The role of Polycomb group proteins throughout development: favoring repression

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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.

Introduction

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 (Priest et al., 1967), histone hypoacetylation (Okamoto et al., 2004), and DNA hypermethylation (Mohandas et al., 1981). Moreover, Xi chromatin is enriched in the variant histone MacroH2A (Costanzi and Pehrson, 1998). Hence, X chromosome inactivation involves multiple interdependent layers of epigenetic repression (Brockdorff, 2002; Csankovszki et al., 2001; Heard, 2004; Plath et al., 2002).
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 (de Napoles et al., 2004; Francis and Kingston, 2001; Lund and van Lohuizen, 2004; Orlando, 2003; Wang et al., 2004).

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 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). 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 (de Napoles et al., 2004; Fang et al., 2004; Wang et al., 2004). The PRC1 protein Bmi1 was originally identified as an oncogenic collaborator with Myc (van Lohuizen et al., 1991), a function in part mediated through repression of the Cdkn2a tumor suppressor locus (Jacobs et al., 1999b; Jacobs et al., 1999a). Bmi1-deficient mice display homeotic skeletal transformations typical for PcG mutations (van der Lugt et al., 1994) and have severe defects in stem cell maintenance in both hematopoietic (Lessard and Sauvageau, 2003; Park et al., 2003) and neuronal tissues (Leung et al., 2004; Molofsky et al., 2003).

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.

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 (Alkema et al., 1997), a clone containing full-length speckle-type POZ protein, SPOP (Nagai et al., 1997), 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 (Furukawa et al., 2003). CULLIN3 together with ROC1 constitute distinct ubiquitin E3 ligases with individual BTB proteins that potentially target ubiquitination of many substrate proteins (Furukawa et al., 2003; Pintard et al., 2003; Xu et al., 2003). Coimmunoprecipitation 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 demonstrates 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 (Furukawa et al., 2003). The variant histone protein MACROH2A1 has recently been reported to interact with the MATH domain of SPOP in GST pull-down assays (Takahashi et al., 2002). Using coimmunoprecipitation 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 ubiquitin. 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 can be found at the end of this chapter and is available as supporting information online at 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 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.

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 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.
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 MACROH2A1-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. (Chadwick and Willard, 2002)). 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 (Saurin et al., 1998; Voncken et al., 1999). 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) (Chadwick and Willard, 2001). 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 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 (Chadwick and Willard, 2002). 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 (Chadwick and Willard, 2002; Fuss and Linn, 2002; O’Keefe et al,
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 1h. 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.
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. (Chadwick and Willard, 2002)). 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 can be found at the end of this chapter and is available as supporting information online at 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 (de Napoles et al, 2004; Fang et al, 2004). 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 ± 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 a 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 (Csankovszki et al, 1999). 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 can be found at the end of this chapter and is available as supporting information online at 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 (Zhang et al, 2004) and cyclin E (Singer et al, 1999), 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 MACROH2A 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 can be found at the end of this chapter and is available as supporting information online at 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, 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 (Csankovszki *et al.*, 2001) and to analyze the involvement of BRCA1 in *Xist* localization to the Xi (Ganesan *et al.*, 2002).

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 24 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. (Csankovszki *et al.*, 2001)). 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 (Csankovszki *et al.*, 2001) 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 posttranslational 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...
XCI involves E3 ligase CULLIN3/SPOP

the moderate percentage of reactivation observed (Csankovszki et al., 2001), this model has been used in other studies and proved to be a reliable tool to analyze X chromosome silencing mechanisms (Csankovszki et al., 2001; Ganesan et al., 2002).

The 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 (Chadwick and Willard, 2001; Costanzi and Pehrson, 2001), 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 (Smith et al., 2004) 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 (Fyodorov et al., 2004; Luo et al., 2004). 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 (de Napoles et al., 2004; Fang et al., 2004). Because Xist-dependent PRC2 recruitment occurs during initiation of Xi but seems to be largely dispensable in differentiated cells (Mak et al., 2004; Silva et al., 2003), 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 can be found at the end of this chapter and is available as supporting information online at 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.

Materials and Methods

Antibodies

Polyclonal antibodies against EZH2 and CBX4/HPC2 were obtained from A. P. Otte (University of Amsterdam, Amsterdam), and the monoclonal RNF2/RING1B antibody was from H. Koseki (RIKEN Research Center for Allergy and
Immunology, Yokohama, Japan). Anti-BMI1, anti-H2A, and anti-MACROH2A1 were from Upstate Biotechnology (Lake Placid, NY); anti-CULLIN3, anti-ubiquitin, and anti-Myc were from Santa Cruz Biotechnology; anti-Flag was from Sigma; anti-HA was from Roche; and anti-Brdu antibody was from DAKO. Rabbit polyclonal antibodies were raised against full-length mouse SPOP-GST fusion proteins by using standard procedures.

Plasmids
The yeast two-hybrid fusion vector and expression vectors for full-length and deletion mutants of BMI1 are described in refs. (Alkema et al., 1997) and (Voncken et al., 1999). Mammalian expression vector for HA-tagged SPOP was a generous gift from Dr. M. Hagiwara (Tokyo Medical and Dental University, Tokyo) (Nagai et al., 1997). Expression vectors for FLAG-tagged and Myc-tagged SPOP were generated by using standard PCR methods and verified by sequencing. A full-length CULLIN3 expression vector was obtained from the IMAGE consortium (5784147). FLAG-tagged CULLIN3 expression vector was generated by using standard PCR methods. FLAG-tagged MACROH2A1 was generated by PCR from the IMAGE clone 4077577. Human ROC1 was obtained from the IMAGE clone 3138751 and cloned by digestion into pGlo-Myc3.1. FLAG-PCNA expression vector was a generous gift from Sebastian Nijman (The Netherlands Cancer Institute). Expression construct for HA-tagged ubiquitin was a generous gift from Thijn Brummelkamp (The Netherlands Cancer Institute).

Construction of the plasmids that contain DNA templates for the synthesis of RNAs under the control of the U6 or the H1 promoters was performed essentially as described (Brummelkamp et al., 2002; Sui et al., 2002). The RNAi sequences were as follows: for MACROH2A1, GGTCACACCCGGCACATCT (Fig. 4) and GGCCCTTGGAGGTAGCTGGAA (Fig. 8); for SPOP, GGAGGAAATGGGTGAAGTCAT (Fig. 4), GGTGGAAGTCAATGGTGGTGT, and AGCTGCTAAGATAGCCTTGTG (Fig. 4); for CULLIN3, GCTAGAATTAATGAAGAAAT, GGTGTACTCGTTATGTTG, and GAATAACAGTGGTCTTAGT (Fig. 4), GGAACTCATTTCCAAGGAC, and GGAGTCAAAGGGCTAACAGAA (Fig. 8); for BMI1, GTATTGTCCTATTTGTGAT; and for GFP, GCTGACCCTGAAGTTCATC. 

Cell Culture, Transfections, and Retroviral Infections
All of the somatic cells were maintained in DMEM supplemented with 10% FBS (GIBCO) under standard conditions. 293HEK cells were transfected by using the calcium-phosphate precipitation method. Phoenix producer cells were used to generate retroviral stocks, and subconfluent mouse embryonic fibroblast cultures were transduced by incubation at 37°C with viral supernatant in the presence of polybrene (4 μg/ml, Sigma). After 6 h, the viral supernatant was diluted with complete medium and left on the cells for 24 h. When indicated, selection was added to the cultures 24 h after the transfection or transduction.

Synchronization of 293HEK cells in S-phase was attained by a double thymidine block as described in ref. (Voncken et al., 1999). At the indicated times after the release of the block, cells were fixed and stained.

Immunoprecipitations and Western Blot Analysis
For immunoprecipitations of proteins 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. (Wang et al., 2004). For detection of endogenous proteins, nuclei were isolated (Wang et al., 2004) and lysed in RIPA buffer.

Micrococcal Nuclease Nucleosome
Cells were harvested by centrifugation at 4°C for 10 min at 150 xg, suspended in buffer A (10 mM Heps, pH 7.9/1.5 mM MgCl2/10 mM KCl/0.5 mM DTT), transfered to a dounce homogenizer, and lysed with ~10 strokes of a type A pestle. Samples were centrifuged at 750 xg for 5 min at 4°C, and nuclei were resuspended in buffer M1 (50 mM Tris-HCl, pH 7.5/0.34 M sucrose/3 mM CaCl2/60 mM KCl/0.5 mM PMSF/4 mM benzamidine/10 μM pepstatin/10 μM leupeptin/1 μg/ml aprotinin). Then, nuclei were digested with 60 units/ml micrococcal nuclease for 10 min at 37°C. The micrococcal nuclease reaction was quenched by addition of EGTA to a final concentration of 50 mM. The digested nuclei were dounced 100 times in a glass dounce homogenizer (tight pestle) and incubated at 4°C for 10 min with 500 mM NaCl. Then, samples were centrifuged at 14,000 xg for 20 min at 4°C, and pellets were diazoyed against EQ buffer (20 mM Heps, pH
In Vitro Ubiquitination Assay

293 cells were cotransfected with pCMV-Sport-Flag-CULLIN3, pCMV-HA-SPOP, and pGlo-Myc-ROC1. Forty-eight hours after transfection, cells were harvested in ELB lysis buffer, sonicated for 10 pulses of 50% at level 4, and centrifuged for 15 min at 14,000 rpm at 4°C. Flag-CULLIN3 immunocomplexes were immobilized on a mixture of protein G and A-agarose beads (Amersham Pharmacia) were washed twice with ELB lysis buffer and twice with PBS. The beads with the immunoprecipitated complex were incubated with 2 μg of purified BMI1 [a generous gift of G. Buchwald (The Netherlands Cancer Institute)] in a ubiquitin ligation reaction mixture containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM NaF, 2 mM ATP, 0.6 mM DTT, 12 μg of ubiquitin (Sigma), 100 ng of rabbit E1 (Boston Biochem), and 900 ng of His-6-UbcH5a (Affinity Research Products) at 37°C for 1 h. The reaction was terminated by heating at 70°C for 10 min in SDS sample buffer (Invitrogen) and resolved by SDS/PAGE, followed by immunoblotting with an anti-BMI1 antibody.

Immunostaining

All somatic cell types and the male mouse ES cell line carrying the Xist cDNA transgene were grown on glass slides or plated onto them the day before the stain, fixed in 4% paraformaldehyde (PFA) for 10 min, and permeabilized for 5 min in 0.2% Triton X-100/PBS. Trophoblast stem cells were plated onto glass slides, permeabilized for 5 min in cold CSB buffer (3 mM MgCl₂/0.3 M sucrose in PBS) containing 0.2% Triton X-100 and fixed in 4% paraformaldehyde for 15 min. Cells were then incubated in blocking solution (5% goat serum/0.2% fish skin gelatin/0.2% Tween 20 in PBS) for 30 min before incubating with primary antibody for 1 h at room temperature in a humidified chamber. After three consecutive 5 min washes in 0.2% Tween 20/PBS, cells were incubated for 1 h with secondary antibody. The cells were subsequently washed with 0.2% Tween/PBS twice and stained with DAPI in PBS followed by two additional washes before mounting in Vectashield mounting medium (Vector Laboratories). For colocalization of PRC1 members and MACROH2A1 with sites of BrdUrd incorporation, unsynchronized cells were incubated for 1 h with medium containing 10 μM BrdUrd. After the immunostaining for PRC1, cells were refixed for 10 min with 4% PFA/PBS at room temperature, washed three times with PBS, and incubated for 15 min with blocking solution. DNA was denatured by a 30 min incubation with 2 M HCl/0.5% Triton X-100 at room temperature. Cells were then washed once with 0.1 M NaB₃O₃ (pH 8.5) for 5 min and twice with PBS for 5 min and then incubated with anti-BrdUrd antibody BU20a for 1 h. After washing twice with PBS for 5 min and once with blocking solution for 5 min, cells were incubated for 45 min with Alexa Fluor 488-conjugated antimouse antibody. After washing the cells three times for 5 min with PBS at room temperature, cells were stained with DAPI and mounted.

Fluorescence in Situ Hybridization (FISH)

In cases when immunostaining was followed by FISH, immunostaining was carried out as described above, with the exception that tRNA and RNase inhibitors were added with the primary antibody. After the final immunostaining wash, cells were fixed with 4% PFA and then dehydrated through a 70-85-100% ethanol series. FISH was performed as described in ref. (Plath et al., 2003). Xist RNA was detected with a fluorescein-labeled single-stranded RNA probe antisense to Xist.

Fluorescence Microscopy and Image Acquisition

Stained cells were viewed by epifluorescence microscopy by using a Zeiss microscope or by confocal laser sequential scanning microscopy using a Leica microscope. Epifluorescent images were captured with a cooled charge-coupled device camera and processed using IMAGE I software and PHOTOSHOP 3.0 (Adobe Systems). Quantification of the percentage of cells was performed with a 40x objective. Over 300 cells per condition were scored from at least eight random fields. Values are means ± SEM.

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. (Csankovszki et al., 2001). 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.

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References


Supporting information

**Fig. 5.** Cullin and Spop ubiquitinate MACROH2A but not other targets. (a) Complex formation of CULLIN3/SPOP results in the creation of an active E3 ubiquitin ligase for MACROH2A but not for PCNA. Immunoprecipitations from transiently transfected 293HEK cells using anti-Flag antibody are shown. The equal expression of all transfected constructs was verified. Arrow indicates bands representing the Ig heavy chain (hc). (b) (Left) 293 cells were transfected with mock, CULLIN3, SPOP, or BMI1 RNAi vectors. 72 h posttransfection, nuclear extracts were prepared and used for Western blot analysis. Western blots probed with anti-CULLIN3, SPOP, and BMI1 antibodies show the efficiency of the RNAi on endogenous protein levels. Endogenous level of RNF2/RING1B was used to demonstrate equal loading. (Right) 293 cells were transfected with mock, CULLIN3, SPOP, BMI1, and MACROH2A1 RNAi vectors. 72 h posttransfection, nuclear extracts were prepared, and acidic extracted histones were used for Western blot analysis. Western blot probed with an anti-MACROH2A1 antibody shows the efficiency of the MACROH2A1 RNAi on the endogenous protein level. Endogenous level of histone H2A was used as loading control. No major changes in overall levels of BMI1 (a) or MACROH2A1 (b) can be detected in extracts from CULLIN3 or SPOP RNAi transfected cells.

**Fig. 6.** BMI1 localization in early stages of X inactivation. (a) Colocalization of Bmi1 and Ezh2 on the Xi in extraembryonic cells. Trophoblast stem cells were stained with Bmi1 and Ezh2 antibodies as indicated. The merged image consisting of Bmi1 (green) and Ezh2 (red) shows that Bmi1 colocalizes with Ezh2 in an Xi-like pattern. (b) Xist RNA is sufficient to recruit the PcG complex to the Xi. Male ES cells that were induced to express Xist RNA from a constitutive promoter for 1 and 2 days were stained for Bmi1 and Ezh2. Bmi1 colocalizes with Ezh2 at the Xi 2 days after induction of the endogenous Xist. Focal accumulation of Ezh2 and Bmi1 is indicated by arrows.
Fig. 7. *XIST* RNA localization is not affected by CUL3 or SPOP RNAi. 293 cells were transfected with mock, CUL3, SPOP, or MACROH2A1 RNAi, selected with puromycin, and fixed 3 days after transfection. Combined *XIST* RNA (green) and MACROH2A1 (red) staining was performed. Nuclear distribution of *XIST* and MACROH2A1 was analyzed by epifluorescence microscopy.
Fig. 8. No significant changes in cell cycle profiles upon RNAi treatments. (a) Cell cycle profile of 293 cells 3 days after transfection with mock, CULLIN3, SPOP, BMI1, or MACROH2A1 RNAi vectors. Cells were fixed and stained with propidium iodide and analyzed by flow cytometry. (b and c) Specificity of RNAi constructs. Additional RNAi vectors targeting CULLIN3, SPOP, and MACROH2A1 were designed and analyzed. As evident from the panels (a, distribution of MACROH2A1; b, reactivation of the X-inactivated GFP transgene), the new RNAi molecules had similar biological activity as those used in Fig. 4.
Fig. 9. BMI1 localization at the Xi is independent of MACROH2A1. 293 cells were transfected with the indicated RNAi vectors, selected with puromycin, and fixed 3 days after transfection. The distribution of the endogenous BMI1 and MACROH2A1 was analyzed by immunofluorescence and epifluorescence microscopy.