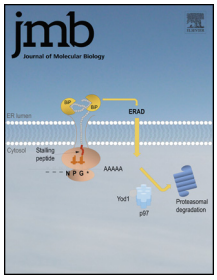

The Third Intron of IRF8 Is a Cell-Type-Specific Chromatin Priming Element during Mouse Embryonal Stem Cell Differentiation

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Abstract

Interferon regulatory factor 8 (IRF8) is a nuclear transcription factor that plays a key role in the hierarchical differentiation of hematopoietic stem cells toward monocyte/dendritic cell lineages. Therefore, its expression is mainly limited to bone marrow-derived cells. The molecular mechanisms governing this cell-type-restricted expression have been described. However, the molecular mechanisms that are responsible for its silencing in non-hematopoietic cells are elusive. Recently, we demonstrated a role for IRF8 third intron in restricting its expression in non-hematopoietic cells. Furthermore, we showed that this intron alone is sufficient to promote repressed chromatin a cell-type-specific manner. Here we demonstrate the effect of the IRF8 third intron on chromatin conformation during murine embryonal stem cell differentiation. Using genome editing, we provide data showing that the third intron plays a key role in priming the chromatin state of the IRF8 locus during cell differentiation. It mediates dual regulatory effects in a cell-type-specific mode. It acts as a repressor element governing chromatin state of the IRF8 locus during embryonal stem cell differentiation to cardiomyocytes that are expression-restrictive cells. Conversely, it functions as an activator element that is essential for open chromatin structure during the differentiation of these cells to dendritic cells that are expression-permissive cells. Together, these results point to the role of IRF8 third intron as a cell-type-specific chromatin priming element during embryonal stem cell differentiation. These data add another layer to our understanding of the molecular mechanisms governing misexpression of a cell-type-specific gene such as IRF8.
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Introduction

Hematopoiesis is a differentiation process initiated from a common precursor known as hematopoietic stem cell. As a result, intermediate progenitor cells are formed in a sequence of cell-fate events leading to lineage commitment to the various types of blood cells. Lineage-specific transcription factors (TFs) are key players in such cell-fate decision processes [1]. Interferon regulatory factor 8 (IRF8) is a crucial determining component in myeloid lineage commitment. This TF is mainly expressed in hematopoietic cells; it is constitutively expressed in monocyte/macrophage linage, dendritic cells (DCs), and B cells and at low levels in resting T cells [2]. It is crucial for monocyte/macrophage/DC development and suppresses granulocyte commitment [3]. IRF8 expression can be further augmented by IFN-γ [3]. IRF8 knockout mice are defective in the ability of myeloid progenitor cells to mature toward monocyte/DC linages [1] and eventually develop chronic myelogenous leukemia-like syndrome [3]. In human, homozygosity mutation at IRF8 gene leads to complete absence of $CD14^+$ and $CD16^+$ monocytes, $CD11c^+$ conventional DCs, and $CD11c^+/CD123^+$ plasmacytoid DCs [4]. Insights to the molecular mechanisms governing IRF8 lineage-specific expression have been detailed (for review, see Ref. [5]). However, the molecular mechanisms that prevent misexpression in non-hematopoietic cells are not well characterized. Repressed chromatin architecture is a key factor ensuring misexpression, yet the molecular components that recruit the chromatin remodeling machineries, such as the Polycomb-group proteins (PcGs), are not well characterized. Recently, we have demonstrated the role of IRF8 third intron as an essential DNA element that is important for eliciting repressed chromatin state at the IRF8 locus in non-

hematopoietic cells [6]. Furthermore, we showed that this intron alone was sufficient to elicit repressed chromatin during the integration of a retroviral reporter gene vector and the subsequent assembly of chromatin. This sustainable gene silencing was lineage specific and occurred only in non-hematopoietic cells. Here, we investigated the role of this intron in determining lineage specification of IRF8 expression during differentiation. Using CRISPR–Cas9, we have generated murine embryonic stem cell (mESC) clones with biallelic deletion of the third intron. Our data clearly show that the IRF8 third intron is a local chromatin primer\organizer that possesses dual negating activities in a cell-type-specific manner. It serves as a nucleation core for repressed chromatin during ESC differentiation to IRF8 expression-restrictive cells such as beating cardiomyocytes. Conversely, it acts as an initiator of active chromatin in IRF8 expression-permissive cells such ESC-derived DCs as well as RAW264.7 (RAW) macrophage cell line.

Results

IRF8 third intron conserved non-coding sequences act in a concerted manner to elicit a repressive effect

The IRF8 third intron is sufficient to elicit the assembly of repressed chromatin upon integration and subsequent silencing of the reporter gene. This was recently demonstrated by cloning the third intron upstream to a luciferase reporter gene cassette in a retroviral vector. This was accompanied by significant increase in repressed chromatin marker, H3K27me3, encompassing the random integration sites [6]. This repressive effect was observed only in IRF8 expression-restrictive cells [NIH3T3 and induced pluripotent stem cell (iPSC)-derived cardiomyocytes] and not in IRF8 expression-permissive cells (RAW). It is important to note that retroviruses integrate mainly into open chromatin structures [7], underscoring the regulatory role of the IRF8 third intron in promoting chromatin remodeling regardless of the viral point of integration. In mammals, regulatory non-coding elements display an extraordinary degree of conservation (for review, see Ref. [8]). Accordingly, IRF8 third intron harbors three conserved non-coding sequences (CNSs) [6] (CNS1–3, Fig. S1) underlining their possible function as regulatory elements within this intron. To study the contribution of each CNS alone to this silencing effect, we have taken a similar approach to that outlined above. For that purpose, a lentiviral vector, in which the third intron or each of the CNSs of IRF8 was cloned upstream to a ubiquitin (Uubic) promoter driving the expression of the reporter gene dsRED, was used (see Fig. 1a for schematic illustration). As a control, 1720 bp from the second intron of the housekeeping gene GAPDH was cloned upstream to the reporter gene cassette as well. NIH3T3 cells were infected and the level of dsRED was measured by flow cytometry, fluorescence-activated cell sorting (FACS), 72 h later. It is clear from Fig. 1b that the IRF8 third intron elicited 5-fold decrease in the reporter gene expression in comparison to the second intron of GAPDH, confirming our previously reported results [6]. This third intron repressive effect is further underscored due to the fact that this experimental system is different from that

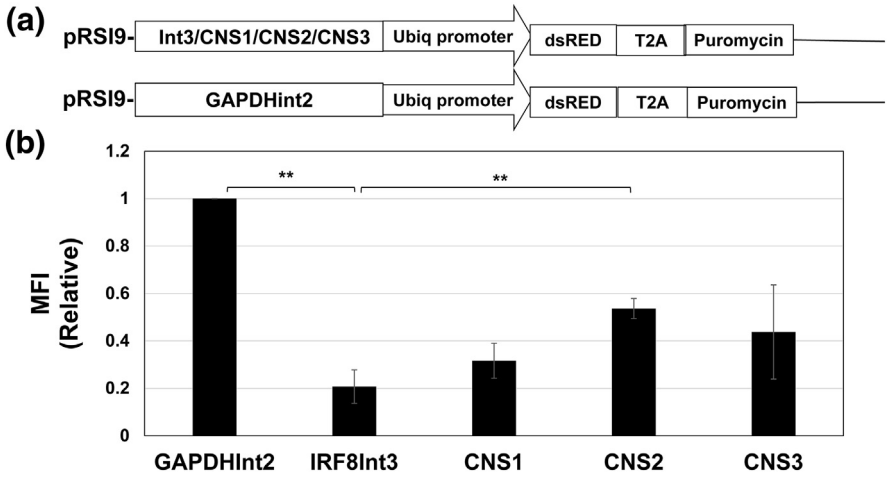

Fig. 1. Each of the third intron CNSs elicits a moderate repression of a reporter gene, regardless of the point of integration. (a) Schematic illustration of the lentiviral reporter construct, pRSI9, harboring IRF8 third intron (IRF8Int3) or each of the CNSs 1–3 (top) or harboring GAPDH second intron GAPHInt2 (bottom). (b) 1.25×10^5 NIH3T3 cells were seeded in 6-well plates and subsequently were transduced separately with constructs described in panel A. Seventy-two hours later, percentage of MFI was determined using FACS Aria. Results were normalized to integrated copy number by RT-qPCR using primers corresponding to dsRED. MFIs are shown, and values are mean ± AVEDEV (n > 3). Student's *t* test, $*p < 0.05$, $**p < 0.01$.

published before [6], yet yielding similar results. All three components of this reporter system are changed: a lentiviral instead of retroviral vector, and different reporter cassette (promoter and reporter gene) highlighting the third intron genuine cis-regulatory activity. Furthermore, each CNS alone also exhibited significant repressive effect on the fluorescence level of dsRED when cloned upstream to the reporter gene cassette. However, the repressive effect of the whole third intron was significantly higher than that of each CNS (Fig. 1B). Taken together, these results point to a cooperative repression role of mainly CNS1 and CNS3 and to a lesser extent of CNS2.

The IRF8 third intron exerts persistent silencing of the reporter gene expression

The ability of the third intron alone to elicit gene silencing *via* chromatin remodeling is reminiscent of the silencing effect of the *Drosophila* cis-acting Polycomb response elements (PREs; for review, see Ref. [9]). PREs alone are sufficient to mediate silencing of a reporter gene. Furthermore, deletion of PREs leads to alleviation of reporter expression [10]. To this end, we used a lentiviral reporter vector as described above except that the IRF8 third intron is flanked by VloxP sequences. This excisable intron was cloned upstream to the dsRED reporter gene cassette (VloxPInt3). As a control, we also cloned the second intron of GAPDH (GAPDHInt2) as schematically illustrated in Fig. 2a and detailed under Materials and Methods. As expected, a 5-fold reduction in mean fluorescence intensity (MFI) of the reporter gene was observed with IRF8 third intron harboring or not harboring VloxP as compared to GAPDHInt2-transduced cells (Fig. 2). However, unlike the reported data for the PRE cis-element, excision of IRF8 third intron in the transduced cells using the VCre recombinase did not alleviate the dsRED expression even when cells were cultivated for a long period following excision, to allow for chromatin reorganization (Fig. 2b). These results are consistent with our previously reported results using BAC-reported system [6], where only ectopic (*in vitro*) deletion of the third intron alleviated the reporter gene expression, while *in situ* removal of the third intron from the integrated BAC had no effect on the repressed state of the reporter gene. Together, the third intron acts as an independent priming element for the assembly of repressed chromatin regardless of the site of integration. Once repressed chromatin is initiated, it probably elicits an epigenetic memory, and thus, the subsequent deletion of the third intron is ineffective.

IRF8 third intron acts as a cell-type-specific cis-acting repressor during ESCs differentiating only in IRF8 expression-restricted cells

Our results point to the role of IRF8 third intron as a cis-acting regulator that affects local chromatin state. To elucidate its role as chromatin modulator during cell differentiation in lineage-specific manner, we

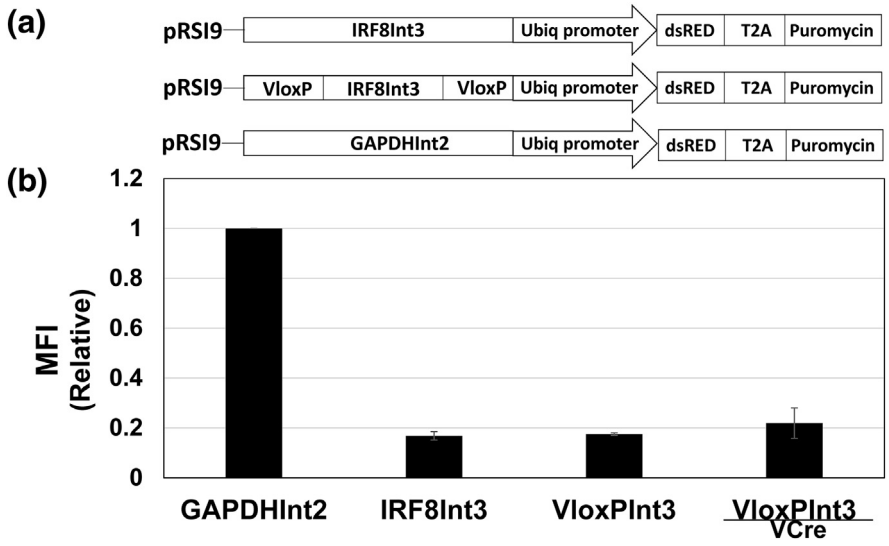

Fig. 2. IRF8 third intron elicits long-lasting repression of a reporter gene, and its genomic deletion does not salvage its expression. (a) Schematic illustration of the lentiviral reporter construct, pRSI9, harboring IRF8 third intron (IRF8Int3), the third intron flanked by VloxP sites (VloxPInt3), and GAPDH second intron GAPHInt2 (GAPDHInt2). (b) NIH3T3 were transduced with either pRSI9-IRF8Int3, pRSI9-VloxPInt3, and pRSI9-GAPDHInt2 (see details under Materials and Methods). Subsequently, cells transduced with pRSI9-VloxPInt3 were re-transduced with pMSCV-VCre to excise the integrated third intron. Following the third intron removal, cells were cultivated for at least seven generations (~1 week), and MFI was determined by FACS. Relative values are shown by means ± AVEDEV ($n \geq 3$). Student's *t* test, $p < 0.01$.

studied its effect on a reporter gene expression and chromatin state in mESCs and following the differentiation to either cardiomyocytes [11,12] or DCs [13] (see supporting data in Fig. S2). For that purpose, mESCs were infected with a retroviral vector harboring the IRF8 third intron (pMSCV-IRF8int3) or GAPDH second intron (pMSCV-GAPDHint2) upstream to a luciferase gene driven by the Nramp1 promoter, and cell differentiation was induced as outlined above (see schematic illustration in Fig. 3a). It is clear from Fig. 3b that in ES cells transduced with retroviral vector harboring the third intron of IRF8 or the second intron of GAPDH, the luciferase expression levels were not significantly different, although 20% reduction was observed in cells transduced with the third intron. However, upon differentiation of these transduced ES cells to cardiomyocytes, a 5-fold reduction in luciferase activity was noted only in cells transduced with the IRF8 third intron and not with the control GAPDH second intron (Fig. 3B). Unlike cardiomyocytes, the differentiation of the transduced ES cells to DCs did not result in third intron-mediated repressive effect on the reporter gene activity. Similar luciferase levels in cells harboring the IRF8 third intron upstream to the reporter gene or harboring the control GAPDH second intron were observed (Fig. 3B). Taken together, these results recapitulate our published results using iPSCs [6], but further extend our understanding of the regulatory role of the third intron during the differentiation of ES cells to DCs, which was not possible with iPSCs. Collectively, our results highlight the role of IRF8 third intron as an initiator of repressed chromatin during ES cell differentiation only in IRF8 expression-restrictive cells. These results underscore the role of the IRF8 third intron as a cell-type-specific modulator of chromatin architecture. It acts as a priming element that recruits the cellular machinery only in expression-restrictive cells, resulting in the formation of repressed chromatin and presumably subsequent establishment of epigenetic memory.

Genomic deletion of IRF8 third intron reveals dual activity in a cell-type-specific manner

To test the role of the third intron as a chromatin modulator during ES cell differentiation in its genomic settings, we employed CRISPR/Cas9 system [14] to delete the third intron in mESCs. The CRISPR/Cas9 system was used with two sgRNAs, which target the 5′ and 3′ ends of the third intron (gRNA 1 and 4, respectively; see Fig. S3). Briefly, a two-step protocol was utilized to generate mESC cells with biallelic deletion of the IRF8 third intron. First, the third intron was replaced by mCherry reporter cassette flanked by VloxP sites. Second, Fluorescent cell clones were transduced with VCre encoding retroviral vector resulting in mESC clones with deleted third intron. Specifically, we took advantage of CRISPR/Cas9-mediated homology-directed repair to replace the third intron with a donor DNA segments. Two donor DNA segments were generated harboring 900-bp homology arms as well as two VloxP sites within the homology arms, which flank the 5′ and 3′ sgRNA target sites at the third intron. These DNA segments also harbor an expression cassette of fluorescent reporter (mCherry), but varied in the drug resistance selection genes: puromycin or hygromycin. In order to enrich for targeted integrants and to rule out random integration

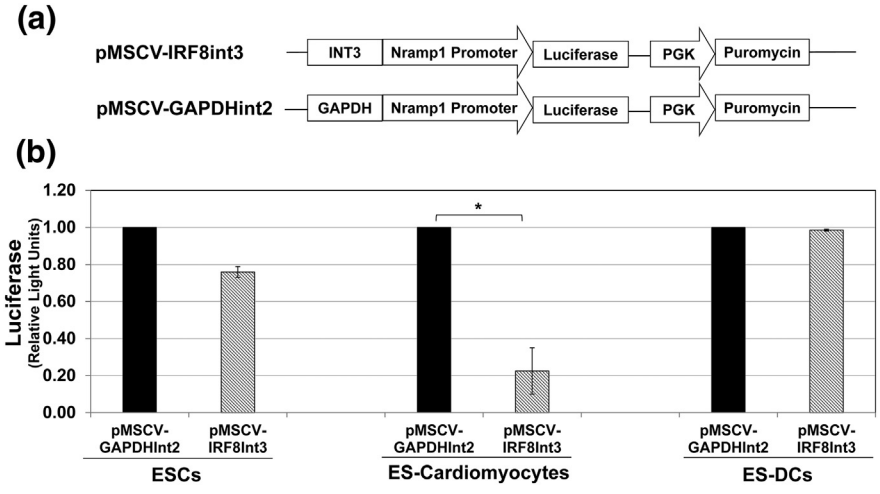

Fig. 3. IRF8 third intron elicits reporter gene repression only in differentiated ESCs to cardiomyocytes and not to DCs. (a) Schematic illustration of the retroviral vectors reporter construct, pMSCV, harboring IRF8 third intron (IRF8Int3) and GAPDH second intron GAPHInt2 (GAPDHInt2). (b) mESCs were transduced with either pMSCV-IRF8Int3 or pMSCV-GAPDHInt2 reporter constructs. Subsequently, transfected cells were differentiated toward cardiomyocytes or DCs (as detailed in Materials and Methods). Luciferase levels were measured, and relative luciferase activity was calculated for the non-differentiated and differentiated ESCs. Values are shown by means ± AVEDEV. Student's *t* test, $*p < 0.05$.

of the donor plasmids in the genome, cells were transfected with the two gRNAs (Fig. S3), Cas9 encoding vector and the two donor DNA segments, and cultivated without drug selection for 2 weeks. Subsequently, the growth medium was supplemented with puromycin and hygromycin for additional week. To evaluate correct integration by homology-directed repair, single clones, resistant for the two antibiotics, were picked and the 5′ and 3′ junctions were assayed by PCR (for details, see Fig. S4). Clones 16 and 21 harboring the biallelic third intron replacement cassette were further verified by DNA sequencing (data not shown) and taken for further study. For "clean" deletion of the third intron, clones 16 and 21 were transduced with pMSCV-VCre to remove the reporter\selection cassettes. Three days later, cells were harvested and non-fluorescent cells were sorted by FACS. Subsequently, individual clones were isolated and the deletion of the third intron was verified by PCR (Fig. S5) and by DNA sequence analysis (data not shown). Initially, IRF8 mRNA level in these third intron-deleted clones was compared to that of WT mESCs. Surprisingly, deletion of IRF8 third intron resulted in elevated expression of IRF8 in these undifferentiated mESC clones (Fig. 4, WT *versus* clones 16 and 21). To determine the role of the third intron on IRF8 expression level and chromatin state during differentiation, mESCs and their third intron-deleted clones were triggered to differentiate toward cardiomyocytes and DCs. Cells were subsequently harvested and relative expression of IRF8 was determined. In addition, the chromatin state of the IRF8 coding region in the differentiated cells in comparison to undifferentiated cells was analyzed by chromatin immuno-precipitation (ChIP)–qPCR using antibody directed against H3K27me3 post-translational modification (PTM), a common marker for repressed chromatin. As evident from Fig. 5, the expression of the endogenous IRF8 was further elevated when the third intron-deleted clones differentiated to beating cardiomyocytes compared to mESC-derived cardiomyocytes in which the third intron is intact (Fig. 5a, clones 16 and 21, 3- and 4-fold, respectively). Furthermore, ChIP analysis of these clones revealed a significant reduction in repressive H3K27me3 modification in these clones in the IRF8 coding region in comparison to control (Fig. 5b). These results suggest that the removal of the third intron led to chromatin remodeling in the IRF8 coding region and the subsequent alleviation of IRF8 expression in cardiomyocytes. Surprisingly and in contrast, when the same clones, 16 and 21, were triggered to differentiate to DCs, IRF8 mRNA expression level was significantly lower than in the corresponding mESC-derived DCs (Fig. 5c). This reduction is consistent with the observed enrichment of repressive chromatin, as indicated by increased H3K27me3 histone PTM in the IRF8 coding region in these third intron-deleted clones in comparison to WT mESC-derived DCs (Fig. 5d). This suggests that during the differentiation of mESC toward DCs, which are IRF8 expression-permissive cells, the third intron acts as an enhancer element. This explains the change in repressed chromatin level and the subsequent reduction in IRF8 expression level when this intron is completely deleted.

Taken together, the IRF8 third intron is essential for chromatin repression and subsequent deposition of epigenetic memory during differentiation in IRF8 expression-restrictive cells such as mESC-derived cardiomyocytes, resulting in restricted IRF8 expression in non-hematopoietic cells. Surprisingly, our results suggest a possible role of the IRF8 third intron as an enhancer element during mESC differentiation to IRF8 expression-permissive cells such as DCs. Interestingly, such enhancer activity was not observed with just the third intron using retro\lentiviral reporter gene vectors as elaborated above.

To further demonstrate the role of the third intron as an enhancer in permissive cells and in a single cell clone before and following removal of the third intron, we used BAC-IRF8 reporter assay as previously

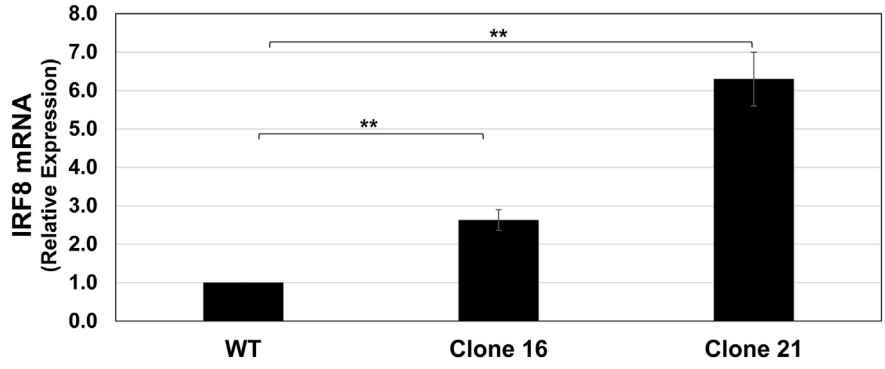

Fig. 4. Relative IRF8 expression in mESC stable clones before and following genomic deletion of IRF8 third intron. RNA was extracted from WT mESC and clones in which IRF8 third intron was deleted (clones 16 and 21). Relative mRNA expression levels of IRF8 were determined by RT-qPCR. Values are shown by means ± AVEDEV. Student's *t* test, $^{**}p < 0.01$.

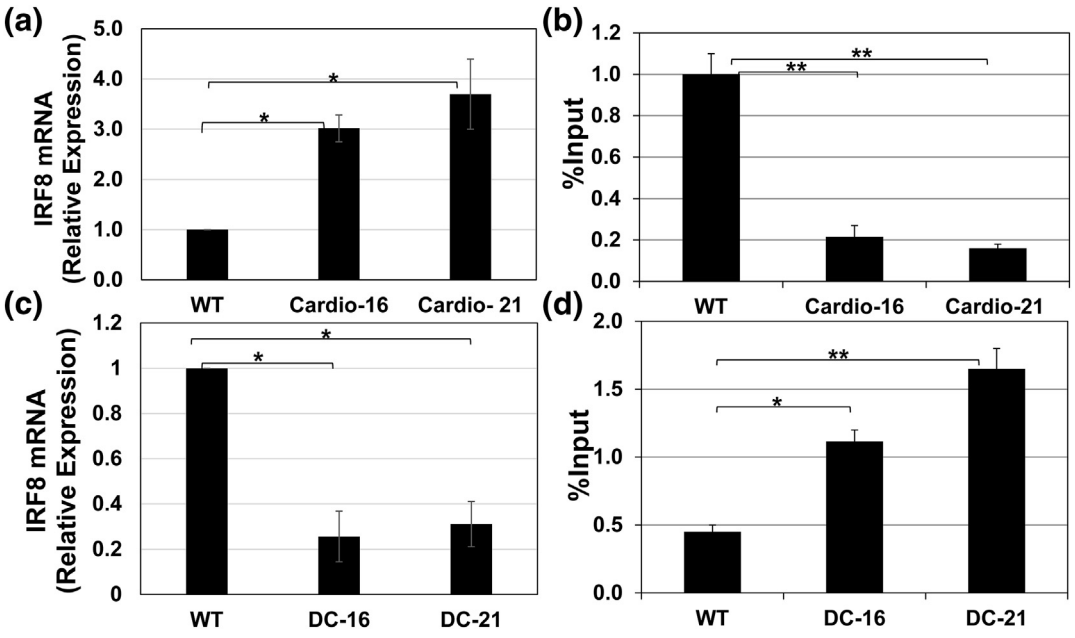

Fig. 5. The fate of IRF8 expression in third intron-deleted ESCs following differentiation to cardiomyocytes and DCs. RNA was extracted from mESC-derived cardiomyocytes (WT and third intron-deleted clones; Cardio-16 and 21) and mESC-derived DCs (WT and third intron-deleted clones; DC-16 and DC-21). Relative mRNA expression levels of IRF8 were determined by RT-qPCR (panels a and c, respectively). mESC-derived cardiomyocytes and mESC-derived DCs described under panels a and c, respectively, were further analyzed for the level of H3K27me3 modification over the IRF8 promoter by ChIP-qPCR analysis (panels B and D, respectively). Percentage (%) of input values are normalized to OCT-4-positive control. Values are shown by means ± AVEDEV. Student's *t* test, $*p < 0.05$, $**p < 0.01$.

described [6]. In principal, RAW cells were transfected with BAC construct harboring the IRF8 locus region in which EGFP cDNA was inserted in IRF8 AUG translation initiation codon. In this BAC IRF8 transgenesis construct, the IRF8 is not transcribed\translated. In addition, the IRF8 third intron in this BAC construct is flanked by two VLoxP sites. This reporter construct, BAC-IRF8.1-VloxP, is authentically reporting on IRF8 lineage-restrictive expression in response to IFN-γ stimulation: fluorescent in permissive cells (RAW and DCs) and non-fluorescent in restrictive cells (NIH3T3 and cardiomyocytes) [6]. This construct was transfected to RAW permissive cells and numerous stable cell clones were isolated. To perform third intron deletion within the cells, clones were transduced with either empty retroviral vector or retroviral vector coding for the VCre gene (detailed under Materials and Methods), and the mean cell fluorescence was subsequently determined by FACS analysis (Fig. 6a). As observed for the mESC-derived DCs, removal of the third intron in RAW clones harboring the BAC-IRF8.1-VLoxP construct, clones 1 and 7, resulted in reduced reporter expression as detected by FACS (Fig. 6a) compared to cells transduced with empty vector. In addition, instead of deleting the third intron within the cells, it was initially deleted in bacteria using VCre expression vector, resulting in BAC-IRF8.1 construct with deleted third intron. This construct was transfected into RAW cells and stable clones were isolated, and their relative fluorescence was compared to a clone with undeleted third intron. As shown in Fig. 6b, the fluorescence of the reporter gene in clones 2 and 3 was almost non-detectable as determined by FACS in comparison to control. Together, it is clear that removal of the third intron after and before transfection of the BAC-IRF8 reporter construct to RAW cells led to a reduction in the fluorescence of the reporter gene, consistent with DCs results shown above. Importantly, deletion of the third intron prior to transfection resulted in a very low level of reporter gene fluorescence. This indicates that the third intron not only operates as transcriptional enhancer but also has a role in priming chromatin assembly in not only in IRF8 expression-restrictive cells but also in IRF8 expression-permissive cells.

Discussion

IRF8 is an orchestrating TF of myeloid cell differentiation and as a myeloleukemia suppressor gene. Its expression is strictly limited to cells of hematopoietic origin. Thus, we focused on unraveling the molecular mechanisms that prevent misexpression in non-hematopoietic cells. Cumulative evidence presented previously [6] and here showed that the third intron

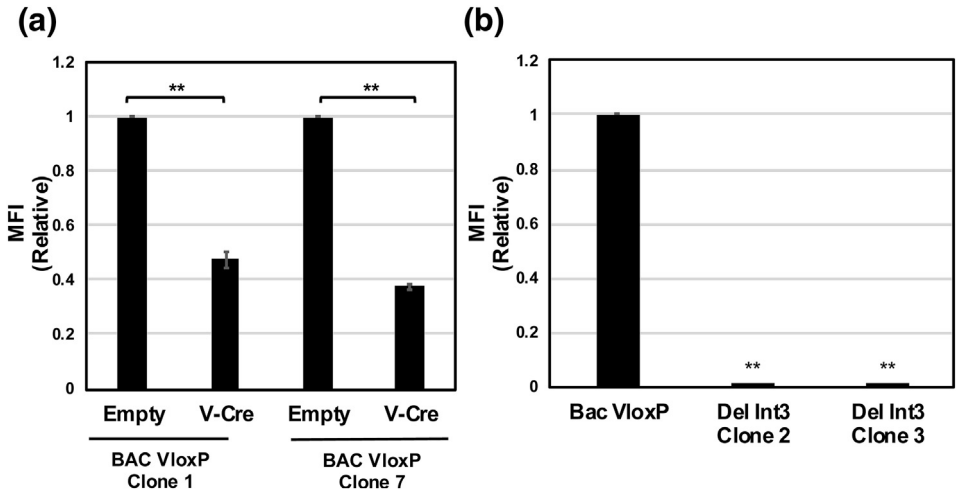

Fig. 6. The effect of IRF8 third intron deletion before and after stable transfection of a BAC driving IRF8 reporter gene expression in RAW cells. RAW cells were transfected with BAC-IRF8.1 VLoxP (see details under Materials and Methods). (a) To induce the third intron deletion within the cells (*in situ*), two different stable clones (clone 1 and clone 7) were transduced with either empty retroviral vector or retroviral vector encoding the V-Cre gene as described under Materials and Methods. (b) The third intron in BAC-IRF8.1 VLoxP construct was initially deleted with the corresponding V-Cre recombinase in *Escherichia coli* (*in vitro*). Subsequently the corresponding BAC DNA was transfected to RAW cells, and stable clones were selected (Del Int3 clone 2 and Del Int3 clone 3). A representative clone harboring 1–2 BAC copies is shown. EGFP fluorescence levels were measured by FACS as detailed under Materials and Methods. MFIs are shown, and values are mean ± AVEDEV (n = 3). Student's *t* test, ** $p < 0.01$.

of IRF8 acts as a chromatin-organizing element. The data suggest that the third intron acts as an initiator of gene repression by affecting chromatin architecture leading to a repressed state. Once a repressed chromatin is established and subsequent deployment of epigenetic memory takes place, the third intron becomes dispensable. This conclusion is backed by two complementing experimental systems. In the first system, utilizing the BAC-IRF8 reporter assay, removal of the intron following stable integration of this BAC construct into the host DNA, had no effect on the repressed state of the reporter gene in expression-restrictive cells. However, if the third intron was initially deleted from this BAC reporter construct and only then stably transfected to IRF8 expression-restrictive cells, the reporter gene was not repressed and was accompanied by an open chromatin architecture [6]. In the second experimental system, the ability of just the third intron to repress the expression of a reporter gene and elicit repressed chromatin in IRF8 expression-restrictive cells was established. Removal of the randomly integrated third intron using VCre recombinase did not alleviate the repression mediated by this intron (Fig. 2B). These results point to the role of IRF8 third intron as a primer or as a "nucleation core" for chromatin condensation/remodeling in expression restricting cells. Furthermore, it suggests that the initial onset of chromatin remodeling is followed by the establishment of an "epigenetic memory." This "memory" is likely to depend on a defined set of histone PTMs [15] that are probably spread along the IRF8 locus. Interestingly, once epigenetic memory is established, it is maintained throughout cell division and transmitted through DNA replication with no further need for the recruiting DNA element [16] or the initiation factor. We hypothesize that the third intron serves as a cue site for the IRF8 locus, providing a platform for cell-type-specific DNA-interacting factors during cell differentiation process. In expression restricting cells, these factors initiate histone PTMs affecting chromatin architecture, followed by further modifications that install the epigenetic memory. These results are supported by previous work by Hansen *et al.* [17], who showed that Polycomb repressive complex 2 (PRC2) binds to and colocalizes with H3K27me3 PTM during ongoing DNA replication. Using heterologous reporter system, it was shown that recruitment of the PRC2 complex to chromatin is sufficient to maintain repression during subsequent cell divisions. The authors suggest that once tri-methylation of H3K27 is established, it recruits the PRC2 complex to maintain the mark at sites of DNA replication, leading to ongoing tri-methylation of H3K27 on the daughter DNA strands. This mechanism ensures maintenance of the H3K27me3 in proliferating cells during DNA replication, thereby preserving chromatin structure and transcriptional programs. Similar observations were reported by Coleman and Struhl [18] demonstrating that H3K27me3, once established at a repressed Drosophila HOX gene, transmits the memory of the

OFF state from one cell generation to the next through multiple rounds of replication.

The third intron CNSs act cooperatively to execute repression effect

The search for cell-type-specific DNA-interacting factors within the third intron is complicated. This intron is abundant with numerous TFs binding sites as evident by GTRD analysis using the ChIP-seq database (see Fig. S6) [19]. As mentioned above, IRF8 third intron contains three conserved nucleotide sequences (CNSs) between human, mouse, and bovine and thus might be engaged with such factors (Fig. S1). In an attempt to narrow down the position of the regulatory element within the third intron responsible for the cell-type-specific repression effect, we studied the effect of each CNS alone. Our results show that while the full-length intron exerts its expected repression, a moderated repression of the dsRED reporter gene was observed when CNS1 and CNS3 were separately cloned upstream to a dsRED transcriptional unit in lentiviral reporter assay (Fig. 1b). In contrast, CNS2 exhibited a lesser repression effect on the fluorescence intensity. While the third intron alone is sufficient to elicit repressed chromatin state, each CNS contributes only partially to the establishment of repressed chromatin (Fig. 1). Together, these results suggest that a combinatorial assembly of DNA-interacting factors along the third intron accounts for the recruitment of the Polycomb complex. These results are similar to the reported study by Vasanthi *et al.* [10], who identified a PRE in the mouse HoxD gene. Unlike the fly PRE, the repressive activity of mouse PRE is more complex and depends on multiple silencing mechanisms, PcG/Trithorax-group proteins (trxG) factors, and heterochromatin structure. Furthermore, the entire length of the PRE is necessary for the repressive activity since overlapped fragments of this repressor element did not add up to that of the full-length fragment. Together, our studies suggest a model whereby multiple CNSs mediate repression of IRF8 gene expression in restrictive cells. These CNSs recruit directly or indirectly the chromatin-repressive epigenetic machinery. We hypothesize that this repressive effect is subsequently spread along the IRF8 locus in an undulating motion. It is tempting to speculate that similar mechanisms repress similar genes with expression limited to macrophages, DCs, and B cells.

IRF8 third intron mediates sustainable repressed chromatin state in expression-restricted cells during mESC differentiation

Once differentiation of ESCs is initiated, lineage specification occurs by the implementation of genome-expression programmers, dictating each cell type a lineage-specific TF repertoire that triggers a set of lineage-specific genes. Consistent with the changes in the global genome activity, changes in histone modification patterns accompany mESC differentiation. Elevation in repressed heterochromatin marks, such as H3K9me3, H3K27me3, and H3K27me2, was also observed during induced mESC differentiation [20,21]. Accordingly, *in-cell* deletion of the IRF8 third intron from genomic integrated BAC or lentiviral reporter constructs does not alleviate the repression elicited by the intron, since the long-term chromatin-repressed architecture is already embedded. However, molecular events taking place during differentiation of mESCs are orchestrating such cell-type-specific chromatin reorganization. Here we show that the IRF8 third intron is not an effective repressor in undifferentiated ESCs. However, it becomes potent when the same cells differentiate to cardiomyocytes and not to DCs (Fig. 5). Together, these results underline the role of this intron as an initiator or priming element of repressed chromatin in IRF8 expression-restrictive cells. These results are in-line with the fact that ESC genome is poised for differentiation (poised chromatin). The chromatin of many genes in both mouse and human ES cells is characterized by co-enrichment of functionally opposite chromatin marks. Chromatin structure consists of regions of H3K4me3 overlapping with H3K27me3 at the transcription start site that maintains genes in uncommitted state [22]. Therefore, when we performed genomic deletion of the third intron in ESCs, we did not expect any effect on IRF8 expression. Surprisingly, elevation of IRF8 expression was already observed in clones 16 and 21 compared to WT mESC (Fig. 4). Based on ENCODE public database [23], IRF8 promoter in mESCs consists of regions decorated with H3K27me3 (Fig. S7A). Therefore, we speculate that IRF8 intra-locus looping promotes regulatory interaction between the third intron and the promoter to maintain poised chromatin structure. Hence, deletion of the third intron may affect the bivalent structure of the IRF8 promoter, resulting in alleviation of IRF8 expression in third intron-deleted ESCs (Fig. 4).

Deletion of IRF8 third intron in mESCs resulted in beating cardiomyocytes with open chromatin structure over the IRF8 gene locus and subsequent elevated IRF8 expression (Fig. 5b and A, respectively). This underscores the role of this intronic element as chromatin organizer or priming element during embryonal differentiation process in IRF8 expression-restrictive cells. It is further supported by the ENCODE database shown in Fig. S7B (upper panel). The repressive chromatin mark, H3K27me, is decorating the whole IRF8 locus in cardiomyocytes. The third intron is not enriched with the open chromatin mark H3k27ac, but interestingly, the immediate promoter is enriched (Fig. S7B). The opposite chromatin marks

decorate the IRF8 locus in DCs: high decoration with H3k27ac at the immediate promoter as well as the third intron and low level H3K273me decoration along the whole locus (Fig. S7B, lower panel). Thus, deletion of the IRF8 third intron leads to significant reduction in H3K273me enrichment in deleted cardiomyocytes (Fig. 5b). This is concurrent with elevated expression of IRF8 (Fig. 5a). However, this elevated expression is not comparable to that of IRF8 mRNA level in mESC-derived DCs (see comparative IRF8 expression Fig. S8). It is important to note that cardiomyocytes express a very low level of IRF8 in comparison to DCs but somewhat higher than mESCs or NIH3T3 cells, where almost no expression is detected (see comparative IRF8 expression Fig. S8). Still the level of IRF8 expression in the third intron deleted cardiomyocytes is about four times higher than that of undeleted cardiomyocytes (Fig. S8). This elevated level of expression is about 2.5 lower than the IRF8 steady-state level in mESC-derived DCs (Fig. S8). This is expected since efficient transcription is dependent not only on chromatin structure but also on transcriptional activation. Essential TFs such as PU.1 determine IRF8 expression level (for review, see Ref. [5]). This essential factor expression level is also very low in mESCs and the derived cardiomyocytes. Together, these results highlight the role of IRF8 third intron as an organizer of chromatin state over the IRF8 locus preventing misexpression in expression-restrictive cells.

IRF8 third intron acts like an enhancer element during ESC differentiation in IRF8 expression-permissive cells

Here we show that the third intron of IRF8 possesses dual activity in a cell-type-specific manner. In addition to the repressive activity, it also acts as an activator in IRF8 expression-permissive cells, such as DCs and RAW macrophage cell line. Accordingly, biallelic deletion of the third intron in differentiated ESCs to DCs led to a significant reduction in IRF8 expression and a significant increase in repressed chromatin marker, H3K27me, as compared to control, WT mESC-derived DCs (Fig. 5c and d, respectively). Similar results were obtained from *in situ* and *in vitro* removal of the third intron in integrated BAC-IRF8 reporter construct in RAW macrophage cell line, where the reporter gene levels were dramatically reduced (Fig. 6a and b, respectively). This highlights the role of IRF8 third intron as an enhancer element governing IRF8 expression during differentiation toward expression-permissive cells. Our observations are also supported by the ENCODE project ChIP-seq data sets of mouse cell lines that clearly demonstrate that H3K4me1 and H3K27ac modifications are co-localized at the third intron of the IRF8 gene in mouse IRF8 expression-permissive cells such as bone marrow-derived macrophage cells (Fig. S7C, upper panel), while in IRF8-restrictive cell line, the third intron is not enriched with these two histone PTMs (mESC and MEF cells; Fig. S7C, middle and lower panels, respectively).

Taken together, these results highly suggest that the third intron also acts as *bona fide* enhancer element essential for IRF8 expression in permissive cells such as ESC-derived DCs and RAW macrophage cell line.

Similar characteristics between IRF8 third intron and PREs

The molecular mechanisms that prevent misregulation of cell-type-specific genes is mediated by transcriptional regulation and chromatin organization. The latter is regulated, in part, by two antagonizing groups of proteins; the PcG and TrxG, which are essential for eliciting repressed or open chromatin, respectively [24,25]. The mechanisms by which these complexes are tethered to a specific genomic locus are not fully understood. However, studies in flies and, to a lesser extent, in vertebrates suggest that some specific genomic DNA sequences, generally termed PREs, are recruiting these chromatin-modifying complexes. These PREs share some sequence characteristics in the fly. However, in mammals, the only solid definition is the ability to elicit chromatin remolding by recruiting PcG and\or TrxG complexes (for review, see Ref. [9]). PRE-kr, regulates expression of the mouse MafB/Kreisler gene [26]. This PRE-kr recruits PcG proteins in mouse F9 cells and represses gene expression in a PcG/trxG-dependent manner. This PRE contains consensus PHO/YY1-binding sites, GAGAG motifs, and a palindromic double PHO binding site. These features are also shared with fly PREs [26]. Furthermore, in repressed state chromatin, PcG proteins are recruited to PRE by sequence-specific DNA binding factors, such as SUZ12, RUNX1, and REST [9,26]. Interestingly, the IRF8 third intron shares the DNA motifs described for MafB PRE. Furthermore, according to the ENCODE data set, it is bound by several such PRE hallmark DNA binding factors such as SUZ12, RUNX1, and REST (see Figs. S9 and S10, respectively), although the binding peaks are not restricted to the third intron only. Thus, the IRF8 third intron harbors some features described for other vertebrate PREs and as such acts as regulatory element that mediates IRF8 silencing during differentiation to expression-restrictive cells. Finally, like PREs, the third intron also acts as enhancer element. However, the binding factors recruiting the TrxG proteins are yet to be determined.

In conclusion, this study provides additional evidence for the role of IRF8 third intron as a PRE-like element during ESC differentiation. This chromatin priming element possesses dual cell-type-specific activity. It acts as a repressor element governing

chromatin state in IRF8 expression-restrictive cells. Conversely, it functions as an activating element that is essential for open chromatin structure in expression-permissive cells. We hypothesize that cell-type-specific DNA-interacting factors bind this region and recruit the chromatin remodeling machinery during cell differentiation. Repressed chromatin machinery, such as PcG proteins, is recruited in expression-restrictive cells, while active chromatin machinery, such as trxG proteins, is recruited in expression-permissive cells. We propose that the third intron serves as a nucleation core or chromatin initiator for these chromatin changes. These changes are subsequently spread bi-directionally in a wave-like (undulating) motion along the IRF8 locus, thus affecting the ability of TFs to interact with the promoter and other regulatory regions. Alternatively, DNA binding factors, yet to be identified, bind to IRF8 third intron and induce the formation of an intra-locus DNA loop and contact the promoter region or the transcription start site [27] and induce chromatin architecture state that affects IRF8 expression in a cell-type-specific manner.

Materials and Methods

Cell lines

NIH3T3 (mouse embryo fibroblast), RAW264.7 (murine monocyte/macrophage-like cell line), and 293FT (human embryonal kidney) were obtained from ATCC, Manassas, VA (CRL-1658, TIB-71, and CRL-3216, respectively). These cell lines were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 2.5 μg/mL amphotericin and 50 μg/mL gentamycin sulfate (Biological Industries, Beit-Haemek, Israel). Mouse embryonic stem cell line (mES-R1) and mitotically inactivated mouse embryonic fibroblasts (MEF) were purchased from Stem Cell Core and Advanced Cell Technologies, Weizmann Institute of Technology, Israel. Undifferentiated ES colonies were cultured on MEF feeder layer, maintained in knockout DMEM (Gibco) supplemented with 15% FCS, 2 mM glutamine, 1% NEAA, 1% penicillin–streptomycin, 0.1 mM β-mercaptoethanol, 0.1% leukemia inhibitory factor (Peprotec), 1 mM sodium pyruvate, 3 mM CHIR99021 (Axon Medchem), 5 mM PD0325901 (Axon Medchem), and 0.1 mg insulin (Biological Industries). mES-derived cardiomyocytes were maintained in Iscove's medium (Biological Industries), supplemented with 20% FCS, 2 mM glutamine, 1% NEAA, 0.5% penicillin–streptomycin, 0.1 mM β-mercaptoethanol, and 1% ascorbic acid. mES-derived DCs were maintained in RPMI-1640 (Sigma-Aldrich R8758) supplemented with 10% FCS, 2 mM glutamine, 1% penicillin–streptomycin, and GM-CSF (10 ng/mL). OP9 cells were maintained in MEM-alpha medium (Gibco) supplemented with 20% FCS.

Fast ChIP

The protocol was adopted from Nelson *et al*. [28]. Briefly, cells were crosslinked with formaldehyde and lysed. Genomic DNA was sheared using Covaris E220 (Covaris, Inc.) and precipitated using monoclonal antibodies recognizing specific histone PTMs. The following antibodies were used: αH3K27me3 (17-622; Upstate) and anti-normal rabbit IgG (ChIP-Ab and kit; Upstate). Following IP, the sample was de-crosslinked and DNA purified. For histone PTM ChIP, the enriched de-crosslinked DNA samples were analyzed using real-time PCR, as described hereafter (for primers sequences used, see Table S1).

Deletions using VCre recombination system

A site-specific recombination system, VCre/VLoxP [29], was used to remove the third intron from the BAC reporter construct and dsRED lentiviral vector as previously described [6]. BAC reporter constructs were transfected to NIH3T3 and RAW cells, and numerous cell clones were collected. dsRED reporter vectors were transduced to NIH3T3; in order to perform third intron deletion within the cells, clones were transduced with either empty retroviral vector or retroviral vector encoding for the VCre gene. Genomic DNA was extracted from each transduced clone, and VCre efficiency was analyzed using real-time qPCR with primer pair flanking the third intron, primer pair from within the third intron, and primer pair targeting tGFP and dsRED for control in BAC and dsRED constructs, respectively (Del int3, IRF8 int3 amplicon8, tGFP and dsRED; Table S1). All clones exhibited at least 70% deletion efficiency (data not shown). Subsequently, the reporter gene expression was analyzed.

Real-time PCR

The primers used for real-time PCR were designed using PrimerExpress™ software (Applied Biosystems; see Supplemental Table S1). Eight hundred nanograms of total RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcriptase kit (Ambion) according to the manufacturer's protocol. cDNA was amplified with two primers for each gene using Power SYBR Green PCR Master Mix (Applied Biosystems) and Applied Biosystems 7300 real-time PCR System according to the manufacturer's instructions. The amplification reaction condition was 95 °C for 15 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 15 s. The estimated amount of transcripts was normalized to GAPDH mRNA expression. The data are presented as the relative expression of the gene of interest compared with GAPDH.

Retroviral luciferase reporter assay

Plasmid reporter constructs were generated as previously described [6]. These plasmids were transfected to mES-R1 cells, and reporter gene assays were performed exactly as previously described [4] before and after ES differentiation to cardiomyocytes and DCs.

Lentiviral dsRED reporter assay

TagRFP reporter gene in the pRSI9 vector (Cellecta) was replaced with dsRED, which amplified from pCMV dsRED (Table S1), and cloned to XbaI and BamHI digestion sites. Lentiviral reporter constructs were generated by PCR amplifying the IRF8 third intron and the GAPDH second intron (1720 bp) with primers flanked by EcoRI sites (see Supplemental Table S1) and sub-cloned to pRSI9-dsRED vector driven by the ubiquitin promoter. The EcoRI site is upstream the ubiquitin promoter generating pRSI9-dsRED-Int3 and pRSI9-dsRED-GAPDHInt2.

NIH3T3 cells were infected, and to ensure chromosomal integration, reporter gene assay was performed 72 h later as described above. dsRED fluorescence intensity was normalized to genomic lentiviral copy number, as determined using qPCR with primers for dsRED and GAPDH as reference.

Differentiation of mESC to cardiomyocytes

Undifferentiated cells were cultured on MEF feeder layer to 50% confluence and transduced with either pMSCV-Luc-INT3 or pMSCV-Luc-GAPDHint2 retroviral vectors for 24 h (as described above). Forty-eight hours later, transduced cells were selected with puromycin (1 μg/mL) for 1 week. To induce differentiation, the standard hanging drops method to derive embryoid bodies (EBs) was used. Undifferentiated mESCs transduced with either pMSCV-Luc-INT3 or pMSCV-Luc-GAPDHint2 were dissociated with 0.25% trypsin EDTA and suspended in differentiation medium composed of Iscove's modified Dulbecco's medium, 20% FCS, 1 mM L-glutamine, 0.1 mM mercaptoethanol, 1% nonessential amino acid, and 1% penicillin–streptomycin. Three-day cultured EBs were transferred to 6-cm Petri dishes (bacteriological grade) and 4 days later, EBs were plated on 0.1% gelatin-coated culture dishes. The appearance of spontaneous beating colonies, indicative of differentiated cardiomyocytes, was monitored under microscope. At least 50% of the differentiated cells were beating cardiomyocytes, and the remaining were fibroblasts. This differentiated mixed cell population did not express IRF-8 and used for further analysis such as luciferase assay, mRNA expression level, and ChIP analysis, as described hereafter.

Differentiation of mESC to DCs

To induce differentiation toward DCs, we used the published protocol [13]. Undifferentiated mES cells (2×10^5) were harvested and cultured on OP9 cells feeder layer with alpha-MEM supplemented with 20% FCS for 48 h. Forty-eight hours later, medium was changed for another 2 days. In the second differentiation step, cells were harvested and cultured on freshly prepared OP9 feeder in alpha-MEM supplemented with 20% FCS including mouse GM-CSF (20 ng/mL). One week later, small round cells, floating or loosely adherent appeared and increased in number. These cells were harvested by pipetting and suspended in RPMI-1640, 10% FCS including mouse GM-CSF (10 ng/mL), and culture in bacterial (non-tissue culture-coated polystyrene). After 1 week, cells of DC-like morphology should appear.

CRISPR/Cas9-mediated deletion of IRF8 third intron

sgRNAs from the 5′ and 3′ ends of the IRF8 third intron were designed using the online tool (http://chopchop.cbu.uib.no/), and highly scored sgRNAs were chosen (sgRNA 1–4).

Each sgRNA was phosphorylated, annealed, and cloned to lentiGuide-Puro (addgene no. 52963) and to pSpCas9(BB)-2A-GFP (PX458) (addgene no. 48138), which digested with BsmbI and BbsI, respectively. In order to create gRNAs with sticky ends complementary to BsmbI and BbsI digestion products, "CACC" and "AAAC" were added to 5′ end of the designed 20-bp sgRNA in the forward and reverse oligomers, respectively. In order to easily isolate ES cells with biallelic deletion of the IRF8 third intron, we replaced the endogenous third intron with a reporter cassette. For this purpose, we generated two donor plasmids harboring 900-bp homology arms harboring VloxP sites, which flank the 5′ and 3′ sgRNA target sites at the third intron. In between these homology arms, we cloned an expression cassette consists of fluorescent reporter (mCherry), a different drug selection gene for each arm (puromycin or hygromycin; see schematic illustrations in Figs. S4A and S5A).

T7 endonuclease assay

In order to verify the efficiency and specificity of the sgRNAs, a T7 endonuclease assay [30] was performed in NIH3T3 cells and the cleavage efficiency was analyzed using Tapestation (Agilent). The two gRNAs (1 and 4) specifically targeted the IRF8 third intron and efficiently cleaved the mismatched PCR products (Fig. S3). The 512-bp PCR fragment surrounding sgRNA1 cleavage site was cleaved to 312 and 200 bp, and the 461-bp PCR fragment surrounding sgRNA4 site was cleaved into 329 and 132 bp.

Flow cytometry

Flow cytometry analysis was performed using BD FACSAria II (BD Bioscience), and data were analyzed using Flowing Software 2 (Cell Imaging Core, Turku Centre for Biotechnology). Unstained WT NIH3T3 and RAW cells were used as negative control for dsRED and EGFP, respectively.

Statistical methods

All experiments were performed in $n \geq 3$ replicates, and values are presented as means ± AVEDEV. Data were compared by unpaired two-tailed Student's *t* test; *p* values <0.05 or <0.01 were considered statistically significant, as indicated in the appropriate figure. When applicable, we employed false discovery rate correction for multiple hypotheses testing, using the Benjamini–Hochberg method [31]. Asterisk indicates *p* values that are significant after correction with $\alpha = 0.05$ or $\alpha = 0.01$.

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Declarations of Interest: None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2018.11.022.

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Abbreviations used:
IRF8, interferon regulatory factor 8; PcGs, Polycomb-group proteins; PRE, Polycomb response elements; TrxG, Trithorax-group proteins; TF, transcription factor; mESC, mouse embryonal stem cell; iPSC, induced pluripotent stem cell; CNS, conserved non-coding sequence; FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity.

References

[1] P. Arnold, A. Scholer, M. Pachkov, P.J. Balwierz, H. Jorgensen, M.B. Stadler, et al., Modeling of epigenome dynamics identifies transcription factors that mediate Polycomb targeting, Genome Res. 23 (2013) 60–73.
[2] D. Kurotaki, T. Tamura, Transcriptional and epigenetic regulation of innate immune cell development by the transcription factor, interferon regulatory factor-8, J. Interf. Cytokine Res. 36 (2016) 433–441.
[3] K. Akashi, D. Traver, T. Miyamoto, I.L. Weissman, A clonogenic common myeloid progenitor that gives rise to all myeloid lineages, Nature 404 (2000) 193–197.
[4] M. Alter-Koltunoff, S. Ehrlich, N. Dror, A. Azriel, M. Eilers, H. Hauser, et al., Nramp1 mediated innate resistance to intraphagosomal pathogens is regulated by IRF-8, PU.1 and Miz-1, J. Biol. Chem. 278 (2003) 44025–44032.
[5] T. Tamura, D. Kurotaki, S. Koizumi, Regulation of myelopoiesis by the transcription factor IRF8, Int. J. Hematol. 101 (2015) 342–351.
[6] M. Khateb, N. Fourier, O. Barnea-Yizhar, S. Ram, E. Kovalev, A. Azriel, et al., The third intron of the interferon regulatory factor-8 is an initiator of repressed chromatin restricting its expression in non-immune cells, PLoS One 11 (2016), e0156812.
[7] R.S. Mitchell, B.F. Beitzel, A.R. Schroder, P. Shinn, H. Chen, C.C. Berry, et al., Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences, PLoS Biol. 2 (2004) 1127–1137.
[8] D. Polychronopoulos, J.W.D. King, A.J. Nash, G. Tan, B. Lenhard, Conserved non-coding elements: developmental gene regulation meets genome organization, Nucleic Acids Res. 45 (2017) 12611–12624.
[9] M. Bauer, J. Trupke, L. Ringrose, The quest for mammalian Polycomb response elements: are we there yet? Chromosoma 125 (2016) 471–496.
[10] D. Vasanthi, A. Nagabhushan, N.K. Matharu, R.K. Mishra, A functionally conserved Polycomb response element from mouse HoxD complex responds to heterochromatin factors, Sci. Rep. 3 (2013) 3011.
[11] T. Hannes, M. Wolff, M.X. Doss, K. Pfannkuche, M. Haustein, J. Muller-Ehmsen, et al., Electrophysiological characteristics of embryonic stem cell-derived cardiomyocytes are cell line-dependent, Cell. Physiol. Biochem. 35 (2015) 305–314.
[12] E.Y. Waese, W.L. Stanford, One-step generation of murine embryonic stem cell-derived mesoderm progenitors and chondrocytes in a serum-free monolayer differentiation system, Stem Cell Res. 6 (2011) 34–49.
[13] S. Senju, S. Hirata, H. Matsuyoshi, M. Masuda, Y. Uemura, K. Araki, et al., Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells, Blood 101 (2003) 3501–3508.
[14] L. Wang, Y. Shao, Y. Guan, L. Li, L. Wu, F. Chen, et al., Large genomic fragment deletion and functional gene cassette knock-in via Cas9 protein mediated genome editing in one-cell rodent embryos, Sci. Rep. 5 (2015) 17517.
[15] E.I. Campos, J.M. Stafford, D. Reinberg, Epigenetic inheritance: histone bookmarks across generations, Trends Cell Biol. (2014) 664–674.

[16] A. D'Urso, J.H. Brickner, Mechanisms of epigenetic memory, Trends Genet. 30 (2014) 230–236.
[17] K.H. Hansen, A.P. Bracken, D. Pasini, N. Dietrich, S.S. Gehani, A. Monrad, et al., A model for transmission of the H3K27me3 epigenetic mark, Nat. Cell Biol. 10 (2008) 1291–1300.
[18] R.T. Coleman, G. Struhl, Causal role for inheritance of H3K27me3 in maintaining the OFF state of a *Drosophila* HOX gene, Science 356 (2017).
[19] I. Yevshin, R. Sharipov, T. Valeev, A. Kel, F. Kolpakov, GTRD: a database of transcription factor binding sites identified by ChIP-seq experiments, Nucleic Acids Res. 45 (2017) D61–D67.
[20] E. Meshorer, T. Misteli, Chromatin in pluripotent embryonic stem cells and differentiation, Nat. Rev. Mol. Cell Biol. 7 (2006) 540–546.
[21] H. Szutorisz, C. Canzonetta, A. Georgiou, C.M. Chow, L. Tora, N. Dillon, Formation of an active tissue-specific chromatin domain initiated by epigenetic marking at the embryonic stem cell stage, Mol. Cell. Biol. 25 (2005) 1804–1820.
[22] K. Sha, L.A. Boyer, The Chromatin Signature of Pluripotent Cells, StemBook, Cambridge (MA), 2008.
[23] K.R. Rosenbloom, C.A. Sloan, V.S. Malladi, T.R. Dreszer, K. Learned, V.M. Kirkup, et al., ENCODE data in the UCSC genome browser: year 5 update, Nucleic Acids Res. 41 (2013) D56–D63.
[24] P.A. Steffen, L. Ringrose, What are memories made of? How Polycomb and Trithorax proteins mediate epigenetic memory, Nat. Rev. Mol. Cell Biol. 15 (2014) 340–356.
[25] J.A. Simon, R.E. Kingston, Occupying chromatin: Polycomb mechanisms for getting to genomic targets, stopping transcriptional traffic, and staying put, Mol. Cell 49 (2013) 808–824.
[26] A. Sing, D. Pannell, A. Karaiskakis, K. Sturgeon, M. Djabali, J. Ellis, et al., A vertebrate Polycomb response element governs segmentation of the posterior hindbrain, Cell 138 (2009) 885–897.
[27] S. Schoenfelder, I. Clay, P. Fraser, The transcriptional interactome: gene expression in 3D, Curr. Opin. Genet. Dev. 20 (2010) 127–133.
[28] J.D. Nelson, O. Denisenko, K. Bomsztyk, Protocol for the fast chromatin immunoprecipitation (ChIP) method, Nat. Protoc. 1 (2006) 179–185.
[29] E. Suzuki, M. Nakayama, VCre/VloxP and SCre/SloxP: new site-specific recombination systems for genome engineering, Nucleic Acids Res. 39 (2011), e49.
[30] L. Vouillot, A. Thelie, N. Pollet, Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases, G3 5 (2015) 407–415.
[31] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. R. Stat. Soc. Ser. B 57 (1995) 289–300.