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Tumorigenesis and Neoplastic Progression

Nf1$^{-/-}$ Schwann Cell-Conditioned Medium Modulates Mast Cell Degranulation by c-Kit-Mediated Hyperactivation of Phosphatidylinositol 3-Kinase

Shi Chen,*† Sarah Burgin,*† Andrew McDaniel,*†‡ Xiaohong Li,*† Jin Yuan,*† Mia Chen,*† Waleed Khalaf,*†‡ D. Wade Clapp,*†‡ and Feng-Chun Yang*†§

From the Department of Pediatrics, Herman B. Wells Center for Pediatric Research,† and the Departments of Microbiology and Immunology‡ and Anatomy and Cellular Biology,§ Indiana University School of Medicine, Indianapolis, Indiana*

Neurofibromatosis type 1 (NF1) is a common genetic disorder and is characterized by both malignant and nonmalignant neurofibromas, which are composed of Schwann cells, degranulating mast cells, fibroblasts, and extracellular matrix. We and others have previously shown that hyperactivation of the c-Kit pathway in an *Nf1* haploinsufficient microenvironment is required for both tumor formation and progression. Mast cells play a key role in both tumorigenesis and neoangiogenesis via the production of matrix metalloproteinases, heparin, and a range of different growth factors. In the present study, we show that tumorigenic Schwann cells derived from *Nf1*$^{-/-}$ embryos promote increased degranulation of *Nf1*$^{+/-}$ mast cells compared with wild-type mast cells via the secretion of the Kit ligand. Furthermore, we used genetic intercrosses as well as pharmacological agents to link the hyperactivation of the p21^{Ras}-phosphatidylinositol 3-kinase (PI3K) pathway to the increased degranulation of *Nf1*$^{+/-}$ mast cells both *in vitro* and *in vivo*. These studies identify the p21^{Ras}-PI3K pathway as a major regulator of the gain in *Nf1*$^{+/-}$ mast cell degranulation in neurofibromas. Collectively, these studies identify both c-Kit and PI3K as molecular targets that modulate mast cell functions in cases of NF1. ***(Am J Pathol 2010, 177:3125–3132; DOI: 10.2353/ajpath.2010.100369)***

Mutations in the *Nf1* tumor suppressor gene cause neurofibromatosis type 1 (NF1), a common autosomal dominant genetic disorder (with an incidence of 1:3500), which is characterized by cutaneous and plexiform neurofibroma formation. Neurofibromin, the protein product of *Nf1,* functions as a GTPase activating protein (GAP) for p21^{Ras} (Ras) by accelerating the hydrolysis of active Ras-GTP to inactive Ras-GDP.[1,2] Neurofibromas are pathognomonic for NF1 and constitute a major source of morbidity in NF1 patients. These complex tumors are composed of high concentrations of Schwann cells, fibroblasts, and degranulating mast cells found in close physical association with each other and within a dense extracellular matrix.

Inflammation and alterations associated with the tumor microenvironment are increasingly recognized as critical components of tumor initiation and progression.[3–5] Studies using genetically engineered mice demonstrated that nullizygosity of *Nf1* in Schwann cells was necessary but not sufficient for neurofibroma formation and that haploinsufficiency of *Nf1* (*Nf1*$^{+/-}$) in non-neuronal lineages of the tumor microenvironment was an additional requirement for tumor progression.[6] Recently, we refined this finding using an *Nf1* conditional knockout model demonstrating that haploinsufficient loss of *Nf1* in the hematopoietic compartment of the microenvironment specifically was required for *in vivo* tumor formation.[7] We further found that c-Kit pathway signaling is critical for the tumor progression.

Mast cells release heparin, histamine, tumor necrosis factor-α, transforming growth factor-β, and metalloproteinases. These mediators alter the extracellular matrix, modulate growth factor presentation to cells within the growing tumor, promote fibroblast proliferation and col-

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S.C. and S.B. contributed equally to this work.

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Current address of W.K.: Vanderbilt University Department of Medicine, Nashville, TN.

Address reprint requests to Feng-Chun Yang, M.D., Ph.D., Indiana University School of Medicine, Cancer Research Institute, 1044 W. Walnut St., R4/427, Indianapolis, IN 46202. E-mail: fyang@iupui.edu.

lagen synthesis, and provide a scaffold for the invasion of blood vessels. However, evaluation of the specific mediators that promote release of these factors from mast cells in the context of neurofibroma development and detailed studies to examine the biochemical pathways that promote this increase in function have not been described. Identification of these degranulation-promoting factors and the biochemical pathways that they activate is important for understanding the pathogenesis of neurofibroma progression and identifying potential molecular targets for treating existing tumors and/or preventing tumor formation.

Previous studies in human neurofibromas have found that *Nf1*$^{-/-}$ Schwann cell conditioned medium contains sevenfold higher concentrations of c-Kit ligand (Kit-L) as well as other growth factors that may enhance mast cell degranulation.[8,9] Studies in human and animal neurofibromas have also observed higher concentrations of Kit-L transcripts within neurofibromas.[10] Here we provide genetic and biochemical evidence that *Nf1*$^{-/-}$ Schwann cell conditioned medium (SCCM) contains a potent degranulation stimulus, Kit-L, that is responsible for the elevated *Nf1*$^{+/-}$ mast cell degranulation. Further, we demonstrate that genetic disruption of the *p85α* regulatory subunit of the class1$_A$ PI3K or the addition of a PI3K inhibitor, Ly294002, is sufficient to abrogate this cellular and biochemical gain of function both *in vitro* and *in vivo*.

Materials and Methods

Animals

Nf1$^{+/-}$ mice were obtained from Dr. Tyler Jacks at the Massachusetts Institute of Technology (Cambridge, MA) in a C57BL/6J.129 background and backcrossed for 13 generations into a C57BL/6J strain.[11] The *Nf1* allele was genotyped as described previously.[9,12–14] C57BL/6J *W41/W41* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The *W41* genotyping was inferred from the characteristic mottled white coat color in *W41/W41* mice and a white abdominal spot on *W41*/+ mice, as described previously.[15,16] *p85α*$^{+/-}$ and *p85α*$^{-/-}$ mice were obtained in a mixed C57BL/6J.129 background from Dr. Lewis Cantley at Harvard University (Boston, MA) and were backcrossed for 12 generations into a C57BL/6J genetic strain. The *p85α* alleles were genotyped by PCR as described previously.[17] All studies were conducted with a protocol approved by the Indiana University Animal Care and Use Committee.

Culture of Bone Marrow-Derived Mast Cells

Bone marrow-derived mast cells (BMMCs) were generated from 6-week-old mice as described previously.[18] In brief, mononuclear bone marrow cells were cultured for 4 weeks in RPMI 1640 containing 1% glutamine (BioWhittaker, Walkersville, ME), 1.5% HEPES (BioWhittaker), 2% penicillin/streptomycin (BioWhittaker), 10% fetal bovine serum (HyClone Laboratories, Logan, UT), and 10 ng/ml recombinant murine interleukin-3 (PeproTech, Rocky Hill, NJ) in a 37°C, 5% CO_2 humidified incubator. The homogeneity of BMMCs was determined by Alcian Blue-Safranin O staining.[9] Furthermore, fluorescence-activated cytometric analysis (BD Biosciences, San Jose, CA) revealed similar forward and side light scatter characteristics and the same percentage of c-Kit$^+$ expression in BMMCs for all murine experimental genotypes (data not shown).

Schwann Cell Culture and Generation of Schwann Cell Conditioned Media

Murine Schwann cells were isolated from WT and *Nf1*$^{-/-}$ mutant mouse embryo dorsal root ganglia at embryonic day 13.5 as described previously.[19] In brief, dorsal root ganglia of the embryos were enzymatically dissociated, and cells from single embryos were plated in a single well of a 12-well tissue culture plate in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY) containing10% fetal bovine serum supplemented with 250 ng/ml nerve growth factor (Harlan Bioproducts for Science Inc., Indianapolis, IN). The medium was changed to serum-free defined N2 medium (Invitrogen) containing 250 ng/ml nerve growth factor and penicillin/streptomycin (1 mM) (BioWhittaker) on the next day. After 5 to 6 days, Schwann cells and neurons were separated from fibroblasts by lifting up the Schwann cell-neuron layers from the dish, leaving the fibroblasts behind. Cells from the same genotype were pooled, and Schwann cells were enzymatically dissociated from the neurons in 0.01% collagenase (Sigma-Aldrich, St. Louis, MO). Cells were centrifuged, resuspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and plated on poly-L-lysine-coated 100-mm diameter cell culture plates (BD, Franklin Lakes, NJ) at a density of approximately 10^6 cells/plate. These cells were considered passage 0. Cells were changed on the next day to Schwann cell growth medium containing 10 ng/ml recombinant human glial growth factor 2 (β-heregulin epidermal growth factor-like domain peptide, Sigma-Aldrich), and 1 mg/ml ***penicillin/streptomycin, with 2 mmol/L forskolin (EMD Biosciences Inc., San Diego, CA) added to promote Schwann cell mitogenesis and suppress fibroblast growth. After 1 week, cells were trypsinized and replated at 10^6 cells/plate (passage 1). Cultures were stained with an antibody directed against S-100 (Sigma-Aldrich), an acidic, calcium-binding protein present in Schwann cells and not fibroblasts, to verify the purity of Schwann cell populations.

β-Hexosaminidase Release Assay

To test whether *Nf1*$^{-/-}$ SCCM affects mast cell function, mast cell degranulation was evaluated by the β-hexosaminidase release assay.[20] In brief, BMMCs were sensitized at 1×10^6/ml in the presence or absence of 1:1000 dilution anti-Ack antibody (eBiosciences, San Diego, CA) in complete RPMI 1640 without cytokines along with 1.5 μg/ml anti-dinitrophenyl (DNP) IgE (clone SPE-7, Sigma-Aldrich) for 2 hours at 37°C in 5% CO_2. Cells were

then washed once in Tyrode's buffer (130 mmol/L NaCl, 10 mmol/L HEPES, 1 mmol/L $MgCl_2$, 5 mmol/L KCl, 1.4 mmol/L $CaCl_2$, 5.6 mmol/L glucose, and 0.05% bovine serum albumin, pH 7.4) and resuspended at 2×10^6/ml in Tyrode's buffer. Cells were then stimulated with either wild-type SCCM, *Nf1*$^{-/-}$ SCCM, or DNP-human serum albumin (HSA) (50 ng/ml, Sigma-Aldrich) and Kit-L (10 ng/ml, recombinant murine stem cell factor, PeproTech) for 5 minutes at 37°C. After the cells were spun down, 30 μl of supernatant was transferred to a 96-well flat-bottom plate. Then 30 μl of 1 mmol/L *p*-nitrophenyl-*N*-acetyl–D-glucosamide was added to each supernatant and mixed before incubation for 1 hour at 37°C. The reaction was terminated by the addition of 200 μl of 0.1 M Na_2CO_3-$NaHCO_3$ buffer, and optical density was read on a plate reader at a wavelength of 405 nm.

Western Blot

WT or *Nf1*$^{+/-}$ mast cells were stimulated with Kit-L (10 ng/ml, PeproTech), WT SCCM, or *Nf1*$^{-/-}$ SCCM for 5 minutes. Cells were then washed with cold PBS, and whole cell lysates were prepared by adding 1× cell lysis buffer (10 mmol/L K_2HPO_4, 1 mmol/L EDTA, 5 mmol/L EGTA, 10 mmol/L $MgCl_2$, 1 mmol/L Na_3VO_4, 50 mmol/L sodium β-glycerophosphate, 10 μg of aprotinin/ml, 10 μg leupeptin/ml, and 1 μg of pepstatin A/ml, pH 7.2). Immunoblot analyses were performed with rabbit antibodies for phospho-Akt (Ser-473) (1:1000, New England Biolab, Ipswich, MA) and total Akt (1:2000, New England Biolabs).

Histological and Immunohistological Analysis

To examine mast cell numbers *in vivo*, mast cell frequency in ear and peritoneal lavage was determined. In brief, mice were sacrificed by CO_2 inhalation. Ears were removed, fixed in buffered formalin, and processed in paraffin-embedded sections. Specimens were stained with Alcian Blue to identify mast cells. Mast cell numbers per 1 mm^2 were quantitated in a blinded fashion. Peritoneal lavage was performed as described previously,[21] with 10 ml of peritoneal lavage fluid concentrated by centrifugation and stained with toluidine blue to quantify total number of mast cells per 10 ml of lavage.

In Vivo *Anaphylaxis Assay*

To evaluate mast cell function *in vivo*, we used a previously described anaphylaxis assay.[9] In brief, WT, *Nf1*$^{+/-}$, *p85*$\alpha^{-/-}$, and *Nf1*$^{+/-}$;*p85*$\alpha^{-/-}$ mice received an intradermal injection of 20 μl of 1:44 dilution of stock monoclonal anti-DNP IgE (clone SPE-7, Sigma-Aldrich) and 50 ng of Kit-L in PBS in the right ear and a PBS injection in the left ear. Twenty hours later, they received a tail vein injection of DNP-HSA (300 μl of a 10 mg/ml DNP-HSA [Sigma-Aldrich]), and 1% Evans blue dye. Thirty minutes later, the mice were sacrificed, and tissue samples were acquired and imaged using an Epson Perfection 4990 photo scanner. Dye was extracted from a 5-mm punch biopsy taken from the sensitization site. These samples were treated with 1 N KOH overnight at 37°C. The next day 900 μl of extraction buffer (85% H_3PO_4, acetone, and H_2O) was added to digested ear, followed by sample agitation and centrifugation. Samples were read at 620 nm with a spectrophotometer.

Statistical Analysis

All *P* values were generated using analysis of variance and post-analysis of variance *t*-test.

Results

Nf1$^{-/-}$ *SCCM Induces Elevated Mast Cell Degranulation in* Nf1$^{+/-}$ *Bone Marrow-Derived Mast Cells*

An increasingly recognized paradigm of tumorigenic cells is their ability to co-opt the normal functions of nonmalignant cells by increasing secretion of growth factors. To test whether paracrine factors produced by Schwann cells promote degranulation, *Nf1*$^{-/-}$ SCCM was used to examine the release of β-hexosaminidase, a preformed enzyme in granules of mast cells that is commonly used as a measure of mast cell degranulation. In four independent experiments, an equivalently low level of β-hexosaminidase release was observed in WT and *Nf1*$^{+/-}$ mast cells in response to WT SCCM (Figure 1A). Both WT and *Nf1*$^{+/-}$ mast cells demonstrated a significant increase in degranulation after stimulation with *Nf1*$^{-/-}$ SCCM compared with stimulation with WT SCCM. However, *Nf1*$^{+/-}$ mast cells demonstrated a 33% higher concentration of β-hexosaminidase release compared with WT mast cells.

Neutralizing Antibody to c-Kit or Genetic Disruption of c-Kit Is Sufficient to Inhibit Mast Cell Degranulation

We have previously reported that *Nf1*$^{-/-}$ Schwann cells secrete greater amounts of Kit-L than WT Schwann cells.[22–24] In addition, we have shown that *Nf1*$^{+/-}$ mast cells have increased degranulation in response stimulation with Kit-L in conjunction with allergen-induced cross-linking of FcεRI by DNP-HSA.[25] To determine whether the increased secretion of Kit-L by *Nf1*$^{-/-}$ Schwann cells was responsible for the increase in degranulation of *Nf1*$^{+/-}$ mast cells after *Nf1*$^{-/-}$ SCCM, we added a c-Kit neutralizing antibody (anti-Ack) to the SCCM before evaluation of mast cell degranulation. Mast cells stimulated with Kit-L/DNP alone were used as a positive control. Kit-L induced a significantly increased amount of β-hexosaminidase release in *Nf1*$^{+/-}$ mast cells compared with that for WT mast cells (Figure 1B). Of importance, the addition of the c-Kit neutralizing antibody to *Nf1*$^{-/-}$ SCCM was sufficient to significantly inhibit degranulation of *Nf1*$^{+/-}$ mast cells to basal levels (Figure 1B), implying

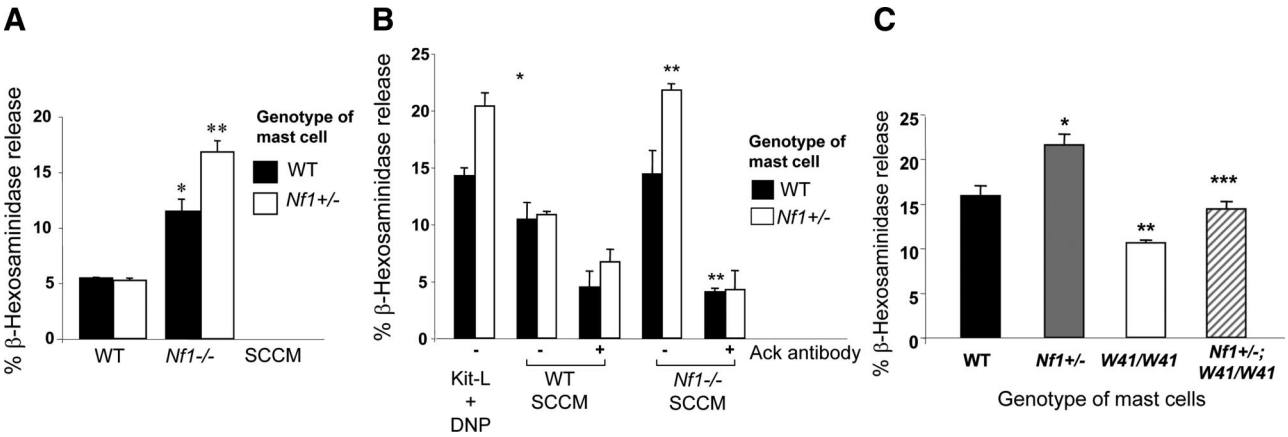

Figure 1. $Nf1^{-/-}$ SCCM significantly promotes $Nf1^{+/-}$ mast cell degranulation. Degranulation of mast cells was assessed by the release of β-hexosaminidase. β-Hexosaminidase activity is measured in the supernatant and the extent of degranulation is reported as a percentage of total cellular β-hexosaminidase activity. **A:** Degranulation of WT and $Nf1^{+/-}$ BMMCs was assessed by the release of β-hexosaminidase after SCCM stimulation. $^{*}P < 0.01$ for WT versus $Nf1^{-/-}$ SCCM. $^{**}P < 0.01$ for $Nf1^{+/-}$ versus WT mast cells stimulated with $Nf1^{-/-}$ SCCM. Data are means ± SEM from triplicate samples in four independent experiments. **B:** β-Hexosaminidase release was measured after stimulation with either Kit-L plus DNP or SCCM (WT or $Nf1^{-/-}$) with or without the addition of Ack. $^{*}P < 0.01$ for WT versus $Nf1^{+/-}$ mast cells stimulated with Kit-L/DNP. $^{**}P < 0.01$ for $Nf1^{+/-}$ versus WT mast cells stimulated with $Nf1^{-/-}$ SCCM and $Nf1^{+/-}$ treated with Ack versus $Nf1^{+/-}$ treated with vehicle. Data are means ± SEM from triplicate samples in four independent experiments. **C:** The $Nf1^{-/-}$ SCCM promotes mast cell degranulation via the c-Kit receptor. Degranulation of BMMCs derived from WT, $Nf1^{+/-}$, *W41/W41*, and $Nf1^{+/-}$;*W41/W41* mice was assessed by the release of β-hexosaminidase after stimulation with $Nf1^{-/-}$ Schwann cell-conditioned medium. $^{*}P < 0.01$ for WT versus $Nf1^{+/-}$ mast cells stimulated with $Nf1^{-/-}$ SCCM. $^{**}P < 0.01$ for WT versus *W41/W41* mast cells stimulated with $Nf1^{-/-}$ SCCM. $^{***}P < 0.01$ for $Nf1^{+/-}$ versus $Nf1^{+/-}$;*W41/W41* mast cells stimulated with $Nf1^{-/-}$ SCCM. Data are means ± SEM from triplicate samples in four independent experiments.

that hypersecretion of Kit-L by $Nf1^{-/-}$ Schwann cells is responsible for the increased degranulation of $Nf1^{+/-}$ mast cells after exposure to $Nf1^{-/-}$ SCCM.

To genetically verify that Kit-L is mediating mast cell degranulation in response to SCCM, $Nf1^{+/-}$ mice were intercrossed with mice containing an inactivating mutation in the c-Kit receptor (*W41/W41*). After generation of mast cells from these intercrossed mice, cells were stimulated with $Nf1^{-/-}$ SCCM, and degranulation was assessed. Consistent with the above data, mast cells from the $Nf1^{+/-}$ mice had significant elevation of degranulation compared with WT mast cells. However, mast cells isolated from mice containing mutations at both the *Nf1* and *W* loci had a reduction in degranulation compared with $Nf1^{+/-}$ mast cells, to a level that was comparable to that of WT mice (Figure 1C), providing genetic confirmation that hyperactivation of the Kit-L/c-Kit pathway in $Nf1^{+/-}$ mast cells mediates the excessive degranulation in response to $Nf1^{-/-}$ SCCM.

Pharmacological Inhibition of the PI3K Signaling Pathway Reduces Kit-L-Mediated Nf1$^{+/-}$ *Mast Cell Gain of Function*

The PI3K pathway is recognized as an important downstream amplifier of both FcεR1 signals and a transducer of c-Kit degranulation signals.[20] To verify that Kit-L stimulation causes hyperactivation of PI3K in $Nf1^{+/-}$ mast cells, AKT phosphorylation at Ser-473 was evaluated as a measure of PI3K activity. As shown in Figure 2A, $Nf1^{+/-}$ mast cells have increased AKT Ser-473 phosphorylation at 5 minutes after Kit-L stimulation compared with WT cells. Furthermore, mast cells treated with the PI3K inhibitor Ly294002 before Kit-L stimulation had reduced AKT Ser-473 phosphorylation. As an initial test to examine whether the c-Kit-mediated increase in degranulation by $Nf1^{+/-}$ mast cells is PI3K-dependent, the cells were incubated with Ly294002 in a similar manner and stimu-

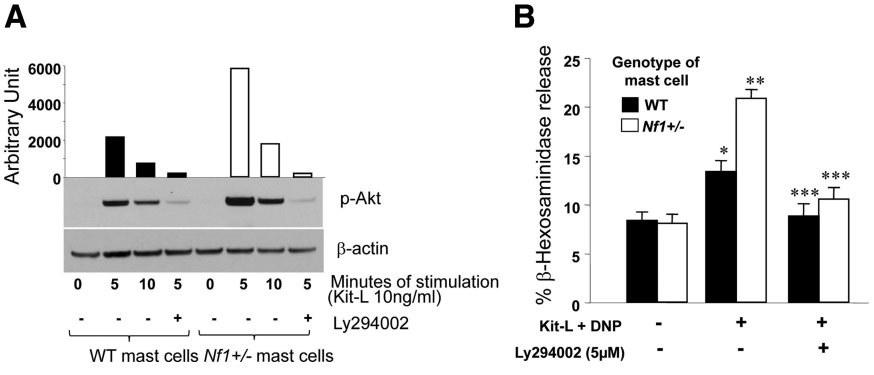

Figure 2. Effect of a pharmacological PI3K inhibitor on $Nf1^{+/-}$ mast cell degranulation. **A:** WT or $Nf1^{+/-}$ mast cells were incubated with or without Ly294002 for the time indicated and then were stimulated with Kit-L. AKT phosphorylation at Ser-473 was examined by Western blot. Data are representative of one of three independent experiments using different primary cell lines. **B:** WT and $Nf1^{+/-}$ mast cells were incubated in the absence or presence of Ly294002 and stimulated with Kit-L/DNP for 15 minutes. β-Hexosaminidase release was examined to determine degranulation. Results are representative of one of four independent experiments performed in triplicate. $^{*}P < 0.01$ for Kit-L-induced WT mast cell degranulation versus basal level. $^{**}P < 0.01$ for $Nf1^{+/-}$ versus WT mast cell degranulation induced by Kit-L. $^{***}P < 0.01$ for WT or $Nf1^{+/-}$ mast cell degranulation mediated by Kit-L with versus without Ly294002 inhibition.

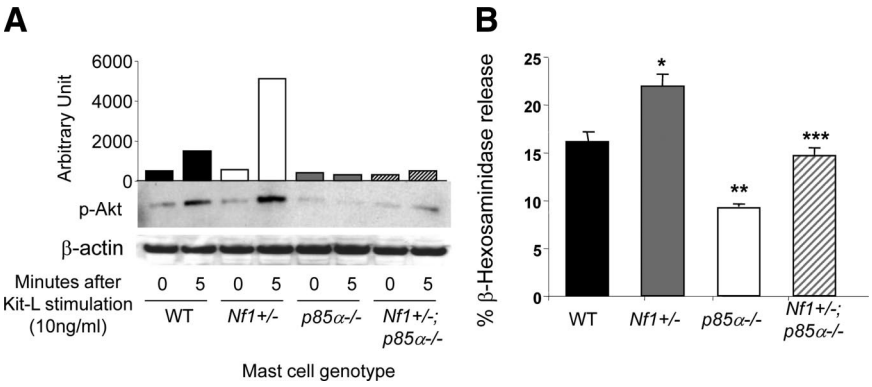

Figure 3. Genetic disruption of *p85α* restores Kit-L-mediated gain of function in $Nf1^{+/-}$ mast cells to WT levels. **A:** Mast cells derived from WT, $Nf1^{+/-}$, $p85\alpha^{-/-}$, and $Nf1^{+/-};p85\alpha^{-/-}$ mice were stimulated with Kit-L, and Akt phosphorylation was examined by Western blot. Data are representative of one of three independent experiments using different primary cell lines. **B:** Mast cells derived from WT, $Nf1^{+/-}$, $p85\alpha^{-/-}$, and $Nf1^{+/-};p85\alpha^{-/-}$ mice were stimulated with Kit-L/DNP for 15 minutes, and mast cell degranulation was examined. Results are representative of one of four independent experiments performed in triplicate. $^{*}P < 0.01$ for $Nf1^{+/-}$ versus WT mast cell degranulation induced by Kit-L. $^{**}P < 0.01$ for $p85\alpha^{-/-}$ versus WT mast cell degranulation induced by Kit-L. $^{***}P < 0.01$ for $Nf1^{+/-}$ versus $Nf1^{+/-};p85\alpha^{-/-}$ mast cell degranulation induced by Kit-L.

lated with Kit-L/DNP to induce degranulation as above. The addition of the PI3K inhibitor was sufficient to produce significant inhibition of degranulation in WT and $Nf1^{+/-}$ mast cells after c-Kit stimulation, demonstrating a crucial role for PI3K in mediating this phenotype (Figure 2B).

Genetic Disruption of PI3K Signaling Pathway Reduces Kit-L-Mediated Gain of Function in Nf1$^{+/-}$ *Mast Cells*

Given the above observations with pharmacological inhibitors, we next sought to independently validate these results using mice containing a disruption in the regulatory subunit of class 1_A PI3K ($p85\alpha^{-/-}$), resulting in the deletion of the p85α subunit but leaving the p55α and p50α isoforms intact,[26] which leads to loss of PI3K signaling. Bone marrow-derived mast cells were generated from the F2 progeny of the intercross of these $p85\alpha^{-/-}$ and $Nf1^{+/-}$ mice, and Kit-L-mediated AKT phosphorylation was measured. Analogous to the data of Figure 2A, abrogation of *p85α* reduces the hyperactivation of AKT at Ser-473 observed in $Nf1^{+/-}$ mast cells to below WT levels (Figure 3A). In a similar manner, genetic disruption of *p85α* in the context of *Nf1* haploinsufficiency significantly decreased degranulation after stimulation with Kit-L/DNP, normalizing β-hexosaminidase release to WT levels (Figure 3B). Taken together, these data supply genetic evidence that PI3K activity is critical in mediating the increase in degranulation of $Nf1^{+/-}$ mast cells in response to Kit-L/DNP.

Genetic Disruption of PI3K Signaling Restored the Gain in Nf1$^{+/-}$ *Mast Cell Functions* in Vivo *to WT Levels*

We have previously demonstrated that $Nf1^{+/-}$ mice have increased numbers of cutaneous mast cells and increased numbers of mast cells in the peritoneal lavage fluid at baseline and in response to Kit-L administration.[20] To determine whether genetic disruption of class1_A PI3K results in corrections of Kit-L-mediated mast cell gains in function *in vivo*, the frequency of mast cells in ear and peritoneal lavage fluid was evaluated using $Nf1^{+/-}$ and $Nf1^{+/-};p85\alpha^{-/-}$ mice.

Representative Alcian Blue-stained histological sections from ears of four genotypes of mice are shown in Figure 4A. Consistent with previous studies, there was a 1.8-fold increase in the number of tissue mast cells in ears from $Nf1^{+/-}$ mice compared with that in WT controls (Figure 4B). A significant reduction in the number of mast cells in the ears of $p85\alpha^{-/-}$ mice was observed compared with that in the ears of WT mice (Figure 4B). Furthermore, loss of PI3K signaling in $Nf1^{+/-};p85\alpha^{-/-}$ mice dramatically reduced the number of mast cells in the ears of these animals (Figure 4B).

Furthermore, a similar result was found in peritoneal lavage as shown in Figure 4, C and D, in which an increased number of mast cells was observed in the peritoneum of $Nf1^{+/-}$ mice compared with that in WT mice, consistent with previous studies.[21] However, we found that loss of *p85α* corrects this phenotype by significantly reducing the number of peritoneal mast cells in $Nf1^{+/-};p85\alpha^{-/-}$ mice (Figure 4D).

To determine whether our *in vitro* degranulation findings are relevant in a more physiological system, we used a previously described passive cutaneous anaphylaxis model[8] to investigate the role of PI3K in regulating Kit-L-dependent mast cell functions. passive cutaneous anaphylaxis produces a profound localized allergic reaction triggered by administration of Kit-L in conjunction with allergen-induced cross-linking of FcεRI. The ears of the mice are first sensitized by intradermal injection of monoclonal anti-DNP IgE. Twenty hours after cutaneous sensitization, degranulation was induced by systemic injection of Kit-L and DNP with Evans blue dye. After 20 minutes, the degranulation response was quantified by measuring extravasation of Evans blue dye into the tissue. This extravasation process is reflective of increased local vascular permeability, a process dependent on mast cell release of histamine and serotonin after degranulation. Representative photographs from treated and untreated ears 20 minutes after stimulation are shown in Figure 4E to illustrate the extravasation of Evans blue caused by Kit-L and DNP. A 1.5-fold increase in extravasation was observed in the ears of $Nf1^{+/-}$ mice after Kit-L/DNP treatment compared with that in WT mice (Fig-

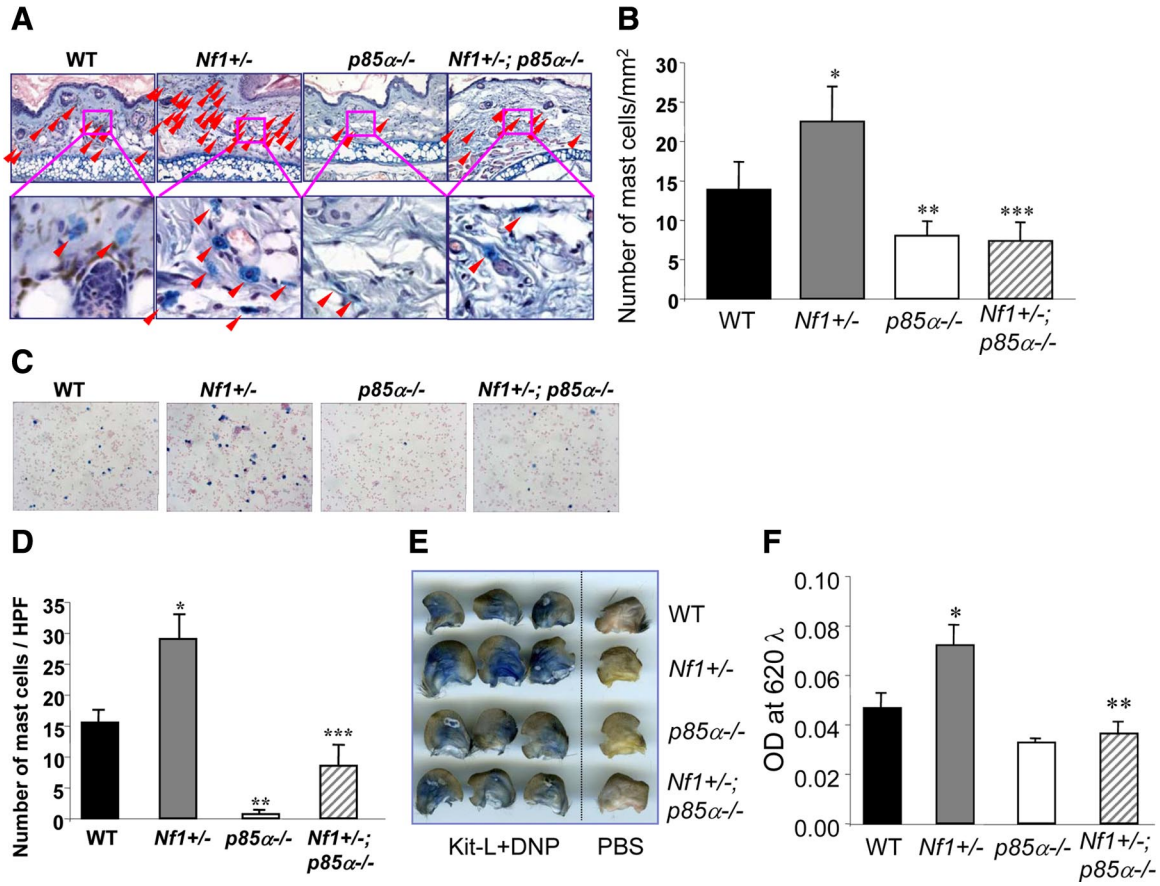

Figure 4. Effect of genetic inactivation of *p85α* on mast cell numbers *in vivo*. **A** and **B:** Ear sections were stained with Alcian Blue, and mast cells were quantitated in a blinded fashion by counting 1-mm^2 sections. Mast cells are identified by **arrowheads**. $^*P < 0.01$ comparing $Nf1^{+/-}$ versus WT mice. $^{**}P < 0.01$ for $p85\alpha^{-/-}$ versus WT mice. $^{***}P < 0.01$ for $Nf1^{+/-}$ versus $Nf1^{+/-}$;$p85\alpha^{-/-}$ mice. **C** and **D:** Representative cytospins from peritoneal lavages stained for mast cells from individual mice of the four *Nf1* and *p85α* genotypes. Peritoneal cells were stained with toluidine blue to quantify the total number of mast cells per peritoneal lavage. $^*P < 0.01$ for $Nf1^{+/-}$ versus WT mice. $^{**}P < 0.01$ for $p85\alpha^{-/-}$ versus WT mice. $^{***}P < 0.01$ for $Nf1^{+/-}$ versus $Nf1^{+/-}$;$p85\alpha^{-/-}$ mice. **E** and **F:** Genetic disruption of *p85α* diminishes PCA *in vivo*. WT, $Nf1^{+/-}$, $p85\alpha^{-/-}$, and $Nf1^{+/-}$:$p85\alpha^{-/-}$ mice were sensitized by intradermal injection of anti-DNP IgE (1:44 dilution, 1 μg/ml) into the right ear (20 μl/injection) and PBS (20 μl/injection) into the left ear. After 20 hours, mice were challenged by intravenous injection of antigen (DNP-HSA) and Kit-L along with Evans blue injection. Photographs of representative IgE-primed (left) and control (right) ears 20 minutes after antigen/Kit-L challenge are shown qualitatively. From each ear, Evans blue was extracted, and the intensity of the dye was measured by absorption at 620 nm. $^*P < 0.01$ comparing $Nf1^{+/-}$ versus WT mice. $^{**}P < 0.01$ for $Nf1^{+/-}$ versus $Nf1^{+/-}$;$p85\alpha^{-/-}$ mice.

ure 4F), indicating increased mast cell degranulation in these animals. Of importance, $Nf1^{+/-}$;$p85\alpha^{-/-}$ mice had significant reductions in Evans blue extravasation compared with $Nf1^{+/-}$ mice, providing *in vivo* support to the hypothesis that Kit-L-mediated hyperactivation of PI3K has a key role in modulating the excessive degranulation in $Nf1^{+/-}$ mast cells.

Discussion

Degranulating mast cells are found at a 10-fold increase in concentration in neurofibromas compared with adjacent areas of unaffected skin[27–29] and are found in close association with Schwann cells, fibroblasts, and blood vessels. The physical proximity of mast cells with these tumor components, together with observations that many factors found in mast cell granules are angiogenic or can alter the extracellular matrix, has led to the hypothesis that mast cell degranulation may promote tumor progression.[30–32] This hypothesis is supported by reports that describe neurofibromas as being highly neoangiogenic with a large amount of extracellular matrix.[9]

Previously, we have demonstrated that $Nf1^{-/-}$ Schwann cells secrete increased amounts of proteins that have been linked to the elevated level of $Nf1^{+/-}$ mast cell degranulation compared with WT Schwann cells, including Kit-L.[9,33] Further, both primary murine $Nf1^{-/-}$ Schwann cells and Schwann cell lines from patients with NF1 secrete high concentrations of Kit-L.[8] Despite the diversity of proteins secreted by $Nf1^{-/-}$ Schwann cells, we provide here genetic and pharmacological lines of evidence to demonstrate that Kit-L is the predominant growth factor in these conditioned media that promotes degranulation of $Nf1^{+/-}$ mast cells. These *in vitro* studies are intriguing, given previous studies demonstrating that Kit-L transcripts are increased in neurofibromas[34] and Kit-L is found in increased concentrations in serum from patients with NF1 9.

Having identified Kit-L as the major paracrine mediator of mast cell degranulation secreted by $Nf1^{-/-}$ Schwann

cells, we next designed experiments to identify the signaling networks downstream of c-Kit responsible for increased degranulation of *Nf1*$^{+/-}$ mast cells. An important priority in studying *Nf1*$^{+/-}$ cells is the identification of specific Ras effector pathways, which are responsible for functional aberrations. Although we have previously shown that multiple Ras effector pathways are altered in *Nf1*$^{+/-}$ mast cells in response to Kit-L,[9,35,36] in this report we provide biochemical, pharmacological, and genetic evidence that c-Kit-mediated hyperactivation of the class 1_A PI3K pathway is specifically responsible for the increased degranulation of *Nf1*$^{+/-}$ mast cells. We show that disruption of the p85 subunit of PI3K results in corrections in mast cell accumulation in tissue and used the passive cutaneous anaphylaxis assay as a model of *in vivo* degranulation to validate the fact that the c-Kit/PI3K pathway regulates this phenotype. This is an important observation because we have previously shown that increased activation of this signaling pathway is also responsible for the increased proliferation and survival of *Nf1*$^{+/-}$ mast cells *in vivo*.[35] Thus, the recruitment, expansion, and now degranulation of mast cells within neurofibromas seem to be mediated via a common cytokine (Kit-L) and a specific Ras effector (PI3K).

In previous studies we found that *Nf1*$^{+/-}$ mast cell progenitors have increased survival, proliferation, and migration in response to recombinant Kit-L.[7] We have recently published studies demonstrating that *Nf1*$^{+/-}$- and c-Kit-dependent bone marrow is necessary for formation and progression of plexiform neurofibromas in the context of *Nf1*$^{-/-}$ Schwann cells, which have previously been shown to be the tumorigenic cells. *Nf1*$^{-/-}$ Schwann cells do not form tumors in the context of WT bone marrow or mast cell-depleted *Nf1*$^{+/-;Wv/Wv}$ bone marrow, but do form tumors in the context of heterozygous *Nf1* bone marrow, highlighting the contribution of mast cells in tumor formation. Further, we have demonstrated that treatment with imatinib mesylate, a known inhibitor of Kit-L/c-Kit signaling, reduced the size and metabolic activity of tumors in an *in vivo* murine model. In the current study, we have identified increased mast cell degranulation resulting from c-Kit-PI3K hyperactivation as a potential mechanism by which imatinib mesylate exerts its tumor-suppressive activity. The novel observation described here that the increased release of tumor-modulating mediators is due to c-Kit-PI3K hyperactivation provides additional rationale for focusing on this pathway as a target for other rational therapeutic agents. Collectively, these studies show that the increase in *Nf1*$^{+/-}$ mast cell degranulation in response to Kit-L is mediated by class 1_A PI3K, and this signaling network is deserving of future studies as a target for neurofibroma therapies. Based on the findings reported here, we are developing murine models using conditional *Nf1* knockout mice with additional disruption at the *p85*α locus to test whether genetic ablation of the c-Kit/PI3K axis is sufficient to prevent the development of plexiform neurofibromas.

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