SUMMARY
Polycomb repressive complex 1 (PRC1) catalyzes lysine 119 monoubiquitylation on H2A (H2AK119ub1) and regulates pluripotency in embryonic stem cells (ESCs). However, the mechanisms controlling the binding of PRC1 to genomic sites and its catalytic activity are poorly understood. Here, we show that Fbxl10 interacts with Ring1B and Nspc1, forming a noncanonical PRC1 that is required for H2AK119ub1 in mouse ESCs. Genome-wide analyses reveal that Fbxl10 preferentially binds to CpG islands and colocalizes with Ring1B on Polycomb target genes. Notably, Fbxl10 depletion causes a decrease in Ring1B binding to target genes and a major loss of H2AK119ub1. Furthermore, genetic analyses demonstrate that Fbxl10 DNA binding capability and integration into PRC1 are required for H2AK119 ubiquitylation. ESCs lacking Fbxl10, like previously characterized Polycomb mutants, cannot differentiate properly. These results demonstrate that Fbxl10 has a key role in regulating Ring1B recruitment to its target genes and H2AK119 ubiquitylation in ESCs.

INTRODUCTION
Polycomb proteins (PcGs) play important roles in stem cell self-renewal and differentiation. Their deregulation or aberrant activity has been tightly linked to cancer development (Bracken and Helin, 2009). PcGs generally form multisubunit chromatin-modifying complexes to maintain transcriptional repression in multicellular organisms. Two main complexes, namely Polycomb repressive complexes 1 and 2 (PRC1 and PRC2), have been described. PRC2 methylates histone H3 on lysine 27 (H3K27). PRC1 was originally purified from D. melanogaster embryos whose core components include Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc), and dRing/Sce (reviewed in Schuettengruber and Cavalli, 2009; Simon and Kingston, 2009). A PRC1-like complex was found to monoubiquitylate histone H2A on lysine 119, catalyzed by dRing in D. melanogaster and Ring1B in mammals (Wang et al., 2004). Therefore, H2A monoubiquitylation has been viewed as a critical component in mediating PcG silencing of developmental control genes (Endoh et al., 2012; Schoeftner et al., 2006; Wang et al., 2004). Genome-wide mapping studies in both D. melanogaster and mammals show an extensive overlap in genome binding sites between PRC2 and PRC1 (Boyer et al., 2006; Bracken et al., 2006; Ku et al., 2008; Lee et al., 2006; Schwartz et al., 2006). Because Pc and its mammalian orthologs (CBXs) bind to H3K27me3, and some studies have shown that chromatin binding of PRC1 is dependent on PRC2, it has been suggested that H3K27me3 is required for PRC1 binding to target genes (reviewed in Sparmann and van Lohuizen, 2006). However, several results, including the recent demonstration that Ring1B-chromatin association and global levels of monoubiquitylated lysine 119 on H2A (H2AK119ub1) are preserved in PRC2-deficient cells (Leeb et al., 2010; Tavares et al., 2012), suggest that this hierarchical recruitment model might be too simplified, and that PRC1 can act independently of PRC2.

Chromatin immunoprecipitation sequencing (ChIP-seq) in mouse embryonic stem cells (ESCs) demonstrated that almost 97% of PRC2 target genes are associated with annotated CpG islands or similar CG-rich regions. Similarly, Ring1B binding also positively correlates with PRC2-positive CpG islands (Ku et al., 2008). The Fbx10/Kdm2b-containing PRC1 (PRC1-Fbx10 complex), known as the dRing-associated factor (dRAF) complex in D. melanogaster (Lagarou et al., 2008) and the BCOR complex in mammals (Gearhart et al., 2006), has been
shown to be important for H2A monoubiquitylation. Interestingly, Fbxl10 contains a CXXC domain, known to have a strong affinity for CpG-rich DNA sequences (reviewed in Deaton and Bird, 2011), and its homolog Fbxl11/Kdm2a has been shown to bind to CpG islands throughout the genome (Blackledge et al., 2010). In Drosophila, dKDM2 has a synergistic effect with PcG in Hox gene silencing (Lagarou et al., 2008). However, the role of mammalian Fbxl10 in regulating H2A monoubiquitylation and PcG-mediated transcriptional silencing is not clear. In this study we have analyzed the relationship between Fbxl10, Ring1B recruitment, and H2AK119 monoubiquitylation in mouse ESCs.

RESULTS

Fbxl10 Is Part of a Noncanonical PRC1, which Is Essential for Global Levels of H2A K119ub1 in ESCs

To characterize the relationship between FBXL10 and PRC1 components, we expressed epitope-tagged FBXL10 in 293FT cells, and by using immunoprecipitation (IP), we confirmed that FBXL10 can bind to the PRC1 members Ring1B, NSPC1, and RYBP, but not to CBX7 (Figure 1A). Next, we performed reciprocal endogenous communoprecipitation (coIP) assays using nuclear extracts prepared from mouse ESCs and antibodies specific for Fbxl10, Ring1B, and NSPC1. As shown in Figure 1B, the three proteins interact weakly with the PRC1-associated protein Rybp. Interestingly, only Ring1B interacts with Cbx7. Thus, Fbxl10, Ring1B, and NSPC1 form a distinct complex in vivo. These results are in agreement with recent biochemical analyses showing that these proteins can interact when overexpressed (Gao et al., 2012). In contrast, overexpressed Fbxl11 does not interact with any tested PRC1 proteins (Figure S1A available online).

To decipher the biological and biochemical roles of Fbxl10, we downregulated Fbxl10 expression in ESCs using short hairpin RNAs (shRNAs) specific for Fbxl10. We identified two Fbxl10 shRNAs that strongly inhibit Fbxl10 expression without leading to detectable changes in the expression of the PRC2 component, Ezh2, the PRC1 component, Ring1B, and Nspc1. As shown in Figure 1B, the three proteins interact weakly with the PRC1-associated protein Rybp. Interestingly, only Ring1B interacts with Cbx7. Thus, Fbxl10, Ring1B, and Nspc1 form a distinct complex in vivo. These results

Figure 1. Fbxl10 Forms a Noncanonical PRC1 Complex, which Is Essential for Global Levels of H2A K119ub1 in ESCs

(A) Anti-FLAG IP of FH-FBXL10 ectopically expressed in 293FT cells, followed by WB using antibodies for the indicated proteins.

(B) CoIP of endogenous Fbxl10, Ring1B, and Nspc1 proteins in E14. For (A) and (B), the input lanes contain 5% of the amount used for IP. The Fbxl10 rabbit polyclonal antibody was used for the IP, whereas mouse monoclonal antibody was used for the WB.

(C) Real-time qPCR analyses for determining the relative mRNA levels of Fbxl10, Ezh2, and Ring1B in Fbxl10-depleted E14 cells using two different shRNA constructs specific for Fbxl10 (shFbxl10-D and shFbxl10-F) and scrambled control (Scr). The error bars denote SD; n = 2.

(D–F) WB analysis of protein lysates and acid-extracted histones prepared from mouse ESCs using antibodies specific for the indicated proteins. β-actin served as a loading control for total protein. H3 served as a loading control for histones. See also Figures S1 and S2.

Molecular Cell

Fbxl10 Recruits PRC1 to CpG Islands

To characterize the relationship between FBXL10 and PRC1 components, we expressed epitope-tagged FBXL10 in 293FT
Ring1B is the catalytic subunit of PRC1 that catalyzes H2AK119ub1 (Buchwald et al., 2006; Wang et al., 2004). To address the contribution of mammalian Fbxl10 to H2A monoubiquitylation, we tested the levels of H2AK119ub1 in wild-type (WT) ESCs as well as in ESCs depleted of various PRC1 subunits. In agreement with other studies, we found that H2AK119ub1 levels are significantly reduced in Ring1b−/− cells (Leeb and Wutz, 2007) and are unchanged when Cbx7 is depleted (Figure 1E). However, depletion of Fbxl10 and Nspc1, but not Fbxl11, led to a significant decrease in H2AK119ub1 levels (Figure 1E; Figures S1B and S1D). As a control for these experiments, we performed complementation experiments by reintroducing human FBXL10, which is not a target of the mouse-specific Fbxl10 shRNAs, and showed that H2AK119ub1 is completely restored (Figure 1F). Moreover, the role of the PRC1-Fbxl10 complex in H2A ubiquitylation is not ESC specific, because the depletion of Fbxl10 in primary mouse embryonic fibroblasts also led to a strong decrease in global levels of H2AK119ub1 (Figure S1E). Taken together, these findings demonstrate that Fbxl10 contributes significantly to H2AK119 monoubiquitylation. Given that Cbx7 has recently been shown to be the major Cbx component of PRC1 in ESCs (Morey et al., 2012), these results also indicate that it is the PRC1-Fbxl10 complex, rather than canonical PRC1, that catalyzes most of the monoubiquitylation of H2AK119 in ESCs.

Fbxl10 Promotes H2A Monoubiquitylation Dependent on a C-Terminal Nspc1-Binding Region

To further characterize the interaction between FBXL10 and proteins in the PRC1 complex, we expressed WT and several mutant versions of FBXL10 in 293FT cells (Figure 2A). Via IP of the overexpressed proteins, we found that the binding to Nspc1 was dependent on 200 amino acids in the C-terminal region, which contains the leucine-rich region (LRR). Although the LRR is conserved in Fbxl11, we did not detect any interaction between Fbxl11 and Ring1B or Nspc1 (Figure S1A). This suggests that specific amino acids in the C-terminal region of Fbxl10 contribute to the assembly of the PRC1-Fbxl10 complex (Figure 2A). Interestingly, we also observed a weaker interaction between Fbxl10 mutated in the CXXC domain, rather than canonical PRC1, that catalyzes most of the monoubiquitylation of H2AK119 in ESCs.

Fbxl10 Recruits PRC1 to CpG Islands

Genome-wide Localization of Fbxl10 in ESCs

Previous data have shown that Fbxl10 binds nonmethylated CpG sequences in vitro (Koyama-Nasu et al., 2007); however, the bona fide target genes of Fbxl10 have not been reported. To determine the genome-wide location of Fbxl10 in mouse ESCs, we generated a ChIP-grade polyclonal antibody for Fbxl10. The specificity of this antibody was confirmed by ChIP-quantitative PCR (qPCR) analyses in Fbxl10-depleted ESCs (Figure S3C). Meanwhile, we also established mouse ESCs expressing FLAG-hemagglutinin (HA)-tagged Fbxl10 (FH-Fbxl10) close to physiological levels of Fbxl10 (Figure S3A). The expression of FH-Fbxl10 did not change the morphology or the expression of pluripotency markers (Figures S3A and S3B). This cell line and WT E14 were used to determine the genome-wide locations of FH-Fbxl10 and endogenous Fbxl10, respectively.

The mapping of Fbxl10 showed that it is mainly enriched around transcription start sites (TSSs) (Figure S3D). Gene annotation of Fbxl10 binding sites, using a false discovery rate of 0.1, showed that FH-Fbxl10 binds in the vicinity of the TSS of 16,412 genes (Table S2). ChIP-qPCR analyses using FLAG antibody in the FH-Fbxl10 and parental control cells confirmed the enrichment of FH-Fbxl10 on the identified targets genes (Figure 3A). In addition, ChIP experiments using the FH-Fbxl10-specific antibody in WT ESCs also showed that endogenous Fbxl10 is significantly enriched at the promoters of the same target genes (Figure 3B). The normalized tag density of ChIP-seq data (tags per million, TPM) also revealed that endogenous Fbxl10 is significantly enriched on Hox gene loci, similarly to ectopic FH-Fbxl10 (Figure S3E). The comparison of the two ChIP-seq data sets using heat maps showed that they display a very similar pattern despite their different densities (different sequencing depth as shown by read counts in Table S1) (Figure 3C). Interestingly, the distribution of Fbxl10 is also very similar to the published binding profile of Fbxl11 (Blackledge et al., 2010) (Figure 3C).

Fbxl10 Binds to CpG Islands throughout the Genome

To examine whether Fbxl10 specifically locates to CpG islands in ESCs, we compared the enriched sites with CpG island elements defined by the UCSC Genome Browser. By analyzing the tag-density enrichment across the TSSs of all CpG island genes (CGI genes) and non-CGI genes for FH-Fbxl10, we found that the majority of Fbxl10 target genes contain a CpG island (n = 13,368) (Figures 3C and 3D). Genes were categorized as either CGI or non-CGI genes depending on whether the gene body (including the area 5 kb upstream of the TSS) overlapped with a UCSC-annotated CpG island or not. Some target genes are classified as non-CGI genes, but they still contain CpG-rich sequences just below the strict threshold for UCSC-annotated CpG islands. For avoiding redundancy, where a CpG island overlapped the TSS of multiple genes or transcripts, only one was recorded. The densities in Figure 3D are scaled according to the counts in each sample (TPM). It is worth noting that the
tag density for Fbxl10 over all targets is closely associated with CpG islands, regardless of whether they are at TSSs, transcription end sites, or intergenic regions (Figure S4A). To further validate the preferential binding on CpG islands, we performed ChIP-qPCR analyses using different primers covering CpG islands or non-CpG islands on a random target gene, Consistent with the previously reported histone demethylase activity (He et al., 2008), Fbxl10 enrichment sites coincide with low levels of H3K36me2 on the tested genes (Figure S5A). However, Fbxl10 depletion does not significantly affect H3K36me2, neither at the global level nor at the specific target loci (Figures S5A and S5B). These results suggest that mechanisms in

Figure 2. FBXL10 Recruits RING1B and NSPC1 to Promoters and Promotes H2AK119ub1 Dependent on its C-Terminal Region

(A) Anti-FLAG IP of FLAG-HA-tagged WT and mutant FBXL10 proteins expressed in 293FT cells. Left panel: 5% of the lysate used for the IP was loaded in each lane. Right panel: Immuno-precipitated material was loaded. Schematic representation of the different FBXL10 mutants, highlighting that a short deletion of the C terminus (ΔLRR) affects the interaction with Nspc1.

(B and D) WB analysis of 293T cells containing an integrated Gal4-luciferase reporter construct and expressing Gal4-FBXL10 WT (B) or ΔLRR (D) fusions dependent on the presence of tetracycline (Tet). The indicated antibodies were used.

(C and E) ChIP-qPCR analyses were performed in mock (Tet−) or tetracycline-treated (Tet+) cells. Immunoglobulin G or M (IgG or IgM) antibody was used as a negative control. Primers specific for the TSS of the luciferase gene were used. The error bars denote SD; n = 2.

Bdnf. The results confirmed that Fbxl10 is specifically enriched at the CpG islands where significant peaks are detected by ChIP-seq (regions A, B, and C; Figure 3E).

As most CpG islands are unmethylated in ESCs, we next investigated the relationship between Fbxl10 occupancy and methylated cytosine (mC) mapped in our previous work (Williams et al., 2011). We found that Fbxl10 and mC distribution are inversely correlated (Figure S4B). To further investigate this observation, we differentiated ESCs to neurons as described (Bibel et al., 2007) and performed Fbxl10 ChIP experiments. The CpG island of Bdnf remains unmethylated, but the islands associated with Lefty1 and Gdf3 are known to become DNA methylated in neurons (Mohn et al., 2008). As shown in Figure S4D, Fbxl10 is enriched at all the promoters in ESCs but is displaced from methylated promoters when differentiated to neurons. The reduced binding levels are not due to a decrease in Fbxl10 expression (Figure S4C), and our results therefore support the notion that DNA methylation status modulates binding of Fbxl10 to DNA.
Figure 3. Fbxl10 Binds to CpG Islands

(A) FLAG ChIP-qPCR analyses in FH-Fbxl10 expressing ESCs. The parental ESCs were used as a negative control.

(B) Fbxl10 ChIP-qPCR analyses in E14 cells with IgG antibody as a negative control. For (A) and (B), TSSs of six repressive genes and three actively transcribed genes were detected. Gapdh promoter (not TSS) served as a negative control.

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addition to Fbxl10 occupancy regulate H3K36me2 status on CpG islands.

**Fbxl10 Colocalizes with Ring1B and H2AK119ub1 on PcG Target Genes but Binds Also to Actively Transcribed Genes**

To investigate chromatin profiles that flank TSSs of Fbxl10-bound genes besides H3K36me2, we performed Ring1B ChiP-seq (Figure S6A). We also compared Fbxl10 binding to published ChiP-seq data for H2AK119ub1 (Brookes et al., 2012), H3K27me3 (Lienert et al., 2011a), H3K4me3 (Schmitz et al., 2011), H3K36me3 (Marson et al., 2008), and RNA polymerase II (RNA pol II) (Lienert et al., 2011b). We found that H3K4me3 is positively correlated with Fbxl10 binding sites (Figures 4A–4C; Figure S5D), with 97% of all Fbxl10 target genes overlapping with H3K4me3 peaks (Figure 4A, Figure S5C, and Table S2). This is also consistent with the fact that unmethylated CpG islands are positively correlated with H3K4me3 (Guenther et al., 2007; Mikkelsen et al., 2007).

Further analysis showed that Fbxl10 targets could be categorized into two major groups (Figure 4A). The first group (n = 5,260) was associated with bivalent domains (Figures 4A and 4B; Figure S5C), a chromatin state characterized by the presence of both H3K4me3 and H3K27me3 (Bernstein et al., 2006; Mikkelsen et al., 2007). Almost all PcG target genes were in this group (positive for H3K27me3, Ring1B, and H2AK119ub1) (Figures 4A and 4B). In contrast, the second group (n = 10,659) was associated only with active marks, including H3K4me3 (at a higher level than the bivalent genes), H3K36me3, and high levels of RNA pol II (Figures 4A and 4C; Figure S5C). For example, both Fbxl10 and Ring1B colocalize on the CpG islands of Wnt1, Wnt10b, Fgf3, and Fgf15 with bivalent marks, but only Fbxl10 is enriched on Arf3 and Fgf4 with active marks (Figure 4D). Fbxl10-binding profiles on these two groups of target genes are very similar (Figure 4D and Figure S5D). Given that PcG proteins may gain or lose target genes during ESC differentiation (Mohn et al., 2008), Fbxl10 may preexist on widespread CGI genes in ESCs and recruit Ring1B to new target genes when they should be silenced or ready to be silenced at specific lineages. Furthermore, it is possible that Fbxl10 plays active roles in the actively transcribed genes by interacting with some coactivators. Taken together, these results show that Fbxl10 can associate with both actively transcribed and poised-repressed target genes, which are mostly occupied by PcG proteins. In this study we focus on the role of Fbxl10 in PcG-bound genes.

**Ring1B and Nspc1 Binding Is Affected by Loss of Fbxl10**

For determining whether Fbxl10 is involved in recruiting Ring1B to target genes, Ring1B ChiP-seq was carried out in Fbxl10-depleted ESCs. For controls, Ring1B ChiP-seq in Suz12+/− and Ring1b+/− ESCs was also performed. By analyzing equal numbers of reads from Fbxl10 ChiP-seq in control and Fbxl10-depleted ESCs in Fbxl10 target genes, we found that the enrichment level of Fbxl10 was significantly decreased in Fbxl10-depleted ESCs compared to controls (Figure 5A). Similarly, we compared equal numbers of reads from Ring1B ChiP-seq data in Ring1B target genes. The distribution of peaks around the TSS was similar in all tested cells, and almost none of the Ring1B target genes were found in the Ring1b−/− cells; however, the tag density was lower in the Suz12+/− or Fbxl10-depleted ESCs (Figure 5B). In comparison with control cells, the Ring1B signal density was decreased approximately 50% in Suz12+/− ESCs (Figure 5B). Significant peaks identified by MACS2 also showed that Ring1B binding was independent of PRC2 in many PcG target genes (Figure S6C, peaks on Hoxa loci and the Cdx2 promoter are shown as examples). By comparing the tag counts under each target peak extracted from the ChiP-seq files, we found that around 48% of Ring1B target sites are significantly affected by loss of Suz12 (Figure S6B and Table S4, log2 < −1). This is consistent with previous findings that PRC1 recruitment is dependent on PRC2 (Ku et al., 2008; Leeb et al., 2010). However, H2AK119ub1 levels are only slightly decreased in Suz12+/− ESCs, even though H3K27me3 is completely erased (Figure S6D). These results are in agreement with the findings that the partial loss of Ring1B binding caused by loss of Suz12 expression does not affect H2AK119 monoubiquitination and that H2AK119ub1 levels are unchanged in Cbx7−/−depleted cells (Figure 1E).

The Ring1B enrichment in Fbxl10-depleted cells also displays a decrease (Figure 5B). Consistent with this, many peaks of Ring1B binding are decreased in Fbxl10-depleted ESCs (Figure 5C). However, different from the effect of Suz12 deletion, the decrease in Ring1B binding in Fbxl10 knockdown ESCs shows an interesting bimodal distribution (Figure S6B). Though only a smaller part of target sites (17%) are significantly affected (log2 < −1), most of target sites are affected in the Fbxl10 shRNA-expressing cells (Table S4). The ChiP-seq results were validated by independent ChiP-qPCR analyses on several target genes (Figure 5D). Moreover, the decrease in Fbxl10 expression also led to a dramatic decrease in Nspc1 binding to the tested genes; however, we did not detect significant differences in the binding of Suz12, H3K27me3, or Cbx7 upon downregulation of Fbxl10 (Figure 5D).

To further corroborate that Fbxl10 and PRC2 are separately involved in Ring1B recruitment, we tested whether less Ring1B is bound at PcG target genes in Suz12+/− ESCs depleted of Fbxl10. According to ChiP-qPCR analyses, Ring1B enrichment...
H3K4me3 IgG FH-Fbxl10 Ring1B H2AK119ub1 H3K27me3 H3K36me3 RNA polII

Mean density (tags/50 bp)

-5 kb TSS +5 kb

Bivalent Targets

H3K4me3-only Targets

103.29

Mean density (tags/50 bp)

-5 kb TSS +5 kb

Bivalent Fbxl10 targets (5,260 genes)

H3K4me3-only targets (10,659 genes)

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remains unchanged in $\textit{Suz12}^{--/--}$ ESCs on some PRC2 target genes (e.g., $\textit{Hoxa10}$, $\textit{Cdx2}$, $\textit{Foxa2}$, and $\textit{Wnt1}$) but is modestly decreased upon downregulation of Fbxl10 in either WT or $\textit{Suz12}^{--/--}$ ESCs (Figure S6E).

**Figure 5.** Fbxl10 Is Required for the Recruitment of Ring1B and Nspc1 and Regulates H2AK119 Ubiquitylation of PcG Target Genes

(A) Mean distribution of tags for Fbxl10 ChIP-seq in control and Fbxl10 knockdown cells (shFbxl10), confirming the specificity of Fbxl10 ChIP assays. Equal reads (3.3 million) are compared throughout Fbxl10 binding sites in control ESCs.

(B) Mean distribution of tags for Ring1B ChIP-seq in control, $\textit{Suz12}^{--/--}$, shFbxl10-expressing, and Ring1b $^{--/--}$ mouse ESCs. Equal reads (5.6 million) are compared throughout Ring1B binding sites in control ESCs. For (A) and (B), the average Fbxl10 or Ring1B binding in 50 bp bins is shown within genomic regions covering ±5 kb of the TSSs.

(C) ChIP-seq binding profiles for Fbxl10 and Ring1B in control and Fbxl10-depleted ESCs. The normalized number of tags (TPM) for each ChIP-seq is given to the left of each graph.

(D) Examples of tracks are shown for designated ChIP-seq binding profiles. The normalized number of tags (TPM) for each ChIP-seq is given to the left of each graph.

(E) ChIP-qPCR analyses of Fbxl10, Ring1B, Nspc1, Suz12, H3K27me3, and Cbx7 occupancy on the CpG islands of seven representative Fbxl10-Ring1B cotargets in control and Fbxl10-depleted (shFbxl10) ESCs. H3K27me3 enrichment is normalized to H3 enrichment. Fgf4 was only targeted by Fbxl10 and served as a negative control for other ChIP assays.

**H2AK119ub1 Is Decreased as a Result of Fbxl10 Depletion**

Next we studied the effects of Fbxl10 depletion on H2AK119ub1 levels in Pcg target genes. Despite the relatively modest

**Figure 4.** Fbxl10 Colocalizes with Ring1B and H2AK119ub1 on PcG Target Genes but Also Binds to Actively Transcribed Genes

(A) Heatmap representation of the binding profiles of IgG, FH-Fbxl10, Ring1B (two assays pooled), H2AK119ub1 (Brookes et al., 2012), H3K27me3 (Lienert et al., 2011a), H3K4me3 (Schmitz et al., 2011), H3K36me3 (Marson et al., 2008), and RNA polII (Lienert et al., 2011b) in mouse ESCs at the indicated Fbxl10 target genes (±5 kb around the TSS). The same color scales (white, no enrichment; blue, high enrichment) were used for all data sets.

(B and C) Merged profiles show the ChIP-seq signals of IgG, FH-Fbxl10, Ring1B, H3K4me3, and RNA polII around the TSS of bivalent Fbxl10 target genes (B) or active H3K4me3-only Fbxl10 target genes (C). Average enrichment ratios within ±5 kb regions around to the TSS were calculated.

(D) Examples of tracks are shown for designated ChIP-seq binding profiles. The normalized number of tags (TPM) for each ChIP-seq is given to the left of each graph.
The CXXC Domain of Fbxl10 Is Required for Its Binding to DNA, for the Recruitment of Ring1B and Nspc1, and for H2AK119 Monoubiquitylation

To test whether the CXXC domain of Fbxl10 is required for the binding of Fbxl10 to target genes and for the recruitment of Ring1B and Nspc1, we stably expressed WT and *CXXC mutant Fbxl10 (both resistant to the shRNA) in Fbxl10-depleted ESCs. Reduction in Ring1B binding (Figures 5B and 5D), we observed a significant decrease in H2AK119ub1 levels in Fbxl10-depleted cells, comparable to the complete loss in Ring1b−/− cells (Figure 5E). This suggests that the active E3 ligase complex for H2A monoubiquitylation in ESCs. In contrast, depletion of Fbxl11 has almost no effect on H2AK119ub1 at target genes, and Ring1B binding even appears to be slightly increased (Figure S6F). Taken together, these results demonstrate that Fbxl10 recruits Ring1B and Nspc1 independent of PRC2 and that the PRC1-Fbxl10 complex is responsible for a substantial part of H2A monoubiquitylation in ESCs.

The CXXC Domain of Fbxl10 Is Required for Reconstitution of the Catalytically Active Complex

Consistent with having the CXXC domain, FBXL10 also expressed FBXL10 DΔLRR in the Fbxl10 knockdown cells. As shown in Figure 2, FBXL10 interacts with PRC1 components through its C terminus. To test how the C terminus of Fbxl10 affects Ring1B recruitment and catalytic activity in vivo, we also expressed FBXL10ΔLRR in the Fbxl10 knockdown cells. Consistent with having the CXXC domain, FBXL10ΔLRR can bind DNA (Figure 6B) but cannot rescue the binding of Ring1B or Nspc1 and H2AK119ub1 levels in target genes (Figures 6C–6E). In contrast, the Ring1B and Nspc1 binding levels are even lower in the *CXXC rescued cells, perhaps because the overexpressed *CXXC DNA-binding mutation of FBXL10 titrates them out of chromatin (Figure 6C). Consistent with the fact that *CXXC cannot bind DNA, it also fails to demethylate H3K36me2 (Figure 6F). These results provide strong evidence that Fbxl10 recruits an active H2AK119ub1 E3 ligase complex to CpG islands dependent on its CXXC domain.

The C Terminus of Fbxl10 Is Required for PRC1 Binding and H2A Ubiquitylation

As shown in Figure 2, Fbxl10 interacts with PRC1 components through its C terminus. To test how the C terminus of Fbxl10 affects Ring1B recruitment and catalytic activity in vivo, we also expressed FBXL10ΔLRR in the Fbxl10 knockdown cells. Consistent with having the CXXC domain, FBXL10ΔLRR can bind DNA (Figure 6B) but cannot rescue the binding of Ring1B or Nspc1 or H2AK119ub1 levels (Figures 6C–6E). Therefore, the C-terminal Nspc1- and Ring1B-binding region of FBXL10 is essential for the reconstitution of an active PRC1-Fbxl10 complex on PcG target genes.

Fbxl10 Is Required for Proper ESC Differentiation

Individual PcG proteins are not required for the proliferation and self-renewal of ESCs but are essential for the ability of ESCs to differentiate (Leeb et al., 2010; Pasini et al., 2007; van der Stoop et al., 2008). To determine the role of Fbxl10 in ESC pluripotency
and differentiation, we challenged the Fbxl10-depleted ESCs to undergo differentiation by forming embryoid bodies (EBs) in suspension cultures. Although the Fbxl10-depleted ESCs did not show detectable proliferation defects (Figure S1B) and formed EBs with an efficiency similar to that of control cells (Figure 7A, day 2), the Fbxl10-depleted EBs are denser and lack central cavities compared to control cells (Figure 7A, day 10). These phenotypes are very similar to the ones recently reported for Rybp knockdown ESCs (Tavares et al., 2012), suggesting there could be overlapping functions among the noncanonical PRC1 complexes. We also induced differentiation of ESCs expressing scrambled or Fbxl10 shRNAs in monolayer culture. After 4 days of differentiation, the control cells develop a flat and spread morphology, whereas the Fbxl10-depleted cells still grow in clusters (Figure S7A). The failure of proper differentiation is consistent with the lack of transcriptional activation of lineage marker genes and the inability to fully silence pluripotency genes (Oct4 and Nanog) during differentiation in the same cells (Figures A and B).
whereas the noncanonical PRC1 complexes play more important roles in H2AK119 monoubiquitylation have been characterized (Gao et al., 2012). Six PCGF proteins in mammals exclusively interact with diverse PcG or non-PcG components in each complex. Although the various PRC1 complexes are believed to catalyze H2AK119 monoubiquitylation, the distinct subgroups do not appear to have equal activity. The canonical PRC1 complex represses gene transcription by compacting chromatin, whereas the noncanonical PRC1 complexes play more important roles in H2A ubiquitylation (Gao et al., 2012). Several laboratories have shown that the PRC1 core components, Ring1B and PCGFs, alone can catalyze H2A ubiquitylation in vitro (Buchwald et al., 2006; Elderkin et al., 2007; Wu et al., 2008), but that other components may be required for their proper activity. However, addition of a CBX subunit to the RING1B-PCGF complex does not promote H2A ubiquitylation in vitro, but Drosophila Kdm2 does (Lagarou et al., 2008; Tavares et al., 2012). The different PCGF proteins in the PRC1 complexes may also exert different biological functions by regulating distinct subsets of target genes in specific cell types and/or developmental stages. Mel18 (Pcgf2) and Nspc1 (Pcgf1) are highly expressed in ESCs (Dietrich et al., 2010; Tavares et al., 2012). Mel18 exists in two distinct PRC1 complexes by interacting with either Cbx or Rybp, whereas Nspc1 only forms a noncanonical PRC1 (Gao et al., 2012). According to our findings, PRC1-mediated H2AK119ub1 is dramatically reduced after depletion of Fbxl10 or Nspc1, suggesting that Mel18 cannot exert a function similar to that of Nspc1 in ESCs.

The function of any protein or complex is determined by its localization, abundance, and activity. The different subunits in each complex may play different roles, thereby fine-tuning the function of the complex. Rybp exists in multiple noncanonical PRC1 complexes, and its loss significantly affects Ring1B stability (abundance); therefore, it is to be expected that downregulation of Rybp leads to a decrease in H2AK119ub1 levels (Tavares et al., 2012). In contrast, Fbxl10 is required for recruitment of Ring1B and Nspc1 to DNA (localization) and, therefore, for the activity of the complex. Importantly, downregulation of Fbxl10 does not affect Ring1B expression and only leads to a slight decrease in Nspc1 expression.

Although PcG target genes contain CpG islands in mammalian cells and the PRC2 complex is directly recruited to CpG islands (Lynch et al., 2012; Mendenhall et al., 2010), it is unclear how PcGs are recruited to these sites. An attractive possibility is that proteins with specificity for CG-rich sequences recruit the PcG proteins to their target genes. Our results show that Fbxl10 recruits catalytically active PRC1 components (Nspc1-Ring1B) to CpG islands dependent on its CXXC domain. Interestingly, how DNA and histone modifications affect the binding of effector proteins to regulate chromatin function has been intensively investigated. For example, Bartke et al. (2010) used an elegant in vitro nucleosome affinity purification approach combined with SILAC (stable isotope labeling by amino acids in cell culture)-based proteomic analysis to identify proteins regulated by DNA and/or histone methylation. Relevant to this study, they found that DNA methylation, irrespective of the histone methylation status, strongly excludes the recruitment of components of the Fbxl10-containing BCOR complex, but not of other PRC1 complexes (Bartke et al., 2010). This is nicely consistent with our work and further supports the notion that DNA methylation inhibits the binding of Fbxl10 to CpG motifs.

Fbxl10 and Fbxl11 share conserved domains, including the JmC and CXXC domains. Consistent with this, we have shown that Fbxl10 binds to CpG islands as Fbxl11 (Blackledge et al., 2010). Interestingly, Fbxl11 does not associate with PcG proteins and its function therefore appears different from that of Fbxl10. However, both proteins catalyze the demethylation of H3K36me2, which is a very abundant modification and is specifically depleted at CpG islands (Blackledge et al., 2010). Although it remains unclear whether H3K36me2 affects PcG binding in vivo, it is tempting to speculate that the demethylase activity of Fbxl10 plays the role of a “fail-safe” mechanism that prevents aberrant dimethylation of H3K36 on CpG islands. H3K36 methylation was recently shown to inhibit PRC2-mediated H3K27 methylation (Yuan et al., 2011), and Fbxl10 could in this way play a role in ensuring that genes associated with H2AK119ub1 are also trimethylated on H3K27.

In addition to Fbxl10 and Fbxl11, Cfp1/Cxxc1 (Thomson et al., 2010) and Tet1 (Williams et al., 2011; Wu et al., 2011) also bind to most CpG islands in ESCs. The convergence of CpG-binding proteins at CpG islands may cooperatively contribute to the establishment of a specialized chromatin state. Generally, these proteins confer an H3K4me3-positive, an H3K36me2-depleted, and a DNA hypomethylation chromatin status, which in turn may confer an important platform for additional levels of chromatin modifications and transcriptional regulation, as discussed above.

Up to 70% of mammalian genes are associated with CpG islands, most of which are free from DNA methylation. CpG islands function as important platforms for adopting the diversity of chromatin states. The default state is permissive to either activation or repression (reviewed in Blackledge and Klose, 2011; Deaton and Bird, 2011). According to our genome-wide mapping study, Fbxl10 occupies almost all CGI genes, including both bivalent and active genes. It directly recruits Ring1B to bivalent genes and promotes H2AK119 ubiquitylation, which may restrain paused RNA pol II at the TSS of bivalent genes (Stock et al., 2007). Recent genome-wide mapping in ESCs showed that H2AK119ub1 is more closely associated with bivalent (i.e., H2AK119ub1 with H3K4me3) chromatin states than H3K27me3 (H3K27me3 with H3K4me3) (97% versus 79%) (Brookes et al., 2012). The target genes under these conditions will not be fully expressed or silenced until PcGs are dissociated by specific activators or by gaining DNA methylation. Interestingly, recent
results have shown that both Ring1A and Ring1B contribute to H2AK119ub1 in ESCs and that the deletion of the two Ring1 proteins leads to increased expression of target genes and loss of ESC proliferation (Endoh et al., 2012). In our study, downregulation of Fbx10 in ESCs resulted in a decrease in H2AK119ub1, which was a bit lower than the one observed in Ring1B knockout (KO) ESCs. As for Ring1B KOs, this decrease in H2AK119ub1 does not lead to defects in ESC proliferation and only has a minor effect on the transcription of the genes we tested (Figure S7C). We speculate that other PRC1 complexes, dependent or independent of residual H2AK119ub1, repress these genes. In other words, additional signals besides the partial removal of H2AK119ub1 are most likely required for the transcriptional activation of PcG-regulated genes. Meanwhile, Fbx10 occupies many non-PcG target genes in ESCs (the second group) that may be targeted by PcG later in differentiated cells. It is interesting to note that PcG recruiter proteins have been shown to display a dual function in activation as well as silencing (Schuettengruber et al., 2009). Thus, it might be important for Fbx10 to preprogram both groups of genes in ESCs and provide a permissive epigenomic landscape for proper differentiation (Figures 7B and S7B). However, Fbx10 may also play a broader role in regulating transcription. Taken together, our findings show that the histone demethylase Fbx10 has an important role in recruiting PRC1 components to CpG islands and in regulating the global levels of H2K119ub1 in ESCs.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation Assays

Mouse ESC lines E14, Suz12−/− (Pasini et al., 2007), and Ring1b−/− (Leeb and Wutz, 2007) were cultured in 2i medium as described (Silva et al., 2008). ESCs were differentiated through the formation of EBs and by removal of LIF, GSK3β, and MEK1 inhibitors, and addition of 10% serum.

shRNAs

Target sequences used in this study were as follows (if not specified, shFbx10-D was used to downregulate Fbx10): Fbx10 shRNA-D: GCCGGCTCATTATTCGCCATAT; Fbx10 shRNA-F: GCGGCTCATTATTCGCCATAT; Fbx11 shRNA: AAGGAGCTGTCTGAAGTTGAG. Target sequences used in this study were as follows (if not specified, shRNAs were listed in Table S3).

Real-Time qPCR and Immunoblotting

Total RNA was extracted with the RNeasy Mini Kit (QIAGEN) and reverse transcribed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems). For obtaining whole-cell protein extracts, cells were lysed in radioimmunoprecipitation assay buffer. Primer sequences and antibodies are listed in Table S3.

IP, ChIP, and ChIP-Seq

IP and the conventional ChIP were performed as described (Pasini et al., 2007). For the ChIP in FH-Fbx10-stable ESCs, a modified ChIP protocol that included a one- or two-step FLAG-HA immunoprecipitation enrichment of chromatin was used (see Supplemental Information). Primer sequences for the amplification of genomic DNA are available in Table S3. ChIP-Seq is described in Supplemental Information. The ChIP-seq data sets used in this study are listed in Table S1.

ACCESSION NUMBERS

The ChIP-seq data sets have been deposited into the Gene Expression Omnibus (GEO) under accession number GSE37930.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.01.016.

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