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Cutting Edge: Drebrin-Regulated Actin Dynamics Regulate IgE-Dependent Mast Cell Activation and Allergic Responses

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Mast cells play critical roles in allergic responses. Calcium signaling controls the function of these cells, and a role for actin in regulating calcium influx into cells has been suggested. We have previously identified the actin reorganizing protein Drebrin as a target of the immunosuppressant 3,5-bistrifluoromethyl pyrazole, which inhibits calcium influx into cells. In this study, we show that Drebrin^{-/-} mice exhibit reduced IgE-mediated histamine release and passive systemic anaphylaxis, and Drebrin^{-/-} mast cells also exhibit defects in FcεRI-mediated degranulation. Drebrin^{-/-} mast cells exhibit defects in actin cytoskeleton organization and calcium responses downstream of the FcεRI, and agents that relieve actin reorganization rescue mast cell FcεRI-induced degranulation. Our results indicate that Drebrin regulates the actin cytoskeleton and calcium responses in mast cells, thus regulating mast cell function in vivo. *The Journal of Immunology*, 2015, 195: 000–000.

Stimulation via the FcεRI stimulation in mast cells results in increased intracellular Ca²⁺, which is critical for the function of mast cells in allergic responses. For example, mice lacking the calcium channel Orail, or the regulator of this channel stromal interaction molecule 1 (STIM1), exhibit severely impaired histamine release and leukotriene production, reduced TNF-α secretion, and an inability to mount an anaphylactic response (1, 2). Actin cytoskeleton reorganization has been implicated in models of calcium release activated channel (CRAC) regulation. Furthermore, mast cells deficient in Wiskott–Aldrich syndrome

protein, a key regulator of F-actin assembly, exhibit diminished Ca²⁺ mobilization, degranulation, and cytokine secretion (3). Nonetheless, little is known about actin-modulatory proteins that are involved in this process. Furthermore, given the connections between increases in intracellular calcium and actin rearrangements, it is not clear whether these defects are related to the former or the latter.

The group of immunosuppressant compounds derived from 3,5-bistrifluoromethyl pyrazole (BTP) can suppress allergy and allergic inflammation, including Ag-induced histamine release and leukotriene production in IgE-primed RBL-2H3 cells, as well as bone marrow–derived mast cells (BMMCs) in vitro and in vivo (4). We have recently determined that BTP2 potently inhibits immune cell activation via modulation of store-operated calcium channel activity, and identified the actin-binding protein drebrin 1 (Dbn1) as a target of BTP (5). We show in this study that Dbn1 regulates systemic anaphylaxis and IgE/FcεRI-induced degranulation in mast cells by regulating actin reorganization and actin dynamics.

Materials and Methods

Generation of Dbn1^{-/-} mice

Wild-type (WT) 129S6/SvEvTac mice were obtained from Taconic, and Dbn1^{-/-} mice on the 129/SV background were generated from a gene-trapped library of mouse ES cells (The Institute for Genomic Medicine, Houston, TX) inserted into intron 8 of the mouse *Dbn1* gene (6), and backcrossed to WT 129S6/SvEvTac to generate WT littermates. Mice were genotyped by PCR using a long terminal repeat reverse primer and primer B that flanks the genomic insertion site of the gene-trap vector: long terminal repeat reverse, 5'-ATAAACCTCTTGCAGTTGCATC-3' (A), 5'-CTTC-ATCTTTGTCAGTCACCAGC-3'; 5'-CCAGTTCTAGGAGAGCCACT-ATC-3' (B). WT mice were littermates of the Dbn1^{-/-} mice used for experiments, all of which were approved by the Institutional Animal Care and Use Committees at The Pennsylvania State University and Cornell University.

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Abbreviations used in this article: BMMC, bone marrow–derived mast cell; BTP, 3,5-bistrifluoromethyl pyrazole; CRAC, calcium release activated channel; Dbn1, drebrin 1; LatB, latrunculin B; SCF, stem cell factor; STIM1, stromal interaction molecule 1; WIP, Wiskott–Aldrich syndrome interacting protein; WT, wild-type.

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Generation of BMMCs

Mouse BMMCs were generated in the presence of IL-3 and/or IL-3/stem cell factor (SCF) as previously described (7), and were routinely >95% positive for FcεRI and c-kit by flow cytometry. Abs were obtained from eBioscience and staining was performed in the presence of Fc block (BioLegend).

In vivo analysis of mast cells

Quantitative analysis of skin mast cells by histological staining was done as previously described (8). Transmission electron microscopy of skin samples was done as previously described (8). Analyses of peritoneal mast cells were performed by flow cytometry.

Measurements of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was monitored as previously described using the Ca^{2+} indicator Fluo-4 (Invitrogen) (4). Mean calcium poststimulation was calculated as the mean of intracellular Ca^{2+} concentration Fluo-4 relative fluorescence measurements poststimulation after subtracting the mean of baseline measurements prestimulation. The slope of calcium decay was calculated as the slope of Fluo-4 relative fluorescence measurements poststimulation fitted by linear regression analysis after subtracting the mean of respective prestimulation baseline measurements.

Western blotting and analysis of mRNA

Lysates from neocortical homogenates of 6- to 10-wk-old mice were analyzed by SDS-PAGE and Western blotting as previously described (7). Analysis of

mRNA was performed as previously described using TaqMan gene expression assay probes for *Dbn1* exons 8–10, with GAPDH as a housekeeping gene and WT set as 1 (Applied Biosystems, Branchburg, NJ) (7).

In vivo histamine release, passive systemic anaphylaxis, and degranulation assays

Analysis of degranulation in vitro and of in vivo histamine release was determined as previously described (7). Passive systemic anaphylaxis was done as previously described (4). Mice were treated with BTP2 i.p. (25 mg/kg) or vehicle (DMSO/ethanol) 1 h before i.v. injection with 8–10 μ g anti-IgE. *Dbn1*^{−/−} mice were only injected with anti-IgE (10 μ g). Mice were then monitored for temperature using a rectal temperature probe.

Statistical analysis

Data represent the mean $\pm$ SEM of at least three independent experiments, with statistical significance analyzed by Student *t* test (unpaired *t* test, two-tailed) or by two-way ANOVA using GraphPad Prism 5, with *p* < 0.05 considered significant.

Results and Discussion

Dbn1−/− mice have reduced numbers of skin and peritoneal mast cells, and exhibit reduced histamine release and passive systemic anaphylaxis

We have previously reported that the actin-binding protein *Dbn1* is a target of BTP2, an immunosuppressant that can inhibit mast cell degranulation and cytokine secretion (4, 5). To examine the role of *Dbn1* in mast cell function, we generated *Dbn1*^{−/−} mice (Supplemental Fig. 1A). *Dbn1*^{−/−} skin tissue-resident mast cells had normal phenotype; however, there was a significant reduction in their density, and in the number of mast cells in the peritoneum, although there was no difference in mast cell density in intestine, trachea, or tongue (Supplemental Fig. 1B–E). We also found that *Dbn1*^{−/−} mice had significantly lower levels of histamine in the serum compared with WT mice during acute IgE challenge in passive systemic anaphylaxis (Fig. 1A). Furthermore, *Dbn1*^{−/−} mice exhibited reduced hypothermia during passive systemic anaphylaxis similar to mice treated with BTP2 (Fig. 1B).

Dbn1 regulates FcεRI-mediated degranulation and intracellular calcium mobilization downstream of FcεRI in BMMCs

We generated BMMCs in vitro to examine whether the reduced degranulation observed in vivo was intrinsic to mast cells. We found that there was no significant difference in differentiation of precursors into c-Kit/FcεRI double-positive mast cells over the 6-wk period in the presence of either IL-3 or IL-3 plus SCF (Fig. 1D), although *Dbn1*^{−/−} exhibited reduced proliferation in response to IL-3, which was rescued by the inclusion of SCF (Fig. 1C). We also found that

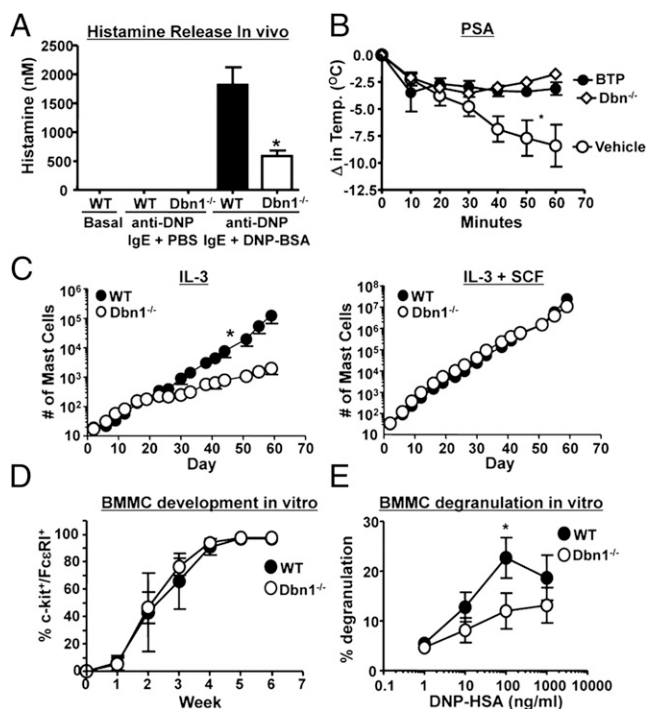


FIGURE 1. *Dbn1*^{−/−} mice exhibit impaired IgE-mediated histamine release in vivo. **(A)** Serum histamine levels of WT and *Dbn1*^{−/−} mice sensitized with anti-DNP IgE, then challenged 48 h later with DNP-human serum albumin (HSA) for 3 min. Control mice were either left untreated or sensitized and injected with PBS alone. Data are expressed as the mean $\pm$ SEM of *n* = 3–5/group. **p* < 0.05. **(B)** Temperature change in WT mice pretreated with BTP or vehicle, or *Dbn1*^{−/−} mice, injected with anti-IgE and monitored for temperature over the indicated time. Data are expressed as the mean $\pm$ SEM of *n* = 6/group. **p* < 0.05 by two-way ANOVA. **(C)** Bone marrow from the indicated mice were differentiated in vitro in the presence of IL-3 (left) or IL-3+SCF (right), and the number of mast cells was determined as indicated (using FcεRI/c-Kit staining and flow cytometry). **p* < 0.05 by ANOVA. **(D)** Percentage of FcεRI/c-Kit double-positive cells in bone marrow cultured in IL-3-supplemented media. **(E)** WT and *Dbn1*^{−/−} BMMCs were sensitized in vitro with anti-DNP IgE, then challenged with DNP-HSA at the indicated concentrations for 1 h. Supernatants were assayed for β -hexosaminidase. Data are expressed as the mean $\pm$ SEM of triplicate experiments. **p* < 0.05 versus WT.

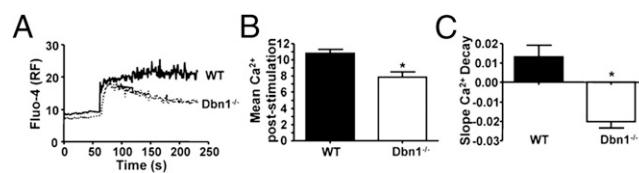


FIGURE 2. *Dbn1*^{−/−} BMMCs exhibit impaired IgE-mediated degranulation and extracellular Ca^{2+} influx in vitro. **(A)** WT and *Dbn1*^{−/−} BMMCs were stimulated as in Fig. 1 in the presence of $CaCl_2$ for the indicated time period. Intracellular Ca^{2+} concentration expressed as Fluo-4 relative fluorescence and representative data of three independent experiments are shown. **(B)** Mean intracellular calcium level and **(C)** the slope of calcium decay poststimulation. Data are expressed as the mean $\pm$ SEM of triplicate experiments. **p* < 0.05 versus WT.

$\text{Dbn1}^{-/-}$ BMMCs exhibited reduced degranulation upon $\text{Fc}\epsilon\text{RI}$ stimulation with IgE/Ag (Fig. 1E). This difference was not due to surface levels of $\text{Fc}\epsilon\text{RI}$ (Supplemental Fig. 2A) or intracellular histamine levels (data not shown). There was no difference in global patterns of tyrosine phosphorylation, or activation of ERK, JNK, or p38 MAPK pathways in BMMCs challenged with Ag/IgE/ $\text{Fc}\epsilon\text{RI}$ (Supplemental Fig. 2B, 2C).

However, whereas WT BMMCs exhibit $\text{Fc}\epsilon\text{RI}$ -induced stable and prolonged increase in intracellular Ca^{2+} , which includes both release from intracellular stores and influx from outside the cells (indicated by a positive slope), $\text{Dbn1}^{-/-}$ BMMCs were defective in this response as indicated by a negative slope, as well as a lower mean increase in intracellular Ca^{2+} (Fig. 2A–C). There was no difference in tyrosine phosphor-

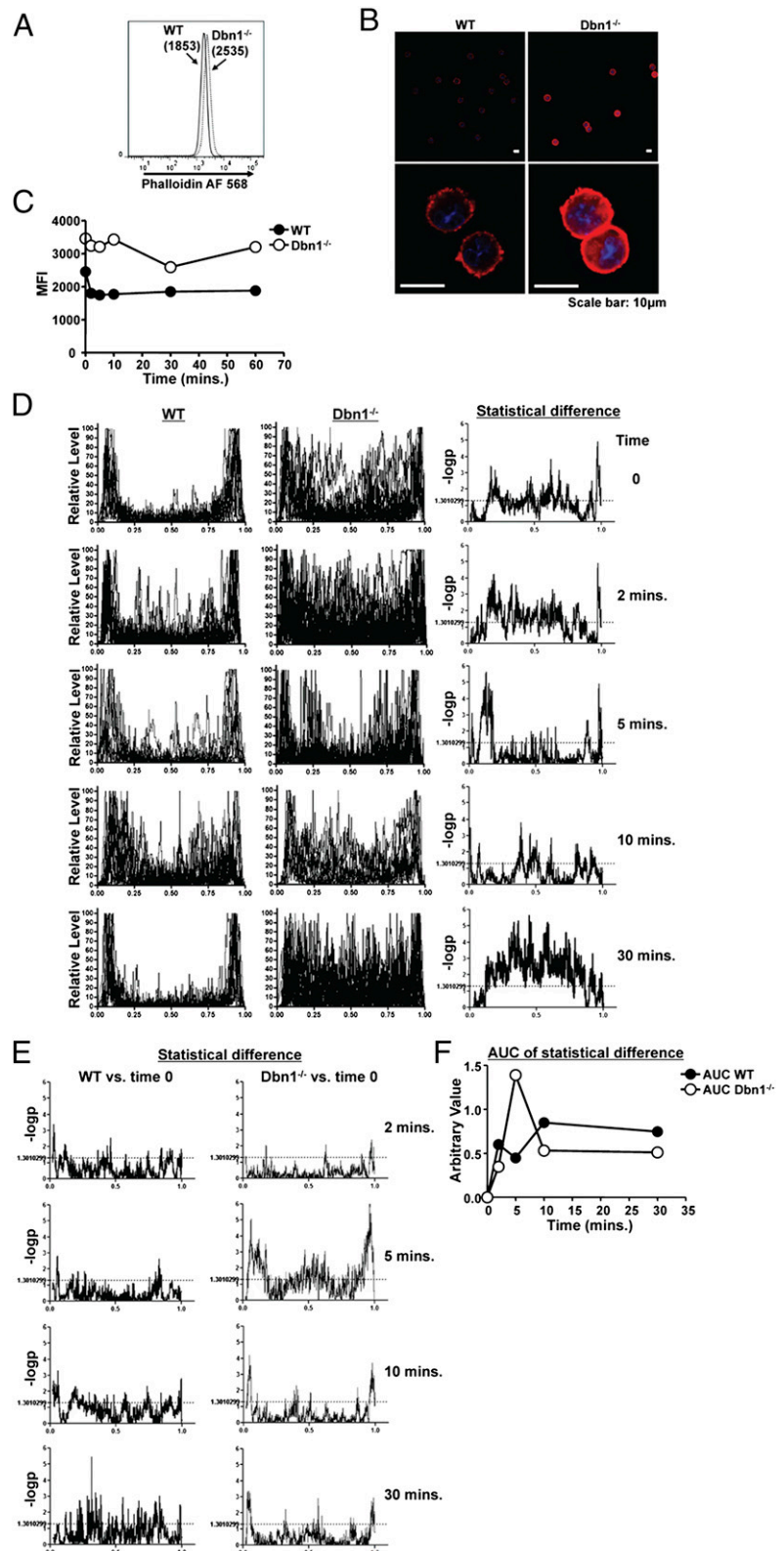


FIGURE 3. $\text{Dbn1}^{-/-}$ BMMCs have altered actin superstructure. (A) WT (solid lines) and $\text{Dbn1}^{-/-}$ (dotted lines) BMMCs were stained with Alexa Fluor-tagged phalloidin and analyzed by flow cytometry. (B) Resting WT (left panels) and $\text{Dbn1}^{-/-}$ (right panels) BMMCs were stained with Alexa Fluor-tagged phalloidin and counterstained with DAPI, then analyzed by confocal microscopy. Scale bar, 10 μm . (C) WT and $\text{Dbn1}^{-/-}$ BMMCs were armed with anti-DNP IgE, then stimulated with DNP-human serum albumin for the indicated time, followed by analysis of F-actin using Alexa Fluor-tagged phalloidin and flow cytometry. (D) Line-scan analysis of cells plotted as a function of cell width (25–35 cells each) and a function of the indicated time after activation with IgE/ $\text{Fc}\epsilon\text{RI}$. Left panels, WT. Middle panels, $\text{Dbn1}^{-/-}$. Right panels, Statistical analysis ($-\log_{10}p$ shown) comparing WT and $\text{Dbn1}^{-/-}$ actin location across the cell. Signals >1.3 are statistically significant ($p < 0.05$). (E) Statistical analysis ($-\log_{10}p$ shown) comparing difference in actin location as a function of the indicated time after activation with IgE/ $\text{Fc}\epsilon\text{RI}$. Left panels, WT. Right panels, $\text{Dbn1}^{-/-}$. Signals >1.3 are statistically significant ($p < 0.05$). (F) Area under the curve (AUC) collected for the statistical analysis shown in (E). Time 0 set at 0.

ylation of PLC γ 1, which regulates Ca²⁺, indicating that the defect in Ca²⁺ mobilization is unlikely to be due to defects in activation of PLC γ 1 (Supplemental Fig. 2D). Further analysis of the effects of these differences in Dbn1^{-/-} BMMCs revealed that Dbn1^{-/-} BMMCs secreted significantly reduced IL-2 and GM-CSF, intracellular calcium-dependent cytokines (9), but not IL-6, after Fc ϵ RI triggering compared with WT BMMCs (Supplemental Fig. 2G).

Dbn1 regulates actin organization in BMMCs

Dbn1 is frequently found to be associated with actin in the dendritic spines of neurons, and has been shown to interact with and induce changes in the actin cytoskeleton when overexpressed (10, 11). Actin has been suggested to play a role in the regulation of Ca²⁺ influx into cells (12–15), and we found that Dbn1^{-/-} BMMCs have higher levels of F-actin (detected by fluorescently tagged phalloidin) than WT cells (Fig. 3A, 3B). Furthermore, although this F-actin was found to be concentrated just under the plasma membrane of resting WT BMMCs, in the absence of Dbn1, it was more distributed inside the cell (Fig. 3B). There was also a statistically significant increase in intracellular F-actin in the absence of Dbn1 (Fig. 3B, 3D). IgE/Fc ϵ RI stimulation of these BMMCs resulted in reduced F-actin in WT cells as previously reported (16), but there was a delay in this change in Dbn1^{-/-} BMMCs (Fig. 3C).

Analysis of the distribution of F-actin in WT and Dbn1^{-/-} BMMCs at rest and after Ag/IgE/Fc ϵ RI triggering revealed WT BMMCs had F-actin primarily, if not exclusively, localized under the regions of the plasma membrane of the cells as indicated by the signal at the edges of the cells (Fig. 3B, 3D, *left panels*). However, this was significantly different in Dbn1^{-/-} BMMCs, which had higher levels of F-actin on the inside of the cell as well (Fig. 3B, 3D, compare *left and middle panels*, with statistical comparison in the *right panels*). IgE/Fc ϵ RI triggering resulted in significant but low-level reorganization of F-actin in WT BMMCs, primarily inside the cell (and not at the plasma membrane) over the time frame of analysis (statistical comparison with time 0; Fig. 3D, 3E). By contrast, IgE/Fc ϵ RI triggering of Dbn1^{-/-} BMMCs resulted in large-scale changes in F-actin structure inside the cell, with changes both inside the cell and at regions near the plasma membrane at 5 min, which transitioned to changes localized near the plasma membrane at 10 and 30 min (statistical comparison time 0; Fig. 3D, 3E). Comparison of the area under the curve as a crude measure of the statistically significant changes revealed that Dbn1^{-/-} BMMCs exhibit more significant changes in F-actin superstructure after IgE/Fc ϵ RI triggering compared with WT BMMCs (Fig. 3F). Note that the absence of Dbn1 did not affect Fc ϵ RI-induced phosphorylation of p21-activated kinase, suggesting that p21-activated kinase is not affected by this pathway (Supplemental Fig. 2F).

To determine whether altering the dynamics of the F-actin superstructure in these cells would rescue the behavior of the Dbn1 mutant cells, we treated Dbn1^{-/-} BMMCs with varying concentrations of latrunculin B (LatB), which interacts with F-actin and induces depolymerization. Note that at these concentrations, F-actin is still present in the cells, unlike the case at higher concentrations (Supplemental Fig. 2E). We found that LatB induces a reduction in F-actin in Dbn1^{-/-}

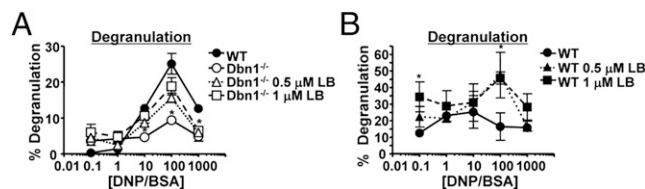


FIGURE 4. Altered actin superstructure in Dbn1^{-/-} BMMCs regulates degranulation independently of increase in intracellular calcium. **(A)** WT and Dbn1^{-/-} BMMCs were sensitized with anti-DNP IgE, then challenged with DNP-human serum albumin (HSA) at the indicated concentrations for 1 h in the presence or absence of 0.5 or 1 μM LatB. Supernatants were assayed for β-hexosaminidase. There was no significant difference between WT and Dbn1^{-/-} BMMCs treated with LatB at the same concentrations of DNP-HSA. Data are expressed as the mean ± SEM of three independent experiments. **p* < 0.05 of vehicle-treated Dbn1^{-/-} versus LatB-treated cells. **(B)** WT BMMCs were sensitized with anti-DNP IgE, then challenged with DNP-HSA at the indicated concentrations for 1 h in the presence or absence of 0.5 or 1 μM LatB. Supernatants were assayed for β-hexosaminidase. **p* < 0.05 of vehicle-treated WT BMMCs versus LatB-treated cells.

cells (Supplemental Fig. 2E) and rescues Fc ϵ RI-induced degranulation, as well as enhances the response of WT cells in vitro in response to Ag cross-linking (Fig. 4). These data indicate that Dbn1 regulates mast cell function by regulating the dynamics and/or location of F-actin in BMMCs, which is required for efficient mast cell responses to Fc ϵ RI triggering.

Our previous identification of Dbn1 as a target for the immunosuppressant BTP, a known regulator of intracellular Ca²⁺ influx into cells (5), along with this work supporting a role for Dbn1 in regulating actin reorganization, Ca²⁺ influx, and mast cell function, adds support to the idea that the actin cytoskeleton plays an important role in regulating this process. We and others have previously reported evidence supporting a role for actin and cytoskeletal rearrangement in the regulation of Ca²⁺ influx into cells (17–19). In B cells, T cells, and mast cells, low concentrations of LatB are able to enhance cell activation and increase in intracellular Ca²⁺ (12, 13, 17). The actin regulators Wiskott-Aldrich syndrome protein, Wiskott-Aldrich syndrome interacting protein (WIP), and WAVE2 have been demonstrated to regulate store-operated channel activity and Ca²⁺ influx into mast cells and/or T cell, and in the case of WIP, regulate actin changes upon activation (3, 12, 20). Our work suggests that similar to the case with WIP, Dbn1 regulates actin changes downstream of the Fc ϵ RI. The ability to modulate the actin superstructure and calcium responses links Dbn1 to the Fc ϵ RI response leading to degranulation by mast cells. Altogether, our data support a model where influx of Ca²⁺ is regulated by modulating actin cytoskeleton in part via Dbn1, leading to mast cell activation and degranulation. Our data validate Dbn1 as a potential target for mast cell-regulated diseases.

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Disclosures

The authors have no financial conflicts of interest.

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Cutting Edge: Drebrin regulated actin dynamics regulates IgE-dependent mast cell activation and allergic responses

Running Title: Drebin regulates mast cell function

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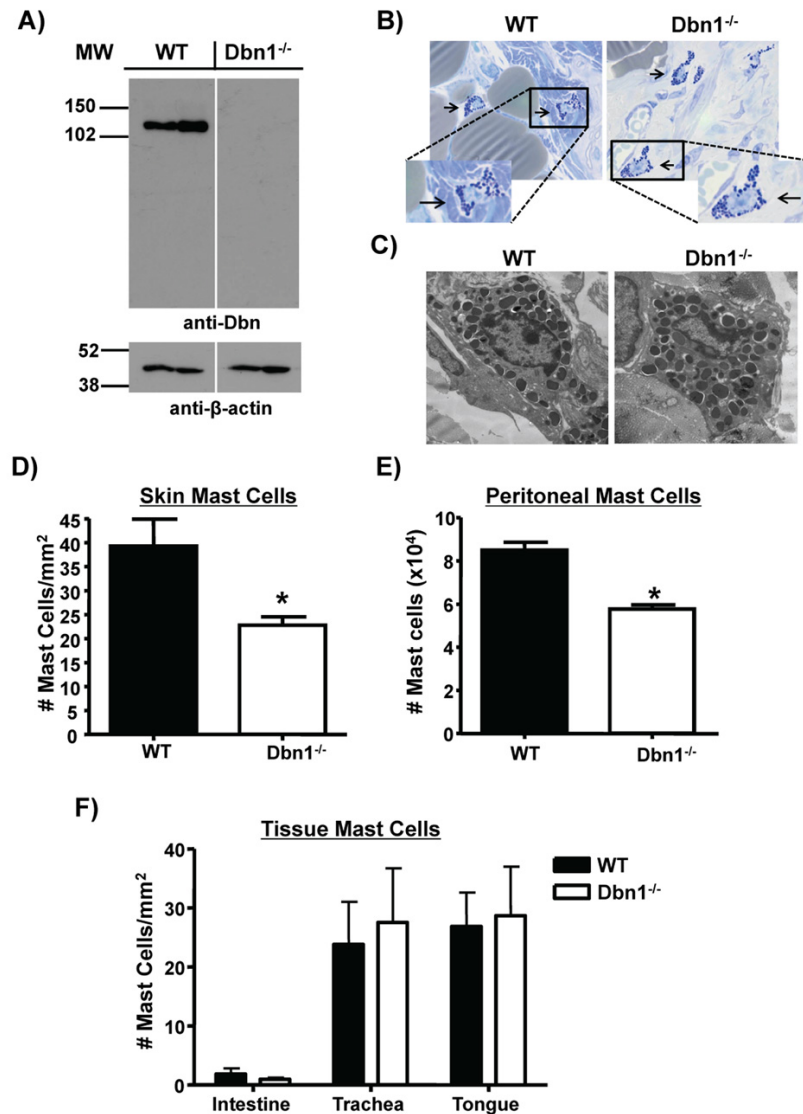
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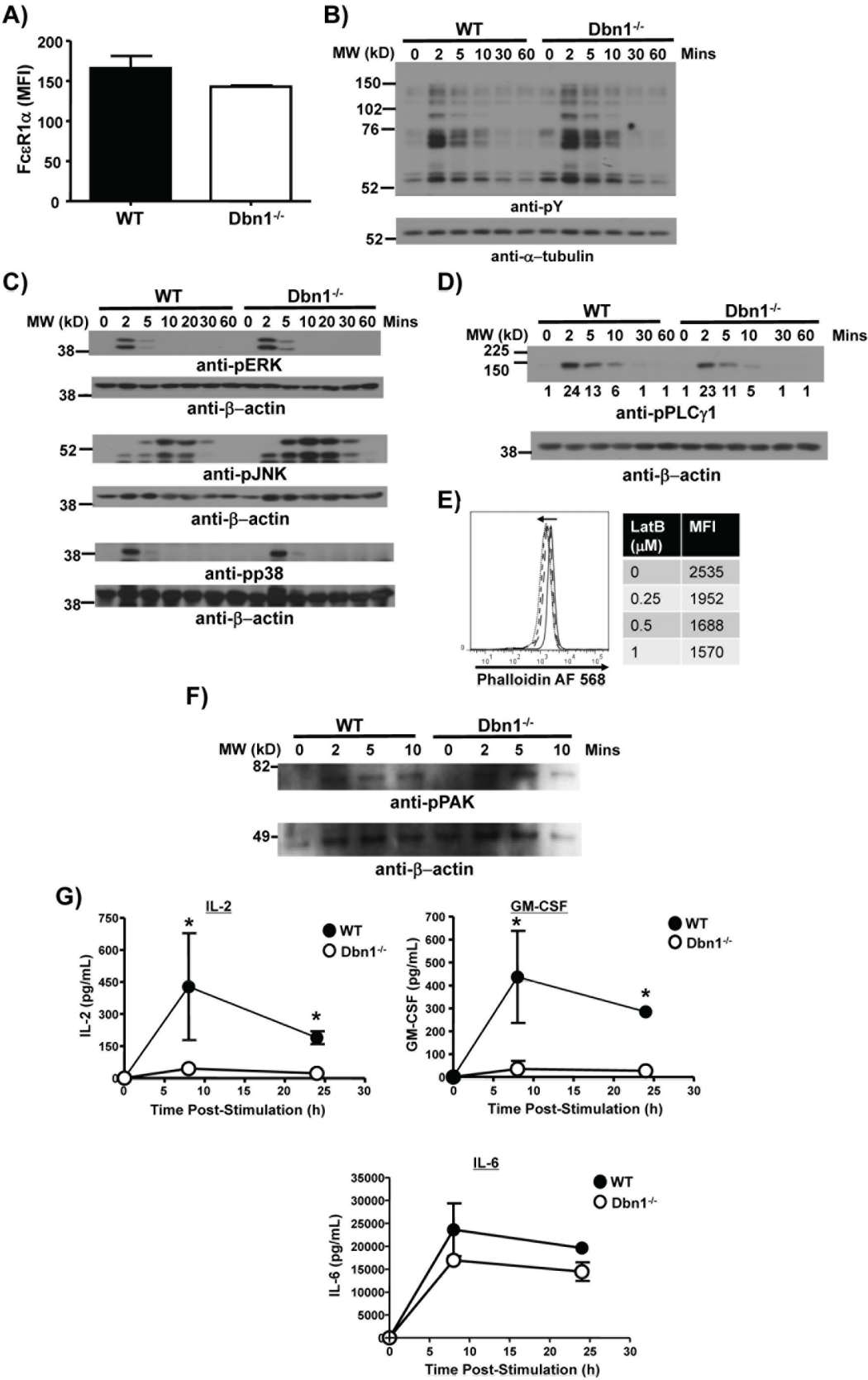
Supplemental Figures 1 and 2

Supplemental Figures



Supplemental Figure 1. Generation of Dbn1^{-/-} mice. **A)** Whole brain lysates from WT and Dbn1^{-/-} mice analyzed by western blot using a pan anti-Dbn1 antibody. Blots were stripped and reprobed with control antibodies for β-actin. **B)** Normal morphology and distribution, but reduction in the number of skin and peritoneal mast cells in the absence of Dbn1^{-/-}. Toluidine blue-stained skin sections from WT and Dbn1^{-/-} mice (6-8 weeks old). Black arrows identify mast cells (20 ×). **C)** Transmission electron micrographs of skin sections from WT and Dbn1^{-/-} mice (6-8 weeks old). **D)** Quantification of the numbers of skin-resident mast cells per

standardized area. **E)** Quantification of the numbers peritoneal mast cells by flow cytometry (FcεRI/c-Kit). **F)** Quantification of the numbers of intestine, trachea and tongue -resident mast cells per standardized area. For skin mast cells: * $p < 0.05$ vs. WT. Data are expressed as the mean $\pm$ SEM of $n = 3$ /group, each representative of the average of individual counts of 3 separate tissue block sections. For peritoneal mast cells: * $p < 0.0001$ vs. WT. Data are expressed as the mean $\pm$ SEM of $n = 6$ /group.



Supplemental Figure 2. No difference in IgE mediated tyrosine phosphorylation in the absence of Dbn1^{-/-} in BMMCs. **A)** MFI of FcεRI on WT and Dbn1^{-/-} BMMCs. *p<0.05. Data are expressed as the mean ± SEM of 3 independent experiments. **B)** WT and Dbn1^{-/-} BMMCs were sensitized *in vitro* with anti-DNP IgE, then challenged with DNP-HSA for the indicated times periods, followed by analysis by western blot for total phosphotyrosine; **(C)** phospho-ERK1/2; phospho-JNK; or phospho-p38 expression. Blots were reprobed with control antibodies for either β-actin or α-tubulin. **D)** WT and Dbn1^{-/-} BMMCs were stimulated as (B) and analyzed by western blot for phospho-PLCγ1. Blots were reprobed with control antibodies for β-actin. Densitometric values indicate fold increase that has been corrected to account for loading levels. **Altered actin superstructure in Dbn1^{-/-} BMMCs regulates degranulation independently of increase in intracellular calcium.** **E)** Dbn1^{-/-} BMMCs were treated with vehicle (solid lines) or varying concentrations of LatB (0.25, 0.5 and 1 μM, dotted lines), followed by staining with AlexaFlour-tagged phalloidin and analysis by flow cytometry. Table; MFI of actin-phalloidin stain at the indicated LatB concentrations. **F)** WT and Dbn1^{-/-} BMMCs were sensitized *in vitro* with anti-DNP IgE, then challenged with DNP-HSA for the indicated times periods, followed by analysis by western blot for anti-phospho-PAK (S199/204). Blots were reprobed with control antibodies for β-actin. **Reduced FcεRI-mediated cytokine production in Dbn1^{-/-} in BMMCs.** **G)** WT and Dbn1^{-/-} BMMCs were stimulated with IgE/anti-IgE for the indicated times and supernatants analyzed the indicated cytokines by multiplex ELISA. *p<0.05, n=2.