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; 5-Aza-dC, 5-aza-2'-deoxycytidine; TSA, trichostatin A; HA, hemagglutinin.

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Fig. 2. BMI1 and MACROH2A1 interact with and are ubiquitinated by the CULLIN3 and SPOP ligase complex. (a) Complex formation among BMI1, SPOP, and CULLIN3 was determined by coimmunoprecipitations from whole-cell lysates followed by Western blot analyses using the indicated antibodies. (b) As for BMI1, complex formation among MACROH2A1, SPOP, and CULLIN3 was determined by coimmunoprecipitations and analyzed by Western blot analyses using the indicated antibodies. (c and d) Complex formation of CULLIN3, SPOP, and either BMI1 or MACROH2A1 results in E3 ubiquitin ligase activity. Immunoprecipitations from transiently transfected 293HEK cells using the indicated antibodies are shown. Equal expression of all transfected constructs was verified (not shown). Arrows indicate bands representing the Ig heavy chain (hc) and monoubiquitinated MACROH2A1. (e) Analyses of nucleosome-associated MACROH2A1 from native 293HEK cells and from a clone overexpressing MACROH2A1-GFP (indicated above the blot). The cells were analyzed for nucleosome-associated MACROH2A1, MACROH2A1-GFP, and ubiquitin by using immunoblotting. To verify the presence of monoubiquitinated MACROH2A1, lysates from cells expressing MACROH2A1-GFP were immunoprecipitated by using an anti-GFP antibody and subsequently blotted for ubiquitin. Ubiquitinated proteins are marked with black dots. (f) *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 coimmunoprecipitate 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 fusion 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. 2e). At present, we cannot discriminate the main role *in vivo* of the polyubiquitinated or monoubiquitinated MACROH2A1, because both modifications can be detected (Fig. 2d 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 $52.6 \pm 6.9\%$ of the 293HEK cells (Fig. 3c). In this cell line, BMI1 staining also reveals discrete perinuclear structures in $7.6 \pm 3.4\%$ of the cells. Notably, colocalization of MACROH2A1 and BMI1 was clearly observed in $5.3 \pm 1.7\%$ 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

Fig. 3. PRC1 nuclear localization in female somatic cell lines. (a–h) Immunofluorescences with the indicated antibodies were performed on U2-OS (a), IMR90 (b), and 293 (c–e) cells. Combined *Xist* FISH and immunofluorescences of 293 cells (f and g) and mouse embryonic female fibroblasts (h). For a and h, colocalization was analyzed by sequential scanning confocal microscopy; for b–g, epifluorescence microscopy was used. (i) PRC1 localization at the Xi is cell cycle-dependent. Asynchronously growing 293 cells were treated with BrdUrd for 1 h. Then, cells were fixed and stained with antibodies against CBX4/HPC2, RNF2/RING1B, or MACROH2A1 and BrdUrd and with DAPI (see *Material and Methods*). Based on the staining of the incorporated BrdUrd into the nascent DNA, different stages of S phase were established. The patterns of distribution of replication sites are shown in *Upper*. More than 200 cells were scored for CBX4/HPC2, RNF2/RING1B, or MACROH2A1 at the Xi and classified into these different stages of the S phase. (j) 293HEK cells were synchronized in S phase with thymidine, labeled with BrdUrd for 15 min, and released from the blockade for the indicated times. Cells were then fixed and stained with anti-BrdUrd antibody and propidium iodide. Cell cycle profile and BrdUrd incorporation were analyzed by flow cytometry. In parallel, cells growing on glass slides underwent the same thymidine treatment. Then, cells were fixed and immunostained. More than 350 cells were scored for the presence of BMI1, RNF2/RING1B, or MACROH2A1 bodies at the Xi.

throughout the nucleoplasm, without specific enrichment at the Xi (data not shown).

MACROH2A deposition into the Xi is influenced by the cell cycle and is most prominent during S phase (34). Because PRC1 and MACROH2A1 colocalization was observed in only a subset of 293HEK cells, we first analyzed RNF2/RING1B and CBX4/HPC2 distribution in S phase. Based on the S-phase staining

pattern defined by the BrdUrd incorporation (34, 37, 38), we observed that RNF2/RING1B and CBX4/HPC2 locate to the Xi chromosomes primarily in early and mid-S phase. Because MACROH2A1 staining of the Xi can be detected during late G₁ and early and mid-S phase, the recruitment of PRC1 to the Xi seems to follow slightly behind that of MACROH2A1 (Fig. 3i and ref. 34). Similar kinetics for the recruitment of BMI1, RNF2/RING1B, and MACROH2A1 to the Xi were 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 CBX4/HPC2 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 $44.5 \pm 3.5\%$ 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 $11.2 \pm 2.0\%$ of the cells. Likewise, in cells containing an SPOP RNAi, only $10.4 \pm 1.9\%$ 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 deviances 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 inactivation

Fig. 4. MACROH2A1 localization and X chromosome inactivation. (a) MACROH2A1 localization in the 293 cell line under different conditions. Cells were transfected with the indicated RNAi vectors, selected with puromycin, and fixed 3 days after transfection. The distribution of MACROH2A1 was analyzed by immunofluorescence and epifluorescence microscopy. (b) Reactivation of GFP in SV-40 T antigen-transformed mouse embryonic fibroblasts with the X-inactivated GFP transgene. The fibroblasts were transfected with different RNAis, selected with puromycin, and allowed to recover. After treatment of the cells with 5-azadC for 4 days and with TSA for 1 day, GFP-positive cells were detected by flow cytometry. (Top and Middle) Representative experiment of the GFP reactivation. (Bottom) Results obtained from more than six independent experiments were statistically analyzed with Student's *t* test.

tion, we made use of a female mouse fibroblast cell line, uXGFP, harboring a silent GFP reporter integrated on the Xi. Similar cells have previously been used to study the synergism of *Xist* RNA, DNA methylation, and histone acetylation in maintaining X chromosome inactivation (5) and to analyze the involvement of BRCA1 in *Xist* localization to the Xi (42). 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 in some degree to other heterochromatic 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 transfected 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 interactions 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 uH2A 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. In addition, MACROH2A1 RNAi treatment does not appear to affect PRC1 Xi 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 pro-

teins. 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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