Polycomb group proteins are essential epigenetic repressors. They form multiple protein complexes of which two kinds, PRC1 and PRC2, are indispensable for repression. Although much is known about their biochemical properties, how mammalian PRC1 and PRC2 are targeted to specific genes is poorly understood. Here, we establish the cyclin D2 (CCND2) oncogene as a simple model to address this question. We provide the evidence that the targeting of PRC1 to CCND2 involves a dedicated PRC1-targeting element (PTE). The PTE appears to act in concert with an adjacent cytosine-phosphate-guanine (CpG) island to arrange for the robust binding of PRC1 and PRC2 to repressed CCND2. Our findings pave the way to identify sequence-specific DNA-binding proteins implicated in the targeting of mammalian PRC1 complexes and provide novel link between polycomb repression and cancer.

Polycomb group (PcG) proteins compose a family of epigenetic repressors that prevent unscheduled transcription of hundreds of developmental genes (1–4). PcG proteins act in concert as multisubunit complexes. These are usually grouped into two evolutionarily conserved classes designated Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2). Several auxiliary complexes, whose repertoire varies between species, further aid PRC1 and PRC2 to achieve robust repression (5).

PRC2 complexes contain a core with four subunits: EZH2 (or related protein EZH1); EED; SUZ12; and RBBP4 (or closely related YAF2 protein) and one of the four PCGF proteins (PCGF1, PCGF3, PCGF5, and PCGF6). Complexes with different PCGF subunits and/or accessory proteins (including PPRC1, PCGF2, and RBBP4) are called non-canonical PRC2 complexes. The PRC2 complexes, including PRC1, can act as E3 ligases and transferase specific to lysine 27 of histone H3 (H3K27me3 produced by PRC2 via the chromosomal binding of their CBX subunits (23–25)). Mutations in genes encoding PRC1 subunits lead to embryonic lethality and misexpression of HOX genes, indicating that PRC1 complexes are essential for PcG repression (26–29).

Other RING2–RING1 complexes contain RYBP (or closely related YAF2 protein) and one of the four PCGF proteins (PCGF1, PCGF3, PCGF5, and PCGF6). Complexes with different PCGF proteins have distinct additional subunits (5, 30). Although PRC1 complexes are integral for PcG repression, the role of other RING–PCGF complexes, sometimes called non-canonical PRC1, is less clear (31–34). All RING–PCGF complexes, including PRC1, can act as E3 ligases in vitro to transfer a single ubiquitin group to lysine 119 of histone H2A (H2AK119) onco-genes (31, 32). To what extent the H2AK119 ubiquitylation is critical for PcG repression is being debated (31, 37, 38).

Much is known about biochemical properties of PRC1 and PRC2, but how they are targeted to specific genes is not well understood. The process is better described for Drosophila, where the PcG system contains fewer variant proteins and has been studied for longer time. In flies, the targeting to specific developmental genes depends on designated polycomb-re-
sponse elements (PREs). These ~1-kb–long elements are the strongest genomic binding sites for PRC1 and PRC2 (39, 40) and are sufficient to generate novel binding sites for both complexes when integrated elsewhere in the genome (41). The pervasive di-methylation of H3K27 within intergenic regions and inactive genes suggests that PRC2 transiently interacts with most of the genome (21, 22). Likewise, the increase of intergenic transcription in cells where PRC1 is depleted suggests that this complex also scans the entire genome (22). In this view, Drosophila PREs are the sites where PRC1 and PRC2 are retained much longer than elsewhere in the genome. A subset of transcriptionally active gene promoters can also retain PRC1 but not PRC2 (42–45). The amount of PRC1 detected at these sites is an order of magnitude lower than at PREs (43–45).

Multiple lines of evidence indicate that Drosophila PREs contain combinations of recognition sequences for different DNA-binding factors. These factors act cooperatively to anchor PRC1 and PRC2, which themselves cannot bind DNA in sequence-specific fashion (41). Recent studies indicate that PREs continue to retain PRC1 when PRC2 and H3K27 methylation are removed by mutation, but PRC2 binding at many PREs is significantly reduced if PRC1 is ablated (46). Consistently, in cells where PRE-equipped Drosophila genes are active, they often lose H3K27me3 and PRC2 but have PRC1 strongly bound at PRE sites (40, 47, 48).

PRC1 and PRC2 complexes are evolutionarily conserved, and target many of the same developmental genes in Drosophila and mammals (4). Therefore, it is likely that the mechanisms that retain PRC complexes at these genes were in place before flies and mammals split from their last common ancestor. To what extent these mechanisms remain similar and which of them have diverged are open questions. The majority of studies in mammalian models have been focused on PRC2 targeting. Their results concur that DNA sequences with a high density of unmethylated CpG di-nucleotides (so-called CpG islands) that lack binding sites for transcriptional activators are sufficient to generate new binding sites for mammalian PRC2 but not PRC1 (49–51). However, in the context of the mouse HoxD locus, factors other than high CpG density appear more important (52). Much less is known about the DNA sequences involved in the targeting of mammalian PRC1. Two studies reported DNA elements capable of autonomous PRC1 targeting (53, 54). Although neither of the elements was mapped to high precision, the two elements appear to be very different. One of them was reported to generate new binding sites for both PRC1 and PRC2 (54), whereas the other seems to be targeting just PRC1 (53).

Here, we establish the human cyclin D2 (CCND2) oncogene as a simple model system to investigate the targeting of mammalian PcG complexes. Using this system, we find that the targeting of PRC1 to CCND2 involves a dedicated targeting element (PTE). This element may further cooperate with an adjacent CpG-island to support the robust binding of PRC1 and PRC2 at repressed CCND2.

Results

PRC1 and PRC2 complexes usually act together to effect epigenetic repression. However, in experiments with cultured Drosophila cells, we noted that some of the PcG target genes, when transcriptionally active, have their PREs bound by PRC1 in the absence of PRC2 and H3K27me3 (40, 48). This fueled our interest to a region ~4.8 kb upstream of the transcription start site (TSS) of the human CCND2 gene. Analyzing previously published chromatin immunoprecipitation (ChiP)–binding profiles (55), we noticed that in the human embryonic teratocarcinoma NT2-D1 cells this region is strongly immunoprecipitated with antibodies against BMI1 and MEL18 but very weakly with antibodies against EZH2 and H3K27me3 (Fig. 1, A and B). This is in stark contrast to all other sites on human chromosomes 8, 11, and 12, profiled by Kahn et al. (55), which show strong precipitation with anti-EZH2 and anti-H3K27me3 antibodies whenever they are strongly precipitated with antibodies against BMI1 or MEL18 (Fig. 1A).

In striking analogy with the PRC1 binding at PREs of transcriptionally active Drosophila genes, in the NT2-D1 cells, the CCND2 gene is highly transcribed (Fig. 1C). In contrast, in TIG-3 human embryonic fibroblasts, the CCND2 gene was reported to be transcriptionally inactive (56) (confirmed by our RT-qPCR measurements in Fig. 1C), decorated with H3K27me3, CBX8, and SUZ12, and up-regulated upon the knockdown of PRC1 and PRC2 (56). This indicates that CCND2 is a regular PcG target gene that, when transcriptionally inactive, acquires the chromatin state characteristic of PcG repression but binds a lot of PRC1 and little PRC2 and H3K27me3 in cells where it is transcriptionally active. Altogether, these observations raised a possibility that the PRC1-binding peak upstream of the CCND2 marks an element that targets PRC1 to this locus.

PRC1 and PRC2 binding to the CCND2 gene in alternative transcriptional states

To explore this possibility, we first performed quantitative ChiP analysis of PRC1, PRC2, and H3K27me3 binding at the CCND2 gene in the NT2-D1 and TIG-3 cell lines. To select informative protein targets for immunoprecipitation, we measured the mRNA levels for genes encoding PRC1 subunits to know which of the alternative variants are available for interrogation in each of the cell lines (Fig. S1). As shown by RT-qPCR, MEL18 mRNA is abundant in NT2-D1 cells and slightly less abundant in TIG-3 cells (Fig. S1). The BMI1 mRNA levels are lower than those of MEL18 but at the same level in both NT2-D1 and TIG-3 cells. RING1 mRNA level is low in both cell lines, but RING2 mRNA is abundant (Fig. S1). Out of five CBX genes implicated in PcG regulation (5), mRNA levels for CBX4, CBX6, and CBX7 are low in both NT2-D1 and TIG-3 cells. CBX8 mRNA is at the edge of the detection in both cell lines, and CBX2 mRNA is abundant in NT2-D1 but barely detectable in TIG-3 cells.

Immunoprecipitations of the formaldehyde cross-linked NT2-D1 chromatin with antibodies against MEL18, BMI1, CBX2, and RING2 give essentially the same results (Fig. 2 and Fig. S2). The ChiP signals peak within a putative PRC1 PTE and recede steeply at both sides to reach a background level halfway between the PTE and the CCND2 TSS. In TIG-3 cells, where CCND2 is transcriptionally inactive, the putative PTE remains the strongest precipitated site with ChiP signals similar to those...
detected in NT2-D1 cells. However, in these cells, in addition to the PTE, the entire upstream region of the CCND2 gene, including the TSS, is also immunoprecipitated, albeit at 10 times lower level. In contrast to PRC1, ChIPs with antibodies against SUZ12 and H3K27me3 give enrichment profiles that differ dramatically between the two cell lines. In NT2-D1 cells, SUZ12 ChIP signals are very weak, compared with that at the positive control ALX4 gene (Fig. 2), which is transcriptionally inactive and bound by PRC1 and PRC2 in both NT2-D1 and TIG-3 cells (55, 57, 58). These are paralleled by weak ChIP signals for H3K27me3. In contrast, in TIG-3 cells, ChIP signals for both antibodies are very strong and on par with those of the ALX4 gene. Strikingly, their profiles do not match those for PRC1. Instead, the SUZ12 and H3K27me3 profiles are broad...
and shifted away from the CpG-poor PTE into the adjacent CpG-rich region (Figs. 2 and 1B). The difference between the PRC1 and SUZ12/H3K27me3 ChIP profiles indicates that a high level of H3K27me3 is not sufficient to retain PRC1 to an extent seen at the putative PTE and that other mechanisms must contribute to PRC1 binding to this element. It also suggests that the putative PTE is not capable of retaining PRC2 as efficiently as activities linked to the adjacent CpG-rich region.

Figure 2. Polycomb proteins and associated histone modifications at the active and repressed CCND2 gene. A, schematic of the human CCND2 locus. Positions of PCR amplicons analyzed in B are shown below the coordinate scale. Note that amplicon 2 corresponds to the summit of MEL18 peak, and amplicon 5 corresponds to the CCND2 TSS (see Tables S4 and S5, for sequence information). B, ChIP profiles of MEL18, RING2, SUZ12, and H3K27me3 in NT2-D1 (left column) and TIG-3 (right column) cells. The immunoprecipitation of the ALX4 gene repressed by PcG in NT2-D1 and TIG-3 cells was used as positive control (for position of PCR amplicons see Fig. S11). The immunoprecipitation of a gene desert region (chromosome 12: 12771096–127713493; Hg19) was used as a negative control. Histograms for both are shown to the right of each graph and are to the same y axis scale. All histograms and graphs show the average and the scatter (whiskers) between two independent experiments.
**PRC1-targeting element of the cyclin D2 oncogene**

If the DNA fragment underneath the CCND2 PRC1 binding peak is a PRC1-targeting element, it should be able to generate new binding sites for PRC1 when integrated elsewhere in the genome. To test this, we cloned the 2.4-kb fragment covering the putative PTE into a lentiviral vector (Fig. 3A) and integrated it back into the genome of NT2-D1 cells by viral transduction.

To distinguish transgenic and endogenous copies of the putative PTE, we identified a small stretch of nucleotides close to the summit of the MEL18/BMI1 peak that shows little conservation within mammalian species and substituted five of those nucleotides in the transgenic copy to create an annealing site for a specific PCR primer (Fig. S3). An analogous construct containing a 2.4-kb fragment from a gene desert region on chromosome 12 showed no binding of PcG proteins and H3K27me3 (Fig. S3) and an empty vector were integrated in parallel as negative controls. The transduced cell lines were genotyped by PCR to validate their identity (Fig. S4 and Fig. S5).

As summarized in Fig. 3B, the 2.4-kb transgenic PTE fragment is immunoprecipitated with antibodies against BMI1 and
MEL18. The precipitation is robust and as strong as that of the endogenous PTE upstream of CCND2. This is in contrast to the transgenic insertion of the negative control fragment from the gene desert (Fig. 3C) or the empty vector (Fig. S5) whose precipitation is very low and close to the ChIP background. The immunoprecipitation of the transgenic 2.4-kb fragment with the antibodies against SUZ12 and H3K27me3 is weak and comparable with that of the endogenous site (Fig. 3B). Here, and in the following experiments mixed (nonclonal) populations of cells with insertions at multiple random genomic locations were used for ChIP assays. Because yields of ChIP reactions are normalized to the amount of input material, comparable immunoprecipitation of transgenic and endogenous PTEs indicates that, at the majority of the insertion sites, the transgenic 2.4-kb PTE fragment is bound by PRC1. Altogether, these observations suggest that the 2.4-kb DNA fragment underneath the CCND2 PRC1-binding peak contains an element capable of autonomous recruitment of PRC1 complexes.

**CCND2 PTE is evolutionarily conserved**

Evolutionary conservation is a good indicator that a regulatory element is functionally important. To investigate this question, we first looked at the evolutionary conservation of the DNA sequence underneath and around CCND2 PTE. Mouse Ccnd2 resides within a large 32-Mbp block of shared synteny between mouse chromosome 6 and human chromosome 12. Alongside with the ORF, which has high DNA sequence conservation, the 10-kb sequence upstream of the Ccnd2 TSS contains multiple blocks predicted as evolutionarily conserved DNA elements (Fig. 4A). One of these blocks corresponds to an ∼300-bp sequence that is directly below the PRC1 binding peak within the human CCND2 PTE.

In mouse F9 testicular teratoma cells, where the Ccnd2 gene is transcriptionally inactive (Fig. 4B), its upstream region is immunoprecipitated with antibodies against Cbx7 and Suz12 (Fig. 4C). This indicates that the mouse Ccnd2 is a PcG target gene. Similar to what is seen in inactive human CCND2 in TIG-3 cells, the ChIP signal for Cbx7 is highest at the site corresponding to the evolutionarily conserved block within the PTE, but the highest Suz12 ChIP signal is shifted from the Cbx7 peak toward the TSS into the CpG-rich area. In contrast, in mouse NIH3T3 cells where the Ccnd2 gene is transcriptionally active (Fig. 4E), the upstream region shows very little precipitation with anti-Suz12 antibodies. However, much like in human NT2-D1 cells, ChIP signals for Ring2 (used to track PRC1 because the Cbx7 gene is not expressed in NIH3T3 cells) are high and peak within the 300-bp sequence orthologous to the human CCND2 PTE (Fig. 4D). To extend the parallel between human and mouse Ccnd2 further, we cloned 3.6- and 1.4-kb fragments covering the putative mouse PTE into the same lentiviral vector used to test the human PTE (Fig. 4A), and we integrated it into the genome of the human NT2-D1 cells by lentiviral transduction. ChIP-qPCR analysis indicates that the AT-rich region common between 3.6- and 1.4-kb fragments is precipitated with antibodies against MEL18, CBX2, and RING2 and weakly with antibodies against SUZ12 (Fig. 4, E and F). This indicates that the mouse Ccnd2 gene is also equipped with a PTE.

**CCND2 PTE is a composite element whose activity depends on specific DNA sequences**

A typical Drosophila PRE is ∼1 kb long and contains recognition sequences for multiple unrelated DNA-binding proteins that cooperate to provide robust PcG targeting. Often it may be subdivided into fragments of a few hundred bp that can still recruit PcG proteins in transgenic assays, but the recruitment is less robust (59–61). Therefore, we wondered whether the CCND2 PTE is smaller than the 2.4-kb fragment tested in our initial transgenic assay and whether the PTE contains a single core recruiting element or multiple weaker elements that cooperate. To address these questions, we examined PRC1 binding by sub-fragments of the 2.4-kb CCND2 PTE (Fig. 5A). ChIP analysis of PRC1 binding in cells transduced with corresponding lentiviral constructs indicates that the 1-kb fragment (PTE 1.2) centered on the summit of the MEL18- and BMI1-binding peak and the larger overlapping fragments (PTE 1.1 and PTE 1.3) bind MEL18, BMI1, and CBX2 as efficiently as the full-length 2.4-kb fragment or the endogenous CCND2 PTE (Fig. 5, B–D).

Further dissection indicates that smaller sub-fragments of PTE 1.2 cannot bind PRC1 as efficiently as the full-length fragment (Fig. 6 and Fig. 5A). When integrated elsewhere in the genome, the left (PTE 1–6) or the central (PTE 6–8) parts are immunoprecipitated with anti-MEL18 antibodies very weakly (Fig. 6, B and C), and their precipitation with anti-BMI1 antibodies is at the edge of detection (Fig. 5A). This is likely, because in NT2-D1 cells BMI1 is less abundant than MEL18 (Fig. 5A). The immunoprecipitation of larger fragments that combine the left and the central parts (PTE 1–8) or the central and the right parts (PTE 6–11) is more efficient (Fig. 6, D and E, and Fig. S8). Consistent with the asymmetric shape of the MEL18/BMI1 ChIP–chip peak (Fig. 18), the PTE 6–11 fragment shows the strongest immunoprecipitation from all PTE 1.2 sub-fragments tested. This suggests that the central and the right parts of the 1-kb PTE have greater contribution to the PRC1 retention. Importantly, the synthetic fragment (PTE 1.2Δ) that includes both the left and the right parts of PTE 1.2 but lacks the central part is still immunoprecipitated (Fig. 6F and Fig. 6). Overall, these results indicate that the CCND2 PTE consists of at least three separable modules, all of which contribute to the PRC1 retention with the central and the right modules being more important.

Both sequence-specific DNA-binding activity and cis-acting noncoding RNAs have been implicated in the targeting of mammalian PcG proteins (62). Recent genome annotation indicates two long noncoding RNAs CCND2-AS1 and CCND2-AS2, which originate within the second exon or the first intron of CCND2 (Fig. 1B). They are transcribed in the opposite direction to CCND2 and traverse over the PTE to terminate some 27 kb away from their TSS (63). These IncRNAs are unlikely to play a critical role in retaining PRC1 at CCND2 PTE as their transcription start sites were not included in our lentiviral constructs. Therefore, we sought evidence of a specific DNA-binding activity targeting the PTE 1.2 fragment. The analysis of the PTE 1.2 DNA sequence revealed no potential binding sites for YY1, RUNX1/CFB, REST, and SNAIL.
**PRC1-targeting element of the cyclin D2 oncogene**

**A**

![CpG Conservation](image)

**B**

![% of β-actin](image)

**C**

![F9 cells](image)

**D**

![NIH3T3 cells](image)

**E**

![% input](image)

**F**

![% input](image)
(Fig. S7A), the sequence-specific DNA-binding proteins previously implicated in the recruitment of mammalian PcG proteins (54, 64–66). Consistently, we and others detect no YY1 binding to the CCND2 PTE (55, 67). Mining the ENCODE data shows that the PTE 1.2 fragment does not bind any of the sequence-specific DNA-binding proteins mapped to date (Fig. S7A), although multiple transcription factors bind the adjacent CpG-rich region.

Because we did not find any binding sites for known sequence-specific proteins, we analyzed the CCND2 PTE for sequences that might bind proteins not yet implicated in PcG targeting. We hypothesized that some of the other PcG target genes, represented by high-confidence BMI1/MEL18-binding sites on the three human chromosomes surveyed by ChIP (55), may have elements similar to the CCND2 PTE. In this case, they may use some of the same DNA-binding proteins for PcG targeting and may be distinguished by the same sequence motifs. To test this conjecture, we applied multivariate modeling (68, 69) and searched for all possible 6-nucleotide–long sequence words predictive of high-confidence MEL18/BMI1-binding sites compared with 1000 random sequences not bound by MEL18 or BMI1 and matched for the distance to the closest TSS. This approach yielded two motifs that we dubbed “CGA” and “CGCG” (Fig. 6G and Fig. S7B). Although the CGCG motif is likely an outcome of the close proximity between PTEs and CpG-islands, the CGA motif is interesting. First, it is present right at the summit of the chromosome 12 gene desert was assayed as negative control. In both profiles across the upstream region of the each graph) was used as negative control. Here and in the scale in mm10 genomic coordinates.

To test this hypothesis, we generated a lentiviral construct that contained the PTE 1.2 fragment in which the CG dinucleotide within the CGA motif was replaced with the AA dinucleotide. ChIP-qPCR analysis of transduced NT2-D1 cells showed that antibodies against PRC1 components precipitated the mutated PTE1.2 fragment (PTE1.2mCGA) 4-fold weaker than the WT variant (Fig. 6f and Fig. S8). The reduction of ChIP signals is comparable with that seen after deleting the entire central part of the PTE (PTE1.2A construct). This suggests that the CGA motif makes an important contribution to PRC1 binding within the CCND2 PTE.

In the NT2-D1 cells, where CCND2 is transcriptionally active, the PTE displays strong binding of PRC1 and very weak binding of PRC2 and H3K27me3. In these cells, the adjacent CpG-island binds neither PRC1 nor PRC2 nor H3K27me3. In the TIG-3 cells, where CCND2 is inactive, the PTE retains much PRC1 as in the NT2-D1 cells, and we see weak PRC1 binding (~10-fold lower compared with the PTE) within the adjacent CpG-island. In these cells, the CpG-island is covered with PRC2 and H3K27me3. Taken together our observations argue that H3K27me3 is not sufficient to retain PRC1 to an extent seen at the PTE. At best, the interaction between H3K27me3 and PRC1 may account for the low-level PRC1 binding within the CpG-island.

Although not sufficient to drive the strong PRC1 binding at the PTE, H3K27me3 may still be necessary for it. To address this question, we used the CRISPR/Cas9-mediated genome editing to knock down SUZ12 in the NT2-D1 cells (Fig. S10). To our surprise, the SUZ12 knockdown (Fig. 7, A and C) reduced the PTE immunoprecipitation by the antibodies against PRC1 subunits (Fig. 7B). Western blot analysis argues that the reduced immunoprecipitation of the CCND2 PTE is not due to the lower PRC1 abundance in the SUZ12-depleted cells (Fig. 7A). In fact, the NT2-D1 cells seem to require at least a low level of PRC2 for proliferation as after multiple attempts we failed to recover any cell lines completely deficient for SUZ12. In the clonal isolate used here (S1H6), the level of SUZ12 dropped to a few percent of that in the original NT2-D1 cells and the overall H3K27me3 is reduced about 10-fold (Fig. 7, A and C). Under
these conditions SUZ12 and H3K27me3 are no longer detectable at the CCND2 PTE. At the same time, a very low SUZ12 ChIP signal is still detected at the ALX4 gene (used as a positive control example of a PcG-repressed gene in NT2-D1 cells). With this level of SUZ12 binding, ALX4 shows significant immunoprecipitation with anti-H3K27me3 antibodies, and ChIP signals for CBX2 and MEL18 are not reduced (Fig. 7B). Taken together, these results suggest that the physical presence of PRC2 or its enzymatic activity is required for the robust binding of PRC1 at the CCND2 PTE.

To distinguish between the two possibilities, we inhibited PRC2 methyltransferase activity with a small molecule inhibitor UNC1999 shown to be highly specific for EZH2 and EZH1 (70). Notably, UNC1999 does not lead to degradation of PRC2.
and does not prevent its binding to chromatin (71). Consistent with observations in other cultured cell lines (70, 71), 12-day treatment of NT2-D1 cells with recommended (2 μM) concentration of UNC1999 leads to partial inhibition of the PRC2 activity without reducing the overall levels of PRC2 and PRC1 (Fig. 7D). Under these conditions, ChIP signals for H3K27me3 at the CCND2 PTE drop to a background level but reduce less than 2-fold at the control ALX4 gene. At both sites, the SUZ12 ChIP signals remain unchanged (Fig. 7E). Importantly, following the UNC1999 treatment, the MEL18 and CBX2 ChIP signals decrease 4-fold at the CCND2 PTE but remain unchanged at ALX4 (Fig. 7E). To exclude the possibility that UNC1999 treatment causes indiscriminate loss of proteins bound next to active genes, we tested the binding of transcription factor CTCF upstream of the CLBP, VDAC2, and STK17A genes. According to a published expression array profile of the NT2-D1 cells (58), these genes are expressed within the same range (2–16% of GAPDH) as CCND2. As illustrated by Fig. 7F, the CTCF ChIP signals are not affected by the UNC1999 treatment. Taken together, our experiments suggest that enzymatic activity of PRC2 but not its physical presence are critical for the strong binding of PRC1 to the PTE.
Discussion

Epigenetic repression by polycomb group mechanisms is essential for development of all multicellular animals and is frequently disrupted in cancers (4, 72, 73). Yet, our understanding of how the repression is targeted to specific genes is far from complete. Here, we analyzed the binding of canonical PcG complexes within the human CCND2 gene, which led to the following main conclusions. First, ChIP experiments identified the high-affinity PRC1-binding site upstream of the CCND2 TSS. This site binds PRC1 regardless of whether CCND2 is transcriptionally active or silent, and in both conditions it represents the strongest bound region within the locus. Second, transgenic analyses showed that the DNA of this high-affinity PRC1-binding site is sufficient to generate new PRC1-binding events when integrated elsewhere in the genome, and, therefore it represents a novel PTE. Third, the comparison of PRC1-, PRC2-, and H3K27me3-binding profiles in cells where CCND2 is transcriptionally active with those in cells where CCND2 is repressed indicates that the high level of H3K27me3 is not sufficient to retain PRC1 to an extent seen at the PTE. Hence, other mechanisms must contribute to PRC1 binding at the PTE. Fourth, although H3K27me3 is not sufficient to account for the strong PRC1 binding at the CCND2 PTE, the enzymatic activity of PRC2 is necessary.

Historically, our view of the mammalian PcG system has been influenced by concepts developed in the Drosophila model. One of them is the concept of PREs that, in flies, are found at all developmental genes regulated by PcG mechanisms and serve as high-affinity binding sites for both PRC1 and PRC2. Similar to fly PREs, the CCND2 PTE is short (∼1 kb), modular, and able to generate new binding sites for PRC1 when integrated elsewhere in the genome. However, in contrast to fly PREs, its ability to retain PRC2 is less clear. In cells where CCND2 is silent and bound by large quantities of PRC2, most of PRC2 binds outside the PTE within the adjacent CpG-island. This agrees with the documented ability of CpG-islands to retain PRC2, as long as their DNA is un-methylated and contains no enhancers or promoters engaged in the transcriptional activity (49–51, 74). Consistently, the PTE binds little PRC2 and H3K27me3 when integrated elsewhere in the genome or in cells where CCND2 is transcriptionally active. Altogether, our observations argue that, by itself, the CCND2 PTE is not very efficient in retaining PRC2. We speculate that at CCND2, the PTE and the adjacent CpG-island act in concert to target PRC1 and PRC2 in quantities necessary for repression. In this view, the combination of the PTE and the CpG-island represents the CCND2 PRE.

Similar to Drosophila, where H3K27me3 is often excluded from PREs (40, 75, 76), the tri-methylation of H3K27 is not sufficient to account for the strong binding of PRC1 at the CCND2 PTE. Yet, different from the fly case where PRC1 does not require PRC2 or H3K27me3 to bind PREs (46), the CCND2 PTE relies on the catalytic activity of PRC2 to bind PRC1 efficiently. How the catalytic activity of PRC2 helps the binding of PRC1 at the PTE is not entirely clear. Interactions with H3K27me3, deposited by the small amount of PRC2 present at the PTE, may combine with individually weak interactions between PRC1 and the PTE-bound sequence-specific adapter proteins to yield the robust PRC1 binding. Alternatively, the binding of PRC1 at the PTE may require the global hit-and-run di-methylation of H3K27, which is known to make chromatin refractory to transcription and, possibly, more accommodating for PRC1 binding (22). More work will have to be done to discriminate between the two possibilities.

The discovery of the CCND2 PTE presents new opportunities to study the targeting of mammalian PcG complexes. Of obvious interest is the nature of the DNA-binding proteins that may retain PRC1 at the CCND2 PTE. Another interesting problem that could be addressed using the CCND2 model is the question of what impairs the PRC2 binding to target genes when these are transcriptionally active. Finally, CCND2 is an oncogene (77–79), and multiple lines of evidence link the PcG mechanisms to cancer progression (73, 80). It is still puzzling why some tumor types depend on the overexpression of PcG proteins and others require the loss of PcG function. The oncogenic effect of the overexpression has to some extent been explained by the erroneous silencing of the INK4A/ARF locus (81), but the link between malignant transformation and the loss of PcG proteins remains elusive. We speculate that a failure to repress CCND2 may, at least in part, explain this link. From this, we predict that the CCND2 PTE may be disrupted in some cancers that overexpress CCND2 but have the PcG system intact.

Experimental procedures

Transgenic constructs

Lentiviral constructs were produced by in vitro recombinatorial integration of fragments of interest into the Eco47III site of the pLenti-CMV-TRE3G-eGFP-ICR-Puro or pLenti-ICR-Puro vectors. In vitro recombinational was done using In-Fusion HD system (Clontech). All fragments were amplified using high-fidelity Pfu DNA polymerase (ThermoFisher Scientific) and human or mouse genomic DNA or, in case of CCND2 PTE sub-fragments,
DNA of the PTE 2.4-kb construct as a template. PCR primers and their sequences are indicated in Tables S1 and S2.

pLenti-CMV TRE3G-eGFP-ICR-Puro was generated based on the plenti-CMV TRE3G-eGFP-Puro backbone (82). As the first step, pLenti-CMV TRE3G-eGFP-Puro was cut with HpaI and EcoRI and recombined with two DNA fragments produced by PCR with the following pairs of primers: 5’-TCGAGCGAT-TCGTTAACCT-3’, 5’-AGCCTAGTCTCGTGATCGAT-AAA-3’, and 5’-CAGGAGACTCCGCTGAGTTGCGG-3’; 5’-CTACCCCGTAGAATTCCACGTGGGAG-3’, and DNA of pLenti-CMV TRE3G-eGFP-Puro as a template. This step introduced a unique PmlI site between eGFP and Pac (puromycin resistance gene) and the unique Eco47III site upstream of the above construct as a template.

We originally planned to use the pLenti-CMV TRE3G-eGFP-ICR-Puro–based constructs and integrate them into the NT2-D1 cells that had been modified to express the TetR protein from the constitutive CMV promoter. In theory, this should have allowed us to induce eGFP by adding the doxycycline to the media. Unfortunately, we soon discovered that the CMV promoter is not active in NT2-D1 cells. Therefore, to simplify and reduce the size of the transgenes, we have removed the TRE3G-eGFP part from pLenti-CMV TRE3G-eGFP-ICR-Puro to yield the plenti-ICR-Puro vector (Fig. S5A). This was done by digesting pLenti-CMV TRE3G-eGFP-ICR-Puro with EcoRV and Eco47III and recombining it with the short dsDNA fragment produced by the annealing of the 5’-GATACCGAG-ACTAGGCCTGAGATGGTGGCTACGTGGATC-3’ and reverse 5’-TTTTTCACAAATGGCGCTGTGAGCC-3’, using DNA of the above construct as a template.

For lentiviral infection, cells were plated at a confluence of 40–60% 24 h in advance. Viral supernatant was added in serial dilution to cells in combination with 8 μg/ml Polybrene (Millipore). After overnight incubation, the medium was changed to remove Polybrene. Transduced cells were selected for 14 days by growth on culture medium supplemented with 4 μg/ml puromycin (Invitrogen). To generate the SUZ12 knockout NT2 cell line, the SUZ12/g1.1 (caccgGTTGCGCCGGAGGCTT) and SUZ12/g1.2 (aaacAAGCCGGCCGGCCGAC) DNA oligonucleotides were annealed and cloned into lentiCRISPRv2 plasmid (Addgene catalog no. 52961) linearized by digestion with BsmBI. The construct was introduced in NT2-D1 cells by lentiviral infection, and transduced cells were selected by growth on the culture medium supplemented with 4 μg/ml puromycin (Invitrogen). Cells were cloned, and knockdown was assayed by Western blotting and immunostaining with the antibodies listed in Table S3.

Chip and RT-qPCR analyses

ChIP reactions were performed as described in Refs. 40, 55. The antibodies used for ChIP are listed in Table S3. Total RNA from cultured cells was isolated using TRI Reagent (Sigma) according to the manufacturer’s instructions. cDNA was prepared with the first-strand cDNA synthesis kit (ThermoFisher Scientific) using 2 μg of RNA and random hexamer primers and purified as described (48). qPCR analysis of cDNA and ChIP products was performed essentially as described previously (48, 75) except that the iQ5 real-time PCR detection system (Bio-Rad) and KAPPA SYBR FAST qPCR kit (Kappa Biosystems) were used for all analyses. Wilcoxon signed rank test implemented in R was used to evaluate the statistical significance of the difference in the immunoprecipitation of transgenic and control amplicons (Fig. 4, E and F; test parameters: Wilcox.test (my.data$knockout, my.data$WT, paired “greater”) or the difference in immunoprecipitation of the PTE amplicons in SUZ12 knockdown or PRC2-inhibited cells compared with control cells (Fig. 7, B and E; test parameters: Wilcox.test (my.data$knockout, my.data$WT, paired = TRUE, alternative = “greater”)) or the difference in immunoprecipitation of the PTE amplicons in SUZ12 knockdown or PRC2-inhibited cells compared with control cells (Fig. 7, B and E; test parameters: Wilcox.test (my.data$knockout, my.data$WT, paired = TRUE, alternative = “greater”)).
TRUE, alternative = “less”). To compare gene expression between different cell lines and experimental conditions, the number of cDNA molecules was normalized to the stably and constitutively expressed glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) gene (83, 84). The primers used for qPCR analyses are described in Tables S4–S6.

**Electrophoretic mobility shift assay**

To prepare the nuclear extract, the NT2-D1 cells from four 182.5-cm² T-flasks were disrupted by resuspension in 400 µl of hypotonic Cell Lysis Buffer (5% sucrose, 5 mM Tris-HCl, pH 8, 5 mM NaCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 1 mM PMSF, 2 mM dithiothreitol, 1 × protease inhibitor mixture). After discarding the cytosolic fraction, nuclei were extracted for 1 h at 4 °C with 110 µl of Nuclear Extract Buffer (20 mM HEPES, 20% glycerol, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 1 × protease inhibitor mixture). The nuclear extract was cleared by centrifugation for 20 min at 16,000 × g, at 4 °C, and used immediately or stored at −80 °C.

dsDNA fragments were labeled with [α-32P]dATP (Perkin-Elmer Life Sciences) using PCR and the DNA of the PTE 2.4-kb construct as a template and purified by passing through Illustra MicroSpin S300 HR columns (GE Healthcare). The corresponding PCR primers are indicated in the Table S7.

Binding reactions (final volume of 20 µl) were assembled by combining 5 µl of 4 × binding buffer (80 mM HEPES, pH 7.4–7.9, 20 mM MgCl₂, 20% glycerol, 400 mM NaCl, 4 mM dithiothreitol, 4 mM EDTA), 5–10 µg of nuclear protein, 1–5 µg of salmon sperm DNA (Life Technologies, Inc.), 40 fmol of labeled probe, and 150-fold excess of specific or nonspecific competitor. The binding was allowed to proceed for 20 min at room temperature after which samples were run on 8% acrylamide gel (29:1 acrylamide to bisacrylamide solution) on 1 × TBE for 4–6 h at 160 V. The resulted gels were dried for 2 h and exposed to X-ray film (AGFA Healthcare).

**Computational analyses**

**Definition of bound regions**—The MEL18- and BMI1-bound regions were defined as coordinates of clusters of microarray features that satisfied the following three criteria. (i) Smoothed ChIP/input hybridization intensity ratios of the features were above the 99.8 percentile cutoff; (ii) the maximum distance to the neighboring feature above the intensity cutoff was equal or greater than 200 bp; and (iii) the length of the cluster was equal or greater than 200 bp. The peak center of a bound region was set at the center of the five consecutive microarray features with the highest hybridization intensity values. Regions of 1 kb, centered on overlapping binding peaks of MEL18 and BMI1, were considered as high-confidence MEL18/BMI1-binding sites. CpG-islands were defined using default parameters in the EMBOSS package (85).

**Motif analysis**—The 317 most predictive words from the 1-kb MEL18/BMI1 high-confidence regions derived by multivariate modeling, as described previously (68, 69), were pairwise aligned, and each alignment was assigned a score reflecting the maximum number of identical nucleotides in the alignment. Based on these scores, we generated a hierarchical tree (Euclidian distance and complete linkage) using Cluster 3.0 (86). The tree was divided into eight groups, and the words from each group were realigned using Muscle (87). The aligned words were used to build the position weight matrix (PWM) as described in Ref. 88. As final step, the resulted PWMs were optimized with the Bound/Surveyed Sequence Discrimination Algorithm (89). The prediction of potential binding sites for the YY1, RUNX1/CBFβ, REST, and SNAIL proteins was done as described previously (90).

**External data**—The ChIP–chip profiles of MEL18, BMI1, EZH2, and H3K27me3 on chromosomes 8, 11, and 12 of NT2-D1 cells were from GSE41854 (55).

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