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PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure

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Transcriptional repression mechanisms are important during differentiation of multipotential hematopoietic progenitors, where they are thought to regulate lineage commitment and to extinguish alternative differentiation programs. PU.1 and GATA-1 are two critical hematopoietic transcription factors that physically interact and mutually antagonize each other's transcriptional activity and ability to promote myeloid and erythroid differentiation, respectively. We find that PU.1 inhibits the erythroid program by binding to GATA-1 on its target genes and organizing a complex of proteins that creates a repressive chromatin structure containing lysine-9 methylated H3 histones and heterochromatin protein 1. Although these features are thought to be stable aspects of repressed chromatin, we find that silencing of PU.1 expression leads to removal of the repression complex, loss of the repressive chromatin marks and reactivation of the erythroid program. This process involves incorporation of the replacement histone variant H3.3 into nucleosomes. Repression of one transcription factor bound to DNA by another transcription factor not on the DNA represents a new mechanism for downregulating an alternative gene expression program during lineage commitment of multipotential hematopoietic progenitors.

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Introduction

There is now an increasing appreciation that transcriptional repression is as important as transcriptional activation during normal development (Courey and Jia, 2001). In the hematopoietic system, mounting evidence indicates that multipotential progenitors that give rise to two or more lineage-specific cell types express a 'promiscuous' gene expression program including subsets of genes characteristic of their multiple lineage-restricted progeny (Akashi *et al*, 2000; Graf, 2002). Here, transcriptional repression may be crucial for extinguishing alternative differentiation programs during lineage commitment decisions (Graf, 2002). Inappropriate or untimely transcriptional repression is also thought to be the basis for the block to differentiation in certain hematologic malignancies (Look, 1997).

PU.1 and GATA-1 are two sequence-specific DNA binding proteins that play central roles in the lineage commitment decisions of common myelo-erythroid progenitors (CMPs) to become myeloid progenitors or megakaryocytic-erythroid progenitors, respectively (Graf, 2002). Both proteins act as gene-specific transcriptional activators. Numerous genes that are directly activated by PU.1 or GATA-1 have been identified in myeloid or erythroid cells, respectively.

In addition to their roles as sequence-specific DNA binding proteins, PU.1 and GATA-1 also physically interact. This interaction causes inhibition of the other factor's transcriptional activity (Rekhtman *et al*, 1999; Zhang *et al*, 1999; Nerlov *et al*, 2000). Enforced expression of each factor inhibits the other factor's ability to promote its respective differentiation program (Kulessa *et al*, 1995; Rao *et al*, 1997; Rekhtman *et al*, 1999). Perturbing the balance between the two factors can also lead to leukemia. Activation of PU.1 expression through provirus integration is a critical event in the development of Friend virus-induced erythroleukemias in mice (Moreau-Gachelin *et al*, 1988; Schuetze *et al*, 1992). Leukemogenesis is likely to result, at least in part, from the ability of PU.1 to block erythroid differentiation promoted by GATA-1, because simply providing additional GATA-1 to the erythroleukemia cells causes them to differentiate and undergo proliferation arrest (Choe *et al*, 2003). Thus, an insight into the molecular basis of the mutual antagonism between PU.1 and GATA-1 is important for understanding control processes in normal hematopoiesis and how these processes are disrupted in leukemia.

We report here on the mechanism by which PU.1 represses GATA-1 and thereby inhibits the erythroid differentiation program. We find that PU.1 binds to GATA-1 on its target genes and recruits a repression complex consisting of pRb and histone methyltransferase (HMT) Suv39h. The complex causes methylation of lysine-9 in H3 histones in the vicinity of the genes and binding of heterochromatin protein 1 (HP1). Interestingly, the effects of the repression complex on chromatin are readily reversed by siRNA-mediated silencing of PU.1 expression, which leads to removal of the repression complex and reactivation of the erythroid gene expression program.

Results

PU.1 represses GATA-1 by recruiting pRb, Suv39h and HP1α, leading to histone H3 methylation

Previous work from our laboratory showed that PU.1 interacts directly with both GATA-1 and pRb and that pRb is

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required for PU.1-mediated repression of GATA-1 transcriptional activity (Rekhtman *et al*, 1999; Rekhtman *et al*, 2003). To further investigate the mechanism by which the PU.1–pRb complex represses GATA-1, we used quantitative chromatin immunoprecipitation (qChIP) to interrogate GATA-1 target sites in the regulatory regions of erythroid-specific genes for the presence of components and histone modifications thought to be associated with pRb-mediated repression. ChIP studies were carried out at nine endogenous GATA-1 sites in two types of cell lines. We used MEL cells expressing an inducible form of GATA-1 (GATA-1-ER) fused to the ligand binding domain of the estrogen receptor (ER). In the absence of 17-β-estradiol, these MEL-GATA-1-ER cells are blocked from undergoing erythroid differentiation due to dysregulated expression of PU.1, which binds to endogenous GATA-1 and inhibits its ability to promote differentiation. In the presence of 17-β-estradiol, the GATA-1-ER protein is activated, changing the stoichiometry of total GATA-1 to PU.1 in the cells, causing the cells to differentiate (Choe *et al*, 2003). We also used an interleukin-3-dependent progenitor cell line with multipotential properties isolated from the fetal liver of a PU.1 null mouse. This cell line expresses an exogenous, conditionally active form of PU.1 (PU.1-ER) that when activated by tamoxifen causes the cells to undergo differentiation into macrophages (Walsh *et al*, 2002). These cells allowed us to study PU.1-mediated repression of the erythroid transcriptional program during commitment of multipotential progenitors to myeloid differentiation. By analyzing the dynamic changes occurring during both erythroid and myeloid differentiation, we were able to correlate events associated with PU.1-mediated repression of GATA-1 and also with derepression of GATA-1.

In undifferentiated MEL-GATA-1-ER blasts, GATA-1, PU.1 and pRb were all found to occupy eight of the nine endogenous GATA-1 binding sites (Figure 1A). The one exception was the promoter of the embryonic globin gene (Ey) where we did not detect appreciable levels of any of the proteins including GATA-1 at either stage (Figure 1A). This finding is consistent with the fact that Ey is not expressed in differentiating MEL cells, which express an adult erythroid program (Wright *et al*, 1983). Apparently, Ey is repressed in these cells by a mechanism independent of GATA-1 and PU.1. As expected, we also did not detect GATA-1 or PU.1 or pRb, in the promoter of the G6PD gene (Figure 1A), a housekeeping gene that is expressed in the cells at both stages, as well as in the promoters of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and MyoD genes (data not shown). Importantly, the occupancy of PU.1 and pRb, but not GATA-1, declined during 17-β-estradiol-induced differentiation (Figure 1A), consistent with derepression of the loci (Supplementary Figure 1). Similar results were obtained with MEL cells before and after DMSO-induced differentiation (data not shown). These results are consistent with a model we proposed previously (Rekhtman *et al*, 2003), in which GATA-1 is poised for transcription in undifferentiated MEL blasts, but its activity is repressed by PU.1, which binds to it and tethers the corepressor pRb. When differentiation is triggered, PU.1 levels decline (Rao *et al*, 1997; Rekhtman *et al*, 1999; Choe *et al*, 2003) (Supplementary Figure 1) and GATA-1 is free to stimulate transcription of its target genes.

Several distinct mechanisms of pRb-mediated transcriptional repression have been described, involving recruitment of complexes containing histone deacetylases (HDACs) (Zhang and Dean, 2001) and HMTs (Nielsen *et al*, 2001b; Vandel *et al*, 2001). We reported previously that repression of GATA-1 by PU.1 and pRb is not reversed by treatment with trichostatin A (Rekhtman *et al*, 2003), suggesting that repression may not involve an HDAC complex. Therefore, we determined the level of two types of methylated histone H3 modifications that are associated with repressed chromatin in mammalian cells, methylated lysine 9 (H3K9Me) and methylated lysine 27 (H3K27Me). We did not detect an appreciable level of H3K27Me near any of the nine GATA-1 sites, nor in the G6PD promoter region (data not shown). However, H3K9Me was readily detected in the region of the eight GATA-1 binding sites that were also found to be occupied by GATA-1, PU.1 and pRb in undifferentiated cells (Figure 1B). The presence of H3K9Me at these loci was detected with four different antibodies, including two antibodies reported to be specific for trimethylated H3K9 (see Materials and methods). H3K9Me was not found in appreciable amounts at the Ey or G6PD promoters, which are not occupied by the three protein factors.

pRb has been shown to interact with complexes containing two different types of HMTs, the mammalian polycomb repressive complex containing the Ezh2 HMT that causes methylation of H3K27 (Dahiya *et al*, 2001) and the Suv39h HMT–HP1α complex that causes methylation of H3K9 (Nielsen *et al*, 2001b; Vandel *et al*, 2001). Finding that the repressed loci contain H3K9Me but not H3K27Me suggested that the PU.1–pRb complex may recruit Suv39h and HP1α to repressed GATA-1 targets. qChIP experiments showed that HP1α is present in the region of each of the eight repressed GATA-1 target sites and not in the promoter regions of the Ey and G6PD genes (Figure 1B). Suv39h was found near six of eight of the repressed sites. The two repressed regions that did not contain Suv39h, the promoters of the EKLF and NFE2 genes, instead were found to be occupied by CtBP (Figure 1C). CtBP is a corepressor protein that interacts with pRb (Meloni *et al*, 1999) and is also associated with HMTs G9a and EuHMT, which also methylate H3K9 (Shi *et al*, 2003). Consistent with the absence of H3K27Me at all nine tested GATA-1 sites, we also did not detect Ezh2 at these sites (data not shown). H3K27Me and Ezh2 were detected at the β-minor globin promoter, indicating that both antibodies can detect these epitopes in MEL cell chromatin.

Derepression of GATA-1 target genes during erythroid differentiation leads to disruption of the repression complex and conversion of H3K9Me to H3K9Ac

As discussed, when MEL-GATA-1-ER cells are induced to differentiate with 17-β-estradiol, PU.1 and pRb leave GATA-1 target genes whereas GATA-1 remains at these sites (Figure 1A). qChIP analysis showed that during differentiation Suv39h and HP1α also leave the GATA-1 target sites that they previously occupied in the undifferentiated MEL blasts (Figure 1B). Similarly, CtBP occupancy within the EKLF and NFE2 promoters also declined (Figure 1C). Likewise, during differentiation, the level of H3K9Me declined (Figure 1B), and concomitantly the level of H3K9Ac was markedly increased (Figure 1D). All of these changes were also observed during DMSO-induced differentiation of MEL cells (data not shown). These results suggest that a shift in the stoichiometry between PU.1 and GATA-1, accomplished by activating

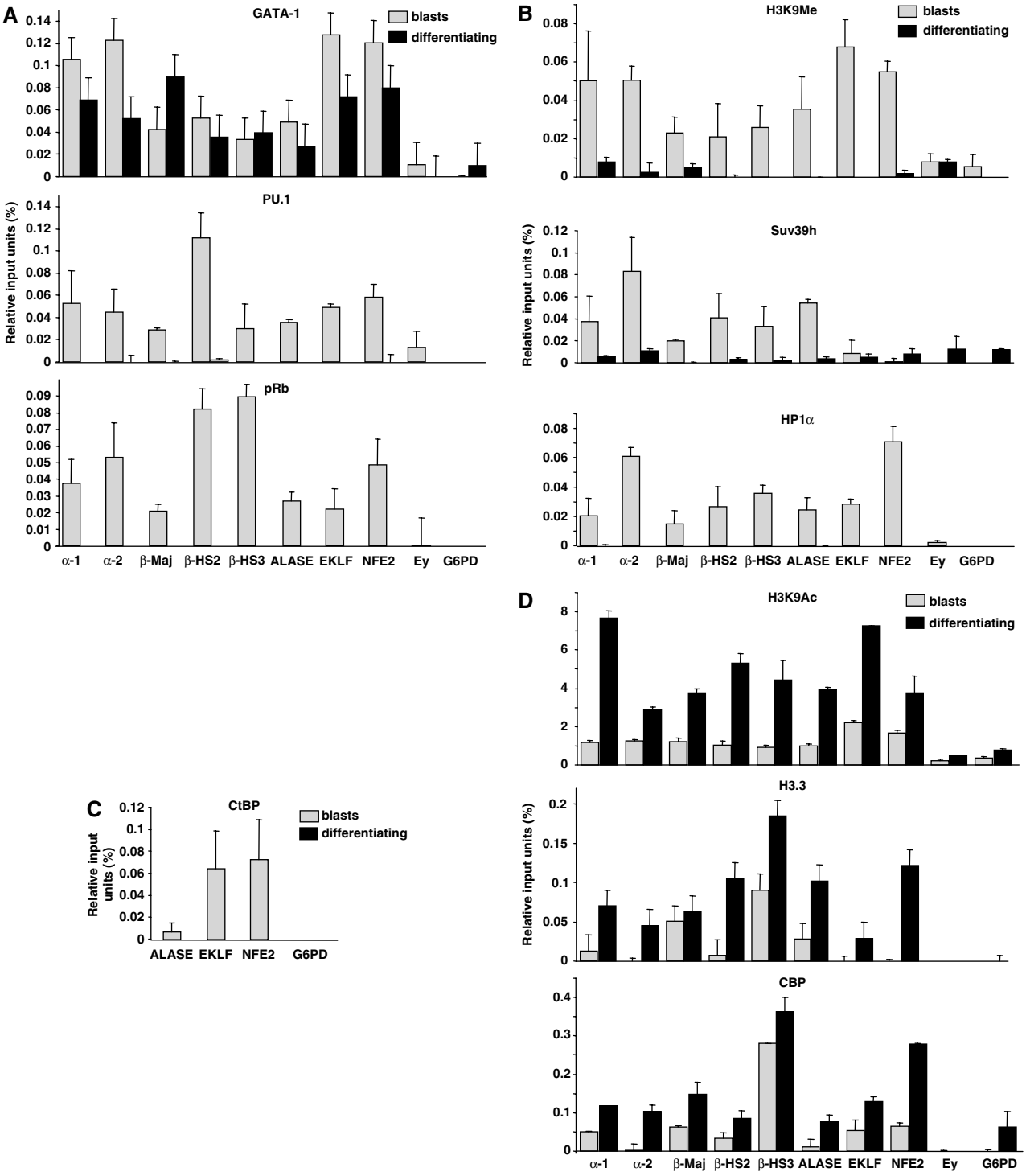

Figure 1 PU.1 represses GATA-1 by recruiting a repression complex consisting of pRb, Suv39h and HP1α, leading to methylation of histone H3 on lysine 9. Erythroid differentiation causes removal of the repression complex leading to conversion of H3K9Me to H3K9Ac and derepression of transcription. (**A–D**) ChIP was performed as described in Materials and methods on crosslinked chromatin from MEL cells expressing a GATA-1-ER fusion protein. The cells were cultured in the absence (gray bars) or presence (black bars) of 10^{-7} M 17-β-estradiol for 3 days. ChIP was carried out with antibodies for the proteins or histone modifications indicated above each panel, as well as control antibodies (see Materials and methods and Supplementary data for a detailed description). The amounts of specific DNA fragments encompassing GATA-1 sites lying within the DNA regions indicated at the bottom of the figures that were present in the immunoprecipitates were quantitated by real-time PCRs. The DNA regions or HS sites containing GATA-1 binding sites at the indicated positions were as follows: α-1 (−189/−192, −252/−255); α-2 (−182/−186, −252/−255); β-major (β-Maj) (−249/−252); β-HS2 (one site, plus one additional ∼0.1 kb upstream and one ∼0.3 kb downstream of the HS2 PCR product); β-HS3 (two sites, plus five additional ∼0.3 kb upstream and ∼0.3 kb downstream of HS3 PCR product); ALASE (−94/−98, −114/−118, plus five additional functional GATA-1 sites within 1.2 kb upstream); EKLF (one site at −65 and additional GATA-1 sites ∼0.8 kb upstream of the PCR product); and p45 NF-E2 (−614/−617, −636/−639, −692/−695, −703/−706) (see Supplementary data for detailed information on the PCR primers used). The bars indicate the percentage of the input DNA fragment in specific immunoprecipitates after subtracting the percentage in immunoprecipitates using control antibody. Error bars indicate the standard deviations of duplicate PCRs. Similar results were obtained in three repeat experiments.

exogenous GATA-1-ER in the cells, triggers events that lead to a decline of PU.1, which in turn causes removal of the repression complex containing pRb, Suv39h (or CtBP) and HP1α from the bound GATA-1. Loss of the repression complex containing the HMT allows for conversion of H3K9Me to H3K9Ac (see below).

Methylation of histone H3 on lysine 9 (H3K9Me) is considered to be a relatively stable histone modification (Peterson and Laniel, 2004). One possible mechanism for reducing H3K9Me after removal of the HMT from the specific loci might involve histone exchange with the replication-independent H3.3 variant (Ahmad and Henikoff, 2002). H3.3 gene expression is induced during MEL cell differentiation (Krimer *et al*, 1993; Supplementary Figure 1). We found by qChIP that its presence increases specifically in the region of the eight GATA-1 target sites as they undergo derepression (Figure 1D). The H3.3 content near these sites during differentiation is much higher than near the G6PD promoter, which is active in MEL cells before and during differentiation. The histone acetyltransferase CBP is known to interact with GATA-1 (Blobel *et al*, 1998). We observed increased occupancy of CBP near each of the derepressed GATA-1 target sites (Figure 1D), suggesting that its recruitment during differentiation may lead to acetylation of the H3K9 residue in nucleosomes in these regions.

PU.1 uses the same mechanism to repress the erythroid transcriptional program during myeloid differentiation

To determine whether PU.1 uses the same mechanism seen in MEL cells to repress the erythroid program during myeloid differentiation, we employed PU.1−/− multipotential progenitor cells that express a PU.1-ER fusion protein. Treating these cells with tamoxifen causes them to undergo differentiation into macrophages (Walsh *et al*, 2002). Prior to differentiation, the cells express mRNA transcripts of the GATA-1 and GATA-2 genes (Walsh *et al*, 2002; Figure 2B), as well as several other erythroid-specific genes, including α- and β-globins, ALAS-E, EKLF and p45NF-E2 (Figure 2B). When the cells are treated with tamoxifen, expression of these genes is rapidly downregulated, whereas the mRNA of Mac-1, a macrophage-specific marker, is rapidly increased. qChIP analysis showed that GATA-1 is present at the same eight GATA-1 target sites detected in undifferentiated MEL blasts (Figure 2A). However, in contrast to the repressed loci in MEL cells in which PU.1 is constitutively expressed, these regions in the progenitor cells that do not express active PU.1 are associated with nucleosomes containing H3K9Ac instead of H3K9Me. Upon activation of PU.1-ER with tamoxifen, these loci become occupied by PU.1, pRb, Suv39h and HP1α. Furthermore, occupancy by GATA-1 continues even though the genes become repressed (Figure 2A). Concomitantly, the levels of H3K9Ac decline and nucleosomes containing H3K9Me become associated with these loci (Figure 2A). These results indicate that PU.1 uses the same mechanism we found in MEL cells, to repress erythroid-specific genes during commitment of multipotential progenitors to myeloid differentiation.

Binding of the repression complex to GATA-1 target sites requires occupancy by GATA-1

Promoter regions and other regulatory sequences contain binding sites for numerous protein factors. Therefore, events occurring in such regions during differentiation may not simply reflect actions associated with a single DNA binding protein. To address this potential complexity, we also studied by ChIP the protein occupancy and histone modifications at a very simple GATA-1 reporter gene integrated into MEL cell chromatin at a defined location. By using the method of recombinase-mediated cassette exchange (RMCE) (Feng *et al*, 1999), we were able to compare the properties of the reporter with that of a reporter containing mutated GATA-1 sites at the same locus. In this way, we could identify events specifically associated with the GATA-1 binding site.

The reporter gene (αD3Luc) is driven by a promoter consisting of a 65 bp DNA sequence from the α-globin promoter containing two closely spaced GATA sequences (GATAaGATA) (Evans and Felsenfeld, 1991). Protein occupancy at this promoter before and during differentiation was compared with that of a companion promoter (αD4) containing a mutated sequence (CTGAaCTGA) that does not bind GATA-1 (Evans and Felsenfeld, 1991), integrated at the same locus. All four components of the repression complex (PU.1, pRb, Suv39h and HP1α) were found, along with GATA-1, at the αD3 promoter in undifferentiated MEL cells (Figure 3). The promoter was also associated with nucleosomes containing H3K9Me. None of these components, or H3K9Me, was found at the mutant αD4 promoter (Figure 3). These results show that the recruitment of all of the components of the repression complex and the resulting methylation of H3K9 are dependent upon an intact GATA-1 binding site in the locus targeted for repression. Consistent with observations described above for endogenous loci, we observed that all of the components of the repression complex, but not GATA-1, leave the αD3 promoter during induced differentiation and that H3K9Me is converted to H3K9Ac (Figure 3). Furthermore, during differentiation, histone H3.3 becomes associated with nucleosomes at αD3, and CBP is recruited to the locus. The appearance of both components during differentiation was dependent upon an intact GATA-1 site (Figure 3). Thus, these results with a model GATA-1-responsive promoter fully support the data obtained with endogenous GATA-1 targets. Because no other erythroid-specific DNA binding proteins are known to bind the αD3 promoter, the absence of the components of the repression complex at the mutant αD4 promoter implies that the repression complex is built upon a platform of GATA-1 bound to its binding site (see Discussion).

The repression complex is localized to the promoter region of GATA-1 target genes

HP1 is involved in position effect variegation (PEV) in *Drosophila*, a phenomenon in which expressed genes are variably silenced when they become located, by for example chromosomal rearrangements, near constitutive heterochromatin. It is thought that silencing occurs by spreading of the silent, heterochromatin structure into the region of the rearranged locus. To determine whether the repression complex containing Suv39h and HP1α is localized near to the GATA-1 site or whether it spreads into the surrounding chromatin, we performed qChIP studies at ∼2 kb intervals over a 35 kb region at the α-globin locus. This locus contains two α-globin genes, α-1 (containing two GATA-1 binding sites: −189/−192, −252/−255) and α-2 (also containing two GATA-1 binding sites: −182/−186, −252/−255), separated

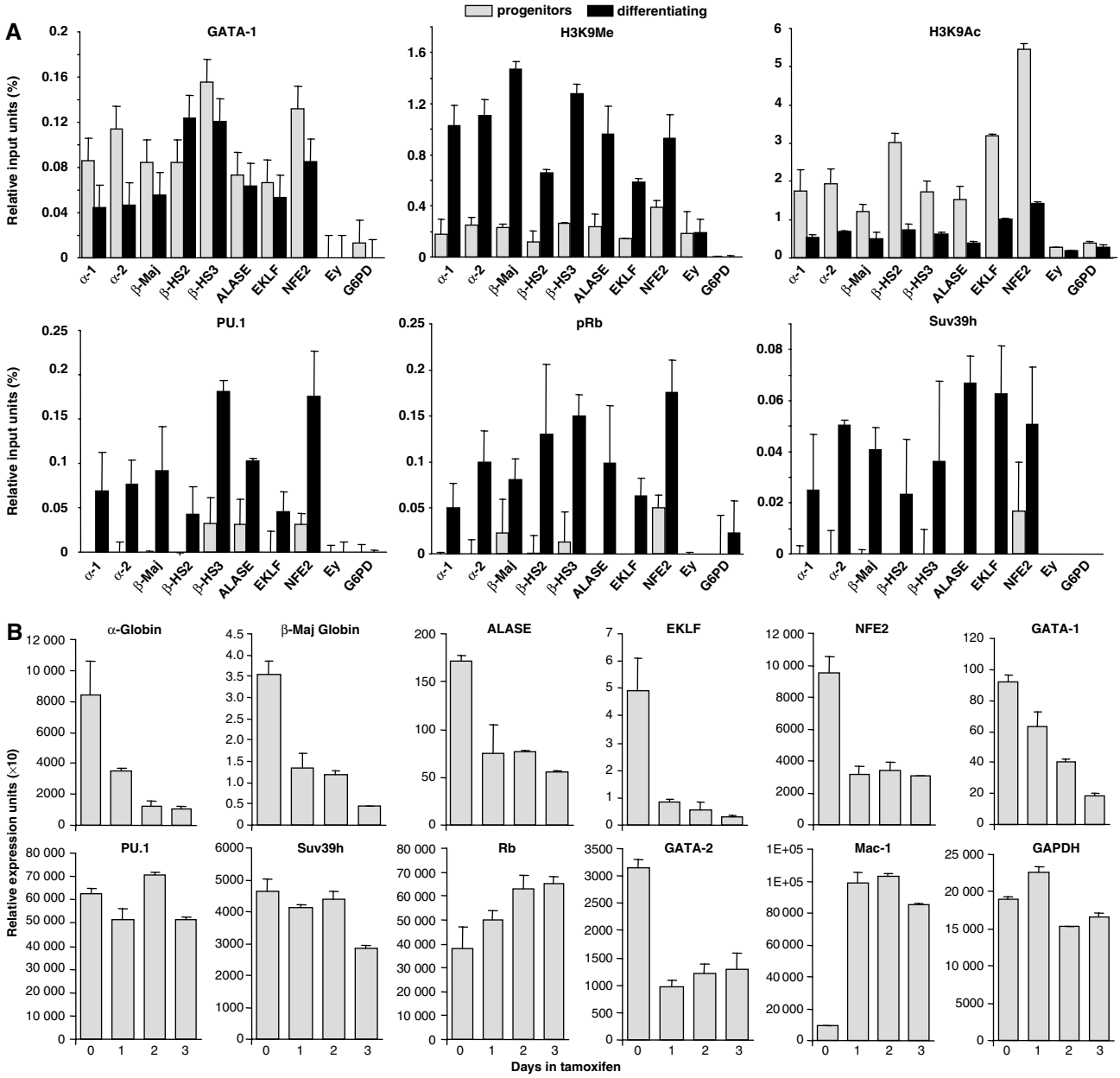

Figure 2 PU.1 recruits a complex consisting of pRb, Suv39h and HP1α leading to methylation of histone H3 on lysine 9 at repressed GATA-1 target genes during myeloid differentiation. (**A**) ChIP was performed on crosslinked chromatin from PU.1−/−, IL-3-dependent cell line expressing a PU.1-ER fusion protein (Walsh *et al*, 2002). The cells were grown in the absence (gray bars) or presence (black bars) of 10^{-7} M tamoxifen for 24 h. Other details are as described in the legend to Figure 1 and Materials and methods. Similar results were obtained in two repeat experiments. (**B**) RT–PCR analysis of mRNA levels during differentiation of PU.1−/−, IL-3-dependent cells expressing PU.1-ER. Cells were cultured in the presence of 100 nM tamoxifen for the indicated number of days. Total RNA was isolated and analyzed for the indicated mRNAs by quantitative RT–PCR as described in Materials and methods and Supplementary data. The relative expression of each of two other housekeeping genes (β2-Microglobulin and HPRT) was also relatively constant during tamoxifen treatment, similar to that of GAPDH.

by about 12 kb. qChIP with the GATA-1 antibody showed it to be highly localized to the two α-globin promoters at this locus (Figure 4A). GATA-1 was neither detected in appreciable amounts in the coding regions of the two genes, nor in the intergenic sequences between and on either side of the two genes. The occupancy of PU.1, pRb and Suv39h showed a very similar, highly localized distribution to the two promoter regions (Figure 4A). As expected based upon the data in Figure 1, occupancy of PU.1, pRb and Suv39h in the promoter regions declines during differentiation, whereas GATA-1 continues to occupy the promoters (Figure 4A). The distribution of HP1α in the region is somewhat broader than that of the other proteins. As expected, it is not present in these regions in the differentiating cells (Figure 4B). The distribution of H3K9Me-containing nucleosomes is even broader. Methylated H3K9 residues were readily detected as much as 4 kb upstream of the α-1 promoter and ∼2 kb on either side of the α-2 promoter. However, they were not detected in appreciable amounts at two positions between the two genes, nor ∼8 kb upstream of the α-1 promoter (in the zeta-globin gene) or ∼6 kb downstream of the α-2 gene. As expected, methylated H3K9 residues were not detected at

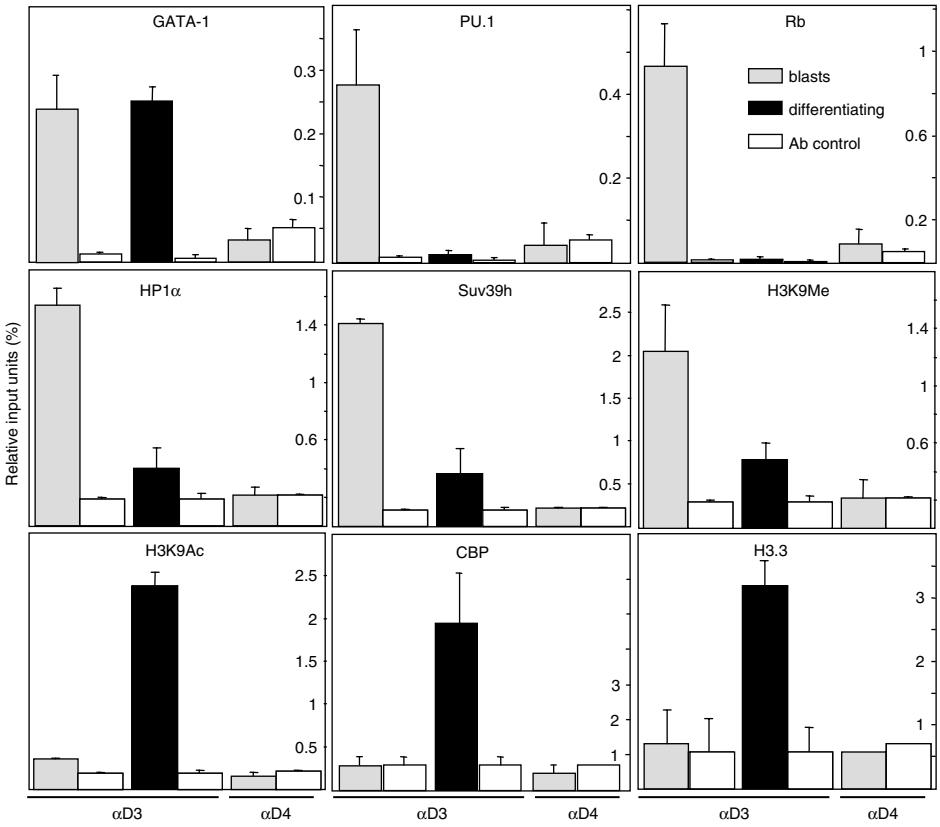

Figure 3 Binding of the repression complex and histone methylation of an exogenous GATA-1 target gene integrated in cellular chromatin requires an intact GATA-1 binding site. A GATA-1-responsive reporter gene (αD3) or a reporter gene with mutated GATA-1 binding sites (αD4) was integrated at the RL5 locus in MEL cells (Feng *et al*, 1999) by RMCE as described in Supplementary data. ChIP was performed on crosslinked chromatin from cells of each type of cell line after culture in the absence (gray bars) or presence (black bars) of 2% DMSO for 3 days. Other details are as described in the legend to Figure 1 and Materials and methods. The bars indicate the uncorrected percentages of the input DNA fragment in specific immunoprecipitates, including the values obtained with the appropriate isotype control antibodies (white bars). Values for αD4 in differentiating cells (not shown) were similar to those for αD4 in blasts. Similar results were obtained in three repeat experiments.

any position in the locus in differentiating cells. Instead, they were replaced by nucleosomes containing acetylated H3K9 (Figure 4B). These regions were also occupied by H3.3 in the differentiating cells (Figure 4B). These results indicate that the repression complex is highly localized, possibly to a single nucleosome at the GATA-1 site in the promoter, whereas the elements typical of heterochromatin structure, HP1α and H3K9Me, occupy a broader but still quite limited region around the GATA-1 site (see Discussion).

Removal of the repression complex, reversal of the histone H3 repressive mark and derepression of GATA-1 target genes by siRNA knockdown of PU.1

PEV silencing of genes by HP1 in *Drosophila* is stably maintained over many cell divisions. To determine whether the effects of the PU.1–Rb–Suv39h–HP1α repression complex on GATA-1 target genes are stable or whether they can be reversed simply by inhibiting PU.1 synthesis, we transfected MEL cells with a double-stranded PU.1-specific siRNA oligonucleotide. Cells treated for 48 h with the PU.1-specific siRNA or a control siRNA were assayed by ChIP for protein occupancy and the state of histone H3K9 modifications at multiple endogenous GATA-1 target sites. PU.1-specific siRNA treatment led to a marked decline of PU.1 mRNA levels within 48 h (Figure 5C). This decline was accompanied by removal of PU.1 from the GATA-1 target sites (Figure 5A). Removal of PU.1 also led to removal of pRb, Suv39h (or CtBP) and HP1α from the sites. In contrast, GATA-1 remained bound to its sites. Concomitant with the leaving of all of the components of the repression complex, methylated H3K9 residues were lost and H3 molecules containing K9Ac were now present at the sites (Figure 5B). These changes were accompanied by the appearance of the H3.3 histone variant and the histone acetyltransferase CBP at the sites. The changes in chromatin structure and protein occupancy were also accompanied by derepression of transcription of α- and β-globin genes, as measured by accumulation of both types of globin pre-mRNAs and their mature mRNAs, as well as accumulation of the mRNAs from several other GATA-1 target genes that were repressed in the cells before siRNA treatment (Figure 5C). The levels of derepression of these genes were comparable to that achieved by treatment for the same time with 2% DMSO, a potent chemical inducer of MEL cell differentiation. These results indicate that the effects of the repression complex organized by PU.1 upon GATA-1, including the histone H3K9Me repressive mark, are reversed upon removal of PU.1.

It was also of interest to determine if the effects of the repression complex could be reversed by inhibiting Suv39h synthesis. Accordingly, we transfected MEL cells with an

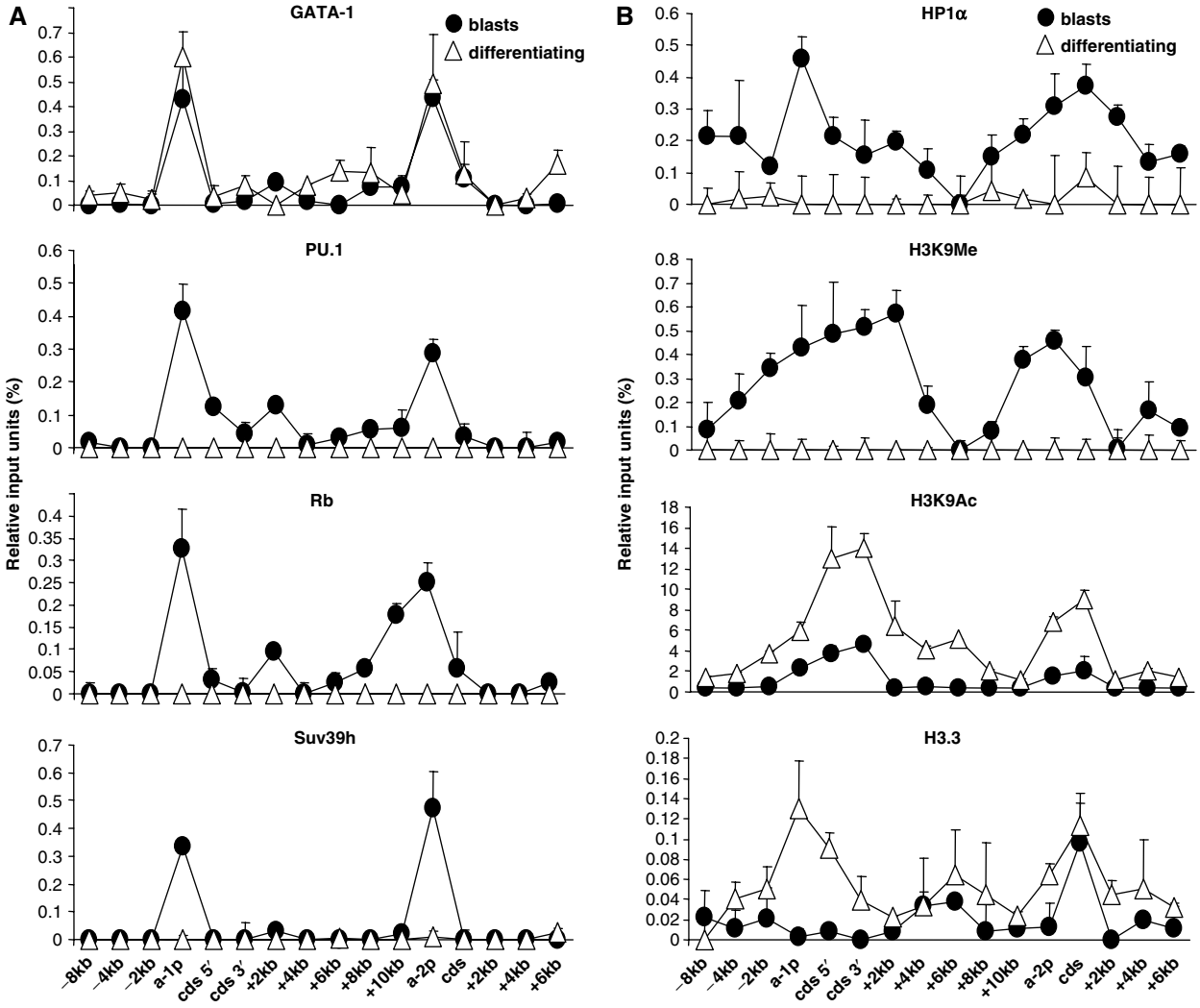

Figure 4 Distribution of the components of the repression complex and histone modifications over the α-globin locus. (**A, B**) ChIP was performed on crosslinked chromatin from MEL cells expressing a GATA-1-ER fusion protein. The cells were cultured in the absence (filled circles) or presence (open triangles) of 10^{-7} M 17-β-estradiol for 3 days. The amounts of 16 different specific DNA fragments (~100 bp in length) indicated at the bottom of the figure that were present in immunoprecipitates were quantitated by real-time PCR. The DNA fragments were localized within the α-1 and α-2 globin gene promoters (α-1p and α-2p, respectively), α-1 coding sequences (cds5′, cds3′), α-2 coding sequences (cds), or at the approximate indicated distances upstream or downstream of α-1p and α-2p. Other details are as described in the legend to Figure 1 and Supplementary data. Similar results were obtained in three repeat experiments.

siRNA oligonucleotide directed against the mRNAs of both Suv39h1 and Suv39h2. Suv39h siRNA treatment led to a decline in Suv39h occupancy at the α-1, α-2, β-major and ALAS-E promoters and at βHS2 (Supplementary Figure 2A). Concomitantly, the level of H3K9Me and occupancy by HP1α at these regions also declined. The specificity of the Suv39h RNA interference is demonstrated by finding that the reductions in H3K9Me and HP1α were not seen at the EKLF and NFE2 promoters where CtBP is present instead of Suv39h in MEL blasts. Reduction of Suv39h, H3K9Me and HP1α at α-1, α-2, β-major, ALAS-E and βHS2 is also accompanied by a marked increase in H3.3-containing nucleosomes. However, Suv39h siRNA treatment led to only a relatively modest increase in globin transcripts (Supplementary Figure 2C), compared with the large increase observed with PU.1 RNA interference (Figure 5C). We think that the failure to achieve full derepression of transcription may be due to the fact that, despite reduced occupancy of Suv39h and HP1α and the decline in the levels of H3K9Me, there is no significant increase in CBP occupancy or the level of H3K9Ac at these loci (Supplementary Figure 2B), whereas both of these parameters increase with PU.1 RNA interference (Figure 5B). Failure to recruit CBP and to acetylate H3 histones might be due to the continued presence of PU.1 and Rb that remain at the loci even after removal of Suv39h (see Discussion).

Discussion

As summarized in Introduction, recent evidence indicates that the physical interaction between PU. 1 and GATA-1 and the resulting mutual antagonism of each factor's transcriptional activity play an important role in the proper development of the myeloid and erythroid compartments of the hematopoietic system. Regulation of the stoichiometry of the two factors in multipotential progenitor cells is likely to

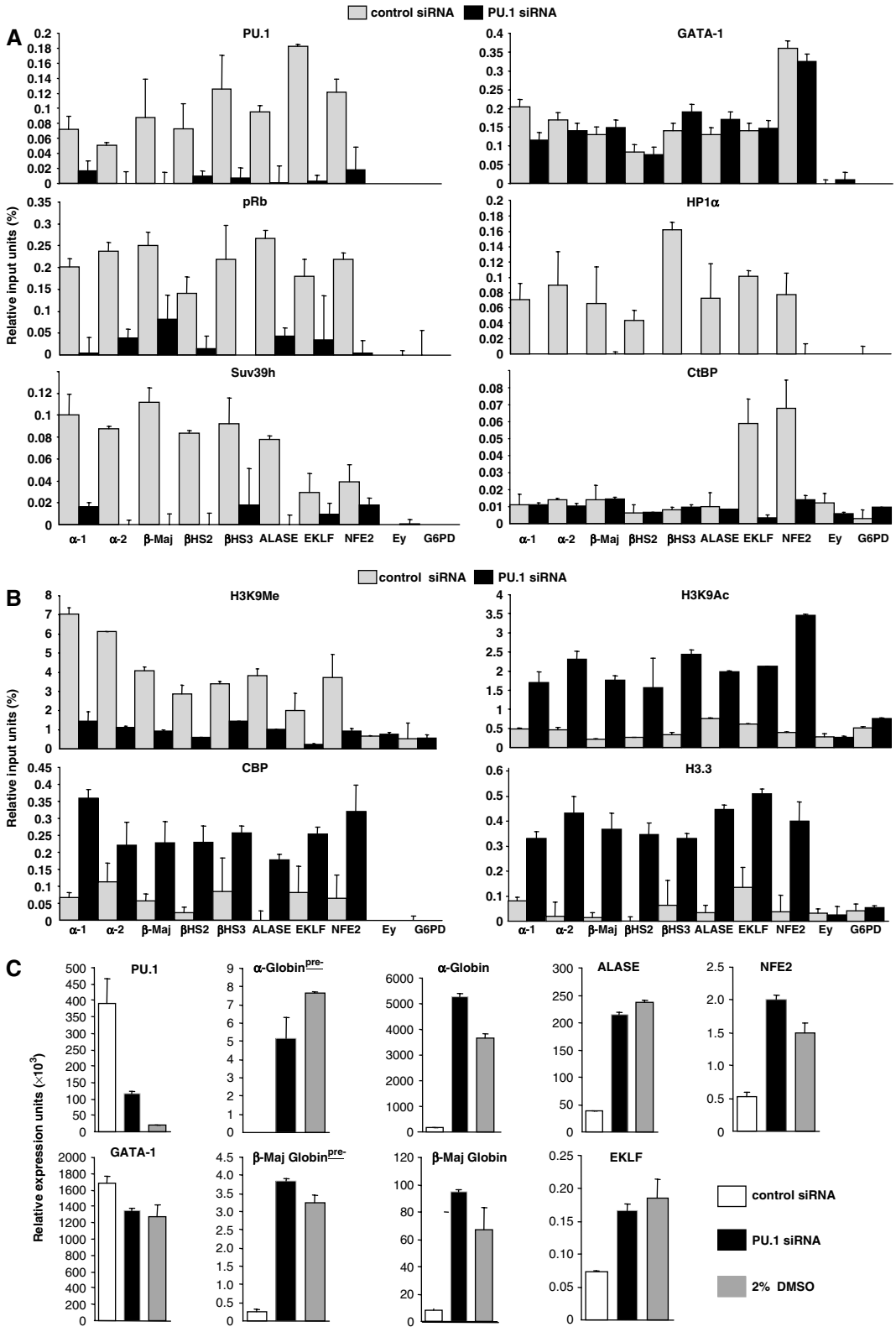

Figure 5 PU.1 RNA interference leads to loss of the repression complex and histone H3 lysine 9 methylation concomitant with derepression of GATA-1 target genes. (**A, B**) ChIP was performed on crosslinked chromatin from MEL cells that were treated for 48 h with a double-stranded, PU.1-specific siRNA (black bars) or control oligonucleotide (white bars) as described in Supplementary data. (**C**) RT–PCR analysis of mRNA levels after siRNA treatment. Total RNA was isolated from the cell cultures described in (A) and (B) prior to crosslinking and analyzed for the indicated mRNAs by quantitative RT–PCR as described in Materials and methods. For comparison, mRNA levels were also measured in an RNA preparation from MEL cells treated with 2% DMSO for 48 h (gray bars). Globin pre-mRNA (pre) levels were measured with pairs of intron-specific PCR primers. The relative expression of each of three housekeeping genes (GAPDH, β2-Microglobulin and HPRT) was approximately the same in the three samples (data not shown).

be a key determinant in lineage commitment decisions. The mutual antagonism of the two factors may also be involved in shutting down the alternative factor's transcriptional program as multipotential progenitors undergo maturation into committed myeloid or erythroid cells. The results reported in Figure 2 provide some of the first direct support for this concept. The stoichiometry of PU.1 and GATA-1 can also be very significant in leukemia. Activation of PU.1 expression in erythroblasts is a critical event in development of murine erythroleukemia and rebalancing of PU.1–GATA-1 levels by expression of exogenous GATA-1 in the erythroleukemia cells leads to terminal erythroid differentiation and loss of tumorigenicity (Choe *et al*, 2003).

In the current work, we used both multipotential progenitor cells and erythroleukemia cells to investigate the mechanisms by which PU.1 represses GATA-1 target genes. Our results show that PU.1 represses GATA-1 by binding to it on DNA and recruiting a repression complex consisting of pRb, HP1α and, in most loci examined, the HMT Suv39h. This leads to methylation of lysine 9 in histone H3 in nucleosomes in the vicinity of the GATA-1 binding site (Figure 6A). The model depicted in Figure 6A is strongly supported by the studies carried out with a simple GATA-1-responsive reporter gene integrated in cellular chromatin by RMCE (Figure 3). It is also supported by studies in which PU.1 was knocked down by siRNA (Figure 5). The former studies in which reporter genes with either functional or mutant GATA-1 sites were compared prove that the repression complex is bound on a platform of GATA-1 bound to its binding site. The latter studies show that PU.1 is also essential for organizing the repression complex and histone methylation at repressed GATA-1 target genes. Under circumstances in which PU.1 levels are much lower than those of GATA-1, for example during erythroid differentiation or in PU.1−/− progenitors, the repression complex is not bound to GATA-1, lysine 9 in histone H3 is acetylated and the gene is transcribed (Figure 6B). Our evidence indicates acetylation of H3 may be brought about by recruitment of CBP, a histone acetyltransferase that has been shown to interact with GATA-1 and to stimulate its transcriptional activity (Blobel *et al*, 1998; Hung *et al*, 1999). Additionally, we show that the repressed state can be reversed, as for example during PU.1 knockdown or erythroid differentiation of MEL cells, whereupon the methylation mark on H3K9 is lost and H3 molecules acetylated on lysine 9 are found at the derepressed loci. Our data indicate that this change involves incorporation of the variant core histone H3.3 into the nucleosomes (Figure 6B).

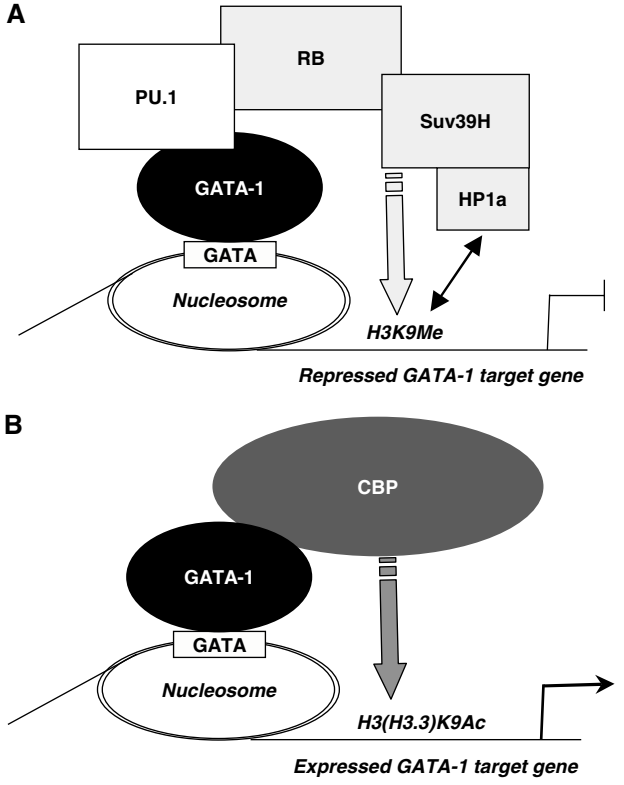

Figure 6 Model of PU.1-mediated repression of GATA-1 and derepression upon downregulation of PU.1 levels. The figures indicate the proteins and histone modifications present near a GATA-1 binding site in chromatin when PU.1 levels are sufficient to cause repression (**A**) and when they are reduced, leading to derepression (**B**). Protein–protein interactions established by previously published data are indicated by overlaps between two objects. The binding of HP1α to H3K9Me (arrow) and the ability of Suv39h and CBP to cause methylation and acetylation (broken arrows), respectively, are also established by references cited in the text. See text for details.

In normal development, the model depicted in Figure 6A is most likely applicable at an early stage during myeloid commitment of CMPs. These cells express a number of erythroid-specific genes at a low level, including GATA-1 (Akashi *et al*, 2000; also see Figure 2B). Because GATA-1 positively regulates its own expression (McDevitt *et al*, 1997), the effect of PU.1-mediated repression in such cells will be amplified, leading to a rapid shut-off of the erythroid program during myeloid commitment. It should be noted that PU.1 also interacts strongly with GATA-2 (Rekhtman *et al*, 1999). GATA-2 is thought to regulate the proliferation and differentiation of very immature hematopoietic cells (Heyworth *et al*, 1999). Thus, it is also possible that the model depicted in Figure 6A, with GATA-2 substituted for GATA-1, is applicable to events occurring in very early hematopoietic cells, in which PU.1 may oppose GATA-2 function and thereby promote maturation of such cells to the next stage.

Methylation of histone H3 on lysine 9 or lysine 27 is generally associated with transcriptionally inactive chromatin states. We did not detect H3K27Me at any of the repressed loci using antibodies from two different sources. In contrast, we readily detected H3K9Me at these loci, with four different antibodies, including two antibodies reported to be specific for trimethylated H3K9. Three groups of HMTs capable of methylating H3K9 have been characterized to date, including two very closely related HMTs Suv39h1 (Rea *et al*, 2000) and Suv39h2 (O'Carroll *et al*, 2000), G9a (Tachibana *et al*, 2001) and G9a-related protein (GLP, also known as EuHMT1 in humans; Ogawa *et al*, 2002) and SETDB1/ESET (Yang *et al*, 2002; Schultz *et al*, 2002). Because mice lacking either both Suv39h HMTs, G9a or ESet exhibit quite different developmental abnormalities, it has been suggested that the three groups of HMTs play distinct roles in regulating chromatin structure during development (Peters *et al*, 2001; Tachibana *et al*, 2002; Dodge *et al*, 2004). For example, it has been suggested that G9a and GLP together play a dominant role in regulating developmental gene expression by causing

mono- and dimethylation of H3K9 in euchromatin (Tachibana *et al*, 2002, 2005), whereas Suv39h1 and Suv39h2 together are primarily involved in trimethylation of constitutive heterochromatin in pericentric regions (Peters *et al*, 2003; Rice *et al*, 2003). However, our results suggest that the functions of the two Suv39h HMTs and trimethylation of H3K9 are not confined to constitutive heterochromatin and that they very likely have an important role in regulating gene expression in euchromatin during hematopoietic development.

Our results also implicate HP1α in PU.1-mediated repression of GATA-1. HP1α is known to interact with the Suv39h HMTs (Aagaard *et al*, 1999). It also recognizes specifically trimethylated H3K9 residues in pericentric heterochromatin, where it has been suggested to form a stable macromolecular network that mediates chromatin compaction over large regions of the chromosome (Eissenberg and Elgin, 2000). However, our studies of the distribution of HP1α and methylated H3K9 residues across the α-globin locus (Figure 4B) indicate that they are confined to just a few kilobases on both sides of the GATA-1 sites where the repression complex is bound. Thus, at the α-globin locus, we did not observe the same type of spreading of HP1α and methylated H3K9 that appears to occur in heterochromatin, where it is thought to be responsible for silencing genes that become positioned near heterochromatin through chromosomal translocations. Still, the distributions of HP1α and methylated H3K9 are broader than the highly localized distribution of Suv39h (and GATA-1, PU.1 and pRb; compare Figures 4A and B), suggesting a possible 'tracking' type of process in which the Suv39h enzyme moves along the chromatin, producing methylated H3K9 residues that are then stably bound by HP1α.

The methyl groups on lysine 9 of H3 are considered to be stable modifications compared with some other histone modifications (Berger, 2002; Kouzarides, 2002). It has been suggested that trimethylation of lysine 9 in H3 could permanently mark a gene for inactivity, as might occur for example during commitment to terminal differentiation. However, we found that the amounts of H3K9Me present in the promoter regions of repressed GATA-1 target genes are greatly reduced after treating MEL cells with a PU.1 siRNA oligonucleotide or a differentiation-inducing agent (17-β-estradiol or DMSO) for only 24 h. An alternative to metabolism of the methyl groups on H3K9 is histone exchange. Recent studies in *Drosophila* indicate that the histone H3 variant, H3.3, is rapidly incorporated into chromatin upon transcriptional induction (Schwartz and Ahmad, 2005, and references therein). H3.3 is a replication-independent variant that is synthesized throughout the cell cycle in mammalian cells (Ahmad and Henikoff, 2002). It is induced during MEL cell differentiation (Krimer *et al*, 1993; Supplementary Figure 1). We found that changing the stoichiometry of PU.1 and GATA-1, either by PU.1 RNA interference or differentiation induction, leads to deposition of H3.3 specifically at GATA-1 target genes and not at other active genes (Figures 1D, 3 and 5B), coincident with loss of H3K9Me and acquisition of H3K9Ac. These results support the concept that repressed genes that have been marked by methylation of H3K9 can indeed be reactivated by removal of the repression complex followed by histone exchange.

The precise mechanism by which the repression complex recruited by PU.1 inhibits transcription of GATA-1 target genes remains to be determined. Our studies of the consequences of interfering with Suv39h synthesis suggest that the mechanism may involve several steps. We found that reducing the occupancy of Suv39h at repressed loci caused the expected decrease in the level of H3K9Me and HP1α occupancy at these loci, but unlike interfering with PU.1 synthesis, CBP was not recruited to the loci, the level of H3K9Ac was not increased and the gene transcription was not fully derepressed. We think that the continued presence of PU.1 and/or Rb at these loci (Supplementary Figure 2B), even after removal of Suv39h (and the decline in the level of H3K9Me and loss of HP1α), prevents CBP recruitment and CBP-mediated acetylation of nucleosomes. PU.1 has also been reported to inhibit the activity of CBP in acetylating histones (Hong *et al*, 2002). Thus, PU.1 and Rb may repress GATA-1 on its target genes by simultaneously recruiting a complex that creates a repressive chromatin structure and by blocking an activity needed to produce an active chromatin structure.

Materials and methods

Cell culture

MEL cells (clone DS19) or MEL cells expressing a GATA-1-ER fusion protein were cultured and induced to differentiate with either 2% DMSO or 10^{-7} M 17-β-estradiol, as described previously (Choe *et al*, 2003). PU.1−/− cells, IL-3-dependent progenitor cells expressing a PU.1-ER fusion protein, were cultured and induced to differentiate with 10^{-7} M tamoxifen as described (Walsh *et al*, 2002). MEL cells containing an integrated copy of either the αD3 Luc or αD4 Luc reporter gene (Evans and Felsenfeld, 1991) at the RL5 locus (Feng *et al*, 1999) were prepared by RMCE, as described in Supplementary data. siRNA interference with PU.1 synthesis was carried out as described in Supplementary data.

qChIP

Chromatin crosslinked with formaldehyde was prepared essentially as described (Takahashi *et al*, 2000), with a few modifications described previously (Rekhtman *et al*, 2003). In some experiments, chromatin was sheared by continuous sonication for 8 min in a dry ice ethanol-cooling bath, instead of using 16 30 s pulses. DNA fragments 200–400 bp in length were obtained by both methods. Immunoprecipitations and determinations of the amounts of specific DNA fragments in immunoprecipitates were performed as described in Supplementary data.

mRNA expression analysis

Total RNA was isolated from approximately 10^6 cells with Trizol (Invitrogen) according to the manufacturer's instructions. The RNA was further purified by treatment with 5 U of DNase I (Promega) at room temperature for 30 min, followed by re-isolation with Trizol. cDNA was synthesized and analyzed for the content of specific mRNA sequences by qPCR as described in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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