent with the increased secretion of monocyte-

or macrophage-attracting chemokines, such as CCL2, CCL3, and CCL4, after treatment of mast cells with 2NP (Fig. 1D). Whole-mount immunohistochemical analysis of the skin also revealed these differences (Fig. 4, B and C), and skin mast cells from 2NP-treated mice produced greater amounts of CCL2 than did those in the skin of DNP-treated mice (fig. S7). Thus, low-affinity stimulation of FcεRI results in an inflammatory response marked by a shift in the monocyte or macrophage/neutrophil ratio.

Collectively, our findings demonstrate that differences in the affinity of antigen and antibody interactions are discriminated by receptors through qualitative changes in molecular signals resulting in distinct outcomes. This discriminatory ability of receptors may extend beyond the immune system.

References and Notes

- H. G. Dohlman, J. W. Thorner, *Annu. Rev. Biochem.* **70**, 703–754 (2001).
- M. Behar, N. Hao, H. G. Dohlman, T. C. Elston, *PLOS Comput. Biol.* **4**, e1000197 (2008).
- J. P. Kinet, *Annu. Rev. Immunol.* **17**, 931–972 (1999).
- S. J. Galli, M. Tsai, A. M. Piliponsky, *Nature* **454**, 445–454 (2008).
- C. Isersky, J. Rivera, S. Mims, T. J. Triche, *J. Immunol.* **122**, 1926–1936 (1979).
- H. Metzger, *J. Immunol.* **149**, 1477–1487 (1992).
- N. L. Andrews *et al.*, *Immunity* **31**, 469–479 (2009).
- U. Blank, J. Rivera, *Trends Immunol.* **25**, 266–273 (2004).

- D. R. Jackola, L. K. Pierson-Mullany, C. L. Liebler, M. N. Blumenthal, A. Rosenberg, *Mol. Immunol.* **39**, 367–377 (2002).
- C. Torigoe, J. K. Inman, H. Metzger, *Science* **281**, 568–572 (1998).
- E. Razin, *Methods Enzymol.* **187**, 514–520 (1990).
- C. R. Monks, B. A. Freiberg, H. Kupfer, N. Sciaky, A. Kupfer, *Nature* **395**, 82–86 (1998).
- A. Grakoui *et al.*, *Science* **285**, 221–227 (1999).
- V. Parravicini *et al.*, *Nat. Immunol.* **3**, 741–748 (2002).
- M. Vig *et al.*, *Nat. Immunol.* **9**, 89–96 (2008).
- A. M. Gillfillan, J. Rivera, *Immunol. Rev.* **228**, 149–169 (2009).
- H. Hong *et al.*, *Blood* **110**, 2511–2519 (2007).
- G. Gomez *et al.*, *J. Immunol.* **175**, 7602–7610 (2005).
- J. H. Lee *et al.*, *J. Immunol.* **187**, 1807–1815 (2011).
- M. Rosas, B. Thomas, M. Stacey, S. Gordon, P. R. Taylor, *J. Leukoc. Biol.* **88**, 169–180 (2010).

Acknowledgments: Supported by the intramural research program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases and by its Laboratory Animal Care and Use Section and Flow Cytometry Group, Office of Science and Technology. All data are provided in the main paper and supplementary materials. The authors declare no conflicts of interest.

Supplementary Materials

www.sciencemag.org/content/343/6174/1021/suppl/DC1
Materials and Methods

Figs. S1 to S7

Movies S1 to S6

References (21–34)

8 October 2013; accepted 17 January 2014

Published online 6 February 2014;

10.1126/science.1246976

Cell Surface ABP1-TMK Auxin-Sensing Complex Activates ROP GTPase Signaling

Tongda Xu,^{1,2,3} Ning Dai,^{4*} Jisheng Chen,¹ Shingo Nagawa,^{1,3} Min Cao,² Hongjiang Li,^{1,5,6} Zimin Zhou,² Xu Chen,^{5,6} Riet De Rycke,⁵ Hana Rakusová,^{5,6} Wuyi Wang,^{3,7†} Alan M. Jones,⁸ Jiří Friml,^{5,6,9} Sara E. Patterson,⁷ Anthony B. Blecker,^{4‡} Zhenbiao Yang^{1,3,10§}

Auxin-binding protein 1 (ABP1) was discovered nearly 40 years ago and was shown to be essential for plant development and morphogenesis, but its mode of action remains unclear. Here, we report that the plasma membrane–localized transmembrane kinase (TMK) receptor–like kinases interact with ABP1 and transduce auxin signal to activate plasma membrane–associated ROPs [Rho-like guanosine triphosphatases (GTPase) from plants], leading to changes in the cytoskeleton and the shape of leaf pavement cells in *Arabidopsis*. The interaction between ABP1 and TMK at the cell surface is induced by auxin and requires ABP1 sensing of auxin. These findings show that TMK proteins and ABP1 form a cell surface auxin perception complex that activates ROP signaling pathways, regulating nontranscriptional cytoplasmic responses and associated fundamental processes.

Auxin regulates nearly all aspects of plant development and behavior and impinges on a great variety of responses involving cell polarization, expansion, division and differentiation. Exactly how this small-molecule hormone achieves this multitude of diverse roles is largely unexplained, although it may be perceived by multiple functionally distinct auxin perception and signaling systems (1–6). Members of the nu-

clear TIR1/AFB F-box protein auxin receptor and AUX/IAA co-receptor families modulate nuclear gene transcription in response to various auxin concentrations (1–4).

Independently of the TIR1 family, auxin-binding protein 1 (ABP1) was proposed to perceive extracellular auxin to regulate a plethora of plasma membrane or cytoplasmic responses not necessarily involving gene transcription (6–18).

ABP1 may also coordinate with the TIR1/AFB pathway to regulate gene transcription (16, 19). ABP1 is essential for early embryogenesis, root development, leaf expansion, cell morphogenesis, and subcellular distribution of PIN auxin transporters (6, 8, 9, 12, 13, 15–18, 20, 21). ABP1 is required for the auxin-dependent activation of ROPs [Rho-like guanosine triphosphatases (GTPases) from plants] at the plasma membrane,

¹Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA.

²Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, 117604 Singapore. ³Shanghai Center for Plant Stress Biology, Shanghai Institutes for Biological Sciences, The Chinese Academy of Sciences, Shanghai 200032, China. ⁴Department of Botany, University of Wisconsin, Madison, WI 53706, USA. ⁵Department of Plant Systems Biology, VIB and Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Gent, Belgium. ⁶Bertalanffy Foundation Building, Institute of Science and Technology Austria, Am Campus 1, 3400 Klosterneuburg, Austria. ⁷Department of Horticulture, University of Wisconsin, Madison, WI 53706, USA. ⁸Departments of Biology and Pharmacology, University of North Carolina, Chapel Hill, Chapel Hill, NC 27599, USA. ⁹Mendel Centre for Plant Genomics and Proteomics, Masaryk University, CEITEC MU, CZ-625 00 Brno, Czech Republic. ¹⁰Shanghai Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, The Chinese Academy of Sciences, Shanghai 200032, China.

*Present address: Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA. †Present address: Ceres, 1535 Rancho Conejo Boulevard, Thousand Oaks, CA 91320, USA. ‡Deceased. §Corresponding author. E-mail: yang@ucr.edu



Supplementary Materials for

**Molecular Editing of Cellular Responses by the High-Affinity Receptor
for IgE**

Ryo Suzuki, Sarah Leach, Wenhua Liu, Evelyn Ralston, Jörg Scheffel, Weiguo Zhang,
Clifford A. Lowell, Juan Rivera*

*Corresponding author. E-mail: juan_rivera@nih.gov

Published 6 February 2014 on *Science Express*
DOI: 10.1126/science.1246976

This PDF file includes:

Materials and Methods

Figs. S1 to S7

Captions for movies S1 to S6

References

Other Supplementary Material for this manuscript includes the following:

Movies S1 to S6

Materials and Methods

General Reagents

Mouse anti DNP-specific IgE (H1 DNP- ϵ -26.82) was produced as described (21). Briefly, DNP- and 2-nitrophenyl (2NP)-conjugated Fab fragments of bovine IgG were prepared by conjugation of the ϵ NH₂-caproate derivative of either DNP or 2NP hapten to Fab fragments and the conjugated product was purified by gel-filtration and dialysis (10). All the antigens used in this study were conjugates at a ratio of 4, DNP or 2NP, to 1 Fab fragment. Octyl β -D-glucopyranoside, pepstatin A, hydroxytamoxifen were from Sigma-Aldrich. Ovalbumin (OVA) was from InvivoGen. Mouse anti OVA-specific IgE was from AbD Serotec. PP2 was from EMD Millipore. Imatinib was from Selleckchem. Fluo-4 AM, and Fura Red AM were from Molecular Probes. Murine IL-3 and SCF were from PeproTech. FBS and RPMI 1640 were from Invitrogen.

Mice, BMDC cultures, IgE sensitization, and cell activation

Bone marrow was isolated from femurs of sex and age-matched (8- to 12-wk-old) wild type (WT), *lyn*^{-/-}, *fyn*^{-/-}, and RosaCreERT2-Syk^{floxed/floxed} mice that were maintained and used in accordance with National Institutes of Health guidelines and the animal study proposal approved by the institutional animal care and use committee of NIAMS, NIH. Bone marrow from previously described *lat2*^{-/-} and *lat1*^{-/-}*lat2*^{-/-} mice (22) was used as well as bone marrow from *lyn*^{-/-}*fgr*^{-/-}*hck*^{-/-} mice (23). BMDCs were cultured as previously described (24). Mature *syk*^{-/-} BMDC were generated by hydroxytamoxifen (2 μ M)-treatment of the bone marrow cultures from RosaCreERT2-Syk^{floxed/floxed} mice. Cultures were monitored by FACScan (Becton Dickinson) for surface expression of Fc ϵ RI and used for experiments when >95% of the cells were Fc ϵ RI⁺. Conditions for IgE sensitization and for cell activation were previously described (24).

Gene modification of BMDCs

For ectopic expressions of genes, retrovirus gene transduction was performed as previously described (25). Viral supernatants were produced by transfecting the packaging cells Plat-E with Fgr or Hck in the pMX vector using Lipofectamine 2000 (Invitrogen). Cells were also mock-transfected with pMX empty vector as a negative control. After infection, cells were washed and allowed to grow in IL-3- and SCF-containing medium for 2 days (as above) before initiating selection of transduced cells with 3 $\mu\text{g/ml}$ puromycin (Invitrogen). Following two weeks of selection, cells were analyzed for Fc ϵ RI expression and used when >95% of the cells expressed this receptor.

For transient gene transfection, Nucleofection (Amaxa) of BMDCs was performed according to the manufacturer's instructions with minor modification. After BMDCs were washed twice in PBS, 5×10^6 cells were resuspended in 100 μl of mouse macrophage nucleofection solution (Amaxa). Fluorescent protein conjugated-target protein vectors (5 μg) were added per 5×10^6 cells, and the samples were transferred into certified cuvettes (Amaxa) and transfected by nucleofection program (Y-001).

Measurement of degranulation, cytokine, chemokine, and leukotrienes

Degranulation was determined by the release of the granule enzyme, β -hexosaminidase as previously described (24). Degranulation is expressed as the percent of total cellular β -hexosaminidase in the supernatant. Cytokine and chemokine release was measured from BMDC (2×10^6 cells) stimulated with DNP or 2NP for 3h. Release of TNF- α , IL-6, IL-13, CCL2, CCL3 and CCL4 was determined by Bio-Plex Pro cytokine assays (BioRAD). For leukotriene (LTB $_4$) production, cells were stimulated with the indicated concentrations of antigen for 30 min at 37°C in HEPES-BSA buffer containing 10 $\mu\text{g/ml}$ indomethacin (Sigma Aldrich). The amount of LTB $_4$ in the supernatants was measured using a competitive ELISA (R&D Systems).

To measure CCL2 production in tissue mast cells, frozen sections of Passive Cutaneous Anaphylaxis (PCA)-treated tissue were fixed with 4% paraformaldehyde solution and blocked

with PBS supplemented with 7.5% Blokhin II (Aves Lab. Inc) for 1 hr. Sections were incubated with FITC conjugated anti-CCL2 (Novus Biologicals) or FITC conjugated isotype control (Novus Biologicals) for overnight at 4°C. The sections were washed with PBS supplemented with 0.1% Tween 20 and subsequently stained with Texas Red conjugated-avidin for mast cell detection. After washing in PBS, tissue slides were embedded in mounting media (Vector Laboratories, Inc.) and imaged on a Zeiss LSM 780 microscope (Carl Zeiss). Fluorescence intensity measurements of CCL2 in mast cells were performed as a region of interest by MIPAV software.

Immunoprecipitation and immunoblotting

For Ag stimulation, BMMCs were sensitized at 37°C for 3 hrs with 1µg/ml IgE in RPMI without cytokines. Cells were washed and stimulated at 37°C with DNP (30 ng/ml) or 2NP (3000 ng/mL) in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 20 mM Hepes, 5.6 mM glucose, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.05% BSA, pH 7.4). Stimulation time varied with the type of experiment as indicated. Cell lysates were prepared by incubation of 20×10^6 cells in 1ml of BSS buffer containing 1.0% NP-40 and 60 mM Octyl β-D-glucopyranoside on ice for 15 min essentially as described (24). Lysates were clarified by microcentrifugation for 10 min at maximum speed at 4°C, and supernatants collected. For immunoprecipitation of IgE-bound FcεRI, cells were solubilized in lysis buffer (0.5% Triton X-100, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 5 mM Na₄P₂O₇, 5 mM NaF, 0.5 µM pepstatin A, 1x proteinase inhibitor cocktail (Roche)) for 15 min at 4°C. Supernatants were incubated with goat anti-mouse IgE (SouthernBiotech) for 1 hr at 4°C followed by incubation with protein A Sepharose beads (GE Healthcare) for 1 hr at 4°C. Beads were washed three times with lysis buffer and boiled in SDS-PAGE sample buffer (reducing or non-reducing as indicated) for 5 min. Immunoblotting was as previously described (24, 25). Antibodies used in immunoblotting were obtained from various suppliers: Anti-phospho PLCγ1 (Y783), anti-phospho PLCγ2 (Y759), anti-phospho Syk (Y519 and Y520), anti-phospho-LAT (Y191) antibody (which

recognizes phosphorylated LAT1 and LAT2 (26)) were purchased from Cell Signaling Technology. Anti-phosphotyrosine 4G10 antibody was from Upstate Biotechnology. Anti-Fgr antibody, anti-LAT2 antibody, anti-PLC γ 1 antibody, anti-PLC γ 2 antibody were from Santa Cruz Biotechnology. Monoclonal mouse anti-Fc ϵ RI β (clone: JRK) and monoclonal mouse anti-phospho Fc ϵ RI γ Y47 (clone: γ -pY⁴⁷) was previously described (27-29).

Measurement of changes in intracellular Ca²⁺ and Ca²⁺ influx

The procedure for Ca²⁺ fluorometry on cell populations was previously described (24). For single cell analysis, two Ca²⁺ indicator dyes, Fluo-4 AM/Fura Red AM (Molecular Probes), were used. BMMCs were loaded with Fluo-4 AM (2 μ M) and Fura Red AM (10 μ M) for 30 min at 37°C. The cells were rinsed three times with Tyrodes buffer. A Zeiss LSM-510 META confocal microscope with a 488-nm wavelength light from an argon laser was used for ratio-metric measurements. The fluorescence of Fluo-4 and Fura Red was detected through a band pass filter (505-530 nm) and a long pass filter (>560 nm), respectively. Calcium responses are reported as the ratio of Fluo-4 to Fura Red normalized as a fold increase relative to unstimulated cells. Fluorescence images were collected every 5 sec, and the intensity of fluorescence was quantified using Zeiss LSM-510 META software.

Constructs and Site-specific Mutagenesis

Monomeric versions of fluorescent protein vector (YFP or RFP as indicated) were purchased from Evrogen. Fluorescent proteins were attached to the C termini of mouse Lyn, LAT2, or Fgr. The cDNA encoding mouse wild type Fgr or Hck were cloned into pMX-puro vector. All the constructs were verified by nucleotide sequencing.

Preparation of antigen-containing planar supported lipid bilayers and calculation of antigen density

Planar supported lipid bilayers were prepared essentially as previously described (13, 30, 31). Briefly, Ni-NTA containing lipid bilayers were prepared using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC; Avanti Polar Lipids) and 1,2-Dioleoyl-sn-Glycero-3-[N(5-Amino-1-Carboxypentyl) Imino-diacetic Acid]-Succinyl (Nickel Salt) (DOGS-Ni-NTA; Avanti Polar Lipids) in a 90% DOPC to 10% DOGS-Ni-NTA ratio. Ni-NTA-containing unilamellar vesicles were formed by sonication of the mixed lipids and clarified by ultracentrifugation and filtering. Glass coverslips were cleaned using Nanostrip (Cyantek) and dried. Supported lipid bilayers were prepared from 0.1 mM lipid unilamellar vesicles on freshly cleaned glass coverslips. The soluble antigens DNP-Fab or 2NP-Fab were conjugated to the 12-tandem histidine peptide (ASTGTASACTSGASSTGSHHHHHHHHHHHH; Anaspec) through the cysteine residue using LC-SMCC (succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxy-(6-amidocaproate)) cross linker (Thermo Fisher Scientific) following manufacturer's protocol. The antigen-peptide conjugates were attached to the Ni-NTA containing lipid bilayer by incubating antigen-peptide conjugates with the bilayer for 30 min at room temperature followed by washing. The density of DNP or 2NP molecules on the bilayer was determined by coating the prepared DNP-Fab or 2NP-Fab containing supported lipid bilayers onto 5 μ m diameter silica beads (Bangs Laboratories Inc.) using FITC-conjugated monoclonal antibodies to DNP or bovine IgG in flow cytometric analysis. The fluorescence intensity observed was normalized by QuantumTM FITC MESF calibration beads (Bangs Laboratories Inc.) according to the manufacturers' instructions and the number of DNP or 2NP molecules was calculated. The density of DNP or 2NP was approximately 200 and 2000 molecules/ μ m², respectively. These numbers are consistent with numbers derived from measurement of only the Fab fragments conjugated to each antigen multiplied by the average haptenation number for DNP or 2NP. These Ag densities also provided similar levels of Fc ϵ RI phosphorylation and functioned identically to soluble antigen in eliciting normal and poor degranulation for DNP and 2NP, respectively. The antigen-containing planar supported lipid bilayers were then used for TIRF imaging. For single IgE-Fc ϵ RI cluster tracking experiment, IgE was labeled with Alexa Fluor 568 or 647 (as indicated) according to the

manufacturers' (Invitrogen) instructions with the modification that all the Alexa Fluor-labeled IgE used in this study was used at a ratio of Alexa Fluor dye to IgE molecule of 0.8~1.2 mol dye/mol IgE molecule.

Total internal reflection fluorescence (TIRF) microscopy

Cells were placed on planar supported lipid bilayers containing DNP or 2NP antigen prepared essentially as described (31) with some minor modifications. TIRF image sequences were acquired at 37°C in an environmental chamber surrounding a Leica DMI6000 microscope-based TIRF system (Leica Microsystems Inc., Buffalo Grove, IL), using an Andor 512 x 512 EMCCD camera (Andor Technology, South Windsor, CT), and a Leica 63 × 1.45 N.A. or Leica 100 × 1.4 N.A. objective lenses. Acquisition was controlled by Leica AF7000 fluorescence software, which automatically adjusts the excitation beam angle to excite fluorophores at the same depth in each channel. The exposure time was 100 msec unless otherwise indicated. Four diode lasers (405 nm, 488 nm, 561 nm, 633 nm) were used for the experiments. TIRF images were analyzed by Imaris (Bitplane AG, Zurich, Switzerland), ImageJ (NIH, Bethesda, MD.; freely available at rsbweb.nih.gov/ij/), MIPAV (Medical Image Processing, Analysis, and Visualization, NIH, Bethesda, MD.; freely available at <http://mipav.cit.nih.gov>), Matlab (Mathworks, Natick, MA) software, as indicated. Quantitative colocalization analyses were performed by using the orthogonal regression algorithms implemented in MIPAV software. Background subtraction was performed before co-localization analysis. Co-localization was denoted as a Pearson's correlation coefficient. The cluster detection, number of clusters, and cluster size measurements were performed in MATLAB 7.11 with a customized program. This program first uses an edge filter to make particle edges brighter, improving detection sensitivity, especially for dim objects. Next the watershed segmentation algorithm is used to detect the clusters, with the step size between adjacent thresholds set as three times the standard deviation of the image brightness. The program analyzes one image out of every 10 frames in each series in order to calculate the average of the cluster number and size. The analysis for single cluster mean square displacement

(MSD), diffusion coefficient, and cumulative probability were performed with the cell tracking software created by Drs. D. Blair and E. Dufresne (<http://physics.georgetown.edu/matlab/>). In brief, single cluster tracking and analysis was performed using Matlab (Mathworks) code based on available tracking algorithms. The resulting trajectories were visually inspected and occasional errors in tracking were manually corrected. The positions of the diffraction-limited spots in the trajectories were refined using a 2D Gaussian fit. Mean square displacements were calculated from positional coordinates as described (31). Briefly, short-range diffusion coefficients were calculated from linear fits (Eq.1) to mean-square displacement data of individual trajectories from the first four data points and plotted in the cumulative distribution graph. To further identify the type of the motion, the mean square displacement (MSD) vs. time plot was fitted with functions for normal diffusion, directed motion with diffusion, and confined motion:

$$\langle r^2 \rangle = 4Dt \quad (\text{Eq. 1})$$

$$\langle r^2 \rangle = 4Dt + (Vt)^2 \quad (\text{Eq. 2})$$

$$\langle r^2 \rangle \sim \langle r_c^2 \rangle / 3 [1 - \exp(-12Dt / \langle r_c^2 \rangle)] \quad (\text{Eq. 3})$$

The fitting equations are obtained from the articles describing single-particle tracking (32-34). $\langle r^2 \rangle$ is the MSD, D is the diffusion coefficient, and $\langle r_c^2 \rangle$ is the size of confinement. The confined motion (Eq.3) yielded the best fit for both DNP and 2NP -stimulated clusters. The average of the square of correlation coefficient for DNP and 2NP, a parameter to quantify the goodness of the curve fit was 0.97 ± 0.042 and 0.99 ± 0.004 , respectively. DNP clusters showed a significantly large diffusion coefficient (0.0087 ± 0.0025) as compared to 2NP clusters (0.0032 ± 0.0005) ($p < 0.0001$). Thus, DNP clusters are more dynamic with a higher diffusion coefficient and thus can more easily escape confinement.

Passive Cutaneous Anaphylaxis (PCA)

Following isoflurane-induced anesthesia, mice were passively sensitized by means of intradermal injection of 75 ng of DNP-specific IgE in 30 μ L of normal saline (0.9% NaCl) in both ears. After 24 hrs, mice were challenged with DNP (1 μ g) - or 2NP (100 μ g) -antigen in 200 μ L of normal saline by retro-orbital injection. Normal saline without Ag were used as a control. Ear thickness was measured and recorded before and after retro-orbital injection with antigen using a dial thickness gauge (Mitutoyo). Mice were euthanized 3 or 12 hrs after challenge, and ear pinnae were collected for histologic and flow cytometric analysis.

To measure mast cell-induced vascular permeability, mice were challenged with the antigens dissolved in 0.5% Evans blue. After 30 min, the mice were euthanized and the ears were collected and minced and incubated in formamide for 1 hour at 55⁰C to extract the Evans blue dye. The amount of dye was measured by absorbance at 620 nm.

Flow Cytometry

Flow cytometric analysis for single-cell suspensions from mouse ears was done on a FACSVerse (BD Biosciences, San Jose, CA). Ears were split in dorsal and ventral halves and exposed to trypsinization for 10 minutes at 37°C (0.15% Trypsin-EDTA, Invitrogen). Ears were then transferred to 20% FBS in RPMI1640. Epidermal sheets were removed, minced, and dissociated by pipetting. The remaining dermal tissue was minced and digested for 15 min at 37°C with gentle shaking in a solution of 0.05% DNase I (Roche) and 0.2 U/mL Liberase TL (Roche). Both epidermal and dermal cells were filtered through 70- μ m cell strainers (BD Biosciences, Schwechat, Austria) and washed in 1X PBS. Single-cell suspensions from both epidermis and dermis were incubated on ice for at least 15 min with Fc block (clone 2.4G2, BioXCell, West Lebanon, NH) to reduce non-specific binding to Fc receptors. Analysis was restricted to viable cells, which were identified by LIVE/DEAD® Fixable Violet or Aqua Dead Cell Stain Kit (Invitrogen). Same numbers of events were observed and analyzed in each experiments. Data acquisition was performed with the BD FACSuite software (BD Biosciences) and analyzed with FlowJo software (Treestar Inc., Ashland, OR). The following antibodies were used for flow

cytometric analysis: anti-CD11b-Alexa Fluor 488 (clone M1/70, BioLegend), anti-Neutrophil[7/4]-PE (Abcam), anti-Ly-6G/Ly-6C (Gr-1)-PerCP-Cy5.5 (clone RB6-8C5, BioLegend), anti-CD11c-PE-Cy7 (clone N418, eBioscience), anti-F4/80-Alexa Fluor 647 (clone CI:A3-1, BioLegend), anti-Ly-6G-FITC (clone 1A8, BioLegend), anti-CD11b-V450 (clone M1/70, BD Pharmingen), anti-Neutrophil [7/4]-FITC (Abcam). Myeloid cell subpopulations were distinguished based on an initial Gr-1⁺ or Gr-1⁻ gate. A CD11b⁺ CD11c⁺ double-positive gate was set on Gr-1⁻, live cells to identify dendritic cells. From the Gr-1⁺ gate, neutrophil [7/4]⁻ cells expressing both F4/80 and CD11b were identified as macrophages. Monocytes and neutrophils were initially identified as Gr-1⁺ (hi) and CD11c⁻. Monocytes were further characterized as CD11b⁺ F4/80⁺ (intermediate), while neutrophils were CD11b⁺ F4/80⁺ (low). The identities of the populations were confirmed by examining Ly-6G and neutrophil [7/4] expression of Gr-1⁺ (hi) CD11c⁻ CD11b⁺ F4/80⁺ cells. Gr-1⁺ (hi) CD11c⁻ CD11b⁺ F4/80⁺ (intermediate) cells expressed neutrophil [7/4] and were Ly-6G⁻. The Gr-1⁺ (hi) CD11c⁻ CD11b⁺ F4/80⁺ (low) cells also expressed neutrophil [7/4], but were Ly-6G⁺.

Histology and Immunohistochemistry

Tissue samples were collected at the end of each *in vivo* experiment and placed in 4% formalin solution (Sigma-Aldrich). Embedding, sectioning, hematoxylin and eosin staining, Masson's trichrome, and toluidine blue staining were done by American Histo Labs Inc (Gaithersburg, MD). Whole-mount immunohistochemical analysis of ear skin was performed as previously described (28). Mice were euthanized and their ears were dissected and separated into dorsal and ventral sheets. The cartilage-free sheets were fixed in 1.0% paraformaldehyde at 4°C overnight and then blocked with blocking buffer (1x PBS supplemented with 0.1% Triton X-100 and 1.0% BSA). Samples were incubated with various antibodies overnight (28) at 4°C. After washing in blocking buffer, samples were embedded in Fluoromount G mounting media (SouthernBiotech) and imaged on a Zeiss LSM780 microscope (Carl Zeiss). The following antibodies were used for whole-mount immunohistochemical analysis: FITC-conjugated Ly-6G (clone 1A8, BD

Biosciences), Alexa Fluor 488 conjugated MOMA-2 (AbD Serotec), APC-conjugated anti-CD31 (BD Pharmingen), Texas Red-conjugated Avidin.

Statistical Analysis

Results were analysed using GraphPad Prism (GraphPad Software Inc, California, USA). The statistical significance of differences between groups was determined by unpaired, two-tailed Student's *t*-tests or by One-way Analysis of Variance (ANOVA) as appropriate. Two-way ANOVA was used to examine the overall dose-effects of antigen affinity on degranulation and the effect of antigen affinity on ear swelling. Differences were considered significant if the *P* value was ≤ 0.05 with the confidence intervals of 95%. All results are shown as mean $\pm$ SEM of at least 3 independent experiments.

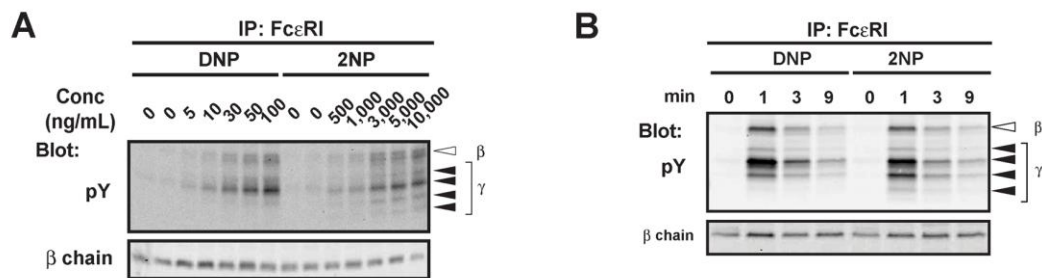


Fig. S1. BMMCs show similar FcεRI phosphorylation but not the same degranulation in response to DNP- or 2NP-treatment. (A) Dose-response of FcεRI phosphorylation (under non-reducing (NR) conditions) in BMMCs after DNP- or 2NP-treatment reveals similar phosphorylation and 30 and 3000 ng/ml, respectively. **(B)** Kinetics of FcεRI phosphorylation (NR conditions) induced by DNP (30 ng/ml) and 100-fold higher concentration of 2NP antigen (3000 ng/ml) is similar. Data in **(A)** and **(B)** is representative of 6 individual experiments.

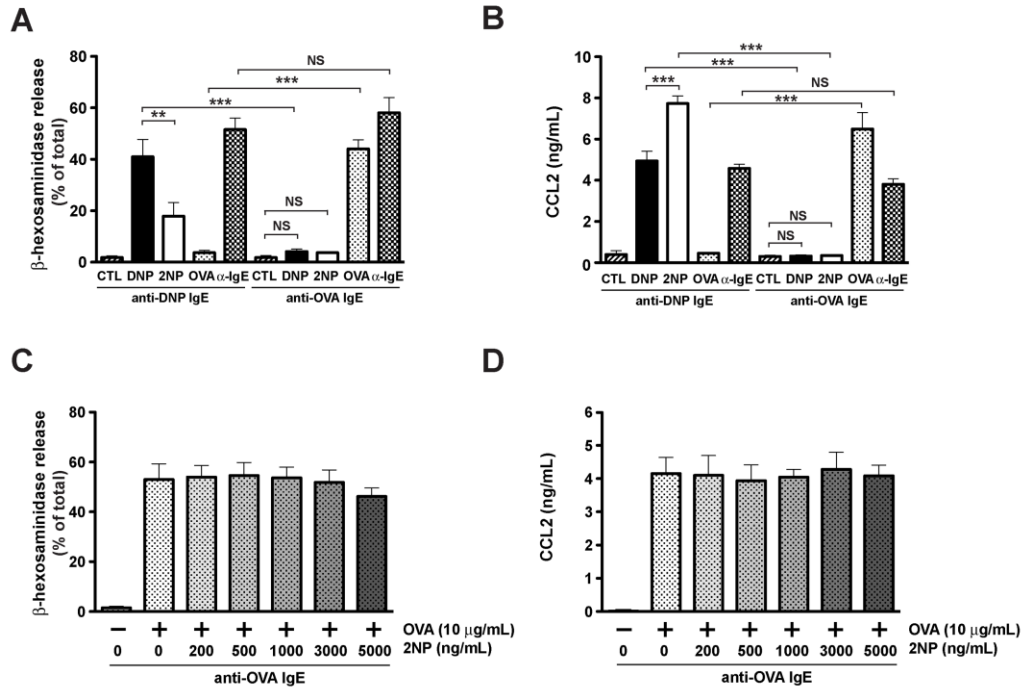


Fig. S2. 2NP-induced BMMC responses requires DNP-specific IgE and 2NP-treatment does not alter mast cell degranulation or CCL2 production induced by OVA/anti-OVA specific IgE-stimulation of FcεRI. Cells were sensitized with DNP-specific IgE or OVA-specific IgE and challenged either DNP (30 ng/ml), 2NP (3000 ng/ml), OVA (10 μg/ml), or anti-IgE (5μg/ml). Degranulation (**A**) or CCL2 production (**B**) was measured. Cells were sensitized with OVA-specific IgE alone and subsequently challenged with OVA alone or OVA and 2NP simultaneously at the indicated concentrations and degranulation (**C**) or CCL2 production (**D**) was measured. NS=not significant, **p<0.01, *** p<0.001, one-way ANOVA. Data was from at least 3 individual experiments.

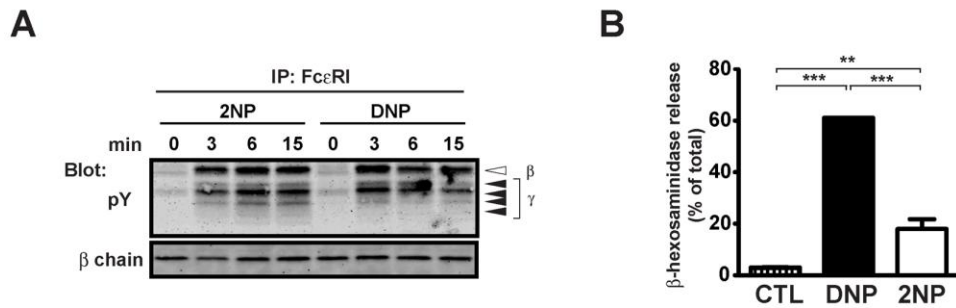


Fig. S3. DNP or 2NP antigens imbedded in planar supported lipid bilayers behave similarly to soluble antigens eliciting similar FcεRI phosphorylation but different mast cell degranulation responses. **(A)** Kinetics of FcεRI phosphorylation induced by DNP- or 2NP-coated planar supported lipid bilayers used to stimulate WT BMMCs. FcεRI was immunoprecipitated from mast cells and resolved proteins were immunoblotted with antibodies to phospho-tyrosine (under non-reducing conditions). Data is representative of 3 individual experiments. **(B)** Degranulation as measured by the release of β-hexosaminidase from DNP- or 2NP-coated planar supported lipid bilayer stimulated WT mast cells. Data is mean ± SE from at least 4 individual experiments. **p<0.01, *** p<0.001, one-way ANOVA.

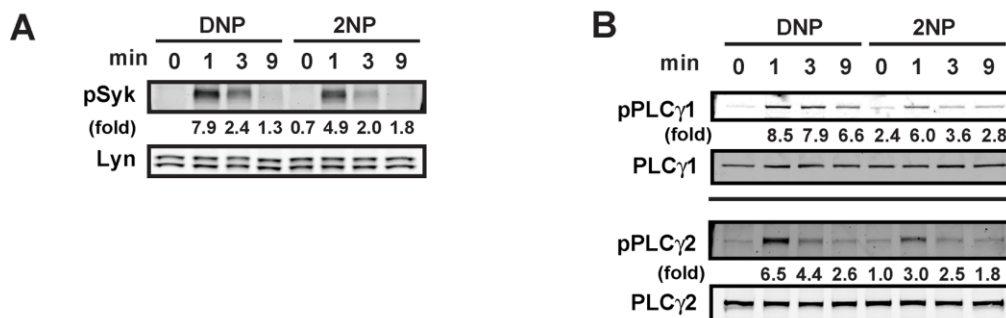


Fig. S4. Phosphorylation of Syk, PLC γ 1, and PLC γ 2 is reduced in 2NP-treated BMMCs relative to DNP-treated cells. (A) Kinetics of Syk (Y519 and Y520) phosphorylation following DNP- or 2NP-treatment of BMMCs. **(B)** Kinetics of PLC γ 1 (Y783) and PLC γ 2 (Y759) phosphorylation as in (A). Fold increase is normalized to DNP (0 min).

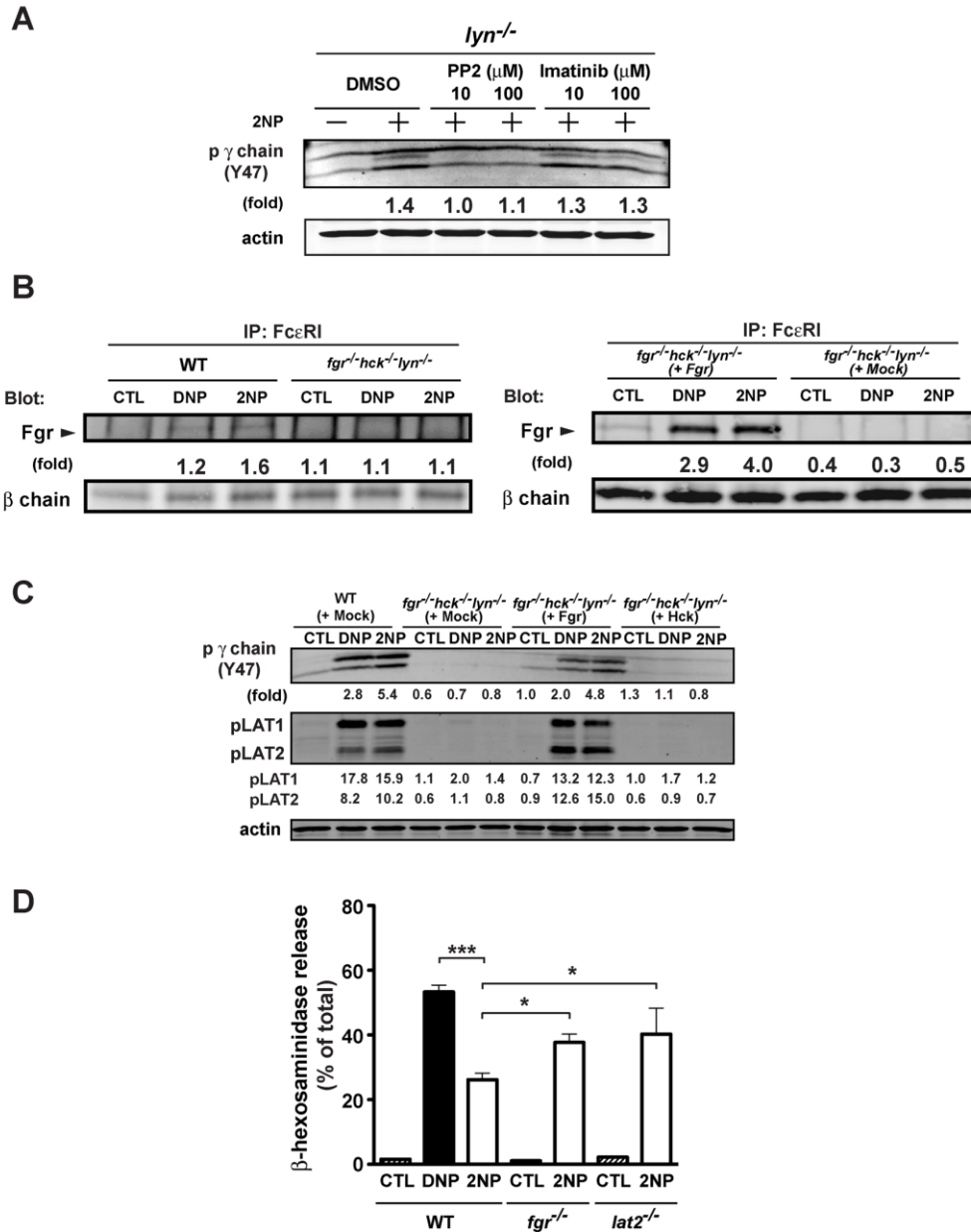


Fig. S5. The residual FcεRI phosphorylation seen in the absence of Lyn is SrcPTK dependent: Src PTK Fgr co-immunoprecipitates with FcεRI and contributes to its phosphorylation. Fgr and LAT2 negatively regulate mast cell degranulation. (A) Lyn-null (*lyn*^{-/-}) BMMCs were incubated with the Src kinase selective inhibitor PP2 and imatinib (which inhibits Kit) for 30 min (at indicated concentrations) followed by stimulation with 2NP for 1 min. Cells were lysed and processed for immunoblotting. 2NP-induced tyrosine phosphorylation of FcεRIγ Y47 in *lyn*^{-/-} BMMCs was suppressed by the Src kinase inhibitor,

PP2, but not imatinib. **(B)** Immunoprecipitation of FcεRI from WT BMMCs (left) or from *fgr*^{-/-} *hck*^{-/-} *lyn*^{-/-} BMMCs expressing Fgr or a Mock vector control (right) after DNP- or 2NP-treatment reveals co-immunoprecipitation of Fgr. Resolved proteins were immunoblotted with antibodies to Fgr. Data shown is representative of 3 individual experiments. **(C)** Phosphorylation of FcεRI (p γchain, Y47), LAT1, and LAT2 prior to or after DNP- or 2NP-treatment (1 min) in cells expressing Fgr, Hck, or control (CTL) vector (mock transfected) relative to *fgr*^{-/-} *hck*^{-/-} *lyn*^{-/-} or WT cells. Fold increase normalized to WT CTL. **(D)** Loss of Fgr or LAT2 enhanced 2NP-induced degranulation responses of mast cells relative to 2NP-treated WT cells. *p<0.05, *** p<0.001, one-way ANOVA. Data was collected from 3 individual experiments.

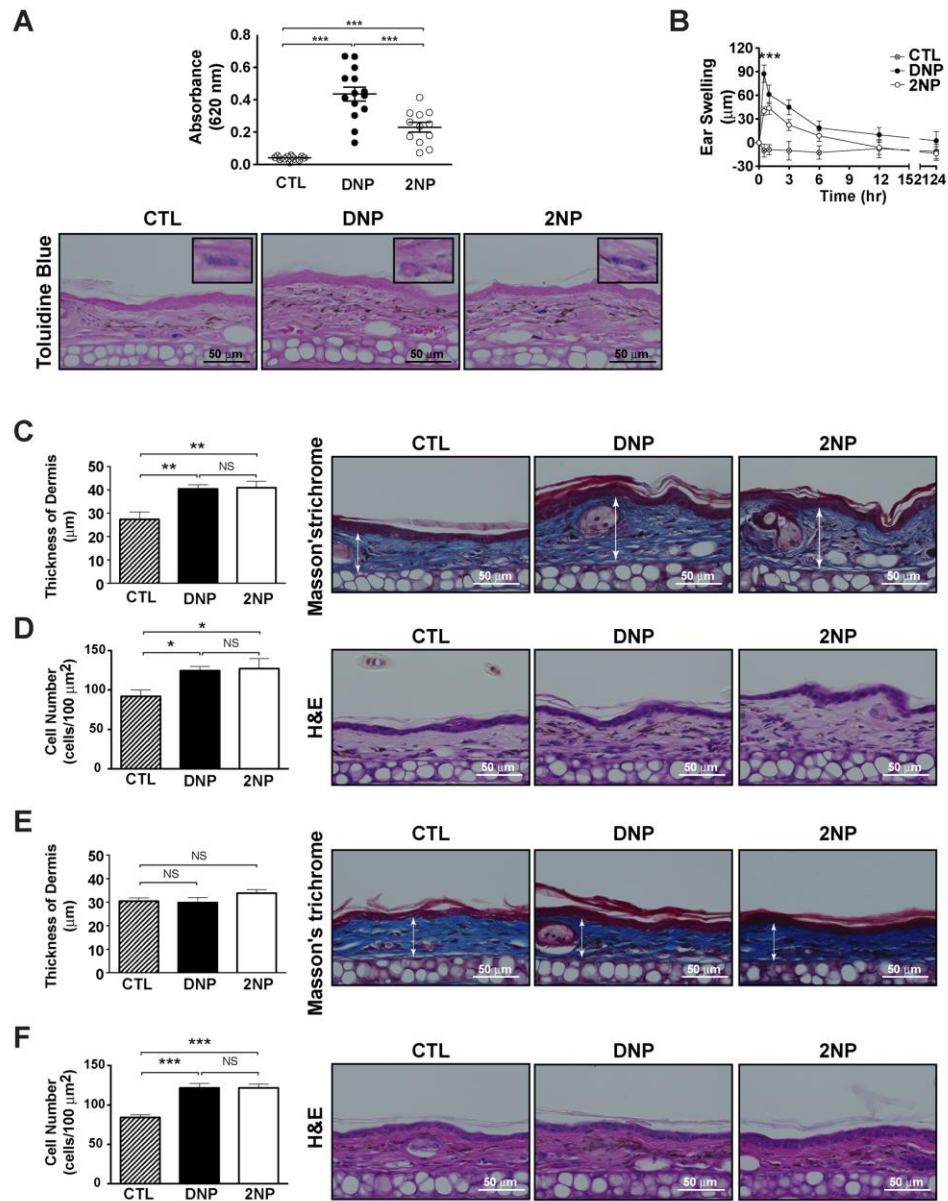


Fig. S6. Treatment of mice with DNP or 2NP in a passive cutaneous anaphylaxis model elicits an inflammatory response. (A) Vascular permeability following passive cutaneous anaphylaxis (PCA) in WT mice treated with DNP or 2NP is increased as measured by Evan's blue dye extravasation in ear tissue. Mice were locally injected (intradermal) with 75 ng of anti-DNP IgE into the ears. After 24 hrs, a systemic injection (retro-orbital) with 1μg DNP or 100μg 2NP in 5.0% w/v Evans blue dye was done. After 30 min, mice were euthanized and ears were collected and Evans blue dye was extracted from ear tissue. Samples were measured for dye

content at 620 nm. *** $p < 0.001$, one-way ANOVA. For visualization of tissue mast cells, ear sections were obtained from DNP- or 2NP -injected or saline-injected ears (as a negative control) at 3 hrs post-challenge. Ear sections were stained with Toluidine blue. Enlarged mast cell images are shown in insets. Representative images are shown, six to eight ears were analyzed per group. Bar = 50 μm . **(B)** Changes in ear swelling after retro-orbital injection of DNP or 2NP into mice 24 hrs after the localized intradermal ear injection with anti-DNP IgE. No ear swelling was observed in the normal saline injected ears (CTL). Significant difference in ear swelling between DNP- or 2NP-treated mice was observed 30min after injection. *** $p < 0.001$, two-way ANOVA. **(C)** Quantitation of dermal thickness in Masson's trichrome stained ear sections 3 hrs post-injection with indicated antigen. Ear sections were stained with Masson's trichrome. Double-headed white arrows delineate thickness of dermis. Representative images are shown for each with six to eight ears analyzed per group. ** $p < 0.01$, one-way ANOVA. Bar = 50 μm . **(D)** Quantitation of cell infiltration at 3 hrs after DNP- or 2NP-injection. Representative images of H&E-stained ear sections from DNP- or 2NP- injected WT mice at 3 hrs. Six to eight ears were analyzed per group. * $p < 0.05$, one-way ANOVA. Bar = 50 μm . **(E)** Quantitation of dermal thickness in Masson's trichrome stained ear sections as in (C), 12 hrs post-injection. No significant difference was observed in dermal thickness. Six to eight ears were analyzed per group. NS = not significance, Bar = 50 μm . **(F)** Quantitation of cell infiltration as in (D) 12 hrs post DNP- or- 2NP injection. Representative images of H&E-stained ear sections are shown. Six to eight ears were analyzed per group. *** $p < 0.001$, one-way ANOVA. Bar = 50 μm .

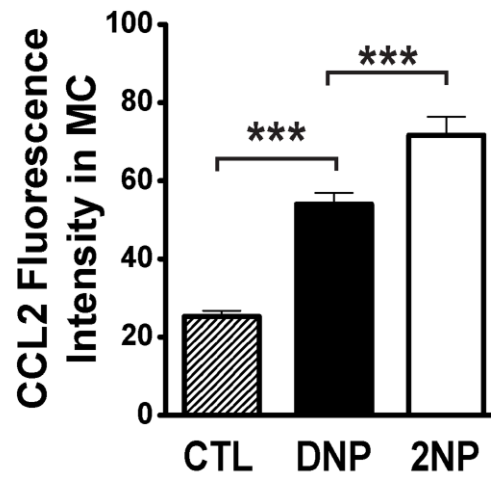


Fig. S7. CCL2 production is significantly elevated after passive cutaneous anaphylaxis (PCA) in the mast cells of 2NP-treated relative to DNP-treated mice. Quantitation of CCL2 fluorescence intensities in ear tissue mast cells after control (normal saline), DNP- or 2NP-challenge in PCA as performed by double-labeled immunohistochemistry. Images from whole mount tissue were randomly taken from six to eight ears per group. Data is mean $\pm$ SE from an analysis of more than 100 cells from control-, DNP-, or 2NP-challenged mice. *** $p < 0.001$ one-way ANOVA.

Movie S1. Real-time TIRF movie showing the mobility of an individual IgE-FcεRI microcluster as labeled with Alexa Fluor 568-conjugated IgE on WT BMMCs placed on planar supported lipid bilayers containing DNP. The display rate is 30 frames per sec. Lines in movie show the trajectories of IgE-FcεRI microclusters.

Movie S2. Real-time TIRF movie showing the mobility of an individual IgE-FcεRI microcluster as labeled with Alexa Fluor 568-conjugated IgE on WT BMMCs placed on planar supported lipid bilayers containing 2NP. The display rate is 30 frames per sec. Lines in movie show the trajectories of IgE-FcεRI microclusters.

Movie S3. Real-time TIRF movie showing the co-localization of IgE-FcεRI microclusters with LAT2-RFP expressed in *lat2*^{-/-} BMMCs. Cells were placed on planar supported lipid bilayers containing DNP. LAT2 (Green), Alexa Fluor 647-conjugated IgE (Red), and merge (Yellow) images are shown in the movies. Movies show 1.0 sec intervals with a display rate of 30 frames per sec.

Movie S4. Real-time TIRF movie showing the co-localization of IgE-FcεRI microclusters with LAT2-RFP expressed in *lat2*^{-/-} BMMCs. Cells were placed on planar supported lipid bilayers containing 2NP. LAT2 (Green), Alexa Fluor 647-conjugated IgE (Red), and merge (Yellow) images are shown in the movies. Movies show 1.0 sec intervals with a display rate of 30 frames per sec.

Movie S5. Real-time TIRF movie showing the distribution and co-localization of IgE-FcεRI microclusters with Lyn-YFP and Fgr-RFP in *fgr*^{-/-}*hck*^{-/-}*lyn*^{-/-} BMMCs. Cells were placed on planar supported lipid bilayers containing DNP. Alexa Fluor 647-conjugated IgE (Cyan), Lyn (Green), and Fgr (Red) images are shown in the movies. Movies show 1.0 sec intervals with a display rate of 20 frames per sec.

Movie S6. Real-time TIRF movie showing the distribution and co-localization of IgE-FcεRI microclusters with Lyn-YFP and Fgr-RFP in *fgr^{-/-}hck^{-/-}lyn^{-/-}* BMMCs. Cells were placed on planar supported lipid bilayers containing 2NP. Alexa Fluor 647-conjugated IgE (Cyan), Lyn (Green), and Fgr (Red) images are shown in the movies. Movies show 1.0 sec intervals with a display rate of 20 frames per sec.

References

1. H. G. Dohlman, J. W. Thorner, Regulation of G protein-initiated signal transduction in yeast: Paradigms and principles. *Annu. Rev. Biochem.* **70**, 703–754 (2001).
[doi:10.1146/annurev.biochem.70.1.703](https://doi.org/10.1146/annurev.biochem.70.1.703) [Medline](#)
2. M. Behar, N. Hao, H. G. Dohlman, T. C. Elston, Dose-to-duration encoding and signaling beyond saturation in intracellular signaling networks. *PLOS Comput. Biol.* **4**, e1000197 (2008). [doi:10.1371/journal.pcbi.1000197](https://doi.org/10.1371/journal.pcbi.1000197) [Medline](#)
3. J. P. Kinet, The high-affinity IgE receptor (FcεRI): From physiology to pathology. *Annu. Rev. Immunol.* **17**, 931–972 (1999). [doi:10.1146/annurev.immunol.17.1.931](https://doi.org/10.1146/annurev.immunol.17.1.931) [Medline](#)
4. S. J. Galli, M. Tsai, A. M. Piliponsky, The development of allergic inflammation. *Nature* **454**, 445–454 (2008). [doi:10.1038/nature07204](https://doi.org/10.1038/nature07204)
5. C. Isersky, J. Rivera, S. Mims, T. J. Triche, The fate of IgE bound to rat basophilic leukemia cells. *J. Immunol.* **122**, 1926–1936 (1979). [Medline](#)
6. H. Metzger, Transmembrane signaling: The joy of aggregation. *J. Immunol.* **149**, 1477–1487 (1992). [Medline](#)
7. N. L. Andrews, J. R. Pfeiffer, A. M. Martinez, D. M. Haaland, R. W. Davis, T. Kawakami, J. M. Oliver, B. S. Wilson, D. S. Lidke, Small, mobile FcεRI receptor aggregates are signaling competent. *Immunity* **31**, 469–479 (2009). [doi:10.1016/j.immuni.2009.06.026](https://doi.org/10.1016/j.immuni.2009.06.026) [Medline](#)
8. U. Blank, J. Rivera, The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol.* **25**, 266–273 (2004). [doi:10.1016/j.it.2004.03.005](https://doi.org/10.1016/j.it.2004.03.005) [Medline](#)
9. D. R. Jackola, L. K. Pierson-Mullany, C. L. Liebeler, M. N. Blumenthal, A. Rosenberg, Variable binding affinities for allergen suggest a ‘selective competition’ among immunoglobulins in atopic and non-atopic humans. *Mol. Immunol.* **39**, 367–377 (2002).
[doi:10.1016/S0161-5890\(02\)00108-6](https://doi.org/10.1016/S0161-5890(02)00108-6) [Medline](#)
10. C. Torigoe, J. K. Inman, H. Metzger, An unusual mechanism for ligand antagonism. *Science* **281**, 568–572 (1998). [doi:10.1126/science.281.5376.568](https://doi.org/10.1126/science.281.5376.568) [Medline](#)

11. E. Razin, Culture of bone marrow-derived mast cells: A model for studying oxidative metabolism of arachidonic acid and synthesis of other molecules derived from membrane phospholipids. *Methods Enzymol.* **187**, 514–520 (1990). [doi:10.1016/0076-6879\(90\)87058-B](https://doi.org/10.1016/0076-6879(90)87058-B) [Medline](#)
12. C. R. Monks, B. A. Freiberg, H. Kupfer, N. Sciaky, A. Kupfer, Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82–86 (1998). [doi:10.1038/25764](https://doi.org/10.1038/25764)
13. A. Grakoui, S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, M. L. Dustin, The immunological synapse: A molecular machine controlling T cell activation. *Science* **285**, 221–227 (1999). [doi:10.1126/science.285.5425.221](https://doi.org/10.1126/science.285.5425.221) [Medline](#)
14. V. Parravicini, M. Gadina, M. Kovarova, S. Odom, C. Gonzalez-Espinosa, Y. Furumoto, S. Saitoh, L. E. Samelson, J. J. O'Shea, J. Rivera, Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* **3**, 741–748 (2002). [Medline](#)
15. M. Vig, W. I. DeHaven, G. S. Bird, J. M. Billingsley, H. Wang, P. E. Rao, A. B. Hutchings, M. H. Jouvin, J. W. Putney, J. P. Kinet, Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. *Nat. Immunol.* **9**, 89–96 (2008). [doi:10.1038/ni1550](https://doi.org/10.1038/ni1550)
16. A. M. Gilfillan, J. Rivera, The tyrosine kinase network regulating mast cell activation. *Immunol. Rev.* **228**, 149–169 (2009). [doi:10.1111/j.1600-065X.2008.00742.x](https://doi.org/10.1111/j.1600-065X.2008.00742.x) [Medline](#)
17. H. Hong, J. Kitaura, W. Xiao, V. Horejsi, C. Ra, C. A. Lowell, Y. Kawakami, T. Kawakami, The Src family kinase Hck regulates mast cell activation by suppressing an inhibitory Src family kinase Lyn. *Blood* **110**, 2511–2519 (2007). [doi:10.1182/blood-2007-01-066092](https://doi.org/10.1182/blood-2007-01-066092) [Medline](#)
18. G. Gomez, C. Gonzalez-Espinosa, S. Odom, G. Baez, M. E. Cid, J. J. Ryan, J. Rivera, Impaired FcεRI-dependent gene expression and defective eicosanoid and cytokine production as a consequence of Fyn deficiency in mast cells. *J. Immunol.* **175**, 7602–7610 (2005). [Medline](#)

19. J. H. Lee, J. W. Kim, K. Kim, H. S. Kim, H. J. Park, D. K. Park, A. R. Kim, B. Kim, M. A. Beaven, K. L. Park, Y. M. Kim, W. S. Choi, The Src family kinase Fgr is critical for activation of mast cells and IgE-mediated anaphylaxis in mice. *J. Immunol.* **187**, 1807–1815 (2011). [doi:10.4049/jimmunol.1100296](https://doi.org/10.4049/jimmunol.1100296) [Medline](#)
20. M. Rosas, B. Thomas, M. Stacey, S. Gordon, P. R. Taylor, The myeloid 7/4-antigen defines recently generated inflammatory macrophages and is synonymous with Ly-6B. *J. Leukoc. Biol.* **88**, 169–180 (2010). [doi:10.1189/jlb.0809548](https://doi.org/10.1189/jlb.0809548) [Medline](#)
21. F. T. Liu, J. W. Bohn, E. L. Ferry, H. Yamamoto, C. A. Molinaro, L. A. Sherman, N. R. Klinman, D. H. Katz, Monoclonal dinitrophenyl-specific murine IgE antibody: Preparation, isolation, and characterization. *J. Immunol.* **124**, 2728–2737 (1980). [Medline](#)
22. M. Zhu, S. Koonpaew, Y. Liu, S. Shen, T. Denning, I. Dzhagalov, I. Rhee, W. Zhang, Negative regulation of T cell activation and autoimmunity by the transmembrane adaptor protein LAB. *Immunity* **25**, 757–768 (2006). [doi:10.1016/j.immuni.2006.08.025](https://doi.org/10.1016/j.immuni.2006.08.025) [Medline](#)
23. C. J. Fitzner-Attas, M. Lowry, M. T. Crowley, A. J. Finn, F. Meng, A. L. DeFranco, C. A. Lowell, Fcγ receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. *J. Exp. Med.* **191**, 669–682 (2000). [doi:10.1084/jem.191.4.669](https://doi.org/10.1084/jem.191.4.669) [Medline](#)
24. R. Suzuki, X. Liu, A. Olivera, L. Aguiniga, Y. Yamashita, U. Blank, I. Ambudkar, J. Rivera, Loss of TRPC1-mediated Ca²⁺ influx contributes to impaired degranulation in Fyn-deficient mouse bone marrow-derived mast cells. *J. Leukoc. Biol.* **88**, 863–875 (2010). [doi:10.1189/jlb.0510253](https://doi.org/10.1189/jlb.0510253) [Medline](#)
25. T. Yamashita, R. Suzuki, P. S. Backlund, Y. Yamashita, A. L. Yergey, J. Rivera, Differential dephosphorylation of the FcRγ immunoreceptor tyrosine-based activation motif tyrosines with dissimilar potential for activating Syk. *J. Biol. Chem.* **283**, 28584–28594 (2008). [doi:10.1074/jbc.M802679200](https://doi.org/10.1074/jbc.M802679200) [Medline](#)
26. S. Iwaki, J. Spicka, C. Tkaczyk, B. M. Jensen, Y. Furumoto, N. Charles, M. Kovarova, J. Rivera, V. Horejsi, D. D. Metcalfe, A. M. Gilfillan, Kit- and FcεRI-induced differential phosphorylation of the transmembrane adaptor molecule NTAL/LAB/LAT2 allows

- flexibility in its scaffolding function in mast cells. *Cell. Signal.* **20**, 195–205 (2008).
[doi:10.1016/j.cellsig.2007.10.013](https://doi.org/10.1016/j.cellsig.2007.10.013) [Medline](#)
27. D. Alvarez-Errico, Y. Yamashita, R. Suzuki, S. Odom, Y. Furumoto, T. Yamashita, J. Rivera, Functional analysis of Lyn kinase A and B isoforms reveals redundant and distinct roles in FcεRI-dependent mast cell activation. *J. Immunol.* **184**, 5000–5008 (2010). [doi:10.4049/jimmunol.0904064](https://doi.org/10.4049/jimmunol.0904064) [Medline](#)
 28. R. Suzuki, S. Leach, B. Dema, J. Rivera, Characterization of a phospho-specific antibody to the Fcε receptor γ chain, reveals differences in the regulation of Syk and Akt phosphorylation. *Antibodies* **2**, 321–337 (2013). [doi:10.3390/antib2020321](https://doi.org/10.3390/antib2020321)
 29. J. Rivera, J.-P. Kinet, J. Kim, C. Pucillo, H. Metzger, Studies with a monoclonal antibody to the β subunit of the receptor with high affinity for immunoglobulin E. *Mol. Immunol.* **25**, 647–661 (1988). [doi:10.1016/0161-5890\(88\)90100-9](https://doi.org/10.1016/0161-5890(88)90100-9) [Medline](#)
 30. A. A. Brian, H. M. McConnell, Allogeneic stimulation of cytotoxic T cells by supported planar membranes. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6159–6163 (1984).
[doi:10.1073/pnas.81.19.6159](https://doi.org/10.1073/pnas.81.19.6159) [Medline](#)
 31. P. Tolar, J. Hanna, P. D. Krueger, S. K. Pierce, The constant region of the membrane immunoglobulin mediates B cell-receptor clustering and signaling in response to membrane antigens. *Immunity* **30**, 44–55 (2009). [doi:10.1016/j.immuni.2008.11.007](https://doi.org/10.1016/j.immuni.2008.11.007)
[Medline](#)
 32. W. Liu, H. Won Sohn, P. Tolar, T. Meckel, S. K. Pierce, Antigen-induced oligomerization of the B cell receptor is an early target of FcγRIIB inhibition. *J. Immunol.* **184**, 1977–1989 (2010). [doi:10.4049/jimmunol.0902334](https://doi.org/10.4049/jimmunol.0902334) [Medline](#)
 33. M. J. Saxton, K. Jacobson, Single-particle tracking: Applications to membrane dynamics. *Annu. Rev. Biophys. Biomol. Struct.* **26**, 373–399 (1997).
[doi:10.1146/annurev.biophys.26.1.373](https://doi.org/10.1146/annurev.biophys.26.1.373) [Medline](#)
 34. P. K. Mattila, C. Feest, D. Depoil, B. Treanor, B. Montaner, K. L. Otipoby, R. Carter, L. B. Justement, A. Bruckbauer, F. D. Batista, The actin and tetraspanin networks organize receptor nanoclusters to regulate B cell receptor-mediated signaling. *Immunity* **38**, 461–474 (2013). [doi:10.1016/j.immuni.2012.11.019](https://doi.org/10.1016/j.immuni.2012.11.019) [Medline](#)