Stable X chromosome inactivation involves the PRC1 Polycomb complex and requires histone MACROH2A1 and the CULLIN3/SPOP ubiquitin E3 ligase


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X inactivation involves the stable silencing of one of the two X chromosomes in XX female mammals. Initiation of this process occurs during early development and involves Xist (X-inactive-specific transcript) RNA coating and the recruitment of Polycomb repressive complex (PRC) 2 and PRC1 proteins. This recruitment results in an inactive state that is initially labile but is further locked in by epigenetic marks such as DNA methylation, histone hypoacetylation, and MACROH2A deposition. Here, we report that the E3 ubiquitin ligase consisting of SPOP and CULLIN3 is able to ubiquitinate the Polycomb group protein BMI1 and the variant histone MACROH2A. We find that in addition to MACROH2A, PRC1 is recruited to the inactivated X chromosome in somatic cells in a highly dynamic, cell cycle-regulated manner. Importantly, RNAi-mediated knock-down of CULLIN3 or SPOP results in loss of MACROH2A1 from the inactivated X chromosome (Xi), leading to reactivation of the Xi in the presence of inhibitors of DNA methylation and histone deacetylation. Likewise, Xi reactivation is also seen on MacroH2A1 RNAi under these conditions. Hence, we propose that the PRC1 complex is involved in the maintenance of X chromosome inactivation in somatic cells. We further demonstrate that MACROH2A1 deposition is regulated by the CULLIN3/SPOP ligase complex and is actively involved in stable X inactivation, likely through the formation of an additional layer of epigenetic silencing.

Bmi1 | SPOP/CULLIN3 E3 ligase | X inactivation | Polycomb silencing

Induction of facultative heterochromatin is a tightly regulated process in which specific loci are packaged into heterochromatin in a manner that depends on cell type. The best known example is the condensation and inactivation of one of the X chromosomes in cells of female mammals. Initiation of X inactivation occurs during early embryonic development, when the noncoding RNA Xist (X-inactive-specific transcript) coats the inactive X chromosome and the initial cis-spread triggers a stepwise series of alterations in chromatin structure that culminate in formation of facultative heterochromatin. The stably inactivated X chromosome (Xi) bears several hallmarks of constitutive heterochromatin, such as delayed replication kinetics (1), histone hypoacetylation (2), and DNA hypermethylation (3). Moreover, Xi chromatin is enriched in the variant histone MacroH2A (4). Hence, X chromosome inactivation involves multiple interdependent layers of epigenetic repression (5–8).

Polycomb group (PcG) proteins are epigenetic gene regulators acting in large multimeric protein modules. Biochemically, PcG proteins separate into two distinct complexes. In human cells, the initiation core complex [Polycomb repressive complex (PRC) 2] contains EZH2, EED, and SUZ12, and the maintenance core complex (PRC1) consists of BMI1, RNF2/RING1B, EDR1/HPH1, and CBX4/HPC2, among other mammalian homologues of the Drosophila proteins Posterior sex combs, dRing1, Polyhomeotic, and Polycomb. PcG complexes interact with chromatin at target genes to impose gene repression, which is thought to be mediated through deacetylation, methylation, and ubiquitination of canonical core histones (9–13).

The role of PcG proteins in the initiation of X chromosome inactivation has started to be unveiled. One of the earliest Xist RNA-dependent events is the recruitment of PRC2, which methylates lysine 27 of histone H3 (14–17). This signal is likely recognized by the Rnf2/Ring1b, Rnf110/Mel18, and Phc2/ Mph2 PRC1-containing complex, and Rnf2/Ring1b, in turn, monoubiquitinates H2A both in embryonic and extraembryonic stem cells (9, 13, 18).

The PRC1 protein Bmi1 was originally identified as an oncogenic collaborator with Myc (19), a function in part mediated through repression of the Cdkn2a tumor suppressor locus (20, 21). Bmi1-deficient mice display homeotic skeletal transformations typical for PcG mutations (22) and have severe defects in stem cell maintenance in both hematopoietic (23, 24) and neuronal tissues (25, 26).

To better understand BMI1 functions, we performed yeast two-hybrid screens using BMI1 as a bait and found SPOP as an interacting protein. Here, we describe an E3 ubiquitin ligase consisting of SPOP and CULLIN3 that is able to ubiquitinate the PcG protein BMI1 and the variant histone MACROH2A1. We also report that the PRC1 proteins BMI1, RNF2/RING1B, and CBX4/HPC2 are recruited to the Xi in a cell cycle-dependent manner. Importantly, functional analysis revealed that SPOP and CULLIN3 are required for MACROH2A1 deposition at the Xi and, together with MACROH2A1, for the maintenance of stable X chromosome inactivation.

Materials and Methods

Antibodies. Detailed information about antibodies can be found in Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

Plasmids. The yeast two-hybrid fusion vector and expression vectors for full-length and deletion mutants of BMI1 are described in refs. 27 and 28. Details on other plasmids used can be found in Supporting Materials and Methods.

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Abbreviations: PcG, Polycomb group; PRC, Polycomb repressive complex; Xi, inactivated X chromosome; Xist, X-inactive-specific transcript; S-Aza-dC, S-aza-2′-deoxycytidine; TSA, trichostatin A; HA, hemagglutinin.

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Cell Culture, Transfections, and Retroviral Infections. All of the somatic cells were maintained in DMEM supplemented with 10% FBS (GIBCO) under standard conditions. Details on transfection, transduction, and the generation of retroviral stocks can be found in Supporting Materials and Methods. Synchronization of 293HEK cells in S phase was attained by a double thymidine block as described in ref. 28. At the indicated times after the release of the block, cells were fixed and stained.

Immunoprecipitations and Western Blot Analysis. For immunoprecipitations of protein complexes, transiently transfected 293HEK cells were lysed in ELB buffer (0.1% Triton X-100/250 mM NaCl/50 mM Tris, pH 7.4/1 mM EDTA/protease and phosphatase inhibitors). Before immunoprecipitation, lysates were precleared by using protein A-Sepharose beads. Stringent lysis was performed by using RIPA buffer (0.15 mM NaCl/0.05 mM Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS).

Acidic histone purification was basically performed as described in ref. 9. For detection of endogenous proteins, nuclei were isolated (9) and lysed in RIPA buffer. Micrococcal nuclease, nuclease and in vitro ubiquitination procedures can be found in Supporting Materials and Methods.

Flow Cytometry and Analysis of GFP Expression. Flow-cytometric analysis was performed on single-cell suspensions after staining the cells under standard conditions with propidium iodide and directly fluorochrome-conjugated monoclonal antibodies against BrdUrd. For the analysis of GFP expression by FACS, transformed mice carrying a GFP transgene on the Xi were used as described in ref. 5. These cells were either mock-infected or infected with RNAis specific for SPOP, MACROH2A1, or CULLIN3 and selected with puromycin for 3 days. After selection, the cells were exposed to 5-aza-2’-deoxycytidine (5-Aza-dC) (4 days at 300 nM) and to 500 nM trichostatin A (TSA) for the last 24 h. The cells were subjected to FACS analysis counting a minimum of 100,000 cells per sample.

Results and Discussion

To identify proteins interacting with BMI1, we performed two-hybrid screens with full-length BMI1 as bait. Among several previously described interacting proteins (27), a clone containing full-length speckle-type POZ protein, SPOP (29), was identified (data not shown). The SPOP protein encodes an N-terminal MATH domain and a C-terminal BTB/POZ domain, the latter of which was recently shown to interact with CULLIN3 (30). CULLIN3 together with ROC1 constitute distinct ubiquitin E3 ligases with individual BTB proteins that potentially target ubiquitination of many substrate proteins (30–32). Immunoprecipitation experiments using epitope-tagged expression constructs verified the interaction between BMI1 and SPOP and identified the domains involved (Fig. 1 a and b). Full-length SPOP, as well as an N-terminal part including the MATH domain, was found to interact with BMI1, whereas BMI1 uses both its central and C-terminal parts to bind to SPOP (Fig. 1c). Immunostainings of endogenous SPOP in HeLa cells demonstrate that SPOP is predominantly nuclear, with few prominent spots in each nucleus (Fig. 1d). Costainings show that BMI1 in these cells distributes into a fine-speckled nuclear pattern, partially overlapping with SPOP (Fig. 1d).

To investigate whether BMI1 can form a complex with SPOP and CULLIN3 in vivo, we reconstituted the complex in 293HEK cells. We find that BMI1 readily immunoprecipitates both hemagglutinin (HA)-SPOP and CULLIN3, and, conversely, CULLIN3 immunoprecipitates BMI1 (Fig. 2a). Complex formation depends on the presence of SPOP, in accordance with BMI1 binding to the MATH domain of SPOP (Fig. 1b) and previously published data showing SPOP–CULLIN interaction by means of the BTB/POZ domain of SPOP (30). The variant histone protein MACROH2A1 has recently been reported to interact with the MATH domain of SPOP in GST pull-down assays (33). Using immunoprecipitation from transiently transfected 293HEK cells, we verified and extended this observation to show that MACROH2A1 also forms a complex with CULLIN3 and SPOP (Fig. 2b).

We next analyzed whether the assembled protein complexes constitute active E3 ligases for ubiquitination. Upon immunoprecipitations of BMI1 (Fig. 2c) or MACROH2A1 (Fig. 2d) from stringent cell lysates, ubiquitination of both proteins was readily detected, whereas PCNA remained unmodified under the same conditions (Fig. 5a, which is published as supporting information on the PNAS web site), indicating specificity. Importantly, for both BMI1 and MACROH2A1, ubiquitination depends on expression of both CULLIN3 and SPOP. Because we were not
products are indicated with asterisks.

ubiquitin molecules ligated to the BMI1 protein. BMI1 protein degradation samples were resolved on SDS without E1, E2, the immunoprecipitated E3 ligase complex, and ubiquitin, and

fected 293 cells is indicated with

ubiquitin. Ubiquitinated proteins are marked with black dots. (Fig.

In vitro ubiquitination of BMI1 by CULLIN3/SPOP/ROC1. E3 ligase complex was obtained by transfection of 293 cells with FLAG-CULLIN3, HA-SPOP, and Myc-ROC1 expression vectors and immunoprecipitation with anti-FLAG antibody (see Supporting Materials and Methods). Purified BMI1 was incubated with or without E1, E2, the immunoprecipitated E3 ligase complex, and ubiquitin, and samples were resolved on SDS/PAGE. Immunoprecipitation with nontransfected 293 cells is indicated with –. Black dots indicate the number of ubiquitin molecules ligated to the BMI1 protein. BMI1 protein degradation products are indicated with asterisks.

able to communoprecipitate BMI1 and MACROH2A1, the data suggest that these proteins likely reside in independent CULLIN3/SPOP complexes or only interact transiently (data not shown). Hence, we conclude that SPOP functions as a substrate-specific adaptor protein capable of tethering BMI1 and MACROH2A1 to CULLIN3, resulting in ubiquitination of BMI1 and MACROH2A1. Because ubiquitination appears to be a prerequisite for proteasomal degradation of many proteins, we analyzed the effect of RNAi-mediated knock-down of CULLIN3 or SPOP (Fig. 5b) on the overall levels of BMI1 and MACROH2A1 in the 293HEK cell line. No significant changes in protein stability were observed, suggesting that ubiquitination serves regulatory functions other than protein degradation of BMI1 and MACROH2A1 (Fig. 5b).

We next analyzed the ubiquitination status of nucleosome-associated MACROH2A1, focusing on endogenous MACROH2A1 from nucleosomes purified from 293HEK cells or on MACROH2A1-GFP protein from nucleosomes of a 293HEK cell line with stable and moderate overexpression of the MACROH2A1-GFP transgene. The nucleosomal preparations were analyzed by immunoprecipitation and Western blotting for MACROH2A1, GFP, and ubiquitin. Endogenous MACROH2A1, as well as the MACROH2A1-GFP fusion protein, were found to be ubiquitinated and migrate mostly at the size of a monoubiquitinated protein species. In addition, polyubiquitination of MACROH2A-GFP fusion protein from the nucleosome fraction could also be detected (Fig. 2f). At present, we cannot discriminate the main role in vivo of the polyubiquitinated or monoubiquitinated MACROH2A1, because both modifications can be detected (Fig. 2 d and e and ref. 34).

Polyubiquitination of endogenous BMI1 could not be detected, suggesting that only a small amount of the protein undergoes this modification. To confirm direct BMI1 ubiquitination by the CULLIN3/SPOP E3 ligase, we performed an in vitro ubiquitination assay. As Fig. 2f shows, BMI1 was readily ubiquitinated in vitro by this E3 ligase, indicating that BMI1 is a bona fide target of the CULLIN3/SPOP/ROC1 complex.

Because both MACROH2A1 and BMI1 are processed by the same CULLIN3/SPOP-dependent ubiquitination mechanism, we analyzed whether the proteins colocalize in somatic cells. In the osteosarcoma cell line U2-OS, PcG proteins have been found to associate predominantly in large protein aggregates (Polycomb bodies) coinciding with amplified regions of α-satellite repeat DNA (28, 35). In these cells, MACROH2A1 distributes in a fine-grained pattern throughout the nucleus, but, in addition, is found to be strongly associated with the Polycomb bodies (Fig. 3a). Because the large Polycomb bodies are most often seen in tumor cell lines, we also analyzed the distribution pattern of BMI1 and MACROH2A1 in early passage female IMR90 primary human lung fibroblasts. In IMR90 cells, BMI1 localizes to a small-speckled pattern with few bright spots (Fig. 3b), whereas staining for MACROH2A1 reveals the well-characterized pattern of Xi enrichment. Intriguingly, we find that BMI1 can also be detected as small foci at the Xi decorated with MACROH2A1 in a small subset of cells (<1%) (Fig. 3b).

Next, we used the 293HEK cell line, which contains a single active X chromosome and a variable number of inactive X chromosomes (one to four copies in different cells) (36). Immunostainings performed in unsynchronized 293HEK cells clearly revealed that MACROH2A1 shows a pattern consistent with enrichment on the inactive X chromosomes in 52.6 ± 6.9% of the 293HEK cells (Fig. 3c). In this cell line, BMI1 staining also reveals discrete perinuclear structures in 7.6 ± 3.4% of the cells. Notably, colocalization of MACROH2A1 and BMI1 was clearly observed in 5.3 ± 1.7% of the cells (Fig. 3c).

To investigate whether additional PRC1 members associate to the Xi, we carried out stainings for RNF2/RING1B and CBX4/HPC2 on asynchronous 293HEK cell cultures. Similar to BMI1, RNF2/RING1B and CBX4/HPC2 could also be identified on the Xi (Fig. 3d and data not shown), indicating a more general role for the PRC1/PcG complex rather than a function unique to BMI1. Double staining for BMI1 and RNF2/RING1B revealed that these two proteins colocalize at the Xi in the same subset of cells, suggesting that they are recruited together at the same Xi (Fig. 3e). Combined RNA FISH for the XIST RNA with immunological detection of BMI1 (Fig. 3f) or RNF2/RING1B (Fig. 3g) in 293HEK cells and for the Xist RNA and Rnf2/Ring1b in mouse embryo fibroblasts confirmed the Xi association (Fig. 3h). CULLIN3 staining revealed general localization...
Methods

DNA, different stages of S phase were established. The patterns of distribution of BMI1, RNF2 and MACROH2A1 were immunostained. More than 350 cells were scored for the presence of BMI1 staining at the Xi of 293HEK cells, we first analyzed RNF2 localization in female somatic cell lines. (Fig. 3a–h). RNF2 and MACROH2A1 staining at the Xi was obtained with cells synchronized in early S phase by a double thymidine block and subsequently released from the blockade (Fig. 3j).

We next investigated whether BMI1 recruitment to the Xi occurs late in development and so is restricted to differentiated somatic cells or whether, by contrast, BMI1 is also involved in early stages of X inactivation. By using trophoblast stem cells and a mouse XY embryonic stem cell line in which the endogenous Xist gene is under the control of a tetracycline-inducible promoter, we could observe that BMI1 was also allocated to the Xi after Ezh2 recruitment (Fig. 6, which is published as supporting information on the PNAS web site). Recent reports indicate that a subset of PRC1 proteins (Rnf2/Ring1B, Rnf110/Mel18, and Phc2/Mph2) are transiently enriched on the Xi in early development (13, 18). Our work supports and extends these results with the observed dynamic recruitment of BMI1, RNF2, RING1B, and MACROH2A1 to the inactive Xi in somatic cells, further highlighting the potential role of PRC1 in X chromosome inactivation.

To explore a mechanistic link between the CULLIN3/SPOP ubiquitin ligase complex and the Xi localization of MACROH2A1, RNAi expression vectors targeting CULLIN3 and SPOP were used (Fig. 5b). In 293HEK cells transfected with a control RNAi vector directed against GFP, staining for MACROH2A1 clearly reveals the Xi chromosomes in 44.5 ± 3.5% of the cells (Fig. 4a). Importantly, RNAi-mediated knock-down of CULLIN3 abolished correct localization of MACROH2A1 to the Xi, with residual Xi staining visible in only 11.2 ± 2.0% of the cells. Likewise, in cells containing an SPOP RNAi, only 10.4 ± 1.9% of the cells had a noticeable MACROH2A1 staining at the Xi (Fig. 4a). Previous studies showed that Macroh2a1 deposition to Xi requires Xist RNA (39). To rule out any effect on XIST localization, combined RNA FISH and immunodetection of MACROH2A1 were performed and indicate that XIST coating of the Xi is not affected in cells with CULLIN3 or SPOP knock-down (Fig. 7, which is published as supporting information on the PNAS web site). In contrast to the CULLIN3 and SPOP RNAi effects, BMI1 RNAi did not lead to major changes in MACROH2A1 localization (Fig. 4a). Notably, no changes in the overall levels of MACROH2A1 or BMI1 could be detected, excluding gross effects on protein stabilization (Fig. 5b). Because CULLIN3 reportedly is involved in the regulation of a number of proteins, among them DNA topoisomerase I (40) and cyclin E (41), CULLIN3 knock-down could have pleiotropic effects and potentially cause cell-cycle arrest. To rule out that the delocalization of MACROH2A1 that we observe in CULLIN3 knock-down cells is due to aberrant cell-cycle arrest in specific stages of the cycle lacking MACROH2A1 enrichment at the Xi, the cell-cycle profile of 293HEK cells treated with RNAi vectors against GFP and CULLIN3 was analyzed by using FACS analysis. We did not observe major cell-cycle deviations as a result of RNAi treatment 3 days after the transient transfections (Fig. 8a, which is published as supporting information on the PNAS web site). We conclude that both CULLIN3 and SPOP are required for correct localization of MACROH2A1 to the Xi, and the data thus strongly suggest that ubiquitination events are involved in specific MACROH2A1 localization, although we currently cannot discriminate whether this effect is direct or indirect.

To investigate the functional significance of the CULLIN3/SPOP E3 ubiquitin ligase complex in X chromosome inactiva-

![Image](https://www.pnas.org/cgi/doi/10.1073/pnas.0408918102)
with the X-inactivated GFP treatment of the cells with 5-azadC for 4 days and with TSA for 1 day, different RNAis, selected with puromycin, and allowed to recover. After Hernandez-Munoz cells have previously been used to study the synergism of harboring a silent GFP reporter integrated on the Xi. Similar tion, we made use of a female mouse fibroblast cell line, uXGFP, MACROH2A1 localization and X chromosome inactivation. (5) and to analyze the involvement of BRCA1 in Xist localization to the Xi. uXGFP cells were transduced with retroviral vectors carrying RNAi cassettes targeting Cullin3, Spop, and MacroH2A1 along with a puromycin marker gene. After puromycin selection, the cells were split into two populations, one of which was treated with the demethylating agent 5-Aza-dC for 4 days, followed by a 12-h treatment with the histone deacetylase inhibitor TSA. Reactivation of the silent GFP was subsequently quantified by using FACS analysis. Mock-transduced uXGFP cells have a low spontaneous rate of Xi reactivation, which can be only slightly increased (1%) by treating the cells with 5-Aza-dC in combination with TSA (Fig. 4b and ref. 5). Similarly, no significant reactivation could be detected in cells in which Macroh2a1 has been stably suppressed by using the RNAi vector alone. However, when the same culture was treated with both 5-Aza-dC and TSA, a prominent reactivation of the X chromosome in 5.6% of the cells could be detected. This result is in line with the previously reported prominent synergism between loss of Xist and 5-Aza-dC in X reactivation (5) and suggests that loss of Macroh2a1 upon Xist deletion mediates this reactivation. It further provides direct evidence that Macroh2a1 acts as an additional epigenetic silencing mechanism acting on top of DNA methylation and post-translational modification of canonical histones (Fig. 4b). Notably, in the presence of 5-Aza-dC and TSA, RNAi-mediated knock-down of CULLIN3 and SPOP also results in Xi reactivation in a significant proportion (4.5% and 4.1%, respectively) of the analyzed cells (Fig. 4b). To nullify the possibility that off-target effects of the RNAis were responsible for the observed Xi reactivation, additional RNAi cassettes were analyzed and found to have similar effects (Fig. 8 b and c).

Although it is formally possible that the GFP transgene may be subject not only to Xist RNA-mediated silencing but also to some degree to other heterochromatin silencing mechanisms, which may explain the moderate percentage of reactivation observed (5), this model has been used in other studies and proved to be a reliable tool to analyze X chromosome silencing mechanisms (5, 42).

GFP reactivation data are in accordance with the disappearance of Xi-associated MACROH2A1 in 293HEK cells transduced with the RNAi constructs against CULLIN3 and SPOP (Fig. 4a) and point to a pivotal role for the CULLIN3/SPOP ubiquitin ligase complex in allocating MACROH2A1 to the Xi. Importantly, this study suggests that MACROH2A1 deposition at the Xi is not a mere consequence or correlate of silencing; instead, it points to direct functions of MACROH2A1 in stable X chromosome inactivation. Finally, it will be interesting to know whether MACROH2A2, another member of the MACROH2A core histone family with similar, although not identical, nuclear distribution (36, 43), might behave similarly to MACROH2A1 and could therefore function as a redundant mechanism for X chromosome inactivation.

Here, we report that BMI1 and MACROH2A1 are able to bind specifically to the substrate recognition domain of the ubiquitin E3 ligase consisting of SPOP and CULLIN3. The finding that BMI1 and MACROH2A1 can be processed by means of the same ubiquitination machinery and are recruited to overlapping subnuclear locations in a highly dynamic S-phase-dependent fashion could account, at least in part, for the recently reported Xi-specific enrichment in ubiquitinated proteins (44) and is suggestive of a functional link between PcG-mediated transcriptional repression and deposition of variant histones. Interestingly, a number of recent studies have identified intersections between the PRC1 PcG proteins and the machineries responsible for DNA replication and histone deposition (45, 46). Furthermore, RNF2/RING1B has been shown to ubiquitinate the histone H2A, and both RNF2/RING1B and H2A are transiently enriched in the inactive X chromosome in the initiation stage of the X silencing (13, 18). Because Xist-dependent PRC2 recruitment occurs during initiation of Xi but seems to be
largely dispensable in differentiated cells (47, 48), it will be of great importance to determine whether the PRC1 complex functions to maintain PcG-mediated silencing of the Xi after Xist and the PRC2 proteins have left the Xi.

We show that knock-down of CULLIN3 or SPOP impairs localization of MACROH2A1 without affecting overall stability of MACROH2A1 or BMI1, suggesting that ubiquitination of MACROH2A1 is involved in localizing MACROH2A1 to the inactive X chromosome through either a direct or indirect mechanism. Furthermore, we demonstrate that the presence of Macroh2a1 on the Xi is causally related to chromosome silencing. We observe prominent X chromosome reactivation accompanied with loss of Macroh2a1 in the presence of inhibitors of DNA methylation and histone deacetylation only. We observe prominent X chromosome reactivation accompanying with loss of Macroh2a1 in the presence of inhibitors of DNA methylation and histone deacetylation only. In addition, MACROH2A1 RNAi treatment does not appear to affect PRC1 X localization (Fig. 9, which is published as supporting information on the PNAS web site), nor does BMI1 RNAi affect MACROH2A1 localization in a major way, although we cannot rule out that such effects might be masked because of functional redundancy by MACROH2A2 or by BMI1-related PRC1 proteins. Therefore, we propose that MACROH2A1 constitutes an additional epigenetic layer of transcriptional silencing, acting in synergy with other repressive imprints such as DNA methylation, histone H3 and H4 deacetylation, and methylation of lysines 9 and 27 of histone H3.

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