Maintenance of gene silencing by the coordinate action of the H3K9 methyltransferase G9a/KMT1C and the H3K4 demethylase Jarid1a/KDM5A

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Chromatin remodeling is essential for controlling the expression of genes during development. The histone-modifying enzyme G9a/KMT1C can act both as a coactivator and a corepressor of transcription. Here, we show that the dual function of G9a as a coactivator vs. a corepressor entails its association within two distinct protein complexes, one containing the coactivator Mediator and one containing the corepressor Jarid1a/KDM5A. Functionally, G9a is important in stabilizing the Mediator complex for gene activation, whereas its repressive function entails a coordinate action with the histone H3 lysine 4 (H3K4) demethylase Jarid1a for the maintenance of gene repression. The essential nature of cross-talk between the histone methyltransferase G9a and the demethylase Jarid1a is demonstrated on the embryonic *E^{y}-globin* gene, where the concurrent introduction of repressive histone marks (dimethylated H3K9 and dimethylated H3K27) and removal of activating histone mark (trimethylated H3K4) is required for maintenance of gene silencing. Taken together with our previous demonstration of cross-talk between UTX and MLL2 to mediate activation of the adult *β^{maj}-globin* gene, these data suggest a model where "active" and "repressive" cross-talk between histone-modifying enzymes coexist on the same multigene locus and play a crucial role in the precise control of developmentally regulated gene expression.

beta-globin locus | epigenetics | erythroid differentiation | hemoglobin

Histones are subjected to a number of posttranslational modifications that play important roles in regulating diverse cellular processes. These modifications are highly dynamic and are established through a competition between "writer" enzymes that introduce the modifications and "eraser" enzymes that remove them (1). Proper coordination between histone-modifying enzymes is critical for the regulation of gene expression (2, 3). For example, it has been shown that the histone H3 lysine 4 (H3K4) methyltransferases MLL3/4 (also called KMT2C/B) and the H3K27 demethylase UTX (also called KDM6A) are part of the same protein complex and coordinate their histone-modifying functions to activate the expression of specific genes through the concurrent introduction of the active histone mark trimethylated H3K4 (H3K4me3) and removal of the repressive histone mark H3K27me3 (4–6). These results underline the importance of cross-talk between histone methyltransferase and demethylase enzymes to activate transcription.

G9a (also called Ehmt2 or KMT1C) and its homolog GLP (also called Ehmt1 or KMT1D) have been identified as major euchromatic methyltransferases. These enzymes work as heterodimers to introduce monomethyl and dimethyl modifications on histone H3 at Lys-9 [single- or dimethylated H3K9 (H3K9me1/me2] (7–10). In addition, several studies have shown that G9a and GLP mediate dimethylation of H3K27, both in vitro (11) and in vivo (12–14).

It has been well established that G9a can repress transcription by the introduction of repressive histone modifications (H3K9me2 and H3K27me2) and/or the recruitment of DNA methyltransferases (reviewed in 14, 15). In addition, G9a can activate transcription of specific genes through a mechanism that does not require its methyltransferase activity (12, 16–19). It is currently unclear how G9a can act to mediate both the activation and repression of transcription. In addition, we do not know whether the G9a-GLP heterodimer interplays with other histone methylating/demethylating enzymes.

Results

G9a and GLP Associate with the Coactivator Complex Mediator and the Corepressor Protein Jarid1a. To identify proteins that interact with the G9a-GLP heterodimer, we performed immunoprecipitation (IP) experiments using anti-GLP Abs and normal IgG as a negative control. Endogenous proteins were immunoprecipitated from nuclear extracts of murine erythroid (MEL) cells and identified by mass spectrometry (MS) (Fig. 1*A* and Dataset S1). Validating our approach, G9a and GLP themselves were identified, as well as a number of previously known G9a-interacting partners, including Wiz (20), DNMT1 (21), and HP1γ (22, 23). In addition, several previously unknown G9a-GLP interacting proteins, including both coactivators and corepressors, were identified, which is consistent with the dual role of G9a in regulating transcription (Fig. 1).

Among the coactivators that interact with G9a-GLP, 17 subunits of the Mediator complex, which is a fundamental component of the RNA polymerase II (Pol II) transcriptional machinery (24), were identified (Fig. 1). This result is consistent with our previous finding that G9a interacts with Pol II (12) and with the previously shown interaction between G9a and Med12 (25). Furthermore, we confirmed the association of G9a-GLP with the Mediator complex by both independent G9a and GLP IPs revealed by Western blot (Fig. 1*B*) and by a reciprocal Med1 IP in which Med12, G9a, and GLP were detected (Fig. 2*B*, *Right*).

In addition to the coactivator complex Mediator, several corepressor proteins were identified as G9a-GLP–associated partners, including the DNA methyltransferase DNMT1, the histone deacetylases HDAC1 and HDAC2, and HP1γ. Furthermore, the H3K4me3 demethylase Jarid1a (also called KDM5A or Rbp2) (26–28) was identified as a previously unknown G9a-GLP–interacting protein (Fig. 1).

In an attempt to separate the various corepressors that associate with G9a and GLP, we performed size exclusion chromatography on nuclear extracts. Consistent with G9a and GLP being part of a heterodimer (10), these two proteins coelute in the same two gel filtration fractions. Strikingly, the main peak of Jarid1a comigrates

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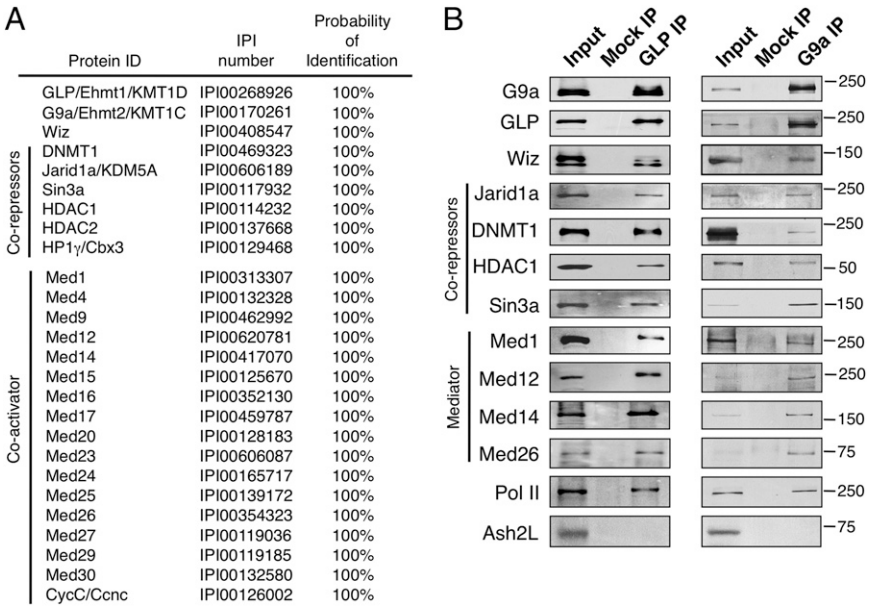

Fig. 1. Identification of Jarid1a and the Mediator complex as previously undescribed G9a- and GLP-interacting proteins. (*A*) MS analysis of a GLP immunoprecipitate. The probability of identification was determined using ProteinProphet (44). IPI, International Protein Index. (*B*) Confirmation of the interaction of GLP and G9a with Jarid1a and the Mediator complex. Proteins immunoprecipitated via Abs against GLP and G9a were analyzed by Western blot. A mock IP with normal IgG was used as a negative control. Abs used for Western blot (*Left*) and molecular masses (*Right*; in kilodaltons) are indicated.

with the main peak of G9a and GLP (Fig. 2*A*, fractions 10 and 11). This is in contrast to other G9a-interacting proteins, such as DNMT1 (21), DNMT3a/b (29), and Wiz (20), or Jarid1a-interacting proteins Ezh2 and Suz12 (30, 31), which coelute with G9a and GLP but are present predominantly in lower molecular mass fractions (Fig. 2*A*, fractions 13–16). This result suggests that Jarid1a and the G9a-GLP heterodimer might be tightly associated in the cell. To address this possibility further, we performed a reciprocal IP using Abs against endogenous Jarid1a. We observed both G9a and GLP in the Jarid1a IP fraction, which confirms the interaction between these proteins (Fig. 2*B*). To determine whether the interaction between G9a and Jarid1a is direct, we incubated recombinant (rec) purified G9a and Jarid1a proteins and performed a G9a IP. As shown in Fig. 2*C*, rec G9a specifically pulled down rec Jarid1a (but not the control rec Ubc4), demonstrating a direct interaction between these proteins. Taken together, these results indicate that G9a, GLP, and Jarid1a are tightly associated within the same protein complex that is distinct from the core PRC2 complex containing Ezh2 and Suz12 (32).

Because these results were obtained using a mouse MEL cell line, we wanted to determine whether the interaction between G9a-GLP and Jarid1a is conserved in other cell types. To address this question, the G9a, GLP, and Jarid1a IPs, as well as the size exclusion chromatography experiments, were repeated with nuclear extract prepared from undifferentiated murine embryonic stem (mES) cells. The association between these proteins was reproduced in mES cells (Fig. S1), suggesting that the interaction between the G9a-GLP heterodimer and Jarid1a is conserved in cell types of different cell fate potential.

Cross-Talk Between G9a and Jarid1a Is Important for the Maintenance of Gene Repression. The interaction between G9a and Jarid1a is particularly interesting in light of the cross-talk between histone methyltransferase and demethylase that has been implicated in the regulation of transcription. Indeed, it has been shown previously that the interaction between the H3K4 methyltransferases MLL3/4 and the H3K27 demethylase UTX is important for the activation of transcription (4–6). In this context, the detected interaction between G9a and Jarid1a suggests that cross-talk between methyltransferase and demethylase might also be important to maintain gene repression through a mechanism that involves the concurrent introduction of repressive histone marks (H3K9me2 and H3K27me2) by G9a and removal of active histone mark (H3K4me3) by Jarid1a. To test this possibility, we studied the mechanism of gene repression on the β-globin locus. This locus comprises several *β-globin* genes that are differentially regulated during development, including the embryonic gene E^y, which is repressed, and the adult gene β^{maj}, which is actively transcribed in differentiated MEL cells (33–36). Furthermore, it was shown previously that G9a maintains the E^y gene in a repressed state in a methyltransferase-dependent manner (12).

To determine whether Jarid1a is also involved in maintaining the E^y gene in a repressed state, stable MEL clones were generated in which the shRNA-mediated knockdown (KD) of Jarid1a can be induced on doxycycline (Dox) treatment. We found that although the KD of Jarid1a (Fig. 3*A*) does not affect the proliferation of cells during erythroid differentiation (Fig. S2), it leads to derepression of the embryonic E^y *globin* gene (Fig. 3*B*, *Left*), suggesting that Jarid1a is involved in maintaining this gene in a silenced state. In contrast, Jarid1a KD has no effect on transcription of the adult β^{maj} *globin* gene (Fig. 3*B*, *Right*). To determine whether Jarid1a directly represses E^y, the binding of Jarid1a to the promoter of this gene was analyzed by ChIP. Jarid1a was found to bind to the E^y gene promoter but not to the β^{maj} promoter or the distal enhancer HS2 in differentiated erythroid cells (Fig. 4*A*). In addition, Jarid1a binding was lost on Jarid1a KD (Fig. 4*A*), which validates the specificity of the Jarid1a Ab. Interestingly, Jarid1a binding to the E^y promoter is also decreased on the KD of G9a (Fig. 4*B*), emphasizing the importance of the interaction between G9a and Jarid1a for the binding of Jarid1a to chromatin. We note, however, that in the absence of G9a, Jarid1a binding to the E^y promoter is not completely lost (compare Fig. 4 *A* and *B*). This residual weak binding could be due to the association of Jarid1a with other chromatin-associated proteins, such as GLP, which continues to interact with Jarid1a even in the absence of G9a (Fig. S3). Importantly, control experiments demonstrate that the KD of Jarid1a does not affect the amount of G9a (at the transcript and protein levels) and, similarly, that the KD of G9a does not affect the amount of Jarid1a in the cell (Fig. 3*A*). To determine whether

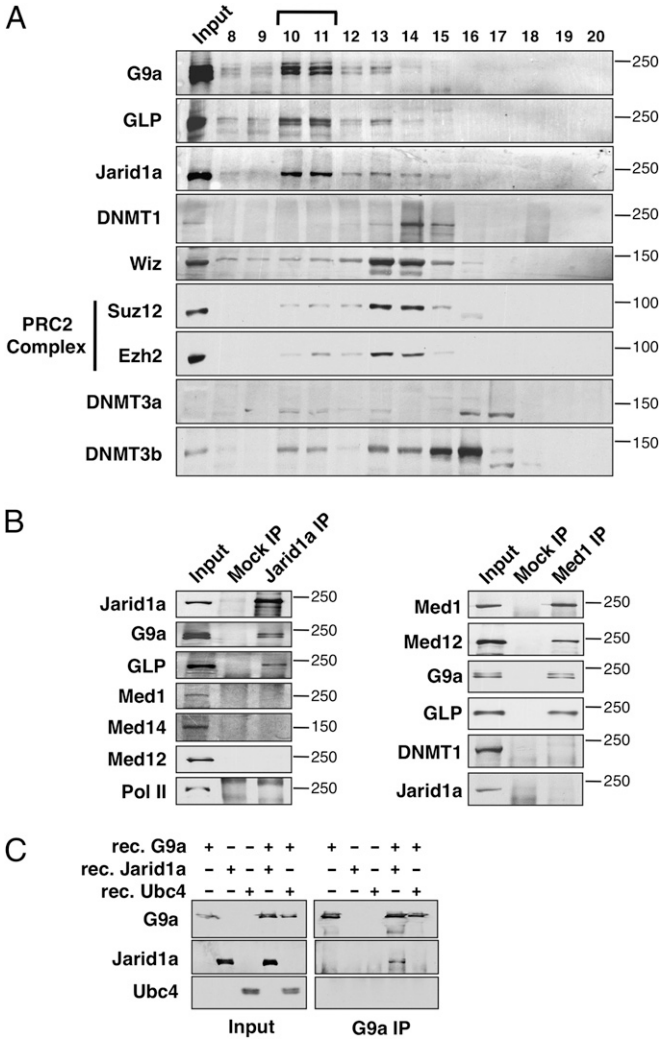

Fig. 2. Jarid1a is tightly associated with the G9a-GLP heterodimer. (*A*) G9a, GLP, and Jarid1a coelute by size exclusion chromatography. A nuclear extract was separated on a Superose-6 column, and the presence of specific proteins in different fractions revealed by immunoblotting with Abs indicated on the left. Molecular masses (in kilodaltons) are indicated on the right. (*B*) G9a-GLP heterodimer associates with Jarid1a and the Mediator complex in a mutually exclusive manner. Proteins immunoprecipitated via Abs against Jarid1a and Med1 were analyzed by Western blot. Mock IPs with normal IgG were used as negative controls. Abs used for Western blot (*Left*) and molecular masses (*Right*; in kilodaltons) are indicated. (*C*) Jarid1a interacts directly with G9a. Rec purified Jarid1a or Ubc4 was incubated with rec purified G9a before IP with G9a Abs. Immunoprecipitated proteins were revealed by immunoblotting with the Abs indicated on the left.

Jarid1a actively demethylates H3K4me3 on the repressed E^y gene, we performed an H3K4me3 ChIP experiment. Depletion of Jarid1a results in increased levels of H3K4me3 on the E^y promoter (but not on the β^{maj} promoter), whereas the H3K9me2 mark is not affected (Fig. 4*C*). We note that the level of increase in H3K4me3 observed on E^y on Jarid1a KD is equivalent to the H3K4me3 increase measured on the *Sdf1* gene in Jarid1a$^{-/-}$ mouse embryonic fibroblasts (MEFs) (26). Taken together, these results indicate that Jarid1a is involved in actively demethylating H3K4me3 on the E^y gene's promoter.

Finally, to determine whether G9a and Jarid1a work together to maintain the E^y gene in a repressed state, we generated clones in which double KD of G9a and Jarid1a is induced on Dox treatment. Study of these clones revealed that the G9a/Jarid1a double KD has an additive effect on the derepression of E^y compared with either single KD on its own (Fig. 3; confirmation of this result in a clone expressing a shRNA targeting a different region of the Jarid1a transcript is shown in Fig. S4), indicating that G9a and Jarid1a work in combination to maintain the E^y gene in a transcriptionally silenced state. Taken together, these results show that cross-talk between the methyltransferase G9a and the demethylase Jarid1a occurs on the E^y *globin* gene and is important to maintain this gene in a repressed state.

G9a-Dependent Binding of the Mediator Complex. We have shown previously that G9a participates in the activation of adult β^{maj} *globin* gene transcription in a methyltransferase-independent manner (12). In contrast, Jarid1a does not appear to regulate transcription of the β^{maj} gene (Fig. 3*B*), suggesting that G9a could be present in distinct coactivator and corepressor complexes. To test this possibility, we performed IPs of both Jarid1a and Mediator. As shown in Fig. 2*B*, G9a, but not the Mediator complex (Med1, Med12, and Med14 subunits), coimmunoprecipitates with Jarid1a. Reciprocally, Jarid1a is not detected in the G9a complex immunoprecipitated with a Med1 Ab (Fig. 2*B*). This shows that G9a interacts with separate coactivator and corepressor complexes containing Mediator and Jarid1a, respectively.

Because the G9a–Jarid1a repressive complex is restricted to the transcriptionally silent E^y gene (Fig. 4*A*), we next examined the localization of the G9a–Mediator complex on the β-globin locus that comprises both the silent E^y *globin* and the active β^{maj} *globin* genes by ChIP. This experiment showed that the Mediator complex subunits Med1, Med12, Med14, and Med17 are bound to the active β^{maj} gene but not to the repressed E^y gene (Fig. S5). Furthermore, G9a is important for binding of the Mediator complex to the β^{maj} gene because Mediator ChIP signals are decreased on G9a KD (Fig. S5). Taken together, these results show that G9a is important for the assembly and/or stability of the Mediator complex to the promoter of the active β^{maj} gene promoter but not to the repressed E^y gene.

Discussion

Cross-Talk Between a Histone Methyltransferase and a Histone Demethylase in the Maintenance of Gene Silencing. The establishment of tissue-specific gene expression programs during differentiation and development is influenced by changes in histone modifications. Gene activation encompasses the coordinated removal of histone marks that are refractory to the transcriptional process and introduction of histone marks that are permissive to transcription (37). Coordination of this process is permitted by several mechanisms of cross-talk between histone modifications, including the association of different histone-modifying enzymes within the same protein complex (2, 3). The prime example of cross-talk facilitating gene activation is the association between UTX and MLL3/4, which work together within the same protein complex to remove the repressive mark H3K27me3 and introduce the active mark H3K4me3, respectively (4–6). In this paper, we have identified previously undescribed cross-talk between two histone-modifying enzymes that play major roles in the maintenance of gene silencing: the H3K9/K27 methyltransferase G9a (14, 15) and the H3K4 demethylase Jarid1a (38). We show that these proteins interact directly and are part of the same protein complex in both erythroid cells and mES cells. Furthermore, our results show that the G9a–GLP–Jarid1a complex is distinct from the core PRC2 repressive complex that contains Ezh2 and Suz12, consistent with the previous finding that Jarid1a and the PRC2 complex bind to largely nonoverlapping genomic regions (31). Functionally, the results show that G9a stabilizes the binding of Jarid1a to chromatin and that these two enzymes work together to maintain gene repression through introduction of the repressive histone marks H3K9/27me2 and removal of the active histone mark H3K4me3. Thus, the maintenance of gene silencing is an active process permitted by cross-talk between the histone methyltransfer-

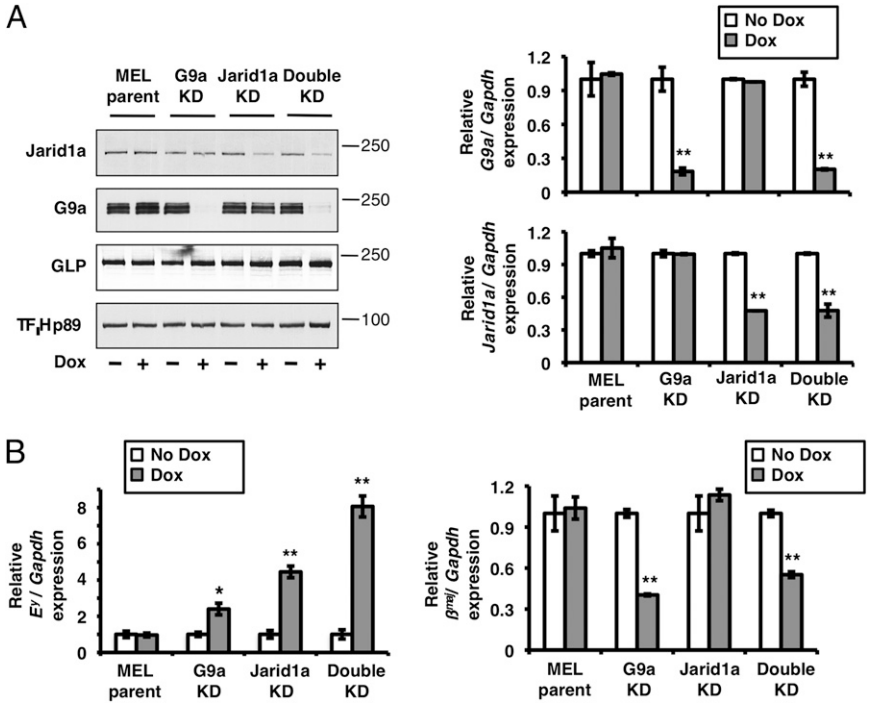

Fig. 3. Jarid1a and G9a cooperate in the maintenance of gene repression. (*A*) Establishment of stable MEL cell clones in which the single KDs of Jarid1a or G9a and the double KD of Jarid1a plus G9a are induced by Dox-mediated expression of specific shRNAs. This figure shows the results using Jarid1a shRNA1 (*SI Materials and Methods*). The levels of Jarid1a and G9a in Dox-treated (Dox) vs. untreated (No Dox) cells were analyzed at the protein level by Western blot of nuclear extracts using the indicated Abs (*Left*) and at the mRNA level by real-time RT-qPCR (*Right*). MEL parent represents a control cell line with no induction of shRNA on Dox treatment. (*Left*) Molecular masses of proteins (in kilodaltons) are indicated on the right. (*Right*) Transcript levels are normalized to *GAPDH* with the ratio observed in the absence of Dox set to 1. (*B*) Additive effect of the double Jarid1a plus G9a KD on derepression of the E^{γ} *globin* gene. Transcripts levels were measured by real-time RT-qPCR in Dox-treated/untreated cells. Transcript levels are normalized to *GAPDH* with the ratio observed in the absence of Dox set to 1. (*A* and *B*) Average values from triplicate experiments are represented with error bars corresponding to SDs. $*P < 0.05$; $**P < 0.01$.

ase G9a and the demethylase Jarid1a. Furthermore, this mechanism may be generalized to additional Jarid proteins, because G9a is also present in a Jarid1c-containing complex (39).

Cross-Talk Between Histone-Modifying Enzymes Differentially Regulates Gene Expression Within a Multigene Locus. G9a plays a crucial role as a "gatekeeper" of cell identity by protecting cells from spurious

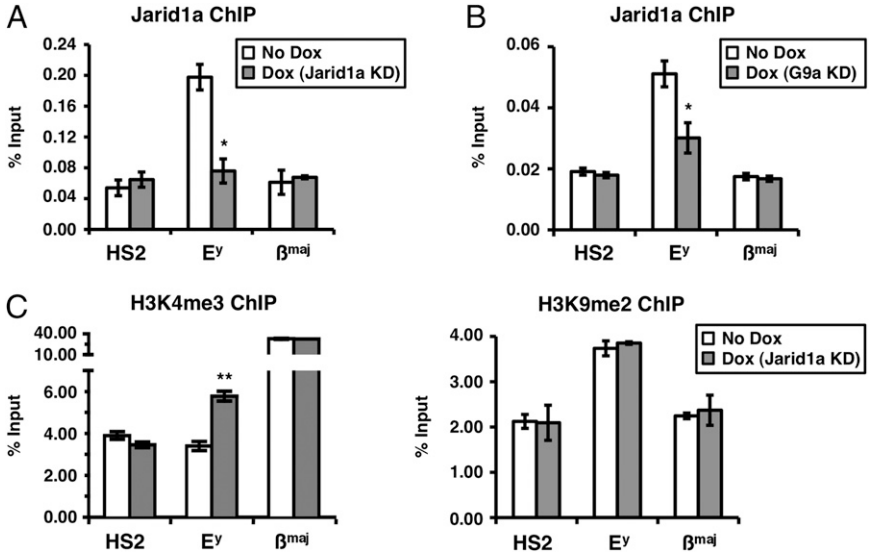

Fig. 4. Jarid1a maintains the globin gene E^{γ} in a repressed state through demethylation of H3K4me3. (*A*) Jarid1a binds to the E^{γ} promoter in erythroid cells. Jarid1a ChIPs were performed in the presence of normal (No Dox) or reduced (Dox) levels of Jarid1a. (*B*) G9a stabilizes the binding of Jarid1a to the E^{γ} promoter. Jarid1a ChIPs were performed in the presence of normal (No Dox) or reduced (Dox) levels of G9a. (*C*) KD of Jarid1a leads to an increase in H3K4me3 on the E^{γ} promoter. Native ChIPs were used to measure the levels of H3K4me3 and H3K9me2 in the presence of normal (No Dox) or reduced (Dox) levels of Jarid1a. (*A*–*C*) ChIPs were revealed by real-time qPCR using Taqman probes located at the HS2 site of the locus control region and at the promoters of the E^{γ} and β^{maj} genes. Error bars represent SDs calculated from triplicate experiments. $*P < 0.05$; $**P < 0.01$.

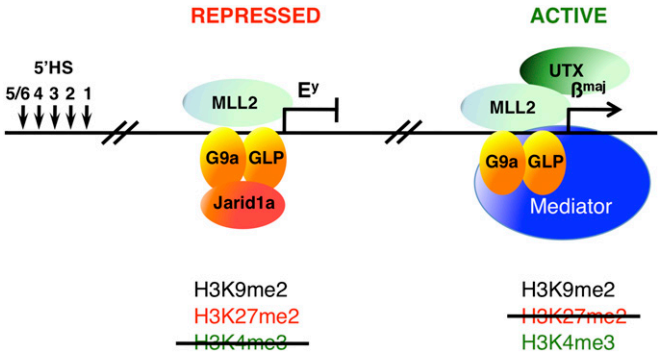

Fig. 5. Model of the regulation of transcription on the β-globin locus through cross-talk between histone methyltransferases and demethylases. In adult erythroid cells, the embryonic globin gene E^y and the adult globin gene β^{maj} are both bound by the histone methyltransferases G9a-GLP, which introduce the repressive marks H3K9me2 and H3K27me2, and MLL2, which introduces the active mark H3K4me3. What differentiates these two genes is the presence of specific demethylases. Indeed, on the E^y gene, the coordinate actions of the H3K4me3 demethylase Jarid1a and the H3K9/K27 methyltransferase G9a-GLP allow for the maintenance of a transcriptionally repressed state. In contrast, on the β^{maj} gene, the demethylase UTX actively removes the repressive mark H3K27me2, whereas the methyltransferase MLL2 trimethylates H3K4, allowing full activation of this gene in erythroid cells. 5′HS1–6 represents the distal β-globin locus control region.

expression of genes from other lineages (15). For example, G9a prevents the expression of neuronal genes in nonneuronal cell types (40) and the expression of pluripotency genes in differentiated cells (29). Similarly, G9a controls the developmental timing of gene expression within specific lineages, as exemplified by the derepression of neuronal progenitor genes in G9a-ablated adult neurons (40). In addition, G9a has a dual role in regulating transcription on the β-globin locus, where it is involved in both repressing the embryonic gene E^y (through the introduction of repressive histone marks H3K9me2 and H3K27me2) and activating the adult gene β^{maj} (in a methyltransferase-independent manner) (12). However, it was not clear how G9a acts as both an activator and a repressor. The present study shows that the dual function of G9a entails an association with two physically distinct complexes: the coactivator Mediator and the corepressor Jarid1a. The existence of these distinct repressor and activator complexes is recapitulated on the β-globin locus, where the G9a–Mediator complex is bound to the active β^{maj} gene, whereas the G9a–Jarid1a complex is bound to the repressed E^y gene (Fig. 5). On the β^{maj} promoter, G9a stabilizes binding of the Mediator complex (Fig. S5) to enable formation of the preinitiation complex and maximal transactivation of this gene (12) (Fig. 3*B*). In contrast, on the E^y promoter, G9a is required for stable binding of Jarid1a (Fig. 4*B*) and for the maintenance of the E^y gene in a repressed state through cross-talk with Jarid1a (Fig. 5). Future studies are required to determine what regulates the association of G9a with Jarid1a vs. Mediator.

Previously, it was shown that the H3K4 methyltransferase MLL2/KMT2D is bound to both the silenced gene E^y and the active gene β^{maj} in differentiated erythroid cells (41). However, the MLL2-dependent H3K4me3 mark was detected on the β^{maj} gene but not on the E^y gene. At this point, it was not clear whether MLL2 is enzymatically inactive on the E^y gene or whether the H3K4me3 mark is dynamically removed. The finding here that Jarid1a demethylates H3K4me3 on the E^y gene promoter suggests that MLL2 could be enzymatically active at this location but that the H3K4me3 mark is removed by Jarid1a. In contrast, the adult β^{maj} gene, which is highly enriched for H3K4me3, is not bound by Jarid1a. Instead, this gene is bound by another demethylase (i.e., UTX), which actively removes the G9a-dependent repressive mark H3K27me2, thereby allowing activation of the β^{maj} gene (12). The presence of both MLL2 and UTX on the adult β^{maj} gene suggests that cross-talk between these histone-modifying enzymes is mediating activation of this gene. Therefore, there are at least two different examples of cross-talking between histone methyltransferases and demethylases that take place on the β-globin locus, one that activates transcription and one that represses transcription (Fig. 5, model).

Finally, it is interesting to note that although the repressed E^y gene and the active β^{maj} gene are bound by both the histone methyltransferases MLL2 and G9a, the active vs. repressed status of these genes correlates with the presence of the histone demethylases (UTX vs. Jarid1a).

Taken together, these results reveal that "active" and "repressive" cross-talk of histone-modifying enzymes coexists on the same multigene locus and plays a crucial role in the precise control of developmentally regulated gene expression.

Materials and Methods

Abs. Abs used in this study are described in *SI Materials and Methods*.

Nuclear Extraction and IPs. Nuclear extraction and IPs were performed as described (41), except that proteins were eluted at 37 °C by 30 min of vortexing at 200 × *g* in a 6-M urea buffer containing 0.05% SDS, 50 mM Tris (pH 8.3), and 5 mM EDTA. Eluted proteins were analyzed by Western blot and/or digested with trypsin and analyzed by liquid chromatography (LC)-tandem MS (MS/MS). Before performing IPs for Western blot analysis, ethidium bromide was added to the nuclear extract at a final concentration of 100 μg/mL.

LC-MS/MS. LC-MS/MS was performed on a linear trap quadrupole (LTQ) mass spectrometer with a nanospray ion source and Surveyor HPLC (Thermo). Data analysis was performed using the Trans-Proteomics Pipeline (TPP), version 4.0 (42), with SEQUEST and the International Protein Index mouse database (43). A probability of identification for each protein was determined using the ProteinProphet algorithm (44) integrated in the TPP.

Size Exclusion Chromatography. Nuclear extracts from MEL and mES cells were resolved by size exclusion chromatography using a Superose-6 10/300 GL column on an AKTA Explorer FPLC system following the instructions of the manufacturer (Amersham). Fractions of 0.5 mL were collected, precipitated with trichloroacetic acid (TCA), and analyzed by Western blot.

MEL Clones with Inducible KD of Jarid1a and G9a. MEL clones with Dox-inducible G9a KD were described previously (12). MEL clones with an inducible KD of Jarid1a and clones with inducible double KD of G9a plus Jarid1a were established as previously described (12). Details are provided in *SI Materials and Methods*. KD was induced by treating cells with 5 μg/mL Dox for 4 d.

ChIPs. Histone ChIPs were performed using a native ChIP protocol (45). X-ChIPs were performed as described (41). Real-time quantitative PCR (qPCR) was done on a Rotorgene 6000 (Corbett Research) using TaqMan probes and primers. ChIP qPCR signals are presented as a percentage of input. Statistical significance was determined using a Student *t* test. Abs used for ChIP and primers/probes used for qPCR are discussed in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Abs. ***Abs used for Western blot.*** From Santa Cruz Biotechnology, we used anti-$TF_{II}Hp89$ (sc-293), anti-Med1(sc-8998), anti-Med12 (sc-5372), anti-Med17 (sc-12453), anti-Med26 (sc-137196), anti-Sin3a (sc-767), anti-DNMT3a (sc-20703), and anti-DNMT3b (sc-20704). From Perseus Proteomics, we used anti-G9a (PP-A8620A-00) and anti-GLP (PP-B0422-00). From Abcam, we used anti-Med12 (ab70842), anti-Med14 (ab72141), anti-DNMT1 (ab87656), and anti-Suz12 (ab12073). From Active Motif, we used anti-Ezh2 (39103). From Bethyl, we used anti-Jarid1a (a300-8974). From Covance, we used Abs against nonphosphorylated Pol II (8WG16). From Upstate/Millipore, we used anti-His tag (05-531) to detect Ubc4 and anti-HDAC1 (06-720). From ProSci, Inc., we used anti-Wiz (6117). The Ash2L Ab was described previously (1).

Abs used for immunoprecipitation. The Abs used for immunoprecipitations (IPs) were the same as described above. In addition, for mock IPs, we used normal rabbit (sc-2027) and mouse (sc-2025) IgG from Santa Cruz Biotechnology.

Abs used for ChIP. The Abs used for ChIP were the same as described above, with the following exceptions. From Abcam, we used anti-Jarid1a (ab65769). From Santa Cruz Biotechnology, we used anti-Med12 (sc-5372) and anti-Med17 (sc-12453). In addition, from Abcam, we used anti-trimethylated histone H3 lysine 4 histone mark (H3K4me3; ab8580) and anti-dimethylated H3K9 (H3K9me2; ab1220). For mock ChIPs, we used normal rabbit (sc-2027), mouse (sc-2025), and goat (sc-2028) IgG from Santa Cruz Biotechnology.

Real-Time RT-Quantitative PCR Analysis. Total RNA was isolated using the RNA STAT-60 (TEL-TEST, Inc.) according to the manufacturer's instructions. One-step real-time RT-quantitative PCR (qPCR) was done on a Rotorgene instrument 6000 (Corbett Research) using TaqMan probes and primers. Statistical significance was determined using a Student *t* test.

Primers and Probes Used for Real-Time RT-qPCR. For *G9a*, the following primers and probes were used:

Forward primer: AAAACCATGTCCAAACCTAGCAA
Reverse primer: GCGGAAATGCTGGACTTCAG
TaqMan probe: FAM-ACAGCCTCCAATCCCTGAGAAGC-GG-BHQ1, which incorporates a fluorophore (FAM) and blackhole quencher (BHQ1)

For *Jarid1a*, the following primers and probes were used:

Forward primer: CATGGGCTCTAGTCTCTATGTGGA
Reverse primer: GCCTGCTGGAGCTCTTGCT
TaqMan probe: FAM-TACCTGAATTGCCCCGACT-BHQ1

For *Ey*, the following primers and probes were used:

Forward primer: GCAAGAAGGTGCTGACTGCTT
Reverse primer: GTAGCTTGTCACAGTGCAGTTCACT
TaqMan probe: FAM-TGGAGAGTCCATTAAGAACCTA-GACAACCTCAAGTC-MGBNFQ

For β^{maj}, the following primers and probes were used:

Forward primer: GAAGGCCCATGGCAAGAAG
Reverse primer: GCCCTTGAGGCTGTCCAA
TaqMan probe: FAM-TGATAACTGCCTTTAACGATGG-CCTGAATCA-MGBNFQ, which incorporates a minor groove binder (MGB) and nonfluorescent quencher (NFQ)

For *GAPDH*, the following primers and probes were used:

Forward primer: TTGTGGAAGGGCTCATGA
Reverse primer: CATCACGCCACAGCTTT
TaqMan probe: FAM-CATGCCATCACTGCCACCCA-BHQ1

Primers and Probes Used for Real-Time qPCR. For HS2, the following primers and probes were used:

Forward primer: CAGAGGAGGTTAGCTGGGCC
Reverse primer: CAAGGCTGAACACACCCACA
TaqMan probe: FAM-AGGCGGAGTCAATTCTCTACTCCC-CACC-BHQ1

For E^y, the following primers and probes were used:

Forward primer: CTTCAAAGAATAATGCAGAACAAAGG
Reverse primer: CAGGAGTGTCAGAAGCAAGTACGT
TaqMan probe: FAM-ATTGTCTGCGAAGAATAAAAGG-CCACCACTT-BHQ1

For β^{maj}, the following primers and probes were used:

Forward primer: CTGCTCACACAGGATAGAGAGGG
Reverse primer: GCAAATGTGAGGAGCAACTGATC
TaqMan probe: FAM-AGCCAGGGCAGAGCATATAAG-GTGAGGT-BHQ1

G9a-Jarid1a Direct Interaction. Recombinant purified G9a was a kind gift from S. Pradhan (New England Biolabs). Recombinant purified Jarid1a was purchased from BPS Bioscience (catalog no. 50110). His-tagged Ubc4 was produced in bacteria and purified by Ni^{2+} chromatography. The G9a-Jarid1 direct interaction experiment was performed as previously described (2).

Cell Culture. Murine erythroid (MEL) cells (clone 745) were cultured in RPMI containing 10% (vol/vol) FBS, 1% glutamine, and antibiotics, and they were differentiated toward the erythroid lineage by adding 2% (vol/vol) DMSO for 4 d as previously described (3). E14 murine embryonic stem (mES) cells (4) were cultured on 0.1% gelatin-coated tissue culture plastic in DMEM supplemented with 15% (vol/vol) FBS, 10^3 U/mL leukemia inhibitory factor, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/mL penicillin/streptomycin, and 10^{-4} M β-mercaptoethanol. Cells were routinely passaged using 0.05% trypsin every 2–3 d. mES cells were grown to ∼70% confluency and harvested using TrypLE cell dissociation reagent (Gibco).

Establishment of MEL Clones with Inducible Knockdown of Jarid1a and G9a. To obtain stable MEL clones with a single knockdown (KD) of Jarid1a, we first modified the pGJ10 doxycycline (Dox)-inducible shRNA expression vector (described in 1) to replace the neomycin (Neo)-resistance gene by a puromycin (Puro)-resistance gene. For this purpose, the pGJ10 expression vector was restriction-digested at ClaI/SmaI sites to remove the Neo gene. The Puro DNA fragment was obtained by amplification using gene-specific forward (5′ AAAGAATCGATCAACCATGACCGAGTACAAGC 3′) and reverse (5′ AACCCGGGTCAGGCACCGGGCTTG 3′) primers that inserted ClaI/SmaI restriction sites at the 5′ and 3′ ends of the gene, respectively. The Puro fragment was then inserted into the ClaI/SmaI site of the digested pGJ10 vector. This vector is named pGJ10-puro. Two different shRNA sequences targeting Jarid1a

(shRNA1: 5′ GATCCCGAGGCAATGACTAGAGTAATTCA-AGAGATTACTCTAGTCATTGCCTCTTTTTGGAAA 3′ and shRNA2: 5′ GATCCCGCCAAACTCGACA AGTAAATTCAA-GAGATTTACTTGTCGAGTTTGGCTTTTTGGAAA 3′) were then cloned into pGJ10 and/or pGJ10-puro vector at Bgl II/Not I restriction sites to obtain the pGJ10-Jarid1a-sh1, pGJ10-puro-Jarid1a-sh1, and the pGJ10-puro-Jarid1a-sh2 vectors. These vectors were then transfected separately into the MEL/tetracycline repressor (TR) cell line (1) that expresses high levels of the TR. After screening for G418- and/or Puro-resistant cells, stable clones that display a Dox-inducible KD of Jarid1a were selected by Western blot and RT-qPCR. In addition, we verified that these clones are able to efficiently differentiate toward the erythroid lineage in the absence of Dox.

To obtain stable MEL clones with a double KD of G9a plus Jarid1a, the previously described Neo-resistant G9a KD MEL cell line, which expresses anti-G9a shRNA (5′ CCCTGATCTTT-GAGTGTAA 3′) in a Dox-inducible manner (2), was transfected with the pGJ10-puro-Jarid1a-sh1 and the pGJ10-puro-Jarid1a-sh2 vectors, separately. MEL cells that are resistant to both G418 and Puro were selected and screened by Western blot for efficient Dox-inducible double KD of G9a plus Jarid1a.

1. Demers C, et al. (2007) Activator-mediated recruitment of the MLL2 methyltransferase complex to the beta-globin locus. *Mol Cell* 27(4):573–584.
2. Chaturvedi CP, et al. (2009) Dual role for the methyltransferase G9a in the maintenance of beta-globin gene transcription in adult erythroid cells. *Proc Natl Acad Sci USA* 106(43): 18303–18308.
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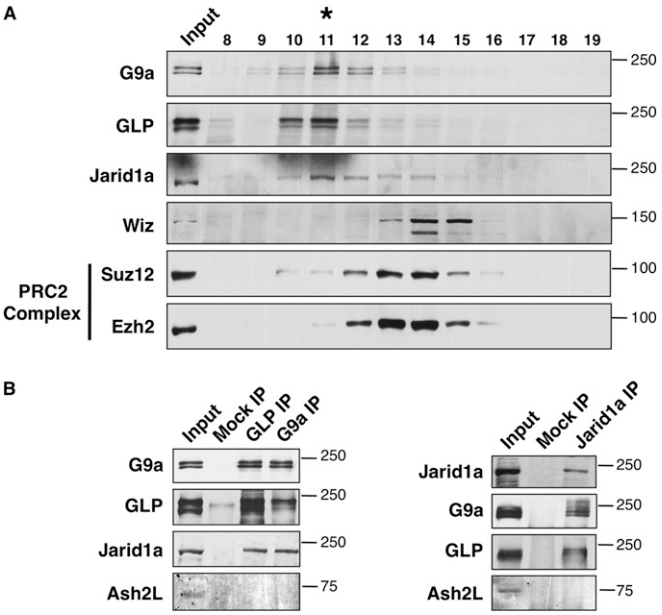

Fig. S1. Jarid1a associates with the G9a-GLP heterodimer in mES cells. (*A*) G9a, GLP, and Jarid1a coelute by size exclusion chromatography. A mES cell nuclear extract was separated using a Superose-6 column, and the presence of specific proteins in different fractions was revealed by immunoblotting with Abs indicated on the left. Molecular masses (in kilodaltons) are indicated on the right. (*B*) G9a and GLP associate with Jarid1a in mES cell nuclear extracts. Proteins immunoprecipitated via Abs against endogenous G9a, GLP, and Jarid1a were analyzed by Western blot. Mock IPs with normal IgG were used as negative controls. Abs used for Western blot (*Left*) and molecular masses (*Right*; in kilodaltons) are indicated.

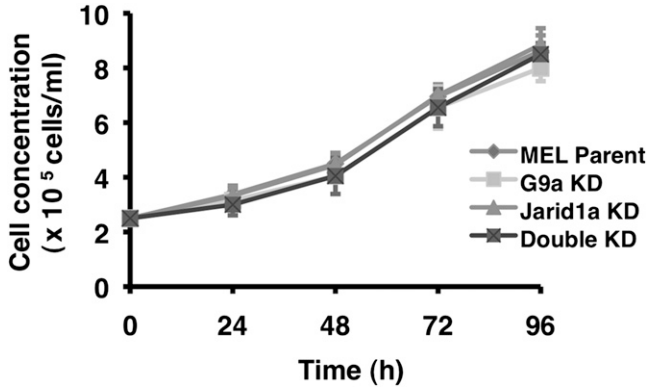

Fig. S2. KDs of Jarid1a and G9a and double KD of Jarid1a plus G9a have no effect on cell growth during erythroid differentiation. Dox-treated MEL cells were counted every 24 h after induction of differentiation (0 h).

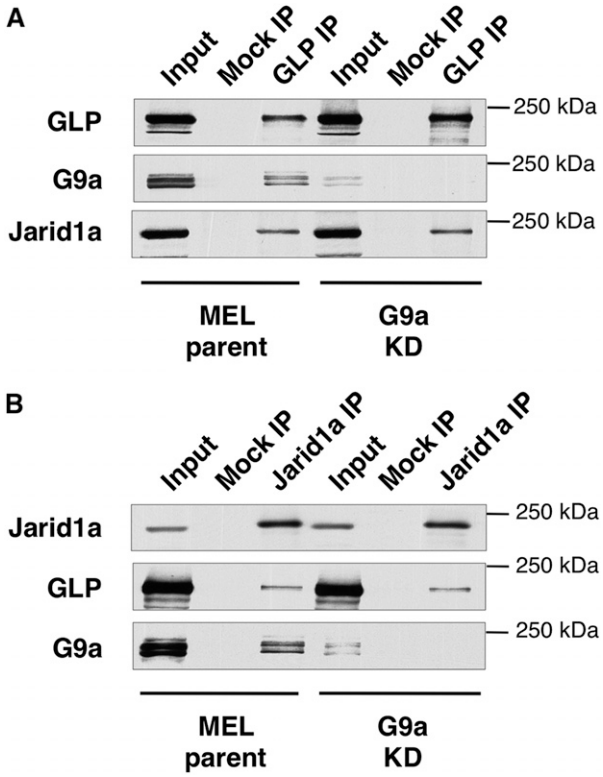

Fig. S3. Jarid1a interacts with GLP in the absence of G9a. (*A*) GLP and associated proteins were immunoprecipitated from a MEL nuclear extract containing a normal (MEL parent) or reduced (G9a KD) amount of G9a. (*B*) Jarid1a and associated proteins were immunoprecipitated from a MEL nuclear extract containing a normal (MEL parent) or reduced (G9a KD) amount of G9a. Mock IPs with normal IgG were used as negative controls. Immunoprecipitated proteins were analyzed by Western blot. Abs used for Western blot (*Left*) and molecular masses (*Right*; in kilodaltons) are indicated.

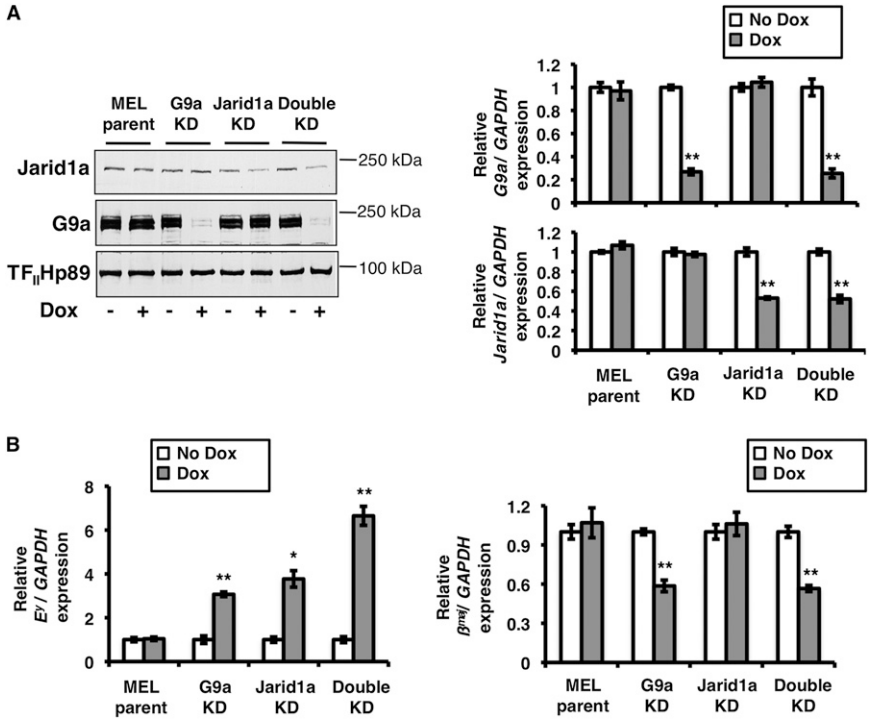

Fig. S4. Jarid1a and G9a cooperate in the maintenance of gene repression. (*A*) Establishment of stable MEL cell clones, where the single KDs of Jarid1a or G9a and the double KD of Jarid1a plus G9a are induced by Dox-mediated expression of specific shRNAs. This figure shows the results using Jarid1a shRNA2 (*SI Materials and Methods*). The levels of Jarid1a and G9a in Dox-treated (Dox) vs. untreated (No Dox) cells were analyzed at the protein level by Western blot of nuclear extracts using indicated Abs (*Left*) and at the mRNA level by real-time RT-qPCR (*Right*). MEL parent represents a control cell line with no induction of shRNA on Dox treatment. (*Left*) Molecular masses of proteins (in kilodaltons) are indicated on the right. (*Right*) Transcript levels are normalized to *GAPDH* with the ratio observed in the absence of Dox set to 1. (*B*) Additive effect of the double Jarid1a plus G9a KD on derepression of the E^y *globin* gene. Transcript levels were measured by real-time RT-qPCR in Dox-treated/untreated cells. Transcript levels are normalized to *GAPDH* with the ratio observed in the absence of Dox set to 1. (*A* and *B*) Average values from triplicate experiments are represented with error bars corresponding to SDs. $*P < 0.05$; $**P < 0.01$.

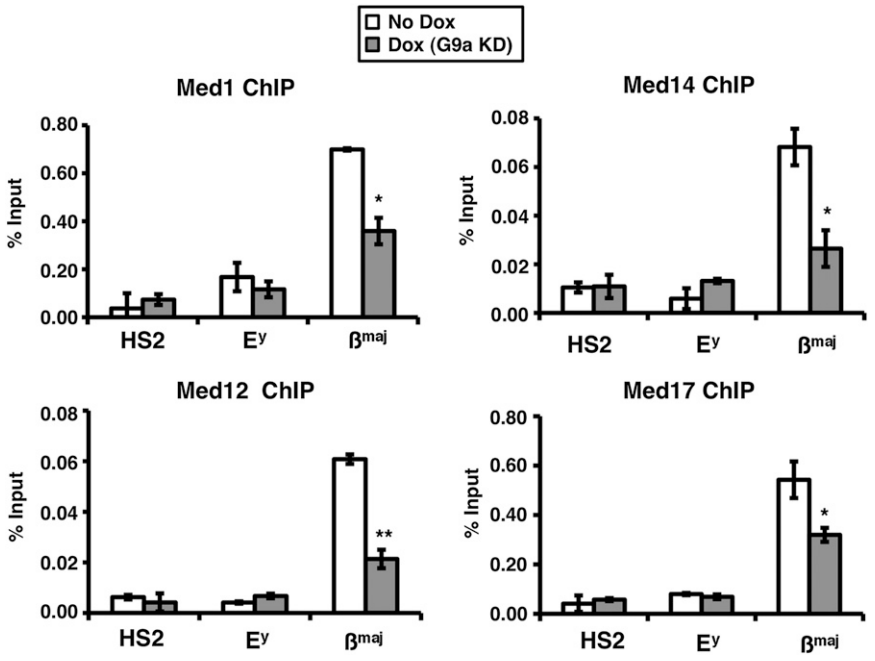

Fig. S5. G9a stabilizes binding of the Mediator complex to the promoter of the β^{maj} *globin* gene in MEL cells. ChIP experiments using Abs against the indicated Mediator complex subunits were performed in the presence of normal (No Dox) or reduced (Dox) levels of G9a. ChIPs were revealed by real-time qPCR using Taqman probes located at the HS2 site of the locus control region and at the promoters of the E^y and β^{maj} genes. Error bars represent SDs calculated from triplicate experiments. $*P < 0.05$; $**P < 0.01$.

Other Supporting Information Files

Dataset S1 (XLSX)