Epigenetic and Transcriptional Architecture of Dendritic Cell Development

Running title: Transcription Factor Circuitry in Dendritic Cells

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Key Points

1. Dynamics of gene expression, histone modification and transcription factor occupancy during DC development from hematopoietic stem cells.

2. Description of basic architecture of how DC transcription factors are wired to drive DC development**.**

Abstract

Dendritic cells (DC) represent professional antigen presenting cells with a pivotal role in both immunity and tolerance induction. They develop from hematopoietic stem cells through successive steps of lineage commitment and differentiation. Here we describe the dynamics of gene expression, histone modification and transcription factor occupancy during DC development, from multipotent progenitors (MPP) to DC restricted common DC progenitors (CDP), and further into classical DC (cDC) and plasmacytoid DC (pDC). Specific H3K4me1, H3K4me3 and H3K27me3 marks in CDP reveal a DC-primed epigenetic signature, which is maintained and reinforced during DC differentiation. Epigenetic marks and transcription factor PU.1 occupancy increasingly coincide upon DC development, advocating for PU.1 acting as a pioneer transcription factor in DC. We developed a computational approach by integrating PU.1 occupancy and 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 circuitry also includes positive feedback loops inferred for individual or multiple factors that stabilize the distinct stages of DC development. In summary, our study provides the basic epigenetic and transcriptional architecture of DC development.

Introduction

Dendritic cells (DC) represent specialized immune cells that develop from hematopoietic stem cells.[1,2] 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.[1,3,4] 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.[1,2,5] First, multipotent hematopoietic stem/progenitor cells (MPP) 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.[1,3,6-13] Furthermore, hematopoietic master regulators, such as the transcription factors PU.1 and Gfi1, were shown to regulate DC development.[3,6,14,15] 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.[16,17] Specific histone methylations impact on gene transcription: Histone H3 lysine 4 trimethylation (H3K4me3) and H3 lysine 27 trimethylation (H3K27me3) at gene

promoters are associated with gene activation and repression, respectively.[18] Many key developmental genes have bivalent modification where large domains of repressive H3K27me3 coexist with small domains of activating H3K4me3.[19,20] These genes are poised for either activation or repression during differentiation.

Epigenetic modifications also regulate hematopoiesis. 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.[17,21,22] For example, specific histone modifications control T cell development and erythropoiesis.[23,24] Additionally, global histone modification and transcription factor occupancy in inflammatory DC stimulated with lipopolysaccharide and in monocyte derived DC and pDC were also studied.[22,25] Specific histone modifications are used for identification of enhancers and/or cis-regulatory modules.[26] For instance, H3 lysine 4 monomethlyation (H3K4me1) marks genomic regions that indicate primed enhancers. Recent genomic studies on the dynamics of blood cell formation from hematopoietic stem cells covered all conventional hematopoietic lineages, but did not include DC.[27]

Here we generated global maps of H3K4me1, H3K4me3, H3K27me3 and PU.1 occupancy in MPP, CDP, cDC and pDC by chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-seq). We report on the dynamics of histone modifications and transcription factor binding during DC development and construct a DC regulatory circuitry for DC commitment and subset specification.

Materials and Methods

Cell culture

Culture of progenitor cells from mouse bone marrow and their differentiation into DC were done as previously described.[5] 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.[5] (GSE22432). DNA microarray data of Miller et al.[11] were retrieved from GSE15907. Data were analyzed as previously described[5] (see supplemental information).

Chromatin immunoprecipitation and deep sequencing (ChIP-seq) analysis

Chromatin immunoprecipitation (ChIP) assays were performed as described[28] with minor modifications (see supplemental information). The ChIP-seq data are available from NCBI GEO series GSE57563 and GSE64767. The PU.1 ChIP-seq data were retrieved from GSE21953, GSE31233, GSE21621 and GSE36104. The ChIP-seq data of Irf4, Irf8 and Stat3 in cDC and of Irf8 and Tcf4 in pDC were downloaded from GSE36104, GSE53311, GSE27161, GSE62702 and GSE43876, respectively. Detailed sequencing data analysis is described in supplemental information.

Identification of PU.1 co-binding transcription factors and construction of DC regulatory networks

An integrative approach was designed to detect the transcription factor motifs that are highly enriched in PU.1 peaks. The corresponding workflow is implemented at the Regulatory Genomics toolbox (www.regulatory-genomics.org). Briefly, we focused on differentially expressed transcription factors upon DC development and collected their sequence motifs

from public databases. We then performed motif search around PU.1 differential peaks to detect putative transcription factor binding sites. Here we only considered PU.1 differential peaks close to up-regulated genes in the same cell types. Next, we determined 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. Finally, the identified PU.1 co-binding transcription factors and selected key DC regulators from the literature[6,29-35] were used to build lineage-specific transcription factor networks and DC regulatory circuitry (see details in supplemental information).

Results

Lineage-specific transcriptional signatures in DC development

MPP were induced to differentiate *in vitro* into CDP (DC commitment) and further into cDC and pDC (DC subset specification; Figure 1A).[5,36] 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 (Figure 1A). A total of 3194 genes were differentially expressed between the differentiation stages (fold change > 2, p value < 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; Figure 1A). Among them, 210 genes encode transcription factors. These include many critical DC regulators, such as Irf4, Irf8, Batf3, Relb, Id2, Spib and Tcf4 (also known as E2-2).[3] 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 (Figure 1B). These clusters match very well to gene expression profiles calculated from *in vivo* sorted DC progenitors and DC (supplemental Figure 1).[11]

The MPP cluster contains Gata2, Gfi1 and Tal1, which are down-regulated from MPP to CDP and have low or no expression in DC. This reflects the gradual restriction of development from early hematopoietic progenitors towards DC committed progenitors and DC (Figure 1B). The pan-DC cluster comprises genes, such as Flt3, with important roles in both cDC and pDC (Figure 1B). The cDC and pDC clusters identify DC subset-specific genes, such as Id2, Irf4 and Zbtb46 for cDC and Irf1 and Tcf4 for pDC. Taken together, our analysis captures known and putative DC regulators and thus provides the basis for investigating the underlying epigenetic and transcriptional architecture of DC commitment and subset specification.

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

Epigenetic modifications, including specific histone modifications, impact on gene expression and thus on cell identity and function.[37] Therefore, to gain further insights into the mechanisms of DC development, we determined how histone marks (H3K4me1, H3K4me3 and H3K27me3) and PU.1 binding relate to stage-specific gene clusters in DC development by ChIP-seq for MPP, CDP, cDC and pDC (Figure 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 (Figure 1B-C). Importantly, the enhancer mark H3K4me1 and PU.1 binding were detected in both progenitor and DC specific genes and followed the pattern of H3K4me3. Moreover, PU.1 occupancy was observed for all stage-specific gene clusters in CDP, suggesting that CDP acquire 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.[17,37,38]

To extend the observation of DC epigenetic priming in CDP, we focused on genes differentially expressed during DC commitment (Figure 1A). Upon MPP-CDP transition, up-regulated genes acquire H3K4me1, H3K4me3 and PU.1 in CDP, whereas down-regulated genes acquire H3K27me3 or bivalent marks without obvious change in PU.1 occupancy (supplemental Figure 2A-B). Importantly, these patterns are maintained in differentiated DC (cDC and pDC), indicating epigenetic DC priming of lineage-specific gene promoters and enhancer regions. Additionally, genes with an increase of H3K4me3 (e.g. Cd74) during DC commitment are associated with immune related functions, such as immune response and leukocyte activation (supplemental Figure 2C-D).

Further analysis of histone marks and PU.1 occupancy during DC subset specifications reveals that the DC-primed epigenetic signatures in CDP are enhanced upon CDP-cDC or CDP-pDC transition, which is particularly prominent for H3K4me1 and PU.1 occupancy

(supplemental Figure 3A-B). For example, the cDC gene Cd83 shows an increase in H3K4me1, H3K4me3 and PU.1 occupancy during CDP-cDC transition and is repressed in pDC due to bivalent modifications (supplemental Figure 3C). The epigenetic profile of the pDC gene Siglec-H during CDP-pDC transition changed accordingly (supplemental Figure 3D).

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.

Next, we focused on the analysis of individual representative genes, including Gfi1, Flt3, Id2 and Irf8 for progenitor, pan-DC, cDC and pDC affiliated genes, respectively (Figures 1D and 2). All these genes are implicated in DC development based on gene knockout studies.[1,3] Flt3 is a key cytokine receptor for DC development and regulated by PU.1.[6] Flt3 promoter shows prominent H3K4me3 signals, which increase upon differentiation (Figure 1D). Concomitantly, H3K27me3 signals decrease from MPP to DC lineages. H3K4me1 and PU.1 peaks reveal 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 (Figure 1D). The progenitor gene Gfi1 has prominent H3K4me3 signals at the promoter in MPP and acquires H3K27me3 upon DC differentiation, thus forming a bivalent modification (Figure 2). A similar pattern was observed for Cepba. cDC genes (Id2, Batf3) show an accumulation of H3K4me3 and PU.1 signals in promoter or enhancer regions, respectively, in cDC. The pDC gene Spib shows 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 show particular prominent H3K4me3 occupancy at promoters in DC, which increase during DC development (Figure 2). In summary, our analysis reveals the dynamics of stage-specific histone marks and PU.1 binding that control the DC differentiation program.

The instructive role of PU.1 in DC development

PU.1 (encoded by Sfpi1 gene) represents a master regulator in hematopoiesis with a prominent role in multiple cell fate decisions, including DC development.[6,14] PU.1 occupancy in differentially regulated genes during DC commitment and specification is prominently up-regulated (supplemental Figures 2A and 3A-B), suggesting that PU.1 has a determining function in establishing the DC lineage. Principal component analysis of our PU.1 ChIP-seq data of DC in comparison to PU.1 data of multiple hematopoietic lineages[22,39-41] reveals a DC-specific developmental pathway induced by PU.1 (Figure 3A). Interestingly, CDP cluster with DC and are positioned distant from MPP, indicating that CDP exhibit a DC-primed PU.1 binding profile. cDC and GM-DC are positioned very close to CDP, which supports the hypothesis that differentiation of cDC represents the default pathway of DC development.[3,6,29]

Genome-wide analysis of PU.1 ChIP-seq data reveals a preference for PU.1 binding to gene bodies and intergenic regions in MPP, CDP, cDC and pDC (supplemental Figure 4A). This is consistent with previous studies and PU.1 function as an enhancer factor.[17,22] Additionally, a prominent increase of PU.1 binding is observed in the promoter region from MPP to CDP and cDC (supplemental Figure 4A). 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 compared to MPP (Figure 3B). A similar trend was observed in GM-DC ChIP-seq data[22] (supplemental Figure 4B). These results suggest that PU.1 acts as a pioneer transcription factor to recruit chromatin modifiers and to initiate DC lineage commitment. Moreover, the genomic distribution of PU.1 peaks is different for cDC and pDC (supplemental Figure 4A), indicating that PU.1 might be also involved in the regulation of DC lineage diversification.

Next, we analyzed the dynamics of PU.1 peaks during DC development and the associated PU.1 motifs (Figure 3C). A total of 19,944 MPP and 23,054 CDP differential peaks were detected. MPP peaks reveal an alternative de-novo motif with a weak GGAA sequence,

while CDP peaks show the classical PU.1 motif (UP00085)[42], containing an ETS binding site (GGAA core site; Figure 3C). Similarly, PU.1 occupancy is also different between cDC and pDC. The PU.1 binding sequence in cDC resembles the classical PU.1 motif, while the pDC binding sequence has a similar motif with less specificity (Figure 3C). These results are in support of DC-primed PU.1 binding profiles in CDP. Furthermore, PU.1 binding was significantly associated with transcriptional activation of lineage-specific genes in CDP, cDC and pDC (Figure 3D), indicating a positive role of PU.1 in DC lineage fate determination.

Identification of PU.1 co-binding transcription factors

Transcription factor networks control hematopoietic cell differentiation, including DC development.[3,4,11,43,44] Genome-wide approaches, interrogating gene expression, ChIP-seq data and transcription factor binding motifs, have been used to identify gene regulatory network.[22,45] To identify PU.1 co-binding partners in DC development, we designed an integrative computational approach to analyze sequences around PU.1 peaks of differentially expressed genes (see Methods; supplemental Figure 5).

In total, 27 transcription factors (represented by 31 motifs) are significantly enriched at different stages of DC development (Figure 4A). Transcription factors with opposing expression and enrichment patterns (supplemental Figure 6) were excluded from further analysis. Twenty transcription factors, including PU.1 itself (red in Figure 4A), were predicted as PU.1 partners and classified into 5 stage-specific clusters. Irf8 and Ets-domain transcription factor Spib (Cluster I; Figure 4A) are known interaction partners of PU.1 and also implicated in DC differentiation.[3] Irf8-deficient mice lack many mature DC subsets. Spib is also indispensable for DC development, particular in pDC.[46] Both factors show a significant enrichment in MPP, CDP and the two DC subtypes, indicating that they undergo composite binding with PU.1 in each step of DC development.

Upon DC commitment a panel of transcription factors, such as Stat1, Klf4, Egr1, Irf1, Runx1, Irf4, and Ets1, are enriched in CDP (Cluster II; Figure 4A). This indicates that PU.1 initiates the DC program by recruiting or cooperating with multiple DC regulators, including known and novel PU.1 interacting factors. For example, up-regulation of Id2 expression is accompanied by an increase of PU.1 binding in the Id2 promoter from MPP to CDP (Figure 4B). 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 subset specification, PU.1 is predicted to collaborate with distinct sets of transcription factors to restrict the developmental program towards to either cDC or pDC, such as Rel/Rela/Nfkb1 and Irf5 for cDC and Tcf4 for pDC (Clusters III, IV and V; Figure 4A). The Id2 gene, a prototype cDC marker, contains specific PU.1 peaks at promoter and distal regions in cDC, which are associated with binding sites of Jun/Ap1, Rel/Nfkb, Irf8, Klf4 and Egr1 (Figure 4B). Conversely, the pDC-affiliated gene Irf1 contains PU.1 binding regions, which harbor the binding sites of Tcf4, a prototype pDC transcription factor (Figure 4B). Furthermore, many cDC or pDC specific regulators identified in this analysis were found to be enriched in CDP (Figure 4A), such as Fos/Jun/Ap1 and Tcf4, again supporting the notion of DC priming in CDP.

PU.1 centered regulatory circuitry of DC development

We then constructed cell-specific transcription factor regulatory networks (Figure 4C). In each network, nodes represent the potential PU.1 co-binding partners (Figure 4A) and selected key DC regulators (e.g., Flt3, c-Kit and Id2).[3,11] 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 stage-specific networks reflect how dynamics of PU.1 composite binding drives DC development. In CDP, PU.1 recruits a core set of transcription factors (e.g., Irf8, Klf4, Runx1, Egr1 and Stat1) to activate the expression of DC marker genes (e.g., Id2, Csf1 and Tcf4; Figure 4C). In cDC and pDC, DC subtype-specific transcription factors collaborate with PU.1 to define cDC or pDC identity (Figure 4C). For example, Rel/Nfkb and Irf4 exclusively co-bind with PU.1 in cDC, whereas Tcf4, Ets1, Irf1 and Spib only cooperate with PU.1 in pDC.

Auto-regulatory feedback loops are important building blocks of transcriptional regulatory networks.[47,48] PU.1 was shown to control hematopoietic development by forming auto-regulatory loops.[49,50] Intriguingly, several positive auto-regulatory loops of key DC genes are captured in our networks (Figure 4C; highlighted in gray). The auto-regulatory loop of Irf8 in CDP indicates that Irf8 induces its own transcription, emphasizing 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.[51] Similarly, an auto-regulatory loop was also observed for Irf4 in cDC, which is in accordance with Irf4 function in cDC development.[3]

We then proceeded to integrate the four stage-specific transcription factor networks in one DC regulatory circuitry (Figure 5A). Each regulator in the circuitry is positioned at a specific stage of DC development in which it is involved. Additionally, some of the positive or negative interactions between DC regulators were included based on the literature.[6,29-35] For example, PU.1 was observed to inhibit Gata1/2 activation, which led to specific myeloid cell fates.[52] Accordingly, this inhibition of Gata2 by PU.1 is displayed in the circuitry.

PU.1/Sfpi1 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; Figure 5A). 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.[10,53]

Additionally, the cDC marker Irf4 is predicted to be regulated by multiple transcription factors, including PU.1, Irf8, Stat3 and Irf4 itself (Figure 5A). ChIP-seq data demonstrate PU.1, Irf4, Irf8 and Stat3 co-binding in the enhancer region of Irf4 gene (Figure 5B), which provides experimental evidence for the predicted interactions within the network. Furthermore, Irf4 binding in the promoter and enhancer regions of Irf4 gene (Figure 5B) supports the auto-regulatory loop inferred for Irf4. Importantly, the positive auto-regulatory loops inferred for PU.1, Irf8, Klf4, Irf4, Spib and Tcf4 may provide the basic regulatory mechanisms to stabilize the DC program.

Multiple feedback loops were observed between pDC factors, such as Irf1, Ets1, Spib and Tcf4 (pDC sub-network in Figure 5A), yet such feedback loops were not shown for cDC factors (Fos/Jun/Batf3, Rel/Relb/Nfkb, Id2 and Irf4). ChIP-seq data demonstrate PU.1, Irf8 and Tcf4 co-binding in the enhancers of Spib and Irf1, two key pDC genes (Figure 5C, supplemental Figure 7). Collectively, our study reveals multiple interactions among the Irf1, Ets1, Spib and Tcf4 transcription factors, which 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 require 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 and H3K27me3 for MPP commitment towards CDP and specification into cDC and pDC. We demonstrate that stage-specific expression of key DC regulators, 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 show 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 in line with our data on the DC-primed transcriptional signature in CDP.[5] Additionally, changes of bivalent domains lead to activation/repression of the DC-primed gene signature in CDP and of DC subset-specific genes in DC, suggesting an important role of bivalent marks in DC commitment and specification.

PU.1 represents a pioneer transcription factor in hematopoietic cell development that acts in concert with other lineage specific factors.[17,22,38] DC progenitors and DC subsets express PU.1 and knockout mice demonstrated the impact of PU.1 on DC development.[54,55] 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.[6,36] PU.1 also induces chromatin remodeling of the Irf8 gene that encodes an important transcription factor for DC development.[14] 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.[22] PU.1 was also positioned at the top of this transcription factor network. However, compared to Garber et al.[22], 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 found 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[3], 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 describe the basic architecture of the DC transcriptional regulatory program, which drives commitment of hematopoietic stem cell and their differentiation into DC. 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.

Authorship

Contribution: QL, HC, IGC, KH, KS and MZ designed the study. HC and SM performed cell sorting and ChIP analysis. QL, ICG, EGG and SH performed bioinformatic analysis; BB, BH, HS and VB performed genome sequencing; QL, HC, ICG, KS and MZ drafted and wrote the manuscript. All authors contributed to and approved the final manuscript.

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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 expressed genes between a pair of cell states is given in red. **(B)** Differentially expressed 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, H3K27 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, H3K27me, 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. Histone modifications and PU.1 binding dynamics of key DC transcriptional regulators during DC development.

Occupancy for H3K4me1, H3K4me3, H3K27me3 and PU.1 and mRNA expression (log2 expression) of DC progenitor genes (Gfi1 and Cebpa), cDC genes (Id2, Batf3) and pDC genes (Tcf4, Spib, Irf7r and Irf1) in MPP, CDP, cDC and pDC. Irf8, a central DC transcription factor, is also shown. Arrow indicates the direction of transcription.

Figure 3. PU.1 transcription factor binding in DC development.

(A) Principal component analysis of genome-wide PU.1 binding profiles in MPP, erythroid 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 both H3K4me1 and H3K4me3, and enhancers if marked only with H3K4me1. **(C)** Differential PU.1 peaks between MPP versus CDP (left) and cDC versus pDC (right) 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)[42] are shown. **(D)** The proportion of PU.1 target genes with differential PU.1 peaks close to differentially regulated genes in progenitors (MPP versus CDP) and DC subsets (cDC versus 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 4. Identification of lineage-specific transcription factor regulatory networks.

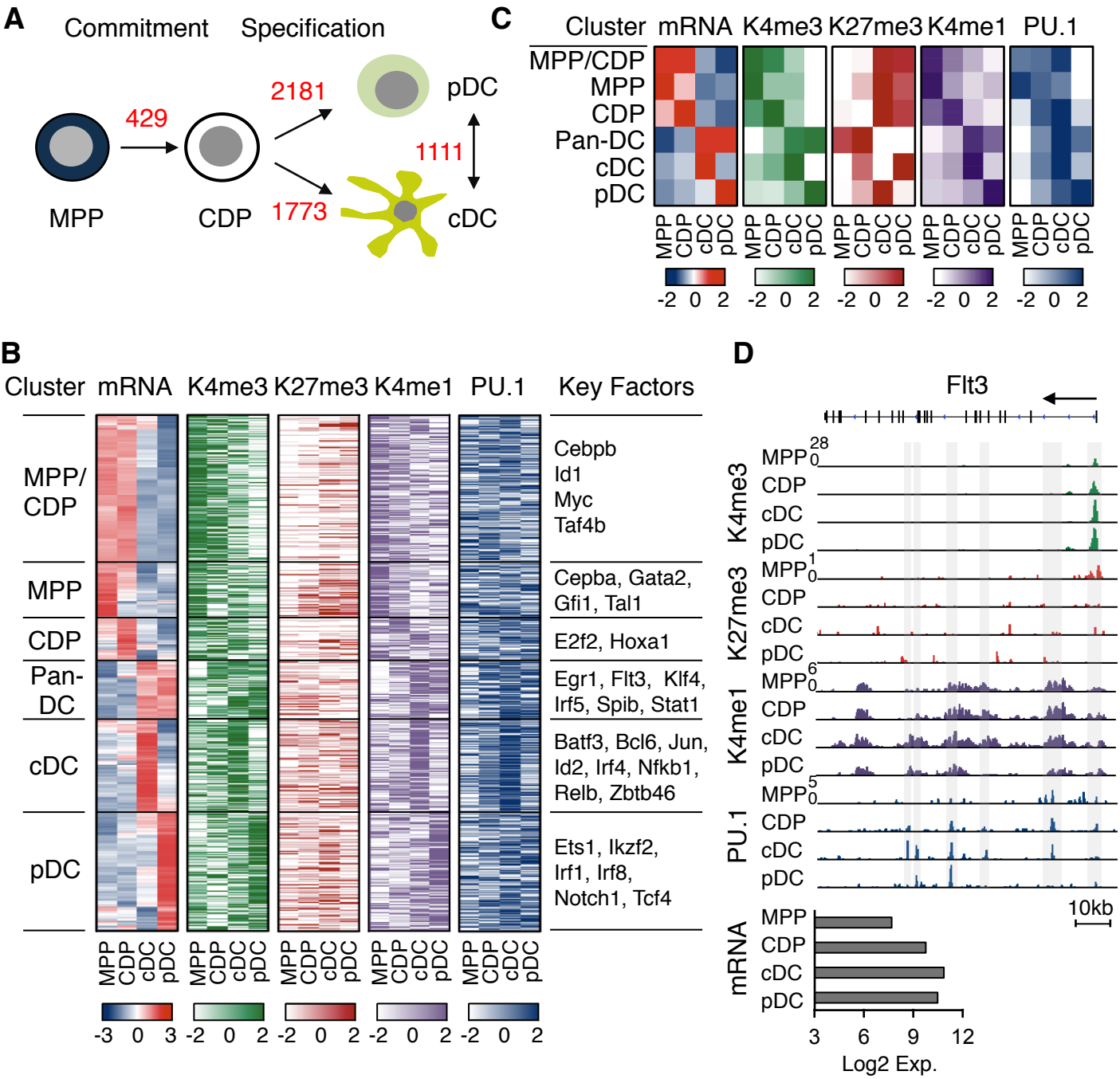
(A) Heat map depicts the enrichment of transcription factor motifs (row) in MPP, CDP, cDC and pDC (column). *P* values are plotted and color-coded using a continuous spectrum from gray (*p* value > 0.05) to blue (*p* value < 0.05). Twenty transcription factors (24 motifs) that are considered as potential 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)** Stage-specific transcription factor regulatory networks. Nodes represent identified Sfpi1/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 the literature (white) were included in the analysis.[6,29-35] 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 an auto-regulatory feedback loop of the indicated transcription factor. The gray edges show regulatory links predicted in at least one of the networks.

Figure 5. The regulatory circuitry of DC development.

(A) 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.[6,29-35] The pDC sub-network of Irf1, Ets1, Spib and Tcf4 is shown (gray box). **(B)** Occupancy of PU.1, Irf4, Irf8 and Stat3 in the enhancer region (H3K4me1) of Irf4 gene in cDC (highlighted in gray) verify the predicted transcription factor binding sites. The ChIP-seq data of Irf4, Irf8 and Stat3 in cDC are from GSE36104, GSE53311 and GSE27161. (**C**) Occupancy of PU.1 and Irf8 in Spib enhancer region (H3K4me1) in pDC (highlighted in gray) is in line with the predicted co-binding of Irf8 and PU.1. The PU.1 peak in mouse pDC is mapped to human genome using UCSC liftOver tool and shown as blue bar. The PU.1 peak collocates with Tcf4 in human pDC and sequence predicted transcription factor binding sites for PU.1, Tcf4, Spib, Irf1 and Irf8 (highlighted in gray). The ChIP-seq data of Irf8 and Tcf4 in pDC are from GSE62702 and GSE43876. Predicted transcription factor binding sites (TFBS), green.

Figure 1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Log2 Exp.

Figure 2

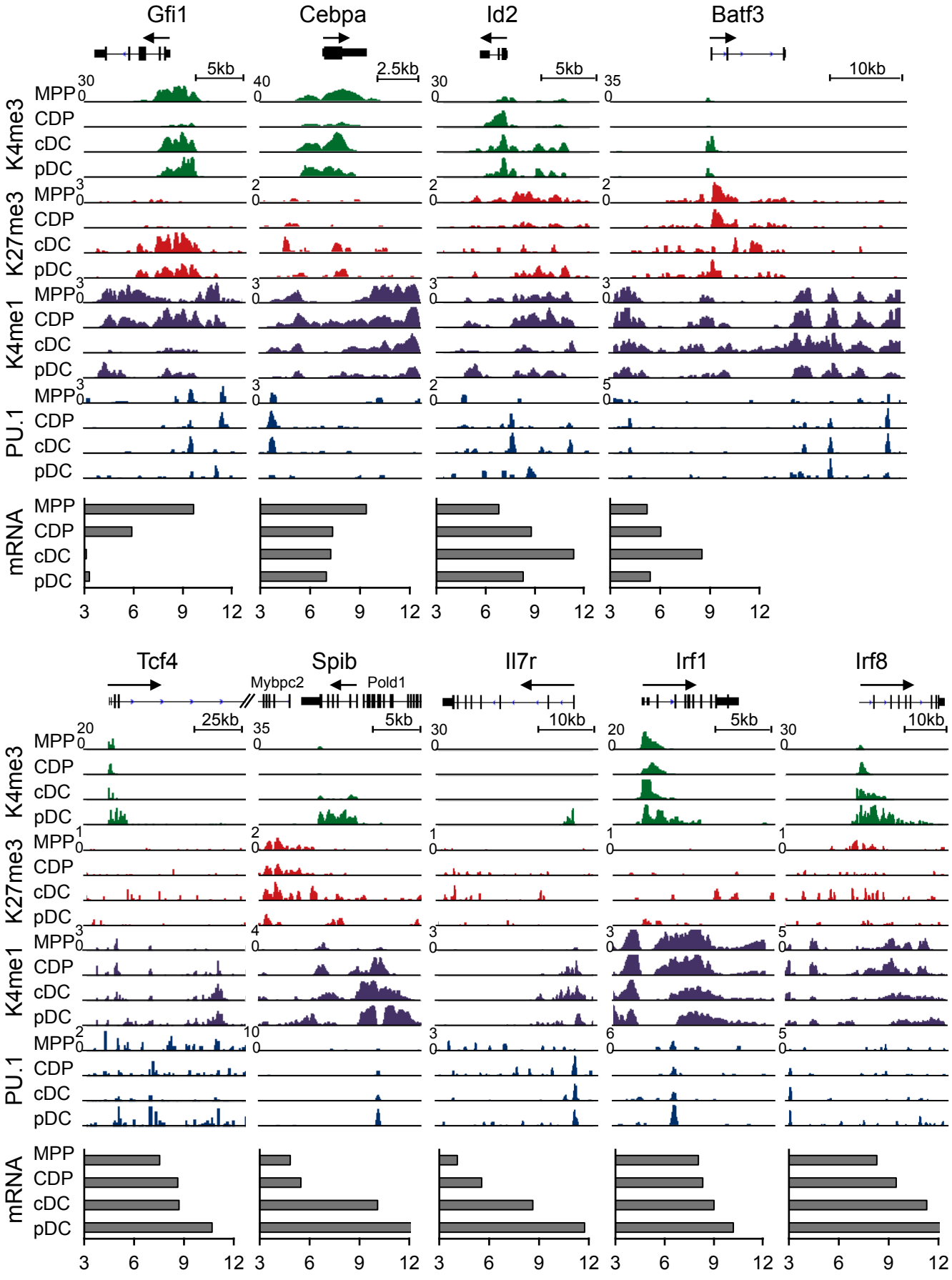
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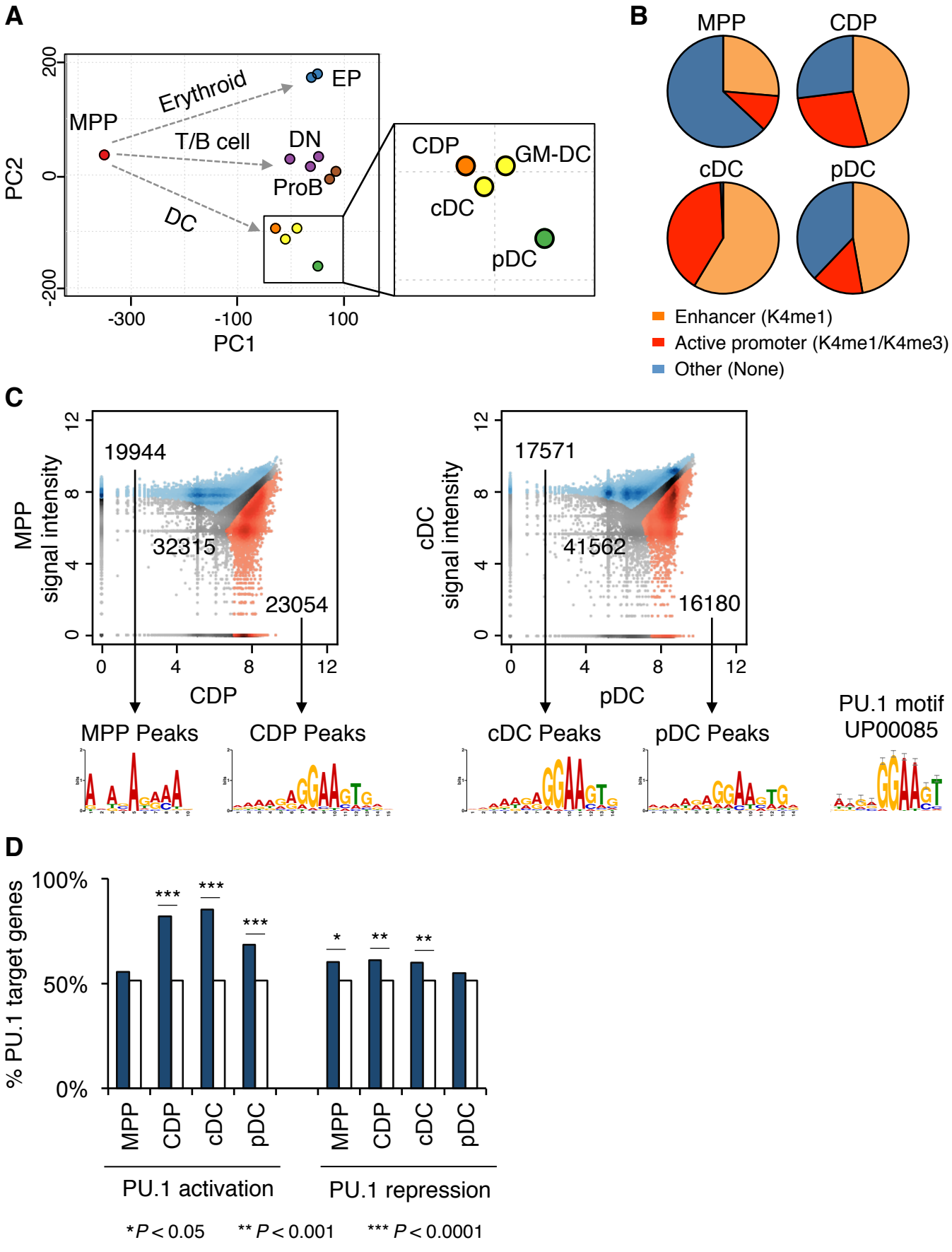
Figure 3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GM-DC
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cDC
pDC
Enhancer (K4me1)
Active promoter (K4me1/K4me3)
Other (None)
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32315
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CDP
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CDP Peaks
cDC signal intensity
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16180
pDC
cDC Peaks
pDC Peaks
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D
% PU.1 target genes
100%
50%
0%
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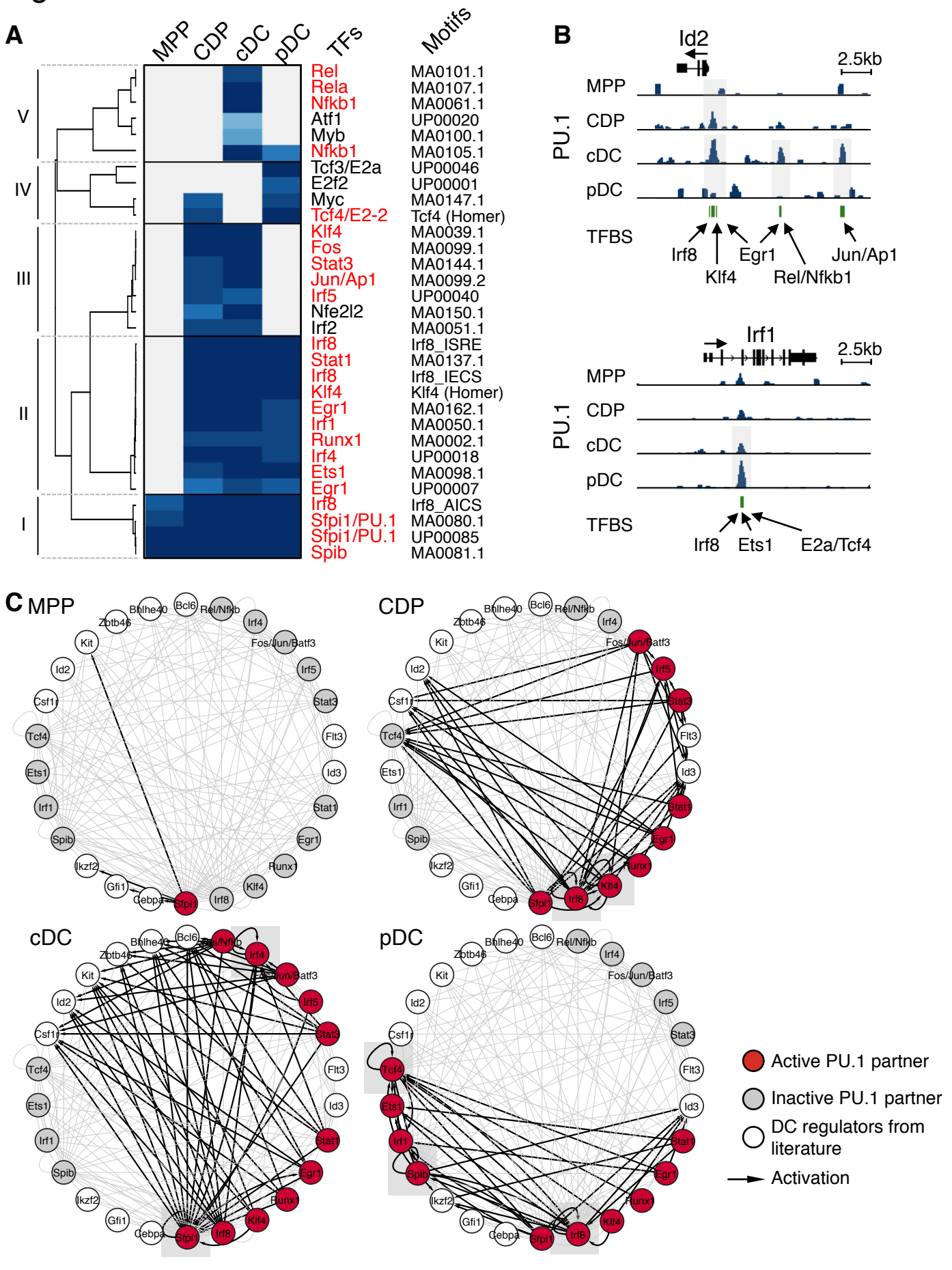
** P < 0.001
*** P < 0.0001

Figure 4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Egr1
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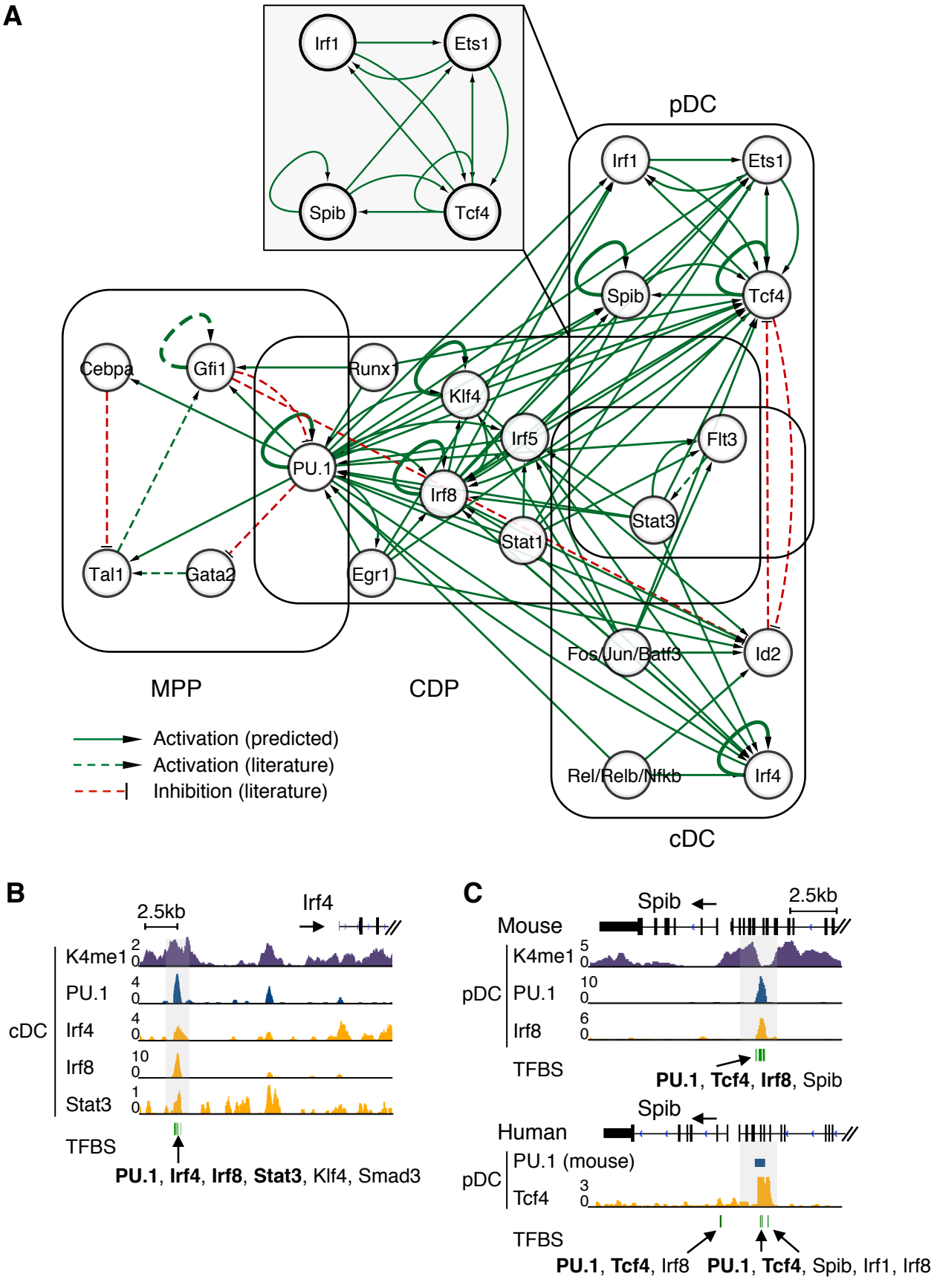
Active PU.1 partner
Inactive PU.1 partner
DC regulators from literature
Activation

Figure 5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Inhibition (literature)
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