PU.1 Level-Directed Chromatin Structure Remodeling at the Irf8 Gene Drives Dendritic Cell Commitment

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SUMMARY

Dendritic cells (DCs) are essential regulators of immune responses; however, transcriptional mechanisms that establish DC lineage commitment are poorly defined. Here, we report that the PU.1 transcription factor induces specific remodeling of the higher-order chromatin structure at the interferon regulatory factor 8 (Irf8) gene to initiate DC fate choice. An Irf8 reporter mouse enabled us to pinpoint an initial progenitor stage at which DCs separate from other myeloid lineages in the bone marrow. In the absence of Irf8, this progenitor undergoes DC-to-neutrophil reprogramming, indicating that DC commitment requires an active, Irf8-dependent escape from alternative myeloid lineage potential. Mechanistically, myeloid Irf8 expression depends on high PU.1 levels, resulting in local chromosomal looping and activation of a lineage- and developmental-stage-specific cis-enhancer. These data delineate PU.1 as a concentration-dependent rheostat of myeloid lineage selection by controlling long-distance contacts between regulatory elements and suggest that specific higher-order chromatin remodeling at the Irf8 gene determines DC differentiation.

INTRODUCTION

During myelopoiesis, self-renewing hematopoietic stem cells (HSCs) differentiate into broadly myeloid-committed progenitors (common myeloid progenitors [CMPs]), which further differentiate into two major branches: polymorphonuclear phagocytes, comprising granulocytes as well as mast cells, and mononuclear phagocytes, comprising monocytes, macrophages, and dendritic cells (DCs) (Hume, 2008; Geissmann et al., 2010; Liu and Nussenzweig, 2010; Kushwah and Hu, 2011). Although the separation process of granulocytes from monocytes is relatively well studied, it is poorly understood how myeloid precursors specify into DCs. DCs are a heterogeneous cell population with a critical role in immune response and self-tolerance (Steinman and Cohn, 1973; Merad and Manz, 2009). In the current model, DCs are replenished from macrophage-DC progenitors (MDPs) harboring both macrophage and DC potential (Fogg et al., 2006; Geissmann et al., 2010). MDPs are thought to directly yield common DC progenitors (CDPs) (Onai et al., 2007). CDPs are a source of both conventional DCs (cDCs) and plasmacytoid DCs (pDCs).

A number of transcription factors, such as PU.1, SpiB, Tcf4 (E2-2), Batf3, Id2, and Irf8 (also known as interferon consensus sequence binding protein [ICSBP]) have been implicated in DC development (Watowich and Liu, 2010; Carotta et al., 2010; Schotte et al., 2004; Cisse et al., 2008; Hildner et al., 2008; Hacker et al., 2003). However, it is currently unknown if and how these factors drive the initiation of DC commitment from CMPs. Therefore, we aimed to decipher transcriptional mechanisms initiating DC fate selection. For this purpose, we chose to explore the molecular function and transcriptional regulation of the Irf8 gene. Irf8−/− mice have profoundly depleted pDCs and CD8α+ cDCs (Tsujimura et al., 2002, 2003b; la Sala et al., 2009) but generate more neutrophils and develop a syndrome that resembles human BCR-ABL+ chronic myeloid leukemia (Holtschke et al., 1996; Schiavoni et al., 2002; Tsujimura et al., 2003a; Ginhoux et al., 2009). Moreover, mutations inactivating the Irf8 DNA binding domain have recently been shown to cause human DC immunodeficiency (Hambleton et al., 2011).

Here, we generated Irf8 reporter mice and identified the progenitor stage at which DC lineages separate from alternative myeloid lineages. We show that Irf8 is required for the production of this progenitor and reveal that the initiation of Irf8 expression is controlled by PU.1-induced higher-order chromatin remodeling.

RESULTS

Irf8 Separates the Early DC from Alternative Myeloid Lineage Programs

Irf8−/− mice lack CD8α+ cDCs and pDCs but maintain normal CD11b+ cDCs, as has been reported previously (Figures S1A
Figure 1. Irf8 Expression Is Required for Transcriptional Identity of Early DC Progenitors

(A) The Cx3cr1GFP reporter allele was introduced into the Irf8−/− background, and Cx3cr1GFP−/− against c-kit in Lin−/−Sca-1−/IL7Rα−/− gated BM cells was analyzed by FACS.

(B) Total numbers of indicated cell populations of Irf8−/−::Cx3cr1GFP−/− and Irf8+/+::Cx3cr1GFP−/− mice are shown ± SD, n = 3–5 each. LSKs were Lin−/−Sca1−/−c-kit+ cells, GMPs were Lin−/−Sca1−/−c-kit+CD34−/−FcγRII/IIIhighGFP−, and CMPs were Lin−/−Sca1−/−c-kit+CD34−/−FcγRII/III−/−*GFP−/−.

(C–D) FACS of MDP-derived cells after 7 days in methylcellulose cultures with 10 ng/ml GM-CSF and 40 ng/ml IL-4. We plated 1,000 flow-sorted MDPs and pooled colonies for analysis. *, p ≤ 0.05; **, p ≤ 0.005; ***, p ≤ 0.001; n.s., not significant; n = 3 each.

(E) Heat maps showing downregulated (yellow) or upregulated (blue) genes in MDPs−/− (Lin−/−Sca-1−/−c-kit+M-CSFR+; C0) of Irf8−/− mice. Differentially expressed genes were grouped into DC, neutrophil, monocyte, or shared DC and monocyte signature genes, as indicated by the color code on the right side. For detailed information about signature genes, see Experimental Procedures, Figure S1F, and Tables S1–S5.

(F) Relative distribution of upregulated myeloid signature genes in Irf8−/−−/− and in Irf8+/+ MDPs−/−.

(G) A density plot showing log2 fold changes of probe hybridization of Irf8−/−−/− versus Irf8+/+ MDP progenitors for all DC, neutrophil, and monocyte signature genes. A right shift indicates higher expression in Irf8−/− MDPs, and a left shift indicates lower expression.

To address whether Irf8 affects MDP fate by skewing transcriptional lineage programs, we isolated MDPs−/− from Irf8−/−−/− and Irf8+/+ mice for genome-wide expression profiling. In the Irf8−/−−/− progenitors, the expression of 104 genes was increased, and the expression of 117 genes was decreased by more than 2-fold (p < 0.05). Unbiased categorization of the differentially expressed genes for different phagocyte lineage-associated gene signatures (Figure S1F and Tables S1–S4) revealed that the Irf8−/−−/− progenitors expressed many DC and/or macrophage genes but few neutrophil genes (Figures 1E and 1F). A loss of Irf8 reversed this distribution in that the expression of DC and shared DC and macrophage genes were reduced, but the expression of neutrophil genes was enhanced. Notably, Irf8 deletion had only minimal effects on macrophage gene expression. Moreover, analysis of the expression of the entire signature gene sets within the Irf8−/−−/− and Irf8+/+ progenitors revealed that Irf8 deficiency resulted in an overall weaker expression of the DC signature,
tracing Irf8 expression at the single-cell level is crucial for understanding the transcriptional separation of the DC from macrophage potential. Because the data showed that Irf8 ablation functionally and transcriptionally separated DC from macrophage potential of MDPs, we reasoned that tracing Irf8 expression at the single-cell level is important for elucidating the transcriptional separator of the DC from macrophage potential.

Tracing Irf8 Expression by Generation of Reporter Mice

We conclude that Irf8 is important for MDP transition to the CDP by orchestrating the shift of neutrophil-to-DC gene expression programming. Irf8 deficiency did not change the MDP macrophage program, indicating that Irf8 also acts as a transcriptional separator of the DC from macrophage potential.

By isolating a phage artificial chromosome (PAC; Mouse PAC Library RPCI-21 296K2) comprising 130 kb of chromosome 8, including all Irf8 exons with flanking sequences of approximately 81.3 kb 5' and 28.3 kb 3'. A reporter cassette with an internal ribosomal entry site linked to a VENUS yellow fluorescence marker (ires-VENUS) was inserted into the 3' untranslated region of Irf8 exon 9 (Figure 2A), resulting in the expression of a wild-type (WT) Irf8 protein and the VENUS reporter from a bicistronic marker (ires-VENUS) was inserted into the 3' untranslated region and a 2.2 kb fragment containing the ∼50 kb enhancer was flanked by loxP sites (black arrowheads). The Irf8 gene exon structure (black boxes), the transcription start site (TSS), and the orientation of transcription (arrow) are indicated.

Quantitative RT-PCR (qRT-PCR) showing mean Irf8 mRNA expression values ± SD in peripheral blood neutrophils (CD11b+Ly6c+M-CSFR) and B cells (B220+IgM+) and splenic macrophages (CD11b+M-CSFR), cDCs (CD11c+C220), and pDCs (CD11c+C220) all sorted from nontransgenic mice.

VENUS expression in Irf8<sup>loxPloxP</sup> transgenic mice (red) and nontransgenic littermates (shaded) derived from line 88. Cell populations were gated as in Figure 2C with the addition of BM erythrocytes (Ter119<sup>−</sup>). The analysis was performed four times with similar results. Mean fluorescence intensity (MFI) is given for VENUS expression of indicated populations. See also Figure S2.

Figure 2. Generation and Validation of Irf8-VENUS Reporter Mice

(A) A schematic map of the genomic PAC clone containing base pairs 123,197,900–123,308,928 of murine chromosome 8 (assembly version NCBI37/mm9), which includes the Irf8 gene. An ires-VENUS cassette was inserted in the Irf8 3' untranslated region and a 2.2 kb fragment containing the ∼50 kb enhancer was flanked by ioxP sites (black arrowheads). The Irf8 gene exon structure (black boxes), the transcription start site (TSS), and the orientation of transcription (arrow) are indicated.

(B) Left, the distribution of VENUS expression in the BM of transgenic mice (line 88). Right, protein from flow-sorted VENUS<sup>−</sup> (neg.) and VENUS<sup>+</sup> (pos.) fractions were subjected to western blotting and probed with antibodies specific to the indicated proteins.

(C) Quantitative RT-PCR (qRT-PCR) showing mean Irf8 mRNA expression values ± SD in peripheral blood neutrophils (CD11b+Ly6c+M-CSFR) and B cells (B220+IgM+) and splenic macrophages (CD11b+M-CSFR), cDCs (CD11c+C220), and pDCs (CD11c+C220) all sorted from nontransgenic mice.

(D) VENUS expression in Irf8<sup>loxPloxP</sup> transgenic mice (red) and nontransgenic littermates (shaded) derived from line 88. Cell populations were gated as in Figure 2C with the addition of BM erythrocytes (Ter119<sup>−</sup>). The analysis was performed four times with similar results. Mean fluorescence intensity (MFI) is given for VENUS expression of indicated populations. See also Figure S2.
were restricted to VENUS+ cells, indicating that the PAC reported Irf8 expression with high stringency. Moreover, the lineage-specific expression pattern of the VENUS reporter closely paralleled endogenous Irf8 expression (Figures 2C, 2D, S2G, and S2H). Hence, the reporter mice properly reflected in vivo Irf8 gene expression.

The Irf8VENUS Reporter Marks a Subpopulation of the MDP

Next, we analyzed Irf8 reporter expression in early hematopoietic precursors. HSCs, multipotent progenitors (MPPs), and common lymphoid progenitors (CLPs) had uniform VENUS levels, indicating that all these cells expressed Irf8 (Figures 3A and S3A). In contrast, only a small subset of myeloid progenitors (MPs) expressed VENUS (representing ~0.03% of all nucleated BM cells). This subset (designated Irf8VENUS+ MPs) was comprised of proliferating cells of typical undifferentiated progenitor phenotype that were essentially restricted to the BM (Figures 3B–3D and S3B). Irf8VENUS− MPs gave rise to Irf8VENUS+ MPs in short-term culture assays, but not the other way around, indicating that there is a lineage relationship between both populations, Irf8VENUS+ MPs being downstream of this relationship (Figure 3E).

Next, we explored the relationship between Irf8VENUS+ MPs and Cx3cr1GFP+ MDPs by crossing Irf8VENUS+ mice. Whereas all Cx3cr1GFP− myeloid progenitors were VENUS− and all Cx3cr1GFP+ CDPs were VENUS+, approximately half of the Cx3cr1GFP+ MDP population expressed the VENUS reporter (Figure 3F). Thus, the MDP comprises two subfractions that can be separated on the basis of Irf8VENUS expression.

Irf8VENUS+ MPs Are Early DC-Restricted Precursors

To evaluate the differentiation potential of Irf8VENUS+ MPs, we performed transplantation experiments in mice. We injected 25,000 FACS-sorted Irf8VENUS+ or Irf8VENUS− MPs (Figure 4A, S4A, and S4B) or, as a control, 25,000 CDPs (all CD45.2+), into
The tail veins of sublethally irradiated congenic mice (CD45.1+) and analyzed their cellular progeny 11 days posttransplantation. Irf8Venus+ MPs gave rise to 1%–2% nucleated cells in the spleens and ~0.3% in the BM of recipients (Irf8Venus− MPs, ~3% in spleens and ~0.9% in BM; CDPs, 0.8% in spleens and 0.1% in BM). In the spleen, we exclusively detected CD11c+MHCII+B220+ cDC progeny of Irf8Venus+ MPs, whereas, in the BM, we also found donor-derived CD11c+ B220+ pDCs (Figure 4B). Irf8Venus+ MPs completely lacked any CD11c− B220+ B cell, Ly6c+M-CSFR+ neutrophil, or Ly6c+−M-CSFR+ monocyte and macrophage potential. Irf8Venus− MPs differentiated into neutrophils, monocytes and macrophages, cDCs and pDCs, but not into B cells. CDPs produced cDCs and pDCs as described before (Onai et al., 2007). Both CD8α+ and CD8α− pDCs were produced by CDPs and Irf8Venus+ MPs at similar frequencies, suggesting that both progenitors are within the same differentiation pathway (Figure S4C). Furthermore, we confirmed a lack of Irf8Venus+ MP differentiation potential into macrophages by the absence of F4/80, MOMA-1, MARCO, and SIGNR1 expression on VENUS+ donor-derived splenocytes (Figure S4D). Moreover, we corroborated DC-restricted differentiation potential of Irf8Venus+ MPs by transplantation into nonirradiated recipient mice and analysis after 7 days (Figure 4C).

To further determine the DC differentiation potential of Irf8Venus+ MPs, we performed in vitro culture experiments in the presence of GM-CSF and FLT3 ligand (FLT3L) for 7 days. Supporting the in vivo results, Irf8Venus− MPs almost exclusively produced CD11c+MHCII+ DCs with a typical spread-out morphology, whereas Irf8Venus+ MPs produced much fewer DCs (Figures S4F–S4G).

Altogether, these experiments demonstrated that the Irf8Venus reporter marked an early BM progenitor with exclusive DC differentiation potential.

A Distal Enhancer Drives Myeloid Irf8 Promoter Activity

The data above indicated that the onset of myeloid Irf8 expression phenotypically marks and functionally determines the developmental stage of DC lineage selection in the BM. To decipher regulatory mechanisms preceding DC fate choice, we explored how Irf8 expression is initiated. Given that lineage-specific gene expression is primarily orchestrated through gene-distant regulatory elements (Heinz et al., 2010), we computationally identified a number of different evolutionarily conserved noncoding sequences (CNSs) upstream of Irf8 (Figure 5A).

We performed reporter assays in stably transfected RAW264.7 myeloid cells and NIH 3T3 fibroblasts to test whether these CNSs were able to enhance the activity of the Irf8 proximal promoter in chromatin context. Both the Irf8 promoter alone and

Figure 4. Irf8Venus+ Myeloid Progenitors Are Early cDC Precursors

(A) The experimental strategy of myeloid progenitor transplantation. Initial gating of Irf8Venus+ MPs was extended by gating on FcyRII/III+ cells to avoid cell-sort contamination with HSCs. See also Figure S3B.

(B) FACS of CD45.2+ donor-derived splenocytes and BM cells 11 days after intravenous transplantation with Irf8Venus+ MPs (Lin−Scal+IL7Rx+ c-kit+FcyRII/III/Irf8-Venus+), Irf8Venus− MPs (Lin−Scal+IL7Rx− c-kit+FcyRII/III/Irf8-Venus−), or CDPs (Lin−Scal− c-kit+M-CSFR+Flt3+) into sublethally irradiated (6 Gy) recipient mice. Results are representative of five independent experiments for a total number of eight CDP− (average cells per recipient = 500 spleen, 200 BM), 12 each Irf8Venus+ MP− (average cells per recipient = 1,000 spleen, 300 BM), or Irf8Venus− MP− (average cells per recipient = 2,000 spleen, 1,000 BM) transplanted mice, respectively. Mean values of at least five mice ± SD are given.

(C) FACS of CD45.2+ donor-derived splenocytes and BM cells 7 days after intravenous transplantation with Irf8Venus+ MPs or Irf8Venus− MPs into non irradiated recipient mice. See also Figure S4.
the promoter in combination with the −38 kb or −16 kb CNSs were unable to drive reporter gene expression. However, the addition of the −11 kb CNS led to a 20-fold increase in promoter activity in RAW264.7 cells, and the addition of the −50 kb CNS increased the activity 750-fold (Figure 5B). No enhancer function was observed in NIH 3T3 cells.

The −50 kb Enhancer Controls Irf8 Expression in MDPs

Because the −50 kb CNS displayed the highest enhancer activity in vitro, we tested whether this element was required for Irf8 expression in vivo. We modified the Irf8VENUS reporter PAC by flanking a 2.2 kb fragment containing the −50 kb CNS with loxP sites and generated transgenic animals (see above).

We crossed two of these transgenic lines (87 and 88) with CMV-cre deleter animals (Schwenk et al., 1995) to generate a germine deletion of the −50 kb element (designated Irf8VENUS−50kbΔ mice) (Figure 5C). We confirmed proper excision, copy number maintenance, and genomic integrity of the Irf8VENUS−50kbΔ transgene in the resulting offspring (Figures S5A–S5C).

Irf8VENUS−50kbΔ mice demonstrated a tight cell-type- and developmental-stage-specific requirement of the −50 kb enhancer for Irf8 expression. Deletion of the −50 kb enhancer decreased VENUS expression only mildly in HSCs and MPPs (both included in the LSK fraction), B cells, and pDCs more strongly in CD8α+ cDCs and most profoundly in MDPs* (Figures 5D, 5E, and S5D).
Next, we explored the functional relevance of the −50 kb element for MDP* formation by introducing the Irf8VENUS−50kb+ or Irf8VENUS−50kbΔ transgenes into the Irf8-deficient background. Importantly, the Irf8VENUS−50kbΔ transgene completely restored normal Irf8+ MDPs* (Figure 5F). In contrast, the Irf8VENUS−50kbΔ transgene had 54% less efficient restoration capacity.

Altogether, genetic experiments in mice provide in vivo evidence for a specific role of the −50 kb element in the control of Irf8 expression in MDPs.

### Higher-Order Chromatin Structure Remodeling at the Irf8−50 kb Enhancer

An important question was why the −50 kb enhancer had such a specific effect on Irf8 expression in DC progenitors. We addressed this issue by exploring the higher-order chromatin structure at the Irf8 locus using chromosomal conformation capturing (3C). First, we screened the Irf8 upstream CNSs for spatial three-dimensional contacts with the proximal Irf8 promoter region in RAW264.7 cells. We observed that the −50 kb enhancer and the −16 kb CNS, but not the −38 or −11 kb CNSs, were in spatial proximity with the Irf8 promoter, thus indicating a specific activating chromatin looping architecture at the Irf8 locus (Figure 6A).

To corroborate the observation that the −50 kb enhancer-to-Irf8 promoter contact existed in primary cells, we used a quantitative 3C (q3C) protocol on FACS-sorted cells. Indeed, the −50 kb enhancer was found in physical contact with the Irf8 promoter in MDPs* but is not found to be in physical contact in MPs (Figure 6B). Moreover, comparison across cell types demonstrated that −50 kb enhancer contact with the Irf8 promoter was strongest in MDPs* but decreased thereafter in their cDC, macrophage, and pDC progeny and was undetectable in B cells (Figure 6C). Thus, the −50 kb enhancer was brought into physical contact with the Irf8 gene by tightly controlled remodeling of its higher-order chromatin structure. The dynamics of this remodeling closely paralleled the onset of Irf8 expression during...
Figure 7. PU.1 Expression Induces −50 kb Irf8 Enhancer Activity and Chromatin Remodeling
(A) Two adjacent sequence-conserved PU.1 binding motifs are located in the −50 kb element of vertebrates.
(B) The −50 kb element is transcriptionally active and binds PU.1 in cDCs, but not in B cells. ChIP results in the indicated cell types with antibodies recognizing PU.1, acetylated histone H3K9 (H3K9ac), and IgG as control are shown. Specific primers that amplify the −50 kb region by real-time PCR were used. Values normalized to input are given ± SD of three different reactions.
(C and D) Reporter assay in RAW264.7 cells stably transfected with the pcP2-50kb-Irf8prom-luc luciferase construct are shown.
(D) Cells were either mock transfected or transiently transfected with constructs expressing shRNAs against PU.1 or a scramble control, along with a GFP marker to allow flow cytometric sorting of transfected cells. The mock control was set as 100%. The experiment was repeated twice with similar outcomes. Mean values ± SD are given; p = 0.003.
(E) q3C shows increased interaction frequency of the −50 kb fragment with the Irf8 promoter 8 hr after 4-hydroxytamoxifen (OHT) induction of PU.1 expression in PU.1+/−/ERT-PU.1 cells (PU.1ER). n = 3 experiments with similar outcomes. All data were normalized as in Figure 6B. Interaction in PU.1+/−/ERT-PU.10 hr induced was set to 1. Mean values ± SD are given; p = 0.003.
(F) qRT-PCR of Irf8 and PU.1 mRNA expression comes. Mean values ± SD are given; p = 0.005.
(G) qRT-PCR of Irf8 and PU.1 reporter expression in MHCII−/− cells transduced with retrovirus expressing GFP alone, Irf8-GFP, or PU.1-GFP. Cells were kept in liquid culture for 8 days with 100 ng/ml Flt3L and were gated on GFP-expressing cells for analysis. n = 3 replicates per condition were analyzed.

** , p ≤ 0.005 and *** , p ≤ 0.001.

myeloid differentiation and, thus, provided an explanation for the specific effect of −50 kb enhancer deletion on DC progenitors in mice.

**PU.1 Binding Is Necessary for −50 kb Enhancer Activity and Induces Chromatin Remodeling**

A computational motif search identified two directly adjacent PU.1-binding consensus motifs that were highly conserved among vertebrate species (Figure 7A). Indeed, PU.1 occupied these sites in primary DCs but did not occupy these sites in B cells (Figure 7B). PU.1 occupancy was associated with cell-type-specific H3K9 acetylation, indicating a transcriptional regulatory activity of the −50 kb element in DCs. In the RAW264.7 cell-line model, mutations in either one of these PU.1-binding sites or knockdown of PU.1 by small hairpin RNA (shRNA) diminished −50 kb enhancer activity as well as endogenous Irf8 expression (Figures 7C, 7D, and 5A). Furthermore, activation of a 4-hydroxytamoxifen (OHT)-responsive PU.1-ERT fusion protein in a PU.1−/− myeloid progenitor cell line (termed PU.1ER cells) revealed PU.1 as a direct activator of Irf8 expression (Figures S6B–S6D).
To investigate whether PU.1 controls chromatin looping of the −50 kb enhancer to the Irf8 promoter, we performed q3C assays in PU.1ER cells and found that PU.1 induction rapidly led to a marked increase in looping frequency (Figure 7E).

−50 kb Enhancer Chromatin Structure Remodeling Requires a High PU.1 Level

Intriguingly, although the expression of both Irf8 and PU.1 increased to similar levels in MDPs, the expression of Irf8 was absent in MPs, whereas the expression of PU.1 was low but readily detectable in MPs (Figure 7F), suggesting that high PU.1 levels are required to trigger Irf8 transcription. To directly test this, we quantified Irf8 expression in myeloid progenitors from PU.1 hypomorphic mice that expressed PU.1 at a 5-fold lower level than the corresponding WT cells (Rosenbauer et al., 2004). Indeed, these progenitors showed almost a complete lack of Irf8 transcripts, indicating that the reduced PU.1 level provided by the hypomorphic alleles was insufficient for Irf8 expression (Figure 7G). In line with this, shRNA-mediated gradual reduction of PU.1 levels in the RAW264.7 model impaired −50 kb enhancer-to-Irf8 promoter contact formation (Figure 7H).

Altogether, these data indicate that PU.1 can function as a concentration-dependent trigger of Irf8 chromatin remodeling and transcription.

PU.1 Fails to Drive DC Differentiation in the Absence of Irf8

PU.1 is known to induce DC differentiation (Carotta et al., 2010; Guerriero et al., 2000). To test whether PU.1 requires Irf8 to induce DC development, we transduced c-kit+ BM progenitors from Irf8−/−; mice with retroviruses expressing either PU.1 or Irf8 along with GFP from bicistronic mRNAs or with a retrovirus expressing GFP only. GFP+ cells were sorted and cultured with Fit3L to support generation of DCs. Ectopic PU.1 failed to drive development of MHCII+CD11c+B220− cDCs and MHCII+CD11c+B220+ pDCs of Irf8−/− progenitors, whereas Irf8 restoration rescued the capacity to produce both DC populations (Figures 7I, S6E, and S6F). This result indicates that PU.1 requires the presence of Irf8 to induce DC differentiation.

DISCUSSION

DCs are related to, but clearly distinct from, macrophages (Merad and Manz, 2009; Geissmann et al., 2010), and the identification of cellular precursors of the DC lineage has been the focus of intense research (Ginhoux et al., 2009; Fogg et al., 2006; Manz et al., 2001; Carotta et al., 2010; Liu et al., 2009; Waskow et al., 2008). Taking advantage of a newly generated Irf8 reporter mouse, we report a distinct Irf8-expressing subfraction of the MDP (Fogg et al., 2006). This Irf8VENUS+ population had the exclusive capacity to differentiate into DCs, but not into monocytes or macrophages, in vivo. Although the cDC potential of Irf8VENUS+ MPs was strong, the pDC potential was weaker than that of CDPs. One likely explanation for this difference could be that Irf8VENUS+ MPs require more time than CDPs to fully replenish pDCs. This, along with their higher c-kit level, supports the idea that Irf8VENUS+ MPs reside upstream of CDPs. However, the question of whether Irf8VENUS+ MPs are a separate population or whether they overlap with the earliest CDPs requires further investigation. In any case, the identification of Irf8VENUS+ MPs as an initial DC precursor within the formerly identified MDP population is instrumental in deciphering the earliest molecular events initiating DC development.

Irf8-reporter-based marking of this early DC stage is associated with functional Irf8 dependency in the production of DC progenitors. MDPs isolated from Irf8−/− mice had maintained macrophage and monocyte differentiation capacity but completely lacked DC potential in vitro. A recent study suggested that Irf8 is required for the production of DC progenitors and for the restriction of GMP expansion (Becker et al., 2012). By introducing the Cx3cr1GFP reporter into Irf8−/− mice, we revealed that Irf8 deficiency reduced DC development by impairing the transition of MDPs into CDPs. In contrast, GMPs were not affected. The early differentiation block in Irf8−/− mice appears surprising, given that not all DC populations are absent in these animals. However, our data showed that some progenitors can transit into the CDP stage even in the absence of Irf8. These cells may be biased to replenish certain DCs. Alternatively; Becker et al. (2012) have shown that Irf8−/− progenitors are less able to replenish all DC populations in chimeric mice, suggesting that homeostatic proliferation may also contribute to the maintenance of CD11b+ DCs in Irf8-deficient animals.

Irf8−/− MDPs showed a lower expression of DC-associated genes but a higher expression of neutrophil-associated genes and an unchanged expression of macrophage-associated genes. This finding indicated that the initiation of DC differentiation choice requires an active, Irf8-dependent shift from neutrophil-to-DC gene expression in MDPs. In light of these data, it appears most likely that the excessive production of neutrophils leading to the development of granulocytic leukemia in Irf8−/− mice may be the result of this DC-to-neutrophil “conversion” of the MDP. It will be interesting to see whether a similar mechanism also underlies the development of Irf8-mutated human DC immunodeficiency, which can be accompanied by high neutrophil counts as well (Hambleton et al., 2011).

A chromatin-based mechanism driving the molecular commitment of early myeloid progenitors toward DC differentiation has remained elusive. Here, we were able to pinpoint a region located 50 kb upstream of the Irf8 gene as the major enhancer driving Irf8 expression during DC progenitor formation in vivo. Deletion of the −50 kb enhancer from the engineered Irf8 PAC led to reduced reporter gene expression in MDPs, but not in stem cells, pDCs, or B cells. Accordingly, DC progenitor development was impaired in the absence of the −50 kb enhancer.

Different models of how distal regulatory elements communicate with proximal gene regulatory regions have been proposed (Wittkopp and Kalay, 2012; Bulger and Groudine, 2011). One of these models suggests that enhancers are placed in physical proximity to promoters through chromosomal looping (Heermann, 2011). Indeed, on the basis of 3C technology, there are now a number of examples known that support the looping model (Palstra et al., 2003; Tolhuis et al., 2002; Apostolou and Thanos, 2008; Spilianakis et al., 2005; Ling et al., 2006). We found that looping of the −50 kb enhancer to the Irf8 promoter occurred with high specificity with regard to the lineage and developmental stage and, at least in a cell-line model, required
PU.1 as a coordinator. Although it is not yet clear whether PU.1 controls Irf8 chromatin looping directly, our findings suggest a concentration-dependent model in which high PU.1 amounts are required to drive Irf8 locus looping and transcription. Such a model is supported by the recent finding that high PU.1 levels are also needed to drive Fit3 expression in cDC progenitors (Carotta et al., 2010). Although our retroviral transduction assays have shown that PU.1 requires Irf8 as a downstream target to drive DC development, Irf8 could not substitute PU.1 in DC differentiation (see Figure 7A) (data not shown). Again, this is similar to the inability of Fit3 to rescue PU.1-deficient DC production (Carotta et al., 2010). Altogether, these results suggest that PU.1 controls multiple independently acting molecular pathways to drive DC development. This idea is supported by our results showing that PU.1 controls Fit3 expression independently of Irf8 (data not shown).

In summary, we have isolated an initial Irf8+ DC progenitor. In this progenitor, Irf8 transcriptionally organizes the separation of the DC program from that of other myeloid lineages, including the monocyctic program. PU.1 induces Irf8 expression by remodelling its higher-order chromatin configuration to loop a distant cis-enhancer into physical proximity to the Irf8 promoter.

EXPERIMENTAL PROCEDURES

Mice and Cell Lines
All mice in this research were studied on a C57Bl/6 background. C57Bl/6 wild-type mice were from Charles River Laboratories. Irf8−/− and URE+/− mice were generated as described before (Hoitischke et al., 1996; Rosenbauer et al., 2004). Irf8Venus+PAC reporter mice were generated by pronuclear injection of the engineered murine Irf8-PAC (Figure S1A) into WT C57Bl/6 fertilized oocytes. Injection was conducted at the Max Planck Institute of Molecular Cell Biology and Genetics. PAC+ animals were identified by FACS and PCR (primers are available on request). Three independent PAC lines were established and used for this study. Two of these lines were bred to CMV-Cre mice (data not shown).

Microarrays
Lin−IL7Rx−Sca1−c-kit−“M-CSFR”+ progenitors were sorted from BM of three independent pools of Irf8−/− and Irf8+/− mice, respectively. Every pool consisted of cells from three to four animals at the age of 8–12 weeks. Additionally, monocyes and macrophages (CD11b+Ly6C+M-CSFR+), neutrophils (CD11b+Ly6C−M-CSFR−), and total DCs (CD19+CD11c+) were sorted from the spleens of three independent pools of C57Bl/6 WT mice, each consisting of three animals at the age of 8–12 weeks. RNA was extracted according to the NUsense Micro Kit (QIAGEN) protocol. High-quality RNA (Rin > 8.9) was assessed by employing the Agilent 2100 Bioanalyzer. For linear amplification of RNA, a strategy of two rounds of reverse transcription followed by T7 promoter-dependent in vitro transcription was applied with the Ovation Pico WTA System (NuGEN) according to manufacturer’s instructions. For each sample, 10 µg of amplified RNA sample was labeled and hybridized in triplicate to a 24-slide cartridge Affymetrix Mouse Genome 430 2.0 Array according to the manufacturer’s instructions.

Chromatin Immunoprecipitation
Chromatin immunoprecipitation (ChIP) was performed employing the Low-Cell ChIP Kit (Diagenode). The antibodies used in this study included anti-PU.1 (T-21X, Santa Cruz Biotechnology), anti-IgG (Millipore, 12-370), and anti-acetyl-histone H3 (Millipore, 06-599). Immunoprecipitates were quantified with SYBR Green quantitative PCR (qPCR) on a 7300 Real-Time PCR System (Applied Biosystems). Results were calculated as percentage of the input sample, and nonspecific IgG control is shown. Primer sequences are available upon request.

Chromosome Conformation Capturing
3C was essentially conducted according to Dekker et al. (2002). In brief, chromatin of 1 × 10⁶ cells was crosslinked by a 10 min treatment with 5.4% formaldehyde followed by quenching with glycerine and digestion with Ncol (New England Biolabs; 800 U, treated overnight). A fraction was removed as a no-digestion control; the remaining sample was diluted 45 times and religated with T4 DNA Ligase (NE Biolabs). For the analysis of ex vivo populations 1 × 10⁶ flow-sorted progenitor cells, we applied a protocol by Dostie and Dekker (2007) and made minor modifications according to Staber et al. (2013). 3C material was analyzed by nested PCR or by qPCR with a TaqMan probe spanning the Ncol site between the Irf8promoter fragment and the −50kb enhancer. Relative crosslinking frequencies were calculated after normalization to total DNA content as quantified at the −50 kb enhancer site. Additionally, relative crosslinking frequencies were calculated after normalization to chromosomal looping at the Gapdh locus, as described before (Spilianakis et al., 2005).

qPCR reactions were performed in triplicate and each experiment was repeated independently up to three times. Primers and probes were validated employing the digested and religated Irf8-PAC DNA. Digestion efficiency was calculated as in Hagege et al., (2007). Only samples with digestion efficiency > 90% were used. Sequences are available upon request.

Cell Transfections and Luciferase Assays
Stably transformed reporter cell lines were generated by electroporation in RAW264.7 and NIH 3T3 cells with a Gene Pulser Xcell (Bio-Rad). Post-linearized reporter plasmids were coelectroporated with a plasmid carrying a puromycin resistance gene. The cells were subsequently kept under puromycin selection for 3 weeks. For the expression of firefly luciferase, 5 × 10⁵ cells of stably transformed pools or single clones were analyzed with the Dual-Luciferase Reporter Assay System (Promega) according to the recommendations of the manufacturer.

RAW264.7 cells were transfected with shRNA-GFP constructs (see Supplementary Information). GFP+ cells were sorted 48 hr after transfection, and 5 × 10⁵ cells were analyzed with the Dual-Luciferase Reporter Assay System.
Statistical Analysis
Unpaired Student’s t test was carried out to determine the statistical significance of experimental results. All experiments shown were replicate at least two to three times with similar results, unless indicated differently in the figure legends.

ACCESSION NUMBERS
Microarray data have been deposited in the Gene Expression Omnibus (GEO) at accession number GSE45467. For microarray data processing, see Supplemental Information.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, six figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.04.007.

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