**Epigenetic Program and Transcription Factor Circuitry** **of** **Dendritic Cell Development**

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**ABSTRACT**

Dendritic cells (DC) develop from hematopoietic stem cells through successive steps of lineage commitment and differentiation. Multipotent progenitors (MPP) are committed to DC restricted common DC progenitors (CDP), which differentiate into specific DC subsets, classical DC (cDC) and plasmacytoid DC (pDC). Here we described the dynamics of transcription level, histone modifications and transcription factor PU.1 occupancy ongoing during DC development. The results reveal a DC-primed epigenetic signature in CDP, including specific H3K4me1, H3K4me3 and H3K27me3 marks, which are maintained and/or reinforced during differentiation into cDC and pDC. Epigenetic marks and PU.1 occupancy increasingly coincide upon DC development, advocating for PU.1 acting as a pioneer transcription factor in DC. We thus integrated PU.1 occupancy with global gene expression to devise a PU.1 centered regulatory circuitry for DC commitment and subset specification. The circuitry provides the transcription factor hierarchy that drives the sequel MPP-CDP-cDC/pDC, including Irf4, Irf8, Tcf4, Spib and Stat factors. The anatomy of the circuitry, including positive feedback loops inferred for individual or multiple factors, provides the basic regulatory architecture, which stabilizes the distinct stages of DC lineage commitment (CDP) and DC subsets specification (cDC and pDC).

**INTRODUCTION**

Dendritic cells (DC) represent specialized immune cells that develop from hematopoietic stem cells (Shortman and Naik 2007; Merad et al. 2013). DC are widely distributed in both lymphoid and non-lymphoid tissues and bridge innate and adaptive immune responses. DC function builds on their capacity to capture, process and present antigens to T cells. DC are divided into distinct subsets according to their localization, phenotype, and function (Belz and Nutt 2012; Satpathy et al. 2012; Merad et al. 2013). Lymphoid tissues contain classical/conventional DC (cDC) and plasmacytoid DC (pDC), which represent the main DC subsets. Peripheral organs contain migratory tissue DC, which capture antigens and migrate to lymphoid organs for antigen presentation to T cells.

DC development from hematopoietic stem cells comprises two critical steps: DC commitment and DC subset specification (Shortman and Naik 2007; Felker et al. 2010; Merad et al. 2013). First, multipotent hematopoietic stem/progenitor cells are committed towards the DC lineage, which yields the DC-restricted common DC progenitor (CDP). Second, CDP further develop into the specific DC subsets, cDC and pDC. cDC are specialized for antigen processing and presenting, while pDC produce large amounts of type I interferon e.g. in response to viral infections.

Genome-wide gene expression and gene knockout studies in mice identified several critical regulators for DC commitment and subset specification, such as Flt3, Stat3, Id2, Irf8 and Tcf4 (Hacker et al. 2003; Cisse et al. 2008; Robbins et al. 2008; Carotta et al. 2010; Jackson et al. 2011; Belz and Nutt 2012; Miller et al. 2012; Sere et al. 2012; Merad et al. 2013). Furthermore, hematopoietic master regulators, such as the transcription factors PU.1 and Gfi1, were shown to regulate DC development (Rathinam et al. 2005; Carotta et al. 2010; Belz and Nutt 2012; Schonheit et al. 2013). However, how the various transcription factors interact to regulate DC development has remained elusive.

Epigenetic mechanisms regulate cell development, identity and function. For example, specific epigenetic landscapes define pluripotency and control lineage committed differentiation (Mikkelsen et al. 2007; Heinz et al. 2010). Histone methylation represents one of the most important epigenetic modifications. Recent studies suggest that specific histone methylation patterns impact on gene transcription (Zhou et al. 2011). For example, histone H3 lysine 4 trimethylation (H3K4me3) and H3 lysine 27 trimethylation (H3K27me3) at gene promoters are associated with gene activation and repression, respectively. H3 lysine 9 trimethylation (H3K9me3) is associated with heterochromatin and transcriptional silencing. Many key developmental genes have bivalent modification where large domains of repressive H3K27me coexist with small domains of activating H3K4me3 (Azuara et al. 2006; Bernstein et al. 2006). These genes are poised for either activation or repression during differentiation.

Epigenetic modifications also regulate hematopoiesis. Accordingly, current efforts on high-throughput mapping of histone modifications and transcription factor binding are directed towards elucidating the regulatory codes that drive lineage commitment and differentiation. Wei et al. demonstrated that histone modifications control specificity and plasticity in T cell linage fate determination (Wei et al. 2009). Wong et al. showed that gene expression during terminal erythropoiesis is mediated by distinct epigenetic modifications (Wong et al. 2011). Garber et al. studied global histone modification and occupancy of multiple transcription factors in DC stimulated with lipopolysaccharide (Garber et al. 2012). Specific histone modifications are also used for identification of enhancers and/or cis-regulatory modules (Shlyueva et al. 2014). For instance, H3 lysine 4 monomethlyation (H3K4me1) marks genomic regions that indicate primed enhancers. So far, the dynamics of histone modifications during DC commitment and subset specific has not been studied.

We previously established a two-step culture system that faithfully recapitulates DC development *in vitro.* First, multipotent progenitors (MPP) and CDP are amplified from bone marrow with a specific stem/progenitor cell cytokine factor cocktail and then second, differentiated with Flt3 ligand (Flt3L) into cDC and pDC subsets (Felker et al. 2010). Here we generated global maps of H3K4me1, H3K4me3, H3K9me3, H3K27me3 and PU.1 occupancy in DC progenitors and differentiated DC by chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-seq). The integrative analysis of ChIP-seq data and gene expression data allowed us to construct a DC regulatory circuitry for DC commitment and subset specification.

**RESULTS**

**Linage-specific transcriptional signatures** **in DC development**

MPP were induced to differentiate *in vitro* into CDP (DC commitment) and further into cDC and pDC (DC subsets specification; Fig. 1A) (Felker et al. 2010). To explore the dynamics of transcription profiles in DC commitment and specification, we determined changes in gene expression between MPP, CDP, cDC and pDC by analyzing DNA microarray data (Fig. 1A). A total of 3194 genes were differentially expressed between the various differentiation stages (fold change > 2, FDR < 0.05). An increasing number of genes was found to be differentially regulated during DC commitment (429 genes) and DC subset specification (1773 and 2181 genes; Fig. 1A). Among them, 210 genes encode transcription factors. These include many critical DC regulators, such as Irf4, Irf8, Batf3, Relb, Id2, Spib and Tcf4 (E2-2) (Belz and Nutt 2012). Differentially expressed genes were categorized by their gene expression profiles in six cell-specific clusters: progenitors (MPP/CDP), MPP, CDP, Pan-DC (cDC/pDC), cDC and pDC cluster (Fig. 1B). These clusters match very well to gene expression profiles calculated from *in vivo* sorted DC progenitors and DC subsets (Supplemental Fig. S1) (Miller et al. 2012).

The MPP cluster contains Gfi1, Gata2 and Tal1, which were down regulated from MPP to CDP and had low or no expression in DC. This reflects the gradual restriction of development from early hematopoietic progenitors towards DC committed progenitors and DC (Fig. 1B). The pan-DC cluster comprises genes, such as Flt3, with important roles in both cDC and pDC (Fig. 1B). The cDC and pDC clusters identify DC subset-specific genes, such as Id2, Irf4, Zbtb46 for cDC and Tcf4 (E2-2) for pDC. Taken together, our analysis captures known and putative regulators of DC development and thus provides the basis of investigating the underlying transcriptional architecture of DC commitment and subsets specification.

**Global maps of histone modification and PU.1 binding in DC development**

To gain further insights into the mechanisms of DC development, we generated genome-wide maps of four histone modifications (H3K4me1, H3K4me3, H3K9me3, H3K27me3) and of the transcription factor PU.1 by ChIP-seq for MPP, CDP, cDC and pDC (Fig. 1B, C). Signals of histone modifications of each cell type were directly compared for all genes with the respective gene expression data (Supplemental Fig. S2). H3K4me3 was found to associate with active promoters and gene transcription, i.e., genes with high H3K4me3 levels relate to high gene expression. Similarly, the enhancer mark H3K4me1 positively correlated with gene expression at both promoters and 3’ ends. The repressive mark H3K9me3 at promoters associated with inactive genes, and H3K27me3 at promoter and 3’ ends also correlated with inactive genes (Supplemental Fig. S2). All these results are consistent with previous studies in other cell systems (Wei et al. 2009; Karlic et al. 2010; Weishaupt et al. 2010; Costa et al. 2011) and emphasize the impact of specific histone modifications on DC development. Interestingly, H3K9me3 at 3’ ends showed a reverse correlation with gene expression for all investigated cell types, suggesting an alternative role of H3K9me3 in DC development and this will be discussed in more detail below.

Next, we determined how H3K4me1, H3K4me3, H3K27me3 marks and PU.1 binding relate to stage-specific gene clusters in DC commitment and DC subset specification (Fig. 1B, C). The active mark H3K4me3 in MPP and CDP was confined to progenitor genes, while H3K27me3 was observed for these genes in cDC and pDC. Conversely, H3K4me3 in cDC and pDC was observed for DC genes, while H3K27me3 was seen for these gene in progenitors (Fig. 1B, C). Importantly, H3K4me1 and PU.1 binding were detected in both progenitor and DC specific genes and followed the pattern of the active mark H3K4me3. Moreover, PU.1 occupancy was observed for all stage-specific gene clusters in CDP, suggesting that CDP acquired a DC-primed PU.1 binding profile during DC commitment. This observation is also in line with the role of PU.1 as a pioneer transcription factor in cell fate specification (Heinz et al., 2010; Zaret and Carroll, 2011) (Chen and Dent 2014).

To further extend these observations, we focussed on the analysis of individual representative genes, including Flt3, Gfi1, Id2 and Irf8 for progenitor, DC, cDC and pDC affiliated genes, respectively (Fig. 1D, Supplemental Fig. S3). All these genes are implicated in DC development based on gene knock out studies (Merad et al. 2013). Flt3 is a key cytokine receptor for DC development and regulated by PU.1 (Carotta et al. 2010). Flt3 promoter showed prominent H3K4me3 signals, which increased upon differentiation (Fig. 1D). Concomitantly, H3K27me3 signals decreased from MPP to DC lineages. H3K4me1 and PU.1 peaks revealed multiple enhancer regions in the body of the Flt3 gene in CDP, cDC and pDC, which relate to an increase of Flt3 expression upon DC differentiation (Fig. 1D). The progenitor gene Gfi1 had prominent H3K4me3 signals at the promoter in MPP and acquired H3K27me3 upon DC differentiation, thus forming a bivalent modification (Supplemental Fig. S3). A similar pattern was observed for Cepba. cDC genes (Id2, Batf3) showed an accumulation of H3K4me3 and PU.1 signals in promoter or enhancer regions, respectively, in cDC. The pDC gene Spib showed pronounced H3K4me3 and PU.1 signals at the promoter in pDC. Similar chromatin profiles were also observed for the pDC genes Tcf4 and Il7r. The Irf family genes Irf1 and Irf8 showed particular prominent H3K4me3 occupancy at promoters in DC, which increased during DC development (Supplemental Fig. S3).

In summary, our analysis revealed the dynamics of stage-specific histone marks and PU.1 binding that control the DC differentiation program.

**CDP acquire DC-primer epigenetic signature upon DC lineage commitment**

DC commitment from MPP to CDP represents a determining step in DC development (Fig. 1A). Surprisingly, upon MPP-CDP transition about 10,000 genes showed a conserved H3K4me3 mark at promoters, while only about 1,000 genes showed a loss or acquisition of the H3K4me3 mark (Supplemental Fig. S4). As expected, these specific changes in H3K4me3 promoter occupancy resulted in the reduction or induction of gene expression. About 1500 and 2600 promoters contained H3K27me3 and bivalent marks respectively, and changes of these marks upon DC commitment had little influence on gene expression (Supplemental Fig. S4B, C).

Genes that acquired H3K4me3 upon DC commitment showed substantial changes in expression and exhibited a functional enrichment of immune-related gene ontology terms (Fig. 2A and Supplemental Fig. S4A). For example, Cd74 (MHC class II invariant chain) showed a primed H3K4me3 signature in CDP and a further increase in H3K4me3 in cDC and pDC (Fig. 2B). Thus, the DC-primed histone marks appear to be crucial for initiating the transcriptional program, which further leads to the establishment of the DC lineages.

To extend our observations, we focused on genes differentially expressed in MPP and CDP (429 genes, Fig. 1A, Fig. 2C). 218 genes were up regulated upon MPP-CDP transition and showed a prominent increase in H3K4me1, H3K4me3 and PU.1 occupancy and a decrease in H3K27me3 mark (Fig. 2C, D). Importantly, this pattern was maintained in differentiated DC (cDC and pDC), indicating epigenetic DC priming of lineage-specific gene promoters and enhancer regions. Conversely, 211 genes were down regulated and this correlated with a loss in H3K4me1 and H3K4me3, and an increase in H3K27me3 occupancy (Fig. 2C, D). Taken together, during MPP-CDP transition, up regulated genes acquired H3K4me1, H3K4me3 and PU.1, whereas down-regulated genes acquired H3K27me3 or bivalent marks without obvious change in PU.1 occupancy. This also suggests that the increase of PU.1 occupancy is the one of the driving forces for DC commitment (see also below Fig. 4A, Supplemental Fig. S6A).

Next, we proceeded to analyze epigenetic profiles in DC subset specification. Genes with abundant expression in cDC compared to pDC, such as c-kit and Batf3, exhibited an enrichment of the H3K4me3 mark and PU.1 binding in cDC versus pDC (Fig. 2E, Supplemental Fig. S5). Interestingly, in pDC these genes showed a reduction of the active mark H3K4me3 and a deposition of the repressive mark H3K27me3, thereby restricting gene expression towards cDC. The same mechanism is used to direct gene expression towards pDC, as observed for Pacsin1 (Fig. 2E, Supplemental Fig. S5). Additionally, lineage-specific H3K4me1 and PU.1 marks associated with cDC or pDC genes correlated with transcriptional activation (Supplemental Fig. S5), suggesting the cell-type specific enhancers are involved in the diversification of DC subsets.

Collectively, CDP acquire DC lineage-specific epigenetic signatures during DC commitment. These lineage-primed H3K4me1/H3K4me3 and PU.1 marks initiate the transcriptional program, and thus drive DC commitment and subset specification, while H3K27me3 mark restricts alterative developmental options.

**H3K9me3 marks occur at 3’ end of active genes**

H3K9me3 occurs in large genomic regions and is associated with compacted chromatin and gene poor areas (Wen et al. 2009). However, H3K9me3 occupancy was observed at promoters of highly expressed cancer-associated genes and has also been implicated in transcription elongation and alternative splicing (Vakoc et al. 2005; Wiencke et al. 2008)(Zhou et al., 2014), suggesting additional function of H3K9me3.

Here we observed that H3K9me3 at 3’ ends is associated with genes expressed at intermediate or high levels, in particularly in cDC and pDC (for all genes: *R* = 0.77 and *R* = 0.56, respectively; for differentially expressed genes: *R* = 0.87 and *R* = 0.79, respectively; Supplemental Fig. S2). To extend this observation, we examined the H3K9me3 mark in larger regions, comprising ±5kb around TSS and 3’ ends (Fig. 3A). The top 2000 genes with high expression showed an over-representation of H3K9me3 at the 3’ ends for MPP, CDP, cDC and pDC. For example, the progenitor associated gene Gfi1 showed an enrichment of H3K9me3 mark at 3’ end in MPP and CDP but not in DCs. cDC or pDC affiliated genes, such as Id2 and Il7r, exhibited higher H3K9me3 levels in the respective DC subsets in comparison to MPP and CDP (Fig. 3B). Therefore, our analysis revealed the enrichment of H3K9me3 marks at 3’ ends of active genes, suggesting that H3K9me3 might play an additional role e. g. in transcriptional activation, elongation and/or alternative splicing of key DC genes.

**The instructive role of PU.1 in DC development**

PU.1 represents a master regulator in hematopoiesis with a prominent role in multiple cell fate decisions, including DC development (Carotta et al. 2010; Schonheit et al. 2013). Principal component analysis of our PU.1 ChIP-seq data of DC development and PU.1 data of multiple hematopoietic lineages from previous studies demonstrated three distinctive development pathways: from MPP towards erythroid cells; T/B cells and DC (Fig. 4A). Interestingly, CDP clustered with DC (i.e., cDC, GM-CSF induced bone marrow derived DC; GM-DC and pDC) and were distant from MPP, indicating that CDP exhibited DC-primed PU.1 binding profiles. cDC and GM-DC were positioned very close to CDP, which supported the hypothesis that differentiation of cDC represents the default DC development pathway (Ghosh et al. 2010; Belz and Nutt 2012).

Genome wide analysis of PU.1 ChIP-seq data revealed a preference for PU.1 binding to gene bodies and intergenic regions in MPP, CDP, cDC and pDC (Supplemental Fig. S6A). This is consistent with observations in previous studies and PU.1 function as an enhancer factor (Heinz et al. 2010; Garber et al. 2012). Additionally, a prominent increase of PU.1 binding events was observed in the promoter region from MPP to CDP and cDC (Supplemental Fig. S6A). Next, we used H3K4me1 and H3K4me3 peaks to define cell specific promoters and enhancers. An increasing number of PU.1 peaks in CDP, cDC and pDC were found in active promoter and enhancer regions, which were significantly enriched compared to those in MPP (Fig. 4B). A similar trend was observed in GM-DC ChIP-seq data obtained from the literature (Garber et al. 2012) (Supplemental Fig. S6B). These results suggest that PU.1 plays a positive role in DC lineage commitment. Moreover, cDC and pDC showed differences in PU.1 occupancy (Supplemental Fig. S6A), indicating that PU.1 might be also involved in the regulation of DC lineage diversification.

Next, we analyzed the dynamics of the differential PU.1 peaks in MPP, CDP, cDC and pDC and the associated PU.1 motifs (Fig. 4C). A total of 19,944 MPP and 23,054 CDP differential peaks were detected. Interestingly, MPP peaks revealed an alternative de-novo motif with a weak GGAA sequence, while CDP peaks showed the classical PU.1 motif (Robasky and Bulyk 2011) containing an ETS binding site (GGAA core site; Fig. 4C). Similarly, PU.1 occupancy was also different in cDC and pDC. The PU.1 binding sequence in cDC resembles the classical PU.1 motif, while the pDC binding sequence has a similar consensus sequence but lower information content (Fig. 4C). These results are in further support of DC-primed PU.1 binding profiles in CDP (see above).

To investigate the role of PU.1 as a transcriptional activator, we compared the inter-relationship of differential PU.1 peaks to differentially expressed genes in the same cell types, e.g., CDP differential peaks next to CDP differential genes. We found that PU.1 binding was significantly associated with transcriptional activation of lineage-specific genes in CDP, cDC and pDC (Fig. 4D). This indicates a positive role of PU.1 in regulating DC lineage fate determination. Additionally, we investigated the role of PU.1 as a repressor by comparing differential PU.1 peaks next to differentially expressed genes in opposing cell types, e.g., CDP differential peaks next to MPP differential genes. Our results suggest that PU.1 might also be involved in transcriptional repression of stem cell-related genes or early hematopoietic genes in MPP and its progeny (Fig. 4D). Taken together, differential PU.1 binding regulates DC development mainly by activating DC lineage-specific genes.

**Identification of PU.1 co-binding transcription factors**

Hematopoietic cell differentiation is controlled by a complex transcription factor network. It has been demonstrated that PU.1 defines macrophage and B cell identities by cooperating with different cell type-restricted transcription factors (Heinz et al. 2010). It is therefore conceivable that DC development is regulated by the interactions of PU.1 and DC lineage-specific transcription factors. To identify key PU.1 co-binding partners during DC commitment and specification, we designed an integrative computational approach to analyze sequences around PU.1 peaks of differentially expressed genes (see Methods section, Supplemental Fig. S7). Briefly, we focused on differentially expressed transcription factors upon DC development and collected their sequence motifs from public databases. Then we performed motif search around PU.1 differential peaks to detect putative transcription factor binding sites. As we assume PU.1 has an activating role in DC development, we only consider PU.1 differential peaks close to up-regulated genes in the same cell types. Finally, one-tailed Fisher’s exact test was applied to measure the enrichment of transcription factor binding sites around differential PU.1 peaks for each cell type, in order to identify cell specific PU.1 co-binding partners.

In total, 27 transcription factors (represented by 31 transcription factor motifs) were significantly enriched at different stages of DC development (Fig. 5A). Transcription factors with contradictory expression and enrichment patterns (Supplemental Fig. S8) were excluded from further analysis. 20 transcription factors including PU.1 itself (labeled in red in Fig. 5A) were considered as the potential PU.1 partners. For example, interferon regulatory factors (Irf8) and the Ets-domain transcription factor (e.g., Spib) are known regulators of DC differentiation. Irf8-deficient mice lack many mature DC subsets (Belz and Nutt 2012). Spib is also indispensable in DC development, particular in pDC (Nagasawa et al. 2008). Both factors showed a significantly enrichment in MPP, CDP and two DC subtypes, indicating that they potentially form composite binding with PU.1 in each step of DC development (Fig. 5A, Cluster I).

Upon DC commitment a panel of transcription factors, such as Stat1, Klf4, Egr1, Irf1, Runx1, Irf4, and Est1, was enriched in CDP (Fig. 5A, Cluster II), indicating that PU.1 initiated the DC program by recruiting or cooperating with multiple DC transcription factors. For example, up-regulation of Id2 expression was accompanied by an increase of PU.1 binding in the Id2 promoter from MPP to CDP (Fig. 5B). Within this PU.1 binding region, Irf8, Klf4 and Egr1 binding sites were detected, suggesting that these factors are recruited by PU.1 to promote Id2 expression.

Upon DC specifications, PU.1 is predicted to collaborate with distinct sets of transcription factors to restrict the development program towards to either cDC or pDC, such as Rel/Rela/Nfkb1 and Irf5 for cDC and Tcf4/E2-2 for pDC (Fig. 5A, Clusters III, IV and V). The Id2 gene, a prototype cDC marker, contained specific PU.1 peaks at promoter and distal regions in cDC, which were associated with binding sites of Jun/Ap1, Rel/Nfkb, Irf8, Klf4 and Egr1 (Fig. 5B). Conversely, the pDC-affiliated gene Irf1 contained PU.1 binding regions, which harbored the binding sites of Tcf4/E2-2, a prototype pDC transcription factor in pDC (Fig. 5B). Furthermore, many cDC or pDC specific regulators identified in this analysis were found to be enriched in CDP (Fig. 5A), such as Fos/Jun/Ap1 and Tcf4, again supporting the notion of DC priming in CDP.

**PU.1 centered regulatory circuitry of DC development**

To better understand the interrelationship among individual PU.1 co-binding factors, four lineage-specific transcription factor regulatory networks were constructed (Fig. 5C). In each network, nodes represent the potential PU.1 co-binding partners (Fig. 5A) and selected key DC regulators from the literature (e.g., Flt3, Kit and Id2) (Belz and Nutt 2012; Miller et al. 2012). An edge between two nodes indicates that a particular transcription factor activates its target gene, i.e., (i) the transcription factor is enriched in the respective cell type, (ii) the target gene is differentially expressed during DC commitment (MPP versus CDP) or DC subset specification (cDC versus pDC) and (iii) there is a transcription factor binding site at the differential PU.1 peak close to the target gene.

Different topologies and connectivity densities of the four lineage-specific networks reflect how dynamics of PU.1 composite binding drives DC development. Upon lineage commitment, PU.1 recruited a core set of transcription factors (i.e., Irf8, Klf4, Runx1, Egr1 and Stat1) to activate the expression of DC marker genes (e.g., Id2, Csf1 and Tcf4) in CDP (Fig. 5C). Upon specification, the DC subtype-specific transcription factors collaborated with PU.1 to define cDC or pDC identity (Fig. 5C). For instance, Rel/Nfkb and Irf4 exclusively co-bound with PU.1 in cDC, whereas Tcf4, Ets1, Irf1 and Spib only cooperated with PU.1 in pDC.

Auto-regulatory feedback loops are important building blocks of transcriptional regulatory networks (Kielbasa and Vingron 2008; Kueh et al. 2013). PU.1 was previously observed to control hematopoietic development by forming auto-regulatory loops (Okuno et al. 2005; Leddin et al. 2011). Intriguingly, several positive auto-regulatory loops of key DC genes were captured in our networks (Fig. 5C, highlighted in gray). For example, the auto-regulatory loop of Irf8 in CDP indicates that Irf8 induces its own transcription, indicating the important function of Irf8 in DC commitment. An auto-regulatory loop of Irf8 was also observed in pDC, which is in line with Irf8 being abundantly expressed in pDC and required for pDC development (Tsujimura et al. 2003). Similarly, an auto-regulatory loop was also observed for Irf4 in cDC, which is in accord with Irf4 function in cDC development (Belz and Nutt 2012).

We then proceeded to integrate the four stage-specific transcription factor networks in one DC regulatory circuitry (Fig. 6). Each regulator in the circuitry is positioned at a specific stage of DC development (MPP, CDP, cDC and pDC) in which it is involved. Additionally, some of the positive or negative interactions between DC regulators were included based on the literature (Laiosa et al. 2006; Carotta et al. 2010; Ghosh et al. 2010). For example, PU.1 was also observed to inhibit Gata1/2 activation, which led to specific myeloid cell fates (Zhang et al. 1999; Walsh et al. 2002). Accordingly, this inhibition of Gata2 by PU.1 was displayed in the circuitry.

Sfpi1/PU.1 acts as a central hub of the DC regulatory circuitry, inducing interactions with early hematopoietic transcription factors (e.g., Gfi1, Cebpa and Tal1) and DC transcription factors (e.g., Irf family genes). During MPP-CDP transition, PU.1 induces a genetic program comprising Flt3, Stat1/3, Irf5/8, Klf4 and Egr1, to establish and maintain DC lineage fate. Additionally, PU.1 appears to restrict MPP-CDP transition by directly or indirectly inhibiting alternative lineage fates, e.g., by inhibiting Gata2. Following DC commitment, CDP can undergo two different developmental options: the subset specification into cDC and pDC. DC subset-specific factors control the antagonized developmental pathways leading to either cDC or pDC. For example, high expression of the cDC marker Id2 inhibits the pDC gene Tcf4 and vice versa, resulting in cDC or pDC development, respectively (Cisse et al. 2008; Li et al. 2012).

The positive auto-regulatory loops observed for PU.1, Irf8, Klf4, Irf4, Spib and Tcf4 provide the basic regulatory mechanisms to stabilize the DC program. More intriguingly, there are also the feedback loops between pDC factors, such as Irf1, Ets1, Spib and Tcf4 (pDC sub-network in Fig. 6A), but such feedback loops are not observed between cDC factors (Fos/Jun/Batf3, Rel/Relb/Nfkb, Id2 and Irf4). This might be indicative for reinforcement of the pDC program and underpins the hypothesis that cDC is the default DC development pathway. Branching off the default pathway and establishing the pDC subset requires specific regulatory mechanisms to stabilize the pDC program.

**DISCUSSION**

The dynamics of specific histone modifications determine lineage fate decisions in hematopoiesis, including DC development. Here we generated high-resolution genome-wide chromatin maps of H3K4me1, H3K4me3, H3K9me3, and H3K27me3 for MPP commitment towards CDP and specification into cDC and pDC subsets. We demonstrate that stage specific expression of key DC transcription factors, including PU.1, Irf1, Irf8, Batf3, Spib and Tcf4, is associated with specific histone modifications in promoter and enhancer sequences, which undergo precise changes in DC development. Genome-wide analysis led us to devise a PU.1 centered regulatory circuitry, which provides the basic architecture of how DC transcription factors are wired to drive DC development.

Genes that are increasingly expressed during DC development showed an increase in H3K4me1 and H3K4me3 marks and a decrease in H3K27me3, as expected. Interestingly, gain in H3K4me1 or H3K4me3 marks on DC genes activates a DC-primed gene expression profile in CDP, which results in DC lineage commitment. These results are very much in line with our data on the DC-primed transcriptional signature in CDP (Felker et al. 2010). We also observed bivalent domains in multiple genes, which changed upon DC development. For example, the frequency of bivalent marks H3K4me3 and H3K27me3 decreased during MPP-CDP transition, which coincides with DC commitment and activation of the DC-primed gene signature in CDP.

Specific histone modifications and gene activity correlated in DC progenitors and DC subsets, in line with the activating role of H3K4me1/H3K4me3 and the repressive role of H3K9me3/H3K27me3 in gene expression (Bernstein et al. 2006; Wei et al. 2009; Zhang et al. 2012). However, we also found that H3K9me3 is enriched at the 3’ end or the body of active genes. This result indicates that H3K9me3 plays roles in both heterochromatin and euchromatin (Black and Whetstine 2011) and might have additional functions beyond acting as a repressive mark. Such further H3K9me3 functions might include transcription elongation (Vakoc et al. 2005) or alternative splicing (Zhou et al. 2014).

PU.1 represents a pioneer transcription factor in hematopoietic cell development that acts in concert with other lineage specific factors (Heinz et al. 2010; Zaret and Carroll 2011; Garber et al. 2012). DC progenitors and DC subsets express PU.1 and knockout mice demonstrated the impact of PU.1 on DC development (Dakic et al. 2005; Nutt et al. 2005). PU.1 controls Flt3 cytokine receptor expression and Flt3/Stat signaling induces PU.1 expression, thus generating a self-reinforcing auto regulatory loop that drives DC development (Carotta et al. 2010; Chauvistre et al. 2014). PU.1 also induces chromatin remodeling of the Irf8 gene that encodes an important transcription factor for DC development (Schonheit et al. 2013). Our genome-wide analysis of PU.1 occupancy is very much in line with the instructive role of PU.1 in DC development. First, we identified an alternative PU.1 motif in MPP compared to CDP, cDC and pDC. Second, PU.1 is predicted to associate with stage-specific transcription factors during the sequel MPP-CDP-cDC/pDC. Both PU.1 binding to stage-specific cis-regulatory elements and recruitment of stage-specific co-binding transcription factors translate into activation of specific target genes. PU.1 co-binding transcription factors include key DC regulators, such as Irf family members (i.e., Irf1, Irf4, Irf5 and Irf8), Klf4, Spib and Tcf4. For example, the co-binding of Tcf4 and PU.1 was specifically observed in pDC, while Irf4 and PU.1 co-binding was confined to cDC. Accordingly, PU.1 has a central position in DC regulatory circuitry and is involved in both DC lineage commitment and subsets specification.

Previous work proposed a layered transcription factor network for inflammatory DC stimulated with lipopolysaccharide (Garber et al. 2012). PU.1 was also positioned at the top of this transcription factor network. However, compared to Garber et al., our regulatory circuitry reveals the transcription factor architecture for the entire sequel MPP-CDP-cDC/pDC, thus covering multiple developmental stages of DC development. Additionally, our regulatory circuitry describes the successive stages of DC development in the steady state.

Different transcription factors were predicted to cooperate with PU.1 to induce DC lineage commitment (MPP-CDP) and subset specific (CDP-cDC/pDC), such as Irf4, Rel/Nfkb, Fos/Jun/Batf3 and Id2 for cDC or Tcf4, Ets1, Irf4 and Spib for pDC. Most importantly, several auto-regulatory feedback loops were identified for key DC genes, such as Irf4, Irf8, Klf4, Tcf4 and Spib. These loops reinforce the expression of stage-specific transcription factors and lock cells in specific differentiation stages, thereby leading to the overall stabilization of the network. Additionally, in keeping with cDC being considered as the default DC developmental pathway (Belz and Nutt 2012), we suggest a specific pDC subnetwork (Tcf4, Spib, Irf1 and Ets1) containing multiple feedback loops. This pDC circuitry is predicted to allow pDC to branch off from the cDC default pathway and to stabilize pDC identify.

In summary, here we propose the basic architecture of the DC transcriptional regulatory program, which drives DC development. We provide a comprehensive characterization of the interplay between individual key DC regulators at specific stages of DC development, which is expected to pave the way for specific tailoring of DC development and function.

**METHODS**

**Cell culture**

Culture of progenitor cells from mouse bone marrow and their differentiation into DC were done as previously described (Felker et al. 2010). MPP, CDP, cDC and pDC were obtained by FACS sorting (FACSAria, BD Biosciences) and used for RNA preparation and chromatin immunoprecipitation (ChIP).

**Gene expression analysis**

Our DNA microarray data of MPP, CDP, cDC and pDC are from Felker et al. (Felker et al. 2010) (GSE22432). DNA microarray data of Miller et al. [18] were retrieved from NCBI GEO (GSE15907). Data were analyzed as previously described (Felker et al. 2010). Differentially expressed genes between two cell types were detected using limma *t*-test with criteria of fold change > 2 and *p* values < 0.05. Raw *p* values were adjusted by Benjamini-Hochberg multiple test correction (Benjamini and Hochberg 1995). To generate linage-specific clusters, all differentially expressed genes were subjected to fuzzy c-mean algorithm (Schwammle and Jensen 2010) and further aggregated into 6 clusters according to their gene expression patterns. Clusters were depicted in heat map format. The boxplot analysis of gene expression data was done in R.

**Chromatin immunoprecipitation and deep sequencing (ChIP-seq)**

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Dahl and Collas 2008) with minor modifications. Briefly, sorted cells were cross-linked at a concentration of 2 \* 106 cells/ml with 1% formaldehyde for 6 min at room temperature. Cross-linking was stopped with 0.125 M glycine. Chromatin sonification into 200-400 bp fragment size was done with Bioruptor with cooling device (Diagenode) at 4°C for 10 min with 30 s pulse/pause cycles. Sheared lysates were clarified by centrifugation (12,000g, 10 min, 4°C). 10 µl Dynabeads Protein A (Life Technologies) was preincubated with either 1 µg IgG control (Santa Cruz Biotechnology) or specific antibodies for H3K4me1 (Abcam, ab8895), H3K4me3 (Diagenode, pAb-003-050), H3K9me3 (Diagenode, pAb-056-050), H3K27me3 (Diagenode, pAb-069-050) or PU.1 (Santa Cruz, sc-352). For immunoprecipitation, sheared chromatin of 1 \* 106 cells was added to the preincubated beads over night at 4°C. Chromatin complexes were isolated by magnetic bead selection and washed with RIPA and TE buffer. Chromatin complexes were digested with 50 µg/ml RNase (Roche) at 37°C for 30 min. Immunoprecipitated DNA was purified using QIAquick PCR Purification Kit according to the manufacturer's protocol (Qiagen). DNA concentration of immunoprecipitated DNA was determined by using Qubit dsDNA HS Assay kit (Life Technologies). Libraries were prepared and subjected to deep-sequencing on the Illunima platform according to the manufacturer's protocols. The sequencing data are available from NCBI GEO series GSE57563.

**Sequencing data analysis**

Short reads of the ChIP-seq experiments were aligned to the mouse reference genome (NCBI37/mm9) using Bowtie. The aligned ChIP-seq data sets of transcription factor PU.1 and the enhancer mark H3K4me1 were then processed to identify genomic regions with sequence enrichment described as peaks using MACS software (version 1.4.2) (Zhang et al. 2008). Peaks of H3K4me3, H3K9me3 and H3K27me3 were identified using SICER, a spatial clustering approach for the identification of larger ChIP-enriched regions (Zang et al. 2009).

The signals of specific histone modifications (H3K4me1, H3K4me3, H3K9me3 and H3K27me3) for gene centric analysis (Fig. 1B, 1C, 2C, S2, S5) were obtained by calculating the average tag density in ±1kb interval centered around transcription start site (TSS) and 3’ end for each Refseq annotated gene. The signals of PU.1 and the enhancer mark H3K4me1 were calculated by the average tag density of ±50kb centered around TSS. Principal component analysis (PCA) was done by prcomp in R package stats.

To detect differential PU.1 binding, the PU.1 peaks in MPP, CDP, cDC and pDC were merged to generate a complete set of peaks. The coverage of reads within the peaks in the complete set was used to estimate the peak signal. The differential PU.1 peaks between pairs of cell types (MPP versus CDP and cDC versus pDC) were detected using exact empirical test from EdgeR (Robinson et al. 2010). Peaks with low read support (i.e., counts-per-million < 3.0) were excluded from further analysis. A peak was considered differential if it produced a significant fold change and *p* value < 0.01 after Benjamini-Hochberg multiple test correction (Benjamini and Hochberg 1995). *De novo* motif detection for PU.1 was performed with MEME-ChIP (Bailey et al. 2010) by providing 200 bp regions around the summits of differential peaks. The motif with highest number of binding sites was reported.

**Identification of PU.1 co-binding transcription factors**

An integrative approach was designed to detect the transcription factor (TF) motifs that are highly enriched in PU.1 peaks. The corresponding workflow is implemented at the Regulatory Genomics toolbox ([www.regulatory-genomics.org](http://www.regulatory-genomics.org)) and depicted in Supplemental Fig. S7. First, differentially expressed genes upon DC commitment (MPP versus CDP) and specification (cDC versus pDC) were detected described as above (Supplemental Fig. S7A). Second, transcription factor motifs were collected from Jaspar (Portales-Casamar et al. 2010), Uniprobe (Robasky and Bulyk 2011) and Homer (Heinz et al. 2010) (Supplemental Fig. S7B). AICS Irf8 motif was obtained by applying the tool MEME-ChIP with default parameters (Bailey et al. 2010) to Irf8 ChIP-seq peaks (Marquis et al. 2011). The Irf8 ISRE and IEACS motifs were obtained from the sequences provided in Kanno et al. (Kanno et al. 2005). The motifs of transcription factors with low gene expression or low variation upon DC development were excluded from further analysis. Next, peaks were assigned to genes if they were in the proximal promoter (1kb upstream of the TSS) and in the gene body (Supplemental Fig. S7C). To detect distal peaks, we also associated peaks when they were 50 kb around the TSS and there was no other gene in between the TSS and the peak. Binding site detection was then performed within PU.1 differential peaks close to differentially expressed genes on the same cell type. All differential peaks were corrected to have uniform size, i.e., 250 bp +/- the peak summit. Motif search was based on Biopython (Cock et al. 2009), utilizing the distribution of the information content of each motif to define a bit score threshold on the basis of a false discovery rate (FDR) test (Supplemental Fig. S7D). We used the FDR value of 0.1 for all binding sites. Next, random binding sites were obtained by iteratively performing binding site detection within randomly sampled regions with 500 bp. The set of random regions had 50 times more peaks then the number of differential peaks. Finally, we employed a one-tailed Fisher’s exact test to measure if the proportion of differential PU.1 peaks close to differentially expressed genes with at least one transcription factor binding site is higher than the proportion of binding sites in random regions. The test was repeated for all motifs and cell-specific differential peaks. Final *p* values were corrected using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). The corrected *p* values (or enrichment scores) were visualized in heat map format (Fig. 5A and Supplemental Fig. S7E). The transcription factors with *p* value < 0.05 were predicted as PU.1 co-binding partners.

**Construction of DC regulatory networks**

The PU.1 co-binding transcription factors identified above and the key DC regulators selected from literature (Laiosa et al. 2006; Carotta et al. 2010; Ghosh et al. 2010) were used to build lineage-specific transcription factor networks (Fig. 5C and Supplemental Fig. S7F). For MPP and CDP networks, differentially regulated genes during DC commitment (MPP versus CDP) were considered. For cDC and pDC networks, differentially regulated genes during DC subset specification (cDC versus pDC) were considered. In each network, nodes represent transcription factors or genes that are connected by edges if the transcription factor (source) is predicted to bind to the gene (source) in the previous analysis. Binding sites of transcription factors enriched in the particular cellular context are depicted in black. In addition, nodes are color-coded differently, i.e., enriched transcription factors in red; non-enriched transcription factors in gray; selected key genes in white. Networks were generated by Cytoscape (Shannon et al. 2003).

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**DISCLOSURE DECLARATION**

**FIGURE LEGENDS**

**Figure 1**

**Gene expression, H3K4me1, H3K4me3, H3K27me3 and PU.1 occupancy in DC development.**

(A) Schematic representation of DC commitment from MPP to CDP and DC subset specification from CDP to cDC or pDC. The number of differentially regulated genes between a pair of cell states is given in red. (B) Differentially regulated genes were clustered according to their expression in MPP/CDP, MPP, CDP, pan-DC, cDC and pDC as indicated. Heat map representation of gene specific mRNA expression (red, high expression; blue, low expression) and the respective H3K4me1, H3K4me3, H3K27me3 and PU.1 occupancy is shown (dark colors indicate high occupancy; light or white colors indicate low or no occupancy). H3K4me3 and H3K27me3 signals at promoter regions (TSS±1kb); H3K4me1 and PU.1 signals at distal regions/enhancers (TSS±50kb). Key regulatory factors are listed. (C) For each of the six clusters, mRNA expression, H3K4me1, H3K4me3, H3K27me3 and PU.1 occupancy were calculated using the geometric mean of the levels of respective genes and are shown in heat map format. Color code as in (B). (D) Occupancy for H3K4me1, H3K4me3, H3K27me3, PU.1 and mRNA profile (log2 expression) of Flt3 gene in MPP, CDP, cDC and pDC. Promoter and enhancer regions with PU.1 binding are indicated (gray).

**Figure 2**

**Epigenetic control of stage-specific genes in DC development.**

(A) Enriched gene ontology terms of genes, which gain H3K4me3 from MPP to CDP. (B) H3K4me3 occupancy and mRNA profiles of Cd74 gene (MHC class II invariant chain), which is up regulated upon DC development. (C) Boxplot analysis of mRNA, H3K4me3, H3K27me3, H3K4me1 and PU.1 occupancy of up or down regulated genes from MPP to CDP (218 and 211 genes, respectively). Changes across DC development (MPP, CDP, cDC and pDC) are shown. H3K4me3 and H3K27me3, TSS±1kb; H3K4me1 and PU.1, TSS±50kb. (D) The percentage of genes with H3K4me3, H3K27me3 or both (bivalent domain) or no modifications is shown. All genes, top panel; Up and down regulated genes between MPP and CDP, middle and low panel, respectively. (E) c-Kit, Batf3 and Pascin1 genes show high mRNA expression in MPP, cDC and pDC, respectively, and dynamic changes of bivalent modification (H3K4me3 and H3K27me3, gray box). Heat map is color-coded as in Fig. 1B.

**Figure 3**

**H3K9me3 modifications at gene ends positively correlate with gene expression.**

(A) Average density of H3K9me3 over 10 kb region at TSS or 3’ gene ends in MPP, CDP, cDC and pDC is shown. Red line, 2000 most abundantly expressed genes in the respective cell types; blue line, 2000 less abundantly expressed genes; Black dotted line, average of all genes. (B) H3K9me3 modification pattern of Gfi1, Id2 and Il7r genes in each investigated cell type. The peaks located at 3’ gene ends are highlighted in gray. The mRNA levels of each gene are depicted in heat map format. Red, high expression; blue, low expression.

**Figure 4**

**PU.1 transcription factor binding in DC development.**

(A) Principal component analysis of genome-wide PU.1 binding profiles in MPP, erythoid progenitor cells (EP), T/B lymphoid cells (double negative T cells, DN; pro B cells, ProB) and DC progenitors and subsets (CDP, cDC and pDC; GM-CSF derived DC; GM-DC). The DC cluster is highlighted. (B) PU.1 binding peaks occurring in enhancers or active promoters in MPP, CDP and cDC and pDC are shown. Regions were defined as active promoters if marked with H4K4me3 and enhancers if marked with H3K4me1. (C) Differential PU.1 peaks between MPP versus CDP and cDC versus pDC are depicted in blue and red as indicated. Non-differential peaks are colored in gray. De-novo PU.1 motifs calculated for cell type specific peaks and the classical PU.1 motif (UP00085, Uniprobe [31]) are shown. (C, left), differential PU.1 peaks in MPP and CDP; (C, right), differential PU.1 peaks in cDC and pDC. (D) The proportion of PU.1 target genes with differential PU.1 peaks close to differentially regulated genes in progenitors (MPP verse CDP) and DC subsets (cDC verse pDC) are shown in percent (filled bars). The percentage of PU.1 targets in all genes was used as background control (open bars). The Fisher’s exact test was employed to calculate the enrichment of PU.1 targets.

**Figure 5**

**Identification of linage-specific transcription factor regulatory networks.**

(A) Heat map depicts the enrichment of TF motifs (row) in MPP, CDP, cDC and pDC (column). *P* values are plotted and color-coded using a continuous spectrum from gray (*p* value > 0.05) to blue (*p* value < 0.5). 20 TFs (24 motifs) that were considered as potential PU.1 co-binding partners are labeled in red. Clusters I to V are indicated. (B) PU.1 occupancy of Id2 and Irf1 loci are shown for MPP, CDP, cDC and pDC. Differential PU.1 peak regions are highlighted by gray boxes. Green lines indicate transcription factor binding sites (TFBS) predicted inside differential PU.1 peak regions. (C) Linage-specific transcription factor regulatory networks. Nodes represent identified PU.1 co-binding partners from (A) that are active (red) or inactive (gray) in MPP, CDP, cDC and pDC. Selected key DC regulators from literature (white) were included in the analysis. In MPP and CDP networks, differentially regulated genes during DC commitment (MPP versus CDP) were considered. In cDC and pDC networks, differentially regulated genes during DC subset specification (cDC versus pDC) were considered. A directed edge from node a to node b (black) indicates that the binding site of node a is found in the differential PU.1 peak region close to node b. A self-loop edge (grey box) indicates auto-regulatory feedback loop of the indicated transcription factor. The gray edges show regulatory links predicted in at least one of the networks.

**Figure 6**

**The regulatory circuitry of DC development.**

The network illustration depicts the organization of the integrated DC regulatory circuitry. Nodes represent key regulatory factors involved in DC development. A directed edge from factor a to factor b indicates an active function (green) or inhibition function (red) of factor a to factor b. Dotted edges represent additional interactions obtained from the literature. The pDC sub-network of Irf1, Ets1, Spib and Tcf4 is shown (gray box).

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