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Crosstalk between histone modifications maintains the developmental pattern of gene expression on a tissue-specific locus

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Genome wide studies have provided a wealth of information related to histone modifications. Particular modifications, which can encompass both broad and discrete regions, are associated with certain genomic elements and gene expression status. Here we focus on how studies on the β-globin gene cluster can complement the genome wide effort through the thorough dissection of histone modifying protein crosstalk. The β-globin locus serves as a model system to study both regulation of gene expression driven at a distance by enhancers and mechanisms of developmental switching of clustered genes. We investigate recent studies, which uncover that histone methyltransferases, recruited at the β-globin enhancer, control gene expression by long range propagation on chromatin. Specifically, we focus on how seemingly antagonistic complexes, such as those including MLL2, G9a and UTX, can cooperate to functionally regulate developmentally controlled gene expression. Finally, we speculate on the mechanisms of chromatin modifying complex propagation on genomic domains.

Key words: β-globin, histone methyltransferases, histone demethylases, NF-E2, spreading, locus control region, chromatin, transcription, G9a, MLL2, UTX

Abbreviations: HMT, histone methyltransferase; H3K4me1/2/3, mono-, di-, or tri-methylated histone H3 at lysine 4; H3K9me2/3, di- or tri-methylated histone H3 at lysine 9; H3K27me2/3, di- or tri-methylated histone H3 at lysine 27; H3K9Ac, acetylated histone H3 at lysine 9; H4R3me2, dimethylated histone H4 at arginine 3; LCR, locus control region; HS2, hypersensitive site 2; LOCKs, large organized chromatin K9 modifications; NF-E2, transcription factor composed of MafK and NF-E2p45; MLL2, mixed lineage leukemia 2; PRMT1/5, protein arginine methyltransferase 1/5; DNMT3a, DNA methyltransferase 3a; Pol II, RNA polymerase II; ncRNA, non-coding RNA; GLP, G9a-like protein

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Genome Wide Studies Reveal Novel Aspects of Transcriptional Regulation

Post-translational modifications of histones are well established to play important roles in the regulation of gene expression.[1,2] Recent, systematic, genome-wide mapping of multiple histone modifications, carried out in a number of cell types and across different developmental stages, has been instrumental in revealing novel features of transcriptional regulation (reviewed in refs. 3 and 4). For example, using a previously established "chromatin signature" for enhancers, which is based on the simultaneous enrichment of monomethylated histone H3 at lysine 4 (H3K4me1) and depletion of trimethylated histone H3 at lysine 4 (H3K4me3),[5] Heintzman and colleagues have identified a surprisingly high number of novel putative enhancers in the human genome (e.g., 36,589 active enhancers in HeLa cells).[6] Strikingly, unlike the chromatin state at promoters, which remains similar across different cell types, histone modifications at enhancers appear highly specific across cell lines that represent distinct lineages and stages of differentiation and they correlate strongly with cell-type specific gene expression programs. Presumably, these enhancers were not recognized previously owing to their distal location from gene promoters: this study showed that the vast majority of enhancers are located several kilobases away from the 5' end of genes, either within introns or intergenic regions. Associating these distal elements to the gene(s) they functionally regulate presents a daunting task, however the wide representation and cell-type specificity of enhancers in modulating gene expression illustrates their functional importance.

Not all histone post-translational modifications have a predictive value. However, in combination with other markers such as transcription factor binding or DNAse I hypersensitivity, they can be used for the identification of functional elements within the genome.[7]

Indeed, specific modifications correlate well with particular regions of active, poised or repressed genes. For example, H3K4me3 peaks within a 1 kb region immediately downstream of transcription start site at both active and poised genes.[8] Trimethylation of histone H3 at lysine 36 (H3K36me3) on the other hand is a strong marker of exons within the coding regions of active genes.[9]

In addition to these local modifications within genes, genome-wide studies have led to the identification of large domains (ranging anywhere from 10 kilobases to megabases) with chromatin markings extending beyond single genes. Some of these domains are consistent with previously defined regions containing repetitive sequences such as centromeres or telomeres.[10] However, others extend across multiple genes that are sometimes arranged in clusters. These gene-rich domains of continuous histone modifications can consist of repressive histones marks (e.g., trimethylation of histone H3 at lysine 27 (H3K27me3) at Hox clusters[11]), active histone marks (e.g., H3K4me2/3 at Hox clusters[12,13]) as well as marks found on and correlated with both repressed and active genes[14-16] (e.g., dimethylation of histone H3 at lysine 9 (H3K9me2)[17]). Interestingly, on Hox genes clusters, the repressive H3K27me3 and activating H3K4me3 domains are dynamically regulated during development by the antagonizing actions of Polycomb (PcG) and Trithorax (TrxG) group enzymes, respectively.[18,19] Furthermore, large domains of continuous H3K9me2 modification (termed the Large Organized Chromatin K9 modifications or LOCKs) are established by the H3K9 methyltransferase G9a (KMT1C), which are mainly cell-specific and increase with differentiation.[17] Importantly, large modified histone domains may represent the detectable output of a highly dynamic competition between methyltransferases and demethylases that continuously compete to either establish or remove specific histone marks. The co-existence of punctate H3K9me3 marks within active chromatin domains[20] or punctate H3K4me3 marks[21] within a large H3K9me2 domain[22] may reflect a snapshot of the dynamic nature of this process (reviewed in ref. 4).

Resolving the mechanism through which histone marks modulate transcription of differentiation-specific genes is an important challenge, which is further complicated by the co-existence of multiple levels of regulation. These include a structural aspect (i.e., specific marks can alter the overall accessibility of chromatin), a "histone code" aspect (i.e., crosstalk between enzymes that write the marks and those that read them) and a "memory" aspect (i.e., the still contentious possibility that some histone marks are established by inheritance from a progenitor cell[23]). We propose that this mechanistic complexity can most efficiently be resolved by examining the details of regulation at specific genomic loci.

The β-Globin Locus Serves as a Paradigm for the Regulation of Tissue-Specific Gene Expression

For the past few decades, the β-globin locus, which contains clustered β-*like globin* genes expressed at distinct stages of development and only in erythroid cells, has served as a paradigm for the regulation of both spatial and temporal gene expression.[24,25] In the mouse, the first β-*like globin* gene to be expressed in primitive erythroid cells of the yolk sac blood islands is β^{h1} starting at approximately E7.5. When these primitive cells mature in the blood stream, a "maturational" switch occurs whereby β^{h1} is progressively silenced and replaced by the *Ey globin* gene, which becomes predominantly expressed at E10.5.[26] Then starting at E11.5, there is a "developmental" switch when definitive erythroid cells are produced, first from the fetal liver and later from the bone marrow. In contrast to their primitive counterparts, definitive erythroid cells express only the adult β^{maj} and to a lesser extent β^{min} *globin* genes. Therefore, expression of the β-*globin* genes is a highly regulated process, both spatially and temporally.

Many aspects of transcriptional regulation revealed by the recent, aforementioned genome-wide studies are recapitulated on the β-globin locus in differentiating adult erythroid cells. This includes a large, differentiation-dependent, G9a-mediated domain of H3K9me2 covering the ~100 kb locus[22] which coexists with punctate H3K4me3 and acetylated histone H3 at lysine 9 (H3K9ac) marks localized at the 5'coding regions of the active β-*globin* genes[21] (**Fig. 1**). There are also "medium-sized" 10 kb domains comprised of H3K4me2 and H3/H4ac that encompass the active β-*globin* genes.[27] In addition, there is a well-characterized distal enhancer, called HS2,[28] the H3K4me1 enhancer signature of which is established early on in multipotent hematopoietic progenitors.[29] HS2 is part of the Locus Control Region (LCR), an upstream 20–30 kb regulatory sequence, which is required for high-level expression of the β-*globin* genes in differentiated erythroid cells[30] and works notably by increasing the transition from transcription initiation to elongation.[31] Finally, the active β-*globin* genes co-localize with the LCR in the three-dimensional nuclear space of erythroid cells,[32] and associate with active transcription factories[33] and other active genes.[34] This observation suggests an important role for genome architecture in the regulation of gene expression.

It is currently unclear how the developmental program of gene expression is achieved on this locus. Initial proposals included the β-*globin* genes competing for binding to the LCR, with the gene closest to the LCR being activated in priority; this model, however, is difficult to reconcile with the recent finding that the murine β-*like globin* genes are not organized in their temporal order of expression during development[26] (**Fig. 1**). Recent studies are uncovering the role of epigenetics in modulating developmentally regulated gene expression and deciphering this role will be facilitated through studies of extensively characterized loci such as the β-globin locus.

Epigenetic Mechanisms Regulate the Developmental Specificity of β-Globin Gene Expression

During differentiation of erythroid progenitors from the definitive lineage, the "adult" β-*globin* gene β^{maj} is activated to extremely high levels (at least 100 fold activation)[35] while the embryonic genes *Ey* and β^{h1} are maintained in a repressed state. Here we discuss recent experiments that have revealed a complex crosstalk of

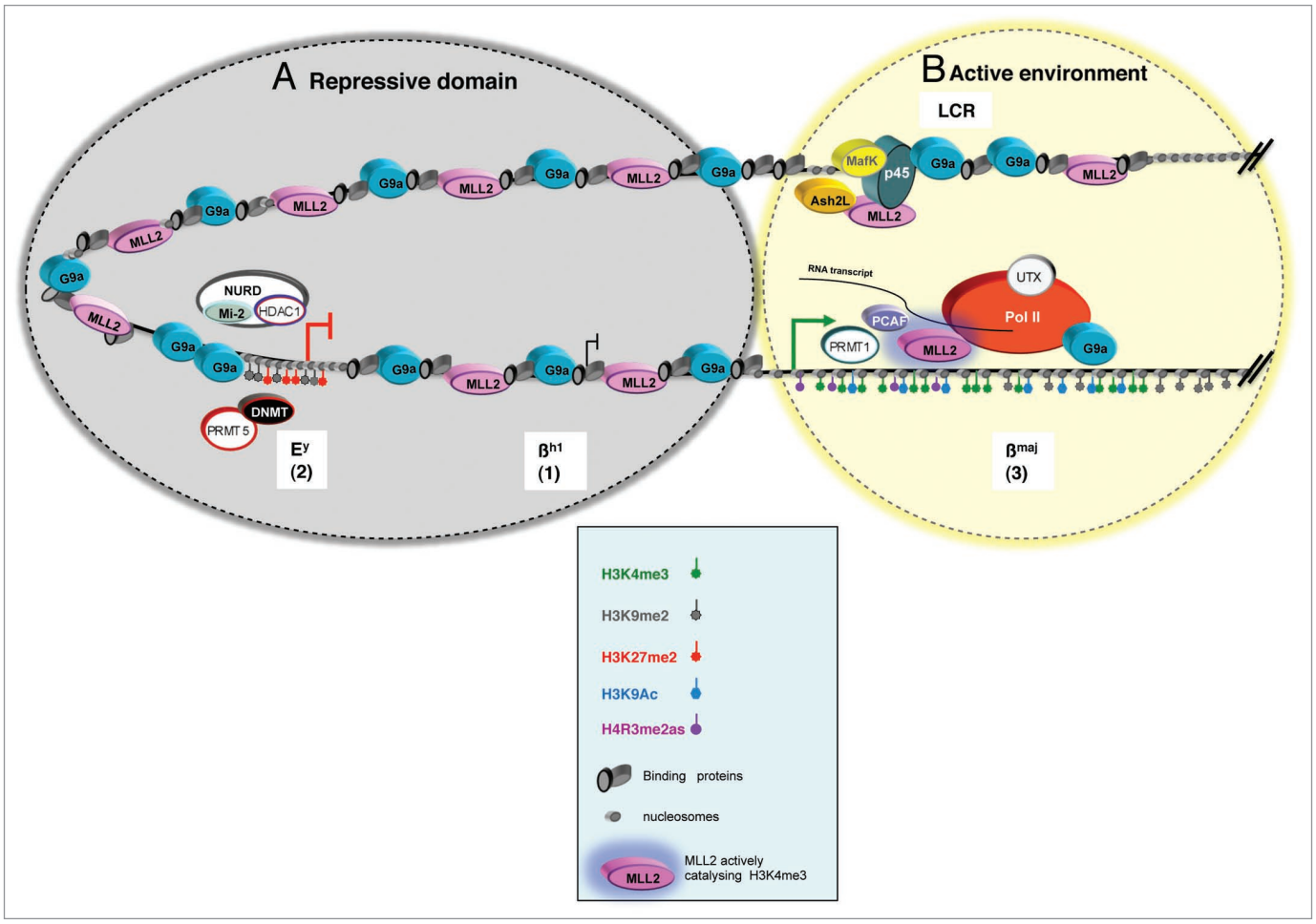

Figure 1. Model for epigenetic control of β-*globin* locus transcriptional regulation in adult differentiating erythroid cells. NF-E2 recruits MLL2 and G9a histone methyltransferases to the HS2 region of the LCR enhancer. MLL2 and G9a spread the length of the locus to modulate gene expression. (A) Repressive domain: A G9a dependent H3K9me2 domain occurs over the many tested regions of the β-globin locus in differentiating definitive erythroid cells. In addition to H3K9me2, G9a also catalyses H3K27me2, which together maintain embryonic (E^y) globin in a repressed state. This repression may be facilitated by chromatin remodeling and histone deacetylase activity via NuRD complex recruitment as well as *symmetrical* H4R3me2 catalysed by PRMT5, which recruits DNMT3a. MLL2 is also present over many tested regions of the locus but in the repressive domain no H3K4me3 is detected, indicating that MLL2 here is catalytically inactive. Embryonic β^{h1} is also repressed, however G9a alone is not sufficient to mediate repression of this gene. (B) Active environment: Looping between the LCR and the β^{maj}-*globin* gene promoter occurs in adult cells to activate gene expression to very high levels. MLL2 is catalytically active at this region, with high levels of H3K4me3 present in the 5' coding region, and is required for high level β^{maj}-*globin* transcription. PRMT1 catalyses *asymmetric* H4R3me2 which facilitates GCN5/PCAF catalysis of H3K9Ac. UTX actively removes repressive H3K27me2 marks to assist transcriptional activation. G9a is required for high level β^{maj}-*globin* gene expression through mechanisms that involve binding to the Pol II, recuitment and/or stabilization of Pol II and preinitiation complex formation. G9a is also responsible for H3K9me2 at the 3' coding region of β^{maj}-*globin*. Numbers below the gene indicate the order of expression during development.

histone modifications involving both large domains of continuous modifications and punctate marks that collaborate to permit maintenance of the developmental pattern of gene expression in adult erythroid cells.

The β-globin locus contains multiple binding sites for transcription factors, both at the LCR and at the promoters of the β-*globin* genes.[28] At the LCR, binding of transcription factors results in the formation of DNase I hypersensitive sites (HSs). Many transcription factors, both hematopoietic-specific (e.g., GATA1, EKLF, TAL1, NF-E2) and ubiquitously expressed (e.g., SP1, USF), have been shown to bind specifically to these sites and to recruit cofactors with chromatin-remodeling or histone-modifying activities (reviewed in ref. 36). Among them, the basic leucine zipper (bZIP) heterodimer NF-E2 (composed of MafK and NF-E2p45 subunits) forms on the β-globin enhancer HS2 via an exchange of MafK heterodimerization partner from the repressor Bach1 to the activator NF-E2p45.[37] This exchange is triggered upon erythroid differentiation by a combination of heme-mediated degradation of Bach1 via the ubiquitin-proteasome pathway[38,39] and increased accumulation of NF-E2p45 protein through inhibition of the JNK pathway, thereby blocking NF-E2p45 proteasomal degradation.[40]

Our recent studies have shown that this assembly of the NF-E2 activator complex permits the recruitment of two histone methyltransferases (HMTs) MLL2 (KMT2D)[21,41] and G9a (KMT1C)[22] to the HS2 site. Interestingly, in contrast

to NF-E2p45, which remains localized mostly at the LCR during differentiation, the MLL2 and G9a enzymes spread over the length of the locus, covering all the regions we have tested including both the embryonic and adult β-*globin* genes[21,22] (**Fig. 1**). While MLL2 and G9a display competing HMT activities, the G9a-dependent H3K9me2 mark appears to represent the dominant state, as indicated from both an in vitro methyltransferase assay (using unmodified histone H3) and in vivo on the β-globin locus.[21,22] Indeed in differentiated cells, despite MLL2 presence across the locus, MLL2-dependent H3K4me3 marks are restricted to the 5' coding region of the transcribed β^{maj} gene (**Fig. 1**). The rest of the β-globin locus lies within a large G9a-dependent H3K9me2 domain. This domain has a repressive function since knockdown of G9a, which results in a concomitant decrease in H3K9me2 mark, leads to reactivation of the embryonic gene E^{y} in differentiating erythroid cells.[22] Therefore, this H3K9me2 domain contributes to maintaining the E^{y} gene in a repressed state upon differentiation of erythroid cells of the definitive lineage.[22] Importantly, this domain is conserved in human adult cells on the embryonic and fetal β-*globin* genes.[42] Interestingly, this H3K9me2 domain on the β-globin locus might be akin to the previously mentioned LOCKs[17], in that it is G9a-dependent, increases during differentiation and correlates with gene repression.

Further insight into this G9a-dependent repressive domain comes from rescue experiments using a methyltransferase-defective G9a mutant, which confirmed that the repressive function of G9a is dependent upon its methyltransferase activity. Our findings also indicate that G9a is able to mediate H3K27me2 in addition to H3K9me2 on the E^{y} *globin* gene.[22] Accordingly, we found that the bulk levels of the repressive H3K27me2 histone modification are reduced upon G9a knockdown in erythroid cells. In addition, this mark, heavily enriched on the E^{y} *globin* gene during differentiation, is reduced with G9a knockdown. This suggests that the H3K27me2 mark is also involved in maintaining E^{y} *globin* in a repressed state. Consistent with this finding,[22] G9a can methylate H3K27 in vitro.[43,44] Furthermore, knocking down the H3K27 methyltransferases EZH2 and EZH1 does not lead to reactivation of E^{y} upon induction of erythroid differentiation, indicating that these methyltransferases are not involved in the repression of E^{y} *globin*.[22]

The HMT PRMT5, which mediates the symmetric methylation of histone H4 at arginine 3 (H4R3me2s), has been shown to maintain the human fetal γ-*globin* gene in a repressed state in adult human erythrocytes.[45] These authors have shown that PRMT5 interacts directly with the DNA methyltransferase DNMT3A and that the PRMT5-catalysed H4R3me2s mark facilitates DNMT3A binding to the human fetal γ-*globin* gene promoter, in turn leading to DNA methylation and repression of this gene in adult erythrocytes. It is possible that a similar PRMT5/DNMT3A-mediated process is involved in the formation of DNA hypermethylated domains at the embryonic genes in definitive murine erythroid cells.[46] In addition to histone methylation, nucleosome remodeling and histone deacetylation are likely important for maintaining the mouse embryonic gene in a repressed state, in a similar fashion to the repression of human fetal γ-*globin* gene, where the transcription factors BCL11A,[47] GATA-1, FOG-1,[47,48] and Ikaros[49] are involved in silencing γ-*globin* through a mechanism linked to NuRD complex component (Mi-2 and HDAC1) recruitment to the γ-*globin* gene promoters.[48,49] Notably, in mouse erythroid cells BCL11A forms a complex with all NuRD components, suggesting that this mechanism might be conserved between mouse and human adult erythroid cells.[47] While additional repressors of the mouse embryonic gene E^{y} have been identified (e.g., SOX6,[50] and DRED[51]), it is not known whether they are involved in recruiting histone or DNA modifying enzymes to the β-globin locus.

Interestingly, even though E^{y} is repressed in definitive progenitor cells, additional mechanisms seem to be required to reinforce this repression when the adult β-*globin* genes become highly activated. Indeed, G9a-dependent H3K9me2 and H3K27me2 marks increase during differentiation of definitive erythroid progenitors.[22] It would be interesting to determine whether H3R4me2s and PRMT5 also increase during differentiation of definitive erythroid progenitors. While we have shown that knockdown of EZH1 and EZH2 does not lead to reactivation of E^{y} transcription,[22] it remains to be determined whether polycomb complexes PRC1 or PRC2 associate with the β-globin locus in erythroid progenitors. In this context, it is interesting to note that PRC2-complex subunits EZH2 and SUZ12 are not associated to the β-globin locus in non-erythroid cells.[52]

While the embryonic β-*globin* genes are maintained in a repressed state in definitive erythroid cells, their adult counterparts are activated at extremely high levels during differentiation. Epigenetic studies point towards a two-step mechanism including (1) anti-repression and (2) activation.

Anti-repression. Mechanisms appear to be in place to overcome the repressive H3K27me2 and H3K9me2 marks at the adult β^{maj}-*globin* gene promoter in differentiating cells. Firstly, we found that the H3K27me2 demethylase UTX[53] is recruited to the β^{maj} *globin* promoter and actively removes H3K27me2,[22] (**Fig. 1**). Importantly, UTX function is directly linked to gene activation since knocking down UTX in differentiated cells leads to a decrease in β^{maj}-*globin* transcription, concomitant with increased H3K27me2 on this gene promoter. This shows that UTX antagonises the repressive effect of G9a at the adult β^{maj}-*globin* gene. Secondly, we noticed that G9a and the H3K9me2 mark do not increase on the β^{maj}-*globin* promoter upon erythroid differentiation.[22] This is in contrast to other locations on the β-globin locus, including the coding region of the active β^{maj} gene where H3K9me2 and G9a both increase during differentiation, particularly at the 3' end of the gene.[14,22] Together with our finding that G9a can interact with RNA polymerase II (Pol II),[22] these results suggest that upon erythroid differentiation, G9a is prevented from accumulating on the β^{maj} promoter via a Pol II-mediated transfer to the coding region. It remains to be determined whether this transfer would play a role in the methyltransferase-independent activating function of G9a that both our lab[22] and the Stallcup lab[54]

have observed. In addition, these results emphasize that the H3K9me2 mark and presence of G9a are not necessarily associated with transcriptional repression when localized outside of promoter regions and are consistent with previous findings that methylated H3K9 occurs in transcribed regions of active genes.[14]

Activation. The activation of β^{maj}-*globin* upon erythroid differentiation occurs through a process that includes deposition of the MLL2-mediated H3K4me3 mark at a restricted location in the 5' coding region of this gene.[21] Indeed, knockdown of MLL2 results in decreased β^{maj}-*globin* transcript concomitant with a decrease in H3K4me3 enrichment on the β^{maj}-*globin* coding region. The removal of H3K27me2 by UTX and the low levels of H3K9me2 at the β^{maj} promoter might be important for this MLL2-mediated methyltransferase activity. Indeed, cooperativity between H3K4 methylation and H3K27 demethylation has previously been reported (reviewed in ref. 55). Interestingly, a recent genome-wide study showed that the presence of MLL-catalysed H3K4me3 potentiates the H3K9ac mark on genes regulated by MLL complexes.[56] This is consistent with our previous results showing that H3K9ac overlaps with the H3K4me3 mark on the active β^{maj} gene in differentiating erythroid cells.[21] Interestingly, evidence points towards the histone acetyltransferases GCN5/PCAF (KAT2A/B) as the enzyme(s) responsible for H3K9 acetylation in the 5' coding region of the active β^{maj} gene. Indeed, GCN5/PCAF acetyltransferases but not p300/CBP (KAT3A/B) are able to acetylate H3K9 on nucleosomes.[57] Furthermore, a recent study has shown that the PRMT1-mediated *asymmetric* dimethylation of histone H4 at arginine 3 (H4R3me2as) facilitates the binding of PCAF and subsequent H3K9 acetylation* on β^{maj}-*globin*[58] (*the authors of this study have used the Upstate 06-599 antibody, S Huang, University of Florida, personal communication). This antibody was raised against H3K9acK14ac, but recognizes predominantly H3K9ac.[59,60]

The activation of β^{maj}-*globin* also requires G9a, the mechanism of which is independent of functional methyltransferase activity.[22,54] It appears that G9a is required for β^{maj}-*globin* transcriptional activation through recruitment and/or stabilization of core transcription components including RNA polymerase II, TFIIF and TFIIH, however the exact mechanism of this is unknown.

In summary, these studies have revealed a highly dynamic picture in which several epigenetic processes and histone modifying enzymes work together to maintain the embryonic β-*globin* genes in a repressed state while simultaneously activating the adult β-*globin* genes during definitive erythroid differentiation. From the evidence, it appears that the maintenance of E^y repression in definitive differentiating erythrocytes involves the dimethylation of H3K9 and H3K27 by G9a, which is recruited to the β-globin locus by NF-E2. It may also require *symmetric* H4R3me2 by PRMT5, which in turn facilitates DNMT3A-mediated DNA methylation. Activation of the adult β-*globin* genes involves demethylation of H3K27me2 by UTX, histone methyltransferase independent coactivator function of G9a, MLL2-catalysed H3K4me3 (which notably has been shown to stabilize TFIID binding[61]), and H3K9Ac by GCN5/PCAF, in a process that is facilitated by *asymmetric* H4R3me2 by PRMT1 (**Fig. 1**).

The findings that *asymmetric* H4R3me2 facilitates gene activation while *symmetric* H4R3me2 correlates with transcriptional repression emphasize the intricate nature of the histone code. While we are starting to understand the role of some histone marks in the regulation of β-*globin* transcription during erythroid differentiation, an important challenge going forward will be to determine the precise relationship of these different marks with respect to each other.

Recruitment and Propagation of Histone Methyltransferase Complexes on the β-Globin Locus

How are enzymes that establish or remove histone marks targeted to specific genomic locations? While "recruitment" and "binding" of histone-modifying enzymes are often considered the same phenomenon, we favor the idea that they represent functionally distinct processes. The recruitment step requires a sequence specific DNA-binding transcription factor (or non-coding RNA) that guides the histone-modifying complex to a specific location (**Fig. 2**). Binding following the recruitment step involves anchoring to chromatin, which is facilitated (or prevented) by specific interactions between a "reader" polypeptide (e.g., bromodomain, chromodomain, PHD finger, WD40 repeats) and particular histone marks (reviewed in ref. 62).

In addition, some histone-modifying enzymes can spread beyond the site to which they are recruited (**Fig. 2**), covering large domains of chromatin, even entire chromosomes. Propagation of these histone-modifying enzymes from a nucleation site occurs through various mechanisms, both transcription-dependent and transcription-independent. For example, the repressive mammalian polycomb complex PRC2,[19] (containing SUZ12, EED, EZH2 and RbAP46/48), which methylates H3K27, is recruited to specific sites via interaction with DNA-binding proteins such as OCT4,[63] YY1 (the mammalian homolog of drosophila PRE-binding protein Pho)[64,65] or non-coding RNAs such as HOTAIR[66] and Xist.[67] Following its recruitment, PRC2 extends laterally, covering broad regions of chromatin that co-localize with H3K27me3.[68-70] It has recently been shown that the chromatin binding of PRC2 entails association of the WD40 domain of EED to H3K27me3 and H3K9me3 and subsequent allosteric activation of EZH2 methyltransferase activity.[71] Enzymes that establish histone marks associated with active genes can also spread beyond their recruitment site via either binding to the elongating RNA Pol II (e.g., the H3K36 methyltransferase HYPB/SET2 (KMT3A)[72]) or binding to active histone marks. For example, the drosophila male specific lethal (MSL) complex, which is involved in dosage compensation via increased transcription on the single male X chromosome, first recognizes a DNA sequence motif within chromatin entry sites (CES)[73] and subsequently spreads bidirectionally in the presence of active transcription.[74] This spreading occurs through specific recognition of H3K36me3 by the chromodomain of MSL3.[75,76] Importantly, examples have also been described where

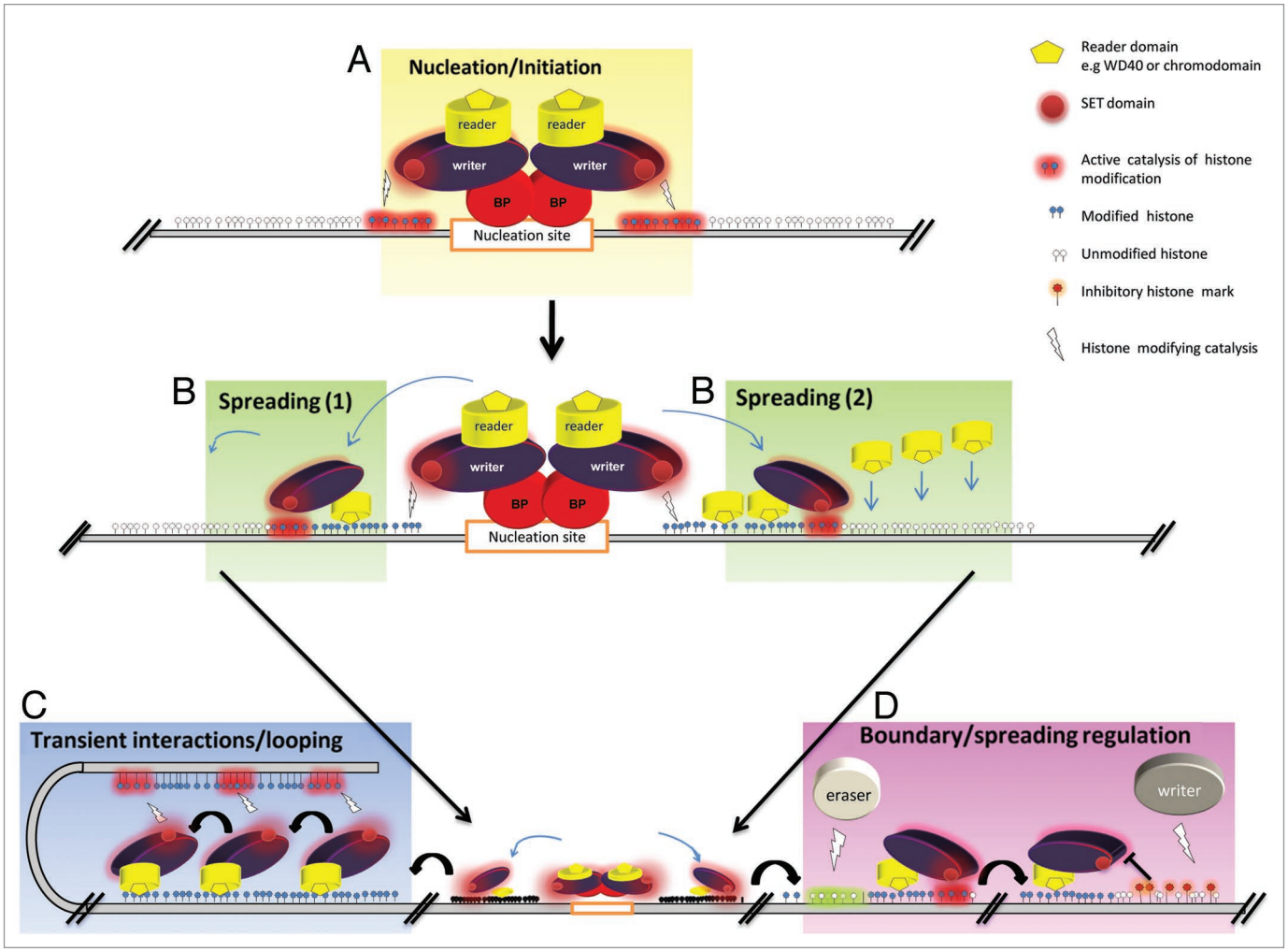

Figure 2. Model for Spreading: For illustrative purposes we show an example of bidirectional spreading. (A) A complex is nucleated at a particular chromatin region by mechanisms such as DNA sequence recognition, non-coding RNA (ncRNA) transcripts or by cooperation of protein complexes bound on a number of DNA elements. This complex or a subsequent complex it attracts will contain a histone modifying complex containing both 'reader' and 'writer' domains. (B) The recruited complex modifies nearby nucleosomes. These modified nucleosomes represent binding sites for a 'reader' subset of the complex resulting in either (1) the direct movement of the 'writers' with the 'readers' to the adjacent nucleosomes or (2) the extraction of the 'writer' portion from that recognition complex to be picked up by nearby chromatin bound 'reader' factors. Either general mechanism results in cycles of binding of the complex, in modification of adjacent nucleosomes and in the progressive spread of the histone modifying complex and the resultant chromatin state. (C) In addition to linear spreading of a complex along a chromatin domain, HMT complexes could also transiently associate with and modify other chromatin domains in close physical proximity (looping). (D) Every step in the process of spreading may be regulated by other factors encountered by the complex in transit. For example, this could include 'eraser' complexes that remove the histone mark, which is the 'reader' recognition motif, and thereby limit the performance of the spreading complex or act as a 'boundary' factor. Other factors could prevent further spread by inducing an inhibitory chromatin state, such as catalyzing histone modifications that hinder the 'writer' activity. Factors may also locally limit the histone modifying activity while not preventing the spread of the complex (not shown). BP: Binding protein (with specificity for DNA/RNA).

both transcription-dependent and -independent mechanisms can work together to establish binding of a complex over large domains. This is the case notably at centromeres in fission yeast, which require transcription of ncRNAs and the RNAi machinery for their assembly into heterochromatin and proliferation of the H3K9me2 mark.[77] In this case, it has been shown that genes located downstream of the Pol II promoter that drives synthesis of the centromeric transcripts are silenced through a mechanism of transcription-mediated spreading of heterochromatin marks through association of RNA-induced transcriptional silencing complex (RITS) with the transcribed ncRNAs. Conversely, genes located upstream of the Pol II promoter are not transcribed but they are also silenced through spreading of the same RITS complex, occurring in a transcription-independent manner via the Tas3 subunit self-oligomerization.[78] This illustrates that with even the same complex, the spreading mechanism can be vastly different depending on the chromatin context.

Our investigations on adult erythroid differentiation revealed that spreading also occurs on the β-globin locus. Specifically, chromatin immunoprecipitation followed by locus wide qPCR uncovered spreading of the HMTs MLL2 and G9a from

a NF-E2-dependent nucleation site on the LCR.[21,22] As described in the previous section, MLL2 spreads across the locus and locally catalyses H3K4me3 at discrete coding regions of the active β^{maj} *globin* gene; G9a is recruited at the same site as MLL2 and spreads across the locus to regulate the embryonic β-*globin* gene *E*^{y} (repression) and the adult β-*globin* gene (β^{maj}) (activation). Spreading of MLL2 and G9a at the many tested regions across the locus was dependent on functional NF-E2p45, suggesting that this NF-E2p45-bound HS2 region acts as a nucleation site for these HMT complexes. However, it is also possible that G9a and MLL2 could be recruited to additional "entry sites" along the β-globin locus. The mechanism(s) through which MLL2 and G9a propagate on the β-globin locus is not clear but we can speculate on mechanisms based on some of the previously described examples.

G9a forms a heterodimer with the related GLP protein.[79] Both G9a and GLP are able to mediate dimethylation of H3K9 via their SET domain. Strikingly, G9a and GLP are also able to bind specifically to their own products (H3K9me1 and H3K9me2) via centrally located ankyrin repeats, which form a characteristic methyl lysine-binding module.[80] The spreading mechanism of the G9a/GLP heterodimer has been speculated to involve binding to the methyl lysine product followed by conversion of adjacent histones to product and so on.[81] Other key indications come from the finding that G9a ankyrin repeats have a slight preference for H3K9me2, while the ankyrin repeats of GLP slightly prefer H3K9me1;[80] hence one mode of spreading may involve the GLP subunit of the heterodimer recognizing H3K9me1 and G9a placing the dimethyl mark. We find that deletion of NF-E2p45 abrogates G9a recruitment and spreading on the β-globin locus. What remains to be investigated is if mutation in the ankyrin repeats that is deficient for methyl lysine binding allows G9a recruitment but abrogates G9a spreading from the LCR nucleation region.

MLL2 is part of a multi-protein complex containing several core subunits (including ASH2L, DPY30, RbBP5, WDR5, CFP1/CGBP) that are important to regulate its H3K4 methyltransferase activity.[82-85] Interestingly while several subunits of this complex (i.e., MLL2, ASH2L and DPY30) are recruited to the β-globin LCR in a NF-E2p45-dependent manner, only MLL2 was found to spread the length of the locus.[21] A possible mechanism through which MLL2 could be forced out of the MLL2 complex at the LCR is suggested by studies performed on the related MLL1 complex, which associates with identical core binding partners. It has been shown in vitro that WDR5 interacts with MLL1 via its histone H3-binding pocket and that the WDR5-MLL1 interaction is preferentially competed by mono- or dimethylated H3K4.[86] We speculate that a similar mechanism might occur at the LCR, where the H3K4me2 mark, present at this location,[27] could lead to the displacement of MLL2 from its interacting partner WDR5, thereby initiating spreading of the MLL2 subunit. It remains to be established whether WDR5 remains at the LCR upon erythroid differentiation or travels with MLL2 across the β-globin locus. While spreading of MLL2 within the coding region of active genes might entail interaction with the elongating Pol II[13,87,88] such a mechanism is unlikely to occur within regions between the LCR and the active genes since treatment with the inhibitor of transcriptional elongation DRB (5,6-dichlorobenzimidazole riboside) does not abrogate MLL2 binding from the LCR to the promoter of the active β^{maj}-*globin* gene (Brand M, unpublished observation). At this point, it is unclear how MLL2 spreads between the LCR and the active β^{maj}-*globin* gene. MLL2 contains a CxxC domain, which mediates binding to non-methylated CpG-rich DNA,[89,90] however the region between the LCR and β^{maj} promoter is quite heavily DNA methylated in adult cells.[46] While it has been proposed that MLL2 could bind to methylated H3K4 through its PHD domains,[91] H3K4me2,[27] and H3K4me3,[21] marks are not continuous across the β-globin locus in adult cells. Therefore, it is unlikely that MLL2 uses these domains for self-propagation. The mechanism(s) through which MLL2 spreads from the LCR to the active β-globin gene remains to be determined.

Regardless of the exact mechanism through which the HMTs G9a and MLL2 propagate on the β-globin locus upon erythroid differentiation, it is now clear that "spreading" or "linking" occurs on the β-globin locus during erythroid differentiation[21,22] as previously proposed,[92] and co-exists with a "looping" mechanism[32] that can bring LCR-bound factors and promoters-bound factors in physical proximity within active transcription factories or active chromatin hubs. While both looping and spreading can transmit transcriptional regulatory signals at a distance, we propose that spreading of transcriptional complexes across the entire β-globin locus provides a unique opportunity for co-regulation of clustered genes that are expressed at different times during development. Such a mechanism could explain the maintenance of the developmental specificity of β-globin expression.

Concluding Remarks

Antagonism between the repressive G9a and activating MLL2 methyltransferases appears important for maintaining the developmental pattern of β-*globin* gene expression in adult erythroid cells. In this model, the dominant G9a-mediated H3K9me2 mark would maintain a repressive state across the locus. Conversely, the H3K4me3 activity of MLL2 could be selectively established at specific β-globin genes through a mechanism dependent on the activity of other histone-modifying enzymes (e.g., the histone acetyltransferases GCN5/PCAF[58] and CBP,[37] the arginine methyltransferase PRMT1,[58]) that may not spread on the β-globin locus but rather are enriched selectively at the β-globin LCR and at the promoter of the specific gene they activate. Important challenges for the future will be to decipher precisely how crosstalk mechanisms between histone marks and transcriptional cofactors regulate the expression of clustered genes (including the β-*globin* genes) during differentiation and development, and to correlate the relationship between genome architecture and chromatin modifications.

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