Diverse factors are involved in maintaining X chromosome inactivation

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X chromosome inactivation (XCI) is the most dramatic example of epigenetic silencing in eukaryotes. Once established, the inactivated X chromosome (Xi) remains silenced throughout subsequent cell divisions. Though the initiation of XCI has been studied extensively, the protein factors involved in Xi silencing and maintenance are largely unknown. Here we report the discovery of a diverse set of 32 proteins involved in maintenance of Xi silencing through a genome-wide RNAi screen. In addition, we describe the mechanistic roles of two proteins—origin recognition complex 2 (Orc2) and heterochromatin protein 1 (HP1α)—in Xi silencing. Immunofluorescence studies indicate that Orc2 and HP1α localize on Xi in mouse cells. Depletion of Orc2 by shRNA leads to the loss of both Orc2 and HP1α localization on Xi. Furthermore, the silencing of genes on Xi is disrupted in both Orc2- and HP1α-depleted cells. Finally, we show, using ChIP assay, that the localization of HP1α and Orc2 to the promoter regions of Xi-silenced genes is interdependent. These findings reveal a diverse set of proteins involved in Xi silencing, show how Orc2 and HP1α impact Xi silencing, and provide a basis for future studies on the maintenance of Xi silencing.

X chromosome inactivation (XCI) is the molecular mechanism that ensures equivalent gene dosage of the X-linked genes between XX female and XY male mammals (1–4). This process initiates at an early stage of embryogenesis with the expression of the X-inactive–specific transcript (*Xist*) noncoding RNA, which then coats the inactivated X chromosome (Xi) in *cis* and facilitates the spreading of silencing factors to the entire Xi (5, 6). XCI represents a dramatic example of epigenetic silencing in mammals, which results in silencing of most of the genes on the entire Xi. Once established, Xi silencing is maintained for subsequent generations. Compared with studies on the initiation of Xi silencing, relatively few studies have been performed to understand how Xi silencing is maintained during mitotic cell divisions.

Cytogenetic studies indicated that several epigenetic marks are enriched on Xi. For instance, noncoding RNA Xist is highly enriched on Xi. Following the coating of Xi by Xist-RNA, several histone marks associated with euchromatin, such as H3K9ac, H3K4me2, and H3K4me3, are lost and several repressive marks, including H3K27me3, H3K9me2, H4K20me1, and H2Aub1, start to appear on Xi (7–10). Of these histone marks, H3K27me3 catalyzed by PRC2 appears to coat a large portion of mouse Xi (10). In addition to these changes in histone modification, DNA on Xi is hypermethylated. Interestingly, mutations in *PRC2* or the *Xist* gene alone have little effect on silencing of genes on Xi after initiation of Xi silencing (3); such findings suggest that these factors are not involved in the maintenance of Xi silencing. Alternatively, these factors function in synergy for Xi silencing, and mutations in each alone will not affect Xi silencing dramatically. Indeed, a later study using combinations of inhibitors against histone deacetylases and DNA methyltransferases as well as mutations on *Xist* indicated that histone hypoacetylation, Xist RNA, and DNA methylation work in synergy to maintain Xi silencing (11). Thus, maintenance of Xi silencing likely involves multiple silencing mechanisms.

A screen for the mutants that impact position effect variegation (PEV) in *Drosophila* identified about 60 mutants, and most of these mutants affect chromosome inheritance and maintenance (12). However, the identity of many of these mutated genes is not known. As Xi silencing involves mechanisms as intricate as PEV, one would expect that many protein factors would be involved in Xi silencing. However, to date, very few protein factors involved in Xi silencing have been identified. In this report, we described a GFP-based assay for identification of genes involved in Xi silencing. Using this assay and a genome-wide shRNA screen, we identified 94 genes involved in Xi silencing. We validated 46 candidates using an independent set of shRNAs and found 32 proteins were involved in Xi silencing. Many of these factors had not been implicated in Xi silencing previously. Characterization of Orc2 revealed that it impacted Xi silencing, at least partly, through stabilization of HP1α on Xi.

Results and Discussion

Genome-Wide shRNA Screen for Factors Involved in Xi Silencing. Previously we used the GFP transgene integrated at the silent mating-type locus as a reporter gene to identify genes involved in transcriptional silencing in budding yeast (13). Therefore, we decided to use similar approaches to identify genes involved in silencing on Xi. To do this, we first isolated mouse embryonic fibroblasts (MEF) from a female embryo produced by crossing a female wild-type ICR mouse with a male carrying an X chromosome-linked GFP transgene (14) (Fig. 1*A* and Fig. S1*A*). These MEF cells were then immortalized (iMEF) using SV40 large T antigen. GFP– cells were isolated by FACS, and individual clones were verified for the presence of silenced GFP on Xi (Fig. S1*A*). Because both histone deacetylation and DNA methylation are required for Xi silencing (11), we verified these cells further by testing whether Xi-GFP silencing depends on either of these two mechanisms. iMEF cells were treated with the DNA methyltransferase inhibitor 5-aza-2′deoxycytidine (5-aza) and/or the histone deacetylase inhibitor trichostatin A (TSA), and the percentage of GFP+ cells was determined by flow cytometry. Although 5-aza and TSA alone resulted in reactivation of Xi-GFP in a substantial proportion of cells, combined treatment reactivated GFP in a much higher proportion of cells (Fig. 1*B* and Fig. S1*B*). This finding is consistent with the idea that histone deacetylation and DNA methylation synergize to maintain Xi silencing (11). Furthermore, these results demonstrate that Xi-GFP is epigenetically silenced and that the iMEF cell line is suitable for a genome-wide screen of factors involved in Xi silencing.

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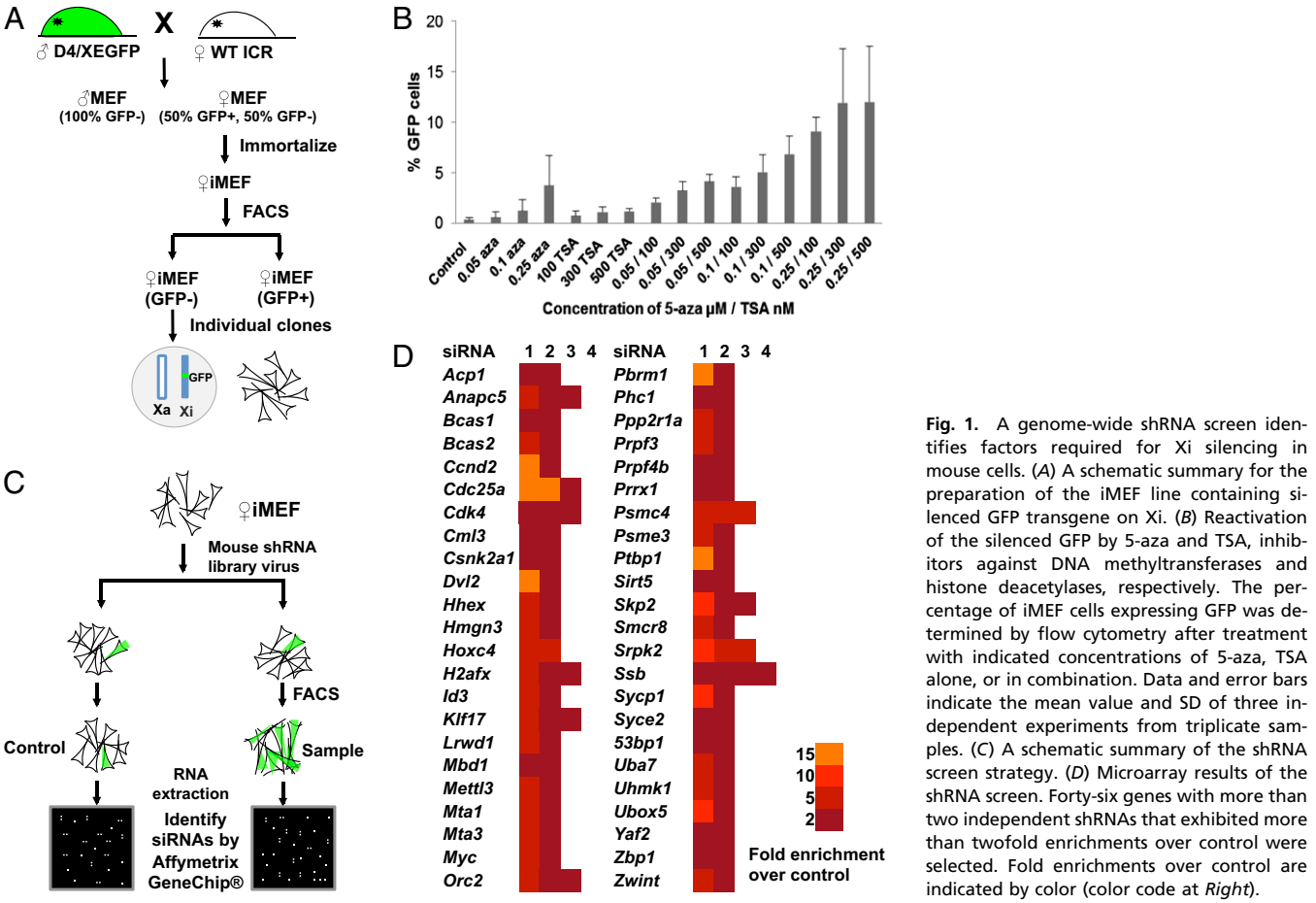

Fig. 1. A genome-wide shRNA screen identifies factors required for Xi silencing in mouse cells. (*A*) A schematic summary for the preparation of the iMEF line containing silenced GFP transgene on Xi. (*B*) Reactivation of the silenced GFP by 5-aza and TSA, inhibitors against DNA methyltransferases and histone deacetylases, respectively. The percentage of iMEF cells expressing GFP was determined by flow cytometry after treatment with indicated concentrations of 5-aza, TSA alone, or in combination. Data and error bars indicate the mean value and SD of three independent experiments from triplicate samples. (*C*) A schematic summary of the shRNA screen strategy. (*D*) Microarray results of the shRNA screen. Forty-six genes with more than two independent shRNAs that exhibited more than twofold enrichments over control were selected. Fold enrichments over control are indicated by color (color code at *Right*).

To perform such a screening (Fig. 1*C*), iMEF cells were infected with a lentiviral-based mouse shRNA library containing 150,000 shRNA against 40,000 genes or EST (15). GFP+ iMEF cells were collected by FACS at 72 h postinfection. Total RNA from the control and GFP+ cells was extracted, reverse-transcribed and used to identify shRNAs that were enriched in the GFP+ cells by Affymetrix GeneChip (Fig. 1*C*). Ninety-four genes, each with at least two independent shRNAs enriched by at least twofold over control, were identified, 46 of which are listed in Fig. 1*D*.

Validation of the Screening Results. We selected 46 genes, based mainly on the availability of an independent set of shRNA against each gene, to confirm to what extent these genes are involved in silencing of Xi-GFP (Fig. 1*D*). Of the 46 proteins tested, depletion of each of 32 proteins with at least one shRNA resulted in elevated GFP expression compared with nontargeting control cells (Fig. 2*A*). In contrast, depletion of Ezh2, the catalytic subunit of the PRC2 complex, had no apparent effect on GFP expression, which is consistent with published results (16). Our results suggest that these 32 proteins are required for silencing of the Xi-linked GFP transgene. Consistent with this idea, increased GFP mRNA levels were detected by real-time PCR (RT-PCR) after depletion of each of the 32 proteins (Fig. 2*B*).

To determine whether any of these 32 proteins are required for silencing of endogenous genes known to be silenced on Xi, we used RT-PCR to analyze gene expression of three X-linked genes (*Hprt*, *Pgk-1*, and *MeCP2*) after depletion of each of the 32 proteins. At least one of the three X-linked genes was up-regulated after depletion of each of the 32 proteins, whereas the expression of *Utx*, which escapes XCI (17), was not altered significantly (Fig. 2*C*). Thus, 32 genes impact silencing of both the GFP transgene as well as endogenous genes on Xi, validating their role in Xi silencing.

The majority of these 32 genes have not previously been implicated in Xi silencing. Gene ontology analysis revealed that most of the genes are involved in RNA processing, cell cycle regulation, gene transcription, and chromatin (Fig. 2*D* and Table S1), suggesting that they impact Xi silencing via distinct mechanisms. To test this idea, we further characterized Orc2, a protein known for its role in DNA replication.

Orc2 Localizes on Xi and Impacts Xi Silencing. Orc2 is a subunit of the origin recognition complex (ORC), a complex that functions in DNA replication initiation (18). In addition, yeast and *Drosophila* ORC has been implicated in transcriptional silencing (19, 20), and human Orc2 has been shown to colocalize with HP1α at heterochromatin foci (21, 22). However, it is unknown whether Orc2 has any role in Xi silencing. We therefore examined whether Orc2 is localized on Xi by indirect immunofluorescence. As reported, trimethylation of H3 lysine 27 (H3K27me3) was highly enriched on Xi at the nuclear periphery (10), and Orc2 was detected at foci that colocalize with DAPI staining (21) (Fig. 3*A*). Importantly, we observed that in interphase, over 85% of cells showed prominent Orc2 staining adjacent to or overlapping with the H3K27me3 focus (Fig. 3*A*) or Xist RNA (Fig. S2). Given the high percentage of cells with an Orc2 focus adjacent to the H3K27me3 focus during interphase, we asked whether Orc2 is localized on the Xi during other stages of the cell cycle. Instead of staining Orc2 in metaphase chromosomes, which might give false-negative signals due to fixation methods (19, 23), we ana-

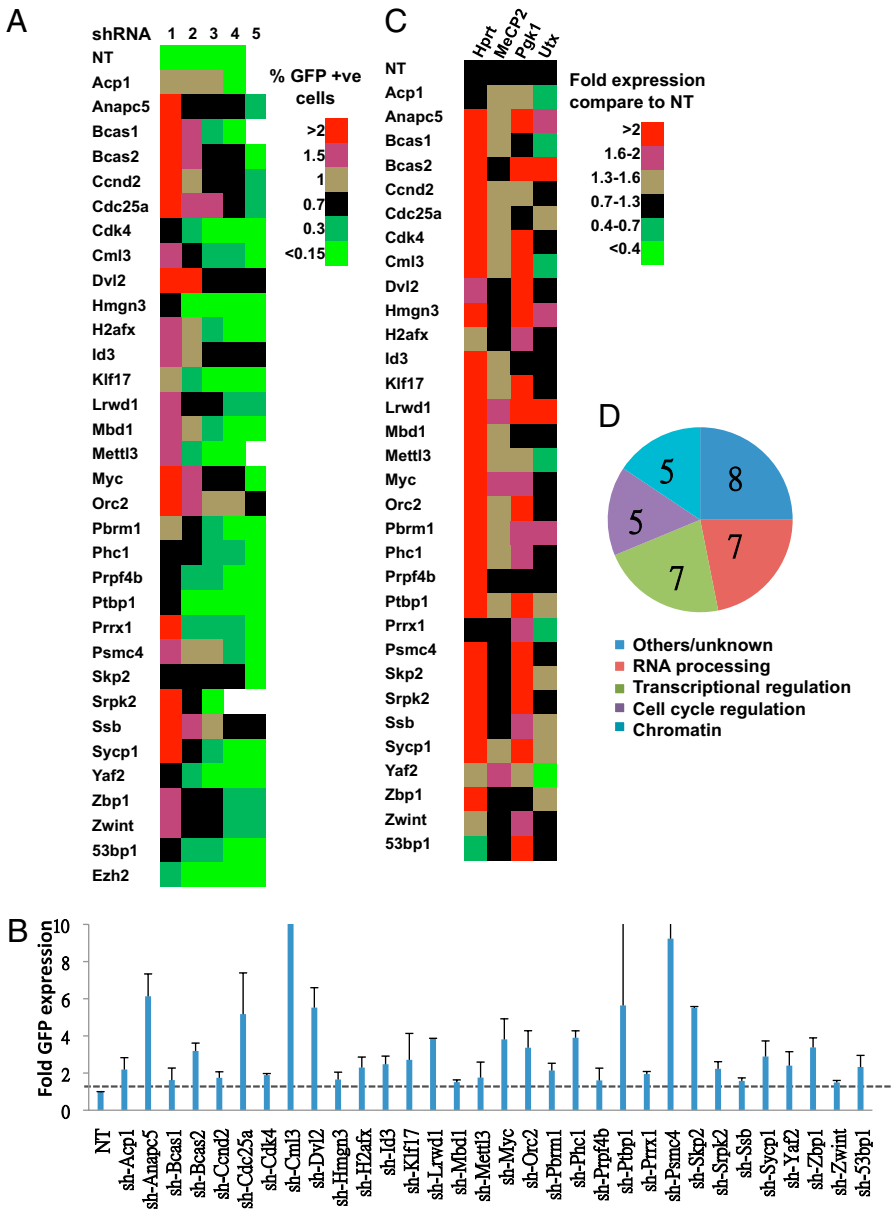

Fig. 2. Verification of genes involved in Xi silencing. (*A*) Thirty-two genes are involved in silencing of Xi. Four or five shRNAs against each of 46 genes shown in Fig. 1*D* were used to knock down each gene individually in iMEF cells, and the percentage of GFP+ cells was determined by flow cytometry. Percentage of GFP+ iMEF for each shRNA was represented by different colors (color code at *Right*). NT, nontargeting shRNA. (*B*) Analysis of the GFP mRNA after depletion of each of the 32 proteins shown in *A*. One shRNA was selected based on the results shown in Fig. 2*A* against each of the 32 proteins for depletion of the corresponding protein. After depletion, RNA was purified and reverse transcribed. The GFP mRNA was quantified by real-time PCR and normalized against β-actin and compared with NT shRNA. Data and error bars indicate the mean value and SD of two independent experiments from duplicate samples. (*C*) Up-regulation of X-linked genes in iMEF after depletion of each of the 32 proteins using shRNA. The level of mRNA for the X-linked genes *Hprt*, *Pgk1*, *MeCP2*, and *Utx* was determined as described above. Fold expression of each gene over NT is represented by different colors (color code at *Right*). (*D*) Pathway analysis of the genes identified in the screen.

lyzed the localization of Orc2 and H3K27me3 on Xi in prophase and prometaphase cells where individual chromosomes can be revealed by DAPI staining (Fig. 3*B*). Orc2 was found at the pericentric heterochromatin on Xi (prophase 34/34 cells counted, prometaphase 24/24). In addition, Orc2 localization on Xi was lost upon Orc2 depletion by shRNA. These results strongly suggest that Orc2 is localized at Xi.

Next, we determined how depletion of Orc2 affected expression of seven additional X-linked genes subjected to XCI (Fig. 3*C* and Table S2). In addition to *Hprt*, *MeCP2*, and *Pgk1* (Fig. 2*C*), depletion of Orc2 also led to increased expression of two other X-linked genes (*Mtmr1* and *Mtm1*), whereas Orc2 depletion had no apparent effect on the expression of four other genes tested (*Mcts1*, *Rpgr*, *Gla*, and *Smc1a*). Interestingly, we observed that depletion of Orc2 also resulted in a slight reduction in expression of the *Ocr1* gene. These results suggest that depletion of Orc2 affects gene expression of a subset of X-linked genes subjected to inactivation. We note that a mutant allele of *SmcHD1*, a protein known to be involved in Xi silencing, also up-regulates only a fraction of X-linked genes in female mice (24), consistent with the idea that multiple mechanisms are involved in silencing X-linked genes.

To understand how depletion of Orc2 affects epigenetic silencing on Xi, we first determined whether Orc2 depletion affects heterochromatin marks such as trimethylation of histone H3 lysine 9 (H3K9me3), H3K27me3, and histone H4 lysine 20 (H4K20me3). Loss of Orc2 had no apparent effect on foci marked by these marks (Fig. 3*A* and Fig. S3). Moreover, there was no significant difference in *Xist* expression as revealed by RT-PCR and *Xist* RNA FISH (Fig. 3*C* and Fig. S4). Consistent with a previous report showing that Orc2 is required for the localization of HP1α on heterochromatin in human cells (21), HP1α localization at heterochromatin in interphase was significantly compromised in Orc2-depleted cells, even though total HP1α levels were unperturbed by Orc2 depletion (Fig. S5). These results suggest that Orc2 may be required for HP1α localization on Xi.

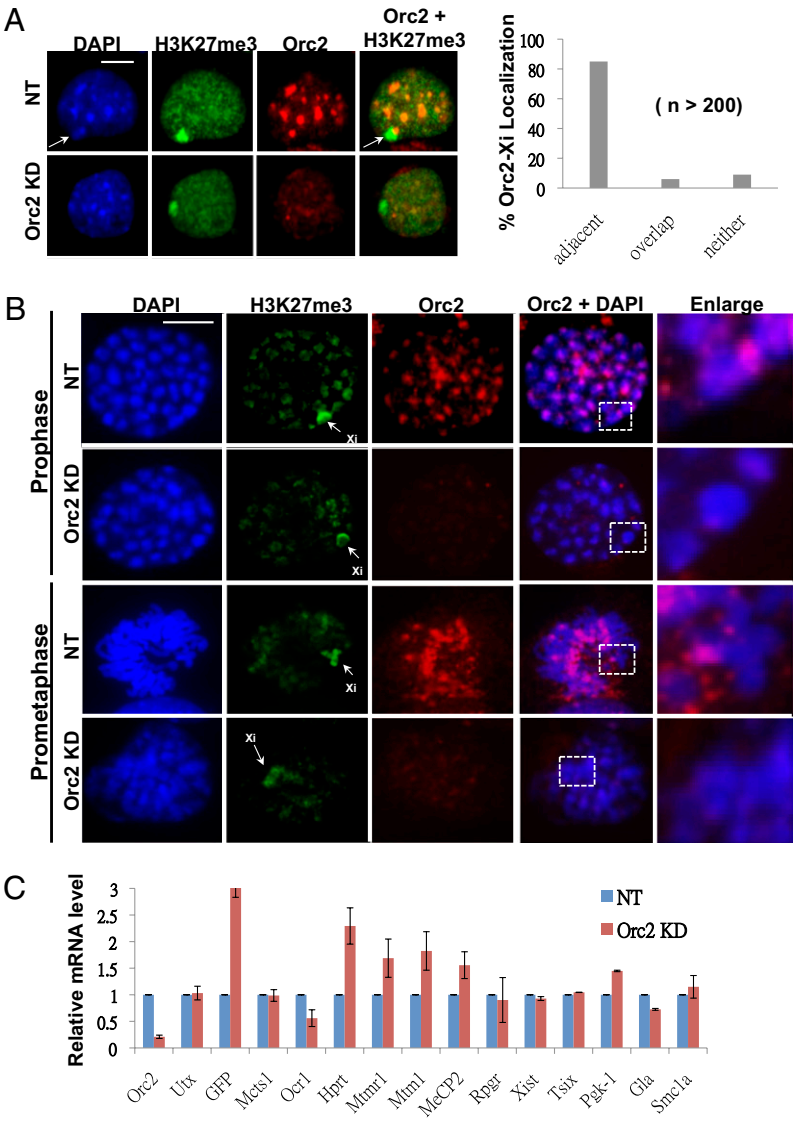

Fig. 3. Orc2 localizes on Xi and impacts Xi silencing. (*A*) An Orc2 focus is adjacent to the H3K27me3 focus based on immunofluorescence staining. iMEF cells were treated with nontargeting shRNA (NT) or Orc2 shRNA to knock down Orc2 (Orc2 KD) and immunostained with antibodies against H3 methylated at lysine 27 (H3K27me3, green) or Orc2 (red). (*Right*) Graph shows percentage of cells with colocalization of Orc2 and H3K27me3. (*B*) Orc2 localizes at the pericentric heterochromatin of Xi in prophase (*Upper*) and prometaphase (*Lower*) in iMEF cells. (Scale bars: 5 μm.) (*C*) Reactivation of the silenced GFP transgene and up-regulation of some X-linked genes in Orc2 shRNA knock down iMEF. X-linked genes *Hprt*, *Mtmr1*, *Mtm1*, *MeCP2*, and *Pgk-1*, as well as the silenced GFP transgene, were up-regulated in Orc2-depleted iMEF. Graph shows the data of mean and SD of real-time RT-PCR from two independent experiments.

HP1α Localizes on Xi and Impacts Xi Silencing. Because it is still controversial whether HP1α is localized on Xi in mouse cells (25), and HP1α was not identified in our screen, we first performed a detailed analysis of the localization of HP1α using immunofluorescence. More than 95% interphase cells (113 of 118 cells counted; Fig. 4*A*, *Upper*) and 100% prophase cells (51 of 51 cells counted; Fig. 4*A*, *Lower*) had a HP1α focus adjacent to the H3K27me3 focus, which is reminiscent of the pericentric heterochromatin localization of Orc2 on Xi. Moreover, depletion of HP1α resulted in loss of HP1α staining (Fig. 4*A*), suggesting that this staining is specific. This result indicates that HP1α is localized on Xi.

To determine whether HP1α is required for Xi silencing, we analyzed how depletion of HP1α affected expression of the GFP transgene and 13 X-linked genes. Depletion of HP1α resulted in increased expression of the GFP transgene as detected by flow cytometry (Fig. S6) and RT-PCR (Fig. 4*B*). Of the 13 X-linked genes tested, the expression of five (*Mcts1*, *Ocr1*, *Hprt*, *Mtmr1*, and *Mtm1*) increased, whereas the expression of seven other genes (*Rpgr*, *Xist*, *Tsix*, *Pak-1*, *Gla*, and *Smc1a*) was not altered to a detectable degree in HP1α depletion cells. *Mtm1*, which is located 69 megabases (Mb) from pericentric heterochromatin of X, is the distal gene that is up-regulated in HP1α-depleted cells. Thus, HP1α is also involved in Xi silencing, possibly at the chromatin region close to pericentric heterochromatin.

Of 14 genes (including the GFP transgene) tested, depletion of Orc2 and HP1α affected expression of most genes (12 of 14) similarly (Figs. 3*C* and 4*B*), suggesting that there is a general trend of Orc2 and HP1α affecting expression of X-linked genes via similar mechanisms. Interestingly, depletion of Orc2 and HP1α also affect expression of two genes (*MeCP2* and *Ocr1*) differently, suggesting that Orc2 and HP1α impact expression of these two genes independently.

To determine if the effect of Orc2 or HP1α depletion on expression of X-linked genes is due to reactivation of genes on Xi

rather than up-regulation from the active X-chromosome, we determined how depletion of Orc2 or HP1α affects expression of *Hprt* using RNA FISH (Fig. S7). In iMEF cells infected with virus for nontarget shRNA control, we observed monoallelic expression of *Hprt*—a single Hprt RNA dot per nucleus (217 of 217 cells counted). In addition, the Hprt RNA signal was far away from the Xist RNA cloud (which marks Xi), indicating that this Hprt RNA signal is from Xa (Fig. S7 *A* and *C*). In Orc2- or HP1α-depleted iMEF, ~9–10% of cells showed biallelic expression of *Hprt*, with one Hprt RNA signal distant from the Xist RNA cloud, and the other close to the Xist RNA cloud, which likely represents the reactivation of the silenced *Hprt* allele on Xi (Fig. S7 *B* and *C*). Together, these results demonstrate that the effect of Orc2 and HP1α depletion on the expression of Hprt is due to reactivation of *Hprt* on silenced Xi and further suggests that Orc2 and HP1α impact Xi silencing in MEF cells.

Next, we determined whether Orc2 is required for stable localization of HP1α on Xi. Depletion of Orc2 resulted in loss of HP1α association with pericentric heterochromatin of Xi (Fig. S8). To confirm the localization of Orc2 and HP1α localization on Xi and the dependence of HP1α localization on Orc2, we performed ChIP assays using antibodies against Orc2 and HP1α and analyzed coprecipitated DNA using real-time PCR. Significantly more Orc2 and HP1α could be detected at the GFP transgene and the promoter of *Hprt*, but not the promoter of *Utx* that escapes Xi silencing (Fig. 4 *C* and *D*). Moreover, depletion

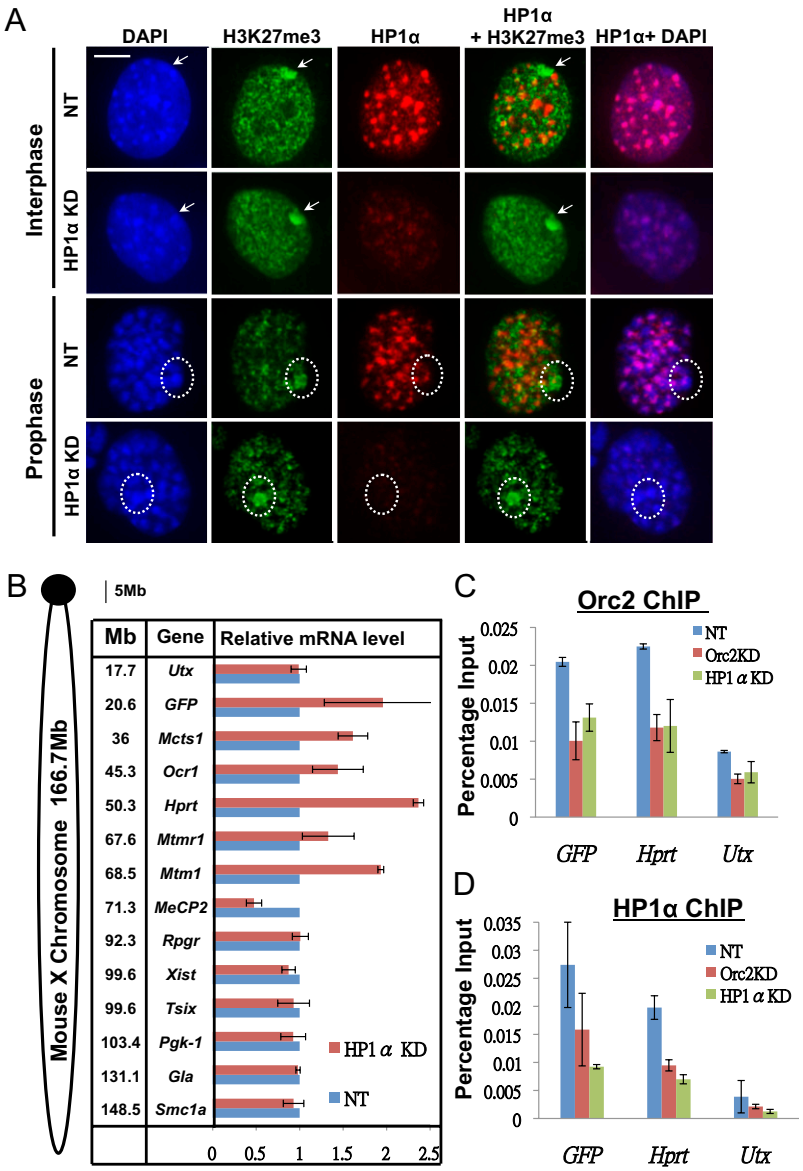

Fig. 4. HP1α localizes on Xi and is required for Xi silencing. (*A*) HP1α localizes adjacent to the H3K27me3 focus in interphase cells (113/118 cells counted; *Upper*) and in prophase cells (*Lower*; 51/51 cells counted). Depletion of HP1α results in a loss of this localization. (*B*) Depletion of HP1α results in increased expression of the GFP transgene and some X-linked genes. The expression of 13 X-linked genes was determined by real-time RT-PCR in cells infected with virus for nontargeting (NT) or HP1α (HP1α KD), and the fold change (KD/NT) of each gene was reported. The distances of each gene from the centromere on mouse X chromosome was shown. Pericentric heterochromatin (black circle) occupies ~6 Mb from centromere on X chromosome. (*C* and *D*) The localization of Orc2 and HP1α on Xi are dependent on the presence of each other. ChIP assays were performed using antibodies against Orc2 (*C*) and HP1α (*D*) in NT control, Orc2 knockdown (Orc2 KD), or HP1α KD. ChIP DNA was analyzed by real-time PCR using primers flanking the GFP transgene and the promoter regions of X-linked genes *Hprt* or *Utx*.

of Orc2 or HP1α resulted in reduced binding of Orc2 and HP1α to the GFP transgene and *Hprt*, respectively. These results provide additional support to the idea that Orc2 and HP1α are localized at Xi. Importantly, the binding of HP1α to the GFP transgene and *Hprt* promoter was significantly reduced in Orc2-depleted cells, confirming that Orc2 was required for localization of HP1α on Xi. Interestingly, we also observed that depletion of HP1α resulted in reduced binding of Orc2 to the GFP and the promoter of *Hprt*, indicating that the localization of Orc2 and HP1α on Xi is interdependent.

Since the first report of XCI by Mary Lyon in 1961 (26), the initiation of XCI has been studied extensively. However, little is known about the maintenance of XCI and the protein factors involved. Our genome-wide RNAi screen uncovers protein factors important for maintenance of gene silencing on Xi. Moreover, we have shown that Orc2 and HP1α are localized to chromatin regions close to pericentric heterochromatin of Xi and are involved in Xi silencing. These results suggest that like human Xi, mouse Xi is also organized into different functional domains (27), such as the ones containing HP1α or H3K27me3. Orc2 and HP1α also function in constitutive heterochromatin, such as pericentric heterochromatin; these findings suggest that some factors identified in this screen have roles in silencing other than Xi silencing. Furthermore, 32 protein factors identified in this study will likely function in several distinct pathways to maintain Xi silencing. For instance, Lrwd1 was recently shown to interact with ORC complex (28, 29). Therefore, Lrwd1 identified in this screen is likely to function in the same pathway as Orc2 in Xi silencing. In addition, we also identified several factors, such as Bcas2 and Prpf4b, which were previously known for their roles in RNA processing, raising the possibility that they may function in the same pathway for Xi silencing as well. In this regard, we note that genes involved in RNA splicing have been shown to be involved in heterochromatin silencing in *Schizosaccharomyces pombe* (30). A recent report suggests that ASF/SF2-dependent Xist RNA processing is important to regulate random X inactivation (31). Therefore, protein factors identified in this study will provoke future analysis of how Xi silencing, as well as silencing at other types of heterochromatin (e.g., constitutive heterochromatin), is maintained in mammalian cells.

Methods

Genome-Wide shRNA Screen. Immortalized female MEF cells bearing the silenced GFP transgene were subjected to a genome-wide shRNA screen. In brief, cells were infected with lentivirus containing a genome-wide mouse shRNA library with 150,000 siRNA covering 40,000 genes (System Biosciences). Cells were collected 72 h postinfection and subjected to FACS sorting to isolate GFP-expressing cells. RNA was isolated from cells, reverse transcribed, and amplified by PCR. Subsequently, Mouse GeneChip Affymetrix hybridization was performed according to the manufacturer's instructions (System Biosciences) using these cDNAs. To validate the primary screening results, independent shRNAs were purchased from Sigma and lentiviruses were produced for infection and knockdown of each of the target genes in iMEF cells.

Other experimental procedures, including cell culture, cells treated with inhibitors against histone deacetylases (HDAC) and DNA methyltransferases (DNMT), real-time PCR coupled with reverse transcription, ChIP assays, immunofluorescence, RNA fluorescence in situ hybridization, primer sequences for genotyping, and real-time PCR and shRNA sequences are included in *SI Methods*.

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