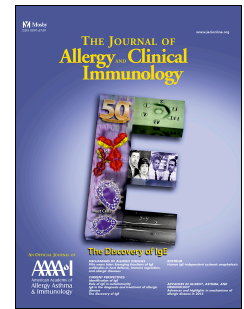


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The transcription factors GATA2 and MITF regulate *Hdc* gene expression in mast cells and are required for IgE/mast cell-mediated anaphylaxis

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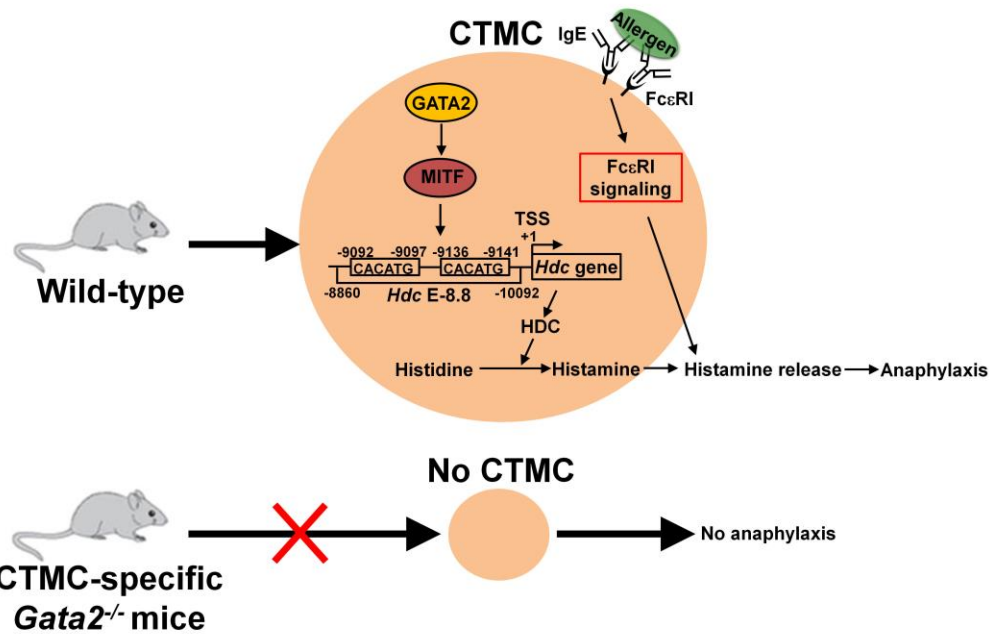
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Transcription factors GATA2 and MITF regulate anaphylaxis



CTMC, Connective tissue mast cell.
 GATA2, GATA binding protein 2.
 FcεRI, high-affinity receptor for the Fc region of IgE.
 HDC, Histidine decarboxylase.
 IgE, Immunoglobulin E.
Hdc E, *Hdc* enhancer.
 MITF, Microphthalmia-associated transcription factor.
 TSS, Transcription start site.

The transcription factors GATA2 and MITF regulate *Hdc* gene expression in mast cells and are required for IgE/mast cell-mediated anaphylaxis

Running title: Transcription factors GATA2 and MITF regulate anaphylaxis

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Background: Histamine is a critical mediator of IgE/mast cell-mediated anaphylaxis. Histamine is synthesized by decarboxylating the amino acid histidine, a reaction catalyzed by the Hdc-gene encoded enzyme histidine decarboxylase. However, regulation of the Hdc gene in mast cells is poorly understood.

Objective: We sought to investigate the in vivo regulation of IgE/mast cell mediated anaphylaxis by transcription factors GATA2 and MITF and the mechanisms by which GATA2 and MITF regulate Hdc gene expression in mouse and human mast cells.

Methods: mice deficient in the transcription factors Gata2, Ahr, Ahrr, or Bhlhe40 were assessed for anaphylactic reactions. Chromatin immunoprecipitation sequencing analysis identified putative Hdc enhancers. Luciferase reporter transcription assay confirmed enhancer activities of putative enhancers in the Hdc gene. The shRNA knock down approach was used to determine the role of MITF in regulating mouse and human HDC gene expression.

Results: Connective tissue mast cell (CTMC)-specific Gata2 deficient mice failed to develop IgE/mast cell-mediated anaphylaxis. GATA2 induced the expression of Mitf, Ahr, Ahrr and Bhlhe40 in mast cells. MITF, but not AHR, AHRR or

57 **BHLHE40, was required for anaphylaxis. MITF bound to an enhancer located**
58 **8.8 kb upstream of the transcription start site of the Hdc gene and directed**
59 **enhancer activity. MITF overexpression largely restored Hdc gene expression in**
60 **the Gata2 deficient-mast cells. In human mast cell line LAD2, MITF was**
61 **required for the HDC gene expression and histamine synthesis.**
62
63 **Conclusion: The transcription factors GATA2 and MITF regulate Hdc gene**
64 **expression in mast cells and are required for IgE/mast cell-mediated anaphylaxis.**

65 **Key messages:**

- 66 ● GATA2 is required for connective tissue mast cell differentiation
- 67 ● GATA2 and MITF regulate IgE-mediated anaphylaxis in vivo
- 68 ● MITF regulates *Hdc* gene expression by directly activating an *Hdc* enhancer

69

70 **Capsule summary**

71 Our study demonstrates that the transcription factors GATA2 and MITF play an

72 essential role in IgE/mast cell-mediated anaphylaxis mainly by regulating *Hdc* gene in

73 mast cells.

74 **Key words:** *mast cell, CTMC, GATA2, MITF, Hdc, enhancer, anaphylaxis, histamine*

75 *synthesis*

76 **Abbreviations used:**

- 77 4HT: 4 hydroxytamoxifen;
- 78 AHR: Aryl hydrocarbon receptor;
- 79 AHRR: Aryl-hydrocarbon receptor repressor;
- 80 BHLHE40: Basic helix-loop-helix family member E40;
- 81 BMMCs: Bone marrow-derived mast cells;
- 82 ChIP-seq: Chromatin immunoprecipitation sequencing;
- 83 CTMC: connective tissue mast cell;
- 84 FACS: Fluorescence activated cell sorting;
- 85 GATA2: GATA binding protein 2;
- 86 H3K4me1: Monomethylation of lysine residue 4 on histone 3;
- 87 H3K27Ac: Acetylation of lysine residue 27 on histone 3;
- 88 HDC: Histidine decarboxylase;
- 89 MMCP1: Mouse mast cell protease 1;
- 90 MITF: Microphthalmia-associated transcription factor
- 91 PCA: Passive cutaneous anaphylaxis;
- 92 PCMCs: Peritoneal Cavity Mast Cells
- 93 pre-BMPs: Pre-basophil and mast cell progenitors;
- 94 PSA: Passive systemic anaphylaxis;
- 95 TNP: 2, 4, 6-Trinitrophenyl;
- 96 TSS: Transcription start site;

97 YFP: Yellow fluorescent protein.

98 INTRODUCTION

99 Anaphylaxis is a serious allergic reaction that is rapid in onset and includes
100 signs and symptoms that involve the skin, gastrointestinal track, respiratory system
101 and cardiovascular system¹. The most severe form of anaphylaxis is anaphylactic
102 shock, which is characterized by hypotension and can cause death¹. Anaphylaxis can
103 be caused by allergies to foods, insect venoms, medications and other agents¹. The
104 incidence of food-induced anaphylaxis has risen at an alarming rate, especially in
105 children, in developed countries during the past several decades and continues to rise²⁻
106 ⁴. Understanding the molecular regulation of anaphylactic shock is an important step
107 in developing effective prevention and treatment.

108 Mast cells are mononuclear protease granule-containing cells that display FcεRI,
109 the high affinity receptor for IgE, on the cell surface. FcεRI is a heterotetramer
110 composed of one IgE-binding α subunit, one membrane-tetraspanning β subunit and a
111 dimer of disulfide-linked γ subunits. Mast cells can be activated by antigen (Ag)
112 crosslinking of specific IgE that is associated with FcεRI. Mast cell activation by
113 IgE/FcεRI crosslinking induces degranulation, with release of inflammatory
114 mediators including histamine, and secretion of both pre-formed and newly
115 synthesized cytokines. While cytokines induce allergic inflammation, histamine has a
116 major role in causing IgE-mediated anaphylactic shock⁵⁻⁷. Mice genetically deficient
117 in mast cells or depleted of mast cells by treatment with anti-stem cell factor antibody
118 are unable to develop IgE-mediated anaphylactic shock^{6,7}. Amelioration of peanut

allergy by treatment with the anti-IgE monoclonal antibody omalizumab supports the importance of IgE/mast cells in mediating human anaphylactic shock⁸.

Histamine is produced by the decarboxylation of the amino acid histidine, a reaction catalyzed by enzyme histidine decarboxylase^{9, 10}. The *Hdc* gene encodes histidine decarboxylase, the rate-limiting enzyme that is essential for mouse and human histamine synthesis^{9, 11, 12}. Mice deficient in the *Hdc* gene fail to synthesize histamine and have reduced or absent IgE-mediated anaphylactic responses¹³⁻¹⁶. There is only limited knowledge of how *Hdc* gene expression is regulated. The transcription factor SP1 binds to a GC box found in both the human and mouse *Hdc* gene promoters^{17, 18}. Several promoter elements that negatively regulate *Hdc* gene transcription have been reported. For example, YY1 and KLF4 negatively regulate the *Hdc* gene by suppressing SP1 in a gastric cancer cell line^{19, 20}.

GATA binding protein 2 (GATA2) is a member of the GATA family of transcription factors. GATA2 has been shown to be critical for survival and proliferation of hematopoietic stem cells^{21, 22}, granulocyte-monocyte progenitor differentiation²³, and basophil and mast cell differentiation^{24, 25}. Recently, we demonstrated that GATA2 plays a critical role in regulating *Hdc* gene expression in mast cells²⁶. However, the in vivo role of GATA2 in regulating IgE/mast cell-mediated anaphylaxis is not clear. Microphthalmia-associated transcription factor (MITF) plays a critical role in mast cell differentiation. A MITF null mutation results in loss of cKIT expression and severely impairs mast cell differentiation in C57BL/6

140 mice^{27, 28}, although this mutation does not affect mast cell differentiation in WB mice
141 because of overproduction of the cKIT ligand stem cell factor in this strain²⁹. We
142 reported that MITF is sufficient to induce the differentiation of common
143 basophil/mast cell progenitor pre-BMPs into mast cells and is required to maintain
144 mast cell identity³⁰. However, it is unknown whether MITF regulates the *Hdc* gene in
145 either mouse or human mast cells. Furthermore, the mechanisms by which GATA2
146 and MITF regulate the *Hdc* gene have not been investigated.

147 In this study, we have investigated the in vivo roles of GATA2 and MITF in
148 IgE/mast cell-mediated anaphylaxis. We determined that GATA2 is required for
149 CTMC differentiation and for maintaining the expression of genes required for
150 anaphylaxis in vivo. MITF, but not other GATA2-dependent transcription factors, is
151 critical for regulating IgE/mast cell-mediated anaphylaxis. We observed that MITF
152 binds the -8.8 *Hdc* enhancer and is important for activities of its enhancer. Our results
153 indicate that the transcription factors GATA2 and MITF control IgE/mast cell-
154 mediated anaphylaxis mainly by regulating *Hdc* gene expression in mast cells.

155 **METHODS**

156 **Mice**

157 Mouse information is described in detail in the METHODS section of this article's
158 Online Repository at www.jacionline.org. All animal experiments were conducted
159 according to protocols approved by the National Jewish Health Institutional Animal
160 Care and Use Committee (Denver, CO).

161

162 **FACS analysis and sorting**

163 Cells prepared from various tissues were stained with fluorochrome-labeled
164 antibodies. Stained cells were acquired by CyAN (DakoCytomation, Glostrup,
165 Denmark) and analyzed using FlowJo software (Tree Star, Ashland, OR). Cell sorting
166 was carried out using a Moflo cytometer (DakoCytomation). A more detailed
167 description of cell surface markers used is included in the METHODS section in this
168 article's Online Repository at www.jacionline.org.

169

170 **Quantitative RT-PCR (qPCR)**

171 qPCR measurements were performed as described in detail under METHODS of the
172 Online Repository. Primer sequences are listed in Table E1 of the Online Repository.
173 qPCR analysis was carried out as described in detail in the METHODS section in this
174 article's Online Repository at www.jacionline.org.

175

176 **Passive cutaneous anaphylaxis (PCA)**

177 Mice were sensitized with IgE anti-2, 4, 6-Trinitrophenyl (TNP) antibody and
 178 challenged with TNP-BSA (300µg /300 µl PBS) and 0.5% Evans blue dye via
 179 intravenous injection. Thirty minutes after challenge, the ears were collected and
 180 incubated in dimethylformamide for 48 hours, after which the absorbance of the
 181 dimethylformamide at 620 nm (OD620 nm) was measured. See the METHODS in
 182 this article's Online Repository at www.jacionline.org for further details.

183

184 **Passive systemic anaphylaxis (PSA)**

185 Mice were sensitized with 10 µg of IgE anti-TNP antibody and challenged with 10 µg
 186 of TNP-BSA. Rectal temperatures of the challenged mice were measured before
 187 challenge (0 min) and at 5, 10, 15, 20, 25, 30, 40, 60, 80, 100 and 120 minutes after
 188 challenge. See the METHODS in this article's Online Repository at
 189 www.jacionline.org for further details.

190

191 **Chromatin Immunoprecipitation Sequencing (ChIP-seq)**

192 DNA fragments associated with histone H3 lysine 4 monomethylation (H3K4me1) or
 193 H3 lysine 27 acetylation (H3K27ac) were immunoprecipitated using respective
 194 specific antibodies obtained from Abcam (Cambridge, MA). The immunoprecipitated
 195 DNA fragments were repaired, ligated to adapters, and size selected to libraries,
 196 which were sequenced as reported previously³¹. Reads were aligned to the mouse

reference genome and peaks were called comparing an immunoprecipitated sample to its corresponding input sample. ChIP-seq data were visualized using the Integrative Genomics Viewer (IGV)^{32,33}. Data may be accessed at the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>, GSE97253). See the METHODS in this article's Online Repository at www.jacionline.org for further details.

Statistical analysis

All the error bars in this report represent standard error of mean (SEM). For ELISA, qPCR, and luciferase analyses, mean $\pm$ SEM were derived from data pooled from two or three independent experiments. Results from each independent experiment are detailed in supplemental figures in this article's Online Repository at www.jacionline.org. Differences between paired samples were analyzed using Student's t test. The changes at various time points before and after challenge in PSA analyses were analyzed using a two-way repeated measure ANOVA.

Other methods including cell culture, histology, ELISA measurements, plasmid construction, adoptive transfer of BMMCs, luciferase reporter assay, retroviral infection, ChIP, and cholera toxin treatment of BMMCs, are included in the METHODS in this article's Online Repository at www.jacionline.org.

217 RESULTS

218 GATA2 is critical for CTMC differentiation and IgE/mast cell-mediated

219 anaphylaxis

220 GATA2 exerts its functions at multiple developmental stages of hematopoiesis.

221 Previously, we reported that *Gata2* mRNA expression is upregulated in common
 222 basophil and mast cell progenitors (pre-BMPs)²⁶. GATA2 is required for the
 223 differentiation of pre-BMPs into mast cells and for maintaining gene expression in
 224 mast cells in vitro²⁶. However, the in vivo roles of GATA2 in mast cell differentiation
 225 and maintenance have not been investigated. To address this, we generated CTMC-
 226 specific *Gata2*-deficient mice by crossing *Gata2*^{f/f} mice to CTMC-specific *Mcpt5*-*Cre*
 227 mice³⁴. *Gata2*^{f/f} *Mcpt5*-*Cre* mice exhibited a nearly complete deficiency of mast cells
 228 in the ear skin, stomach, trachea and peritoneal cavity, while having comparable
 229 number of basophils (Fig 1, A and B) and T cells, B cells, DCs, NK cells, neutrophils
 230 and macrophages (see Fig E1 in this article's Online Repository at
 231 www.jacionline.org). Thus, we refer to *Gata2*^{f/f} *Mcpt5*-*Cre* mice as CTMC-deficient
 232 mice.

233 To analyze whether connective tissue mast cell-deficient mice have compromised
 234 mast cell functions, we performed passive cutaneous anaphylaxis (PCA), an assay that
 235 measures cutaneous mast cell degranulation, as well as passive systemic anaphylaxis
 236 (PSA). We found that CTMC-deficient mice failed to develop PCA and PSA in
 237 response to antigen (Ag) and Ag-specific IgE crosslinking of FcεRI (Fig. 1, C and D).

238 These mice failed to release histamine 5 minutes after antigen challenge (5% of
 239 controls) (Fig 1, *E*). MMCP-1 is a protease predominantly expressed by mucosal mast
 240 cells³⁵. To determine whether CTMC-deficient mice retain the ability to produce
 241 MMCP-1, we examined MMCP-1 levels in the serum of the sensitized and challenged
 242 mice and found that CTMC-deficient mice produced 47% less MMCP-1 than WT
 243 controls (Fig 1, *F*), suggesting that approximately half of mucosal mast cells in
 244 *Gata2*^{f/f} *Mcpt5-Cre* mice are also impaired by the absence of the *Gata2* gene. These
 245 data demonstrate that GATA2 is critical for CTMC differentiation and function in
 246 vivo and suggest that GATA2 also contributes to the differentiation and function of
 247 mucosal mast cells that express MMCP1.

248 To distinguish the role of GATA2 in maintaining the expression of genes that are
 249 required for mediating anaphylaxis from its role in connective tissue mast cell
 250 differentiation, we acutely deleted the *Gata2* gene in differentiated Bone Marrow-
 251 derived Mast Cells (BMMCs) and tested whether *Gata2*^{-/-} BMMCs restore the ability
 252 of CTMC-deficient mice to develop anaphylactic reactions. To prepare *Gata2*^{-/-}
 253 BMMCs, we treated BMMCs cultured from conditional *Gata2* knockout
 254 (*Gata2*^{f/f} *Rosa*^{Yfp/Yfp} *TgCreErt2*^{hemi} mice) or littermate control mice
 255 (*Gata2*^{+/+} *Rosa*^{Yfp/Yfp} *TgCreErt2*^{hemi} mice) with 4 hydroxytamoxifen (4HT) for 11 days
 256 to delete the *Gata2* gene as described previously²⁶. YFP⁺ *Gata2*^{-/-} BMMCs and the
 257 control YFP⁺ *Gata2*^{+/+} BMMCs were FACS-sorted and transferred into recipient
 258 CTMC-deficient mice. Our results show that *Gata2*^{-/-} BMMCs failed to restore IgE-

259 mediated anaphylaxis in CTMC-deficient mice, whereas *Gata2*^{+/+} BMMCs
 260 completely restored the ability of CTMC-deficient mice to develop anaphylaxis (Fig
 261 1, G). Our data establish that GATA2 is essential in maintaining the expression of
 262 genes that are required for anaphylaxis.

263
 264 **The transcription factor MITE, but not AHR, AHRR or BHLHE40, regulates**
 265 ***Hdc* gene expression and IgE/mast cell-mediated anaphylaxis**

266 Our previous study showed that GATA2 is critical for *Hdc* gene expression in
 267 mast cells²⁶. However, it remained unclear whether GATA2 directly regulates the *Hdc*
 268 gene or regulates it indirectly by inducing the expression of *Hdc* gene-regulating
 269 transcription factors. Mast cells express higher levels of *Hdc* transcripts³⁶. Therefore,
 270 we reasoned that transcription factors that are highly expressed in mast cells, but not
 271 in basophils, are more likely to play a role in regulating the *Hdc* gene. We analyzed
 272 our published microarray data that profiled gene expression in mast cells versus
 273 basophils³⁰ and identified 15 transcription factor genes that are expressed to a much
 274 greater extent in mast cells than in basophils. These include *Mitf*, *Ahrr*, *Ahr*, *Hic1*,
 275 *Mecom*, *Bhlhe40*, *Batf1*, *Eid2*, *Id2*, *Mdfig*, *Meis1*, *Mzf1*, *Pgr*, *Tal1* and *Zfx3* (Fig. 2,
 276 A). In this study, we selected transcription factor genes *Mitf*, *Ahr*, *Ahrr* and *Bhlhe40*
 277 for further study because MITF has been shown to play a critical role in mast cell
 278 differentiation and because mice deficient in *Ahr*³⁷, *Ahrr* (NIH Knockout Mice
 279 Project) and *Bhlhe40*³⁸ genes are available, which allowed investigation of the role of

these genes in mast cell differentiation by both gene deletion and gene suppression (shRNA) approaches. Using qPCR, we verified that *Mitf*, *Ahr*, *Ahrr* and *Bhlhe40* mRNA were expressed to a much greater extent in mast cells than in basophils [fold difference (mast cells/basophils): *Mitf* 8.8, *Ahr* 33.2, *Ahrr* 17.2 and *Bhlhe40* 19.8] (Fig. 2, B). Furthermore, expression of these transcription factor genes in mast cells is dependent on GATA2 (percentages of reduction in *Gata2*^{-/-} BMMCs: *Ahr* 79.9%, *Ahrr* 94.5%, *Bhlhe40* 86.8%, and *Mitf* 60.8%) (Fig 2, C).

Mitf null mutant mice on a C57Bl/6 background do not develop mature mast cells^{39, 40}. To determine whether the GATA2-dependent transcription factors MITF, AHR, AHRR and BHLHE40 regulate the *Hdc* gene, we used a shRNA knockdown approach. We examined *Hdc* mRNA expression in BMMCs with knocked down *Mitf* (sh*Mitf*), *Ahr* (sh*Ahr*), *Ahrr* (sh*Ahrr*), or *Bhlhe40* mRNA expression (sh*Bhlhe40*). We found that *Hdc* mRNA expression was significantly diminished (70% reduction) in BMMCs with knocked down *Mitf* mRNA expression (Fig 3, A), but not in BMMCs with knocked down *Ahr*, *Ahrr* or *Bhlhe40* mRNA (Fig 3, B). BMMCs with knocked down *Mitf* mRNA also synthesized less histamine (68% reduction) (Fig 3, A). BMMCs with knocked down *Mitf* mRNA, but not with control shRNA targeting random sequences or with knocked down *Ahr*, *Ahrr*, or *Bhlhe40* mRNA, failed to restore anaphylaxis in CTMC mice (Fig 3, C).

BMMCs are considered to be immature because they contain low levels of histamine^{41, 42}. To determine whether MITF is also required for *Hdc* mRNA

expression and histamine synthesis in mature mast cells, we knocked down *Mitf* mRNA expression in mature peritoneal cavity mast cells (PCMCs) and found that PCMCs with knocked down *Mitf* mRNA expressed 65% less *Hdc* mRNA-and synthesized 75% less histamine than normal control mast cells (Fig 3, D). Knockdown of *Mitf* mRNA expression did not lead to mast cell death or down regulation of *Fcer1a* mRNA expression (see Fig E5 in this article's Online Repository at www.jacionline.org).

To verify the results obtained using the shRNA knockdown approach, we examined the *Hdc* mRNA in mature PCMCs of *Ahr*^{-/-}, *Ahrr*^{-/-} or *Bhlhe40*^{-/-} mice. Consistent with the results obtained using shRNAs, comparable levels of *Hdc* mRNA expression were observed in the mature PCMCs derived from *Ahr*^{-/-}, *Ahrr*^{-/-} or *Bhlhe40*^{-/-} mice and littermate control mice (see Fig E6 in this article's Online Repository at www.jacionline.org). In agreement with these findings, *Ahr*^{-/-} (see Fig E7A), *Ahrr*^{-/-} (see Fig E8A) and *Bhlhe40*^{-/-} mice (see Fig E9A) had normal numbers of mast cells in the ear skin, stomach, trachea and peritoneal cavity. These mice also developed normal PCA (see Fig E7B, Fig E8B and Fig E9B) and PSA (see Fig E7C, Fig E8C and Fig E9C in this article's Online Repository at www.jacionline.org).

A recent study comparing mouse and human mast cell gene signatures found considerable similarity in gene expression between mast cells from these two species⁴³. This observation prompted us to investigate whether MITF regulates the *Hdc* gene in human mast cells. We knocked down human *MITF* mRNA expression in

322 LAD2 human mast cells, which have high levels of *HDC* mRNA and histamine⁴⁴.

323 LAD2 cells with knocked down *Mitf* mRNA expressed 69% less *HDC* mRNA and

324 synthesized 62% less histamine than negative control LAD2 cells (Fig 3, E).

325 Collectively, these results demonstrate that MITF, but not any of the other GATA2-

326 dependent transcription factors, regulates *Hdc* gene expression and histamine

327 synthesis in both mouse and human mast cells as well as IgE/mast cell-mediated

328 anaphylaxis in mice.

329

330 **Identification of putative *Hdc* enhancers**

331 Although our experiments established roles for MITF in regulating the *Hdc*

332 gene and anaphylaxis, they did not provide evidence that MITF directly regulates the

333 *Hdc* gene. To address this, we studied MITF binding of *Hdc* enhancers. Epigenomic

334 studies demonstrate that monomethylation of lysine 4 on histone 3 (H3K4me1) marks

335 genes that are poised to be transcribed, whereas acetylation of lysine 27 on histone 3

336 (H3K27ac) identifies genes that are actively being transcribed. The combined

337 presence of H3K4me1 and H3K27ac modifications predicts enhancer activity⁴⁵⁻⁴⁹. To

338 detect these marks genome-wide, we performed H3K4me1 and H3K27ac ChIP-seq

339 analysis of BMMCs. We used BMMCs because they recapitulate the *Hdc* gene

340 expression in peritoneal mast cells analyzed directly ex vivo. We found two putative

341 *Hdc* enhancers (defined by both H3K4me1 and H3K27ac histone modifications)

342 located -8.8 kb upstream and +0.3 kb downstream from the transcription start site

(TSS) of the *Hdc* gene (indicated in Fig 4, A by red lines). We also note that there are two H3K4me1 peaks located around the +8.5 kb region. However, H3K27ac peaks in the locations were insignificant based on our peak calling statistical analysis (Fig 4, A). Thus, we did not consider these two H3K4me1 peaks further as putative *Hdc* enhancers.

To determine whether the putative *Hdc* enhancers function in *Hdc* gene transcription, we constructed a luciferase reporter gene in which luciferase expression is under the control of the *Hdc* minimal promoter (-44 to +1 relative to the TSS) and the -8.8 or +0.3 *Hdc* enhancer (a schematic diagram is shown in Fig 4, B upper panel). We found that the -8.8 *Hdc* enhancer dramatically increased the activity of the *Hdc* minimal promoter (13.4-fold increase) (Fig 4, B, lower panel), whereas the +0.3 *Hdc* enhancer failed to show significant enhancer activity.

MITF binds to the -8.8 *Hdc* enhancer and drives its activity

To determine whether MITF binds to the -8.8 *Hdc* enhancer, we aligned our H3K4me1 and H3K27ac ChIP-seq data from this region with the published MITF ChIP-seq data⁵⁰ and found one statistically significant MITF-binding peak in the -8.8 *Hdc* enhancer (Fig 5, A). Our ChIP-qPCR analysis of MITF binding in BMMCs revealed that among five predicted MITF-binding sites located within the -8.8 and +0.3 *Hdc* enhancers (Fig 5, A), MITF binding was only detected at two MITF-binding

sites located in the -8.8 *Hdc* enhancer (e.g., MITF-binding sites 3 and 4, a 6.3-fold enrichment found in the combined binding sites) (Fig 5, *B*).

BMMCs and the CFTL-15 mast cells that we used to analyze *Hdc* enhancer activity are considered to be immature mast cells because they contain low amounts of histamine and express fewer receptors for IgE than mature mast cells⁵¹⁻⁵³. To determine whether the binding of MITF to the -8.8 *Hdc* enhancer increases in mature mast cells, we measured MITF binding both in BMMCs that were induced to mature and in mature PCMCs. BMMCs were induced to mature by cholera toxin treatment as reported⁵⁴. We found that *Hdc* mRNA expression levels and histamine content were enhanced 8.9- and 4.7-fold, respectively, by cholera toxin treatment (Fig 5, *C* and *D*). Concomitantly, the binding of MITF to the -8.8 *Hdc* enhancer also increased to levels similar to those of mature PCMCs [Fold enrichment (anti-MITF antibody/IgG): untreated BMMCs 7.4, cholera toxin-treated BMMCs 11.2 and PCMCs 11.7] (Fig 5, *E*).

To determine whether MITF activates -8.8 *Hdc* enhancer activity, we prepared CFTL-15 mast cells with control or knocked down *Mitf* mRNA, followed by transfection of the -8.8 *Hdc* enhancer luciferase reporter gene construct. The -8.8 *Hdc* enhancer lost ~60% of its activity in the presence of knocked down (by 80%) *Mitf* mRNA (Fig 6, *A*). Conversely, overexpression of MITF in a non-mast cell line greatly increased the activity of the -8.8 *Hdc* enhancer (8.2-fold increase) (Fig 6, *B*). Mutations in MITF-binding sites 3 and 4, but not MITF-binding site 5, abolished the

MITF/-8.8 *Hdc* enhancer-driven luciferase reporter gene activity (percentages of reduction: BS3 Mut, 98%; BS4 Mut, 92% and BS345 Mut, 92%) (Fig 6, C). Taken together, our results demonstrate that MITF directly binds to the -8.8 *Hdc* enhancer in immature as well as mature mast cells and activates the enhancer.

MITF is sufficient to fully restore *Kit* and *Fcer1a* gene expression but only partially restores *Hdc* gene expression

Having established that expression of the *Mitf* gene in mast cells is GATA2-dependent and that MITF directly regulates the *Hdc* gene, we investigated whether MITF is sufficient to upregulate *Hdc* gene expression in the absence of the *Gata2* gene. To prepare *Gata2*^{-/-} BMMCs that overexpress MITF (*Mitf*⁺*Gata2*^{-/-} BMMCs), *Gata2*^{f/f} x *ERT2-Cre* BMMCs were retrovirally transduced with recombinant retrovirus for enforced expression of MITF. Next, *Mitf*⁺*Gata2*^{f/f} BMMCs were acutely depleted of the *Gata2* gene by 4HT treatment for 11 days²⁶. Our results confirmed that overexpression of MITF is sufficient to drive the expression of the *Kit* and *Fcer1a* genes (Fig 7, A), but only partially restored *Hdc* gene expression ~63% relative to the wild-type control (Fig 7, B). In contrast, overexpression of GATA2 completely restored *Hdc* gene expression (146% of wild-type) (Fig 7, B). Together, these results indicate that GATA2 regulates *Hdc* expression though inducing MITF, and that additional GATA-2 dependent mechanisms are required for full expression of the *Hdc* gene in mast cells.

406 DISCUSSION

407

408 Our results demonstrate that a GATA2-MITF regulatory circuit controls
 409 IgE/mast cell-mediated anaphylaxis in mast cells. Previous studies established that
 410 GATA2 plays a crucial role in mast cell development. We and others have
 411 demonstrated that GATA2 is essential for the differentiation of pre-BMPs into mast
 412 cells and is required for maintaining the molecular program that defines mast cells in
 413 vitro^{26, 55}. In this study, we present evidence that GATA2 is required for the
 414 differentiation of CTMCs in vivo. Although CTMC-deficient mice failed to release
 415 histamine in response to Ag and Ag-specific IgE crosslinking of FcεRI, these mice
 416 retained the capacity to secrete mast cell protease MMCP1, albeit in reduced amounts.
 417 Our findings are consistent with evidence that CTMCs play a major role in inducing
 418 anaphylaxis⁵⁶. Taking advantage of the CTMC-deficient mice in adoptive transfer
 419 experiments, we demonstrated that GATA2 was essential for maintaining the ability of
 420 mast cells to mediate anaphylaxis in response to Ag and Ag-specific IgE crosslinking
 421 of FcεRI.

422 Our study sheds light on molecular mechanisms by which transcription factors
 423 regulate IgE/mast cell-mediated anaphylaxis. We and others previously confirmed that
 424 GATA2 is the major transcription factor required for maintaining the expression of
 425 many, if not all, mast cell signature genes^{26, 55}. These results indicated that GATA2
 426 initiates a regulatory hierarchy composed of many transcription factors that

427 synergistically control expression of mast cell-specific genes. Here, we show that
 428 GATA2 induces the expression of the transcription factor-encoding *Mitf*, *Ahr*, *Ahrr*
 429 and *Bhlhe40* genes. We also found that the expression of *Ahr*, *Ahrr* and *Bhlhe40*
 430 depends on MITF (Li et. al. unpublished data), indicating that MITF functions at a
 431 higher level within the hierarchy than AHR, AHRR and BHLHE40. Consistent with
 432 this model, MITF is critically involved in regulating the expression of *Hdc*, as well as
 433 additional genes in mast cells (Li et. al. unpublished data). Our data obtained with
 434 both gene deletion and shRNA approaches demonstrate that AHR, AHRR and
 435 BHLHE40 are not required for *Hdc* gene expression. However, we should interpret
 436 these findings with caution. We do not rule out the possibility that AHR, AHRR or
 437 BHLHE40 redundantly regulate the *Hdc* gene in mast cells.

438 Enhancers and their associated transcription factors are critical regulators of cell-
 439 specific gene expression^{49, 57}. We used active histone marks to localize two potential
 440 *Hdc* gene enhancers. We demonstrate that MITF directly binds and activates the -8.8
 441 kb upstream *Hdc* enhancer. Because transfection efficiency of mature primary mast
 442 cells is inefficient, we transfected CFTL-15 mast cells with the *Mitf* and luciferase
 443 reporter genes to analyze *Hdc* enhancer activity. CFTL-15 mast cells are thought to
 444 resemble immature mast cells because they contain low amounts of histamine and
 445 express fewer receptors for IgE than mature PCMCs⁵⁸. Our results provide evidence
 446 that MITF also regulates the *Hdc* gene expression in mature mast cells. MITF binding
 447 to the -8.8 *Hdc* enhancer concomitantly was increased when BMDCs were induced to

448 mature to cells with higher *Hdc* gene expression. Although the increase in MITF
449 binding was moderate compared with the increase in *Hdc* gene expression as BMMCs
450 matured, we propose that this moderate increase in MITF binding might correlate
451 with greater *Hdc* transcriptional output when the MITF binding is put into context
452 with the *Hdc* promoter and other potential *Hdc* enhancers. Furthermore, we
453 demonstrated that *Mitf* gene expression is required to maintain *Hdc* gene expression in
454 mature mast cells. In contrast to the -8.8 enhancer, the +0.3 downstream *Hdc* enhancer
455 lacks detectable enhancer activity. However, the in vivo importance of this enhancer
456 in *Hdc* gene transcription cannot be ruled out by the luciferase reporter gene
457 transcription assay alone.

458 Although mouse and human mast cells share expression of similar genes
459 programs, direct evidence that MITF plays a critical role in regulating *Hdc* gene
460 expression and histamine synthesis in human mast cells lays a solid foundation for
461 future studies of how the human *HDC* gene are transcriptionally regulated. This will
462 enable a more comprehensive understanding of how histamine synthesis and release
463 are controlled.

464 Although MITF fully restored c-Kit and FcεR1α expression, it only restored *Hdc*
465 gene transcription to an average of 63% of wild-type amounts. This suggests that in
466 addition to inducing MITF expression, GATA2 is directly involved in regulating the
467 *Hdc* gene. Feed-forward loop transcription circuits regulate gene expression in many
468 developmental systems⁵⁹. This regulation involves three classes of factors. First, a

primary transcription factor gene (gene product) regulates the expression of a second transcription factor gene. The primary and secondary transcription factors bind the regulatory region of a third (target) gene and cooperatively regulate its transcription⁵⁹. Our data support GATA2, MITF and the *Hdc* gene form a feed-forward loop. In this mode of action, it is possible that in addition to inducing MITF expression, GATA2 maintains the accessibility of the *Hdc* promoter and/or enhancers, and that GATA2 may function by coordinating interactions between promoter and enhancers modules. It is also possible that GATA2 cooperates with MITF to optimally transcribe the *Hdc* gene. Thus, our findings that GATA2 and MITF regulate IgE/mast cell-mediated anaphylaxis mainly by regulating the *Hdc* enhancer add new knowledge how enhancers and their associated transcription factors regulate anaphylaxis.

Genes are typically regulated by a set of transcription factors that bind together to clustered binding sites within an enhancer region^{49, 60, 61}. One of the best-studied examples is the human *IFNB* enhancer, which is activated by multiple transcription factors in response to intracellular signaling pathways⁶². Our findings confirm a central role for MITF in coordinating a cluster of transcription factor-binding sites within the *Hdc* enhancer to regulate histamine synthesis. Future studies will address how *Hdc* enhancers interact with the *Hdc* promoter, as well as how other transcription factors cooperatively regulate the *Hdc* gene.

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489

490

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492

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504 **Figure Legends**

505

506 **FIG 1.** GATA2 is critical for connective tissue mast cell differentiation and IgE/mast
 507 cell-mediated anaphylaxis. **A**, Flow analysis of peritoneal cavity mast cells (MC) and
 508 bone marrow basophils (Ba). Total number of cells (mean $\pm$ SEM, n=3 mice) (*right*
 509 *panel*). **B**, Toluidine blue staining of mast cells (magnification 3 $\times$ 40; insert, 3 $\times$ 100).
 510 Mast cells are indicated by arrows. Average number of mast cells in ten randomly
 511 selected fields (mean $\pm$ SEM, n=3 mice). **C**, PCA analysis, mean $\pm$ SEM, n = 3 mice;
 512 **D**, PSA analysis, mean $\pm$ SEM, n = 6 mice. **E**, ELISA analysis of histamine, mean $\pm$
 513 SEM, n=3 samples from 3 individual mice. **F**, ELISA analysis of MMCP-1 (mean $\pm$
 514 SEM, n=3 samples from 3 individual mice). **G**, PSA analysis of the *Gata2*^{fl/fl}*Mcpt5*-
 515 *Cre* mice reconstituted BMMCs (mean $\pm$ SEM, n=5 mice). Statistical differences were
 516 analyzed by student's *t* test (**A**, **B**, **C**, **E**, **F**) or by ANOVA (**D**, **G**). *: p<0.05; **:
 517 p<0.01; ***: p<0.001.

518

519 **FIG 2.** Transcription factors AHR, AHRR, BHLHE40, and MITF depend on GATA2
 520 for their expression in mast cells. **A**, Microarray analysis of transcription factor
 521 expression profiles in BMMCs versus basophils. **B**, qPCR analysis of mRNA
 522 expression in BMMCs versus basophils. The numbers indicate the fold of difference
 523 in mRNA expression between basophils and BMMCs (mean $\pm$ SEM, n=3 samples
 524 from 3 individual mice). **C**, qPCR analysis of mRNA expression in *Gata2*^{+/+} or *Gata2*^{-/-}

525 ^{-/-} BMMCs (mean ± SEM, n=3 samples from 3 individual mice).

526

527 **FIG 3.** Transcription factors MITE, but not AHR, AHRR or BHLHE40, regulate *Hdc*
 528 gene expression and IgE/mast cell-mediated anaphylaxis. **A**, qPCR analysis of *Hdc*
 529 mRNA expression in BMMCs with knocked down *Mitf* mRNA (sh*Mitf*) or with
 530 shRNA control (shCTRL) (mean ± SEM, n=3 samples from 3 individual mice).
 531 ELISA analysis of the histamine contents in BMMCs with knocked down *Mitf* mRNA
 532 (sh*Mitf*) or with shRNA control (mean ± SEM, n=3 samples from 3 individual mice)
 533 (*right panel*). **B**, qPCR analysis of *Hdc* mRNA expression in BMMCs with knocked
 534 down *Ahr* mRNA (sh*Ahr*), *Ahrr* mRNA (sh*Ahrr*) and *Bhlhe40* mRNA (sh*Bhlhe40*) or
 535 with shRNA control (shCTRL) (mean ± SEM, n=3 samples from 3 individual mice).
 536 **C**, PSA analysis of *Gata2*^{fl/fl} *Mcpt5-Cre* mice reconstituted with BMMCs with knocked
 537 down *Mitf* mRNA (n=4 mice), *Ahr* mRNA (n=3 mice), *Ahrr* mRNA (n=3 mice),
 538 *Bhlhe40* mRNA (n=3 mice) or with shRNA control (n=4 mice). Statistical differences
 539 in PSA analysis were analyzed by ANOVA. *: p<0.05; **: p<0.01. **D**, qPCR analysis
 540 of *Hdc* gene expression (*left panel*) and ELISA analysis of histamine content (*right*
 541 *panel*) in PCMCs with knocked down *Mitf* mRNA (sh*Mitf*) or with shRNA control
 542 (shCTRL) (mean ± SEM, n=3 samples from 3 individual mice). **E**, qPCR analysis
 543 *HDC* gene expression (*left panel*) and ELISA analysis of the histamine contents (*right*
 544 *panel*) in the human mast cell line LAD2 with knocked down *Mitf* mRNA (sh*Mitf*) or
 545 with shRNA control (shCTRL) (mean ± SEM, n=3 transduced samples).

546

547 **FIG 4.** Identification of putative *Hdc* enhancers. **A**, BMMCs were used for ChIP-seq
 548 (duplicate samples were used for H3K4me1 ChIP-seq and triplicate samples were
 549 used for H3K27ac ChIP-seq). H3K4me1 and H3K27ac ChIP-seq data were analyzed
 550 with the Integrative Genomics Viewer. Red bars indicate putative *Hdc* enhancers that
 551 show significant H3K4me1 and H3K27ac modifications. RPM: reads per million
 552 nucleotides; E: enhancer, +0.3 indicates the distance from the beginning of the
 553 downstream *Hdc* enhancer to the TSS of the *Hdc* gene, and -8.8 indicates the distance
 554 from the beginning of the upstream *Hdc* enhancer to the TSS of the *Hdc* gene. **B**,
 555 Scheme of the luciferase constructions (upper panel) and luciferase reporter analysis
 556 of the *Hdc* enhancer activities in the CFTL-15 mast cell line (lower panel). Error bars
 557 represent mean $\pm$ SEM, n=3 transfectants. *HdcMP*: *Hdc* minimal promoter.

558

559 **FIG 5.** MITF binds to the -8.8 enhancer in immature and mature mast cells. **A**,
 560 Enlarged ChIP-seq peak plots of the *Hdc* E-8.8 region and *Hdc* E+0.3. The published
 561 MITF ChIP-seq data were aligned with our H3K4me1 and H3K27ac ChIP data using
 562 the Integrative Genomics Viewer. The black bar beneath the MITF ChIP-seq peak plot
 563 indicates the MITF-binding peak that is statistically significant. The red bars indicate
 564 the positions of consensus MITF-binding sites (BSs). **B**, ChIP-qPCR analysis of
 565 MITF binding (mean $\pm$ SEM, n=2 samples from 2 independent experiments).
 566 BMMCs, anti-MITF antibody or control IgG was used for immunoprecipitation. **C**,

567 qPCR analysis of the *Hdc* mRNA expression (mean $\pm$ SEM, n=3 samples from 3
 568 individual mice); **D**, ELISA analysis of histamine content (mean $\pm$ SEM, n=3 samples
 569 from 3 individual mice); and **E**, ChIP-qPCR analysis of MITF binding in untreated
 570 BMMCs, cholera toxin treated BMMCs, and PCMCs (mean $\pm$ SEM, n=3 samples
 571 from 3 independent experiments). CT: Cholera Toxin.

572

573 **FIG 6.** MITF drives the -8.8 *Hdc* enhancer activity. **A**, Luciferase reporter gene
 574 analysis of *Hdc* enhancer activities in CFTL-15 cells with knocked down *Mitf* mRNA
 575 or shRNA control (mean $\pm$ SEM, n=2 transfectants). **B**, Luciferase reporter gene
 576 analysis of the *Hdc* enhancer activities in HEK293 cells co-transfected with vector
 577 control or the MITF expression construct (mean $\pm$ SEM, n=2 transfectants). **C**,
 578 Luciferase reporter gene analysis of the activity of *Hdc* enhancers that have a mutated
 579 MITF binding site(s) (mean $\pm$ SEM, n=2 transfectants).

580

581 **FIG 7.** MITF is sufficient to fully restore cKIT and FCεRI expression but can only
 582 partially restore *Hdc* gene expression. **A**, Flow analysis of cKIT and FcεRI expression
 583 in *Gata2*^{-/-} BMMCs overexpressing MITF, GATA2 or control vector (CTRL). YFP⁺
 584 GFP⁺ cell populations are shown. Data represent three independent experiments with
 585 similar results. **B**, qPCR analysis of the *Hdc* mRNA expression in *Gata2*^{-/-} BMMCs
 586 overexpressing MITF, GATA2 or a control vector (CTRL). YFP⁺ GFP⁺ BMMCs were
 587 FACS-sorted for qPCR analysis. Error bars represent mean $\pm$ SEM, n=3 samples from

588 3 independent experiments.

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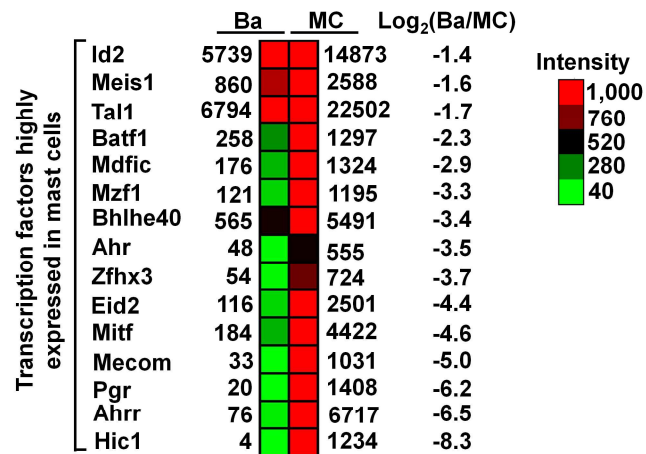
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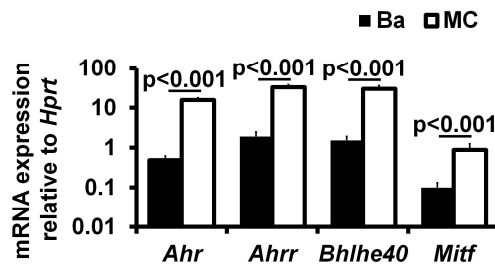
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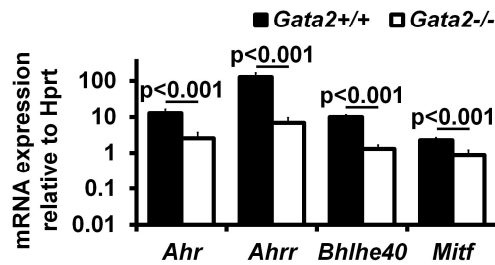
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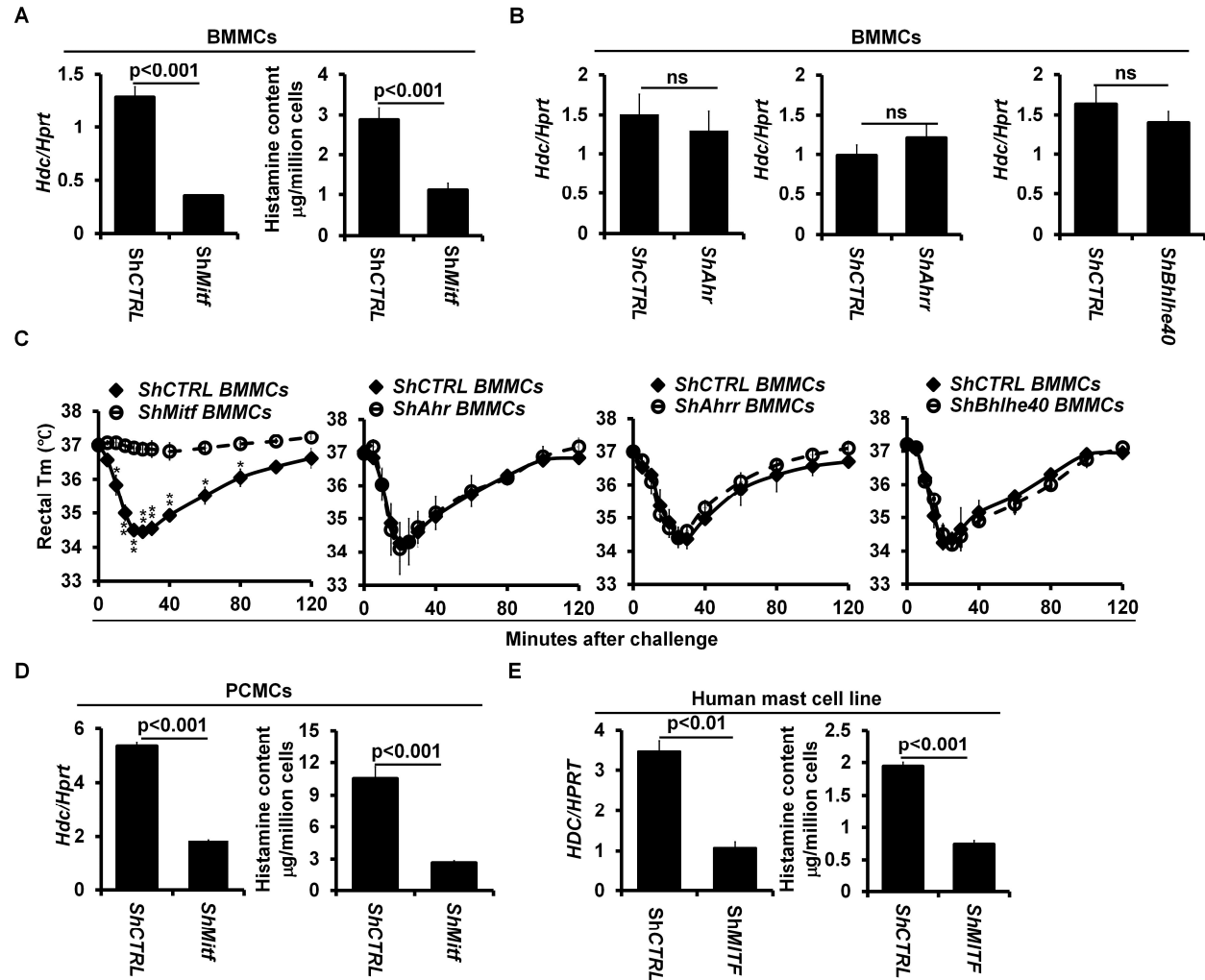


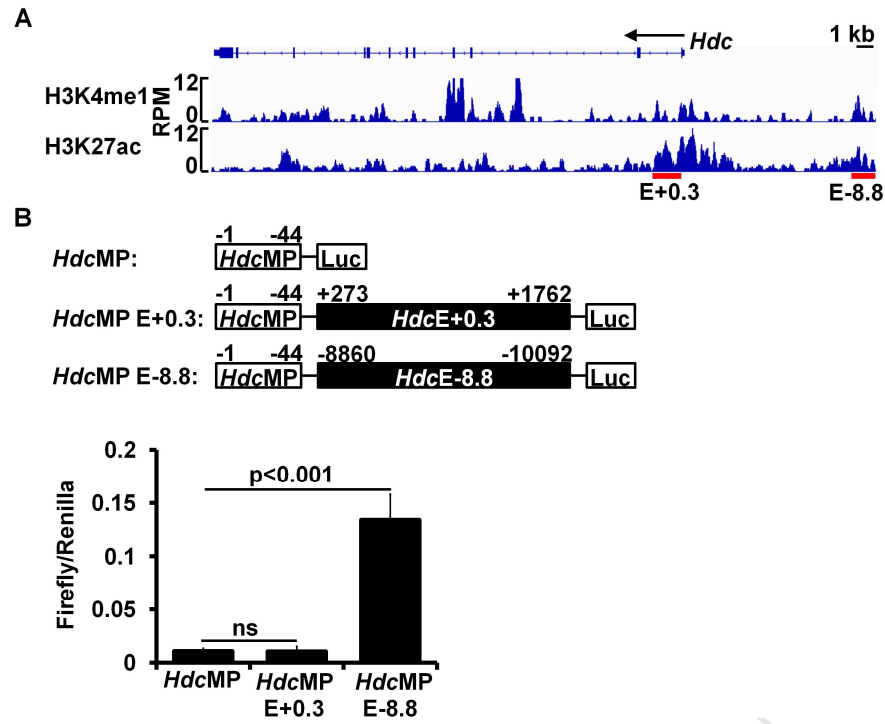
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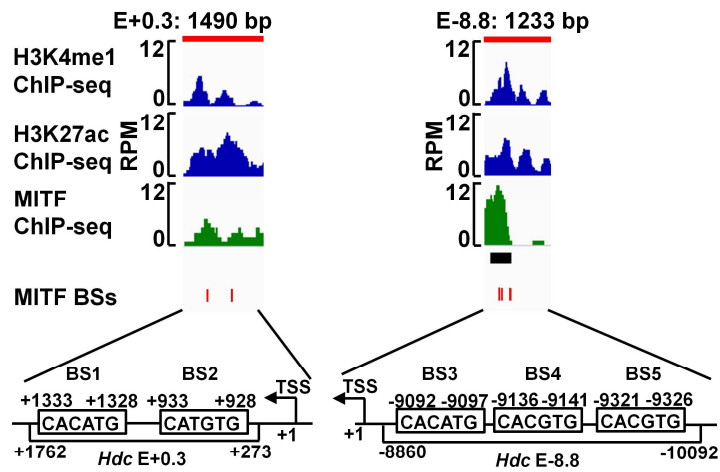
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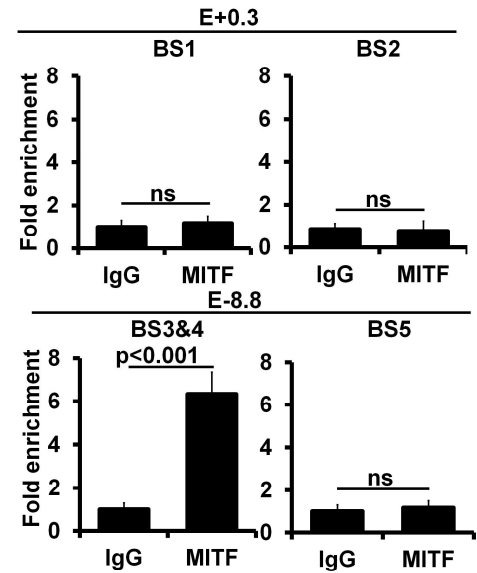




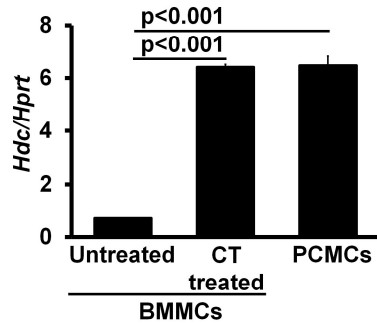
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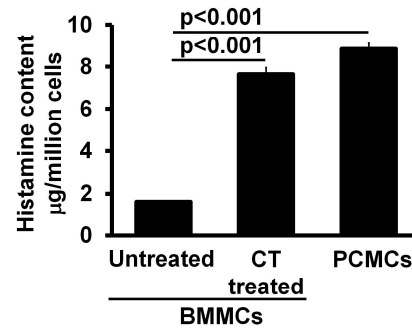
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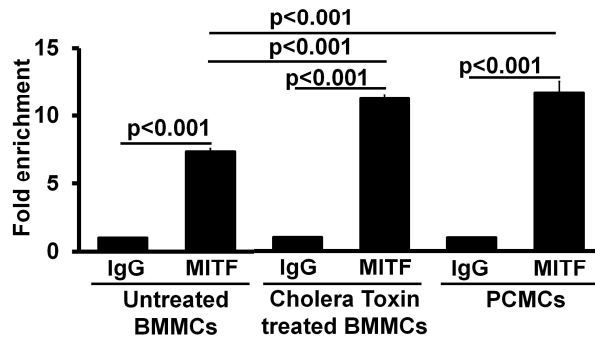
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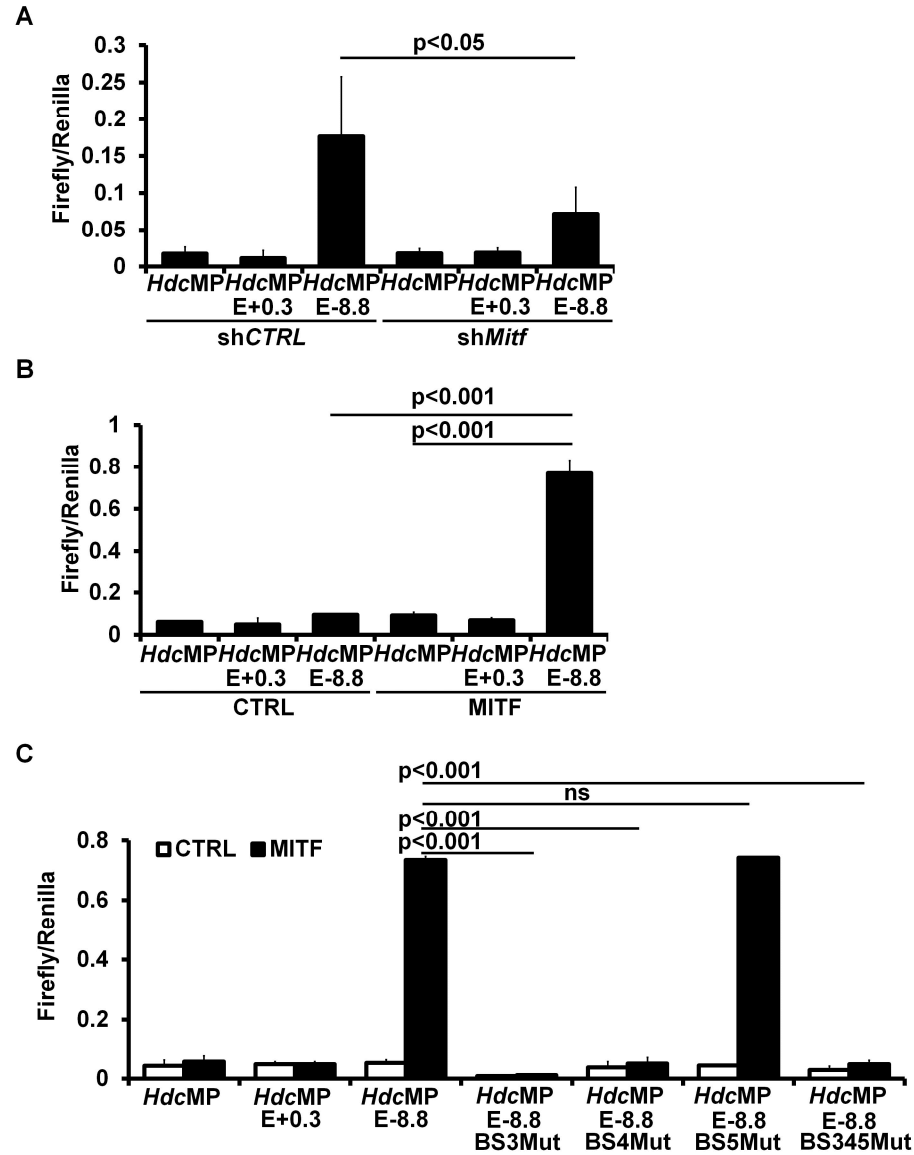


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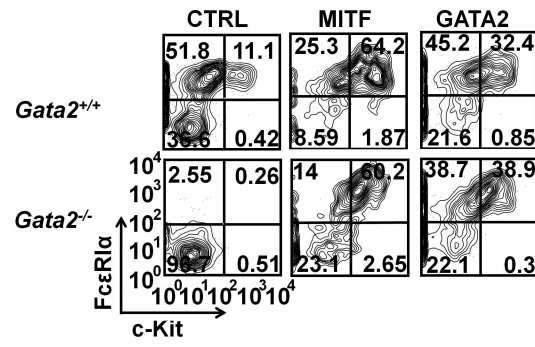


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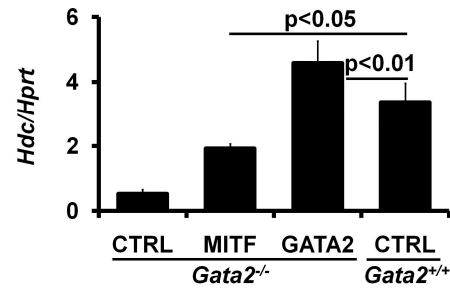




A



B



1 METHODS

2 Mice

3 *Gata2*^{f/f} mice (B6.129-*Gata2*^{tm1Sac/Mmmh})^{E1} were purchased from the Mutant Mouse
 4 Regional Resource Center (the mutant mouse regional resource center at University of
 5 California-Davis, Davis, CA). Inducible *Gata2* knockout mice
 6 (*Gata2*^{f/f} *Rosa*^{Yfp/Yfp} *TgCreErt2*^{hemi} mice) were generated by crossing *Gata2*^{f/f} mice to Cre
 7 activity reporter *Rosa*^{Yfp/Yfp} mice^{E2} [B6.129X1-*Gt(ROSA)26Sor*^{tm1(EYFP)Cos}/J, the Jackson
 8 Lab, Bar Harbor, ME] and inducible Cre mice [B6. Cg-Tg(CAG-cre/*Esr1**)5Amc/J]^{E3}
 9 (the Jackson Lab) as described^{E4}. These mice were backcrossed to the C56BL/6
 10 background for 4 generations. Connective tissue mast cell-specific *Gata2* deficient mice
 11 (*Gata2*^{f/f} *Mcpt5*-Cre mice) were generated by crossing *Gata2*^{f/f} mice to connective tissue
 12 mast cell-specific Cre mice^{E5} [*Mcpt5*-Cre mice, B6-*Tg(Cma1-cre)*/ARoer, provided by Dr.
 13 Axel Roer via Dr. Jim Hagman, Denver, CO]. These mice were backcrossed to the
 14 C56BL/6 background for 5 generations. Mast cell-specific *Ahr* deficient mice
 15 (*Ahr*^{f/f} *Cpa3*-Cre mice) were generated by crossing *Ahr*^{f/f} mice^{E6} (B6-*Ahr*^{tm3.1Bra}/J, the
 16 Jackson Lab) to the mast cell-specific Cre mice^{E7} [*Cpa3*-Cre mice, B6-*Tg(Cpa3-*
 17 *cre)*4Glli/J, the Jackson Lab]. *Ahrr*^{-/-} sperm with the FRT flanked Neo and floxed *Ahrr*
 18 gene (*Ahrr*^{tm1a(KOMP)Wtsi}) were purchased from the NIH knockout mouse project repository
 19 (University of California-Davis, Davis, CA). Our core facility (National Jewish Health,
 20 Denver, CO) performed in vitro fertilization and generated *Ahrr* knockout first mice

(referred to as *Ahrr*^{-/-} mice). *Bhlhe40*^{-/-} mice (B6.129.CD1-*Bhlhe40*^{tm1Rhli}) were generated by Dr. Robert H Lipsky^{E8} and provided by Dr. Mark Mattson of the National Institutes of Health (Bethesda, Maryland). These mice were on a B6.129 mixed genetic background (backcrossed to the C56BL/6 background for one generation). All animal experiments were conducted according to protocols approved by the National Jewish Health Institutional Animal Care and Use Committee.

FACS analysis and sorting

For fluorescence activated cell sorting (FACS) analysis of basophils, mast cells, cells obtained from the bone marrow and peritoneal cavity were stained with the following antibodies: basophils and mast cells were stained with APC-CY7-conjugated anti-c-Kit (2B8) and PE-CY7-conjugated anti-FcεRIα (MAR-1) antibodies; peritoneal mast cells were identified as CD3ε⁻B220⁻ FcεR1α⁺c-Kit⁺; T cells and B cells were stained with PE-CY5-conjugated anti-CD3ε (145-2C11) and PE-CY7-conjugated anti-CD19 (1D3) antibodies, respectively; dendritic cells (DCs) were stained with APC-conjugated anti-MHC Class II (MHC II) (M5/114.15.2) and PE-conjugated anti-CD11c (N418) antibodies; neutrophils and macrophages were stained with PE-conjugated anti-Gr-1 (RB6-8C5) and APC-conjugated anti-CD11b (M1/70) antibodies; NK cells were stained with APC-conjugated anti mouse pan-NK cells (CD49b) (DX5) and PE-conjugated anti-NK1.1 (PK136) antibodies. Dead cells were identified by staining with propidium iodide (PI)

and excluded from all FACS plots. Stained cells were acquired by using CyAN (DakoCytomation, Glostrup, Denmark) and analyzed using FlowJo software (Tree Star, Ashland, OR). The total number of positive cells was calculated by multiplying the total number of cells harvested from the bone marrow or peritoneal cavities by the percentage of positive cells. The YFP⁺ or YFP⁺GFP⁺ BMMCs, and basophils (FcεR1α⁺c-Kit⁻CD49b⁺CD11b⁺) were FACS-sorted using a MoFlo cytometer (DakoCytomation, Glostrup, Denmark). All Abs used for FACS analysis and sorting were purchased from BD Pharmingen (San Diego, CA) or eBioscience (San Diego, CA).

Cell culture

Bone marrow-derived mast cells (BMMCs) were prepared by culturing bone marrow cells from mice in the presence of IL-3 for 4 weeks as described^{E4}. Greater than 99% of BMMCs were mast cells (FcεR1α⁺c-Kit⁺) as determined by FACS analysis. Peritoneal Cavity Mast Cells (PCMCs) were cultured from cells collected from the peritoneal cavity of mice in Iscove's DMEM (10016CV, CorningTM cellgroTM, Manassas, VA) plus 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM beta-mercaptoethanol in the presence of 20 ng/mL IL-3 and 20 ng/mL recombinant murine SCF (250-03, Peprotech, Rocky Hill, NJ). After 4 weeks of culture, over 99% of cells were mast cells (FcεR1α⁺c-Kit⁺). Basophils were prepared as described^{E4}. Briefly, IL-3 (10 µg) and anti-IL-3 antibody (5 µg; MP2-8F8; BD PharMingen) complex (IL-3C) was i.p. injected into

mice 3 days before bone marrow harvest. HEK293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM plus 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin at 5% CO₂ and 37 °C. CFTL-15, a murine IL-3-dependent mast cell line^{E9}, was provided by Dr. Melissa Brown (Northwestern University, Chicago, IL). CFTL-15 cells were cultured in complete RPMI 1640 containing 10% FBS in the presence of 20 ng/ml IL-3^{E10}.

The human mast cell line LAD2 was provided by Dr. Dean Metcalfe (National Institutes of Health, Bethesda, MD)^{E11} and cultured in StemProTM-34 SFM (10639011, Thermo Fisher Scientific, Waltham, MA) plus 2.6% StemPro-34 Nutrient Supplement (GIBCO), 2 mM L-Glutamine (25005CI, CorningTM cellgroTM), 100 units/ml penicillin, and 100 µg/ml streptomycin in the presence of 100 ng/ml recombinant human SCF (300-07, Peprotech)^{E12}.

Histology

Tissues from *Gata2*^{f/f}*Mcpt5*-Cre mice or littermate control *Gata2*^{+/+}*Mcpt5*-Cre mice, *Ahr*^{f/f}*Cpa3*-Cre mice or littermate control *Ahr*^{+/+}*Cpa3*-Cre mice, *Ahr*^{-/-} mice or littermate control *Ahr*^{+/+} mice, *Bhlhe40*^{-/-} mice or littermate control *Bhlhe40*^{+/+} mice, were fixed in 4% paraformaldehyde, cut into 5 µm sections and stained with toluidine blue as described previously^{E4}. Histological images were captured on a Nikon E800 microscope (Nikon, Melville, NY).

81

82 **Passive cutaneous anaphylaxis (PCA)**

83 *Gata2^{f/f}*Mcpt5-Cre mice or littermate control *Gata2^{+/+}*Mcpt5-Cre mice, *Ahr^{f/f}* *Cpa3*-Cre
 84 mice or littermate control *Ahr^{+/+}* *Cpa3*-Cre mice, *Ahr^{r-/-}* mice or littermate control *Ahr^{r+/+}*
 85 mice, *Bhlhe40^{-/-}* mice or littermate control *Bhlhe40^{+/+}* mice, were sensitized by IgE anti-2,
 86 4, 6-Trinitrophenyl (TNP) antibody (100 ng/10 μ l) (IGEL 2a, ATCC, Manassas, VA)^{E13}
 87 or PBS (10 μ l) via intradermal injection. Twenty-four hours later, the sensitized mice
 88 were challenged with TNP-BSA (300 μ g/300 μ l PBS) (T-5050-100, LGC Biosearch
 89 Technologies, Novato, CA) with 0.5% of Evans blue dye via intravenous injection. Thirty
 90 minutes after challenge, ears were collected and incubated in dimethylformamide for 48
 91 hours, which was then measured for absorbance at 620 nm (OD620 nm)^{E14}.

92

93 **Passive systemic anaphylaxis (PSA)**

94 Mice were intraperitoneally sensitized with 10 μ g of IgE anti-TNP antibody (IGEL 2a,
 95 ATCC)^{E13} in 100 μ l PBS. Twenty-four hours later, the sensitized mice were
 96 intraperitoneally challenged with 10 μ g of TNP-BSA in 100 μ l PBS. Rectal temperature
 97 of the challenged mice was measured before challenge (0 min) and at 5, 10, 15, 20, 25,
 98 30, 40, 60, 80, 100 and 120 minutes after challenge as described^{E15}.

99

100 **Histamine measurement**

Plasma was collected 5 minutes after antigen challenge for histamine release measurement. For histamine content measurement, BMMCs, PCMCs, and human mast cell line LAD2 cells were frozen and thawed three times. Histamine in the plasma or cell lysates was measured by using an enzyme immunoassay kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instruction.

Measurement of Mouse Mast Cell Protease 1 (MMCP-1)

Serum was collected 4 hours after antigen challenge. Mouse Mast Cell Protease 1 (MMCP-1) concentrations in the serum were measured with an ELISA kit (Penicuiik, Scotland) according to the manufacturer's instruction.

Lentiviral short hairpin (sh) RNA

shRNA targeting genes of interest (*shMitf*, TRCN0000362536, *shAhr* TRCN0000226005, *shAhrr* TRCN0000445538, and *shBhlhe40* TRCN0000226078) were designed and tested by The RNAi Consortium (TRC) at the Broad Institute and distributed as Mission® shRNA library by Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). ShRNA targeting random sequences was used as a control (*shRNA CTRL*, SHC216-pLKO.5-puro Non-target *shRNA* control). Lentiviral transduction particles were prepared by transfecting HEK293 cells with shRNA plasmid (2 µg) and packaging plasmids (1.5 µg of *pΔ8.9* and 0.5 µg of *pCMV-VSV-G*, Sigma-Aldrich). Lentiviral supernatant was collected and

filtered 48 hours after transfection. Lentivirus was added to BMMCs (1×10^6 cells/ml) or CFTL-15 cells (1×10^6 cells/ml) in a 12-well plate in the presence of polybrene (8 μ g/ml; Millipore, Billerica, MA) and centrifuged at 1,350 g for 90 minutes at room temperature. Transduced cells were then cultured in complete IDMEM (BMMC) or RPMI160 (CFTL-15) in the presence of 20 ng/ml of IL-3 and 2 μ g/ml (BMMC) or 4 μ g/ml (CFTL-15) puromycin (Sigma-Aldrich) for three days. Ten days after puromycin selection, cells were used for experiments.

Adoptive transfer of BMMCs

Gata2^{-/-} and control *Gata2*^{+/+} BMMCs were prepared as described^{E4}. Briefly, *Gata2*^{-/-} BMMCs were cultured from bone marrow of *Gata2*^{f/f}*Rosa*^{Yfp/Yfp}*TgCreErt2*^{hemi} and *Gata2*^{+/+} BMMCs were cultured from control *Gata2*^{+/+}*Rosa*^{Yfp/Yfp}*TgCreErt2*^{hemi} mice for 4 weeks. The cultured BMMCs were treated with 25 nM 4-hydroxytamoxifen (4HT) for 11 days. YFP⁺ BMMCs were FACS-sorted and used as donor BMMCs. Deletion of the floxed *Gata2* gene in the sorted YFP⁺*Gata2*^{f/f}*Rosa*^{Yfp/Yfp}*TgCreErt2*^{hemi} BMMCs was determined to be near 100%. Donor BMMCs with knocked down *Mitf* mRNA, *Ahr* mRNA, *Ahrr* mRNA, *Bhlhe40* mRNA, or with a shRNA control were prepared by using the shRNA approach. BMMCs from bone marrow of *Gata2*^{+/+}*Mcpt5*-Cre mice were transduced with sh*Mitf* lentivirus, sh*Ahr* lentivirus, sh*Ahrr* lentivirus, sh*Bhlhe40* lentivirus, or shRNA control virus. Donor cells (1×10^6 in 200 μ l PBS) were

intraperitoneally injected into *Gata2^{f/f}Mcpt5-Cre* mice. Three days later, the reconstituted mice were analyzed for PSA.

Quantitative PCR (qPCR)

Total RNA from FACS-sorted basophils, cultured BMMCs, PCMCs, or human mast cell line LAD2 cells was isolated using TRIzol® (Ambion Inc, Austin, TX). cDNA was synthesized by reverse transcription. Quantitative PCR (qPCR) was performed in an ABI PRISM 7700 Sequence Detection System. Primer sequences are listed in Table E1.

Relative mRNA amounts were calculated as follows: Relative mRNA amount =

$$2^{[Ct(Sample) - Ct(Hprt)]}.$$

Chromatin immunoprecipitation assay

ChIP analysis of MITF recruitment to the *Hdc* enhancers was performed as described^{E4}.

Anti-MITF antibody (C5) was purchased from Thermo Scientific (Fremont, CA). IgG

control was purchased from Santa Cruz Biotech. Fold enrichment was calculated using

the following equation: Fold Enrichment = $2^{[\Delta Ct(IgG) - \Delta Ct(IP)]}$, where $\Delta Ct(IgG) = Ct(IgG) -$

$[Ct_{(Input)} - \text{Log}_2(\text{Input Dilution Factor})]$, $\Delta Ct(IP) = Ct(IP) - [Ct_{(Input)} - \text{Log}_2(\text{Input Dilution$

Factor)]^{E16}.

Plasmid construction

The *Hdc* minimal promoter (-44 to -1 relative to the transcription start site (TSS) of the *Hdc* gene) was amplified and cloned into a pGL3-basic luciferase vector (Madison, WI) via the BglII and HindIII restriction sites. The -8.8 enhancer (-8860 to -10092 relative to the TSS of the *Hdc* gene) and the +0.3 enhancer (+273 to +1762) were generated by PCR amplification and cloned into the *Hdc* minimal promoter luciferase vector via the KpnI and MluI restriction sites. The MITF binding site mutations were generated by overlapping PCR. The primers used for plasmid constructions are listed in Table E1. All constructed plasmids were verified by sequencing.

Luciferase reporter assay

CFTL-15 cells (5×10^6) were electroporated with 10 μ g of luciferase plasmid and 0.5 μ g of *Renilla* vector at 450 V and 500 microfarads using a Bio-Rad Gene Pulser.

HEK293 cells were transfected with 1 μ g of luciferase plasmid and 0.1 μ g of *Renilla* vector plus MITF expression plasmid or control plasmid using calcium phosphate.

Twenty hours after electroporation or transfection, cells were collected, and luciferase activities were measured by using an InfiniteM1000® microplate reader (Tecan Systems, Inc., San Jose, CA) and the Dual-Luciferase reporter assay system (E1960, Promega).

The transcription activity was normalized as the ratio of luciferase activity divided by *Renilla* activity.

181 **Retroviral infection**

182 Full-lengths *Gata2* and *Mitf* cDNAs were amplified from mouse BMMC mRNA and
 183 cloned into the retroviral expression vector MSCV2.2-Ires-*Gfp* through BglII and NotI
 184 restriction sites. The retroviral particles preparation and infection were conducted as
 185 described previously^{E4}. In brief, bone marrow cells from the control
 186 (*Gata2*^{+/+} *Rosa*^{Yfp/Yfp} *TgCreErt2*^{hemi}, *Gata2*^{+/+}) mice and inducible *Gata2* knockout
 187 (*Gata2*^{f/f} *Rosa*^{Yfp/Yfp} *TgCreErt2*^{hemi}, *Gata2*^{f/f}) mice were spin infected with 1 mL of
 188 recombinant retrovirus containing the *Gata2*, *Mitf*, or *Gfp* (CTRL) gene. Infected cells
 189 were cultured in complete IDMEM in the presence of 20 ng/ml of IL-3 for two days
 190 before they were treated with 25 nM 4HT for 11 days to delete the *Gata2* gene as
 191 described^{E4}. The treated cells were analyzed by FACS analysis or subjected to FACS
 192 sorting. YFP⁺GFP⁺ cells were used for qPCR analysis.

194 **ChIP-seq**

195 DNA fragments associated with H3K4me1 or H3K27ac histone marks were
 196 immunoprecipitated with anti-H3K4me1 antibody (ab8895, Abcam, Cambridge, MA) or
 197 anti-H3K27ac antibody (ab4729, Abcam), respectively. The immunoprecipitated DNA
 198 fragments were repaired, ligated to adapters, and size selected with the Ion Plus Fragment
 199 Library Kit (#4471252, Thermo Fisher Scientific) according to the Ion ChIP-Seq Library
 200 Preparation protocol. Libraries were analyzed for size and quantity using the High

Sensitivity Bioanalyzer Kit (#5067-4626, Agilent Technologies, Santa Clara, CA). The ChIP library was sequenced on an Ion Torrent Proton Semiconductor Sequencer. Reads were aligned to the mouse mm9 reference genome using Torrent Suite v4.4.6 for the H3K4me1 samples and v5.0.4 for the H3K27ac samples using default parameters. Peaks were identified by comparison with matched input DNA using MACS version 2.0.10.20130306 (tag: beta) for H3K4me1 and v2.1.1.20160309 for H3K27ac with a q-value threshold of 0.001 (macs2 callpeak -t .. -c .. -f BAM -g mm -n ... -q 0.001 -B --SPMR)^{E14}. A bedGraph coverage file to display the fold enrichment (FE) of each immunoprecipitated sample with respect to its matched input DNA was created using MACS (macs2 bdgcmp -c ..._control_lambda.bdg -t ..._treat_pileup.bdg -o ..._FE.bedgraph -m FE). ChIP-seq data were displayed using Integrative Genomics Viewer (IGV)^{E17, 18}. The ChIP-seq data have been deposited in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo>, GSE97253). These data will be released to the public upon acceptance of this manuscript.

Cholera toxin treatment of BMMCs

BMMCs were not treated or treated with 0.08 µg/mL cholera toxin (#100B, List Biological Laboratories Inc., Campbell, California) for 48 hours according to the published report^{E19}. Then, the cells were used for qPCR analysis of *Hdc* mRNA expression and ELISA analysis of histamine content.

Figure Legends

FIG E1. Neutrophils (Neu), Macrophages (MΦ), T cells, B cells, Dendritic cells (DCs), and NK cells are not affected in *Gata2^{f/f} Mcpt5-Cre* mice. **A.** Flow analysis of the indicated cell lineages in the bone marrow (Neutrophils and macrophages) and spleen (T cells, B cells, Dendritic cells and NK cells). **B.** Total number of cells (mean ± SEM, n=3 mice). FIG E1 related to FIG 1.

FIG E2. Plasma histamine and serum MMCP-1 levels are decreased in *Gata2^{f/f} Mcpt5-Cre* mice. **A.** ELISA analysis of plasma histamine level (mean ± SEM, triplicates). The left, middle, and right panels show three independent experiments. **B.** ELISA analysis of serum MMCP-1 level (mean ± SEM, triplicates). The left, middle, and right panels show three independent experiments. FIG E2A related to FIG 1E; FIG E2B related to FIG 1F.

FIG E3. Transcription factors AHR, AHRR, BHLHE40 and MITF depend on GATA2 for their expression in mast cells. qPCR analysis of the mRNA expression in BMMCs (MC) versus basophils (Ba) (**A**), and *Gata2^{+/+}* BMMCs versus *Gata2^{-/-}* BMMCs (**B**) (mean ± SEM, triplicates). The left, middle, and right panels each show three independent experiments. FIG E3A related to FIG 2B; FIG E3B related to FIG 2C.

FIG E4. The transfection factor MITF, but not AHR, AHRR or BHLHE40 regulates the

Hdc gene expression. qPCR analysis of the *Hdc* mRNA expression and ELISA analysis of histamine content in BMMCs (**A**), PCMCs (**C**), and human mast cell line LAD2 (**D**) with knocked down *Mitf* mRNA (*ShMitf*) or with shRNA control (*ShCTRL*) (mean $\pm$ SEM, triplicates); **B**. qPCR analysis of the *Hdc* mRNA expression in BMMCs with knocked down *Ahr* mRNA (*ShAhr*), *Ahrr* mRNA (*ShAhrr*), and *Bhlhe40* mRNA (*ShBhlhe40*) or with shRNA control (*ShCTRL*) (mean $\pm$ SEM, triplicates). The left, middle, and right panels each show three independent experiments. FIG E4A related to FIG 3A; FIG E4B related to FIG 3B; FIG E4C related to FIG 3D; FIG E4D related to FIG 3E.

FIG E5. Knockdown of *Mitf* mRNA expression does not lead to mast cell death or down regulation of *FceR1a* expression. **A**. Number of live *ShCTRL* or *ShMitf* PCMCs at different days after puromycin selection (mean $\pm$ SEM, n=3 samples). **B**. qPCR analysis of the *Fcer1a* mRNA expression in *ShCTRL* or *ShMitf* PCMCs (mean $\pm$ SEM, n=3 samples). FIG E5 related to FIG 3.

FIG E6. The transfection factors AHR, AHRR and BHLHE40 are not required for the *Hdc* gene expression in PCMCs. qPCR analysis of the *Hdc* mRNA expression in PCMCs of the indicated mice (mean $\pm$ SEM, triplicates). FIG E6 related to FIG 3.

FIG E7. *Ahr^{f/f}Cpa3-Cre* mice have normal numbers of mast cells and develop passive cutaneous anaphylaxis and passive systemic anaphylaxis normally. **A.** Flow analysis of peritoneal cavity mast cells and total number of peritoneal cavity mast cells (mean $\pm$ SEM, n=3 mice) (*left panel*). Toluidine blue staining of mast cells (magnification $\times 40$; insert, $\times 100$). Mast cells are indicated by arrows. Average number of mast cells in ten randomly selected fields ($\times 40$) (mean $\pm$ SEM, n=3 mice) (*right panels*). **B.** PCA analysis, mean $\pm$ SEM, n=3 mice. **C.** PSA analysis, mean $\pm$ SEM, n=3 mice. FIG E7 related to FIG 3.

FIG E8. *Ahr^{rr/-}* mice have normal numbers of mast cells and develop passive cutaneous anaphylaxis and passive systemic anaphylaxis normally. **A.** Flow analysis of peritoneal cavity mast cells and total number of peritoneal cavity mast cells (mean $\pm$ SEM, n=3 mice) (*left panel*). Toluidine blue staining of mast cells (magnification $\times 40$; insert, $\times 100$). Mast cells are indicated by arrows. Average number of mast cells in ten randomly selected fields ($\times 40$) (mean $\pm$ SEM, n = 3 mice) (*right panels*). **B.** PCA analysis, mean $\pm$ SEM, n = 3 mice. **C.** PSA analysis, mean $\pm$ SEM, n=3 mice. FIG E8 related to FIG 3.

FIG E9. *Bhlhe40^{-/-}* mice have normal numbers of mast cells and develop passive cutaneous anaphylaxis and passive systemic anaphylaxis normally. **A.** Flow analysis of peritoneal cavity mast cells and total number of peritoneal cavity mast cells (mean $\pm$

SEM, n=3 mice) (*left panel*). Toluidine blue staining of mast cells (magnification $\times 40$; insert, $\times 100$). Mast cells are indicated by arrows. Average number of mast cells in ten randomly selected fields ($\times 40$) (mean $\pm$ SEM, n=3 mice) (*right panels*). **B.** PCA analysis, mean $\pm$ SEM, n=3 mice. **C.** PSA analysis, mean $\pm$ SEM, n=3 mice. FIG E9 related to FIG 3.

FIG E10. Identification of putative *Hdc* enhancers. Luciferase reporter analysis of the *Hdc* enhancer activities in the CFTL-15 mast cell line. Error bars represent mean $\pm$ SEM, triplicates. The left, middle, and right panels each show three independent experiments. *HdcMP*: *Hdc* minimal promoter. FIG E10 related to FIG 4B.

FIG E11. MITF binds to the -8.8 enhancer. **A.** ChIP-qPCR analysis of MITF binding (mean $\pm$ SEM, triplicates). **B.** qPCR analysis of the *Hdc* gene expression in untreated or cholera toxin (CT) treated BMMCs, and PCMCs (mean $\pm$ SEM, triplicates). **C.** ELISA analysis of histamine content in untreated or cholera toxin (CT) treated BMMCs and PCMCs (mean $\pm$ SEM, triplicates). **D.** ChIP-qPCR analysis of MITF binding in untreated or cholera toxin (CT) treated BMMCs and PCMCs (mean $\pm$ SEM, triplicates). The left and right panels (**A**) or the left, middle, and right panel (**B**, **C**, and **D**) each show two or three independent experiments, respectively. FIG E11A related to FIG 5B; FIG E11B related to FIG 5C; FIG E11C related to FIG 5D; FIG E11D related to FIG 5E.

FIG E12. MITF drives the -8.8 enhancer activity. **A.** Luciferase reporter gene analysis of *Hdc* enhancer activities in CFTL-15 cells with knocked down *Mitf* mRNA or shRNA control (mean $\pm$ SEM, triplicates). **B.** Luciferase reporter gene analysis of the *Hdc* enhancer activities in HEK293 cells co-transfected with vector control or the MITF expression construct (mean $\pm$ SEM, triplicates). **C.** Luciferase reporter gene analysis of the activity of *Hdc* enhancers that have a mutated MITF binding site(s) (mean $\pm$ SEM, triplicates). The left and right panels (**A**, **B**, and **C**) or the upper and lower panel (**D**) each show two independent experiments. FIG E12A related to FIG 6A; FIG E12B related to FIG 6B; FIG E12C related to FIG 6C.

FIG E13. MITF partially restores *Hdc* gene expression in *Gata2* deficient mast cells. qPCR analysis of the *Hdc* mRNA expression in *Gata2*^{-/-} BMMCs overexpressing MITF, GATA2 or a control vector (CTRL) (mean $\pm$ SEM, triplicates). The left, middle, and right panels each show three independent experiments. FIG E13 related to FIG 7B.

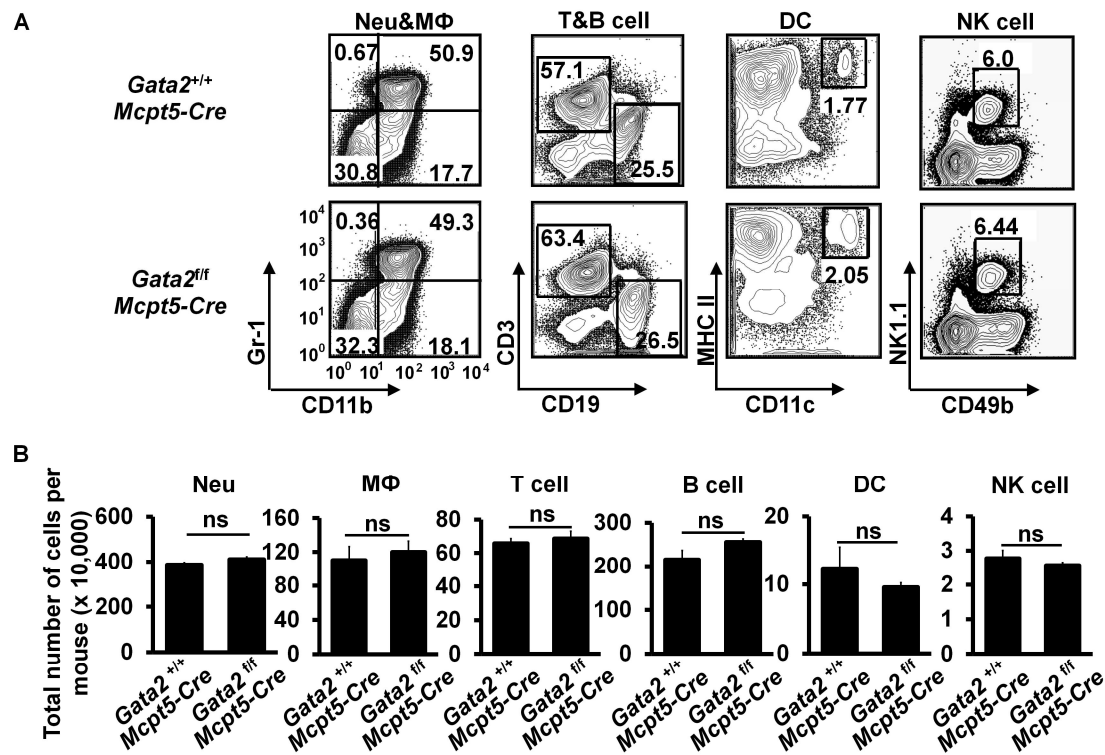
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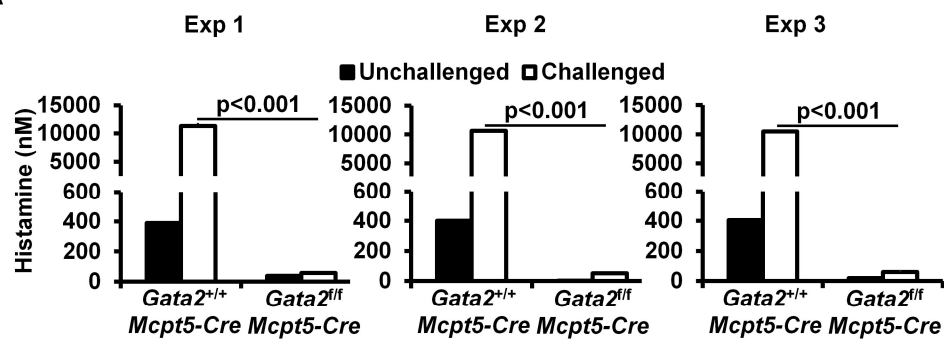
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Table E1 Primer sequences:

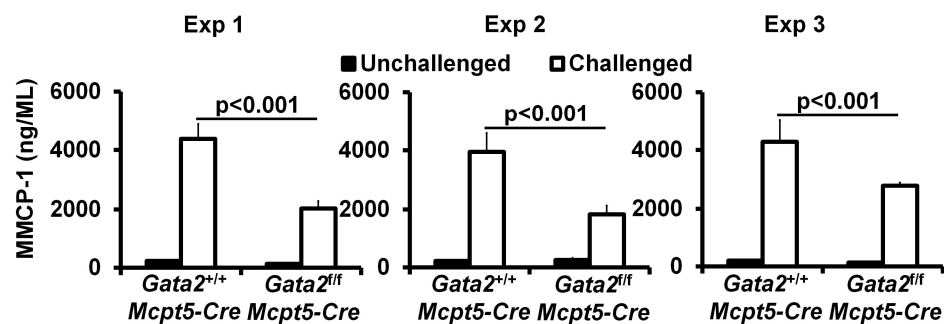
Primers for qPCR and ChIP-qPCR		
Gene	5' primer	3' primer
<i>Mitf</i>	AACCGACAGAAGAAGCTGGA	TGATGATCCGATTACACCAGA
<i>Ahr</i>	AGCAGCTGTGTCAGATGGTG	CTGAGCAGTCCCCTGTAAGC
<i>Ahr</i>	AGAGGAGGACCAGAAGCACA	TCTGTGCTCCAGTTCAGGTG
<i>Bhlhe40</i>	CATGAGAACTCTCGGGACCT	TCTCTTTCAAGTCCACGGCT
<i>Hdc</i>	TTCCAGCCTCCTCTGTCTGT	GGTATCCAGGCTGCACATT
<i>Hprt</i>	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAATTATAGCC
<i>Human HDC</i>	TTCTGCCTGTGGATGACAAC	TAAGCAGCATCGATGTGGAG
<i>Human HPRT</i>	CTCATGGACTAATTATGGACAGGAC	GCAGGTCAGCAAAGAATTTATAGCC
<i>HdcE</i> +0.3BS1 ChIP	GGGGCCACAGTGATGAAAC	AGGAAAATGTGTCCCACTGC
<i>HdcE</i> +0.3BS2 ChIP	GAAGGGTTTCTGGTTGCTCA	CTTGTCACACGGCTTCCAC
<i>HdcE</i> -8.8BS3&4 ChIP	GCAAAGCCTCAAGGTGGTAA	GAAGCTGCCCCGAATACTTGT
<i>HdcE</i> -8.8BS5 ChIP	CTGCAAGCTACATTTTCAGCA	TGTGAGAACAACTGCACTGG
Primers for constructions		
Constructs	5' primer	3' primer
pGL3- <i>Hdc</i> MP	AATAAGATCTGGCTAAAGGAGGGGAATAGG	AATAAAGCTTGCATCTCTTATTTAAGGAGTTCCC
pGL3- <i>Hdc</i> MPE+0.3	AATAGCTAGCGTAGATCAGAGTTTAAACAGGTCCCT	AATAAGA TCTCTTCTTCTTCTTCTTCTTTTTTTTTTTT
pGL3- <i>Hdc</i> MPE-8.8	AATAGGTACCAACCAATTAGACTCCTCATGCCTT	AATAACGCGTAAGGAAGTATAAACGAGGGGTG
BS3 Mut	GTTCTGCTGGAGACAATTAAATACAG	ATTACATCCTGTATTTAATTGTCTCCAG
BS4 Mut	CAGCTGCAGGCGAATTAATGT	GTGAAAGCAACATTAATTGCCT
BS5 Mut	AAGCCGTTGGAGAATTAATTGCT	ACACAAACCCAAGCAATTAATTCTC



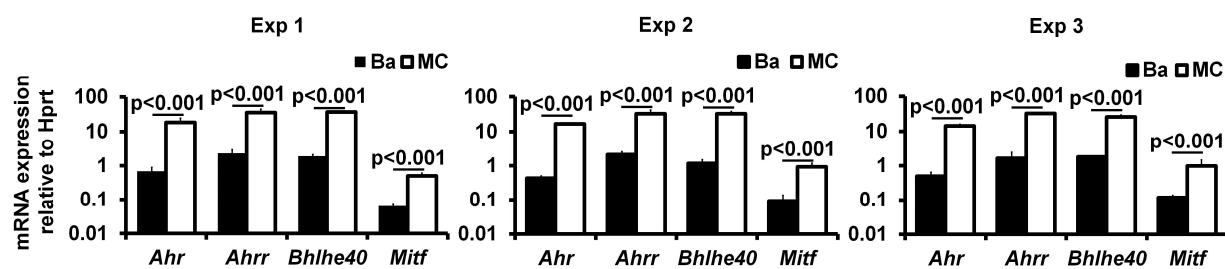
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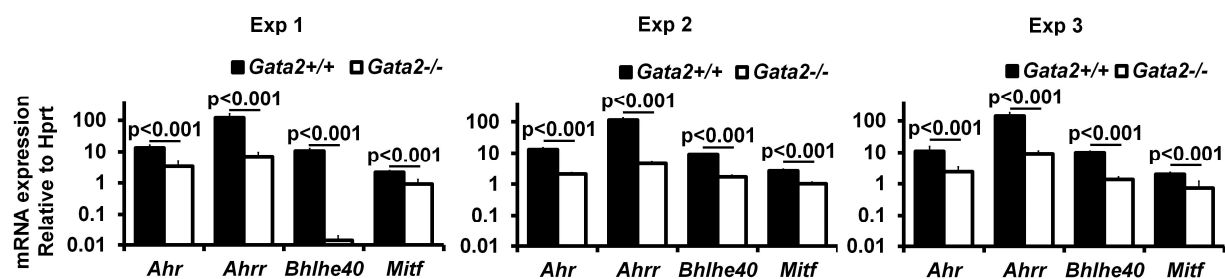
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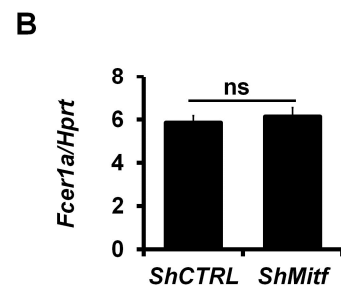
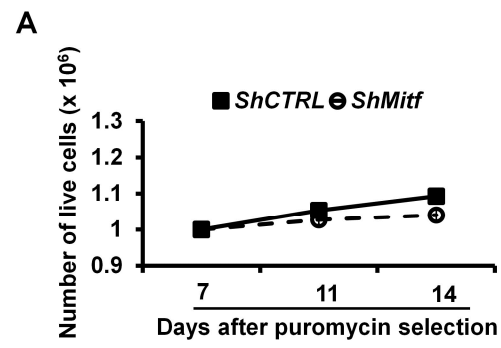


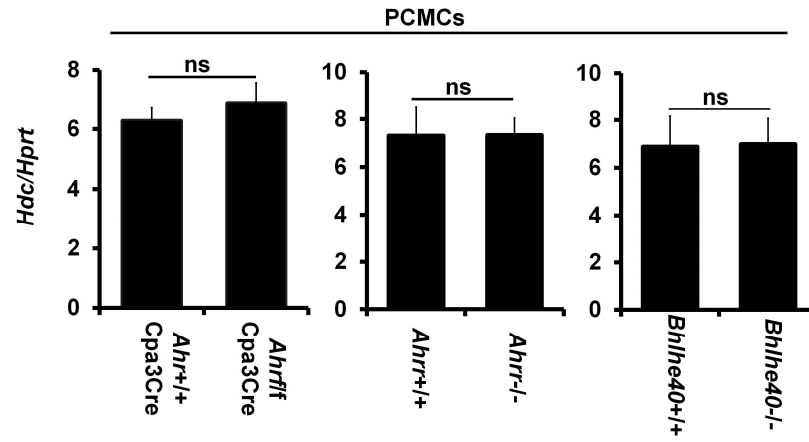
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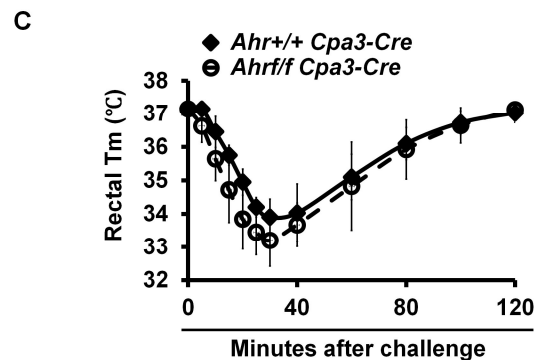
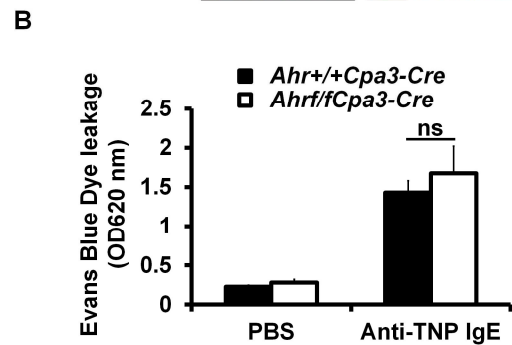
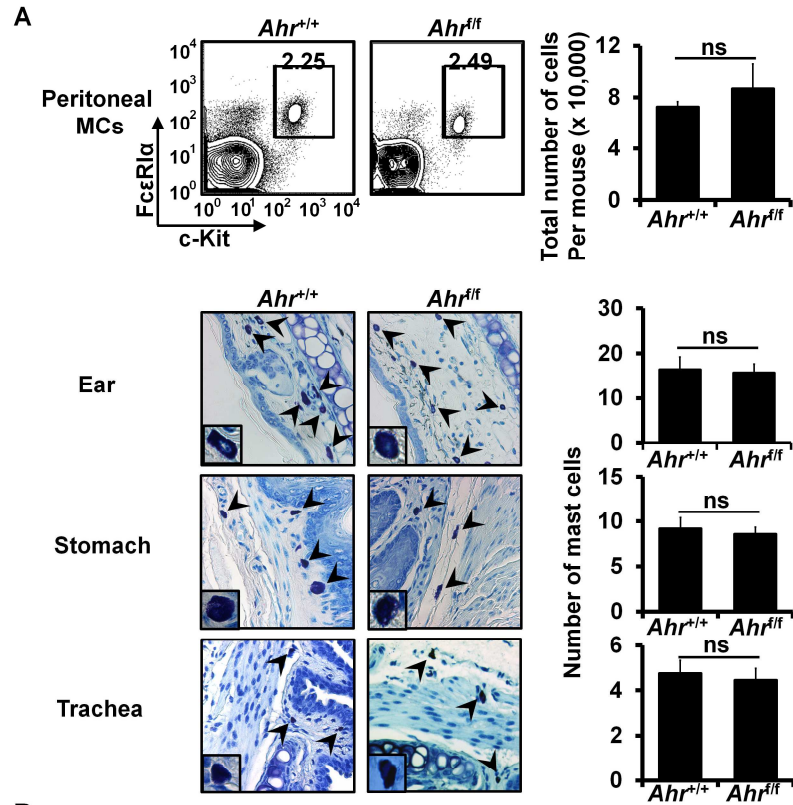


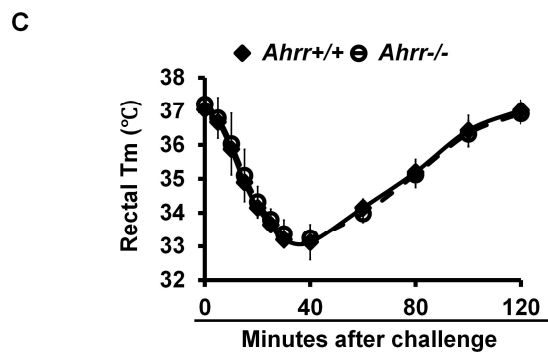
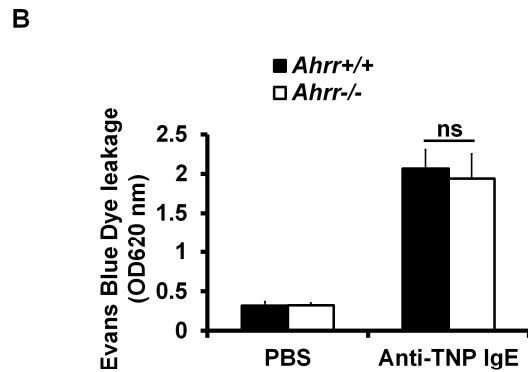
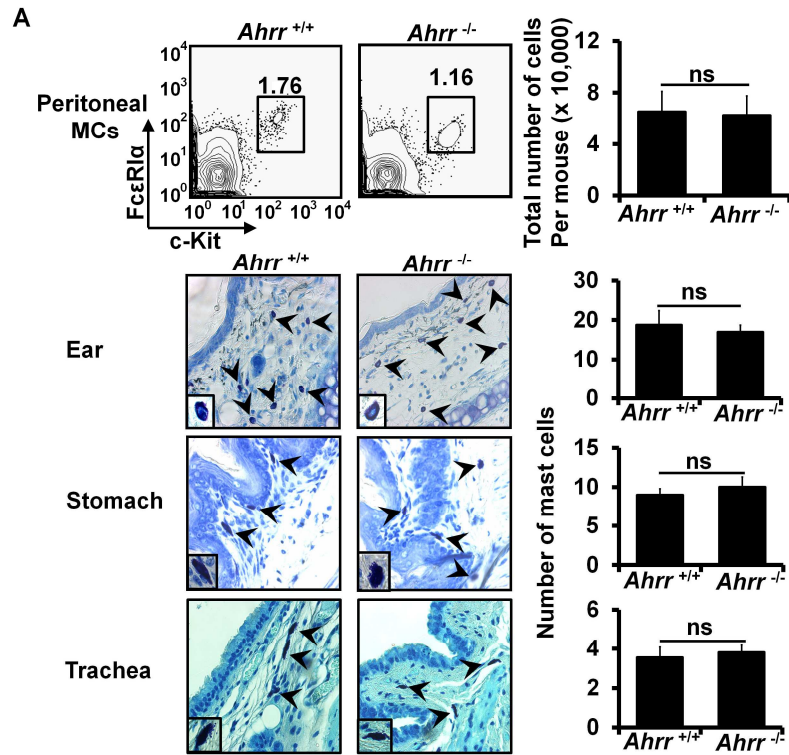
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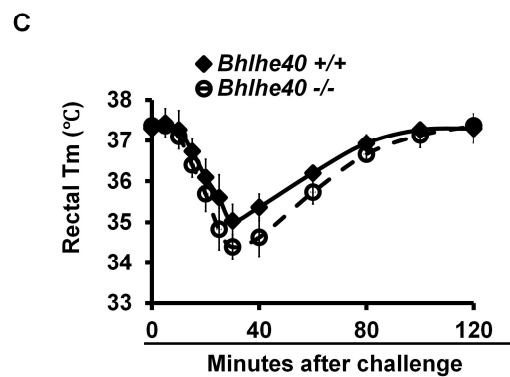
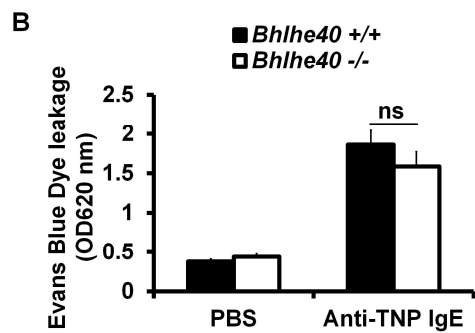
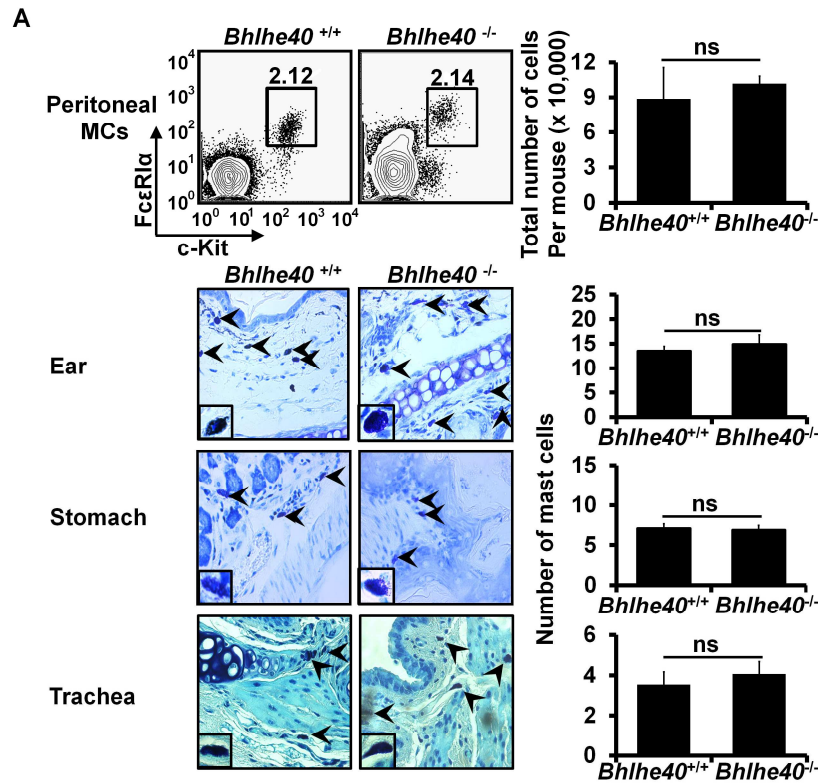


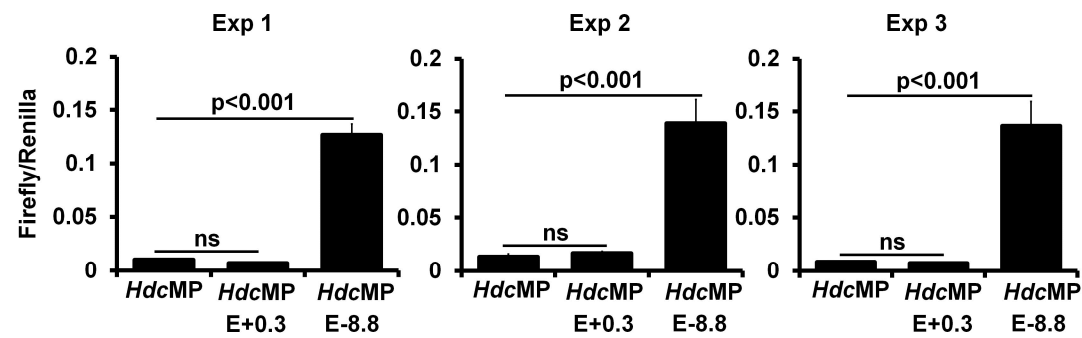




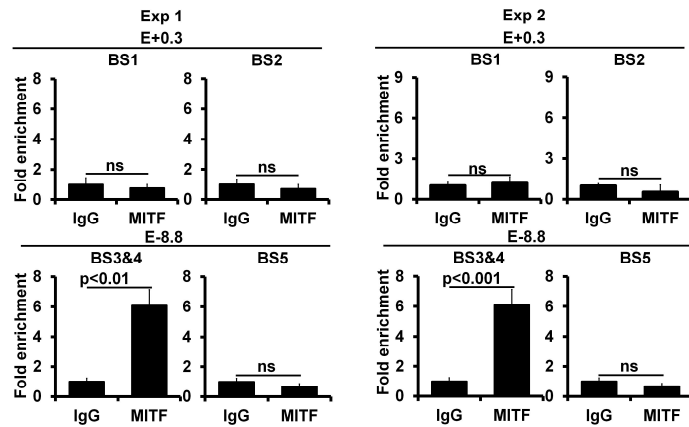




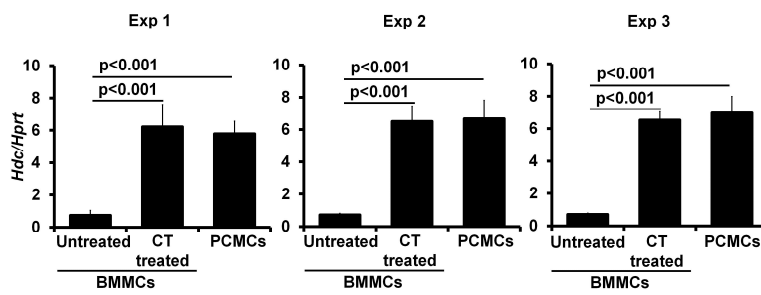




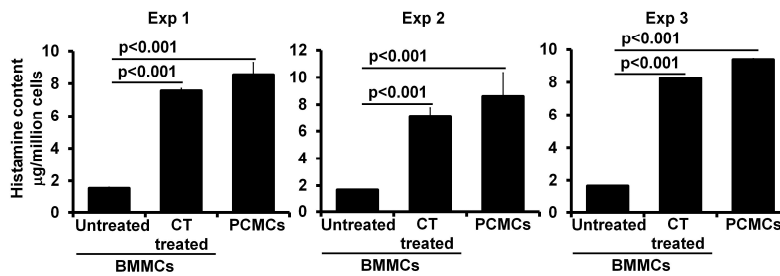
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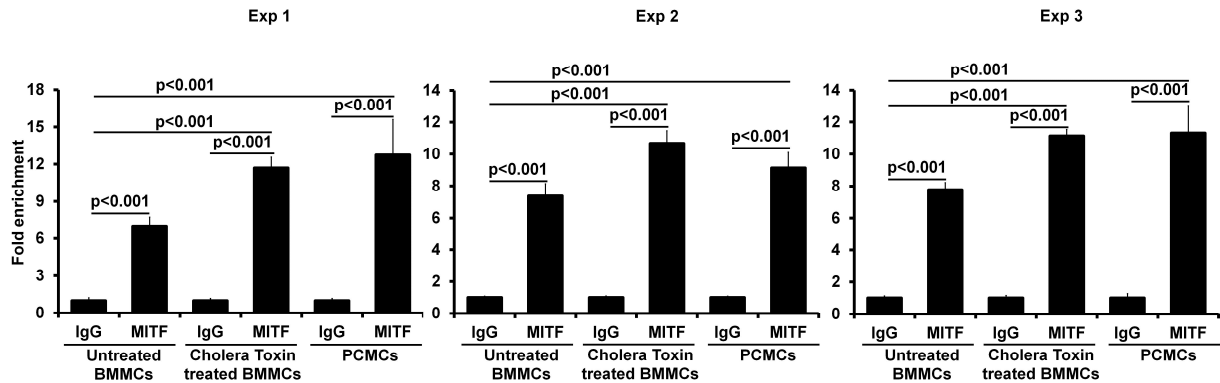
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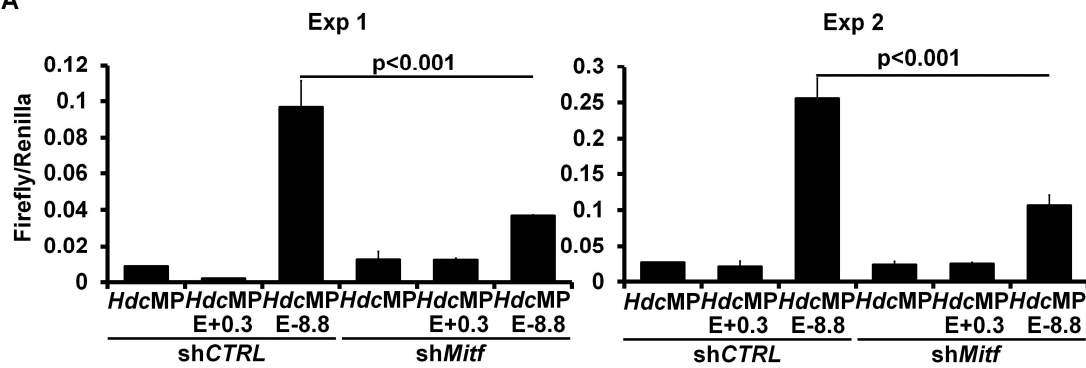
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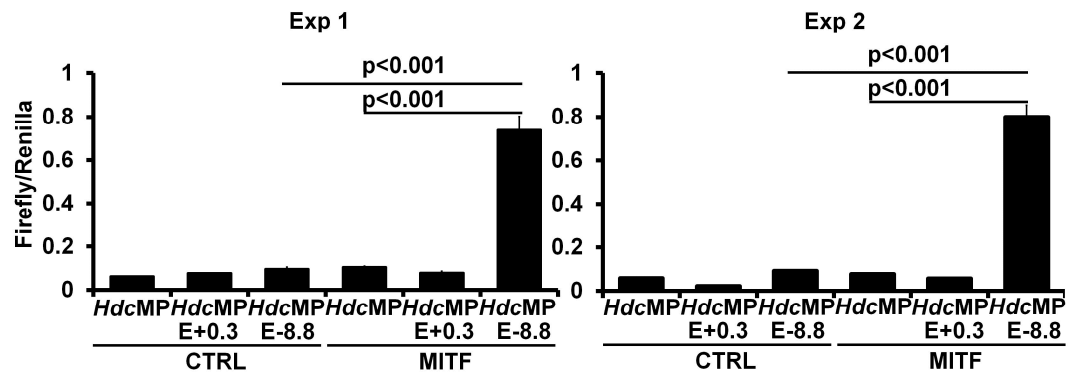
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C

