

**DUAL ROLE OF PU.1 IN ENHANCER PRIMING IN MACROPHAGES**

**By**

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## **ABSTRACT**

### **DUAL ROLE OF PU.1 IN ENHANCER PRIMING IN MACROPHAGES**

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All multicellular organisms arise from a single-celled zygote by the precise execution of a gene expression program which ensures appropriate cell identity. This process is particularly challenging in eukaryotic cells since eukaryotic DNA is packaged by architectural proteins called histones into chromatin, which might act as a barrier to the transcriptional machinery. Macrophages are cells of the immune system which undergo rapid, large scale changes in gene expression in response to bacterial or viral challenge. This makes macrophages an excellent model for studying cell-type specific as well as inducible gene expression. Studies at the genome-wide level have shown that distal regulatory elements like enhancers play an essential role in determining the macrophage inducible response to microbial challenge. Further, lineage-specific transcription factors like PU.1 and C/EBP $\beta$  are known to bind inducible enhancers prior to gene induction in resting macrophages. Earlier studies using genome-wide approaches indicate that PU.1 is able to interact with chromatin, thus functioning as a 'pioneer factor' in macrophages. However, not much is known about the mechanism by which PU.1 keeps enhancers accessible prior to gene induction in resting macrophages. Using bone-marrow derived primary mouse macrophage cells as well as PU.1 deficient cell lines, my work highlights the changes in chromatin associated with PU.1 binding during macrophage differentiation as well as in response to bacterial infection. Using a quantitative nucleosome occupancy assay, we reported that PU.1 binding correlates with low nucleosome occupancy at an inducible

enhancer in resting macrophages. Further upon induction with an appropriate stimulus, nucleosomes are stably evicted from the distal enhancer and the corresponding gene can be induced. More importantly, my results suggest that lack of PU.1 binding renders regulatory regions (enhancers and promoters) of inducible genes susceptible to heterochromatin formation and silencing by Polycomb repressive complex 2 (PRC2) in differentiated macrophages. PRC2-mediated silencing is also associated with an increase in nucleosome occupancy at the target regions and the corresponding genes cannot be induced. Results obtained from this research will provide important insights into the role of lineage-specific transcription factors at regulatory elements both during normal development and disease.

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## KEY TO ABBREVIATIONS

|         |                                             |
|---------|---------------------------------------------|
| AP1     | Activator protein 1                         |
| ASB5    | Ankyrin repeat and SOCS box containing 5    |
| ATP     | Adenosine triphosphate                      |
| BRG1    | Brahma related gene-1                       |
| BAF     | BRG1 or hBRM associated factors             |
| BET     | Bromodomain and extraterminal domain family |
| CCDC64B | Coiled-coil domain containing 64B           |
| C/EBP   | CCAAT/enhancer binding protein              |
| CSFR1   | Colony stimulating factor 1 receptor        |
| DTT     | Dithiothreitol                              |
| EDTA    | Ethylenediaminetetraacetic acid             |
| ETS     | E26 transformation specific                 |
| FBS     | Fetal bovine serum                          |
| HEK293  | Human embryonic kidney cells 293            |
| IFNB1   | Interferon B1                               |
| IL1A    | Interleukin 1A                              |
| IL12B   | Interleukin 12B                             |
| IRF3/8  | Interferon regulatory factor 3/8            |
| ISD     | Interferon stimulatory DNA                  |
| LAP     | Liver-enriched activator protein            |
| LDS     | Lithium dodecyl sulphate                    |
| LIP     | Liver-enriched inhibitory protein           |

|         |                                                                |
|---------|----------------------------------------------------------------|
| NFκB    | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| PCR     | Polymerase chain reaction                                      |
| PEG     | Polyethylene glycol                                            |
| PIC     | Pre-initiation complex                                         |
| RPL4    | Ribosomal protein L4                                           |
| SFFV    | Spleen focus-forming virus                                     |
| SDS     | Sodium Dodecyl sulphate                                        |
| SFPI1   | SFFV protein 1                                                 |
| STFA3   | Stefin 3                                                       |
| SV40    | Simian virus 40                                                |
| SWI/SNF | SWItch/Sucrose Non-Fermentable                                 |
| TCA     | Trichloroacetic acid                                           |
| TLR4    | Toll-like receptor 4                                           |
| TSE     | Tris/Sucrose/EDTA buffer                                       |
| TSS     | Transcription start site                                       |
| XBP     | X-box binding protein                                          |

## CHAPTER 1: INTRODUCTION

## **Lineage-specific TFs are determinants of cellular fate during differentiation**

Mammalian development begins from a single-celled zygote proceeded by a series of cell divisions following which a mature organism is formed. The early stages of a developing embryo have been very well-characterized in the mouse and have distinct morphological events which can be easily distinguished. The execution of this precise cell identity cascade includes a number of processes involving the extracellular signals in the microenvironment as well as cell-intrinsic factors resulting in differential gene expression. However, what lies at the core of this fundamental developmental process is the expression of lineage-appropriate genes with temporal and spatial precision. Lineage-specific transcription factors (TF) which are expressed early in development play a central role in this process. These factors are sequence-specific DNA binding proteins which act alone or in combination with other TFs to ensure appropriate cell identity. Elegant experiments by the Yamanaka group (2) demonstrated that ectopic expression of a combination of four lineage-specific pluripotency TFs was sufficient to reprogram adult somatic cells to pluripotent stem cells, thus highlighting that terminally differentiated cells retain the ability to switch their cell identity. There is active ongoing investigation to determine the process by which TFs which are expressed in different lineages with spatiotemporal precision select their specific target sites on DNA. Genome-wide studies in the last several years have provided a great deal of information about the binding preferences of TFs and their localization in the genome at different developmental stages. Different combinations of lineage-specific TFs can bind cooperatively to enhancers, and the consensus recognized by a combination of TFs is often distinct from that bound by each TF alone, thus highlighting the importance of combinatorial gene regulation (3, 4). Studies using different

cell types have suggested that lineage-specific TFs interact with chromatin which helps in creating a permissive environment for subsequent binding of other TFs during development (5). However, we still lack a complete understanding of the mechanistic basis of lineage-specific TF function, specifically their ability to initiate and maintain cell identity in different developmental contexts (38).

### **Macrophages and blood cell development**

Macrophages are cells of the immune system and are derived from hematopoietic stem cells. Hematopoietic stem cells (HSC) provide an ideal model to investigate both cellular development from a common multipotent progenitor as well as studying the functional response of a specific lineage of cells like macrophages. HSCs are blood cells derived from the hemogenic epithelium and have self-renewal capacity. In the presence of appropriate growth factors, these cells can give rise to all the different blood cell lineages. Among these, macrophages are specialized innate immune system cells derived from the myeloid lineage and require the lineage specific TFs PU.1 and C/EBP $\alpha/\beta$  for their differentiation and commitment (40). Transdifferentiation experiments conducted by the Graf lab led to the interesting finding that B cells, which are terminally differentiated cells of the lymphoid lineage, can be reprogrammed into macrophages by the expression of two key TFs, PU.1 and C/EBP $\beta$  (17).

### **Macrophages and inflammatory gene expression programs**

One of the important functions performed by macrophages as cells of the innate immune system is to provide a rapid and efficient response to microbial challenge. This response

involves a massive change in the transcriptional program with the up-regulation of several hundred genes required to respond to the challenge. Detailed studies by the Natoli and Glass labs have shown that the distal regulatory regions of these inducible genes in macrophages are pre-occupied by lineage-specific factors like PU.1 and C/EBP $\beta$  and are associated with the histone mark H3K4me1 even in unstimulated cells (3, 18). The pre-marked regions, when challenged with lipopolysaccharide (LPS) which is a TLR4 agonist and mimics bacterial challenge, recruit signal-induced transcription factors (like NF $\kappa$ B, AP1, IRF3), which help in subsequent induction of the associated genes (18).

### **Lineage-specific TFs involved in macrophage identity**

A large number of TFs are upregulated during macrophage differentiation from myeloid progenitor cells. Of these, the constitutive factors PU.1 and C/EBP $\beta$  have been widely studied for their roles both during macrophage differentiation as well as in inducible gene expression. Reprogramming studies performed by the Graf group have shown that forced expression of PU.1 and C/EBP $\beta$  is sufficient to convert mouse fibroblasts to macrophage-like cells. The partially reprogrammed cells, while exhibiting some properties of macrophages, have a defective inflammatory response indicating that early binding of TF's to regulatory regions during differentiation might be essential to achieve an appropriate immune response in macrophages (19).

### ***PU.1***

PU.1 belongs to the ETS family of TF's which is comprised of more than 30 different members with highly conserved DNA binding domains. The *PU.1* gene was initially

discovered as a target for insertion of the virus SFFV (20), the PU.1 structural protein is comprised of the ETS DNA binding domain, a transactivation domain and an additional PEST domain which is involved in protein-protein interactions. PU.1 is expressed early during blood cell development and is absolutely essential for the maturation of macrophages, B cells and early T cells (21, 22). PU.1 knockout mice are severely immunocompromised and lack any functional macrophages and B cells (23, 42). Conditionally inducible PUER mice have demonstrated that lack of PU.1 early during development results in a defective inflammatory response and an inability to express some key cytokine genes (24). Genome-wide ChIP-seq binding studies have reported that PU.1 binds to both promoters and distal enhancers in resting bone-marrow derived macrophages (BMDMs), enhancer binding by PU.1 is followed by H3K4me1 deposition and maintains a permissive chromatin structure at these regions to allow subsequent gene induction. In fact, forced expression of PU.1 in non-myeloid cells like fibroblasts is sufficient for PU.1 binding, H3K4me1 deposition and increased accessibility at some macrophage enhancers but not others (18). Further, studies on the IRF8 gene have shown that PU.1 might also be involved in mediating long range looping interactions between distal enhancers and promoters, which brings distal elements which are several kbp away in closer proximity for interaction and facilitates gene expression (19).

### ***C/EBP $\beta$***

C/EBP $\beta$  belongs to the C/EBP family of TFs which is comprised of several other members including C/EBP $\alpha$ , C/EBP $\gamma$ , C/EBP $\delta$  which share substantial homology in the bZIP domain. C/EBP $\beta$  contains an N-terminal activation domain, a basic DNA binding domain and a

leucine zipper domain. C/EBP $\beta$  deficient mice are highly susceptible to bacterial infections and have impaired macrophage function (26). Genome-wide studies in bone-marrow derived macrophages have demonstrated that C/EBP $\beta$  binds to a large number of distal macrophage enhancers and binding to a majority of these enhancers is PU.1-dependent (3). C/EBP $\beta$  exists primarily as three isoforms, LAP1, LAP2 and LIP resulting from alternate translation initiation (27). LAP1 has been suggested to possess a SWI/SNF interaction domain at its N-terminus and interacts with BRG1 in vitro (9), which might explain its ability to function as a 'pioneer' factor in some cell-types. C/EBP $\beta$  overexpression has been demonstrated to convert differentiated B cells into macrophages and this reprogramming is dependent on the presence of a functional PU.1 protein in the reprogrammed cells (17), thus highlighting the essential role played by the two TFs in ensuring macrophage identity and function.

### **Pioneer TFs interact with chromatin during differentiation**

Pioneer factors are a class of lineage-specific transcription factors which are expressed early in development and have the ability to bind chromatinized DNA (5). This property distinguishes pioneer factors from other transcription factors and gives them the unique ability to initiate cell fate decision events early in development. Further studies into the role of pioneer factors at the *Alb1* enhancer in ES cells have revealed that pioneer factor binding might function to protect the local chromatin state by preventing repressive DNA methylation machinery from silencing distal regulatory sites early in development (11). Additionally, some pioneer factors have been reported to alter higher order chromatin structure which might facilitate interaction between distal regulatory regions as well as their

target promoters (12, 13). Initial studies suggested that pioneer factor binding results in an accessible chromatin structure which facilitates binding of other transcription factors and recruitment of the RNA polymerase machinery to achieve context-specific gene expression. Further, this process was assumed to be an intrinsic property of pioneer factors in the absence of ATP dependent chromatin remodeling machinery. In vitro studies have shown that FoxA1 can interact with chromatin in the absence of ATP dependent chromatin remodeling enzymes. This intrinsic property of FoxA1 to access chromatin can be partly attributed to its C-terminal domain which is able to interact with core histones and is required for chromatin opening (5, 6). However, such conclusive evidence is lacking for other factors reported to have pioneer activity. It has been suggested that the pioneer activity of some factors like GATA1 might be attributed to their ability to recruit members of the SWI/SNF chromatin remodeling complex (7). Using an inducible PUER macrophage differentiation cell line as well as shRNA-mediated PU.1 knockdown approaches in bone-marrow derived macrophages, it has been shown that PU.1, a macrophage lineage-specific TF can function as a pioneer factor based on its ability to alter chromatin (3, 37). Studies using the same inducible PUER system have also demonstrated that PU.1 co-immunoprecipitates with members of the BAF complex, known subunits of the SWI/SNF chromatin remodeler, upon its induction using tamoxifen (8). Likewise, another macrophage lineage-specific TF, C/EBP $\beta$  has been reported to possess a SWI/SNF interaction domain and can recruit the BRG1 subunit under in vitro conditions (9). Studies have also suggested that some TFs might exhibit pioneer activity only in certain cell-types, thus indicating that pioneer factors may function in a context dependent manner in addition to their intrinsic properties (for review see 10). Thus, pioneer factors function at multiple levels during

differentiation to create a permissive chromatin environment for subsequent gene induction. How these factors manage to establish and maintain this permissive state in the presence of repressive silencing machinery during differentiation is an open question in the field.

### **Distal enhancers play an essential role in shaping cell fate**

While studies on gene regulation focused on proximal transcription elements like the promoter and gene start sites, distal regulatory elements called enhancers play an essential role in cell fate determination. Initially identified in the SV40 DNA virus, for enhancing  $\beta$ -globin gene expression in cis (14), studies in the last several decades have conclusively established that cis-regulatory modules or enhancers are the major players in cell-type specific gene expression.

Enhancers provide an ideal system for achieving cell-type specificity due to the presence of different combinations of TF binding sites. Genome-wide studies have shown that active enhancers are associated with characteristic histone modifications and binding of certain coactivators like p300 and therefore large scale discovery of enhancers has been aided by the presence of specific marks on enhancers such as H3K4me1, H3K27ac, as well as DNase I hypersensitivity (15), however, functional studies to validate the large number of regulatory elements identified by these approaches have been lacking. The IFNB1 proximal enhancer which assembles an enhanceosome is an example of a well characterized regulatory element of just 55 bp with compact binding sites for at least eight TFs which include both lineage-specific as well as signal inducible TFs. The enhanceosome model for enhancers suggests a high level of cooperativity between individual factors such that the enhancer functions only in the presence of all factors. In fact, mutation studies have

demonstrated that deletion of a single base pair in the 55bp enhanceosome completely abolishes gene induction (16, 41). This highlights an important aspect of enhancer function during development integrating both cell-type specific as well as inducible gene expression signals.

### **Macrophage enhancers play an important role in the inflammatory response**

Genome-wide studies by the Glass and Natoli labs have provided some important insights into the active regulatory elements in macrophage cells (3, 18). Initial studies conducted on bone-marrow derived mouse macrophages mapped the lineage-specific TF's, PU.1 and C/EBP $\beta$  binding as well as the enhancer marks, H3K4me1 and p300 in both resting and LPS-activated macrophages. One of the important highlights of these studies was that a large number of LPS-inducible p300 bound regions were enriched for PU.1 binding, even in resting macrophages, before gene induction. LPS induction resulted in the recruitment and binding of signal-induced TF's like NF $\kappa$ B, AP1, IRF3 to these enhancers which ultimately results in induction of the target genes (18). Thus, lineage-specific TF's are bound to inducible macrophage enhancers in the resting state, creating a permissive environment for subsequent gene induction.

### **PRC2 interacts with enhancers to ensure lineage-specific gene expression**

Polycomb group proteins which were originally discovered in *Drosophila* (28) have been known to play a key role during differentiation and are often associated with repressed genes and heterochromatin. Polycomb group proteins are primarily classified as PRC1 and PRC2 complexes and these two complexes can function in a coordinated manner as well

as independent of each other (for review see 29). PRC2 establishes the H3K27me3 histone mark and PRC1 can recognize this mark and further ubiquitylate histones. Mechanisms used by PRC2 to recognize its target sites on DNA in mammals are still under investigation but certain factors like high density of CpG islands have been reported to promote PRC2 binding (30). A recent study in mammals showed that short sequences (spanning less than two nucleosomes) were sufficient to recruit PRC2 to DNA and high CpG island density alone couldn't account for this recruitment (31). This suggests that additional sequence features might determine PRC2 binding in mammals. In addition, genome-wide approaches have suggested that absence of transcriptional activator motifs in the underlying DNA might predispose to PRC2 binding in mouse Embryonic Stem Cells (ESC's) (32). It remains to be known however, what combined genomic features determine PRC2 targeting during mammalian cellular differentiation. Studies in *Drosophila* have reported that PRC2 binding in some cases might correlate with lower nucleosome turnover (33) suggesting that PRC2 might play a role in stabilizing nucleosomes. Further, it has been established that differentiated cells display a much higher number of H3K27me3 marked nucleosomes when compared to stem cells or progenitor cells (34, 35), however it still remains to be understood how PRC2 binding at distal regulatory regions impacts cell fate during differentiation.

### **Clinical significance**

Macrophages have been demonstrated to play a key role in atherosclerotic plaque formation. Plaque formation mainly occurs through the recruitment of macrophage foam cells which display an 'altered' inflammatory phenotype in atherosclerotic lesions (36).

Studies in mice have shown that PU.1 deficiency in mature macrophages results in an attenuated reaction to endotoxin and a weakened inflammatory response which eventually provides a survival benefit to the mice when challenged with LPS (24). Further, certain inhibitors designed to target the BET bromodomain family of proteins which bind acetylated histones, has been shown to selectively inhibit only certain classes of inducible genes in macrophages, thus providing protection against endotoxic shock caused by an exaggerated response to LPS in macrophages (39). Such clinical studies emphasize the importance of studying the role of the underlying chromatin as well as lineage-specific TFs like PU.1 and C/EBP $\beta$  both during macrophage differentiation as well as prior to inflammatory gene induction.

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## **CHAPTER 2: NUCLEOSOMES ARE STABLY EVICTED FROM ENHANCERS DURING INDUCTION OF CERTAIN PROINFLAMMATORY GENES IN MOUSE MACROPHAGES**

The work described in this chapter was published in the following manuscript:

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## **Abstract**

Chromatin is thought to act as a barrier for binding of *cis*-regulatory transcription factors (TFs) to their sites on DNA and recruitment of the transcriptional machinery. Here we have analyzed changes in nucleosome occupancy at the enhancers as well as at the promoters of pro-inflammatory genes when they are induced by bacterial lipopolysaccharides (LPS) in primary mouse macrophages. We find that nucleosomes are removed from the distal enhancers of IFNB1 and IL12B, as well as from the proximal enhancer of IFNB1, and that clearance of enhancers correlates with binding of various *cis*-regulatory TFs. We further show that for IFNB1 the degree of nucleosome removal correlates well with the level of induction of the gene under different conditions. Surprisingly, we find that the promoter of IL12B is not cleared of nucleosomes when the gene is expressed indicating that removal of promoter nucleosomes is not a regulated step during induction of some genes.

## Introduction

IFNB1 is a pro-inflammatory cytokine and is induced in response to microbial infection in both human and mouse macrophages. It has been extensively studied in human cells, and was shown to contain a conserved promoter proximal enhancer that assembles an enhanceosome when the gene is induced by viral infection (40). Studies in HeLa cells showed that this promoter proximal enhancer is in a nucleosome-free region prior to induction and is flanked by two nucleosomes (1). These authors further showed that upon induction the nucleosome-depleted region is expanded to include the TATAA-sequence of IFNB1 and they suggested that clearance of the TATAA-sequence involved sliding of a nucleosome to a downstream position by SWI/SNF. The mouse gene was recently shown to be regulated not only by the promoter proximal enhancer but also by a distal enhancer located 6 kb downstream of its TSS (45). This region was shown to bind the *cis*-regulatory TFs XBP and IRF3 as well as the co-activator CBP/p300 when IFNB1 was induced by LPS and thapsigargin (TPG), an inducer of ER-stress that enhances expression of certain pro-inflammatory cytokines. Furthermore, a minimal region of 305 bp that encompasses consensus-sites for these TFs was shown to enhance transcription of a reporter gene confirming this region as a *bona fide* enhancer. Similar studies of the IL12B gene performed mostly by Stephen Smale's laboratory have identified a distal enhancer located 10 kb upstream of its TSS (48). The distal enhancers of IL12B and IFNB1 were also classified as enhancers in a recent genome-wide study, which sought to identify functional distal enhancers in mouse macrophages (13).

In this study, we have used a quantitative assay to analyze nucleosome occupancy at the transcriptional regulatory regions of the pro-inflammatory cytokines, IFNB1 and IL12B -

upon their induction in primary mouse macrophages. The quantitative nucleosome occupancy assay uses a wide range of MNase concentrations and detects the distinct digestion rates of the same segment of DNA, when it is naked or associated with a nucleosome, which allows us to derive the fractional occupancy of a genomic region by a nucleosome (4). Pro-inflammatory cytokines are expressed by macrophages as part of the innate immune response to various pathogens (for review see 21). The main TFs that act downstream of these pathways are NF $\kappa$ B, AP1 and IRF3/7, which bind to their target genes upon induction. In addition to these signal-induced TFs, at least two lineage-specific TFs, PU.1 and C/EBP $\beta$ , are also required for expression of these pro-inflammatory genes in macrophages (13, 15, 18).

We have analyzed changes in chromatin architecture at the enhancers of IFNB1 and IL12B as well as their promoters upon LPS induction. We find that nucleosomes in the distal enhancers of both IFNB1 and IL12B are rapidly evicted when the genes are induced. Similar nucleosome removal occurs at the proximal enhancer of IFNB1, which leads to clearance of the adjacent TATA-box and TSS (as had been described for the human gene (1)). In addition, all the nucleosome-depleted regions become associated with *cis*-regulatory TFs and the co-activator p300. Surprisingly, we find that nucleosomes are not removed from the promoter of IL12B when the gene is induced. Our results suggest that enhancers - both distal and proximal - have to be cleared of nucleosomes to allow binding of signal-induced TFs. In contrast, studies with the IL12B promoter indicate that nucleosome clearance at promoters is not a regulated step during induction of some cytokines.

## **Materials and methods**

### ***Primary cell isolation, cell-lines and growth conditions***

Primary cells were isolated from 8-12 week old C57BL/6 mice (NCI) with IACUC oversight. Bone marrow derived macrophages (BMDMs) were generated as described (23) and grown in BMDM medium (60% IMDM medium (Gibco), 30% conditioned medium from L-929 fibroblasts, 10% FBS, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 1X penicillin-streptomycin. LPS induction was performed by adding 1  $\mu$ g/ml LPS from *E. coli* strain EH100 (Ra mutant)(Sigma) to serum-starved BMDMs for the indicated times. Serum starvation was done by growth of cells in incomplete IMDM medium for 1h. Other inducers were ISD (interferon stimulatory DNA) derived from *Listeria monocytogenes*; poly(I:C), synthetic dsRNA that acts as a TLR3 agonist; and poly(dA:dT), a synthetic analog of B-DNA (all obtained from Invivogen). 1  $\mu$ g/ml of either of these inducers was given to BMDMs by transfection with Lipofectamine 2000 (Invitrogen) in an equal volume mixture. Where indicated, thapsigargin (Sigma) was added at 1  $\mu$ M for 1 h to serum-starved cells prior to LPS addition (45). Splenic B-cells were isolated by negative selection with CD43 antibody-coupled Dynabeads according to the instructions of the manufacturer (Life Technologies), with an additional red blood lysis step using lysis buffer (Sigma). For LPS induction B-cells were grown in B-IMDM medium (IMDM medium (Gibco), containing 55  $\mu$ M 2-Mercaptoethanol and 2 mM L-glutamine) for 1.5 h prior to LPS addition for the indicated times. RAW264.7 cells were grown in DMEM medium (Gibco) containing 10% FBS and 1X penicillin-streptomycin.

### ***mRNA determination***

Total RNA was isolated from BMDMs or B-cells using Trizol (Invitrogen/Lifetech). In brief, Trizol was added to cells growing in culture, and Trizol lysates were collected. 400 µl of chloroform was added per 1 ml Trizol lysate, the aqueous phase was extracted, 170 µl isopropanol was added and the mixture was further purified on ReliaPrep RNA Cell Miniprep System columns according to the manufacturer's protocol (Promega). RNA was converted into cDNA according to the protocol described (9) except that High Capacity Reverse Transcriptase was used (Invitrogen/Lifetech) and analyzed by qRT-PCR with specific primer pairs. Primers used can be given upon request.

### ***Chromatin immunoprecipitation***

Chromatin from  $5 \times 10^6$  cells per antibody that had been cross-linked with 0.5% formaldehyde for 10 min was isolated by sonication with a Branson sonifier (10 pulses of 10" at setting 4) in Lysis buffer (50 mM Hepes-KOH, pH 7.5, 1% TritonX-100, 0.1% SDS) and centrifugation for 10' at 21,000 x g. Isolated chromatin was directly diluted with High Salt ChIP buffer and incubated with either 1 µg of anti-Poll antibody (sc-56767), 6 µg anti-TBP (sc-204), 4 µg anti-PU.1 (sc-352), 4 µg anti-C/EBPβ (sc-150), 6 µg anti-NFκB (sc-372), 5 µg anti-c-Jun (sc-45), 6 µg anti-p300 (sc-585) or 10 µg anti-IRF3 (sc-9082) all from Santa Cruz Biotechnologies. 20 µl of Protein A/G magnetic beads (Pierce) were added to the reaction and incubated at 4°C for 2 h. Beads were washed with 280 µl each of TSE buffer (20 mM Tris pH 8.0, 0.1 % SDS, 1 % TritonX-100, 2 mM EDTA), TSE250 (TSE buffer, 250 mM NaCl) and TSE500 (TSE buffer, 500 mM NaCl), Wash buffer III (10 mM Tris pH 8.5, 0.25 M LiCl, 1 % NP-40/Igepal, 1 % deoxycholate, 1 mM EDTA) and TE (10

mM Tris-HCl pH 8.0, 1 mM EDTA) all containing Complete protease inhibitors. Antibody complexes were eluted from the beads with 2 x 100  $\mu$ l Elution buffer (0.1 M NaHCO<sub>3</sub>, 1 % SDS) by incubation for 30' (and 10') at 55°C. Eluates were combined and the cross-link was reversed by incubation at 65°C for 4 h. DNA was purified using a Qiagen PCR purification kit, and analyzed on a Lightcycler 480 (Roche) using primer pairs in the regions indicated in the figures. Sequences of the primers used can be given upon request.

### ***Quantitative nucleosome occupancy assay***

The assay was performed essentially as described in (4) with certain modifications. Cross-linked chromatin from 1 to 3 x 10<sup>7</sup> cells isolated as described for ChIP experiments was incubated in Lysis buffer containing 140 mM sodium chloride with 22 increasing concentrations of MNase (0.001179 U to 20 U, NEB) in the presence of 0.15 mM CaCl<sub>2</sub> for 1 h 30'. DNA was purified as described and quantified using a Roche Lightcycler 480. Digestion data was analyzed using two-state exponential curve-fitting as described (4). Data was normalized to several genomic locations, including a region in the promoter of KIT (3) that was highly protected and a region in the ORF of RPL4. The data was displayed in the IGV genome browser v2.3 (31) and overlays of nucleosome occupancy during a timecourse of LPS induction were created from IGV tracks using Adobe Photoshop.

### ***Genomic DNA isolation***

Genomic DNA was isolated from RAW264.7 macrophages as described (35) and DNA standard curves were created using a 1/3 fold dilution series with the highest concentration yielding qRT-PCR amplification at around cycle 20 for the majority of primer pairs.

### ***qRT-PCR***

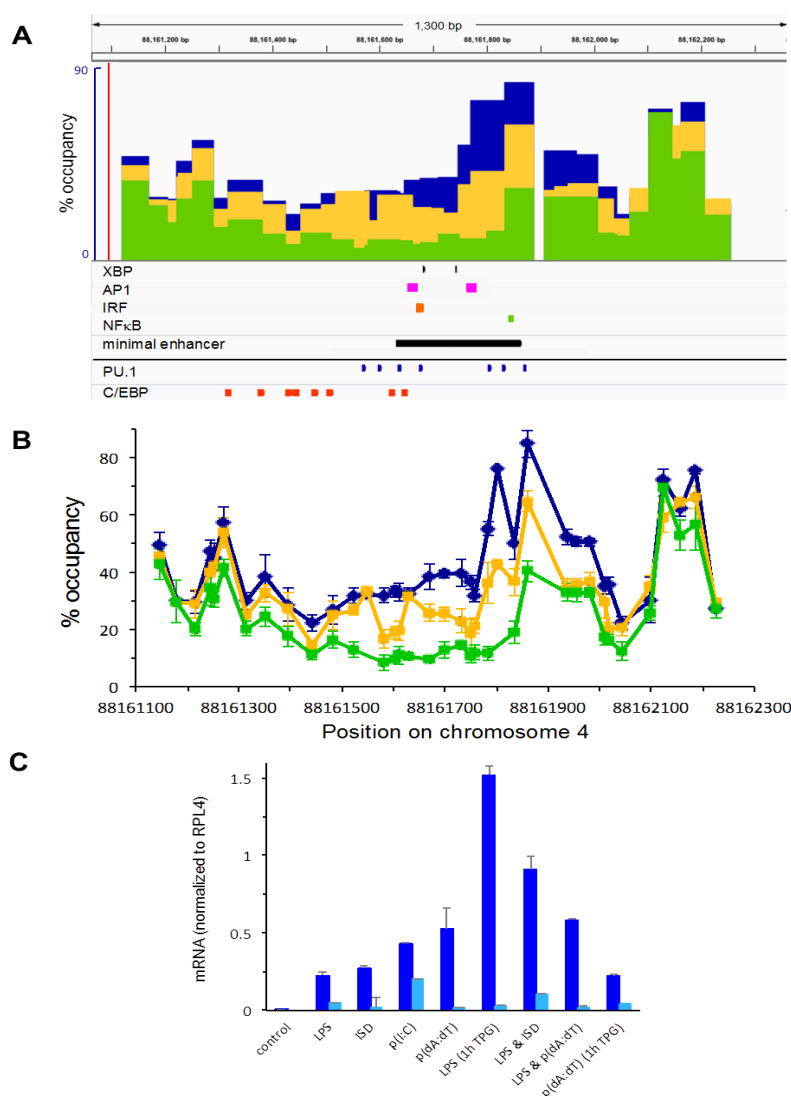
DNA and cDNA was quantified on a Lightcycler 480 (Roche) as described (4) with the following modifications. Primers were designed using the program PCRTiler (12). To verify that only a single amplicon was produced by each primer pair and no primer dimers were formed a  $T_m$ -curve was performed as a quality control for each primer pair at the end of each qRT-PCR run. We also found that addition of 1.5% PEG400 (Fluka) to the qRT-PCR reaction greatly enhanced performance for many primer pairs and led to a greater linear range of the qRT-PCR measurements.

## Results

### *IFNB1*

We found that *IFNB1* was only moderately induced by LPS (Fig. 1C), a result reported by others (27). To further increase induction, we treated macrophages with other inducers of this cytokine either alone or in addition to LPS (Fig. 1C). As shown in Fig. 1C we found transient induction of *IFNB1* with various inducers (*i.e.* ISD, p(I:C), p(dA:dT)) either alone or in combination with LPS. However, the strongest increase in *IFNB1* expression was seen when cells were pre-treated with the ER-stress inducer TPG prior to LPS induction (as described in (45)). We therefore analyzed nucleosome occupancy at the regulatory regions of *IFNB1* upon induction by LPS alone or after pretreatment with TPG. Figure 1A and B shows nucleosome occupancy at the enhancer 6 kb downstream of the TSS of *IFNB1* before LPS induction (blue bars and lines) or 1.5 h after LPS induction with (green) or without (yellow) TPG pretreatment. We find that in resting macrophages the region encompassing the minimal enhancer region defined by Zeng et al. (black bar under the figure, taken from (45)) partially overlaps with a highly occupied nucleosome (80-90%). To the left of this highly occupied sites nucleosome positions are less well defined and occupancy was found to be lower (around 40%). Nucleosome occupancy in this region only slightly decreased when cells were induced with LPS alone for 1.5 h, and did not change upon prolonged induction (data not shown). However, if cells were pretreated with TPG prior to LPS induction, nucleosomes were completely removed from the lowly occupied region (5-10% remaining) and partially from the highly occupied nucleosomal site. The region

**Figure 1. Nucleosome occupancy at the distal enhancer of IFNB1 upon LPS and TPG induction**



**(A) and (B)** Nucleosome occupancy was determined in BMDMs before induction (blue bars and lines), and upon induction with 1μg/ml LPS for 1.5 h with (green) or without (yellow) pretreatment of cells with 1 μM TPG for 1 h. The minimal enhancer region (black bar) and binding sites for XBP, AP1, IRF3 and NFκB identified by (Zeng et al., 2010) are shown in (A). ConSite predicted binding sites for PU.1 and C/EBP are indicated.

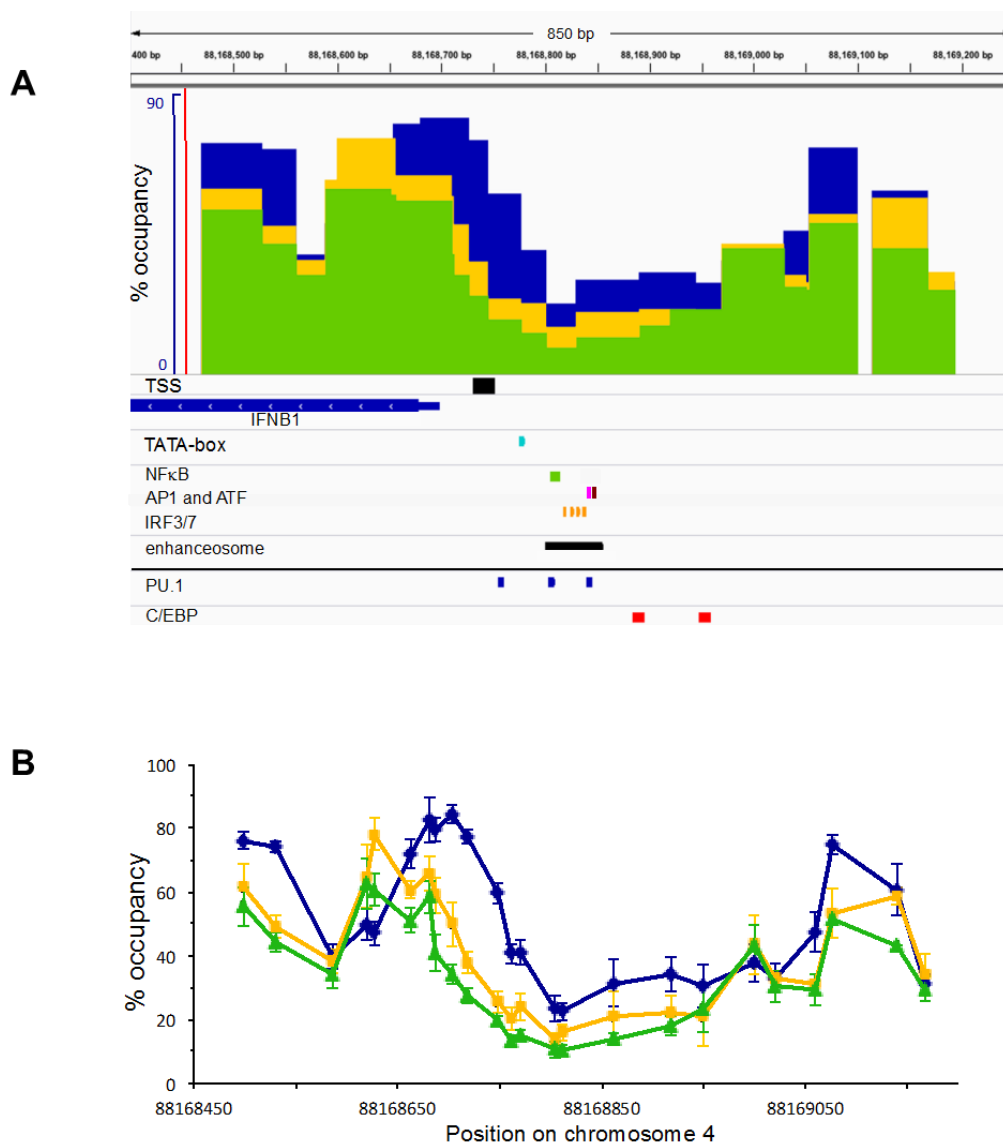
Figure 1 (cont'd)

**(C)** Expression of IFNB1 upon stimulation with different inducers. BMDMs were induced for 3 h (dark blue) or 16 h (light blue) with 1  $\mu$ g/ml of LPS, or 1  $\mu$ g/ml of ISD, p(l:C), or p(dA:dT) added either alone or together with LPS as indicated in the figure. Where indicated cells were pre-treated with the ER-stress inducer TPG for 1 h prior to LPS induction. Data was normalized to the ORF of RPL4.

that was cleared of nucleosomes encompasses binding sites for the TPG-induced TF XBP, as well as for AP1 and IRF3 as indicated in the figure (45). A binding site for NF $\kappa$ B was reported in the region that we find is highly occupied by a nucleosome before induction and becomes partially cleared upon induction. We also identified consensus-sites for PU.1 and C/EBP in the nucleosome-depleted region using ConSite (Fig. 1A).

Figure 2A and B shows nucleosome occupancy at the promoter and the promoter proximal enhancer of IFNB1, which forms an enhanceosome upon induction indicated by the black bar underneath the figure (taken from 24), both prior to (blue bars and lines) and upon LPS induction of the gene with (green) and without (yellow) pretreatment with TPG. We find that the enhanceosome is formed in a region that spans a linker region between two nucleosomes as has been described for the human gene. The nucleosome on the right was found to be lowly occupied (40%) and partly covered the enhancer. The nucleosome to the left was highly occupied (90%) and encompasses the TSS and TATAA-sequence of IFNB1. Upon LPS induction the region that forms an enhanceosome was partially cleared of nucleosomes. Similar to our findings at the distal enhancer of IFNB1 we found that pretreatment of cells with TPG prior to LPS induction led to further depletion of nucleosomes at the proximal enhancer, which became essentially nucleosome-free in the presence of TPG and LPS (5-10%). The nucleosome to the right of the enhanceosome was partially depleted and the nucleosome to the left was shifted to a downstream position, which led to clearance of the TSS and TATAA-sequence as has been described for the human gene (1).

**Figure 2. Nucleosome occupancy at the proximal enhancer and promoter of IFNB1 upon LPS and TPG induction**



**(A) and (B)** Nucleosome occupancy at the proximal enhancer and promoter of IFNB1 was determined as in Fig.1 and analyzed in a region encompassing the proximal enhancer that is conserved in humans and has been shown to form an enhanceosome upon viral stimulation of HeLa cells (40), as well as the 5' region of the IFNB1 ORF.

**Figure 3. Binding of TFs and recruitment of the transcriptional machinery to the distal and proximal enhancers of IFNB1**

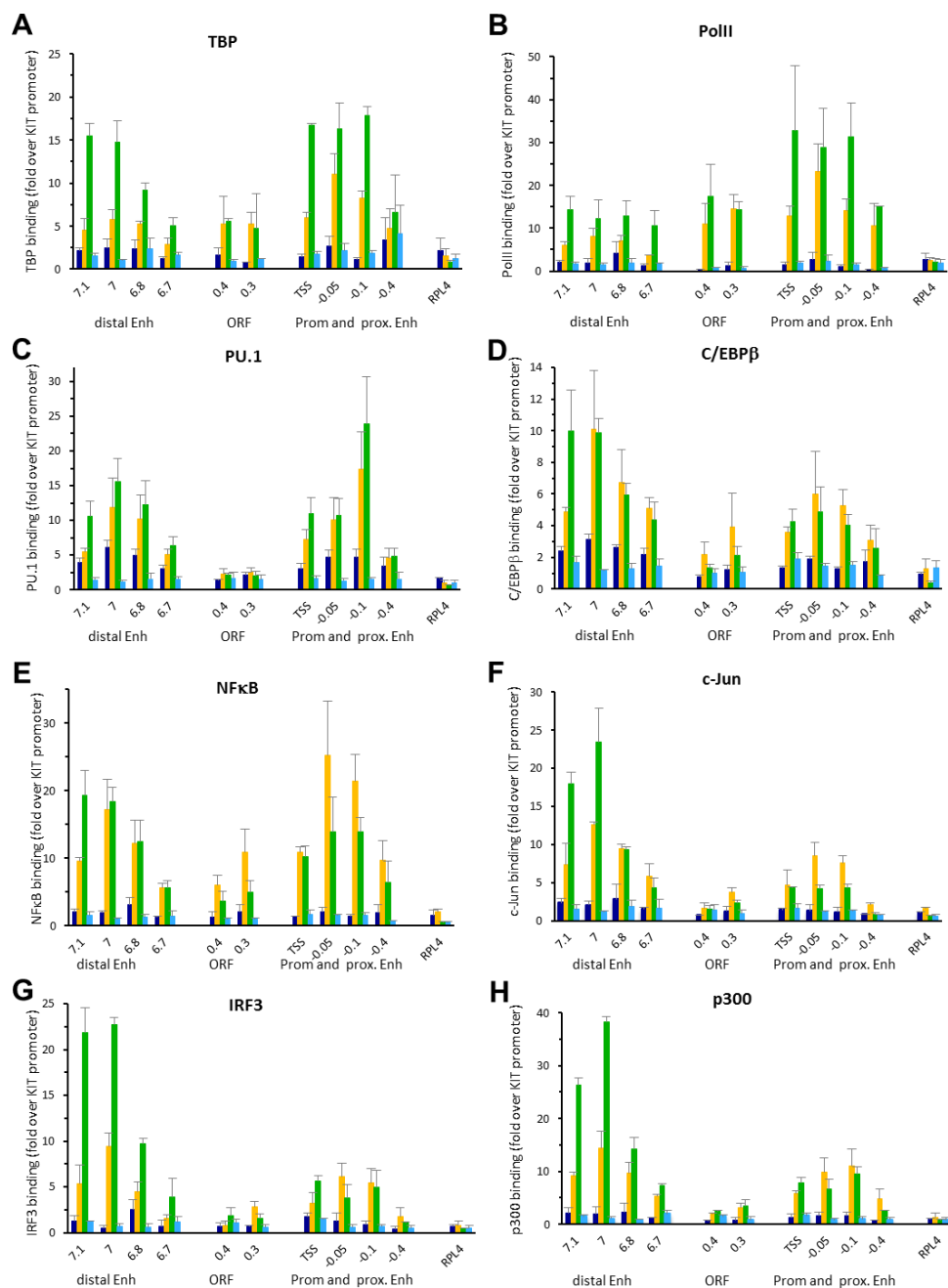


Figure 3 (cont'd)

**(A-H)** ChIP experiments were performed in BMDMs before (dark blue), and upon 1.5 h LPS induction with (green) or without (yellow) pretreatment of cells with TPG, as well as in splenic B-cells (light blue). Binding to a control region in the ORF of RPL4 is shown. The antibodies used in each ChIP experiment are indicated in the figures of panels (A) through (H). Error bars indicate the SEM of at least three independent experiments and statistical significance of binding of these factors to the different regions was determined by Student's T-tests.

### ***TF binding to the distal and proximal enhancers of IFNB1***

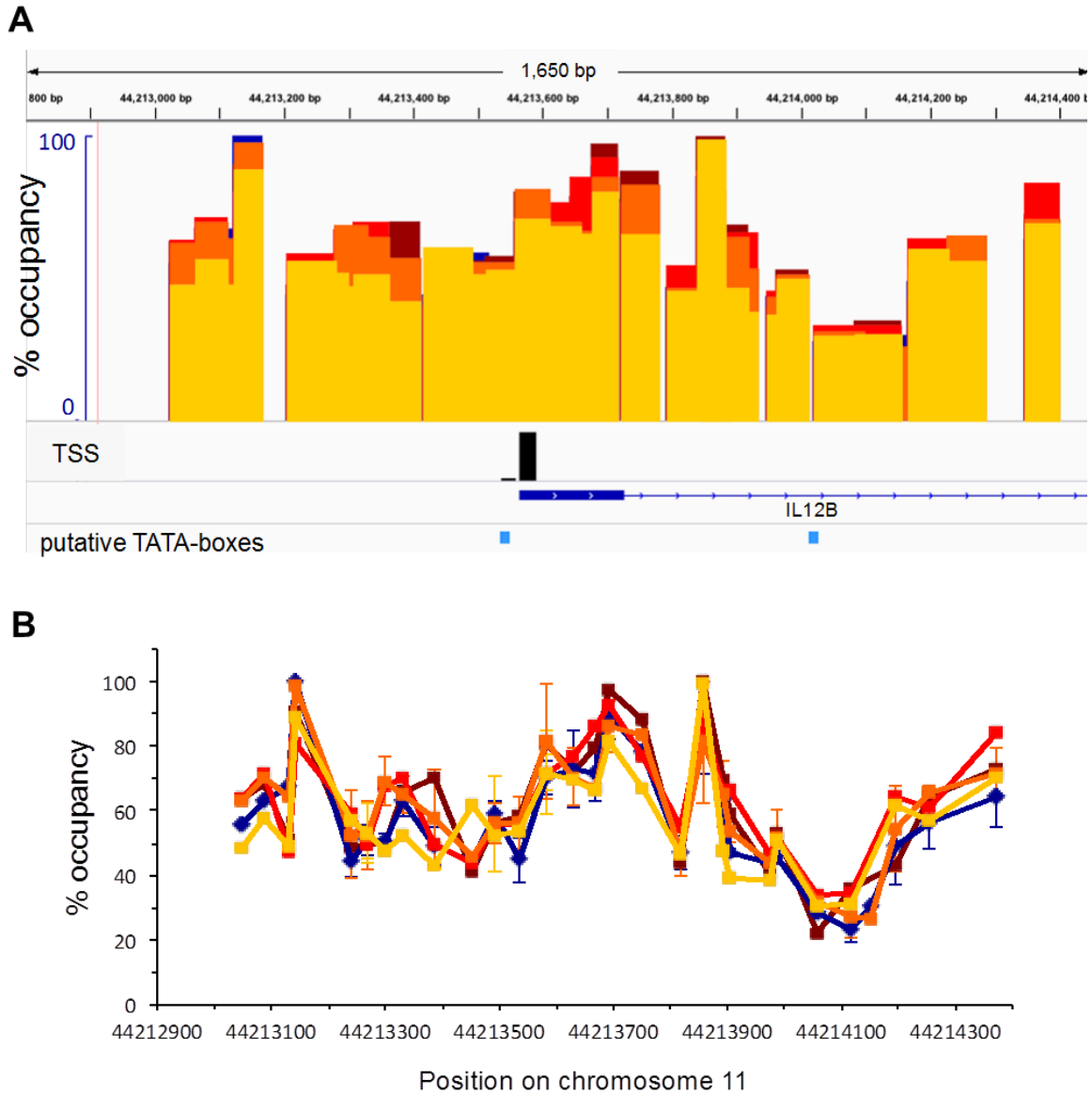
To determine binding of *cis*-regulatory TFs and the transcriptional machinery to the distal as well as to the proximal enhancer and the promoter of IFNB1 upon induction of the gene, we performed ChIP experiments. Fig. 3 shows that all the factors tested were recruited to both the distal as well as to the proximal enhancer of IFNB1. Due to the proximity of the proximal enhancer to the promoter, including the TSS and TATAA-sequence, our ChIP experiments cannot distinguish binding to the promoter and promoter proximal enhancer. As shown in the figure we found more binding of TBP and PolIII to the proximal enhancer/promoter when cells were pretreated with TPG prior to LPS induction (compare green to yellow bars) in agreement with the increase in gene expression we observed (Fig. 1C). We also found binding of TBP and PolIII to the distal enhancer of IFNB1 upon induction. Furthermore, we found that binding of PU.1 to the proximal and distal enhancer increased when cells were pretreated with TPG. C/EBP $\beta$  and NF $\kappa$ B binding did not increase significantly at the distal enhancer upon TPG treatment over levels seen when cells were treated with LPS alone and binding to the proximal enhancer was somewhat decreased. In contrast, we found a significant increase in binding of c-Jun, IRF3 and p300 to the distal enhancer upon TPG pretreatment, while binding to the proximal enhancer remained the same or decreased slightly. We hypothesize that complete nucleosome removal from the distal enhancer after pretreatment of cells with TPG prior to LPS induction (see Fig. 1A and B) facilitated binding of *cis*-regulatory TFs tested under these conditions. While the further increase in nucleosome removal upon pretreatment with TPG at the promoter proximal enhancer was less dramatic than at the distal enhancer, it correlated

with more binding of some TFs (PU.1) and increased recruitment of the transcriptional machinery.

### ***Nucleosome occupancy at the IL12B promoter upon LPS induction***

Figure 4A and B shows nucleosome occupancy at the IL12B promoter including a region 600 bp upstream and 800 bp downstream of the TSS. Surprisingly, we did not find any changes in nucleosome occupancy upon LPS induction (compare blue bars and lines to increasing shades of red). This region was highly occupied by nucleosomes prior to induction (70-100%) and no changes were observed during the 10 h timecourse of LPS induction. The TSS (indicated by the black bar underneath the figure in panel A) was occupied in about 70% of the population and a TATAA-sequence that we identified 28 bp upstream of the TSS (light blue box) was occupied in around 50-60%. We found that a region 400 bp downstream of the TSS that contains a TATAT-sequence was relatively lowly occupied by nucleosomes prior to induction (20-30%), which had initially suggested to us that this downstream region might function to assemble a pre-initiation complex. However, an previous search for TSS that used CAGE-analysis to detect any capped mRNAs had not found any transcription starting from this downstream region, but had instead confirmed the annotated TSS for IL12B (Carninci et al., 2006). We therefore conclude that the upstream TATAA-sequence is used to assemble a PIC.

**Figure 4. Nucleosome occupancy at the IL12B promoter upon LPS induction**



**(A) and (B)** Nucleosome occupancy around the TSS of IL12B in BMDMs was analyzed before induction (blue bars and lines), and after 1.5 h (yellow), 3 h (orange), 5 h (light red) and 10 h (dark red) of growth of cells in the presence of 1  $\mu$ g/ml LPS, using the assay described in 4. The black bar below the data in (A) indicates the TSS (5) and the light blue bars indicate putative TATA-boxes predicted by ConSite.

## Discussion

### ***The promoter and promoter proximal enhancer of IFNB1***

In contrast to our findings at the IL12B promoter we found that the TATAA-sequence in the IFNB1 promoter was cleared of nucleosomes upon induction in primary mouse macrophages as had been described for the IFNB1 promoter in human cells (Fig. 2A and B) (1). This region is adjacent to the proximal enhancer of IFNB1, which became associated with all the TFs we tested as well as with the co-activator p300, when the gene was expressed (see Fig. 3). In HeLa cells the proximal enhancer of IFNB1 has been reported to be completely nucleosome-free prior to induction (1), but we found that in primary BMDMs the corresponding region was lowly occupied by nucleosomes prior to gene expression and became completely cleared of nucleosomes upon induction. The changes in chromatin architecture at the proximal enhancer of IFNB1 were similar to what we observed at the distal enhancer of IFNB1 as well as at the distal enhancers of IL12B and IL1A: thus, the enhancers were only moderately occupied by nucleosomes in resting macrophages and a central region was completely cleared of nucleosomes when the associated genes were induced. The size of the cleared region varied from about 1 nucleosome (at the proximal enhancer of IFNB1) to removal of 2-3 nucleosomes in the distal enhancer of IFNB1 (compare Figs. 1A and 2A). The small size of the nucleosome-free region in the proximal enhancer of IFNB1 is in agreement with formation of an enhanceosome at this site, which forms a highly organized structure with a defined footprint (24). Together, our data suggest that enhancers of pro-inflammatory genes undergo similar changes in nucleosome occupancy regardless of their distance from a TSS, and that clearance of enhancer nucleosomes may be required to allow binding of certain *cis*-

regulatory TFs. Moreover, we hypothesize that nucleosome clearance of the TATAA-sequence of IFNB1 may occur inadvertently due to its proximity to the proximal enhancer. Nevertheless, it remains to be determined whether binding of TBP to a nucleosome-free TATAA-sequence facilitates PIC formation compared to PIC assembly on a TATAA-sequence that is at least partially associated with nucleosomes.

### ***Nucleosome occupancy at the IL12B promoter***

The most surprising result of our study was the finding that the promoter of IL12B was not cleared of nucleosomes when the genes were expressed, while nucleosomes were rapidly removed from the associated distal enhancers. Thus, we found that the TSS of IL12B was occupied in about 70% of the population and remained highly occupied when the gene was induced by LPS (see Fig. 4A and B). Our findings are in agreement with previous studies that revealed only modest changes in restriction enzyme accessibility upon LPS induction of macrophages in a region 100-200 bp upstream of the TSS of IL12B, compared to changes in the enhancer 10 kb upstream (41). Furthermore, these authors did not detect any changes in MNase protection in the IL12B promoter upon LPS induction in accordance with our results. We therefore conclude that removal of nucleosomes from promoters is not a regulated step during induction of these pro-inflammatory genes, while enhancers are rapidly cleared of nucleosomes. It is conceivable that a pre-initiation complex might protect against MNase digestion, but studies in yeast had shown that a PIC does not register as protected in our assay, which requires continuous stretches of at least 45 bp - the minimal size of the amplicons - to be protected against digestion (4). In addition, we found no MNase protection by a PIC at the promoter of IFNB1 when the gene was highly expressed

(Fig. 2A and B) suggesting that MNase protection at IL12B is indeed due to nucleosomes that remain associated with these promoters. Transcription of many inducible genes, including the genes we have studied, has been reported to be highly stochastic (34, 41, 47) and stochasticity may be caused by various factors including the abundance of *cis*-regulatory TFs in individual cells as has been reported for IFNB1 (46). Furthermore, it is conceivable that the presence of competing nucleosomes at TF-binding sites or at promoters may contribute to stochastic gene expression. Our results suggest that in the case of the pro-inflammatory genes we have investigated *cis*-regulatory TFs may not have to compete with nucleosomes for binding to their sites, which are rapidly cleared in an early step during LPS induction. Instead, we hypothesize that assembly of a PIC at the promoters of these genes may have to occur in the face of competing nucleosomes, which may contribute to the highly stochastic expression of IL12B and other cytokines.

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## REFERENCES

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### **CHAPTER 3: THE LINEAGE-SPECIFIC TRANSCRIPTION FACTOR PU.1 PREVENTS POLYCOMB-MEDIATED HETEROCHROMATIN FORMATION AT MACROPHAGE-SPECIFIC GENES**

The work described in this chapter was published in the following manuscripts:

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McAndrew MJ, Gjidoda A, Tagore M, Miksanek T, Floer M (2016) Chromatin remodeler recruitment during macrophage differentiation facilitates transcription factor binding to enhancers in mature cells. *J Biol Chem.* 2016 Aug 26;291(35):18058-71.

## **Abstract**

Lineage-specific transcription factors (TFs) are important determinants of cellular identity but their exact mode of action has remained unclear. Here we show using a macrophage differentiation system that the lineage-specific TF PU.1 keeps macrophage-specific genes accessible during differentiation by preventing polycomb repressive complex (PRC2) binding to transcriptional regulatory elements. We demonstrate that in the absence of PU.1 binding the distal enhancer of a gene becomes bound by PRC2 as cells differentiate, and the gene becomes wrapped into heterochromatin, which is characterized by increased nucleosome occupancy and H3K27 tri-methylation. This renders the gene inaccessible to the transcriptional machinery and prevents induction of the gene in response to an external signal in mature cells. In contrast, if PU.1 is bound at the transcriptional regulatory region of a gene during differentiation, PRC2 is not recruited, nucleosome occupancy is kept low and the gene can be induced in mature macrophages. These results show that one role of PU.1 is to exclude PRC2 and to prevent heterochromatin formation at macrophage-specific genes.

## Introduction

We reported previously that the distal enhancers of the pro-inflammatory cytokines IL12B and IL1A are bound by the macrophage lineage-specific TFs PU.1 and C/EBP $\beta$  in macrophages before LPS induction. It has been further suggested that binding of lineage-specific TFs facilitates subsequent binding of signal-induced TFs and transcription of the associated genes (2, 3). Lineage-specific TFs have also been termed master regulators or pioneer TFs and it has been suggested that they can access their sites in chromatin and keep enhancers accessible (for a review see (4)). To determine whether PU.1 plays a role in keeping macrophage-specific enhancers lowly occupied by nucleosomes during macrophage differentiation and accessible to the transcriptional machinery at a later stage we used a previously developed macrophage differentiation system (5). PU.1 deletion leads to embryonic lethality and severe defects in hematopoiesis, but a hematopoietic progenitor cell-line derived from the fetal liver of a PU.1<sup>-/-</sup> mouse can be propagated by growth in the presence of IL3 (6). More importantly reintroduction of PU.1 as an estrogen receptor-fusion (PUER) allows differentiation of these progenitors into macrophage-like cells and has been used to study the role of PU.1 during macrophage differentiation. One recent study determined the effects of PU.1 binding on nucleosome occupancy in this PUER expressing cell-line and showed that on average PU.1-sites in the genome were more highly occupied by nucleosomes in the absence of PU.1 (3). Another study that used shRNA-mediated knockdown of PU.1 in bone marrow derived macrophages came to a similar conclusion indicating that PU.1 keeps regulatory regions depleted of nucleosomes (7).

In this study, we have analyzed the role of PU.1 in keeping the enhancers of IL1A and IL12B accessible during macrophage differentiation by determining changes in nucleosome occupancy at these sites when PUER is re-expressed in PU.1<sup>-/-</sup> hematopoietic progenitors or when primary macrophages (BMDMs) are differentiated in the presence of shRNAs targeting PU.1. We find that IL1A but not IL12B can be induced by LPS when PUER expressing cells are differentiated into macrophage-like cells by growth in the presence of tamoxifen. Furthermore, we show that nucleosome occupancy at PU.1-sites in the IL1A enhancer is high in PU.1<sup>-/-</sup> cells and decreases as PUER binds to these sites. In contrast, we show that the IL12B enhancer is *less* occupied by nucleosomes in PU.1<sup>-/-</sup> cells than in mature macrophages but PUER cannot bind when cells are grown even for prolonged times in the presence of tamoxifen. Instead, we demonstrate that differentiation of these cells into macrophage-like cells by growth in the presence of tamoxifen leads to heterochromatin formation at the IL12B gene. We find that nucleosome occupancy at the IL12B enhancer increased, and that the whole IL12B locus became associated with PRC2 and H3K27me3. Our results indicate that PU.1 binding to enhancers prevents heterochromatin formation at macrophage-specific genes and keeps these regions accessible to TF binding and recruitment of the transcriptional machinery.

## **Materials and methods**

### ***Primary cell isolation, cell-lines and growth conditions***

BMDMs and splenic B-cells were isolated from 8-12 week old female C57BL/6 mice (NCI) with IACUC oversight, and BMDMs were grown and induced with LPS as described (1) for the times indicated in the figures. The PU.1<sup>-/-</sup> and PUER expressing cell-lines were obtained from Peter Laslo and grown as described (6). In brief, PU.1<sup>-/-</sup> or PUER expressing cells were cultured in IMDM medium without phenol red (Gibco) with 10% FBS, 2 mM L-glutamine, 50  $\mu$ M  $\beta$ -mercaptoethanol, 1x penicillin-streptomycin and 5 ng/ml recombinant mouse IL3 (Life Technologies). Where indicated PUER expressing cells were resuspended in complete medium with 100 nM 4-OHT (Sigma).

### ***shRNA mediated knockdown of SFPI1 in mouse bone marrow cells***

Lentiviral particles containing shRNAs targeting SFPI1 that had been pre-validated by the Broad Consortium (TRC collection MISSION shRNA library, Sigma) or control shRNA targeting firefly luciferase were produced in HEK293T cells. Briefly, HEK293T cells were seeded at a density of  $1 \times 10^7$  cells per 150 mm plate and grown for 24 h in IMDM medium supplemented with 10% FBS, 50  $\mu$ M  $\beta$ -mercaptoethanol, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 1x penicillin-streptomycin. Calcium phosphate-DNA suspension was prepared for each 150 mm plate by drop wise addition of 2 ml HEPES-buffered saline (50 mM HEPES, 280 mM NaCl, 10 mM KCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 12 mM D-glucose, pH 7.05) to 2 ml of a solution containing 0.3 M CaCl<sub>2</sub>, 25  $\mu$ g of target shRNA, 15  $\mu$ g of packaging vector (psPAX2, Addgene) and 5  $\mu$ g of envelope vector (pMD2.G, Addgene), and the mixture was allowed to stand for 3-4 minutes. After replacement of the

IMDM medium the calcium phosphate-DNA suspension was added drop wise to HEK293T cells with gentle mixing and cells were incubated at 37°C for 16 h. Medium was replaced again, cells were grown for 24 h and virus-containing supernatant was collected. Concentrated virus supernatant was either used directly or frozen at -80°C and stored for later use. For lentiviral transductions, bone marrow cells from the femur and tibia from 8-12 week old C57BL/6 female mice were collected as described and grown for 48 h in BMDM medium containing L929 cell-supernatant as a source of M-CSF (1). Cells were infected with lentivirus in the presence of 8 µg/ml polybrene (Sigma) and incubated for 4 h at 37°C. After replacement of the medium cells were grown for 48 h and transduced cells were selected by growth in the presence of 5 µg/ml puromycin for 5 days. Cells were harvested for various experiments as described (1).

### ***Chromatin isolation and Western blotting***

Chromatin isolation was performed essentially as described (24). Briefly,  $2 \times 10^6$  cells that had been transduced with lentivirus bearing shRNAs targeting SFPI1 or untreated control cells were resuspended in 400 µl extraction buffer, which contained 0.2% NP40 but no sodium butyrate. The final chromatin bound fraction was obtained by extraction with 160 µl high-salt solubilization buffer, and DNA-bound proteins were obtained by TCA precipitation. The resulting pellet was washed with acetone and then resuspended in 50 µl of LDS Sample Buffer (Novex Lifetech) containing 2 µl 1M DTT, and samples were heated to 75°C for 10 min. SDS-PAGE was performed on a 4-12% Bis-Tris Plus gel (Novex LifeTech). Western analysis was performed after protein transfer onto a nitrocellulose membrane and quantification of total protein levels by Ponceau Red staining. An antibody detecting PU.1

(sc-352X, Santa Cruz) was used to determine knockdown efficiency. Chemoluminescent signal after incubation with appropriate secondary antibodies was quantified on a ChemiDoc MP Imager (Bio-Rad).

### ***mRNA determination***

RNA was extracted and cDNA prepared and analyzed by qRT-PCR with specific primer pairs as described before (1). Specific primer pairs were designed to quantify mRNA levels in exon 4 for IL12B, exon 7 for IL1A, exon 18 for CSFR1, exon 15 for EMR1, exon 1 for TLR4, Exon 12 for KIT, exon 2 for SFPI1 and exon 1 for C/EBP $\beta$ . Measurements were normalized to RPL4 mRNA levels. Primer pair sequences can be provided upon request.

### ***Chromatin immunoprecipitation experiments***

ChIP experiments were performed as described previously (1) except that for H3K27me3 and Suz12 we diluted isolated chromatin in Low Salt ChIP buffer (20 mM Tris-HCl, 200 mM NaCl, 0.5% TritonX-100, 2 mM EDTA, Halt protease inhibitor cocktail w/o EDTA (Thermo Scientific), pH 8) and incubated with 0.5  $\mu$ g anti-H3K27me3 (C36B11, Cell Signaling), 0.6  $\mu$ g anti-Suz12 (D39F6, Cell Signaling) or 5  $\mu$ l anti-BAF155 (D7F8S; Cell Signaling Technology) respectively. For other ChIP experiments we used 3  $\mu$ g of anti-Med1 (A300-793A, Bethyl Laboratories). Locations corresponding to LPS-inducible enhancers identified by Ghisletti et al. (2) are 6.8 kb upstream of CCDC64b, 7 kb downstream of IL20B, 9.8 kb upstream of IL27, 11.8 kb upstream of STFA3, 63 kb upstream of ASB5, 44 kb upstream of Peli1, 64 kb upstream of IL6 and 3.9 kb upstream of Ccl5. Where indicated data is displayed as fold over an intergenic region located between the IL1A and IL1B genes.

Primer pairs for these and all other amplicons used can be given upon request. For experiments where we compared PU.1 binding in PU.1<sup>-/-</sup> cells to binding in cells expressing PUER or to BMDMs we could not normalize our data in this way, because background levels of PU.1 were also significantly reduced at non-specific regions in the genome. We therefore display our data as %IP, but we adjusted these values for varying levels of input. This allowed us to adjust for slight variations in the total (input) DNA in each ChIP reaction, which we found to affect the %IP obtained in each reaction. While this adjustment did not affect the overall result, it considerably reduced the errors (SEM) of our triplicate biological replicates. Briefly, for each replicate experiment we compared the values for total (input) DNA obtained for each cell-type to total (input) DNA values obtained for BMDMs (grown in the absence of LPS) at each genomic location we measured, and calculated an average deviation of each input from the BMDMs. The %IP values obtained with different antibodies were then adjusted by this average deviation.

### ***Quantitative nucleosome occupancy assay and qRT-PCR***

The quantitative MNase assay was performed and DNA was quantified by qRT-PCR as described before (1, 25). Primer pair sequences can be provided upon request. The MNase assay was performed at least twice for each cell-type and an in depth analysis with overlapping primer pairs covering each region of interest is shown for a representative experiment performed side by side with control cells (BMDMs). Analysis of nucleosome occupancy in PU.1 knockdown cells was performed once after pooling cells obtained from two separate lentiviral transductions with two distinct shRNAs that yielded significant levels of PU.1 mRNA knockdown in replicate experiments. For bar graphs the data is displayed

using the IGB genome Browser and overlays and difference maps were generated by the program.

### ***Data analysis***

Bioinformatic data analysis was performed using HOMER and Venn Diagrams were created with mergePeaks (3). The data sets used for analysis were GSM538004 (PUER peaks in PUER expressing cells grown for 2 days in the presence of tamoxifen)(3), GSM487450 and GSM487449 (PU.1 peaks in resting BMDMs and p300 peaks in BMDMs grown for 2 h in the presence of LPS, respectively)(2), GSM946531, GSM946537, and GSM946523 (H3K27me3 peaks in erythroid progenitors, erythrocytes and megakaryocytes, respectively)(17), GSM721294 and GSM721295 (H3K27me3 peaks in myoblasts and myotubes, respectively)(16), and GSM1412513 (H3K27me3 peaks in 3T3-L1 adipocytes)(26). The overlapping and unique sites identified by mergePeaks can be found in Suppl. Tables 1-3. Cluster analysis was performed manually and a table was created in Excel. For gene ontology analysis of the clusters PANTHER 9.0 Classification System was used, which included a Bonferroni correction for multiple testing (27).

## Results

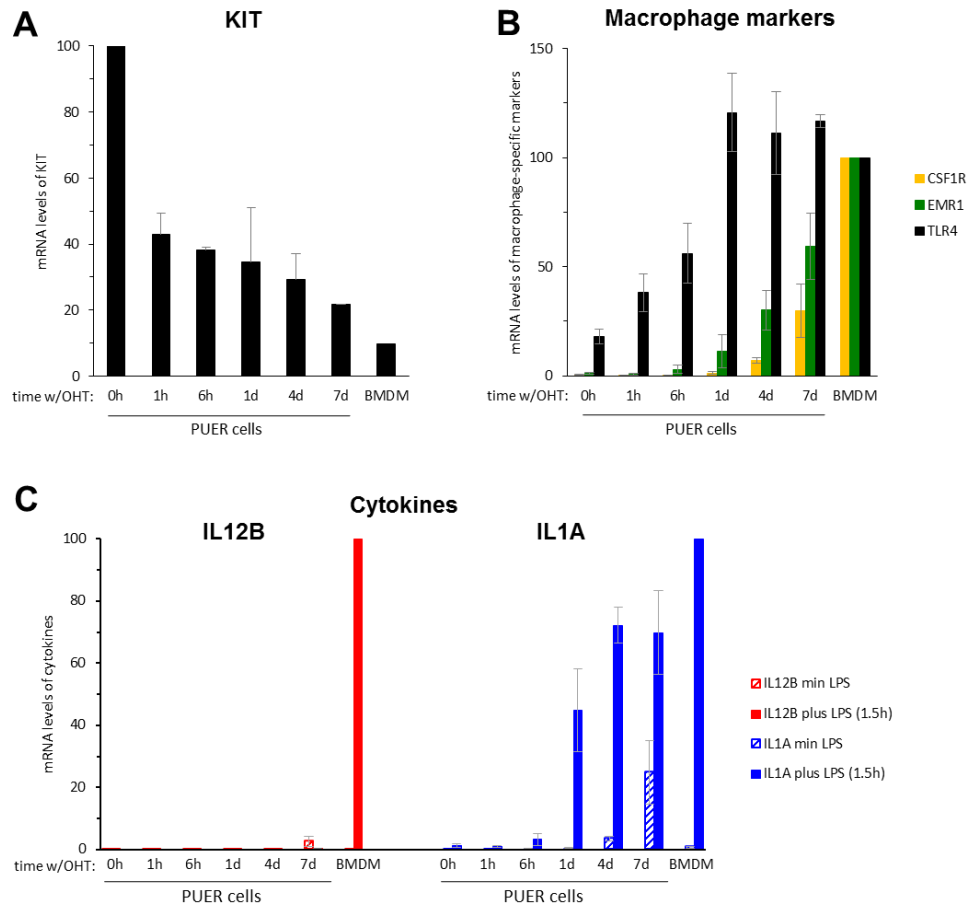
### ***IL1A but not IL12B can be induced by LPS when PUER cells are differentiated into macrophage-like cells***

To investigate the role of the lineage-specific TF PU.1 during macrophage-differentiation we used a previously described inducible PUER cell-line derived from PU.1<sup>-/-</sup> hematopoietic progenitors (kindly provided by Peter Laslo, University of Leeds)(6). Growth of these cells for prolonged times in the presence of tamoxifen and IL3 has been shown to facilitate differentiation into macrophage-like cells. As shown in Fig. 5 we confirmed that growth for as little as 1 h in the presence of tamoxifen led to considerable reduction in expression of the hematopoietic progenitor marker KIT (Fig. 5A). We also confirmed that macrophage-specific markers, including TLR4, EMR1 and CSFR, were upregulated under these conditions (Fig. 5B). However, we noted that while some markers were expressed at levels similar to those found in BMDMs after only 1 day of growth in the presence of tamoxifen (e.g. TLR4), the levels of others (e.g. CSF1R) remained considerably lower even after 7 days. Significantly, we found that IL1A was induced in response to LPS on day 1, whereas IL12B was not induced even when cells had been grown for 7 days in the presence of tamoxifen (Fig. 5C).

### ***Nucleosome binding at the IL1A and IL12B enhancers in PU.1<sup>-/-</sup> hematopoietic progenitors***

To determine whether PU.1 plays a role in keeping the enhancers of IL12B and IL1A accessible to signal-induced TFs we analyzed nucleosome occupancy in PU.1<sup>-/-</sup> hematopoietic progenitors using the quantitative assay described (25). Fig. 6A and B show

**Figure 5. Expression of macrophage-specific genes in PUER expressing cells grown in the presence of tamoxifen**



PUER expressing cells were grown in the presence of tamoxifen for the times indicated and mRNA was quantified as described in *Materials and Methods*. mRNA levels found in BMDMs is shown for comparison. Data was normalized to levels of RPL4 mRNA. **(A)** mRNA levels of the progenitor marker KIT. Levels found in PUER-cells grown in the absence of tamoxifen were set to 100%. **(B)** mRNA levels of the macrophage-specific genes CSF1R (yellow), EMR1 (green) and TLR4 (black). Levels found in BMDMs were set to 100%. **(C)** mRNA of IL12B (red) and IL1A (blue) before (hatched bars) or 1.5 h after LPS addition (solid bars). Levels found in BMDMs grown for 1.5 h in the presence of LPS were set to 100%. Error bars show SEMs for at least three replicate experiments.

**Figure 6. Nucleosome occupancy at the IL1A and IL12B enhancers in PU.1<sup>-/-</sup> progenitors, BMDMs and B-cells**

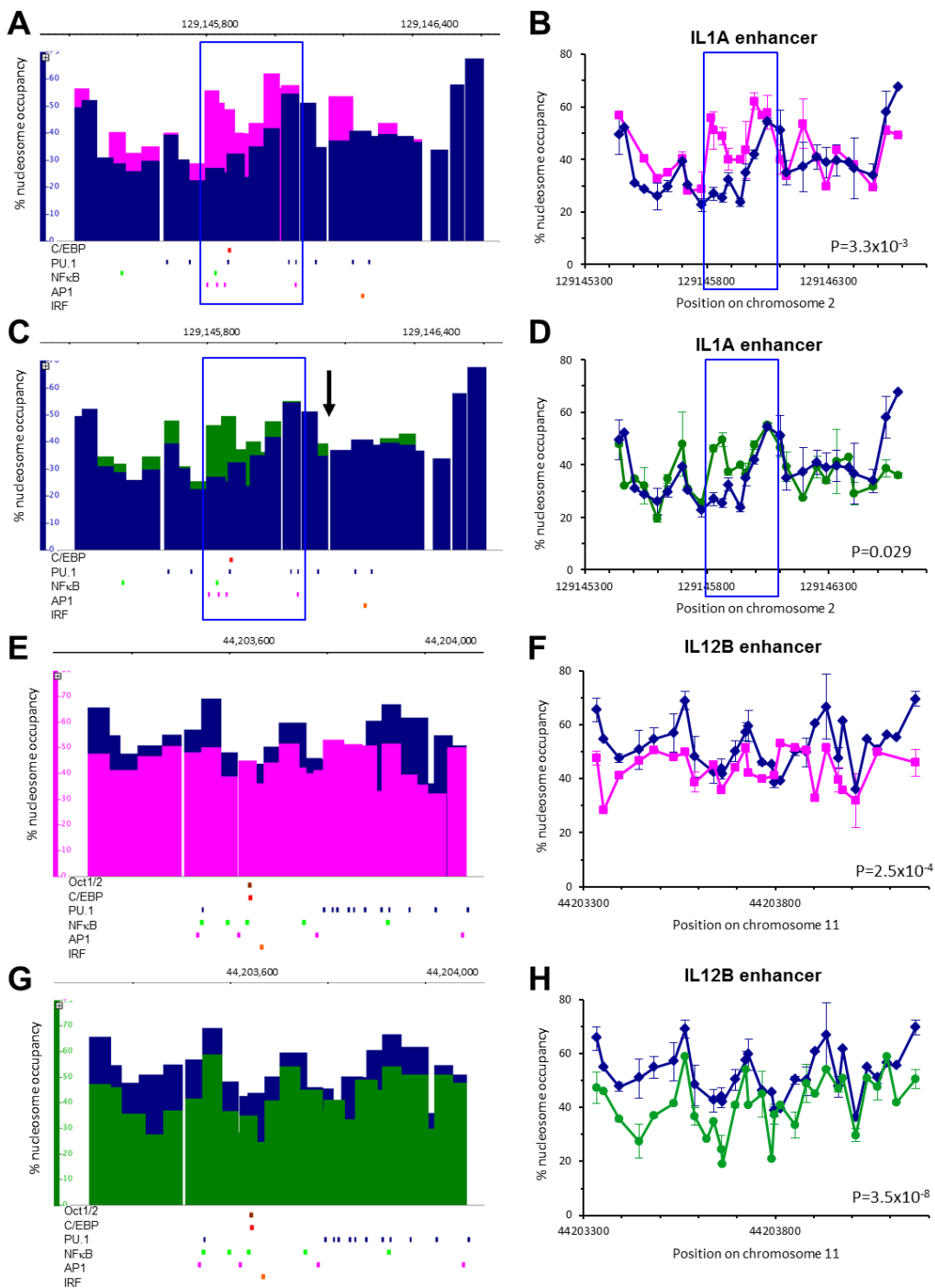


Figure 6 (cont'd)

**(A-D)** Nucleosome occupancy at the IL1A enhancer is shown as bar (A and C) and line graphs (B and D) in BMDMs (blue), PU.1<sup>-/-</sup> progenitors (magenta) and splenic B-cells (green). Occupancy was analyzed in at least three independent experiments for each cell-type and error bars representing the SEM are included in the line graphs of (B) and (D). The blue box in (A-D) indicates the region that is cleared of nucleosomes in LPS-stimulated BMDMs (1), and nucleosome occupancy in this region is significantly different in the PU.1<sup>-/-</sup> or B-cells compared to BMDMs as indicated by the P-values (Student's T-test) shown in the figures. Consensus sites for TFs are indicated underneath the panels. **(E-H)** Nucleosome occupancy at the IL12B enhancer is shown as described for the IL1A enhancer in (A-D). Statistical significance of the differences found over the whole IL12B enhancer region in various cell-types compared to BMDMs is indicated by the P-values (Student's T-test) in the figures.

levels found in BMDMs (compare magenta to blue bars and lines). The strongest increase in nucleosome occupancy was found at a region that becomes completely cleared of nucleosomes when IL1A is expressed, as we had previously demonstrated (1)(region that at the IL1A enhancer nucleosome occupancy around the predicted PU.1 consensus-sites was increased in PU.1<sup>-/-</sup> hematopoietic progenitors compared to marked by the blue box, see also Fig. 8D). These results suggest that PU.1 binding displaces nucleosomes in the IL1A enhancer in agreement with previous results from genome-wide studies (3). PU.1 is also expressed in other hematopoietic cells and we had previously shown that low levels of PU.1 are bound to the IL1A enhancer in splenic B-cells (1). As shown in Fig. 6C and D we found that the pattern of nucleosome occupancy at the IL1A enhancer in B-cells was similar to that found in PU.1<sup>-/-</sup> cells and differed only at one location (indicated by the black arrow in Fig. 6C). The two predicted PU.1-sites flanking this region have the preferred consensus GAGGAA (28), while the first two bases at other predicted sites in the region the becomes depleted of nucleosomes in the IL1A enhancer vary (*i.e.* XXGGAA). Our results suggest that low levels of PU.1 found in B-cells may allow PU.1 binding to the strong PU.1-consensus sites and displace nucleosomes in their vicinity but leave weaker sites unbound and occupied by nucleosomes. High levels of PU.1 found in BMDMs may lead to additional binding of PU.1 to weaker sites and further depletion of nucleosomes.

In contrast to our findings at IL1A we found that the absence of PU.1 had the opposite effect on IL12B. Thus nucleosome occupancy at the IL12B enhancer was *lower* in PU.1<sup>-/-</sup> hematopoietic progenitor cells than in BMDMs (Fig. 6E and F). Nucleosome occupancy at preferred nucleosomal positions was around 45-50% in PU.1<sup>-/-</sup> cells compared to 60-75% in BMDMs (Fig. 6E and F, magenta compared to blue bars and lines).

Lower levels of nucleosome occupancy at the IL12B enhancer were also seen in resting B-cells (Fig. 6G and H). Our results at the IL12B enhancer are in direct contrast to results from genome-wide studies that have analyzed average nucleosome occupancy in the presence and absence of PU.1 after alignment of all PU.1 binding sites (3, 7). Such a view may obscure distinct effects at individual sites. Together our results show that the absence of PU.1 has opposite effects on nucleosome occupancy at the IL1A and IL12B enhancers.

***Nucleosome binding at the enhancers when PU.1 is knocked down in bone marrow derived hematopoietic progenitors***

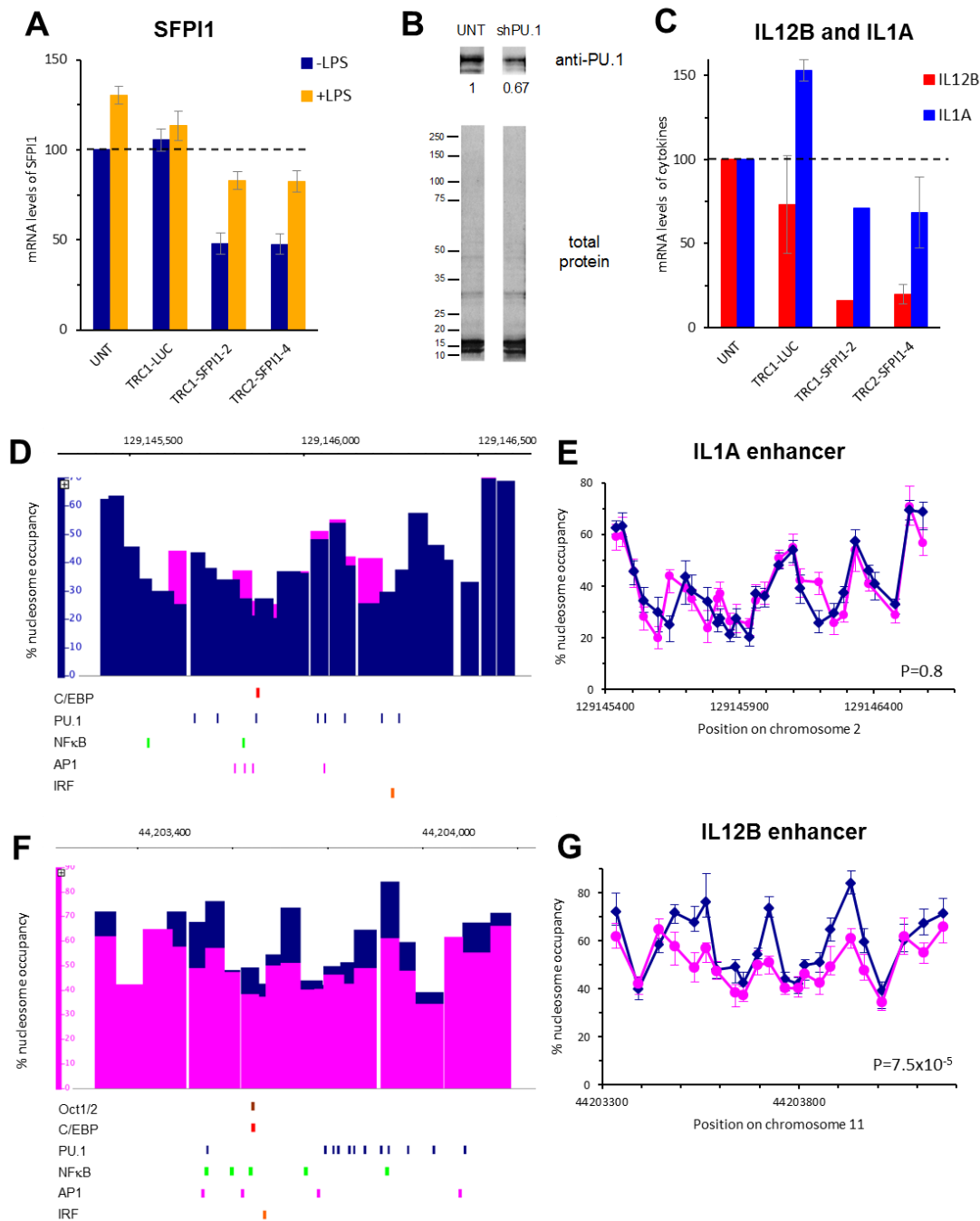
To confirm that the differences in nucleosome occupancy we observed were a consequence of the lack of PU.1 and not due to other secondary effects in the PU.1<sup>-/-</sup> cell-line, we also knocked down PU.1 in primary bone marrow derived hematopoietic progenitors using lentiviral delivery of shRNAs (Fig. 7). For these experiments cells isolated from bone marrow were grown for two days in culture and then transduced with lentivirus expressing different shRNAs against SFPI1, the gene encoding PU.1. Cells were then grown in the presence of M-CSF for 7 more days, which favors differentiation of progenitors into macrophages. We depleted SFPI1 mRNA to about 50% of wild-type levels, an effect similar to that achieved by others in primary macrophages (Fig. 7A)(7). The levels of chromatin-bound PU.1 protein were found to be reduced by 33% in the knockdowns (Fig. 7B). At this level of knockdown we found strong impairment of IL12B induction 1.5 h after LPS addition (10-fold reduction), while the effects on IL1A were less dramatic (20-30% loss)(Fig. 7C). While knockdown of SFPI1 had negligible effects on nucleosome occupancy at the IL1A enhancer that were not statistically significant (P-value=0.8)(Fig. 7D and E),

nucleosome occupancy at the IL12B enhancer was decreased to levels similar to those found in PU.1<sup>-/-</sup> cells (Fig. 7F and G). Our findings at individual enhancers using our quantitative occupancy assay are again in contrast to results from genome-wide studies in PU.1 knocked down BMDMs, which had suggested that on average nucleosome occupancy at PU.1-sites increased in the absence of PU.1 (7).

### ***Knockdown of PU.1 impairs nucleosome removal at the IL12B enhancer upon LPS induction***

We also determined whether the reduced levels of PU.1 present in our partial knockdown affected nucleosome removal at the IL12B enhancer in response to LPS (Fig. 8A and B). We found that nucleosome occupancy was somewhat higher at the IL12B enhancer 1 h after LPS addition when PU.1 was knocked down compared to control cells (Fig. 8A and B). A student's T-test indicates that the increase at IL12B is statistically significant. Nucleosome removal at IL1A was not affected by the PU.1 knockdown (Fig. 8C and D). Together our results demonstrate that our partial PU.1 knockdown affects induction and nucleosome removal at some genes but not others. This is in agreement with previous studies in PU.1 heterozygous mice, which have shown that some macrophage markers as well as certain cytokines are expressed at lower levels when PU.1 levels are reduced (29, 30).

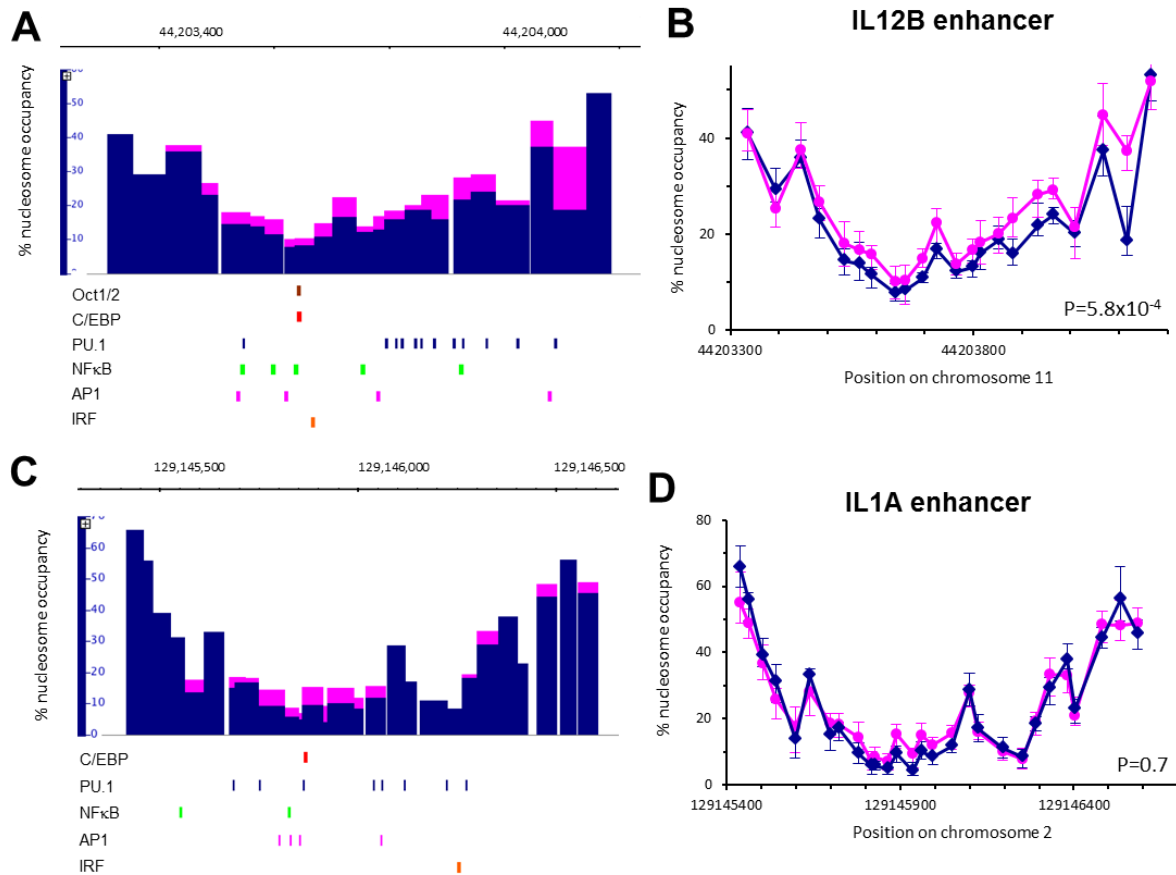
**Figure 7. Gene expression and nucleosome occupancy analyzed in BMDMs differentiated in the presence of specific shRNAs targeting PU.1 or control shRNAs targeting firefly luciferase.**



#### Figure 7 (cont'd)

Bone marrow progenitors were transduced with lentivirus bearing specific or control shRNAs and differentiated in the presence of M-CSF as described in *Materials and Methods*. **(A)** SFPI1 mRNA was quantified in cells grown with (yellow) or without (blue) LPS for 1.5 h and data was normalized to mRNA levels of RPL4. Levels of SFPI1 found in BMDMs grown without LPS were set to 100%. **(B)** PU.1 protein levels in the chromatin-bound fraction of untreated cells and cells knocked down for PU.1 are shown. Cells transformed with either one of the specific shRNAs identified in (A) were pooled and the chromatin-bound fraction was isolated as described in *Materials and Methods* (24). A Western blot probed with an antibody against PU.1 is shown, as well as the total protein in the chromatin-bound fraction detected by Ponceau Red staining of the nitrocellulose membrane. **(C)** mRNA levels of IL12B (red) and IL1A (blue) in cells grown for 1.5 h in the presence of LPS was quantified and levels found in BMDMs were set to 100%. **(D-G)** Nucleosome occupancy at the IL1A (D and E) and IL12B (F and G) enhancers in the absence of LPS was analyzed in cells transduced with specific shRNAs against SFPI1 (magenta) and against luciferase (blue). For these experiments cells transduced with either one of the two SFPI1-specific shRNAs identified in (A) were pooled and analyzed together. The error bar shows the confidence interval of measurements at each genomic location derived from curve-fitting of MNase digestion data as described in (25). Student's T-tests indicate that differences in occupancy at IL12B are statistically significant between PU.1<sup>-/-</sup> cells and BMDMs, while occupancy at IL1A is not statistically significantly different. P-values are indicated in the figure. Comparison of untreated cells and cells treated with shRNAs against LUC at IL12B can be found in Suppl. Fig. 1 and showed no statistically significant difference.

**Figure 8. Nucleosome removal upon LPS induction determined in cells transduced with shRNAs targeting PU.1**



Nucleosome occupancy was analyzed as described in the legend of Fig. 7 in cells transduced with shRNAs against SFPI1 (magenta) or luciferase (blue) 1 h after growth of cells in the presence of LPS. **(A and B)** Nucleosome occupancy at the IL12B enhancer is statistically significantly different in PU.1<sup>-/-</sup> and BMDMs as indicated by a Student's T-test (P-value shown in the figure). **(C and D)** Nucleosome occupancy at the IL1A enhancer is not significantly different.

***PUER binds and facilitates recruitment of other TFs and the transcriptional machinery to the IL1A but not the IL12B enhancer***

We found that PUER bound to the IL1A enhancer when cells were grown for as little as 6 h in the presence of tamoxifen (Fig. 9A, orange bars). After 4 and 7 days of growth in the presence of tamoxifen PUER binding to the IL1A enhancer was comparable to levels of PU.1 found in BMDMs at some locations in the IL1A enhancer (-10.1 kb) but it remained somewhat lower at others (-10.3 kb)(Fig. 9A, compare red and maroon to blue bars). PUER mRNA levels were similar or even somewhat higher in PUER expressing cells compared to BMDMs (Fig. 10A) suggesting that expression of PUER is not limiting in this system. Significantly and in contrast to our findings at IL1A, we did not detect any binding of PUER to the IL12B enhancer even when cells were grown for 7 days in the presence of tamoxifen (Fig. 9A).

As shown in Fig. 9B we also detected high levels of C/EBP $\beta$  binding at the IL1A enhancer when cells were grown in the presence of tamoxifen for as little as 6 h, which further increased on day 4 and 7 (Fig. 9B). Our data indicate that C/EBP $\beta$  binding to the IL1A enhancer in PUER expressing cells was higher than in BMDMs. This result is consistent with higher C/EBP $\beta$  mRNA levels found in PU.1<sup>-/-</sup> progenitors and PUER cells than those present in BMDMs (Fig. 10B). Our results further show that C/EBP $\beta$  binding to the IL1A enhancer is dependent on PUER binding, which is in agreement with genome-wide studies that had indicated that C/EBP $\beta$  binds cooperatively with PU.1 to certain sites (3). Strikingly, we did not detect any C/EBP $\beta$  binding to the IL12B enhancer even after prolonged growth of cells in the presence of tamoxifen (Fig. 9B).

Upon LPS induction, NF $\kappa$ B bound to the IL1A but not the IL12B enhancer in PUER cells that were grown in the presence of tamoxifen and significant levels of NF $\kappa$ B binding were detected after 7 days (Fig. 9C, maroon bars). Similarly, we detected recruitment of the transcriptional machinery as represented by the mediator subunit Med1 to the IL1A but not the IL12B enhancer when cells were stimulated with LPS, and significant levels of Med1 were detected 4 and 7 days after growth of PUER expressing cells in the presence of tamoxifen (Fig. 9D, red and maroon bars). This also led to recruitment of Med1 to the IL1A promoter consistent with DNA looping, which we have suggested may bring the enhancer into proximity of the promoter under conditions that lead to gene expression (1). Furthermore, we detected intermediate levels of Med1 recruitment to the IL1A enhancer in the absence of LPS when cells were grown for 7 days in the presence of tamoxifen consistent with IL1A mRNA production under these conditions (Fig. 9D, maroon bars and Fig. 5C, hatched bars).

***Growth of PUER expressing cells in the presence of tamoxifen and PUER binding leads to lower nucleosome occupancy at the IL1A enhancer***

Our results shown in Fig. 6A and B demonstrated that the IL1A enhancer is more highly occupied by nucleosomes in PU.1<sup>-/-</sup> cells than in BMDMs and suggested that PU.1 binding may be sufficient to displace nucleosomes. To determine if growth of PUER expressing cells in the presence of tamoxifen reduces nucleosome binding at the IL1A enhancer, we analyzed nucleosome occupancy during a timecourse experiment. As shown in Fig. 11A we found that as early as 1 h after addition of tamoxifen nucleosome occupancy at the IL1A enhancer resembled that found in resting BMDMs (compare light orange to blue bars). Our

results and those of others indicate that at this time-point significant PUER-binding can be detected at the IL1A enhancer (3)(Tagore, M. and Floer, M. unpublished data). Significantly, upon prolonged growth of cells in the presence of tamoxifen we detected further removal of nucleosomes from the IL1A enhancer in the absence of LPS, and at 6 h occupancy at the nucleosomal position in the IL1A enhancer indicated by the green arrow in Fig. 11A was about 20% lower than in BMDMs. This decrease in nucleosome binding can also be seen in the difference maps of Fig. 11B, which show the difference in nucleosome binding at each time-point compared to BMDMs. This view shows significant depletion of nucleosomes after 6 h, 4 and 7 days of growth in the presence of tamoxifen even in the absence of LPS (see the area indicated by the blue boxes in Fig. 11A and B)(1). Our gene expression analysis had detected some level of IL1A mRNA in the absence of LPS induction at day 7 (Fig. 5C) as well as recruitment of the transcriptional machinery (see maroon bars in Fig. 9D). We conclude that in PUER expressing cells grown for prolonged times in the presence of tamoxifen IL1A expression becomes partially uncoupled from LPS induction. Further, we also detect recruitment of BAF/PBAF, subunits of the SWI/SNF remodeling complex correlate with PUER binding at the enhancer of IL1A as well as PELI1, IL6 and CCL5, putative macrophage inducible enhancers identified in a genome-wide study (2). Statistically significant BAF155 recruitment and PUER binding was detected as early as 1 h after addition of tamoxifen at IL1A and PELI1 (Fig. 11C and D, orange bars) and further increased with prolonged growth in the presence of tamoxifen to reach significant levels at all four enhancers after 6 h (red bars). The appearance of a BAF155 signal at macrophage-specific enhancers immediately upon PUER binding before other changes have occurred strongly suggests that BAF/PBAF recruitment is mediated by

PU.1, although we cannot exclude the possibility that other factors are involved. Together, our results suggest that upregulation of PU.1 expression during macrophage differentiation induces PU.1 binding and concomitant recruitment of BAF/PBAF to enhancers of macrophage-specific genes, which primes these genes for induction in mature macrophages.

**Figure 9. TF binding and recruitment of the transcriptional machinery**

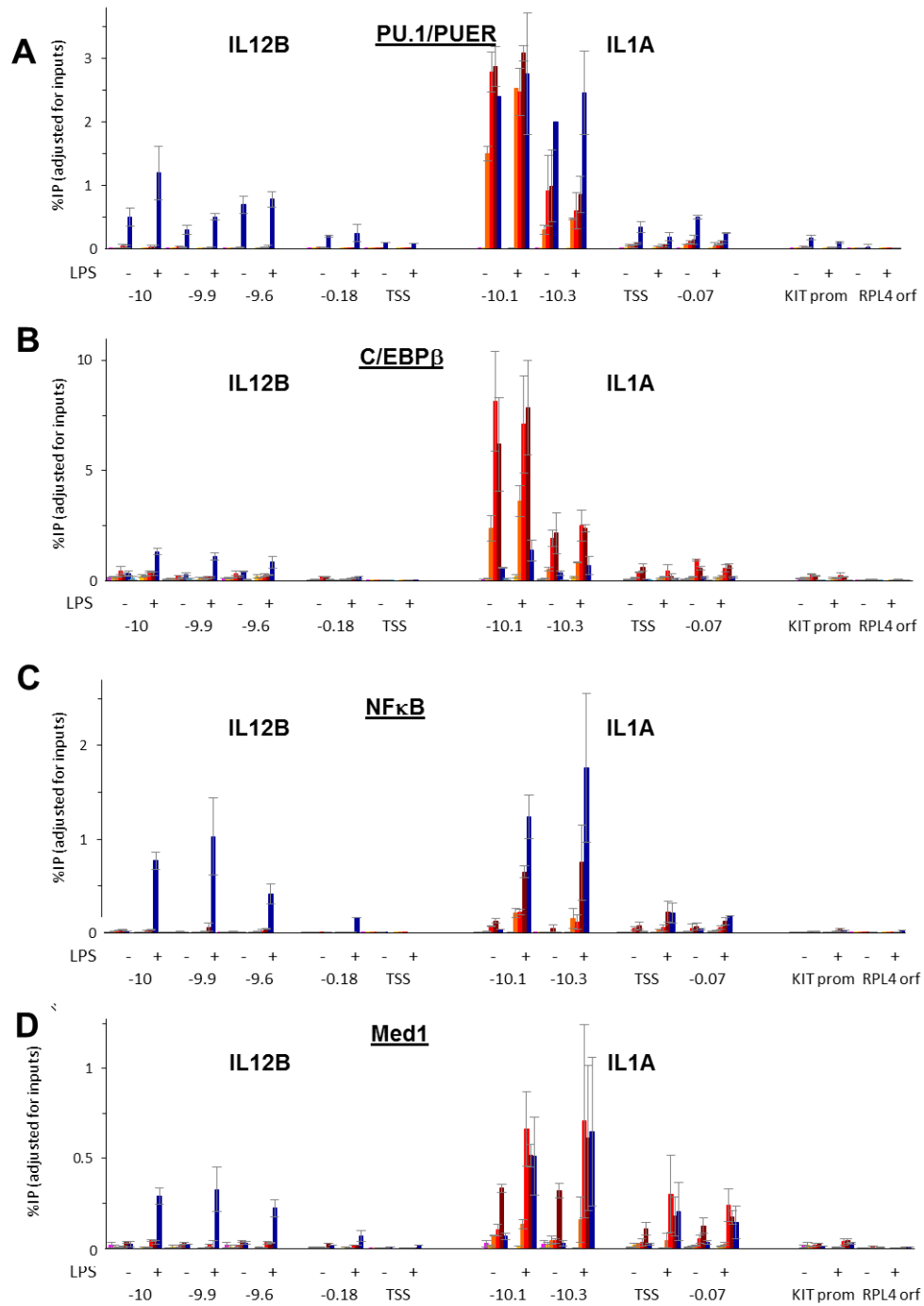
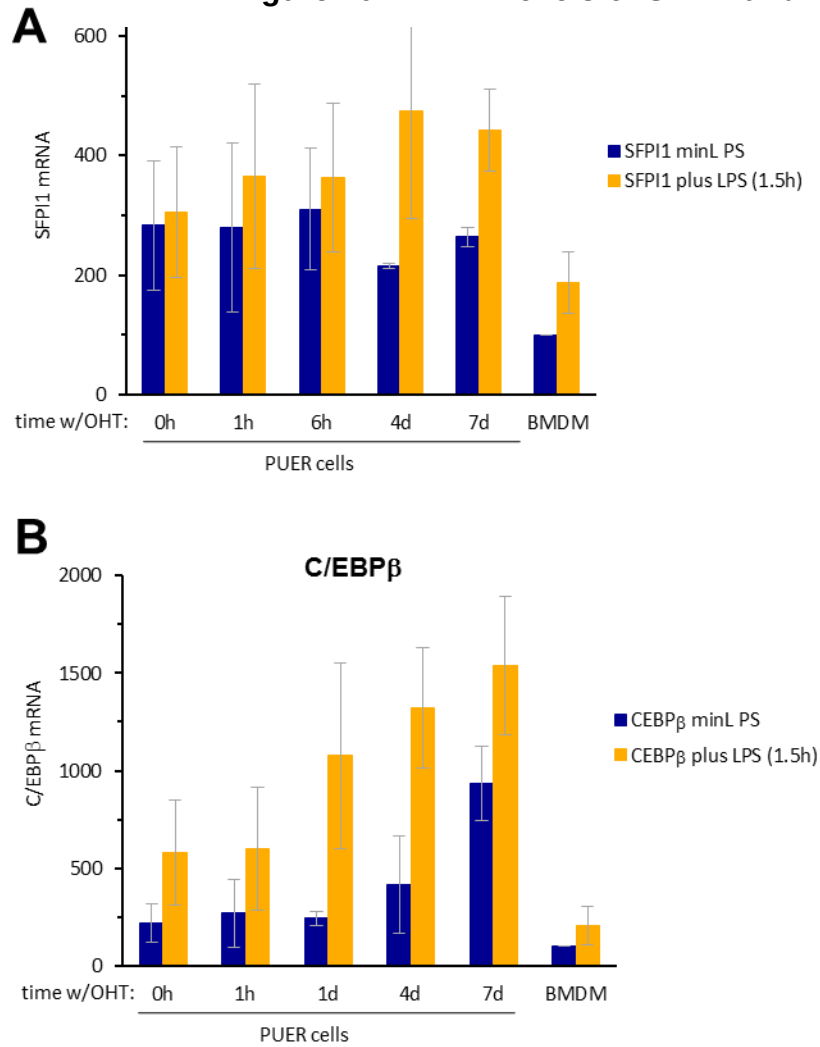


Figure 9 (cont'd)

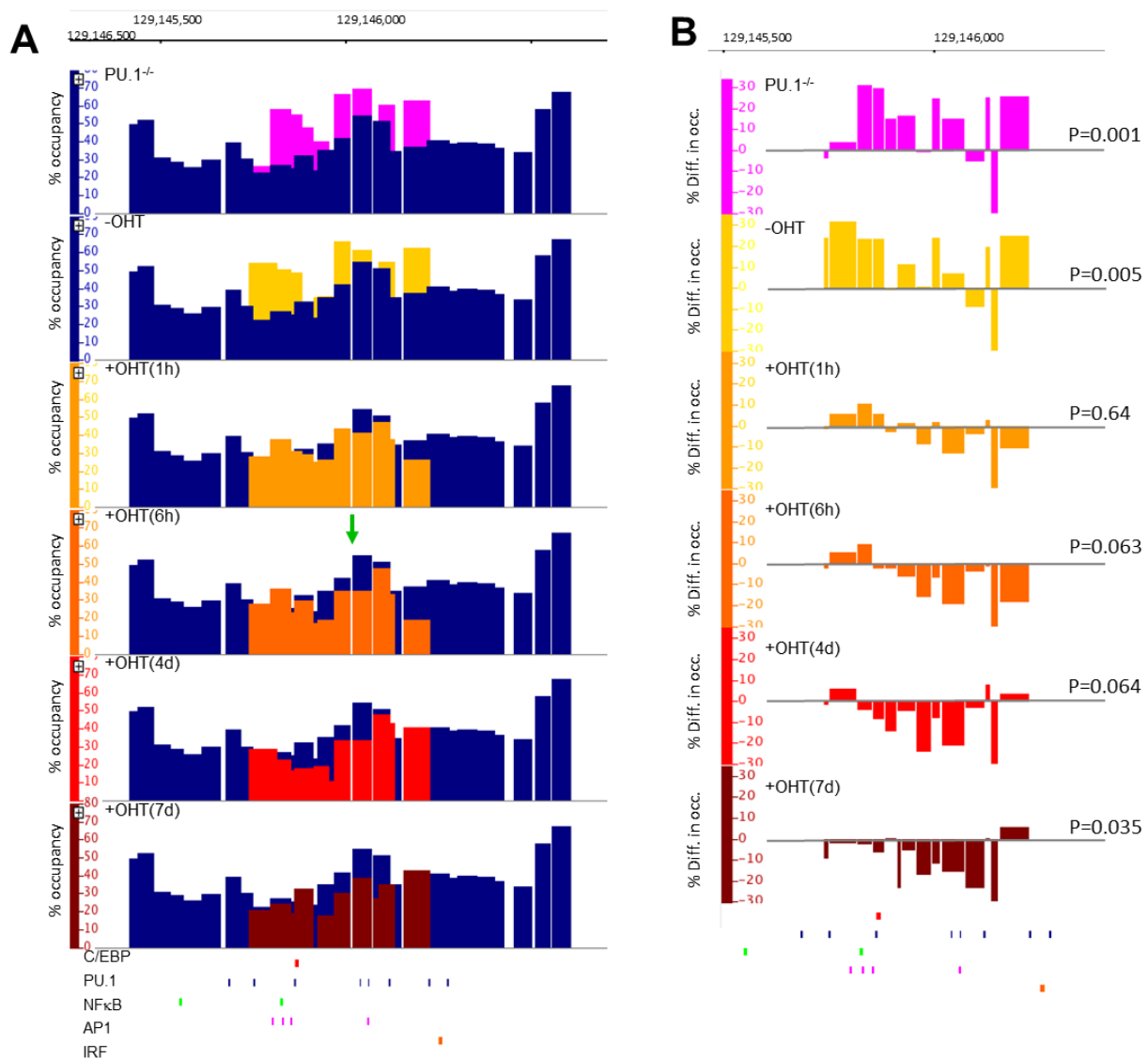
PUER, C/EBP $\beta$ , NF $\kappa$ B binding and recruitment of the transcriptional machinery to the IL12B and IL1A enhancers and promoters analyzed in PU.1<sup>-/-</sup> progenitors (magenta), PUER cells grown in the absence (yellow), or for 6 h (orange), 4 days (red) or 7 days (maroon) in the presence of tamoxifen, and in BMDMs (blue), grown either in the absence or presence of LPS for 1.5 h. Labels underneath each panel indicate the distance from the TSS of IL12B and IL1A respectively. Control locations in the KIT promoter and the ORF of the RPL4 gene are also shown. ChIP data is displayed as %IP adjusted for inputs as described in the *Materials and Methods*. **(A)** PU.1 and PUER binding. **(B)** C/EBP $\beta$  binding. **(C)** NF $\kappa$ B binding. **(D)** Mediator binding as determined with an antibody against Med1.

**Figure 10. mRNA levels of SFPI1 and C/EBP $\beta$**

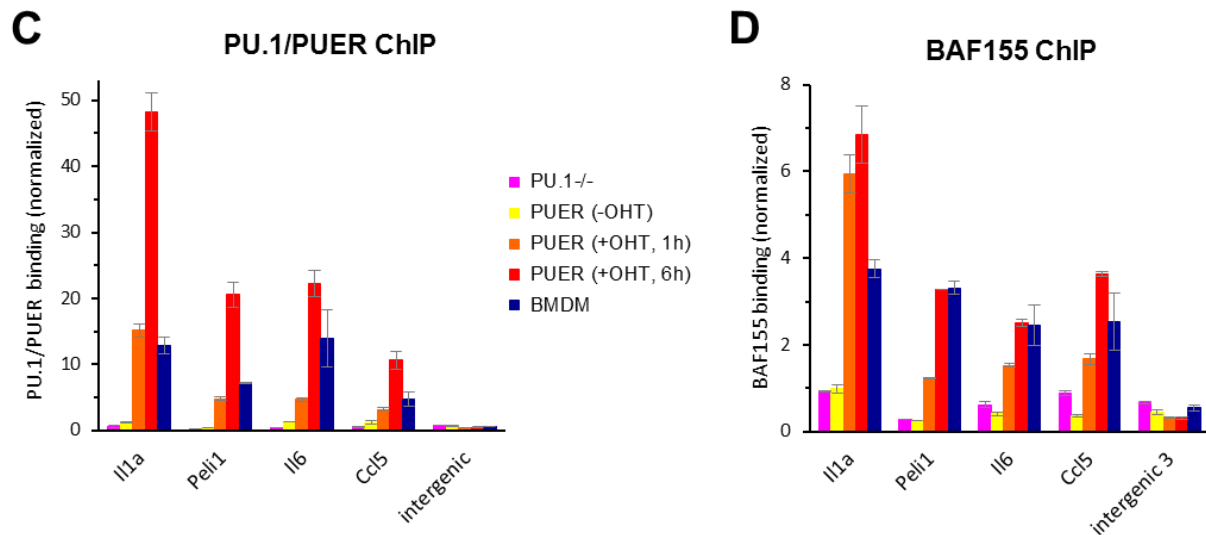


mRNA was analyzed in PUER expressing cells grown for the indicated times in the presence of tamoxifen and in BMDMs, grown for 1.5 h with (yellow) or without LPS (blue) as described in the legend of Fig. 1. mRNA levels found in BMDMs grown in the absence of LPS were set to 100%. **(A)** Expression analysis of PUER and PU.1. **(B)** Expression analysis of C/EBP $\beta$ .

**Figure 11. Changes in nucleosome occupancy at the IL1A enhancer in PUER cells grown in the presence of tamoxifen**



**Figure 11 (cont'd)**



Nucleosome occupancy was analyzed in PU.1<sup>-/-</sup> progenitors (magenta), PUER-cells grown in the absence of tamoxifen (yellow), or for 1 h (light orange), 6 h (dark orange), 4 days (red) or 7 days (maroon) in the presence of tamoxifen, or in BMDMs (blue). All cells were grown in the absence of LPS. **(A)** Occupancy is shown as bar graphs and levels found in BMDMs are shown for comparison. **(B)** Difference maps show changes in occupancy (compared to BMDMs) as cells are grown for increasing times in the presence of tamoxifen. The arrow indicates the nucleosomal position that showed the greatest change in occupancy. **(C)** PU.1/PUER binding at LPS-inducible enhancers of IL1A, PELI1, IL6 and CCL5 (for genomic coordinates of the enhancers see Materials and Methods). ChIP experiments were performed twice and error bars indicate the SEM. **(D)** A BAF155 ChIP was performed with cells as in (C) and a statistical analysis confirmed significance of the differences in BAF155 recruitment as described for PU.1/PUER binding in (C).

***Growth of PUER expressing cells in the presence of tamoxifen leads to heterochromatin formation at the IL12B locus***

In contrast to our findings at IL1A we had shown that the IL12B enhancer was less occupied by nucleosomes in PU.1<sup>-/-</sup> cells and in our partial PU.1 knockdowns (see Fig. 6E and F, Fig. 7F and G). When PUER expressing cells were grown for prolonged times in the presence of tamoxifen, we found that the IL12B enhancer became more highly occupied by nucleosomes (Fig. 12A). Binding at peak nucleosomal positions in the IL12B enhancer (indicated by the green arrows in Fig. 12A) increased as early as 6 h after tamoxifen addition and reached levels similar to those found in resting BMDMs after 7 days. A summary of the changes at the IL12B enhancer is shown in Fig. 12B, which shows that nucleosome occupancy increases over the whole region. Furthermore, a region that includes a cluster of predicted PU.1 consensus sites (indicated by the blue box in Fig. 12A) showed slightly higher nucleosome occupancy in PUER expressing cells grown in the presence of tamoxifen than in BMDMs (the levels of nucleosome occupancy in BMDMs are indicated by the hatched blue lines in each panel of Fig. 12A). We conclude that under these conditions PU.1 was not bound to the IL12B enhancer leading to the formation of nucleosomes at the PU.1 consensus sites.

To determine whether nucleosomes formed at IL12B in the absence of PU.1 were qualitatively different from those formed in the presence of PU.1 we analyzed trimethylation at H3K27 (Fig. 12C). H3K27me3 is a modification often found at genes that are transcriptionally silent and is a mark for facultative heterochromatin. Indeed we detected H3K27me3 at the IL12B enhancer when PUER expressing cells were grown for 4 and 7 days in the presence of tamoxifen. H3K27me3 was also detected at the IL12B promoter,

the ORF and in the intervening region (-7kb) between the upstream enhancer and the promoter suggesting that the whole IL12B locus becomes modified under these conditions. Levels of H3K27me3 found at the IL12B gene locus on day 7 were similar to those found at the promoter of a gene that is repressed as macrophages differentiate (*e.g.* the progenitor marker KIT) or at a gene that is only expressed in another hematopoietic lineage (*e.g.* VPRED2, expressed only in the B-cell lineage)(see Fig. 12C)(31). These results suggest that in the absence of PU.1 binding to the IL12B enhancer the IL12B locus becomes associated with heterochromatin marks during differentiation, a fate normally reserved for genes that are repressed in macrophages.

Our results further showed that the appearance of H3K27me3 correlated with an increase in nucleosome occupancy at IL12B and other sites (Fig. 12D). Thus we found that in PU.1<sup>-/-</sup> progenitors the KIT promoter was occupied by nucleosomes in around 45% of the population and occupancy increased to about 60% when PUER expressing cells were grown for 7 days in the presence of tamoxifen. Occupancy at the KIT promoter was around 90% in BMDMs consistent with high occupancy of this region found in previous studies in a myeloid cell-line where KIT is not expressed (32). Similarly, we found that the VPRED2 promoter was occupied in around 40% of the population in PU.1<sup>-/-</sup> progenitors and occupancy increased to around 65% in PUER expressing cells grown for 7 days in the presence of tamoxifen. The IL12B promoter was already highly occupied in PU.1<sup>-/-</sup> cells and occupancy did not change significantly when cells were grown in the presence of tamoxifen. Together our results indicate that during differentiation regions that are silenced in macrophages become associated with highly occupied nucleosomes that are tri-

methyated on H3K27. We conclude that high nucleosome occupancy and H3K27me3 are hallmarks of facultative heterochromatin.

### ***Nucleosomes at the IL12B locus are tri-methylated on H3K27 by recruited PRC2***

Tri-methylation of H3K27 is mediated by the polycomb repressive complex PRC2 (8-10, 33). As shown in Fig. 12E we detected recruitment of the PRC2 subunit Suz12 to the IL12B enhancer, promoter and ORF when cells were grown for 4 and 7 days in the presence of tamoxifen. Suz12 levels found at IL12B on day 7 were similar to those found at the KIT and VPRED2 promoters, and were negligible at IL1A (Fig. 12D, maroon bars). Together our data indicate that in the absence of PU.1 binding to the IL12B enhancer the polycomb repressive complex PRC2 is recruited when cells are differentiated into macrophage-like cells, which leads to H3K27 tri-methylation. Our data further suggest that polycomb binding and H3K27 tri-methylation spreads over the whole region to encompass the IL12B promoter, the intervening region and the IL12B ORF (see Fig. 12C and E).

### ***Heterochromatin is formed at other LPS-inducible enhancers***

To determine whether other LPS-inducible enhancers fail to bind PUER we examined existing genome-wide data. Heinz, S. et al. had previously identified PUER-bound sites when PUER expressing cells had been grown for 2 days in the presence of tamoxifen (3). We used the HOMER software developed by these investigators to determine the overlap of PUER-bound sites (Fig. 13A, red) with PU.1-bound sites identified in resting BMDMs by Ghisletti, S. et al. (2)(blue). We found that while a large number of sites overlapped, 20,345 sites bound in BMDMs did not bind PUER; this set included the IL12B enhancer. To

determine whether the sites not bound by PUER included LPS-inducible enhancers that function in normal macrophages we compared this dataset with the LPS-inducible p300 peaks that had been identified in BMDMs by Ghisletti, S. et al. (2). These investigators identified 2,350 LPS-induced p300 peaks and concluded that inducible p300 binding was a good mark for LPS-responsive enhancers. We found that of these 2,350 inducible peaks (Fig. 13B, dark red) 942 were bound by PU.1 in BMDMs before LPS-induction (light red). Ghisletti, S. et al. had found a slightly higher number of inducible p300 peaks to coincide with PU.1 binding 2 h after LPS induction (2). Of these 942 PU.1-bound, LPS-inducible p300 peaks 381 failed to bind PUER when it was re-expressed in PU.1<sup>-/-</sup> progenitors (Fig. 13B, blue). Thus we identified a large fraction (40%) of putative PU.1-dependent, LPS-inducible enhancers that is active in BMDMs but did not bind PUER when it was re-expressed in PU.1<sup>-/-</sup> cells and resembled the IL12B enhancer.

We then investigated whether any of these macrophage-specific enhancers that fail to bind PUER in PU.1<sup>-/-</sup> cells was associated with marks of facultative heterochromatin in other cell-types. We compared this set of enhancers to six datasets of H3K27me3 ChIP-seq experiments generated by the Mouse ENCODE consortium in various cell-types, including erythroid progenitors, erythrocytes, and megakaryocytes (16), myoblasts and myotubes (17), and 3T3-L1 adipocytes (26)(Fig. 13C). 210 or 55% of the 381 PU.1-dependent LPS-inducible macrophage enhancers (blue) showed H3K27 tri-methylation in at least one other cell type (yellow). We then performed a clustering analysis of these 381 enhancers according to their H3K27 tri-methylation pattern in different cell-types (Fig. 13D). We divided the 381 peaks into 4 clusters: sites that were tri-methylated on H3K27 in 5-6 cell-types (cluster I), in 2-4 cell-types (cluster II), in any one cell type (cluster III) or sites that

were not tri-methylated (cluster IV). The color intensity from yellow to dark green indicates the fraction of sites that were tri-methylated in each cell-type and cluster. Gene ontology analysis using PANTHER (27) revealed that clusters I and II were over-represented for macrophage activation and immune response genes, while clusters III and IV were over-represented for genes involved in constitutive cellular processes. These results suggest that a large fraction of enhancers that regulate LPS-induced responses associated with immune function in macrophages, become associated with facultative heterochromatin in other cell-types. A list of these and all the other sites identified in Fig. 9 can be found in the Suppl. Tables 1-3.

### ***H3K27me3 at enhancers that cannot bind PUER in macrophage-like cells***

We confirmed H3K27me3 binding at a subset of the PU.1-dependent, LPS-inducible enhancers identified in Fig. 13C in PUER expressing cells that were grown for prolonged times in the presence of tamoxifen (Fig. 13E). The figure shows five enhancers that are between 6.8 kb and 63 kb upstream of the TSS of the nearest LPS-inducible gene and had been assigned to these genes by proximity (2). We also included the distal and proximal enhancers of IFNB1, which we had previously studied (1). IFNB1 was not induced in PUER expressing cells (Tagore, M. and Floer, M., data not shown) and both enhancers behaved like that of IL12B, which is shown for comparison in the figure. We confirmed that neither of these enhancers bound PUER even when PUER expressing cells had been grown in the presence of tamoxifen for 7 days (Fig. 13F). Together our results show that facultative heterochromatin is formed at other enhancers in the absence of PU.1 binding and suggests

**Figure 12. Nucleosome occupancy, polycomb binding and H3K27me3 at IL12B and genes repressed in macrophages**

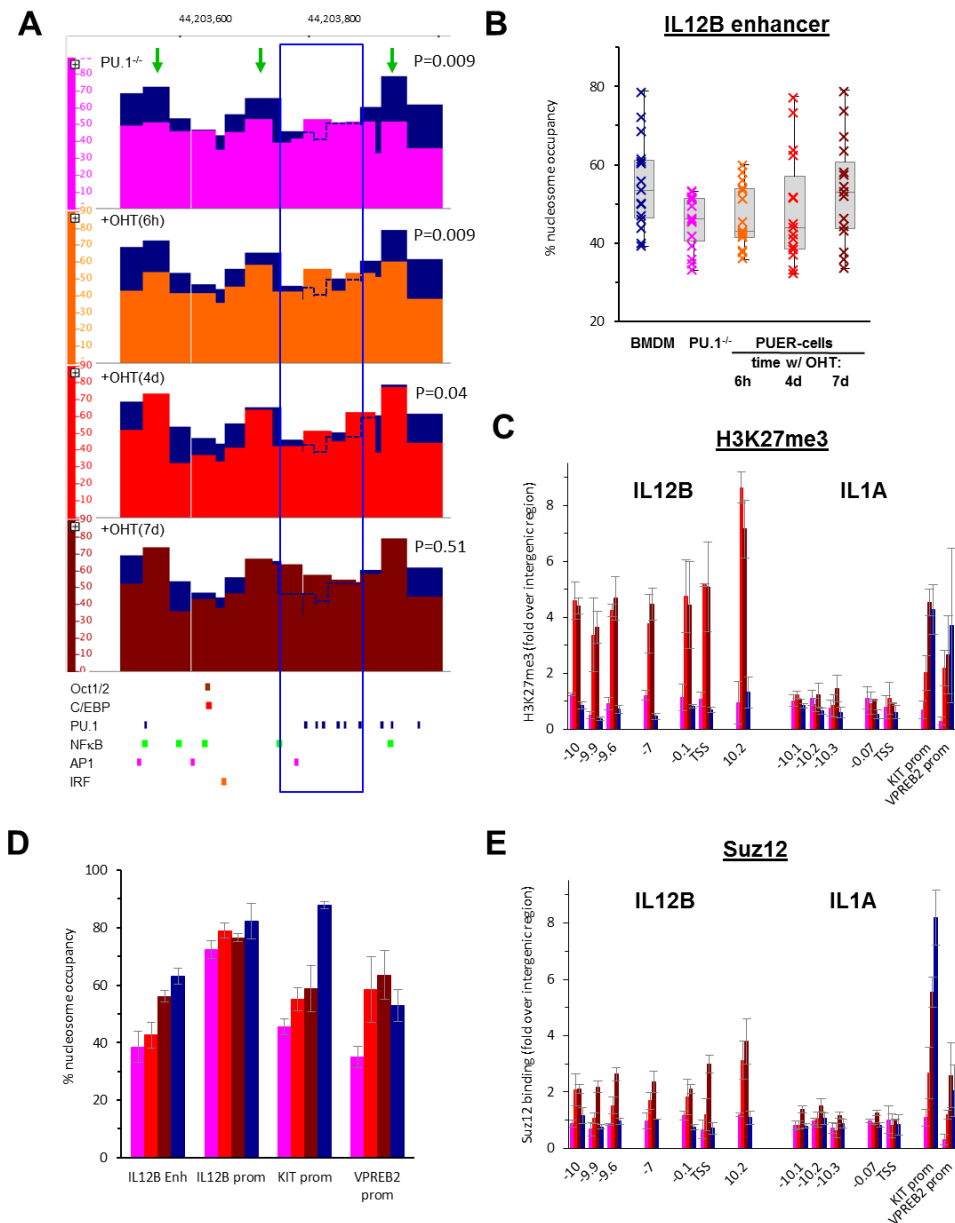


Figure 12 (cont'd)

(A) Nucleosome occupancy at the IL12B enhancer was analyzed in PU.1<sup>-/-</sup> progenitors (magenta), PUER-cells grown for 6 h (dark orange), 4 days (red) or 7 days (maroon) in the presence of tamoxifen, or in BMDMs (blue). Cells were grown in the absence of LPS. The blue box highlights the region that is more highly occupied in PUER-cells grown for prolonged times in the presence of tamoxifen than in BMDMs and the hatched blue lines show the levels of nucleosome occupancy found in BMDMs. Green arrows indicate the peak positions of the three nucleosomes that are removed when BMDMs are stimulated with LPS (1). The timecourse experiment was performed twice and a representative experiment is shown. P-values indicate that PU.1<sup>-/-</sup> and PUER-expressing cells grown for 6 h in the presence of tamoxifen are statistically significantly different from BMDMs over the whole IL12B enhancer regions (Student's T-test). At 4 days and 7 days occupancy resembles that found in BMDMs. (B) Results shown in (A) are summarized in a box plot. (C) H3K27me3 was analyzed by ChIP in cells grown as described in (A). Colors are the same as in (A) and the results from cells grown for 4 and 7 day in the presence of tamoxifen are shown. (D) Nucleosome occupancy at the promoters of KIT, VPRED2 and IL12B in different cell-types was measured as in (A). The average changes at the three nucleosomal peaks in the IL12B enhancer are shown for comparison. Error bars indicate SEMs from two independent experiments. (E) Suz12 binding determined by ChIP in cells as described in (C). ChIP experiments described in (C) and (E) were performed at least three times and error bars (SEM) are shown. Student's T-tests show that differences at IL12B in H3K27me3 and Suz12 binding are statistically significant between PUER expressing cells grown for 4 days or 7 days in the presence of tamoxifen and BMDMs (P<0.01) but not between PU.1<sup>-/-</sup> cells and BMDMs.

**Figure 13. H3K27me3 at LPS-inducible enhancers in the absence of PU.1 binding**

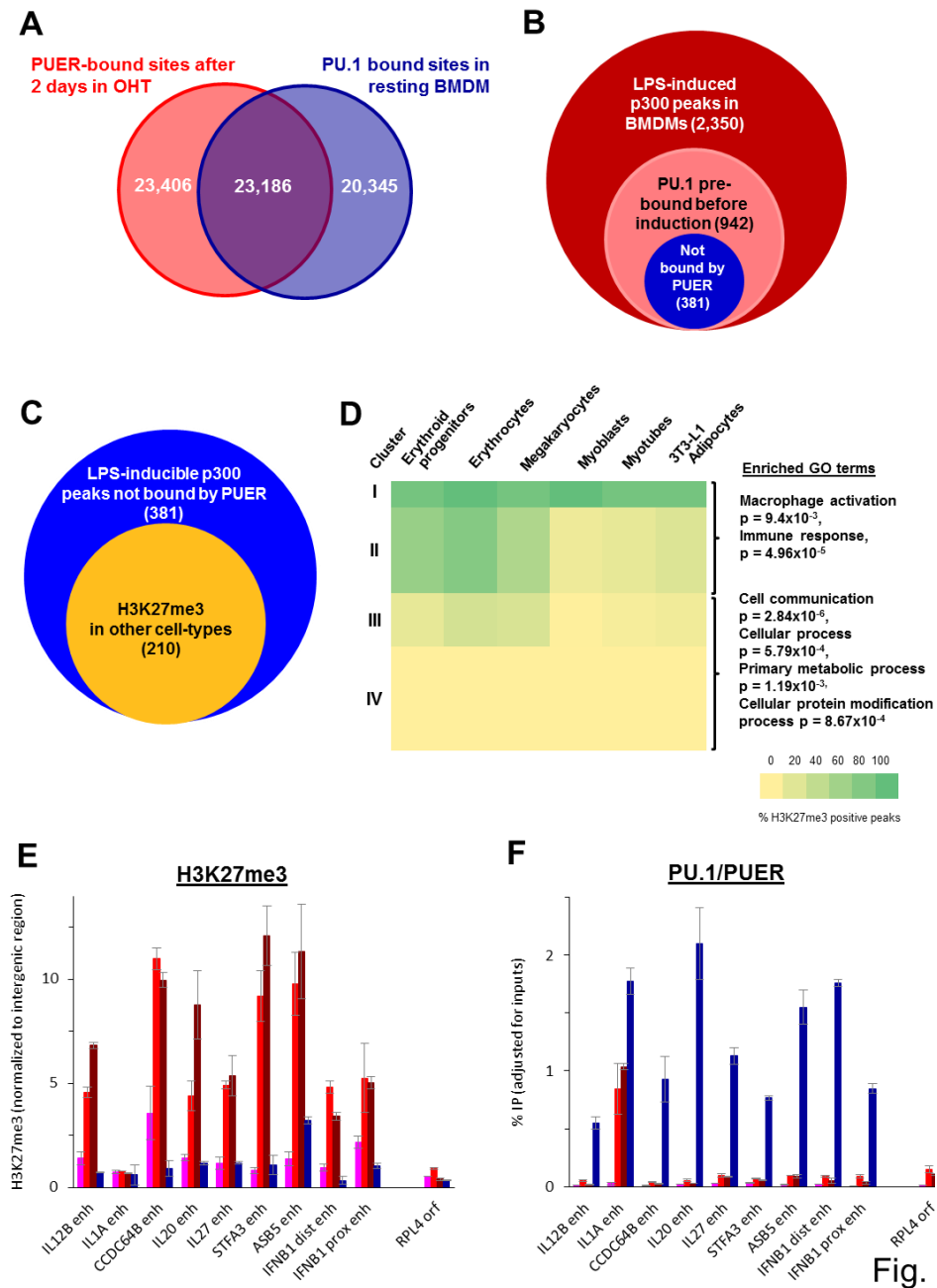


Figure 13 (cont'd)

(A) Overlap of PUER-bound sites identified in PUER expressing cells grown for 2 days in the presence of tamoxifen (3)(red) with PU.1-bound sites identified in resting BMDMs (2)(blue) is shown. (B) Venn diagram shows that of LPS-inducible p300 peaks identified in BMDMs (dark red)(2) a subset was bound by PU.1 in resting BMDMs (light red)(2), but comparison of these sites with the PUER bound dataset of (A) showed that a fraction did not bind PUER (blue). (C) Venn diagram shows that a fraction of sites that did not bind PUER identified in (B)(blue) was associated with H3K27me3 peaks (yellow) identified in the 6 cell-types indicated in (D)(16, 17). (D) Sites identified in (B) were divided into 4 clusters as described in *Materials and Methods*. The scale from yellow to dark green indicates the fraction of H3K27me3 peaks found in each category and cell-type of (C). GO terms enriched in clusters I and II, and III and IV respectively were identified using PANTHER (27). Statistical significance of the enrichment is demonstrated by the P-values shown in the figure. (E) H3K27me3 was analyzed by ChIP as described in the figure legend of Fig. 8C. (F) PU.1 and PUER binding was analyzed in cells as in (E) as described for Fig. 5A. Experiments in (E) and (F) were performed at least twice and error bars (SEM) are shown. Student's T-tests show that differences at all enhancers except that of IL1A are statistically significant between PUER expressing cells grown for 4 days or 7 days in the presence of tamoxifen and BMDMs ( $P < 0.01$ ) but not between PU.1<sup>-/-</sup> cells and BMDMs.

that this is a widespread mechanism that renders macrophage-specific enhancers unresponsive in other cell-types.

## **Discussion**

### ***Heterochromatin formation at macrophage-specific enhancers in the absence of PU.1 binding***

Our results show that a lack of PU.1 binding to the IL12B enhancer, when it was re-expressed as a PUER-fusion to induce differentiation of PU.1<sup>-/-</sup> hematopoietic progenitors, rendered the IL12B gene unresponsive to LPS stimulation in mature macrophages (Fig. 9A and 5C). In the absence of PUER binding C/EBP $\beta$  and signal-induced TFs failed to bind to the enhancer, and the transcriptional machinery was not recruited to the IL12B promoter in response to LPS (Fig. 9C-D). Instead, the IL12B gene locus became wrapped into facultative heterochromatin, as demonstrated by recruitment of PRC2 and tri-methylation of H3K27, and we found that heterochromatin formation was associated with an increase in nucleosome occupancy (Fig. 12A, B and D). Our results further show that other macrophage-specific enhancers that fail to bind PUER in this system also acquired heterochromatin marks (*i.e.* H3K27me3) when cells were differentiated into macrophage-like cells (Fig. 13E and F). Together our findings suggest that one role of lineage-specific or pioneer TFs such as PU.1 is to prevent polycomb binding to regulatory regions of genes that only become induced in fully differentiated macrophages. We propose that PU.1 binding marks these genes for future use and prevents their silencing in macrophages. This idea is consistent with the finding that PU.1 is bound to the regulatory regions of most macrophage-specific genes in macrophages even before they are induced (2, 3, 34). Our

analysis of existing genome-wide data showed that many macrophage-specific enhancers are associated with tri-methylation of H3K27 in other cell-types (16, 17), suggesting that facultative heterochromatin is formed at these enhancers in cell-types that do not express the associated genes.

Our results suggest that heterochromatin formation at the IL12B locus is a consequence of the absence of PUER binding to the distal enhancer of IL12B, and we hypothesize that heterochromatin formation may start from the distal enhancer and spread to the promoter and gene body. While spreading of heterochromatin formation from a distal enhancer remains to be demonstrated, several findings in other systems support the idea that events that occur at the distal enhancers of IL12B and IL1A during differentiation determine the fates of these genes in mature macrophages. Thus previous studies showed that a large number of enhancers is associated with H3K27me3 in different cell-types (16, 17, 26). Moreover, another study demonstrated that erasure of the H3K27me3 mark from a gene was initiated from its upstream enhancer and spread to the promoter and gene body (35). Significantly, we did not detect PRC2 binding or tri-methylation of H3K27 at the IL12B locus in PU.1<sup>-/-</sup> progenitors. Instead these heterochromatin marks appeared only when cells were differentiated into macrophage-like cells (Fig. 12C and E). This result indicates that the absence of PU.1 alone was not sufficient to induce recruitment of polycomb, but suggests that additional events during differentiation triggered PRC2 binding at macrophage-specific genes that failed to bind PU.1. It has been suggested that short DNA-sequences may mediate polycomb binding in mammals, and that polycomb binding is counteracted by TF binding to neighboring sites (36). Whether such PRC2-recruiting sequences exist in the IL12B enhancer remains to be determined.

### ***Differences between the IL12B and IL1A enhancers***

In our partial PU.1 knockdown the levels of nucleosome occupancy at the IL12B enhancer resembled those found in the PU.1<sup>-/-</sup> progenitors (compare Fig. 7F and G to Fig. 6E and F), and IL12B induction by LPS was impaired (Fig. 7C). Under these conditions nucleosomes were less well positioned in the region indicating that PU.1 binding to the IL12B enhancer during macrophage differentiation may lead to the typical pattern of positioned nucleosomes found in BMDMs. We also found that nucleosome removal at the IL12B enhancer in response to LPS was somewhat affected in the partial PU.1 knockdown (Fig. 8A and B) suggesting that PU.1 may play a role in enhancer clearance by, for example, recruiting nucleosome remodelers to the IL12B enhancer. It remains to be determined what keeps the IL12B enhancer lowly occupied by nucleosomes in the PU.1<sup>-/-</sup> progenitors and under the conditions of our partial knockdown, but we speculate that other factors, including other Ets-family TF, may be able to bind to PU.1-sites when PU.1 levels are limiting.

Our results showed that IL1A was not affected by the partial PU.1 knockdown (Fig. 7C, Fig.8C and D) and that PUER binding could be induced at the IL1A enhancer when cells were grown in the presence of tamoxifen. We hypothesize that the strength of the PU.1 consensus-sites in the IL1A and IL12B enhancers is a major distinguishing factor that determines the different responses of the two genes to limiting amounts of PU.1. We had previously shown that PU.1 binding to the IL12B enhancer was about 2-3 fold lower than to the IL1A enhancer as determined by ChIP (also shown in Fig. 9A, blue bars) indicating that the affinity of the IL12B enhancer for PU.1 is lower than that of IL1A (1). These findings suggest that the higher affinity of PU.1 for sites in the enhancer may be one reason why

IL1A is less sensitive to limiting amounts of PU.1 than IL12B, although additional factors may play a role.

***Partial uncoupling of IL1A expression from LPS signaling when PUER-cells were grown in the presence of tamoxifen for prolonged times***

Our studies revealed that the tight regulation of IL1A expression only in response to an inducing signal was lost in PUER expressing cells grown for prolonged times in the presence of tamoxifen. Thus we detected increased basal levels of IL1A mRNA in the absence of LPS after 7 days of growth in the presence of tamoxifen (Fig. 5C). Our findings are consistent with partial nucleosome depletion at the IL1A enhancer under these conditions and recruitment of mediator (Fig. 9D and 11). We speculate that high levels of C/EBP $\beta$  in PUER-cells may play some role in uncoupling IL1A expression from LPS signaling. Prior to LPS induction C/EBP $\beta$  mRNA levels were about 2-fold higher in PUER-cells grown in the absence of tamoxifen than in BMDMs, and C/EBP $\beta$  mRNA levels further increased when cells were grown for prolonged times in the presence of tamoxifen resulting in 10-fold higher levels after 7 days (Fig. 10B). Under these conditions the levels of C/EBP $\beta$  bound at the IL1A enhancer were significantly higher than levels found in BMDMs (Fig. 9B), and we suggest that these high levels of C/EBP $\beta$  may facilitate recruitment of the transcriptional machinery and transcription in the absence of signal-induced transcription factors such as NF $\kappa$ B (see Fig. 9C and D). In contrast, we hypothesize that in normal macrophages C/EBP $\beta$  levels are kept low to create a signal dependent switch. Our results are in agreement with the notion that the lineage-specific TFs PU.1 and C/EBP $\beta$  may play a role in recruitment of the transcriptional machinery. Furthermore, our results suggest that

the IL1A enhancer may function less by assembling an enhanceosome found at other innate immune genes such as IFNB1, which has a very precise structure that is only formed in the presence of all the required TFs (37, 38). Instead, our results show that NF $\kappa$ B is not absolutely required for expression of IL1A and we hypothesize that binding of TFs may occur in a stepwise fashion, and high enough levels of any one of several TFs may result in transcription. Such a billboard model has been suggested for some enhancers, in which a number of different combinations of factors can be arrayed, with variable spacing and stoichiometry, to effect gene expression (39).

#### ***PU.1 and nucleosome binding at PU.1 sites***

Previous studies have suggested that binding of so-called pioneer TFs such as PU.1 may occur to chromatinized DNA, but whether this leads to displacement of nucleosomes or whether PU.1 can bind to its sites on a nucleosomal surface has remained unclear (4). In support of nucleosome displacement genome-wide studies have shown that on average PU.1-sites are less occupied by nucleosomes when PU.1 is bound (3, 7). However, this conclusion was drawn from an analysis performed on average occupancies after alignment of all PU.1-sites. Such an analysis can amplify effects found at individual sites and we found that the results were less clear when we inspected individual PU.1 sites in these genome-wide datasets. Our approach allows a quantitative assessment of the changes in nucleosome occupancy in response to PU.1 binding at the IL1A and IL12B enhancers and is consistent with the notion that PU.1 and nucleosome binding is mutually exclusive. We detected a decrease by about 25% and 20% when PU.1 was bound to the IL1A and IL12B enhancers respectively (Fig. 6A-B, Fig. 11 and Fig. 12A). These findings indicate that PU.1

binding may displace nucleosomes either merely by competing with nucleosomes or by recruiting nucleosome remodelers that disassemble nucleosomes. Our results further suggest that BAF/PBAF is recruited to macrophage-specific enhancers in response to PU.1 binding (Fig. 11C-D). Whether PU.1 directly interacts with BAF/PBAF subunits or whether the interaction is mediated by another factor remains to be determined. C/EBP $\beta$  has been shown to directly interact with BAF/PBAF and to mediate its recruitment in other myeloid cells, suggesting that C/EBP $\beta$  may recruit BAF/PBAF together with PU.1 in macrophages. Alternatively, a PU.1-bound nucleosome may have altered properties such as, for example, less protection against digestion by MNase. We note that our previous studies in yeast have revealed that an altered, partially unwrapped nucleosome is formed by binding of the nucleosome remodeler RSC to a nucleosome at the *UASg* of the *GAL1/10* genes and that formation of this structure facilitates binding of the activator Gal4 to nucleosomal sites without displacing the nucleosome (40). Whether similar structures exist in mammalian cells remains to be determined.

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**CHAPTER 4: PRC2 SPREADS TO INTERGENIC REGIONS DURING MACROPHAGE  
DIFFERENTIATION TO SILENCE NON-LINEAGE SPECIFIC ENHANCERS WHICH  
LACK PU.1 BINDING**

## Introduction

Polycomb genes were first identified in *Drosophila melanogaster*, mainly for their role in transcriptional repression of developmental lineage genes (15, 16). In the subsequent years, mammalian orthologues of the Polycomb genes have been identified and well-studied (17). Studies in the mammalian system using both human and mouse embryonic stem cells (ESC's) have suggested that PRC2 binds to regions with high CpG island density predominantly at gene promoters (18, 19). PRC2 is capable of binding to enhancers and intergenic regions as well, though this has not been well characterized in the mammalian system (20, 21). The mechanisms by which PRC2 is recruited to specific genomic regions called Polycomb response elements (PRE's) were first identified in flies and shown to contain binding sites for specific TF's like GAGA factor (GAF), PHO, Zeste, Grainyhead (Grh) (22, for review see 27). Similar efforts to identify Polycomb response elements in mammals however, have been challenging (for review see 23). Studies using iterative genome-editing approaches reported that high CG density and deletion of transcriptional activator motifs at the Sox2 enhancer was sufficient for PRC2 recruitment (14, 19). Further, PRC2 inhibition using chemical approaches results in increase in histone acetylation levels globally, a mark associated with active enhancers, suggesting that PRC2 might actively repress enhancers (24, 25). These studies have led to models suggesting that PRC2 binding might be a default state of developmental enhancers in the absence of active transcription of the corresponding genes (26).

We previously used a myeloid progenitor cell line derived from the fetal liver of a PU.1<sup>-/-</sup> mouse, containing a tamoxifen-responsive PUER fusion protein, which can be induced

to translocate into the nucleus, upon the addition of tamoxifen. PUER translocation into the nucleus, results in the differentiation of the progenitors into macrophage-like cells, which can respond to microbial challenge (28, 29). We showed that a subset of macrophage enhancers is unable to bind PU.1 in the PUER macrophage-like cells. Further, our detailed studies at the IL12B enhancer revealed that lack of PUER binding results in PRC2 recruitment at the enhancer as well as promoter and gene body and prevented IL12B gene induction. Thus, our studies suggested that one role of lineage-specific TF's like PU.1 is to prevent PRC2 binding to cell-type specific genes. Whether PRC2 binding in the absence of PU.1, initiated at enhancers and then eventually spread to promoters and gene body, remains an open question.

In this study, we use a genome-wide approach to understand how PRC2 binding at silenced genes and their corresponding enhancers initiates during differentiation of the PUER cells into macrophages. We focus our analysis on previously identified putative macrophage enhancers by the Natoli group (10), a subset of which are unable to bind PUER and are devoid of the H3K4me1 mark in PUER cells. Using genome-wide ChIP-seq for the PRC2 mark H3K27me3 in PUER cells at different time points after OHT addition, we show that lack of PU.1 binding results in the binding of PRC2 to a subset of macrophage-specific enhancers, in resting macrophages. PRC2 binds to enhancers only upon differentiation of PUER cells and the fraction of PRC2 bound macrophage enhancers increase as cells are grown for prolonged times in the presence of OHT. Our analysis of published data shows that, this subset of enhancers is unable to produce eRNA transcripts and is associated with H3K27me3 in other myeloid as well as non-myeloid cell types. Thus, lack of PU.1 binding prevents enhancers from acquiring histone marks like H3K4me1 and

prevents enhancer transcription, a feature associated with active enhancers. Our results suggest that in the absence of PU.1 binding these enhancers become silenced by PRC2, and this process might play an important role in preventing aberrant lineage-inappropriate gene expression in committed cell types.

## **Materials and methods**

### ***Primary cell isolation, cell-lines and growth conditions***

BMDMs were isolated from 8-12 week old female C57BL/6 mice (NCI) with IACUC oversight. The PU.1<sup>-/-</sup> and PUER expressing cell-lines were obtained from Peter Laslo and grown as described (1). Where indicated PUER expressing cells were resuspended in complete medium with 100 nM 4-OHT (Sigma).

### ***Chromatin immunoprecipitation (ChIP)***

ChIP experiments were performed as described previously (1) with minor modifications. To increase the resolution of ChIP experiments for H3K27me3 and Suz12, the isolated chromatin was digested with 0.6 U MNase (NEB) for 1 h 30 min in the presence of 0.15 mM CaCl<sub>2</sub>, and the digestion reaction was stopped by addition of 20 mM EDTA. Digested chromatin was diluted 3-fold with Low Salt ChIP buffer (20 mM Tris-HCl, 200 mM NaCl, 0.5% TritonX-100, 2 mM EDTA, Halt protease inhibitor cocktail w/o EDTA (Thermo Scientific), pH 8) and incubated with 0.5 µg anti-H3K27me3 (C36B11, Cell Signaling) or 0.6 µg anti-Suz12 (D39F6, Cell Signaling).

### ***ChIP-seq***

10 ng of purified DNA from ChIP was used to prepare each sequencing library using the NEBNext ChIP-seq library prep kit at the DNA sequencing core facility at the University of Michigan. ChIP-seq libraries were run on a HiSeq 4000 to obtain single-end 50 bp reads. Each ChIP was performed in two biological replicates and was sequenced to obtain a minimum of 60 million reads per condition. For analysis, replicates were combined and raw reads obtained were checked for sequence quality, adapter content, overrepresented sequences and Kmer content using FastQC (2). Trimmomatic (3) was used to remove adapters and low quality sequences and filtered reads were mapped to the mm9 genome using Bowtie2 (4). Mapped reads were analyzed using samtools (5) and the sorted bam files were processed using Deeptools (6) to generate input normalized bigwig files. H3K27me3 ChIP-seq data for various cell-types from other studies were downloaded from GEO database (1) and the SRA files were converted to FASTQ format using the SRA toolkit. All datasets were processed as described using Bowtie2 and Deeptools.

### ***Peak calling and annotation and identification of differentially enriched regions***

Peak calling was performed using SICER 1.1 with the following parameters: 200bp sliding windows, gap size of 600 bp and FDR of 0.01 (7). Peaks were identified from each IP sample as input and MNase-digested chromatin as control for each time point. Peaks obtained were analyzed using GREAT v3 (9) to determine the distance of individual peaks from the TSS, assign individual peaks to the closest gene as well as to perform gene annotation analysis.

Diffreps (8) was used to identify differentially enriched regions using mapped reads from each IP sample as the treatment set and the IP from PUER-cells grown without OHT (0h) as the control dataset. The parameters used were as follows: 200bp window size, with –nsd broad and –meth gt.

### ***Clustering of macrophage enhancers and Heatmaps generation***

Enhancers identified by the Natoli group (10) were processed to obtain a subset of enhancers with PU.1 bound in resting macrophages using HOMER mergePeaks program to look for direct overlaps (11). The enhancers were centered on the site of the PU.1 peaks in BMDM's and clustered by k-means clustering using the computeMatrix tool from Deeptools (6). Clustering was performed using H3K27me3 reads from 24h OHT treated PUER cells, using the reference-point method and including 3 kb flanking regions from each PU.1 peak. The resulting matrix was used to generate heatmaps and average plot profiles for each sample.

## Results

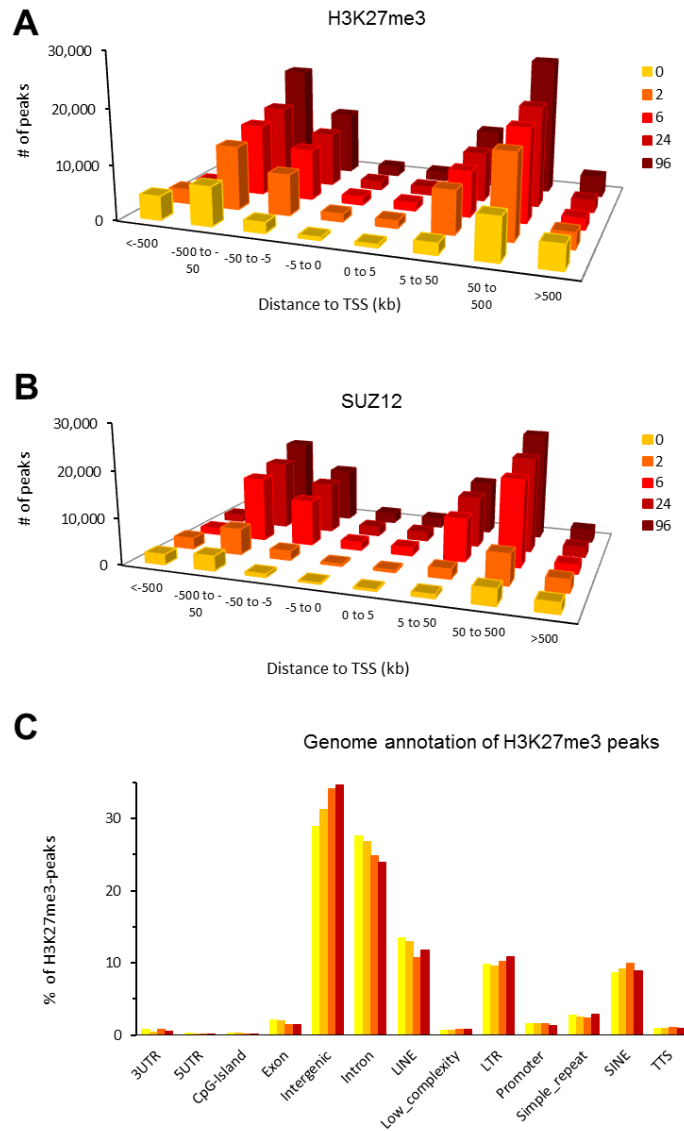
### ***PRC2 binding and tri-methylation of H3K27 increase as PUER cells differentiate into macrophage-like cells***

We and others reported earlier that growth of PUER cells in OHT results in downregulation of hematopoietic progenitor markers (i.e. Kit) as early as 1h and the upregulation of macrophage markers (i.e. EMR1, CSFR1) within 4-7 days after addition of OHT (1, 28). We had previously shown that some macrophage-specific enhancers failed to bind PUER and instead acquire PRC2 (i.e., SUZ12) binding and H3K27me3, suggesting that PU.1 binding may prevent heterochromatin formation at macrophage-specific enhancers. To determine the subset of enhancers that acquire Polycomb we performed a ChIP-seq experiment with antibodies against SUZ12 and H3K27me3 during a time course of tamoxifen treatment of PUER cells. We find that SUZ12 binding and H3K27me3 in the genome increases when PUER cells are grown for prolonged times in the presence of OHT (Fig. 14A and B). Significantly, the majority of PRC2 peaks were localized at distal sites, in intergenic regions and introns and only a small fraction of PRC2 peaks were localized at promoters and CpG islands (Fig. 14A-C). Together our data indicates that as PUER-cells differentiate into macrophage-like cells a growing fraction of the genome acquires PRC2 and H3K27me3. Surprisingly, we find that the newly acquired heterochromatic regions are found mostly outside of CpG island containing promoters.

### ***A fraction of macrophage enhancers acquire H3K27me3 in PUER cells upon differentiation***

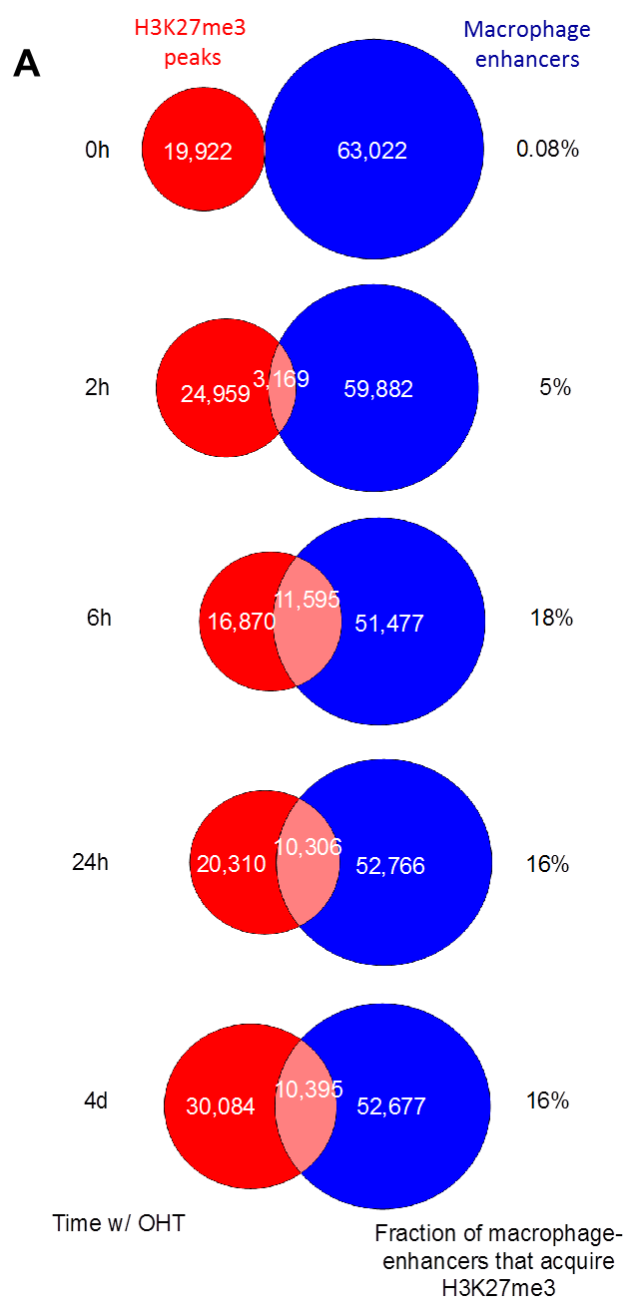
To further characterize the distal intergenic peaks which acquire PRC2

**Figure 14. SUZ12 and H3K27me3 bind to intergenic regions during differentiation**



PUER cells were grown for the indicated time points and H3K27me3 and Suz12 binding locations were identified. Genomic locations obtained from SICER peak calling are distributed in a 3D map based on their distance from the TSS for **(A)** H3K27me3 **(B)** SUZ12 binding. **(C)** Genome-annotation of H3K27me3 peaks at each time-point was performed using HOMER.

**Figure 15. Macrophage enhancers acquire H3K27me3 in PUER-cells grown in OHT.**



**A** Venn diagrams showing overlap between H3K27me3 peaks identified using SICER in PUER cells at each time point (red) with previously identified macrophage enhancers (blue).

and H3K27me3 in OHT-treated PUER cells, we analyzed PRC2 binding at previously identified macrophage enhancers (10). We took all the enhancers identified by Ostuni et al in BMDMs and determined the fraction of enhancers which overlap with the H3K27me3 peaks in OHT-treated PUER cells using HOMER (11). We observe that as early as 2h after addition of OHT, several macrophage enhancer regions acquire H3K27me3, and this fraction increases at later time points (Fig. 15). Further, we observe that while a negligible fraction of these enhancers are PRC2 bound in the PUER progenitor cells, 16% of the putative macrophage enhancers acquire PRC2 upon differentiation into macrophages.

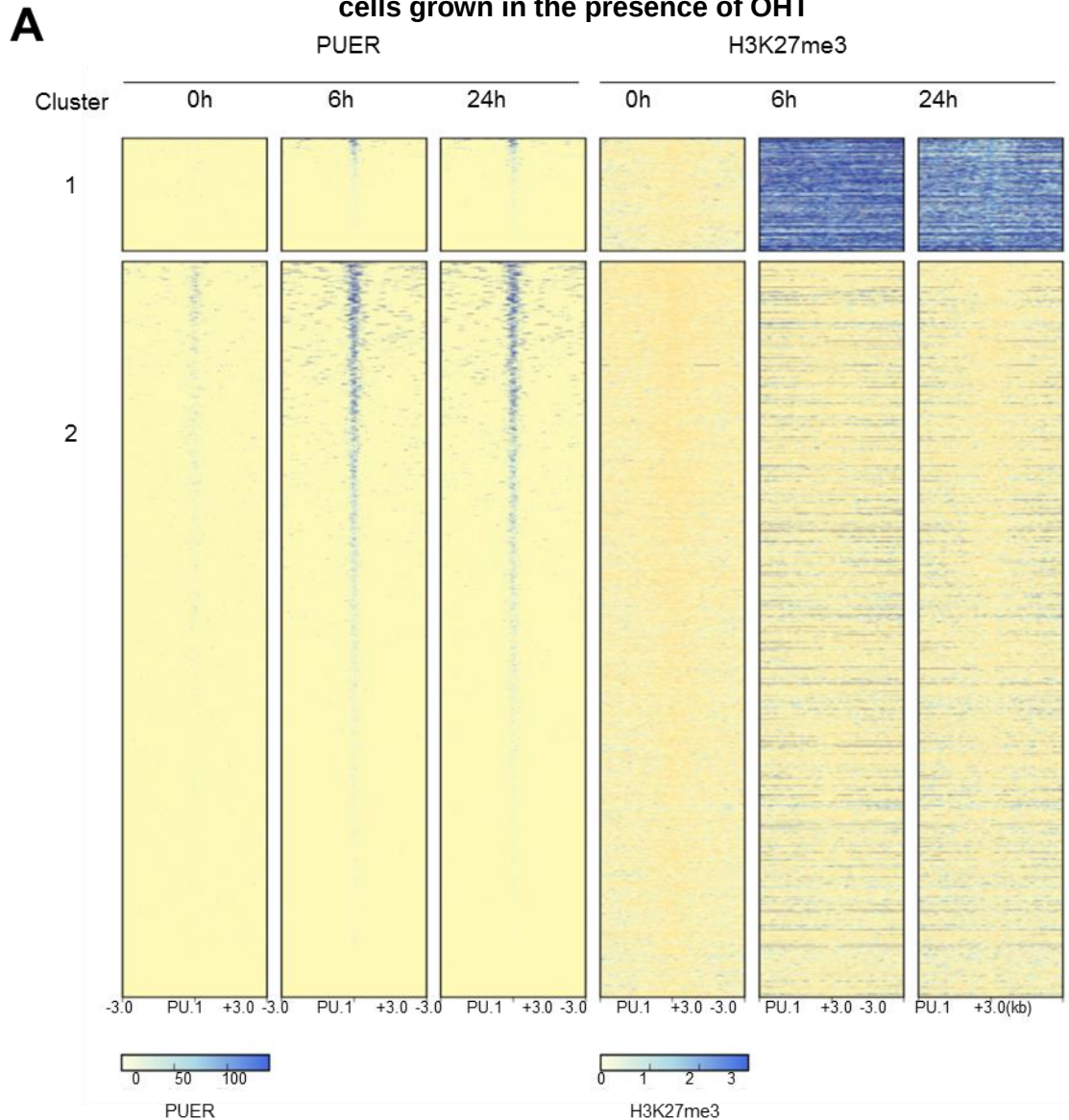
#### ***Macrophage enhancers which acquire PRC2 fail to bind PUER***

We used k-means clustering to further characterize the macrophage enhancers that acquire PRC2 in PUER cells. Enhancers from Ostuni et al. that bound PU.1 in BMDMs were clustered by their H3K27me3 levels in PUER-cells grown for 24 h in the presence of OHT. We found that enhancers that acquire H3K27me3 (cluster 1) have low levels of PUER binding compared to enhancers that fail to acquire H3K27me3 (cluster 2) (Fig. 16). Together our data indicates that a subset of macrophage-specific enhancers acquires PRC2 and H3K27me3, and that PUER binding to these enhancers is either absent or reduced compared to enhancers that do not bind PRC2. We noted that not all the enhancers where PRC2 was absent had detectable levels of PUER at 24h.

#### ***PRC2-bound macrophage enhancers fail to bind other TFs and do not acquire the H3K4me1 mark***

We also analyzed other enhancer marks at these enhancers using

**Figure 16. PUER levels are low at macrophage enhancers that acquire H3K27me3 in PUER-cells grown in the presence of OHT**



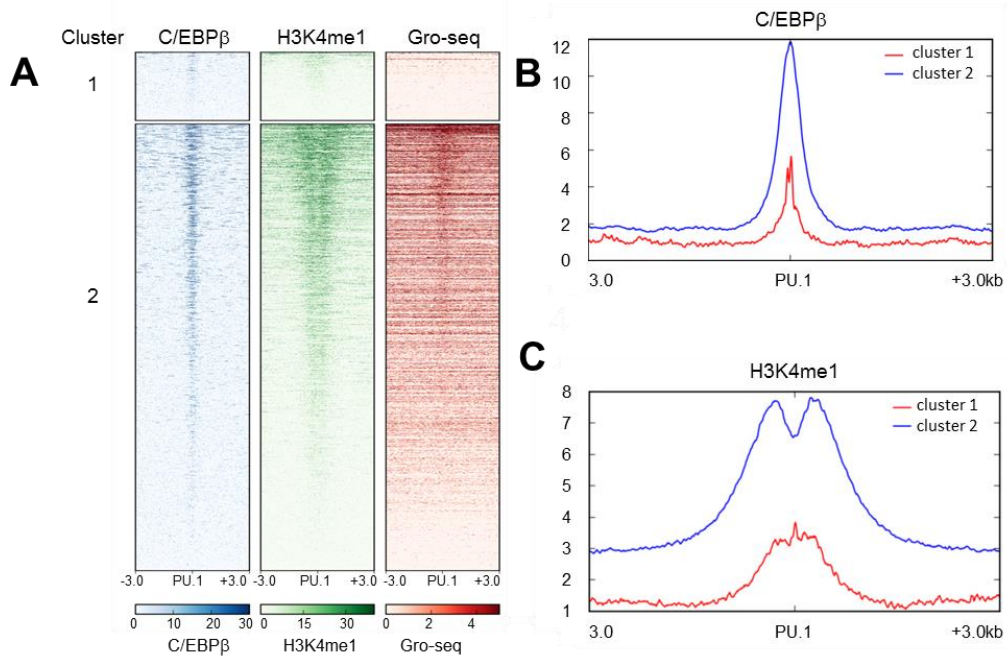
**(A)** Heatmaps show PUER binding in PUER-cells after 0h, 6h and 24h of growth in the presence of OHT (data from Heinz et al. 2010) or H3K27me3 binding at the same timepoints in cells grown in a similar manner (this study) at enhancers that bind PU.1 in BMDMs identified by Ostuni et al. 2013. We used the H3K27me3-signal at the locations of the PU.1-peaks found at these enhancers in BMDMs for initial clustering and found a small cluster of enhancers that acquire polycomb (cluster 1) and a larger cluster that does not (cluster 2). Enhancers in both clusters are shown centered on the location of the PU.1-peaks found in BMDMs.

published ChIP-seq data and found that binding of C/EBP $\beta$  and H3K4me1 is lower in cluster 1 than in cluster 2 (Fig. 17). In addition, we found that these enhancers also failed to produce eRNA transcripts as determined by Gro-seq (data from (12)). In contrast, the enhancers that did not acquire PRC2 and showed significant levels of PUER also acquired significant levels of C/EBP $\beta$ , H3K4me1 and eRNA transcripts upon growth in the presence of OHT. Together, our data suggests that H3K27me3 marked enhancers are silenced and may be refractory to transcription.

### ***Macrophage enhancers that acquire PRC2 are also silenced in other cell types***

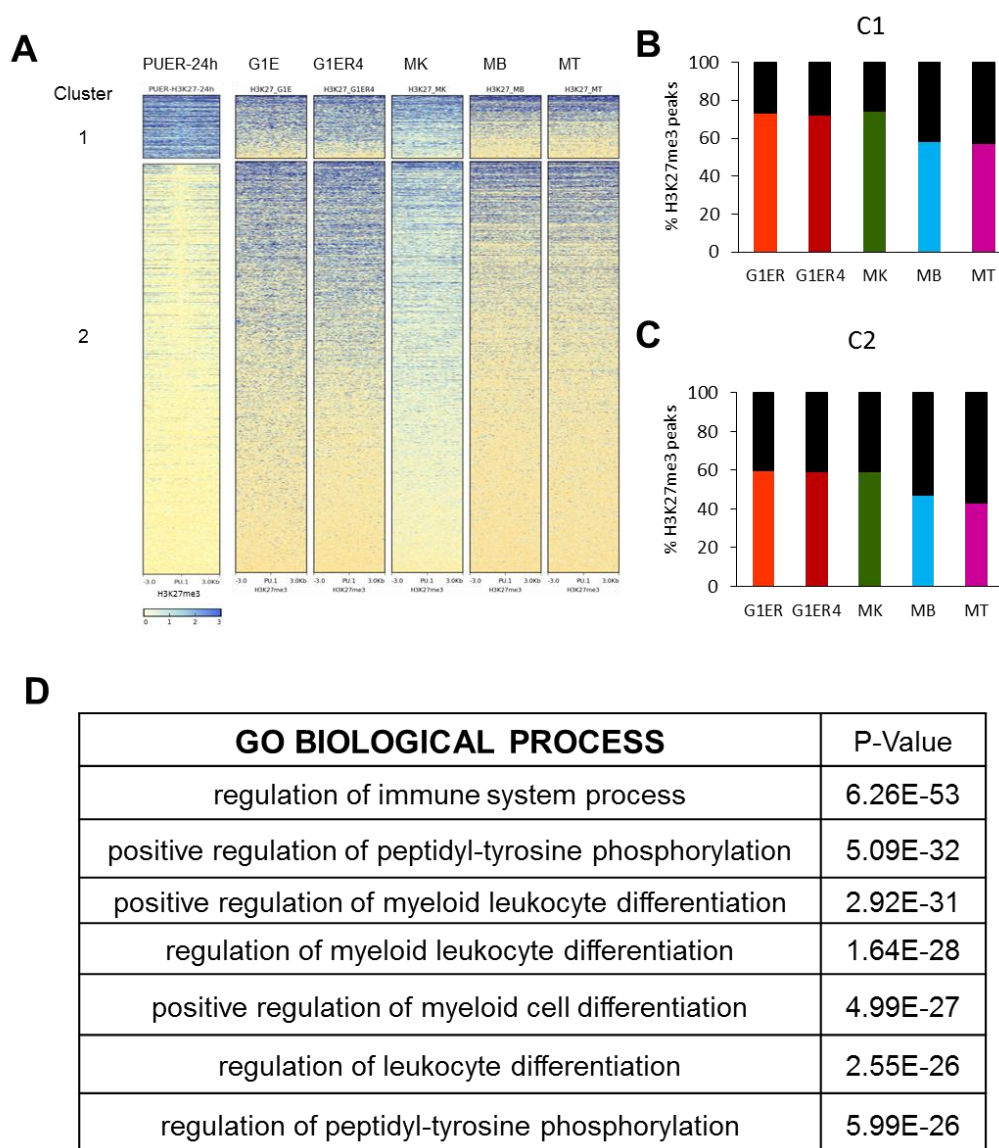
We had previously shown that LPS-inducible macrophage-specific enhancers acquire H3K27me3 in other cell-types (1). We determined whether the subset of macrophage-specific enhancers that acquires PRC2 we identified here also acquires PRC2 in these other cell-types. We observe that cluster 1 enhancers are H3K27me3-marked in other myeloid cell-types (like erythrocytes and megakaryocytes) which do not express PU.1, as well as non-myeloid cells (like myoblasts and myotubes), further validating their role as macrophage lineage-specific enhancers (Fig. 18A and B). Additionally, we find that a fraction of enhancers in cluster 2 which are PUER bound and do not acquire H3K27me3 in PUER cells, also acquire H3K27me3 in other cell types suggesting that alternate lineage enhancers are repressed in different cell-types, possibly to prevent ectopic expression of genes from other lineages (Fig. 18B and C). We further investigated the H3K27me3-marked enhancers in cluster 1 using genome annotation methods to identify the nature of the target genes regulated by the enhancers (9). We find that cluster 1 enhancers are highly enriched for macrophage and immune-cell functions (Fig. 18D).

**Figure 17. C/EBP $\beta$  binding and H3K4me1 is reduced at enhancers that acquire PRC2, and enhancers are less transcribed.**



**(A)** Heatmaps show C/EBP $\beta$ , H3K4me1 and Gro-seq data from Heinz et al.2010 and Kaikkonen et al.2013 in PUER-cells grown for 24h in the presence of OHT at enhancers clustered as in Fig. 16. **(B)** Mean C/EBP $\beta$  binding in each cluster is shown. **(C)** Mean H3K4me1-signal in each cluster is shown.

**Figure 18. Macrophage enhancers that acquire H3K27me3 in PUER-cells grown in OHT are associated with macrophage functions and acquire H3K27me3 in other cell-types.**



**(A)** Heatmaps show H3K27me3 data from PUER-24h OHT cells (this study), Erythroid progenitors (G1E), Erythrocytes (G1ER4), Megakaryocytes (MK), Myoblasts (MB), and Myotubes (MT). Percent H3K27me3 peak overlap of indicated cell-types with **(B)** Cluster 1 and **(C)** Cluster 2 enhancers. Colored bars denote overlapping fraction while black bars denote the non-overlapping fraction in each cell-type **(D)** Gene ontology analysis of cluster 1 enhancers using GREAT (9).

## Discussion

Our studies using the PUER differentiation system demonstrate that PRC2 binds to a large intergenic fraction of the genome as multipotent cells are committed to a specific cell fate (Fig. 14). Previous studies for understanding the role of the PRC2 complex during differentiation have primarily focused on promoters and CpG islands where PRC2 binding correlates with transcriptional repression. Our studies indicate that PRC2 binding might play an important role in silencing alternate lineage enhancers during differentiation, while lineage-specific enhancers are maintained in a permissive state by binding of lineage-determining TF's. Lineage-determining TF's like PU.1 bind to inducible enhancers in resting primary macrophages, have the ability to recruit other TF's, deposit the H3K4me1 mark and a subset of these enhancers produce short eRNA transcripts in the resting stage, even when the associated genes are turned off (Fig. 17A). Our studies show that lack of PUER binding at macrophage enhancers, prevents binding of other lineage-specific TF's like C/EBP $\beta$  as well as deposition of the enhancer mark H3K4me1 (Fig 17A-C). More importantly, in the absence of all these events, a subset of these enhancers are marked by H3K27me3 and show no detectable presence of eRNA transcripts. These findings are reminiscent of an earlier study which suggested that H3K27me3 mark and gene transcription at promoters is mutually exclusive. Further, this study reported that inhibition of transcription resulted in ectopic PRC2 recruitment at repressed promoters. The genes associated with the promoters studied are however constitutively active genes and therefore cannot provide a detailed understanding of the events occurring at promoters prior to gene repression (13). Our studies using inducible enhancers in macrophages, with the target genes turned off in the resting state; provide us with an ideal system to

understand the process by which regulatory elements of inducible genes are silenced during differentiation independent of the transcriptional status of the target genes. Our studies reveal that lineage-specific TF's initiate a cascade of events at enhancers of inducible genes which help poise the genes for later activation upon receiving signals from the environment. But more importantly, these initial events protect the genes from being silenced by the repressive machinery which is highly functional during cell differentiation. It still remains to be investigated whether specific sequence features of enhancers predispose them to PRC2 recruitment in the absence of lineage-specific TF binding.

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## CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

Gene regulation in macrophages stimulated with LPS is a well-studied model for inducible gene expression in mammalian systems. Detailed studies over the last few decades have revealed that the underlying chromatin plays an important role in maintaining a poised state at inducible genes in resting macrophages as well as in activated macrophages exposed to microbial challenge (1). Our studies at individual macrophage enhancers of inducible pro-inflammatory cytokines as well as global genome-wide studies provide important insights into the mechanisms by which macrophage enhancers are primed during differentiation by lineage-specific transcription factors, ensuring a rapid response to infection while also safeguarding the function of the associated genes from the cellular repressive machinery, active during differentiation (2-4).

Our studies in Chapter 2 focused on the inducible enhancers of the pro-inflammatory cytokine, IFNB1. We and others reported that lineage-specific transcription factors PU.1 and C/EBP $\beta$  are bound to inducible enhancers prior to gene induction (5, 6). Lineage-specific TF binding correlated with low nucleosome occupancy at the IFNB1 as well as the IL12B and IL1A enhancers in resting macrophages. Further, upon gene induction, nucleosomes are evicted from the enhancers of IFNB1 and both lineage-specific as well as signal-induced transcription factors are recruited to enhancers. Together our data suggest that the enhancers of pro-inflammatory genes may have to be cleared of nucleosomes to allow binding of these cis-regulatory TFs. It remains to be understood however, if nucleosomes are deposited back at enhancers once an inducible gene is shut down. In fact, it has been demonstrated in yeast, using the GAL4 system, that nucleosomes are not reformed rapidly at regulatory regions when a gene is turned off (12). During inflammation, shutting down gene expression rapidly in the absence of prolonged challenge is a critical

process to prevent tissue damage. Studies in bone-marrow derived macrophages have revealed that immune cells retain a memory of their previous activated state which might help in the rapid re-induction of some cytokines in response to a second challenge (10, 11). Exploring the kinetics of nucleosome re-formation at inducible enhancers upon gene shutdown as well as the kinetics of gene re-induction in response to a second challenge would provide important insights into the mechanism of establishing a 'memory' state of prior transcription in differentiated cells.

In chapter 3, our results show that re-expression of PU.1 in hematopoietic progenitors restored function to certain enhancers of macrophage-specific genes but not others. These findings indicate that lineage-specific TF's may have to be present during the early stages of differentiation and that their expression at a later stage may not be sufficient to reverse changes in chromatin architecture that occur in their absence. We hypothesize that limited accessibility of gene regulatory regions may be the underlying reason for low reprogramming efficiencies during generation of induced pluripotent stem cells (iPSCs) that are an inherent feature of protocols involving ectopic expression of pluripotency factors. Thus it is commonly observed that only a fraction of cells can be reprogrammed into iPSCs by expression of various TFs. Furthermore, it has been suggested that iPSCs are different from embryonic stem cells and may retain a memory of their previous state (7-9). We hypothesize that recruitment of PRC2 and heterochromatin formation at some gene regulatory regions may create a barrier that prevents binding of ectopically expressed TFs and a better understanding of these processes will lead to improved reprogramming protocols in the future.

In chapter 4, our genome-wide approach to investigate PRC2 binding at macrophage enhancers provides some important insight into the interplay between lineage-specific TF binding and the PRC2 machinery during differentiation. We observe that PRC2 spreads to intergenic regions as well as introns during macrophage differentiation from progenitor cells. Further, this spreading silences enhancers that lack lineage-specific TF, PU.1 binding. It remains to be understood if PRC2 binding at enhancers is regulated by other factors during differentiation which might play a role in the recruitment of PRC2 to specific target sites. Further detailed investigations need to be done to explore whether certain intrinsic features of enhancers with respect to their sequence as well as the presence or absence of repressive marks like DNA methylation, might predispose them to PRC2 binding in the absence of marks which distinguish active enhancers.

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