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Polycomb/Trithorax response elements and epigenetic memory of cell identity

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Polycomb/Trithorax group response elements (PRE/TREs) are fascinating chromosomal pieces. Just a few hundred base pairs long, these elements can remember and maintain the active or silent transcriptional state of their associated genes for many cell generations, long after the initial determining activators and repressors have disappeared. Recently, substantial progress has been made towards understanding the nuts and bolts of PRE/TRE function at the molecular level and in experimentally mapping PRE/TRE sites across whole genomes. Here we examine the insights, controversies and new questions that have been generated by this recent flood of data.

Introduction

During the 1990s, studies of the regulation of homeotic genes in the *Drosophila* Bithorax complex (BX-C) uncovered very different behaviour for two classes of cis-regulatory DNA element: initiator elements and maintenance elements (or Polycomb/Trithorax group response elements, PRE/TREs) (Busturia et al., 1989; Chan et al., 1994; Chiang et al., 1995; Simon et al., 1993; Simon et al., 1990) (reviewed by Maeda and Karch, 2006). One can think of these two types of elements as 'shift workers' that use very different strategies to regulate the expression patterns of the same genes at different stages of embryonic development. In the first three hours of development, the initiator elements are in control: the output of each homeotic gene depends on the local concentrations of segmentation gene products (these are activators and repressors that are present in different concentrations at different positions of the embryo). However, a few hours after these homeotic gene patterns have been established, the segmentation gene products decay, and thus the positional information they provide is lost. The transcriptional history of each gene is subsequently maintained throughout the rest of development, and into adulthood, by the ubiquitously expressed Polycomb group (PcG) and Trithorax group proteins (TrxG), which work antagonistically via the PRE/TRE elements to maintain active (TrxG) or silenced (PcG) transcriptional states (Moehrle and Paro, 1994). Although the effects of mutations in the PcG and TrxG genes are seen only after the segmentation gene products decay, the PcG and TrxG proteins themselves appear to associate with PRE/TREs much earlier, so that PRE/TREs are 'preloaded' with PcG and TrxG proteins, ready to maintain the transcriptional states that are set by the transiently acting segmentation gene products (Orlando et al., 1998).

The maintenance of transcriptional memory at PRE/TREs is 'epigenetic'. This term has suffered much overuse and abuse in recent years, but we use here the classical definition given by Ptashne and Gann (Ptashne and Gann, 2002) (p100): "a change in the state of expression of a gene that does not involve a mutation, but that is nevertheless inherited (after cell division) in the absence of the signal (or event) that initiated that change". In the case of PRE/TREs, the information required to turn gene activity off or on after each new cell division is carried on the PRE/TRE, and copied to both new daughter cells at replication and mitosis. The epigenetic nature of PRE/TRE states has been confirmed by several studies that have demonstrated that transgenic PRE/TREs, with their own or foreign promoters, can maintain gene expression states through many cell divisions in the absence of the initial activating or repressing factors (Cavalli and Paro, 1998; Maurange and Paro, 2002; Pelegri and Lehmann, 1994; Poux et al., 1996; Rank et al., 2002).

These studies have shown that PRE/TRE elements have dual potential for the epigenetic maintenance of both activated and silenced states. PRE/TREs also have the potential to switch between these states if experimentally induced to do so by a change in transcriptional status at the promoter (Cavalli and Paro, 1998; Cavalli and Paro, 1999) or by genetic removal of PcG or TrxG proteins (Beuchle et al., 2001; Klymenko and Muller, 2004). These experiments show that the active and silent states are in delicate and dynamic balance with each other, raising the possibility that PRE/TRE switching may play an important role in global developmental transitions (Buszczak and Spradling, 2006; Maurange and Paro, 2002; Ringrose, 2006). Several recent studies in flies and mammals throw light on this issue, showing that PRE/TRE switching indeed plays a vital role in the differentiation of embryonic stem cells (Boyer et al., 2006; Lee et al., 2006), of germ line stem cells (Chen et al., 2005b), in tissue regeneration (Lee et al., 2005), and several other developmental transitions (Bracken et al., 2006). The emerging picture is that PRE/TREs are vital for maintaining the identity of both stem cells and differentiated cells, and that their ability to switch may be essential for orchestrating a delicate balance between proliferation and differentiation during normal development and also in cancer (Fig. 1) (Buszczak and Spradling, 2006; Pasini et al., 2004; Ringrose, 2006; Valk-Lingbeek et al., 2004).

Recent years have seen an explosion of interest in Polycomb and Trithorax regulation, with over 300 research papers and over 100 reviews published in 2005. This has been driven largely by the recent convergence of the Polycomb/Trithorax field with two other rapidly expanding fields: stem cell biology and histone modification. This flood of new information has brought with it many insights, has given birth to several new hypotheses, but has also generated some confusion. Here, we aim to evaluate the new data and to examine some of the currently accepted hypotheses. We will focus on three specific questions about PRE/TREs: (1) What makes a PRE/TRE? (2) When and why do PRE/TREs switch their states during development? (3) How does transcriptional memory survive DNA replication and mitosis? By focusing on questions related to PRE/TREs, we will omit much of the excellent work that has been done on the genetics, biochemistry and cell biology of broader aspects of Polycomb/Trithorax regulation, and on the involvement

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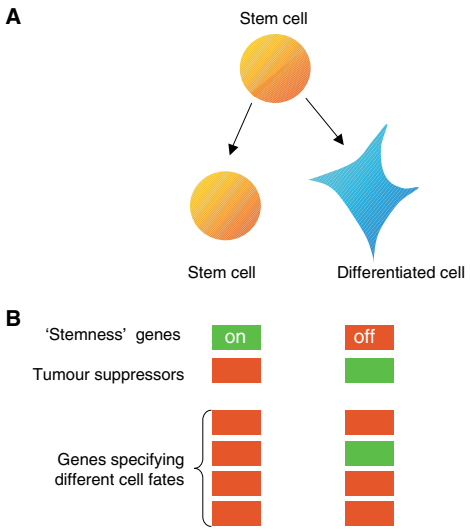

Fig. 1. Polycomb and Trithorax in stem cells and differentiated cells. (**A**) Stem cells have a high capacity to proliferate and to generate different differentiated cell types, and following division can give rise to a new stem cell and a differentiated daughter cell. (**B**) Classes of genes that must be active or silenced in stem cells and differentiated cells are shown. (Left) Tumor suppressors and genes specifying cell fate are silenced in stem cells, whilst genes conferring 'stemness' are active. (Right) The activities of tumor suppressors and 'stemness' genes are reversed in differentiated cells, which have limited proliferation capacity. Most genes that specify different cell fates continue to be silenced in differentiated cells, except for those that are required to specify a given fate. The PcG proteins target many genes of the three classes shown and are essential in stem cells and differentiated cells, both for the maintenance of silent or active states and for the switching of these states upon differentiation (see main text for details).

of the mammalian PcG and TrxG in X-inactivation. We refer readers to several recent reviews that cover these areas of the field in more detail (Bantignies and Cavalli, 2006; Heard, 2005; Ringrose and Paro, 2004).

What makes a PRE/TRE in flies?

PRE/TRE profiling in silico and in vivo

Several years ago, cytological studies anticipated the presence of several hundred PcG/TrxG-regulated loci in the *Drosophila* genome (Chinwalla et al., 1995; DeCamillis et al., 1992; Rastelli et al., 1993; Tripoulas et al., 1996; Zink and Paro, 1989). The handful of PRE/TREs that had been defined experimentally by the turn of this century all share common mechanistic features when taken out of their normal context and tested in transgenic assays. However, alignment of their DNA sequences showed no clear homology and failed to reveal a PRE/TRE consensus sequence that would be useful for identifying other PRE/TREs. We recently designed an alignment-independent algorithm that finds similarities between PRE/TREs, based on favoured pairs of three classes of binding sites for the Gaf (Trl – Flybase)/Pipsqueak (Psq), Zeste and Pho/Pho-like proteins (Ringrose et al., 2003).

At that time, these proteins were the only sequence-specific DNA-binding proteins that had been correlated with PcG/TrxG regulation. The Pho and Pho-like proteins are involved in PcG-mediated silencing at PREs (Brown et al., 2003; Brown et al., 1998; Simon et al., 1992). The Zeste protein plays a role in transcriptional activation of many genes, and appears to participate in both activation and silencing at PRE/TREs (Dejardin and Cavalli, 2004; Hagstrom et al., 1997). The Gaf and Pipsqueak proteins bind to similar DNA sequences and operate in concert at many targets, including the homeotic genes (Decoville et al., 2001; Hodgson et al., 2001; Strutt et al., 1997). Like Zeste, Gaf and Pipsqueak appear to function in both silencing and activation of PRE/TREs (Bejarano and Busturia, 2004; Hagstrom et al., 1997; Decoville et al., 2001; Huang et al., 2002); the roles of these proteins at PRE/TREs are reviewed in detail by Ringrose and Paro (Ringrose and Paro, 2004).

In our bioinformatic approach for PRE/TRE prediction, the algorithm was trained empirically. We found that closely spaced pairs of all three classes of sites were necessary to correctly and significantly predict the PRE/TREs of the BX-C (Ringrose et al., 2003). Using this algorithm for genome-wide prediction, we identified 167 candidate PRE/TRE sequences, and verified a selection experimentally. Three large scale studies of PcG- and Gaf-protein binding in *Drosophila* have now been published (Negre et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006), giving insights into binding profiles in different cell types and at different developmental stages, and also allowing our prediction method to be evaluated in comparison with genome-wide in vivo binding data. Our purpose in this section is to evaluate whether and how these new studies have brought us closer to understanding the sequence requirements for PRE/TREs; the implications of these studies for identifying target genes and studying their regulation will be discussed in a later section.

The three new in vivo analyses have some important differences, which may limit the extent to which they can be directly compared with each other. Schwartz et al. (Schwartz et al., 2006) used chromatin immunoprecipitation (ChIP, see Box 1) on Sg4 cells in culture, and evaluated the entire *Drosophila* genome. Negre et al. (Negre et al., 2006) used ChIP on *Drosophila* embryos, and evaluated 7 Mb of the X chromosome, 3 Mb of chromosome 2L, and several other regions of interest. Tolhuis et al. (Tolhuis et al., 2006) used the DamID (see Box 1) technique on Kc tissue culture cells, and evaluated binding profiles on chromosome 2L, 11 Mb of chromosome 2R, chromosome 4, and 2 Mb of the X chromosome. For those regions that can be compared, these three data sets show some partial overlap (Fig. 2), perhaps owing to the different techniques used. However, the observed differences in binding profiles are also likely to reflect a true shift of PRE/TRE-binding profiles from one cell type to another, and from one developmental stage to another. In this context, it is intriguing that our PRE/TRE prediction dataset contained several hits that are not enriched in any of the in vivo data sets, but whose PRE/TRE status we confirmed by transgenic assays (Ringrose et al., 2003).

Most informative for the question of 'what makes a PRE/TRE?' are the numerous binding sites that did not contain predicted PRE/TREs. Between 73% and 94% of bound sites in the three in vivo studies (Negre et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006) lacked a PRE/TRE prediction (Ringrose et al., 2003), suggesting several possible explanations. First, many of the experimentally defined sites were indeed predicted, but fell slightly below the cut-off used by Ringrose et al. (Fig. 2). Each of the PRE/TRE predictions was given a score, reflecting the number of favoured motif pairs it contained. Predictions were ranked by these scores, and we used a stringent cut-off score of 157 to ensure statistical significance, in order to favour selectivity over sensitivity (Ringrose et al., 2003). Second, PcG proteins may bind to chromatin independently of PRE/TREs; for example, by looping from a PRE/TRE site to a second site (Cleard et al., 2006), or via transient

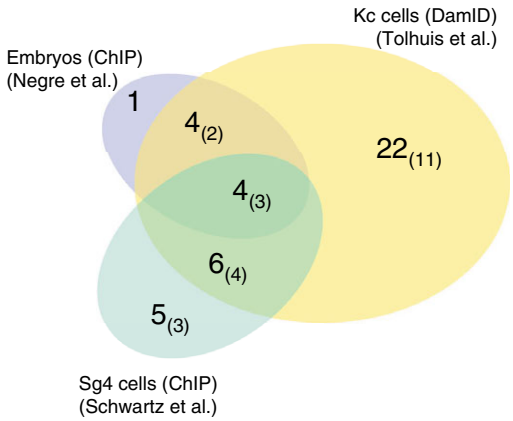

Fig. 2. Overlap between Polycomb targets in three different studies. A comparison of results from three studies which looked at binding profiles for several PcG proteins using tiling path arrays covering all or part of the *Drosophila* genome (Negre et al., 2006; Tollhuis et al., 2006; Schwartz et al., 2006) (see Box 1 for more detail on the techniques used). The diagram compares the regions in common between the three studies (2 Mb of the X chromosome and 3 Mb of chromosome 2L.) The large-type numbers in each field show the number of genes found to be bound by PcG proteins. Subscript numbers in brackets show the number of those genes that have a score of over 70 using PRE/TRE prediction (Ringrose et al., 2003). The score cut-off used in Ringrose et al. (Ringrose et al., 2003) was 157 (see main text for details).

Box 1. Chromatin and DamID: techniques to map binding profiles

In chromatin immunoprecipitation (ChIP), living cells, tissues or embryos are treated with formaldehyde, which covalently crosslinks proteins to nucleic acids (Kim and Ren, 2006). To look at chromatin-binding proteins, crosslinked chromatin is isolated and the DNA is sheared into small pieces. A specific antibody is used to recover the protein of interest and its associated DNA fragments. The enrichment of each DNA fragment over control samples that lack antibody is determined by PCR or by hybridisation to microarrays, and gives an indication of how much of the protein of interest was bound in living cells. The crosslinking reagent is added to living cells and incubated for 10-30 minutes; thus, this technique gives a 'snapshot' of interactions that are occurring at the time the crosslinking is performed. Since all protein and DNA molecules are crosslinked, this technique can also detect indirect protein-DNA interactions, such as those mediated by DNA looping. In DamID, the protein of interest is fused to a DNA methyltransferase (van Steensel, 2005). This fusion protein is then expressed at low levels, either in transgenic animals or, as in the study of Tolhuis et al. (Tolhuis et al., 2006), by the transient transfection of cultured cells. The DNA methyltransferase is thus tethered to the sites at which the protein of interest binds, giving a higher level of DNA methylation at those sites than in control samples, where the methyltransferase is expressed alone. Methylated DNA fragments are subsequently isolated and quantified using microarrays. This technique should in principle detect all the DNA methylation that accumulates during the time that the fusion protein is expressed; thus, it gives a view not only of the abundance of the protein of interest at a given site, but also of the places where that protein has been up to the point the analysis is performed.

non-specific interactions with weak PRE/TRE-like sites. The DamID technique may detect transient interactions that are not detected by ChIP (see Box 1). Indeed, Tolhuis et al. (2006) detect broader domains of Polycomb-binding using DamID than either of the recent ChIP studies (Schwartz et al., 2006; Negre et al., 2006), which is perhaps not surprising given the fact that Polycomb is a highly mobile protein (Ficz et al., 2005).

Third, it is possible that there are classes of PRE/TREs that do not use the Gaf/Psq, Zeste and Pho proteins at all to recruit the PcG and TrxG proteins. Such PRE/TREs may be revealed by systematic computational analysis of the sequences of PcG targets in the new data sets, and should be verified by transgenic assays to rule out the possibility of recruitment by indirect or non-specific binding. However, it should be kept in mind that classes of PRE/TRE might exist that will not function as isolated fragments in transgenic reporter assays, as they might require additional DNA elements or chromatin configurations, and thus would function only in their endogenous environment.

Improved definition of PRE/TREs?

A recent study suggests that although Gaf, Zeste and Pho sites are necessary, they are not alone sufficient to make a PRE/TRE (Dejardin et al., 2005). The authors constructed a synthetic PRE/TRE from Gaf, Zeste and Pho sites embedded in an otherwise unrelated bacterial sequence. This synthetic PRE/TRE showed none of the behaviour typical of transgenic PRE/TREs, such as pairing-sensitive silencing, variegation, and recruitment of PcG proteins. However, the addition of a 14 bp sequence that contained a single binding site for the Dsp1 protein (Fig. 3) gave a synthetic PRE/TRE that now supported some aspects of PRE/TRE function, such as the recruitment of PcG proteins, and PcG-dependent silencing. The Dsp1 protein is involved in regulation of homeotic genes (Decoville et al., 2001), but also regulates many other genes, where it can elicit either activation or silencing, depending on the specific promoter (Brickman et al., 1999; Lehming et al., 1994). Dsp1 binds to a broad range of DNA motifs (Brickman et al., 1999), including the GAAAA motif used by Dejardin et al. (Dejardin et al., 2005). Dejardin et al. suggest a general role for Dsp1 in PcG recruitment and silencing at many PRE/TREs based on the extensive colocalisation of Dsp1 with PcG proteins on polytene chromosomes. However, earlier studies have demonstrated that Dsp1 can also act as a TrxG protein at other homeotic PRE/TREs (Decoville et al., 2001; Rappailles et al., 2005; Salvaing et al., 2006). Thus, although the synthetic PRE/TRE study has shown that Dsp1 is important for silencing at a specific minimal PRE/TRE fragment (Dejardin et al., 2005), it is not clear how this function may be modified by other features of this PRE/TRE that are present in its endogenous context, and how it may be different at other PRE/TREs.

Clues to further pieces in the puzzle of PRE/TRE design come from two other recent studies, showing that the Grainy head (Grh) (Blastyak et al., 2006) and Sp1/KLF DNA-binding proteins (Brown et al., 2005) are each also vital for recruiting the PcG proteins to specific PRE/TREs. However, each of these reports studied only a single PRE/TRE, and colocalisation studies with known PcG or TrxG proteins on polytene chromosomes were not performed, making it difficult to assess whether these proteins are PRE/TRE-specific regulators, or whether they play a more global role. In favour of a global role, one study reported the finding of consensus binding sites for Sp1/KLF in known PRE/TRE elements (Brown et al., 2005); however, these sites are short and rather degenerate and

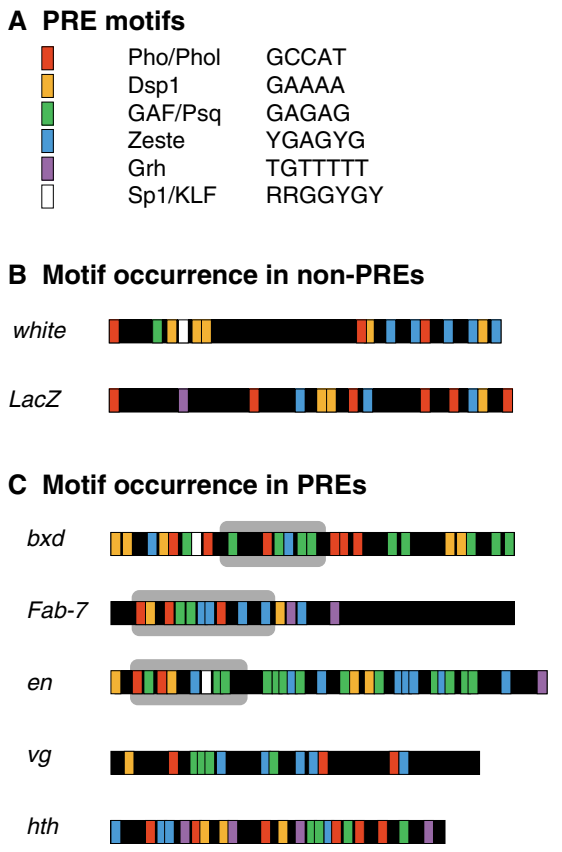

Fig. 3. PRE/TRE motifs and flexibility of PRE/TRE design. (**A**) DNA motifs shown to be important for PRE/TRE function. The Grh (Grainy head) protein binds to several different PRE/TRE sites. The motif shown is that found in PRE/TREs by Blastyak et al. (Blastyak et al., 2006). The Dsp1 protein also has broad DNA-binding specificity (Brickman et al., 1999). The motif shown is that used by Dejardin et al. (Dejardin et al., 2005). Gaf binds the same target sequence as Pipsqueak (Psq), suggesting that the two proteins may compete or cooperate at closely spaced sites. (**B**) Many of these motifs are important for regulating genes that do not have PRE/TREs, for example the *Drosophila white* gene which is regulated by the Zeste protein (600 bp of upstream regulatory region are shown). These motifs are also short and occur randomly in DNA, such as in the bacterial *LacZ* gene (the first 600 bp of the coding sequence are shown). (**C**) PRE/TREs have different combinations of motifs, with no preferred order or number. Shown here are ~600 bp of the bxd and Fab-7 PREs from the *Drosophila* Bithorax complex, and of PRE/TREs from the *Drosophila engrailed* (*en*), *vestigial* (*vg*) and *homothorax* (*hth*) loci. Grey boxes show minimal PRE/TREs where these have been defined (Dejardin et al., 2005; Brown et al., 2005). Flanking sequences contain additional motif clusters which may contribute to the function of these PRE/TREs in their endogenous context.

thus they will occur with a certain frequency at random in any piece of DNA (Fig. 3). In favour of more specific functions, the Grainy head protein is not expressed uniformly during embryogenesis; rather, it shows a highly restricted pattern that changes dramatically during development (Bray et al., 1989). The Sp1/KLF site is bound by several members of the Sp1/KLF family, many of which show tissue-specific expression patterns (Brown et al., 2005). These observations raise the intriguing possibility that PRE/TRE function may be modulated by these factors in different tissues or at different times of development.

Can these new sites help improve PRE/TRE prediction? Rehmsmeier and co workers have recently retrained the PRE/TRE prediction algorithm by incorporating the Dsp1, Grh and Sp1/KLF sites. The inclusion of Dsp1 or Sp1/KLF sites increases the number of experimentally defined Polycomb-binding sites that are hit by a prediction (Fiedler and Rehmsmeier, 2006) (Marc Rehmsmeier, personal communication). To make further analysis more accessible, a new interactive version of the algorithm, jPREdictor, is now available, that enables users to enter their own data and motif definitions and adapt the algorithm for any purpose (Fiedler and Rehmsmeier, 2006) (http://bibiserv.techfak.uni-bielefeld.de/jpredictor).

In summary, much progress has been made in defining new motifs that contribute to PRE/TRE function, but we do not yet know all the rules. A recent study has defined cryptic sequences that have strong nucleosome positioning properties (Segal et al., 2006). This suggests that we may have to look beyond simple DNA motifs to consider also the nucleosome positioning sequences that may modulate the accessibility of those motifs, in order to understand what makes a PRE/TRE.

Finding PRE/TREs in mammals

Genomic PcG profiling

What are the prospects for finding mammalian PRE/TREs? No functional mammalian PRE/TRE has yet been defined, and the search based on sequence criteria alone has been rendered difficult by the lack of mammalian homologues to most of the sequence-specific DNA-binding proteins that act on PRE/TREs in *Drosophila* (Fig. 3). However, three recent reports of genome-wide PcG profiling in mouse (Boyer et al., 2006) and human (Lee et al., 2006) ES cells, and in human embryonic fibroblasts (Bracken et al., 2006) should speed up this search. These three papers each used ChIP (see Box 1) and high resolution oligonucleotide arrays to identify over 500 sites that are targets of several PcG proteins. The future analyses of the DNA sequences of these sites and the comparison of orthologous loci between the mouse and human data sets should provide invaluable insights into the details of PRE/TRE design in mammals.

In this context, Lee et al. (Lee et al., 2006) note that the loci bound by one PcG protein (SUZ12) overlap with several highly conserved regions that had previously been identified by a comparison of vertebrate genomes (Woolfe et al., 2005). There are ~200 genomic regions that contain these highly conserved non-coding elements (HCNEs), but their function is unknown. There is currently some speculation in the literature as to whether these HCNEs might in fact be the long-sought mammalian PRE/TRE elements (Buszczak and Spradling, 2006; Lee et al., 2006). The answer will have to await functional tests of these elements, but several lines of evidence suggest that mammalian PRE/TREs are more likely to be found elsewhere. First, although several of the target loci identified by Lee et al. (Lee et al., 2006) do indeed contain HCNEs, the overlap on a global scale is low: only 8% of HCNE regions were in loci bound by SUZ12. In addition, on a fine scale, the highest peaks of PcG-binding do not appear to correlate strongly with the regions of highest conservation. A study of histone methylation across 61 of these HCNE-containing loci drew similar conclusions: although the H3K27 methylation patterns that are typically produced by the PcG protein EZH2 were indeed enriched at these loci, there was no correlation at the sequence level between the HCNEs themselves and the highest peaks of methylation (Bernstein et al., 2006). This indicates that HCNEs might be involved in other regulatory functions at these

loci, and that the PRE/TREs are not in the regions of highest conservation. Thus the question of what makes a mammalian PRE/TRE remains open.

A clear definition of these elements and how they work will also need functional reporter assays to allow a detailed dissection to be made of the exact sequence requirements for PRE/TRE function, and to identify the sequence-specific DNA-binding proteins that recruit the PcG and TrxG proteins.

When and why do PRE/TREs switch states during development?

Switching upon differentiation: insights from mammalian stem cells

Stem cells are essential not only for generating all tissues during embryonic development (ES cells), but also later in life as a source of new adult tissues (adult stem cells). Stem cells have the potential to take on a wide variety of identities upon differentiation, and have a high proliferation capacity (Buszczak and Spradling, 2006) (Fig. 1). As such, they share certain features with cancer cells (Valk-Lingbeek et al., 2004). The mammalian PcG protein EZH2 is required for ES cells to proliferate in culture (O'Carroll et al., 2001), and mouse knockout studies have demonstrated a role for several of the PRC2 class of PcG proteins in early embryonic development (Valk-Lingbeek et al., 2004).

The PcG proteins BMI-1, MPH1 and MEL-18 are required for the self renewal of various adult stem cell types in vivo (Akasaka et al., 1997; Lessard and Sauvageau, 2003; Molofsky et al., 2003; Ohta et al., 2002). In addition, the aberrant expression of both PcG and TrxG proteins is associated with many types of cancer, underlining their role in keeping cells cycling indefinitely (Leung et al., 2004; Raaphorst, 2003; Rowley, 1998). The tumour suppressor locus, *Ink4a/Arf* (*Cdkn2a* – Mouse Genome Informatics) is an important PcG target in several adult stem cell types. By silencing this locus, PcG proteins have been found to allow these cell types to rapidly proliferate (Gil et al., 2004; Jacobs et al., 1999; Molofsky et al., 2003). A similar mechanism operates in many of the cancer cell lines and tissues that overexpress PcG proteins. However, ES cell proliferation occurs independently of the *Ink4a/Arf* locus, indicating that PcG proteins may keep ES cells proliferating by other means (Molofsky et al., 2004; Valk-Lingbeek et al., 2004).

Indeed, although the recent study of PcG targets in human embryonic fibroblasts identified several tumour suppressors (Bracken et al., 2006), the two mammalian studies of ES cell targets did not (Boyer et al., 2006; Lee et al., 2006). Instead, it appears that most PcG targets in ES cells are regulators of differentiated cell fates. The authors of both studies propose that the PcG proteins keep stem cells in a pluripotent state simply by silencing all the cell fate-specific genes. These genes can nevertheless be activated upon differentiation to confer specific fates, indicating that the repression that is mediated by mammalian PRE/TREs can be relieved, at least at this early stage of development. Whether the TrxG proteins are involved in maintaining this capacity to switch PRE/TREs to an active state in ES cells remains a very interesting question. The identification of 'bivalent chromatin domains' (Bernstein et al., 2006) in mouse ES cells at many of these targets, which carry histone methylation patterns typical of both the PcG and the TrxG, strongly suggests that this may be the case, but confirmation would require mapping of binding sites for the TrxG proteins themselves. The observation that mammalian PRE/TREs are associated with PcG and possibly also with TrxG proteins before differentiation takes place is reminiscent of the early association of PcG and TrxG proteins observed in *Drosophila* (Orlando et al., 1998).

Further insights into the switching behaviour of mammalian PRE/TREs come from the study of Bracken et al. (Bracken et al., 2006). These authors selected specific targets of the PcG protein and looked at their behaviour upon differentiation of neuronal precursors. Intriguingly, the genes that became activated upon differentiation showed a loss of PcG binding, whereas those that were active in precursors nevertheless had high levels of PcG binding and H3K27 methylation. These levels increased only slightly upon differentiation. This suggests that switching on mammalian PRE/TREs is fundamentally different from switching them off. Again, the missing piece in this puzzle may be the TrxG proteins.

Switching upon differentiation: insights from flies

Unfortunately, *Drosophila* does not offer the same wealth of well-defined pluripotent cell lines as in mammals, making it difficult to assess the transition from stem cells to differentiated cells in the same way. However, a recent study has documented PRE/TRE switching in the transition from male germ stem cells to differentiated sperm (Chen et al., 2005b). This study showed that four testis-specific genes, the expression of which drives sperm fate determination, are direct targets of PcG proteins, and that PcG proteins are selectively removed from their promoters upon activation. These four target genes were not found in the genome-wide *Drosophila* PcG-binding studies discussed above (Negre et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006), suggesting that they may be PcG targets only in very specific tissues. The genes studied by Chen et al. (Chen et al., 2005b) are expressed only in testis, and thus may not need to be repressed by PcG proteins in any other cell type. This underlines the importance of tissue specificity. Indeed, many studies have shown genetically that the PcG and TrxG genes have tissue-specific roles (Breen, 1999; Chanas and Maschat, 2005; Janody et al., 2004; Narbonne et al., 2004). It may well be that each type of adult stem cell in *Drosophila* uses a different set of PRE/TREs.

Do the *Drosophila* PcG and TrxG play a similar role to their mammalian counterparts in keeping stem cells and cancer cells proliferating? Again, technical limitations have made it difficult to address this question in cell culture, but studies have identified PcG targets that have a role in proliferation. We predicted several targets with roles in proliferation, and confirmed PRE/TRE status for one of them (*proliferation disrupter*) in a transgenic assay (Ringrose et al., 2003). In addition, a recent study of *Drosophila* S2 cells showed that the cyclin A gene is a PcG target (Martinez et al., 2006), a target which the genome-wide Sg4 cell study did not detect (Schwartz et al., 2006), again strongly suggesting that cell cycle regulation by PcG is cell-type-specific. Indeed, Martinez et al. (Martinez et al., 2006) reported tissue-specific effects of PcG on Cyclin A in *Drosophila* embryos and larvae. Another recent study has shown that when *Delta* is overexpressed in the eye, aberrant overexpression of PcG proteins silences the *Rbf* gene (a homolog of the mammalian retinoblastoma gene), causing severe malignant tumours (Ferres-Marco et al., 2006). *Rbf* was also not detected as a target in the three previously discussed genome-wide binding studies.

In summary, comparisons of the recent *Drosophila* and mammalian data brings us closer to a unified view of the role of PRE/TRE switching in the transition from proliferating stem cells to differentiated cells, but the question of tissue specificity presents a technical challenge that remains to be resolved.

How does transcriptional memory survive DNA replication and mitosis?

Everything's moving

Switching transcription on and off at promoters and enhancers is driven by changes in cellular concentrations of DNA-binding activators and repressors with specific affinities for their binding sites. This results in changes in output at the promoter (Stathopoulos and Levine, 2005). Two recent studies suggest that similar chemical equilibria drive the interaction of PcG proteins with PRE/TREs. Although the PcG proteins themselves are ubiquitously expressed, their affinity for different PRE/TREs appears to be non-uniform. Quantitative fluorescence bleaching studies on *Drosophila* PcG proteins in living embryos and larval tissues have demonstrated that these protein complexes exchange rapidly (within a few minutes) on their chromatin targets (Ficz et al., 2005). The authors examined individual loci in salivary gland nuclei, showing that the PcG proteins exchange with different kinetics at different loci. Importantly, this study also demonstrates that these differences in exchange kinetics cannot be explained simply by different densities of binding sites, suggesting that something intrinsic to each PRE/TRE locus affects the stability of complexes. We have reached a similar conclusion by competition experiments in salivary gland nuclei, and have shown that locus-specific differences in stability correlate well with the transcriptional status of associated genes, with the more stably bound loci being more likely to be silenced (Ringrose et al., 2004). More insights would be gained by observing such exchanges in real time at a single locus with a defined transcription status, but so far these two studies demonstrate that PcG association with PRE/TREs is highly dynamic, and furthermore suggest that the effective affinity of the PcG for each PRE/TRE may determine whether it silences or activates its associated gene.

This idea has implications for how active and silenced states are inherited at PRE/TREs. At the onset of mitosis, the bulk of PcG proteins dissociate from chromatin, and reassociate between anaphase and G1 (depending on the PcG protein) (Buchenau et al., 1998; Miyagishima et al., 2003; Voncken et al., 2005; Voncken et al., 1999). When the PcG proteins rebind to chromatin after mitosis, some property of each PRE/TRE that carries a memory of its activity in the previous cell generation must be there to re-establish the right state of activity. The above studies suggest that this mark may be something that determines the effective affinity of the PcG for the PRE/TRE. But what is this memory made of in molecular terms?

What are cellular memories made of?

How the PcG and TrxG memory system survives the upheavals of DNA replication and mitosis is largely a mystery. The demonstrations in recent years that PcG and TrxG members have distinct enzymatic activities that methylate or ubiquitinate specific histones, that the Polycomb chromodomain binds to specific methylated histone tails in vitro, and that methylation patterns colocalise with PRE/TREs in vivo (Fischle et al., 2003b) have led swiftly to the proposition that modified histones are not only the targeting force for PcG and TrxG recruitment, but are also the signals that silence or activate target genes, and therefore are probably the epigenetic marks that propagate transcriptional memory from one cell generation to the next (Fischle et al., 2003b; Wang et al., 2004a). This idea, though largely unsupported by experimental evidence, has gained such ground in the literature that it appears to be approaching the status of a dogma. For example, histone methylation is often described as a "permanent indexing system" (Fischle et al., 2003a; Fischle et al., 2003b) that "establishes the framework for long-term epigenetic maintenance" (Sims et al., 2003). Although there is evidence in the case of the PcG and TrxG that different patterns of histone modification do accompany active and silenced states (Papp and Müller, 2006; Ringrose and Paro, 2004), whether these modifications are the cause or the consequence of activation and silencing is less clear, and whether they are indeed the principal carriers of information from one cell generation to the next is still an open question.

However, an idea that is unsupported by evidence may nevertheless be right: might modified histone tails indeed be the main carriers of heritable information for the PcG and TrxG? Such a model has three requirements: (1) histone modifications must be able to target PcG and TrxG proteins differentially; (2) different histone modifications must result in silencing or activation; and (3) Histone modifications must be restored before PcG proteins rebind to chromatin after mitosis. During replication, histone octamers are disrupted, parental histone H2A/H2B and H3/H4 dimers are distributed randomly to the two daughter strands, and the difference is made up with new incoming histone dimers that are acetylated but lack any other modifications (Ehrenhofer-Murray, 2004). Thus, immediately after replication, there will be only half of the complement of 'correct' modifications at a given locus, and a number of incorrect modifications that have to be erased. The PcG protein Pho binds specifically to PREs, and can recruit the E(z) methyltransferase to these sites (Wang et al., 2004b). Thus reinstatement of at least this histone modification may require the Pho protein. It is not known whether Pho and E(z) dissociate from chromatin during mitosis, and it is unclear whether the third requirement is fulfilled.

Furthermore, there are several observations that are difficult to reconcile with the first two criteria. First, it is highly unlikely that histone tail modifications are able to globally target PcG or TrxG proteins to PRE/TREs (reviewed by Ringrose and Paro, 2004). For example, chromodomain-swapping experiments have demonstrated that the preference of a given chromodomain for a particular methylated histone tail in vitro is not sufficient to direct a heterologous protein bearing the chromodomain to the sites at which its favoured histone modification is enriched in vivo (Platero et al., 1995; Ringrose and Paro, 2004). In addition, several reports using high resolution mapping in *Drosophila* document a depletion of histones at PRE/TREs, and instead a wide spreading of H3K27 methylation in the flanking regions, whereas the Polycomb group proteins are enriched at PRE/TREs in a very localized fashion (Mohd-Sarip et al., 2006; Papp and Müller, 2006; Schwartz et al., 2006). This pattern has been observed at PRE/TREs of the BX-C, and also at several others in the *Drosophila* genome (Schwartz et al., 2006).

This is strong evidence against histone methylation acting as a global targeting force at PRE/TREs. The histone methyltransferase activity of the PRC2 protein E(z) is nevertheless essential for silencing (Muller et al., 2002). It has been proposed that the Polycomb-H3 methyl lysine interaction may serve instead to fine tune silencing activity by affecting the stability of bound complexes (Ringrose et al., 2004), or to help PcG complexes tethered at the PRE/TRE to track along chromatin in search of the promoter (Papp and Müller, 2006). In contrast to the lack of evidence for histone methylation as the main recruiting force at PRE/TREs, there is ample evidence that PcG and TrxG proteins are targeted to PRE/TREs by interactions with DNA-binding proteins, which provide a platform for the self assembly of complexes at PRE/TREs (Blastyak et al., 2006; Klymenko et al., 2006; Levine et al., 2004; Mohd-Sarip et al., 2005) (reviewed by Muller and Kassis, 2006).

Thus, it could be that the PcG and TrxG proteins are recruited constitutively to all PRE/TREs after mitosis by DNA interactions, but that the state of histone modifications at the PRE/TRE would tell the complexes whether to activate or silence. If this were so, one would expect to see a clear correlation between modifications and transcription status. However, we and others have observed that there is no correlation between histone methylation at PRE/TREs and the transcriptional status of their associated genes (Papp and Müller, 2006; Ringrose et al., 2004). At the promoter, the picture is different: in the case of the homeotic *Ubx* gene, the silent and active states are respectively accompanied by H3K27/K9 and H3K4 trimethylation at the promoter (Papp and Müller, 2006). However, there is strong evidence that it is the PRE/TRE element (or something bound to it), and not the promoter, that carries the information for mitotic inheritance. Transgenic experiments in which the PRE/TRE is deleted by recombination result in a rapid loss of silencing of the reporter gene within a few cell divisions (Busturia et al., 1997; Sengupta et al., 2004). In summary, the available data support a model in which histone modifications at the promoter do reflect silencing and activation, but are unlikely to be the carriers of heritable information at PRE/TREs. How then might information be inherited? Recent data from two studies suggests an elegant solution, in which non-coding RNAs play a central role.

Non-coding RNA enters the memory game

Non-coding transcripts in the BX-C of *Drosophila* were first observed many years ago (Lipshitz et al., 1987; Sanchez-Herrero and Akam, 1989; Cumberledge et al., 1990), and their expression patterns, as detected by in situ hybridisation, correspond to the domains of activation of homeotic genes (Bender and Fitzgerald, 2002; Hogga and Karch, 2002; Rank et al., 2002). Two more recent studies (Sanchez-Elsner et al., 2006; Schmitt et al., 2005) show that this non-coding transcription comes from PRE/TREs, and that it may be the cause, rather than the consequence, of switching a PRE/TRE to the active state. In their study, Schmitt et al. (Schmitt et al., 2005) used transgene constructs that carry a PRE/TRE and an early ubiquitous promoter that drives transcription through the PRE/TRE but not through the flanking reporter gene. This forced transcription through a PRE/TRE was sufficient to activate the reporter gene. The termination of transcription before it passed through the PRE/TRE abolished the activation of the reporter gene, indicating that this PRE/TRE transcription does not serve merely to bring transcription machinery to the reporter gene promoter, but has some effect on the PRE/TRE itself. More recently, Sanchez-Elsner et al. (Sanchez-Elsner et al., 2006) used a tissue-specific analysis of an endogenous homeotic PRE/TRE to show that one of the earliest events in PRE/TRE activation may be the creation of a hybrid between the PRE/TRE DNA and the non-coding RNA it transcribes when it is activated. The RNA component of this proposed hybrid was reported to be necessary and sufficient to trigger many activating events, such as the recruitment of the TrxG protein Ash1, and the activation of the associated gene.

Both of these studies have implications for inheritance: Schmitt et al. (Schmitt et al., 2005) show that non-coding PRE/TRE transcription persists throughout development, suggesting that it may be involved in long-term heritability at PRE/TREs. The authors propose a model in which PcG-mediated silencing occurs by default, and transcription at PRE/TREs is the main force that opposes this silencing, and is required after each round of cell division to reset active PRE/TREs (see Fig. 4). The idea of default silencing, and marking of only active PRE/TREs has been proposed previously (Buchenau et al., 1998). If all PRE/TRE-bearing genes are silenced by default, then only those that must escape this silencing need be marked in any specific way. This active mark must be accurately copied to both new DNA strands upon replication, and it must survive mitosis and give an early start to transcription in the next interphase, before the PcG proteins return and take hold. There is indeed ample evidence for default silencing (Sengupta et al., 2004; Klymenko and Muller, 2004), but what is the nature of the activating mark?

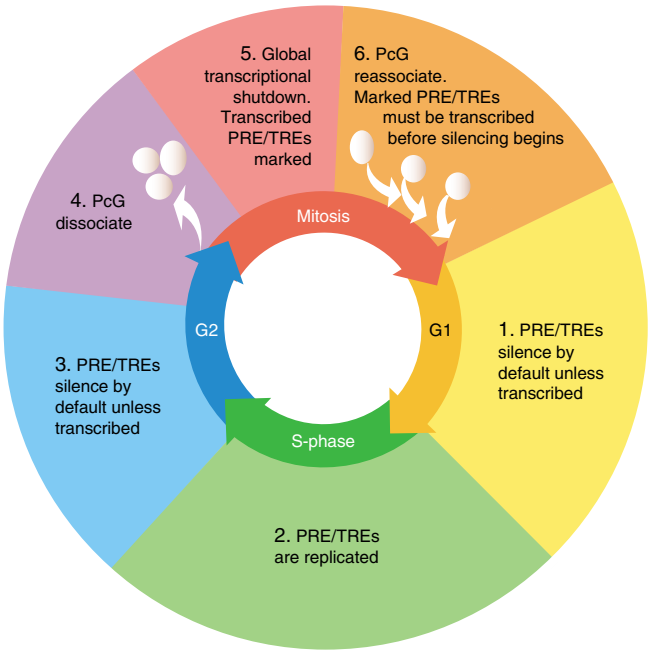

Fig. 4. Model of epigenetic memory at PRE/TREs during the cell cycle. A model based on published findings (Buchenau et al., 1998; Schmitt et al., 2005; Sanchez-Elsner et al., 2006). (**1**) During interphase, PRE/TREs silence by default. Only those PRE/TREs that are transcribed escape this silencing. (**2**,**3**) After replication, transcription through the PRE/TRE continues to counteract silencing. (**4**) At the onset of mitosis, the PcG proteins PSC, PH and PC (Posterior sex combs, Polyhomeotic and Polycomb, respectively) dissociate simultaneously from chromatin (Buchenau et al., 1998). (**5**) During mitosis, there is a global shutdown of transcription. Those PRE/TREs that were transcribed in the previous interphase must somehow be marked. (**6**) The PcG proteins reassociate with chromatin at different points during late mitosis. PSC returns during anaphase, PH in telophase and PC at the beginning of interphase (Buchenau et al., 1998). The transcription of marked PRE/TREs must resume before a functional PcG complex has assembled at PRE/TREs. This would prevent default silencing in the next interphase.

Several potential mechanisms have been proposed, including the idea that histone variants may mark active PRE/TREs (Buszczak and Spradling, 2006; Schmitt et al., 2005). The histone variant H3.3 is deposited preferentially at active loci, independently of replication (Ahmad and Henikoff, 2002; Mito et al., 2005). Thus, if PRE/TRE transcription continues after replication, H3.3 levels could be locally reinstated before entry into mitosis. H3.3 is comparatively enriched in the positive modifications that accompany active transcriptional states (McKittrick et al., 2004). The current idea is that if H3.3 were enriched at transcribed PRE/TREs during interphase, it could be transmitted through mitosis, and may create a chromatin state that favours transcription early in the next interphase. To test this idea, it will be important to distinguish whether PRE/TRE transcription

does indeed continue after replication, and whether this results in the local installation of H3.3. Finally, it will be important to discern whether H3.3 deposition (if it occurs) is simply a consequence of transcriptional activity at PRE/TREs, or whether it is also sufficient to retrigger transcriptional activity after mitosis. This caveat applies to all models invoking histone modifications as both the cause and the consequence of transcriptional activation. What alternatives to this model are there?

First, active PRE/TREs may simply be marked by bound proteins. Although most DNA-binding proteins dissociate from chromatin during mitosis (Martinez-Balbas et al., 1995), some transcription factors, including Gaf and Pipsqueak, do indeed have access to mitotic chromatin (Chen et al., 2005a; Schwendemann and Lehmann, 2002). Second, little attention has been given in the heritability debate to the potential role of DNA structure. During mitosis, DNA compaction increases by up to 10,000-fold (Li et al., 1998). This compaction is accompanied by an increased torsional strain (Castano et al., 1996) and a 10-fold increase in the single-stranded properties of chromatin (Juan et al., 1996). For some genes, mitotic inheritance of transcriptional activity is ensured by single-stranded promoter regions that facilitate transcriptional reinitiation in the next interphase (Michelotti et al., 1997). A similar mechanism may operate at PRE/TREs. PRE/TREs are enriched in AT-rich stretches and potential Z DNA-forming regions (Ringrose et al., 2003). For one PRE/TRE that regulates the homeotic *Ubx* gene, some of these AT-rich motifs have been shown to be required for the correct maintenance of activation (Tillib et al., 1999). These motifs may predispose transcribed PRE/TREs to take up specific stressed conformations that are preserved through mitosis, perhaps stabilised by an RNA-DNA hybrid (Sanchez-Elsner et al., 2006) or by bound proteins, and providing both a physical mark of the memory of transcription and a momentum for its reinitiation. However, all of these ideas relate specifically to the transmission of information through mitosis, and do not address the important issue of how such information would be copied to daughter chromatin upon replication. For any of the above models (including those invoking histone variants), this could only be achieved if PRE/TRE transcription continues after replication during G2, and it will be vital to determine whether this is the case in order to evaluate the plausibility of these various alternative models.

Perspectives

The question that is central to any epigenetic mechanism is: how are activated or silenced states maintained from one cell generation to the next? In the case of PRE/TREs, most of the work in this field has focused on mechanisms of silencing. However, recent work shows that we need to shift this focus, and to understand what maintains activation in the face of a tendency by PRE/TREs to silence by default. To shed light on this issue, it will be important to look closely at the precise timing, during replication and mitosis, of the interactions that occur between PcG proteins, TrxG proteins, non-coding RNAs and the PRE/TREs themselves, at loci with a defined transcriptional status.

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