Dnmt3a restrains mast cell inflammatory responses

Cristina Leoni[a,b], Sara Montagner[a], Andrea Rinaldi[c], Francesco Bertoni[c,d], Sara Polletti[e], Chiara Balestrieri[e], and Silvia Monticelli[a,1]

[a]Institute for Research in Biomedicine, Università della Svizzera italiana (USI), 6500 Bellinzona, Switzerland; [b]Graduate School for Cellular and Biomedical Sciences, University of Bern, 3012 Bern, Switzerland; [c]Lymphoma and Genomics Research Program, Institute of Oncology Research (IOR), USI, 6500 Bellinzona, Switzerland; [d]Oncology Institute of Southern Switzerland (IOSI), 6500 Bellinzona, Switzerland; and [e]Department of Experimental Oncology, European Institute of Oncology (IEO), 20139 Milan, Italy

DNA methylation and specifically the DNA methyltransferase enzyme DNMT3A are involved in the pathogenesis of a variety of hematological diseases and in regulating the function of immune cells. Although altered DNA methylation patterns and mutations in *DNMT3A* correlate with mast cell proliferative disorders in humans, the role of DNA methylation in mast cell biology is not understood. By using mast cells lacking *Dnmt3a*, we found that this enzyme is involved in restraining mast cell responses to acute and chronic stimuli, both in vitro and in vivo. The exacerbated mast cell responses observed in the absence of *Dnmt3a* were recapitulated or enhanced by treatment with the demethylating agent 5-aza-2′-deoxycytidine as well as by down-modulation of *Dnmt1* expression, further supporting the role of DNA methylation in regulating mast cell activation. Mechanistically, these effects were in part mediated by the dysregulated expression of the scaffold protein IQGAP2, which is characterized by the ability to regulate a wide variety of biological processes. Altogether, our data demonstrate that DNMT3A and DNA methylation are key modulators of mast cell responsiveness to acute and chronic stimulation.

DNA methylation | epigenetics | inflammation | mast cells

DNA methylation is an epigenetic process in which a methyl group is covalently linked to a cytosine base in the genomic DNA, predominantly at CpG dinucleotides, yielding 5-methyl-cytosine (5mC). Such a process is carried out by three DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B), and has a critical role in the control of gene expression (1, 2). In general, high levels of DNA methylation are associated with transcriptional silencing (3), especially when present at promoter regions and at repetitive elements (4), although the function of DNA methylation at other genomic features and its correlation with gene expression are more uncertain (3–5). Whereas DNMT1 is thought to be primarily responsible for copying the preexisting methylation to the newly synthesized DNA strand during replication, DNMT3A and DNMT3B display significant affinity also for unmethylated DNA, and are therefore considered de novo methyltransferases (6, 7). DNA methylation is essential during development: Various mouse models have shown that the absence of *Dnmt1* or *Dnmt3b* is embryonically lethal, and mice lacking *Dnmt3a* die within 4 wk after birth because of their failure to thrive (6, 8); in humans, mutations in the *DNMT3A* gene are associated with an overgrowth syndrome with intellectual disability (9). More specific to the hematopoietic compartment, loss of *Dnmt1* in hematopoietic stem cells (HSCs) led to defects in self-renewal, niche retention, as well as altered cell differentiation, especially toward the myeloid lineage (10), whereas loss of both *Dnmt3a* and *Dnmt3b* impaired HSC self-renewal capabilities (11). Importantly, aberrant DNA methylation is a hallmark of many diseases, including autoimmune diseases and especially various types of cancer (4, 5). Mutations in *DNMT3A* have been found in a variety of hematological malignancies (4, 12, 13), including systemic mastocytosis, a clonal proliferative disorder of mast cells (14), pointing toward a role for DNMT3A in modulating mast cell biology. Further correlating DNA methylation with the biology of

mast cells (which are key effector cells in asthmatic and allergic responses), a recent survey compared atopic and asthmatic patients with healthy controls and identified 81 differentially methylated regions (15); the hypomethylated regions included genes such as *IL13*, which is not only crucial in asthma pathogenesis but is also expressed at high levels by mast cells (16). Finally, highlighting the potential relevance of understanding the role of DNA methylation in mast cell biology, altered DNA methylation patterns were identified in patients with mast cell activation disease, a complex disorder characterized by aberrant release of mast cell-derived mediators (17). We therefore set out to investigate the role of DNA methylation in general and DNMT3A in particular in regulating mast cell differentiation and function.

We found that mast cells lacking *Dnmt3a* appeared to be more responsive to stimuli compared with their wild-type counterparts. Among other phenotypes, stimulation with IgE and antigen complexes triggered a significantly stronger acute response in mast cells lacking *Dnmt3a*, including higher cytokine production and increased degranulation capacity. Such phenotypes were recapitulated or exacerbated by treatment of the cells with the demethylating agent 5-aza-2′-deoxycytidine as well as by reducing *Dnmt1* expression, further supporting the notion that DNA methylation-regulated processes are important modulators of mast cell activation. Mechanistically, these effects were likely to be mediated, at least in part, by the dysregulated expression of the scaffold protein IQGAP2 (IQ motif-containing GTPase-activating protein 2), and led to exacerbated in vivo responses in both acute and chronic models of mast cell activation, namely

---

Significance

Methylation of genomic DNA is an epigenetic modification at the interface between genetic information and environmental stimuli underlying many phenotypic variations in the human population as well as the pathogenesis of complex diseases. Accordingly, mutations in the de novo DNA methyltransferase enzyme *DNMT3A* have been identified in a number of diseases, including mast cell-related disorders. However, the role of DNA methylation and DNMT3A in regulating mast cell physiology still needs to be elucidated. Here, we found that *Dnmt3a* plays a critical role in modulating mast cell responsiveness to acute and chronic stimulation, potentially implicating DNA methylation-mediated processes in all types of mast cell-related diseases.

---

passive cutaneous anaphylaxis and oxazolone-induced dermatitis. Our results indicate that appropriate regulation of DNMT3A-mediated processes modulates mast cell responses to environmental stimuli, both in vitro and in vivo, and may be relevant in all types of mast cell activation diseases.

Results

Increased Susceptibility to IgE Stimulation of Mast Cells Lacking *Dnmt3a*. The mRNAs of the three DNA methyltransferases were expressed at relatively high levels in HSCs, and their levels decreased upon differentiation to mast cells (Fig. S1*A*). However, all of them remained expressed at detectable levels in differentiated mast cells, with *Dnmt1* displaying the highest relative expression, *Dnmt3b* the lowest, and *Dnmt3a* expressed at intermediate levels (Fig. 1*A* and Fig. S1*A*). Expression of all *Dnmt*s was induced upon acute stimulation of mast cells with IgE and antigen complexes (Fig. 1*A*), with *Dnmt3a* being the most inducible (about 13.8-fold after 6 h of stimulation), suggesting a

potential role for this enzyme in modulating mast cell responses following activation. Mice genetically deleted for *Dnmt3a* are runt (Fig. S1*B*), and die perinatally within 4 wk after birth (6). We found that differentiation to mast cells was unchanged in the absence of *Dnmt3a*, and the resulting gross phenotype was undistinguishable regardless of the genotype (Fig. S1*C*). However, *Dnmt3a* knockout (KO) mast cells showed a significant increase in proliferation compared with their wild-type (WT) counterparts (Fig. 1*B*). This is in accordance with the fact that *DNMT3A* is frequently mutated in myeloid neoplasms, including systemic mastocytosis (14), and deletion of this enzyme predisposes the cells to myeloid transformation (18). To assess whether such increased proliferation was also associated with increased survival in vitro, mast cells were transiently deprived of the essential survival factor IL-3. Removal of IL-3 from the culture medium led to an overall increase in cell death, which was, however, much more pronounced in the absence of *Dnmt3a* (Fig. 1*C*), potentially pointing toward an increased susceptibility of these
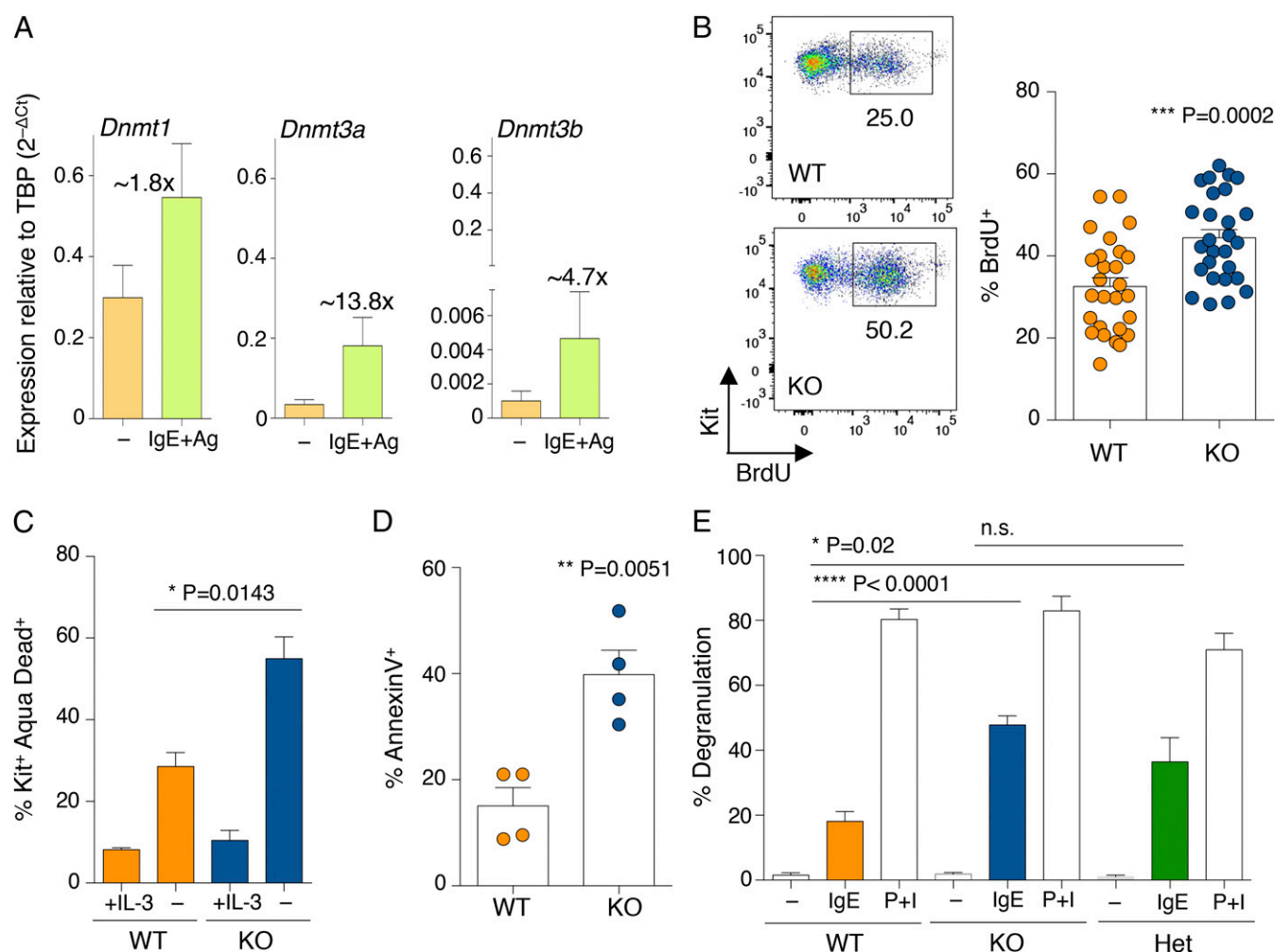

Fig. 1. Mast cells lacking *Dnmt3a* display a complex phenotype. (*A*) WT differentiated mast cells were either stimulated with IgE and antigen (Ag) for 6 h or left untreated. After RNA extraction, expression of the indicated *Dnmt* mRNA was assessed by qRT-PCR. PCR primers used in this study are listed in Table S1. *n* = 5 independent experiments. Mean ± SEM. TBP, TATA-box-binding protein. (*B*) Mast cell proliferation was assessed between weeks 4 and 7 of differentiation by BrdU incorporation. One representative experiment is shown (*Left*); each dot represents one experiment. Mean ± SEM; unpaired *t* test, two-tailed. (*C*) Mast cells were deprived of IL-3 for 48 h prior to staining with an Aqua Dead dye to determine cell survival. Shown is the percentage of dying mast cells (Kit⁺ Aqua Dead⁺) in the absence of IL-3 in *n* = 3 independent experiments. Mean ± SEM; unpaired *t* test, two-tailed. (*D*) Degranulation of mast cells was measured by annexin V staining upon stimulation with IgE and antigen complexes for 30 min. Each dot represents one experiment. Mean ± SEM; unpaired *t* test, two-tailed. (*E*) Mast cell degranulation upon stimulation with either IgE and antigen complexes or phorbol 12-myristate 13-acetate (PMA) and ionomycin (P+I) was measured by β-hexosaminidase release. P+I stimulation was used as a positive control to induce maximum degranulation. *n* = 4 independent experiments. Mean ± SEM; unpaired *t* test, two-tailed. n.s., not significant.

cells to perturbations in their microenvironment. We therefore assessed mast cell responses to physiological stimulation with IgE and antigen. Interestingly, upon acute stimulation, mast cells lacking *Dnmt3a* showed significantly increased ability to release the content of their cytoplasmic granules, as assessed both by annexin V binding [which occurs at sites of secretory granule fusion with the plasma membrane (19–21)] and release of β-hexosaminidase (an enzyme normally stored in cytoplasmic granules) in the culture supernatant (Fig. 1 *D* and *E*). Similarly, production of IL-6, TNF-α, and IL-13 (cytokines secreted by mast cells at high levels in response to IgE stimulation) was also increased in *Dnmt3a*-deleted cells, as assessed both by intracellular staining (Fig. 2 *A* and *B*) and ELISA (Fig. 2*C*).

Dysregulated DNA Methylation Activity Leads to Increased Mast Cell Responses. To further evaluate the impact of DNA methylation on the ability of mast cells to respond to external stimuli, we treated WT and KO cells for 48 h with 5-aza-2′-deoxycytidine (decitabine; DAC), a drug commonly used for the treatment of myelodysplastic syndromes and known to inhibit DNA methyltransferase enzymes (22). Such treatment led to global genomic hypomethylation in mast cells (Fig. 3*A*), which not only correlated with increased capacities of WT mast cells to produce cytokines but also further exacerbated the phenotype displayed by KO mast cells (Fig. 3*B*). To further investigate the role of DNA methylation in mast cell responses, we used shRNAs to downmodulate the expression of *Dnmt1* (*Dnmt3b* was expressed by mast cells only at extremely low levels; Fig. 1*A*). Reducing *Dnmt1* expression provided WT mast cells with increased degranulation capabilities (Fig. 3 *C* and *D*). Such an increase in degranulation was very prominent especially for one of the two shRNAs tested (sh2), which also showed the highest reduction in *Dnmt1* expression (Fig. 3*C*). Interestingly, reduction of *Dnmt1* expression did not further increase the ability of *Dnmt3a* KO cells to degranulate in response to IgE stimulation (Fig. 3*D*), suggesting that *Dnmt3a*

KO cells may have already reached their maximum degranulation capacity in response to IgE and antigen stimulation.

The DNA methylation status of different cell types, including cells of the hematopoietic lineage, with or without *Dnmt3a*, has been previously studied (23–25): First, HSCs lacking *Dnmt3a* showed global 5mC levels that were comparable to those of control cells, with a very modest number of differentially methylated regions (24). In agreement with these findings, we found that overall levels of genomic 5mC were not significantly different in WT and KO mast cells, despite a trend toward slightly diminished levels in KO cells (Fig. S1*D*). Second, the differentially methylated regions identified in *Dnmt3a*-null cells showed substantial hyper- as well as hypomethylation at a variety of genomic regions, with very little correlation between changes in methylation and differential gene expression in the absence of *Dnmt3a* in both mouse and human cells (4, 24, 25). These observations suggest the existence of both direct and indirect effects due to the loss of *Dnmt3a*, and potentially also the existence of DNMT3A activities that are independent of its DNA catalytic activity (26), similar to what has been described for the catalytically inactive DNMT3L (27), some of the DNMT3B isoforms (28), and even for the TET family of DNA-modifying enzymes (29, 30). To gain more insight into the mechanisms that may underlie increased mast cell responses in the absence of *Dnmt3a*, we therefore investigated global gene expression in WT and KO mast cells.

Altered Gene Expression and Chromatin Accessibility in the Absence of *Dnmt3a*. Using Illumina microarrays, we performed gene expression analyses of WT and KO mast cells, either resting or stimulated with IgE and antigen, for 4 and 24 h. Triplicate biological samples were used and data were quantile-normalized. Differentially expressed transcripts were identified by analysis of variance (ANOVA) (P value < 0.05). We found that upon stimulation of KO cells, most transcriptional changes were of
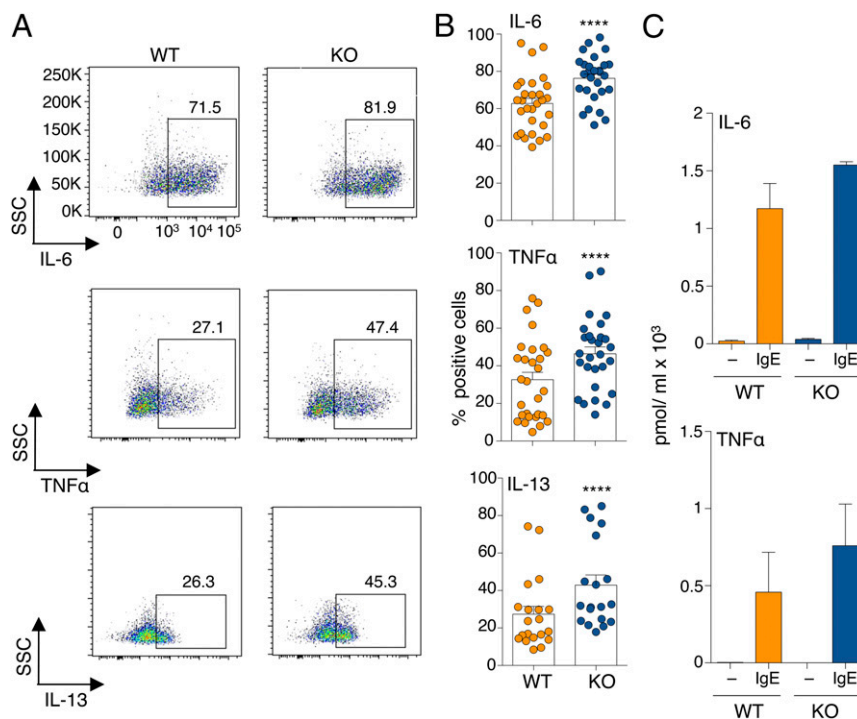

Fig. 2. Increased cytokine production in the absence of *Dnmt3a*. (*A*) Intracellular staining for the indicated cytokines in mast cells stimulated with IgE and antigen for 3 h. One representative experiment is shown. SSC, side-scatter. (*B*) Same as *A*; each dot represents one experiment. Mean ± SEM; paired *t* test, two-tailed (****$P < 0.0001$). (*C*) Release of IL-6 and TNF-α in the supernatant was measured by ELISA after 12 h of stimulation. $n = 4$ independent experiments. Mean ± SEM.
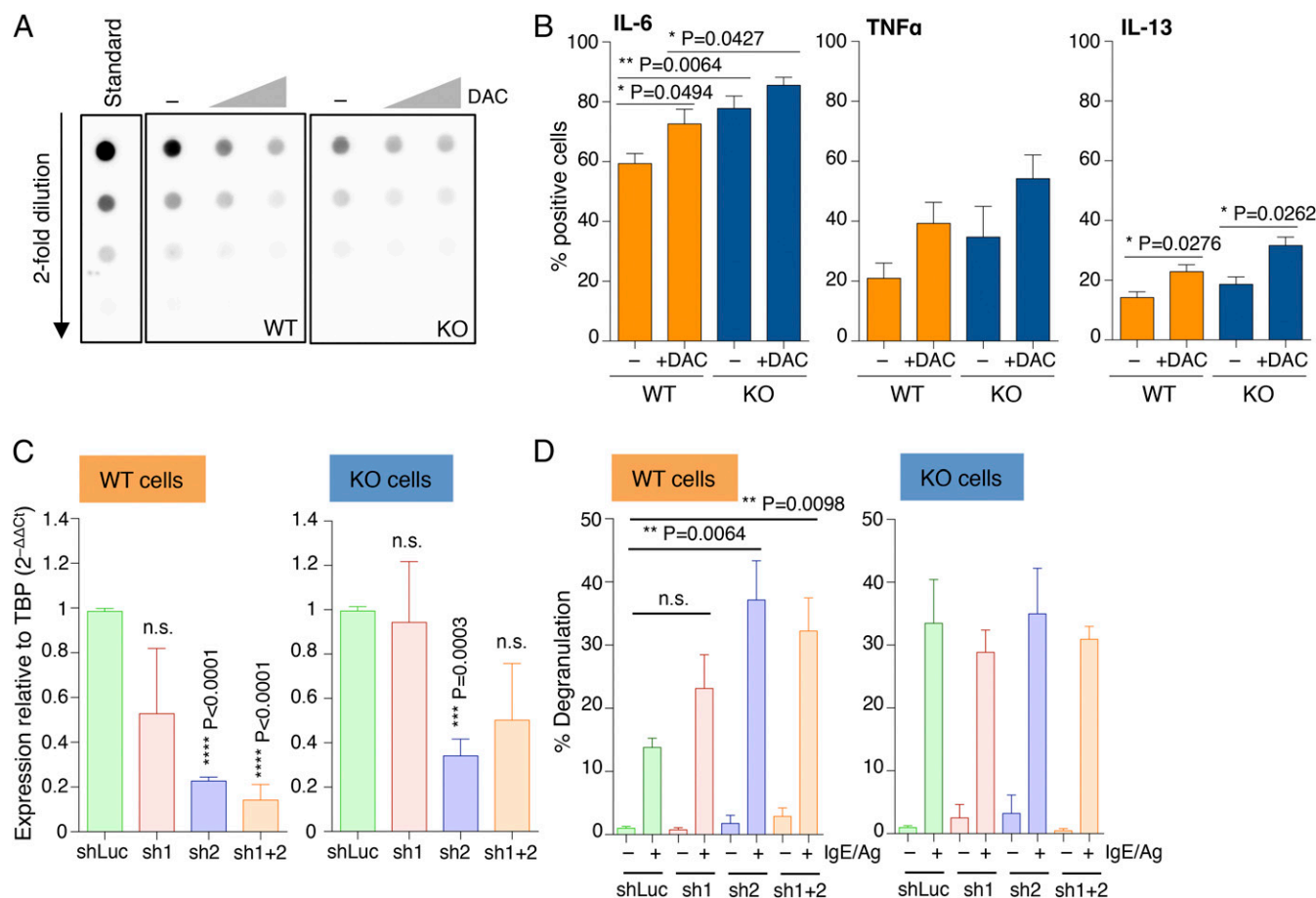
Fig. 3. Increased mast cell responses upon disruption of DNA methylation activities. (*A*) Differentiated mast cells were treated with 0.5 and 5 μM 5-aza-2′-deoxycytidine for 48 h, after which genomic DNA was extracted and overall levels of 5mC were quantified by dot blot. (*B*) Mast cells were treated with 0.5 μM DAC for 3 d before stimulation with IgE and antigen and measurement of cytokine production by intracellular cytokine staining. Mean ± SEM; unpaired *t* test, two-tailed. (*C*) Mast cells were transduced with lentiviral vectors (sh1 and sh2) expressing shRNAs to knock down expression of *Dnmt1*. A vector expressing an irrelevant hairpin (shLuc) was used as control. After puromycin selection of the transduced cells, total RNA was extracted and the extent of *Dnmt1* down-regulation was measured by qRT-PCR. Shown are the compiled results of three independent experiments. Mean ± SEM; unpaired *t* test (relative to the shLuc sample), two-tailed. (*D*) Same as *C*, except that cells were stimulated with IgE and antigen prior to measurement of the extent of degranulation by β-hexosaminidase release. Shown are the compiled results of three independent experiments. Mean ± SEM; unpaired *t* test, two-tailed.

limited magnitude compared with WT cells, and these especially included changes in the levels of expression of many cytokine and chemokine genes (Fig. 4*A*). For instance, the *Tnf* gene was induced 8.7-fold in KO cells and 3.6-fold in WT cells after 4 h (Fig. 4*A*). Similarly, the *Il13* gene was induced 7.9-fold in KO cells and 6.2-fold in WT cells. These findings were also in agreement with the increased cytokine production observed in KO cells by intracellular staining and ELISA (Fig. 2). We did not detect any compensatory increase in *Dnmt1* and/or *Dnmt3b* expression in the absence of *Dnmt3a*. We therefore focused our attention on genes that were differentially expressed between WT and KO cells, regardless of stimulation (Fig. 4*B*). Overall, 2,654 genes were significantly differentially expressed in mast cells in the absence of *Dnmt3a*; of these, only 76 genes were up-regulated at least 1.5-fold and 100 genes were down-regulated at least 1.5-fold, indicating that the extent of transcriptional effects was overall rather subtle. We validated some selected genes by quantitative (q)RT-PCR using independent biological samples (Fig. 4*C*). Genes that were up-regulated in KO cells included *C2*, encoding for the complement component C2 of the classical C3 convertase, *Gpx3* (glutathione peroxidase 3), and *Syce1* (synaptonemal complex central element protein 1), encoding for a protein involved in chromosome segregation (31). Genes that were down-regulated in KO cells included *Hoxb4*, encoding for a

transcription factor (TF) involved in development and a positive regulator of HSC self-renewal (32); the TF *Sox12* (33); and *Iqgap2*, encoding for a scaffold protein with a variety of different functions (34).

To gain a better understanding of the mechanisms that could lead to dysregulated gene expression in the absence of *Dnmt3a*, we performed ATAC-seq (assay for transposase-accessible chromatin sequencing) (35) to assess the overall accessibility of genomic regions in WT and *Dnmt3a* KO mast cells. We found that many regions were more accessible in *Dnmt3a* KO mast cells compared with WT (4,139 regions enriched in KO cells vs. 1,567 regions enriched in WT cells) (Fig. 5*A*). Such regions were similarly distributed in WT and KO cells between promoter, intronic, and intergenic regions (Fig. 5*B*). Most importantly, a GREAT (Genomic Regions Enrichment of Annotations Tool) analysis (36) of the regions specifically enriched in KO cells revealed gene ontology categories that were highly correlated with the phenotype observed in the absence of *Dnmt3a* (Fig. 5*C*), most notably "mast cell degranulation" and "mast cell activation." Moreover, pointing toward dysregulated TF binding and RNA polymerase II (Pol II) recruitment, other significant categories for *Dnmt3a* KO cells included "activating transcription factor binding" and "basal RNA Pol II transcription machinery binding." Overall, these data indicate that the absence of *Dnmt3a*
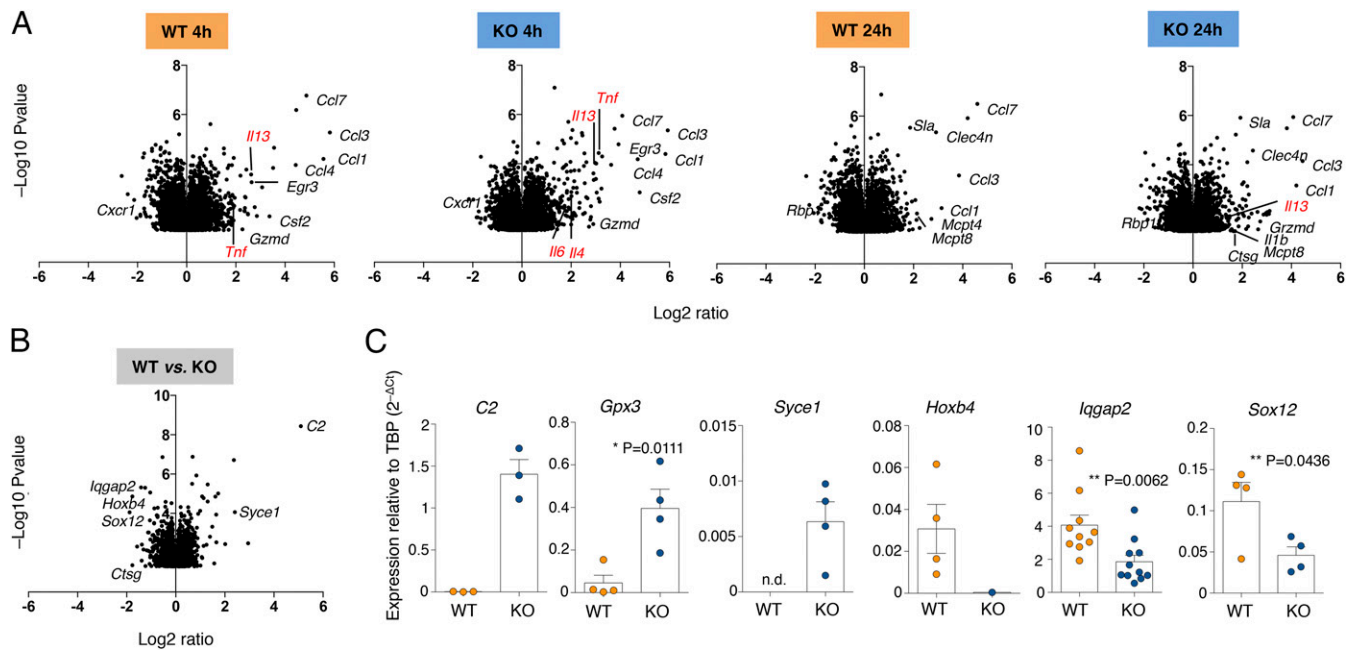
Fig. 4. Altered gene expression in the absence of *Dnmt3a*. (*A*) Mast cells (three independent biological samples) were either left untreated or stimulated with IgE and antigen for 4 or 24 h; RNA was extracted and gene expression was analyzed by microarray. Shown is the effect of stimulation on gene expression in either WT or KO cells. (*B*) Same as *A*, except that genes differentially expressed between WT and KO cells (regardless of stimulation) are shown. (*C*) Selected genes from *B* were validated in independent biological samples by qRT-PCR. Each dot represents one experiment. Mean ± SEM; unpaired *t* test, two-tailed.

results in increased accessibility of genomic regions that may be involved in regulation of mast cell activation.

Reducing *Iqgap2* Expression Is Sufficient to Increase Mast Cell Degranulation. Among the differentially expressed genes in the
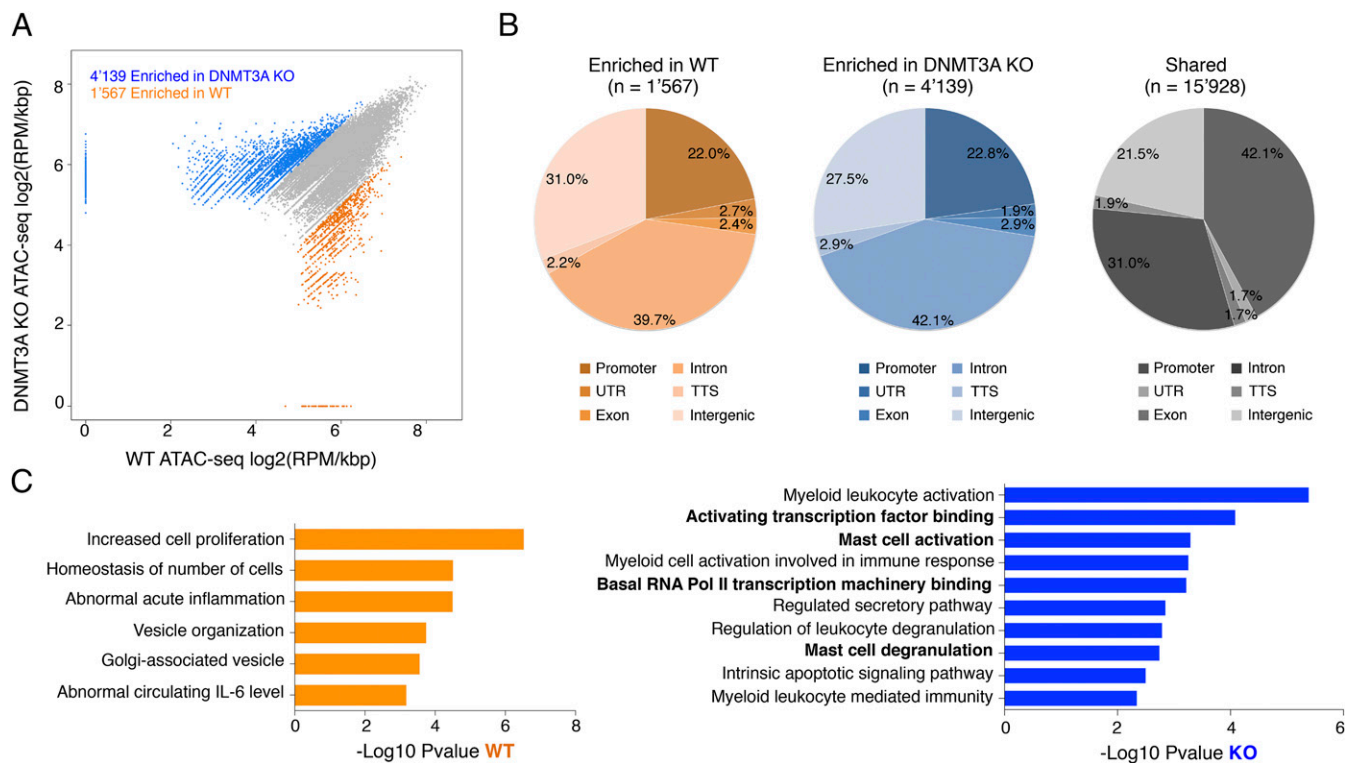

Fig. 5. Chromatin accessibility in WT and KO cells. (*A*) The scatter plot shows ATAC-seq signals as reads per million (RPM) per kilobase at chromatin accessible regions in WT and *Dnmt3a* KO cells. Regions enriched in WT cells are depicted in orange; regions enriched in KO cells are in blue; shared regions are in gray. (*B*) The diagram illustrates the overall distribution of enriched and shared peaks into promoter, untranslated region (UTR), exon, intron, transcription termination site (TTS), and intergenic regions. (*C*) Functional enrichment analysis of the enriched regions was performed using GREAT. The full list of categories is provided in Dataset S1.

absence of *Dnmt3a*, *Iqgap2* showed a large decrease in expression in KO cells (about −54.5%; Fig. 4*C*). IQGAP proteins (IQGAP1, 2, and 3) are widely expressed proteins with the ability to regulate very diverse biological processes, including cytoskeleton dynamics, vesicle trafficking, cell proliferation, intracellular signaling, and TF activity (34). Most importantly, T lymphocytes from IQGAP1-deficient mice displayed increased production of cytokines, and both IQGAP1 and IQGAP2 were shown to interact with the long noncoding RNA *NRON* (noncoding RNA repressor of NFAT), known to interfere with nuclear import of the TF NFAT (37, 38). We therefore hypothesized that altered expression of *Iqgap2* could explain, at least in part, the various phenotypes observed in activated mast cells lacking *Dnmt3a*, including increased cytokine

production and degranulation. First, we measured the expression of the other two family members, *Iqgap1* and *Iqgap3*, and found that, contrary to *Iqgap2*, their expression was slightly increased in KO cells, although in a nonsignificant manner (Fig. 6*A*). IQGAP proteins are, however, known to act as a scaffold for many interacting partners: We therefore assessed the effect of depleting *Iqgap2* itself by transient transfection of siRNAs in WT differentiated mast cells. Two days after transfection, the average reduction in *Iqgap2* expression compared with a control oligonucleotide was ~68% (Fig. 6*B*), indicating effective knockdown to levels comparable to those observed in KO cells (Fig. 4*C*). Importantly, such down-regulation of *Iqgap2* expression corresponded to a significant increase in mast cell degranulation (Fig.
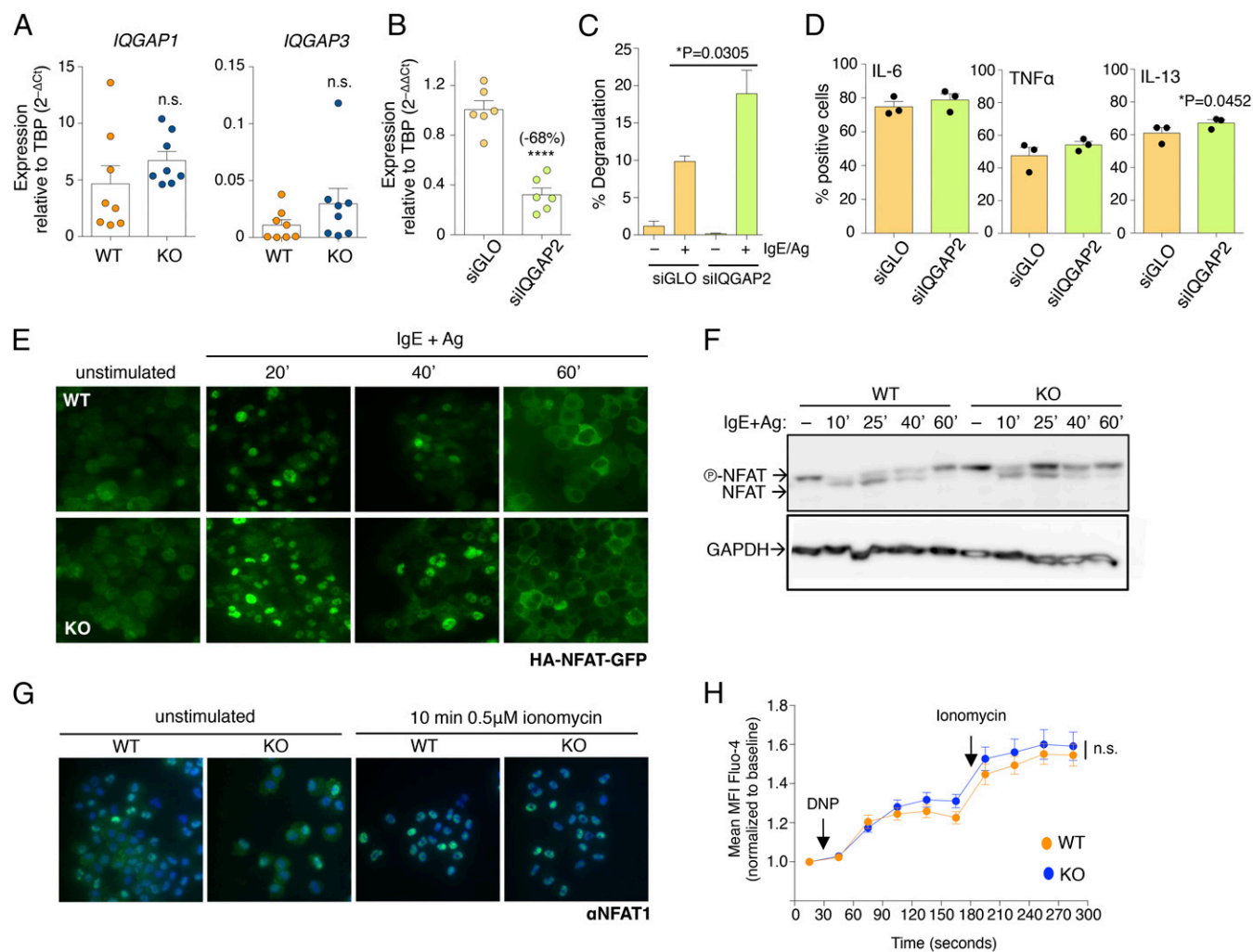

Fig. 6. Reduced expression of *Iqgap2* is sufficient to increase mast cell degranulation. (*A*) Expression of IQGAP family members *Iqgap1* and *Iqgap3* was measured by qRT-PCR in WT and KO mast cells. Each dot represents one experiment. Mean ± SEM; unpaired *t* test, two-tailed. (*B*) Mast cells were transiently transfected with a pool of siRNAs to knock down *Iqgap2* expression. Forty-eight hours after transfection, total RNA was extracted and the extent of *Iqgap2* down-modulation was assessed by qRT-PCR. Each dot represents one experiment. Mean ± SEM; paired *t* test, two-tailed (****$P < 0.0001$). (*C*) Same as *B*, except that transfected cells were stimulated with IgE and antigen, and their ability to degranulate was measured by the release of β-hexosaminidase enzyme. $n = 4$ independent experiments. Mean ± SEM; unpaired *t* test, two-tailed. (*D*) Cells were treated as in *C*, except that cytokine production was measured by intracellular staining. Each dot represents one experiment. Mean ± SEM; paired *t* test, two-tailed. (*E*) WT and KO mast cells were transduced with a lentiviral vector to stably express HA-NFAT1(4–460)-GFP. After selection, cells were stimulated with IgE and antigen for the indicated times, and nuclear translocation of HA-NFAT1(4–460)-GFP was followed over time using a fluorescence microscope. Representative of $n = 8$ independent experiments. (*F*) Same as *B*, except that protein extracts were prepared from transduced cells and the extent of HA-NFAT1(4–460)-GFP dephosphorylation upon stimulation was assessed by Western blot using an anti-HA antibody. (*G*) Nuclear translocation of endogenous NFAT1 upon stimulation of WT and KO cells with 0.5 μM ionomycin was visualized by immunofluorescence using an anti-NFAT1 antibody. Nuclei were counterstained with DAPI. (*H*) Calcium flux was measured by flow cytometry in WT and KO cells loaded with Fluo-4 AM and stimulated with IgE anti-DNP antibody and HSA-DNP antigen as well as ionomycin. Shown are the compiled data of $n = 5$ independent experiments. Mean ± SEM; two-way ANOVA. MFI, mean fluorescence intensity.

6C), whereas cytokine expression showed just a very modest increase, which was statistically significant only for IL-13 (Fig. 6D). Notably, mast cell proliferation and survival were not significantly altered in WT mast cells upon knockdown of *Iqgap2* (Fig. S2A), despite an initial modest increase in cell death, which, however, normalized over time. These data point toward different mechanisms of regulation of acute (degranulation, cytokine production) and homeostatic (proliferation, survival) responses in mast cells.

Because IQGAP1 and IQGAP2 were shown to interfere with nuclear translocation of NFAT (37, 38), and NFAT is required for efficient transactivation of cytokine genes in mast cells (39, 40), we assessed whether nuclear translocation of NFAT was somehow affected in KO cells, due to the altered expression of *Iqgap* family members. First, we transduced WT and KO cells to stably express comparable levels of a reporter NFAT-GFP fusion protein (41), and then we assessed nuclear translocation of the reporter protein with a fluorescence microscope (Fig. 6E). Upon stimulation with IgE and antigen, we observed comparable NFAT nuclear translocation in both WT and KO cells, with comparable kinetics. Moreover, NFAT-GFP nuclear export at later time points of stimulation also appeared to be comparable

in WT and KO cells (Fig. 6E). Similarly, we found that the extent and kinetics of NFAT-GFP dephosphorylation and rephosphorylation upon stimulation with IgE and antigen were also comparable between WT and KO cells by Western blot (Fig. 6F). A similar trend was observed also for endogenously expressed NFAT1 (Fig. 6G), ruling out the possibility that increased NFAT translocation in KO cells could somehow contribute to the increased responsiveness of *Dnmt3a* KO cells. Accordingly, flow cytometry measurements of changes in calcium concentration upon WT and KO mast cell stimulation revealed only a modest trend toward slightly increased Ca^{2+} concentration in KO cells, which was, however, not statistically significant and of insufficient magnitude to alter the kinetics of NFAT translocation (Fig. 6H and Fig. S2B). We also assessed the levels of DNA methylation specifically at the *Iqgap2* locus (Fig. 7A) by restriction enzyme accessibility assay (REAA) and methylated DNA immunoprecipitation (MeDIP). First, by performing REAA analysis, we observed that the gene body of *Iqgap2* was overall methylated in both WT and KO cells, whereas both the core and distal promoter regions were mostly hypomethylated (Fig. 7B). Consistent with the published observation that dif-
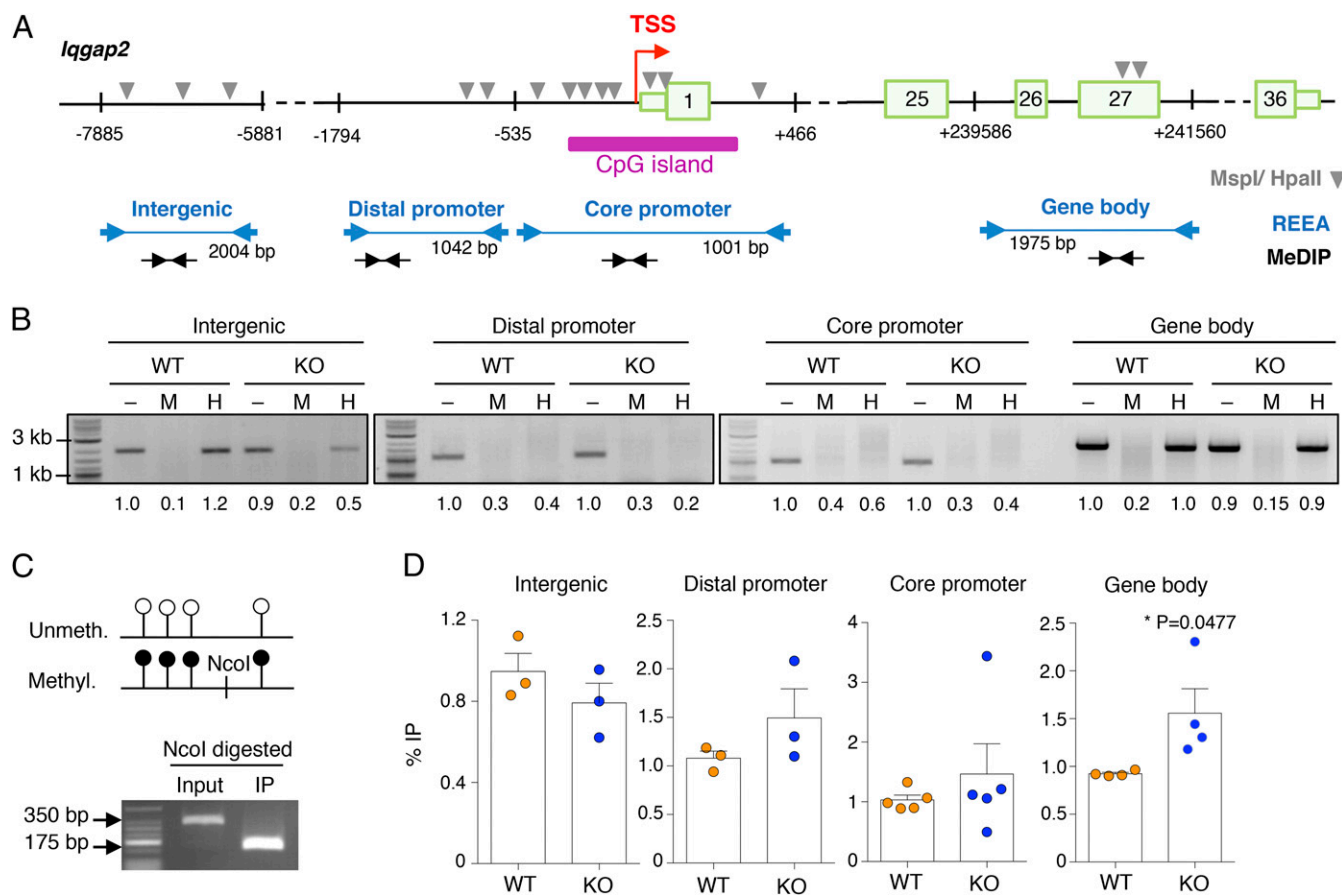

Fig. 7. Methylation analysis of the *Iqgap2* locus. (A) Schematic representation (not to scale) of the *Iqgap2* locus, with the location of the primers used for REAA and MeDIP-PCR indicated, as well as the location of the restriction sites (MspI/HpaII) required for REAA. Numbered boxes represent exons. (B) REAA of the *Iqgap2* locus: Purified genomic DNA of WT and KO cells was either left untreated (–) or digested with the methylation-insensitive enzyme MspI (M) or its CpG methylation-sensitive isoschizomer HpaII (H) before PCR analysis. Representative of n = 2 or 3 independent experiments, depending on the region. Numbers below the bands represent the relative quantification (to input WT) of band intensity. The sizes of the PCR products are indicated in the scheme in A. (C) To check for the efficiency of the IP reaction in all MeDIP experiments, control DNA, containing in vitro methylated and nonmethylated pUC19 plasmid at a ratio of 1:4, was added to every sample before the IP. The combination of plasmid-specific PCR primers with NcoI restriction digestion (site present only in the methylated plasmid) confirmed the specific enrichment of methylated DNA in all MeDIP experiments. Shown is one representative experiment out of at least four. (D) MeDIP-PCR of the *Iqgap2* locus: Sonicated genomic DNA from WT and KO cells was immunoprecipitated with an anti-5mC antibody. Shown is the percentage of immunoprecipitated methylated DNA relative to the input for each region. Each dot represents one independent experiment. Mean ± SEM; unpaired *t* test, two-tailed.

ferentially methylated regions in *Dnmt3a*-null cells showed substantial hyper- as well as hypomethylation at a variety of genomic regions (4, 24, 25), we found that an intergenic region in the proximity of the *Iqgap2* locus was substantially hypomethylated in KO cells (Fig. 7B). Because REAA is not sensitive enough to measure small differences in methylation levels, we performed a MeDIP analysis on these same regions. First, we optimized the IP method using as spike-in a mixture of two plasmids, either unmethylated or in vitro methylated, at a ratio of 4:1. As shown in Fig. 7C, before the IP, a PCR specific for the spike-in plasmids amplified primarily the more abundant unmethylated plasmid, whereas after the IP, we recovered only the methylated form of the plasmid (containing an extra NcoI restriction site that allowed for size discrimination of the PCR product), confirming the specificity of the process. By performing MeDIP on WT and KO mast cells, we found that the distal promoter, core promoter, and gene body regions of *Iqgap2* showed a modest increase in DNA methylation in the absence of *Dnmt3a* (Fig. 7D). At the core promoter region, results were more variable and differences were smaller, most likely due to the fact that this region coincided with a CpG island and was for the most part hypomethylated. The intergenic region appeared to be slightly demethylated in KO cells, concordant with the results of the REAA. Whether such differences in DNA methylation between WT and KO cells are sufficient to justify the observed altered expression of the *Iqgap2* gene remains to be understood. Other mechanisms, direct or indirect, may also lead to altered *Iqgap2* expression in *Dnmt3a* KO cells. Overall, our data suggest that diminished *Iqgap2* expression may contribute, at least in part, to the increased degranulation and cytokine production observed in mast cells lacking *Dnmt3a*, that such phenotypes appear to be NFAT-independent, and that it only partially correlates with changes in DNA methylation at the *Iqgap2* locus.

Exacerbated in Vivo Responses in the Absence of *Dnmt3a* on Acute and Chronic Mast Cell Activation. Because mast cells lacking *Dnmt3a* appeared phenotypically normal but with an increased propensity to respond to stimulation with enhanced cytokine production and degranulation activity, we assessed acute responses (which are primarily dependent on the release of mast cell granules) in an in vivo model of anaphylaxis-associated vascular hyperpermeability (passive cutaneous anaphylaxis) as well as chronic responses in a model of oxazolone-induced dermatitis (Fig. 8A). For passive cutaneous anaphylaxis, we performed adoptive transfer of WT and KO cells in Kit^W-sh/W-sh mice (42) by intradermal injection. Following reconstitution, mast cells were sensitized with IgE–anti-DNP antibody followed by systemic injection of antigen together with Evans blue dye (Fig. 8 A and B). As a measure of mast cell activation, we quantified the cutaneous extravasation of the dye after extraction from the tissue (Fig. 8B). In agreement with the increased responses observed in KO mast cells in vitro, we observed a significantly enhanced response of KO mast cells also during acute responses in vivo (Fig. 8B).

Allergic diseases are usually associated with repeated exposures to the allergen, which over time lead to chronic inflammation and tissue remodeling. Chronic responses in tissues are the result of a complex interplay between stromal cells and various immune cells, including mast cells, which can secrete several mediators with the potential to influence tissue remodeling. Oxazolone induces a T cell-dependent allergic contact hypersensitivity, which also involves mast cells and evolves over time into a chronic allergic inflammatory response that resembles atopic dermatitis (43). We therefore investigated whether the increased inflammatory response of *Dnmt3a* KO mast cells could also play a role during chronic responses. After adoptive transfer of WT and KO mast cells into Kit^W-sh/W-sh mice, the skin was first sensitized with 1% oxazolone, followed by repeated challenges with 0.5% oxazolone (Fig. 8A). We found that also in this chronic model, *Dnmt3a* KO mast cells acted in a more proinflammatory manner compared with their WT counterparts, leading over time to significantly increased ear thickness (Fig. 8C). Because the extent of inflammation in this model was shown to be dependent on mast cell-derived IL-2 production (43),
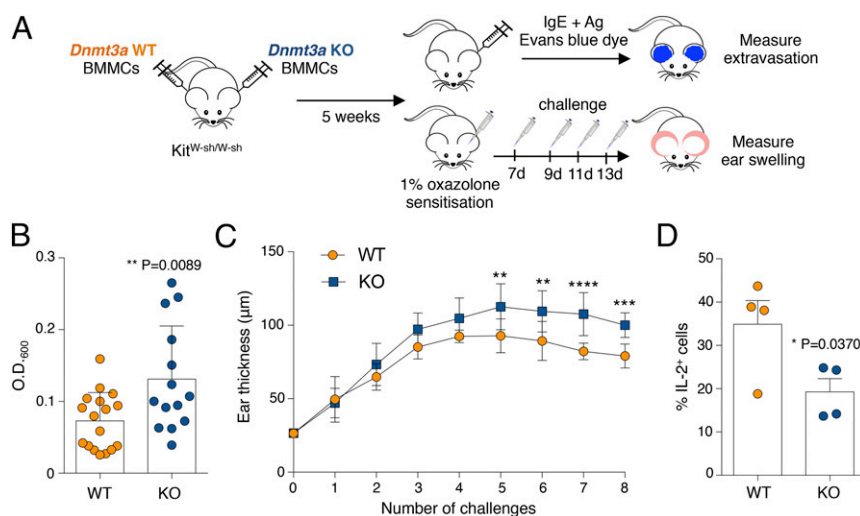
Fig. 8. Increased acute and chronic inflammatory responses in vivo in the absence of *Dnmt3a*. (A) Schematic representation of the experimental design for passive cutaneous anaphylaxis and oxazolone-induced contact sensitivity experiments. BMMCs, bone marrow-derived mast cells. (B) For passive cutaneous anaphylaxis experiments, the ear pinnae of Kit^W-sh/W-sh mice were reconstituted with WT or KO mast cells. After ~5 wk, ears were sensitized intradermally with IgE and then challenged 24 h later by i.v. injection of antigen in the presence of Evans blue dye. The dye was then extracted and measured. Each dot represents one mouse (WT, $n = 17$; KO, $n = 14$). Mean ± SD; unpaired *t* test, two-tailed. (C) Mice were reconstituted as in *B*, except that they were then challenged with 1% oxazolone, followed, 7 d later, by repeated challenges with 0.5% oxazolone. The extent of inflammation was assessed by measuring swelling of the ears. Data include measurements of eight mice (mean ± SD) and are representative of $n = 2$ independent experiments. Comparisons between the groups were performed by two-way ANOVA. **$P = 0.0061$, ***$P < 0.0001$, ****$P < 0.0001$. (D) WT and KO cells were stimulated for 5 h with PMA and ionomycin before intracellular cytokine staining for IL-2 expression. Each dot represents one experiment. Mean ± SEM; paired *t* test, two-tailed.

which has been implicated in the control of allergic diseases, we also measured mast cell-derived IL-2 by intracellular cytokine staining. We found a significant decrease in the ability of *Dnmt3a* KO cells to produce IL-2 (Fig. 8D), strongly suggesting that the capacity of KO cells to produce increased levels of inflammatory cytokines, coupled with the reduced production of IL-2, determines overall exacerbated responses to both acute and chronic stimulation. Overall, our data uncover a role for *Dnmt3a* in restraining mast cell proinflammatory responses.

Discussion

DNMT3A is one of the most frequently mutated genes in hematological malignancies and, due to extensive research, its role in cancer is becoming clearer (44, 45). Although we found a relative increase in the ability of mast cells to proliferate in the absence of *Dnmt3a*, most of the major phenotypes we observed in these cells were actually linked to acute and chronic cell activation, both in vitro and in vivo. Although the role of DNA methylation in general and DNMT3A in particular in allergy, asthma, and mast cell activation disorders is far from being completely understood, several studies have investigated the role of *Dnmt3a* in regulating cytokine production in T lymphocytes (reviewed in ref. 5). For example, the *Il2*, *Il4*, and *Ifng* cytokine genes are regulated by DNA methylation in T cells (46–48) and, in the absence of *Dnmt1* or *Dnmt3a* (but not *Dnmt3b*), T lymphocytes were unable to appropriately silence the expression of *Ifng* or *Il4*, resulting in unrestrained cytokine expression (49–51). T cells lacking *Dnmt3a* also showed increased *Il13* transcription (51), similar to the phenotype we observed in mast cells and, accordingly, these lymphocytes led to increased lung inflammation in a murine model of asthma, further highlighting the importance of DNA methylation in modulating the activity and pathogenesis of complex diseases such as allergy and asthma. Here, we found that not only did loss of *Dnmt3a* lead to enhanced cytokine production in mast cells in response to IgE and antigen complexes but it also led to increased degranulation, suggesting that dysregulated DNA methylation may have a profound effect on mast cell-related diseases.

The complex phenotype resulting from the genetic deletion of *Dnmt3a* is likely the end result of a number of direct and indirect effects acting on the regulation of gene expression at multiple levels. Regulation of gene expression is not a linear one-to-one process but a network of interactions between multiple chromatin- and DNA-modifying enzymes, TFs, and cofactors, as well as posttranscriptional regulators such as RNA-binding proteins and microRNAs (miRNAs), all acting in concert to determine a final outcome (52). For instance, altered DNA methylation patterns can interfere directly with the recruitment of readers of DNA methylation and with the binding of TFs, potentially leading to a cascade of altered TF recruitment and gene expression, which eventually hinders normal cellular functions. Apart from TFs and cofactors able to directly or indirectly bind the chromatin and translate DNA methylation information into a transcriptional outcome, miRNAs represent some of the factors with a complex interplay with DNA methylation. Indeed, not only DNA methylation can alter miRNA expression and consequently expression of downstream genes; miRNAs are also known to fine-tune the ex-

pression of target DNA-modifying enzymes. For example, the miR-29 family was shown to target DNMT1, DNMT3A, and DNMT3B, leading to global DNA hypomethylation and reexpression of tumor suppressor genes in acute myeloid leukemia cells (53). Although our preliminary screening did not identify any major significant modification in the global miRnome of *Dnmt3a* KO mast cells, we identified miR-223 as a dysregulated miRNA in the absence of *Dnmt3a* (Fig. S2C). We found that expression of this miRNA was reproducibly reduced in *Dnmt3a* KO mast cells compared with their WT counterparts, and such reduction could also indirectly contribute to the increased ability of *Dnmt3a* KO mast cells to degranulate in response to acute stimulation. Indeed, down-regulation of miR-223 was recently shown to promote mast cell degranulation in response to IgE stimulation (54).

Although the role of DNA methylation in stabilizing gene expression during development and cell-fate decisions and in maintaining memory of cellular identity is well-established (55, 56), its role during acute stimulation of already-differentiated cells is more nuanced and less understood. In this respect, as mentioned above, T lymphocytes have been studied more extensively than other cell types, showing in general unrestrained expression of genes related to effector functions. Our data provide clear evidence about a role for DNA methylation in general and *Dnmt3a* in particular in restraining responses of differentiated mast cells, suggesting that a more general role of DNA methylation in at least some types of differentiated cells may be to buffer and restrict excessive responses to environmental stimuli.

Materials and Methods

Mice and Cell Cultures. *Dnmt3a* knockout mice were purchased from The Jackson Laboratory, where they were generated as described (57). The *Dnmt3a*-deleted allele lacks exons 13 to 17 of the protein, roughly corresponding to part of the ATRX–DNMT3–DNMT3L domain and part of the catalytic domain, and leads to no detectable expression. Heterozygous intercrosses were used to generate age- and sex-matched KO and WT littermates for use in all experiments shown. Mast cells were differentiated in the presence of IL-3 as the essential survival and proliferation factor, as described (58). All animal studies were performed in accordance with Swiss Federal Veterinary Office guidelines and approved by the Cantonal animal experimentation committee, Dipartimento della Sanità e della Socialità Cantone Ticino (authorization nos. 02/2015 and 07/2015).

Statistical Analysis. Statistical analysis was performed with Prism software (GraphPad). Data are represented as mean ± SEM or SD, and significance was assessed by paired or unpaired Student's *t* test, two-tailed, or two-way ANOVA.

Full materials and methods can be found in *SI Materials and Methods*.

1. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. *Genes Dev* 25(10):1010–1022.
2. Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nat Genet* 33(Suppl):245–254.
3. Schübeler D (2015) Function and information content of DNA methylation. *Nature* 517(7534):321–326.
4. Yang L, Rau R, Goodell MA (2015) DNMT3A in haematological malignancies. *Nat Rev Cancer* 15(3):152–165.
5. Leoni C, Vincenzetti L, Emming S, Monticelli S (2015) Epigenetics of T lymphocytes in health and disease. *Swiss Med Wkly* 145:w14191.
6. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99(3):247–257.
7. Jeltsch A, Jurkowska RZ (2014) New concepts in DNA methylation. *Trends Biochem Sci* 39(7):310–318.
8. Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69(6):915–926.
9. Tatton-Brown K, et al.; Childhood Overgrowth Consortium (2014) Mutations in the DNA methyltransferase gene DNMT3A cause an overgrowth syndrome with intellectual disability. *Nat Genet* 46(4):385–388.
10. Trowbridge JJ, Snow JW, Kim J, Orkin SH (2009) DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells. *Cell Stem Cell* 5(4):442–449.
11. Tadokoro Y, Ema H, Okano M, Li E, Nakauchi H (2007) De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. *J Exp Med* 204(4):715–722.

12. Ley TJ, et al. (2010) DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* 363(25):2424–2433.

13. Shlush LI, et al.; HALT Pan-Leukemia Gene Panel Consortium (2014) Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* 506(7488):328–333.

14. Traina F, et al. (2012) Single nucleotide polymorphism array lesions, TET2, DNMT3A, ASXL1 and CBL mutations are present in systemic mastocytosis. *PLoS One* 7(8):e43090.

15. Yang IV, et al. (2015) DNA methylation and childhood asthma in the inner city. *J Allergy Clin Immunol* 136(1):69–80.

16. Monticelli S, Lee DU, Nardone J, Bolton DL, Rao A (2005) Chromatin-based regulation of cytokine transcription in Th2 cells and mast cells. *Int Immunol* 17(11):1513–1524.

17. Haenisch B, Fröhlich H, Herms S, Molderings GJ (2014) Evidence for contribution of epigenetic mechanisms in the pathogenesis of systemic mast cell activation disease. *Immunogenetics* 66(5):287–297.

18. Zhang X, et al. (2016) DNMT3A and TET2 compete and cooperate to repress lineage-specific transcription factors in hematopoietic stem cells. *Nat Genet* 48(9):1014–1023.

19. Mayoral RJ, et al. (2011) MiR-221 influences effector functions and actin cytoskeleton in mast cells. *PLoS One* 6(10):e26133.

20. Demo SD, et al. (1999) Quantitative measurement of mast cell degranulation using a novel flow cytometric annexin-V binding assay. *Cytometry* 36(4):340–348.

21. Liu S, Nugroho AE, Shudou M, Maeyama K (2012) Regulation of mucosal mast cell activation by short interfering RNAs targeting syntaxin4. *Immunol Cell Biol* 90(3):337–345.

22. Christman JK (2002) 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: Mechanistic studies and their implications for cancer therapy. *Oncogene* 21(35):5483–5495.

23. Bock C, et al. (2012) DNA methylation dynamics during in vivo differentiation of blood and skin stem cells. *Mol Cell* 47(4):633–647.

24. Challen GA, et al. (2011) Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet* 44(1):23–31.

25. Tiedemann RL, et al. (2014) Acute depletion redefines the division of labor among DNA methyltransferases in methylating the human genome. *Cell Reports* 9(4):1554–1566.

26. Koya J, et al. (2016) DNMT3A R882 mutants interact with polycomb proteins to block haematopoietic stem and leukaemic cell differentiation. *Nat Commun* 7:10924.

27. Chedin F, Lieber MR, Hsieh CL (2002) The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. *Proc Natl Acad Sci USA* 99(26):16916–16921.

28. Duymich CE, Charlet J, Yang X, Jones PA, Liang G (2016) DNMT3B isoforms without catalytic activity stimulate gene body methylation as accessory proteins in somatic cells. *Nat Commun* 7:11453.

29. Montagner S, et al. (2016) TET2 regulates mast cell differentiation and proliferation through catalytic and non-catalytic activities. *Cell Reports* 15(7):1566–1579.

30. Williams K, et al. (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* 473(7347):343–348.

31. Costa Y, et al. (2005) Two novel proteins recruited by synaptonemal complex protein 1 (SYCP1) are at the centre of meiosis. *J Cell Sci* 118(Pt 12):2755–2762.

32. Antonchuk J, Sauvageau G, Humphries RK (2002) HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* 109(1):39–45.

33. Hoser M, et al. (2008) Sox12 deletion in the mouse reveals nonreciprocal redundancy with the related Sox4 and Sox11 transcription factors. *Mol Cell Biol* 28(15):4675–4687.

34. Hedman AC, Smith JM, Sacks DB (2015) The biology of IQGAP proteins: Beyond the cytoskeleton. *EMBO Rep* 16(4):427–446.

35. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ (2013) Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods* 10(12):1213–1218.

36. McLean CY, et al. (2010) GREAT improves functional interpretation of *cis*-regulatory regions. *Nat Biotechnol* 28(5):495–501.

37. Sharma S, et al. (2011) Dephosphorylation of the nuclear factor of activated T cells (NFAT) transcription factor is regulated by an RNA-protein scaffold complex. *Proc Natl Acad Sci USA* 108(28):11381–11386.

38. Willingham AT, et al. (2005) A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. *Science* 309(5740):1570–1573.

39. Monticelli S, Rao A (2002) NFAT1 and NFAT2 are positive regulators of IL-4 gene transcription. *Eur J Immunol* 32(10):2971–2978.

40. Monticelli S, Solymar DC, Rao A (2004) Role of NFAT proteins in IL13 gene transcription in mast cells. *J Biol Chem* 279(35):36210–36218.

41. Aramburu J, et al. (1999) Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science* 285(5436):2129–2133.

42. Grimbaldeston MA, et al. (2005) Mast cell-deficient W-sash c-kit mutant Kit[W-sh/W-sh] mice as a model for investigating mast cell biology in vivo. *Am J Pathol* 167(3):835–848.

43. Hershko AY, et al. (2011) Mast cell interleukin-2 production contributes to suppression of chronic allergic dermatitis. *Immunity* 35(4):562–571.

44. Mayle A, et al. (2015) Dnmt3a loss predisposes murine hematopoietic stem cells to malignant transformation. *Blood* 125(4):629–638.

45. Yang L, et al. (2016) DNMT3A loss drives enhancer hypomethylation in FLT3-ITD-associated leukemias. *Cancer Cell* 29(6):922–934.

46. Kersh EN, et al. (2006) Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. *J Immunol* 176(7):4083–4093.

47. Makar KW, et al. (2003) Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. *Nat Immunol* 4(12):1183–1190.

48. Bruniquel D, Schwartz RH (2003) Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat Immunol* 4(3):235–240.

49. Gamper CJ, Agoston AT, Nelson WG, Powell JD (2009) Identification of DNA methyltransferase 3a as a T cell receptor-induced regulator of Th1 and Th2 differentiation. *J Immunol* 183(4):2267–2276.

50. Lee PP, et al. (2001) A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 15(5):763–774.

51. Yu Q, et al. (2012) DNA methyltransferase 3a limits the expression of interleukin-13 in T helper 2 cells and allergic airway inflammation. *Proc Natl Acad Sci USA* 109(2):541–546.

52. Macneil LT, Walhout AJ (2011) Gene regulatory networks and the role of robustness and stochasticity in the control of gene expression. *Genome Res* 21(5):645–657.

53. Garzon R, et al. (2009) MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood* 113(25):6411–6418.

54. Wang Q, et al. (2015) Down-regulation of microRNA-223 promotes degranulation via the PI3K/Akt pathway by targeting IGF-1R in mast cells. *PLoS One* 10(4):e0123575.

55. Bird A (2002) DNA methylation patterns and epigenetic memory. *Genes Dev* 16(1):6–21.

56. Monticelli S, Natoli G (2013) Short-term memory of danger signals and environmental stimuli in immune cells. *Nat Immunol* 14(8):777–784.

57. Skarnes WC, et al. (2011) A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* 474(7351):337–342.

58. Mayoral RJ, Monticelli S (2010) Stable overexpression of miRNAs in bone marrow-derived murine mast cells using lentiviral expression vectors. *Methods Mol Biol* 667:205–214.

59. Deho' L, et al. (2014) Two functionally distinct subsets of mast cells discriminated by IL-2-independent CD25 activities. *J Immunol* 193(5):2196–2206.

60. Rusca N, et al. (2012) MiR-146a and NF-κB1 regulate mast cell survival and T lymphocyte differentiation. *Mol Cell Biol* 32(21):4432–4444.

61. Schepers E, Glorieux G, Dhondt A, Leybaert L, Vanholder R (2009) Flow cytometric calcium flux assay: Evaluation of cytoplasmic calcium kinetics in whole blood leukocytes. *J Immunol Methods* 348(1–2):74–82.

62. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25(4):402–408.

63. Lara-Astiaso D, et al. (2014) Immunogenetics. Chromatin state dynamics during blood formation. *Science* 345(6199):943–949.

64. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15):2114–2120.

65. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9(4):357–359.

66. Li H, et al.; 1000 Genome Project Data Processing Subgroup (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25(16):2078–2079.

67. Zhang Y, et al. (2008) Model-based analysis of ChIP-seq (MACS). *Genome Biol* 9(9):R137.

68. Quinlan AR, Hall IM (2010) BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics* 26(6):841–842.

69. Heinz S, et al. (2010) Simple combinations of lineage-determining transcription factors prime *cis*-regulatory elements required for macrophage and B cell identities. *Mol Cell* 38(4):576–589.

70. Harrow J, et al. (2012) GENCODE: The reference human genome annotation for The ENCODE Project. *Genome Res* 22(9):1760–1774.

71. Fujita PA, et al. (2011) The UCSC Genome Browser database: Update 2011. *Nucleic Acids Res* 39(Database issue):D876–D882.

72. Leoni C, et al. (2015) Reduced DNA methylation and hydroxymethylation in patients with systemic mastocytosis. *Eur J Haematol* 95(6):566–575.