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Perspectives

The CBX family of proteins in transcriptional repression and memory

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For mammals to develop properly, master regulatory genes must be repressed appropriately in a heritable manner. This review concerns the Polycomb Repressive Complex 1 (PRC1) family and the relationship between the establishment of repression and memory of the repressed state. The primary focus is on the CBX family of proteins in PRC1 complexes and their role in both chromatin compaction and phase separation. These two activities are linked and might contribute to both repression and memory.

Keywords. CBX; chromatin; epigenetic memory; nucleosome compaction; phase separation; Polycomb Repressive Complex

1. Introduction to the Polycomb-Group in mammals

It is essential for proper development that master regulatory proteins be stably repressed in cell lineages where their expression would specify an inappropriate cell type. The Polycomb-Group (PcG) of genes encode several protein complexes that accomplish this repression in a wide range of organisms including flies and mammals. This system must have mechanism(s) that repress gene expression and must also have mechanism(s) that generate heritability of that repressed state over numerous cell divisions. This latter aspect, the epigenetic memory of the repressed state, must be secure or clones of cells that have incorrect cell specification would arise. This perspective focuses on recent data indicating a possible link between the mechanisms that repress and the mechanisms that generate epigenetic memory.

Two major families of complexes coordinate to generate repression and epigenetic memory – the Polycomb Repressive Complex 1 (PRC1) and 2 (PRC2) families. The PRC2 family has a methyltransferase (in mammals EZH2 or less frequently EZH1) as a central component and is responsible for creating domains of methylated lysine 27 of histone H3. Both di-(H3K27me2) and trimethylation (H3K27me3) are important for regulation by the PcG (Ferrari *et al.* 2014; Lee *et al.* 2015). The PRC1 family of complexes includes two major classes, one of which is called 'canonical' PRC1 (cPRC1) and contains, as one subunit, a member of the CBX family, a protein that contains a chromodomain that binds to H3K27me3 (figure 1). The 'non-canonical' PRC1 (ncPRC1) complexes do not contain a CBX protein and generate a distinct covalent modification, ubiquitylated histone H2A, which also decorates PcG repressed domains. This ubiquitylation mark stimulates PRC2 methylation of H3K27 (Kalb *et al.* 2014), and thus helps propagate PcG domains (for an in-depth background, see recent reviews (Piunti and Shilatifard 2016; Schuettengruber *et al.* 2017).

While PRC2, cPRC1 and ncPRC1 complexes are conserved in function from flies to mammals, there is a

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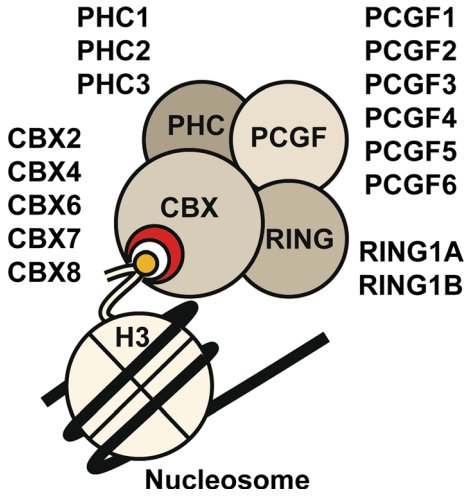


Figure 1. Composition of cPRC1 and its interaction with the nucleosome. Schematic of core components of cPRC1. The red pocket in CBX represents chromodomain. The yellow circle within the chromodomain represents trimethylated lysine 27 on the H3 tail.

significantly greater degree of diversity and complexity in the PcG complexes in mammals, particularly in the PRC1 family (figure 1). For example, mammalian PRC1 complexes can contain six distinct paralogs of the Drosophila Psc protein (called PCGF1-6), five paralogs of the Pc proteins (CBX2,4,6,7,8), and three paralogs of the Ph protein (PHC1,2,3). We review the known differences in function of the CBX proteins and how those differences might generate distinct abilities to repress gene expression, and, also, distinct abilities to remember the repressed state during cell division. We also discuss the possibility that the PHC proteins contribute to these diversities in function of cPRC1 complexes. Finally, we examine data that might provide a link between the mechanisms that repress and the mechanisms that lead to epigenetic memory.

2. The CBX family

The CBX family of proteins is one of the two sets of proteins that are unique to cPRC1, the other being the PHC proteins (Gao *et al.* 2012). The CBX family is named after the Chromobox encoded at its N terminus, which recognizes and binds the modified H3K27me3 that defines PcG domains (Fischle *et al.* 2003; Min *et al.* 2003). Each CBX protein that incorporates into cPRC1 also has a C terminal domain called the 'C box' that interacts to incorporate the CBX protein into the complex (Schoorlemmer *et al.* 1997). These two domains are conserved among CBX proteins (Whitcomb *et al.* 2007). In between these two domains is a central region of varying size that does not show any obvious homology at the amino acid level (figure 2). This central domain, however, is critical for function of the CBX family as discussed below. In this perspective, we focus on CBX2, 4 and 8 as representing CBX members of cPRC1 that are expressed primarily in differentiated lineages. These three proteins share properties that distinguish them from CBX7, which is expressed in pluripotent cells (Morey *et al.* 2012; O'Loghlen *et al.* 2012). CBX6 can also integrate into cPRC1 complexes (Santanach *et al.* 2017), but its biological role has not been widely studied in diverse contexts.

The central domains of CBX2, CBX4, and CBX8 all are predicted to be disordered, all have low complexity, and all have a significant positive charge (figure 3). While the central domain of CBX7 is also predicted to be disordered, it is not as positively charged and far shorter than the disordered region of other CBXs. The charged and disordered central domain is essential for two characteristic functions of cPRC1, chromatin compaction and phase separation. It is intriguing that the central domain has similar characteristics in the CBX proteins that are expressed during lineage specification, but is not found in CBX7, which is expressed primarily in embryonic stem cells which must maintain flexibility in developmental potential. These differences in domain composition might be critical in allowing CBX2, 4 and 8 to maintain a differentiated state while allowing CBX7 to function in cPRC1 complexes that are compatible with plasticity in gene expression.

Distinct differentiated lineages express different combinations of CBX2, 4 and 8. Specific knock-out phenotypes of CBX2 indicated that individual CBX proteins might have non-overlapping functions. In addition to a common PcG mutation phenotype, posterior transformation of axial skeletons (Core *et al.* 1997), CBX2 knock-out mice exhibited male-to-female sex reversal (Katoh-Fukui *et al.* 1998), defects in spleen vasculature (Katoh-Fukui *et al.* 2005), and in bone

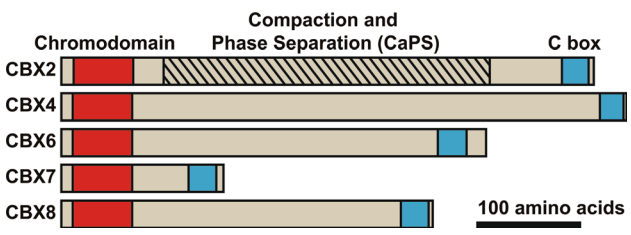


Figure 2. Domain structure of cPRC1-associated mouse CBX proteins. Regions in red represent the chromodomain. Regions in blue represent the C box. The shaded region in the middle of CBX2 is the Compaction and Phase Separation (CaPS) domain.

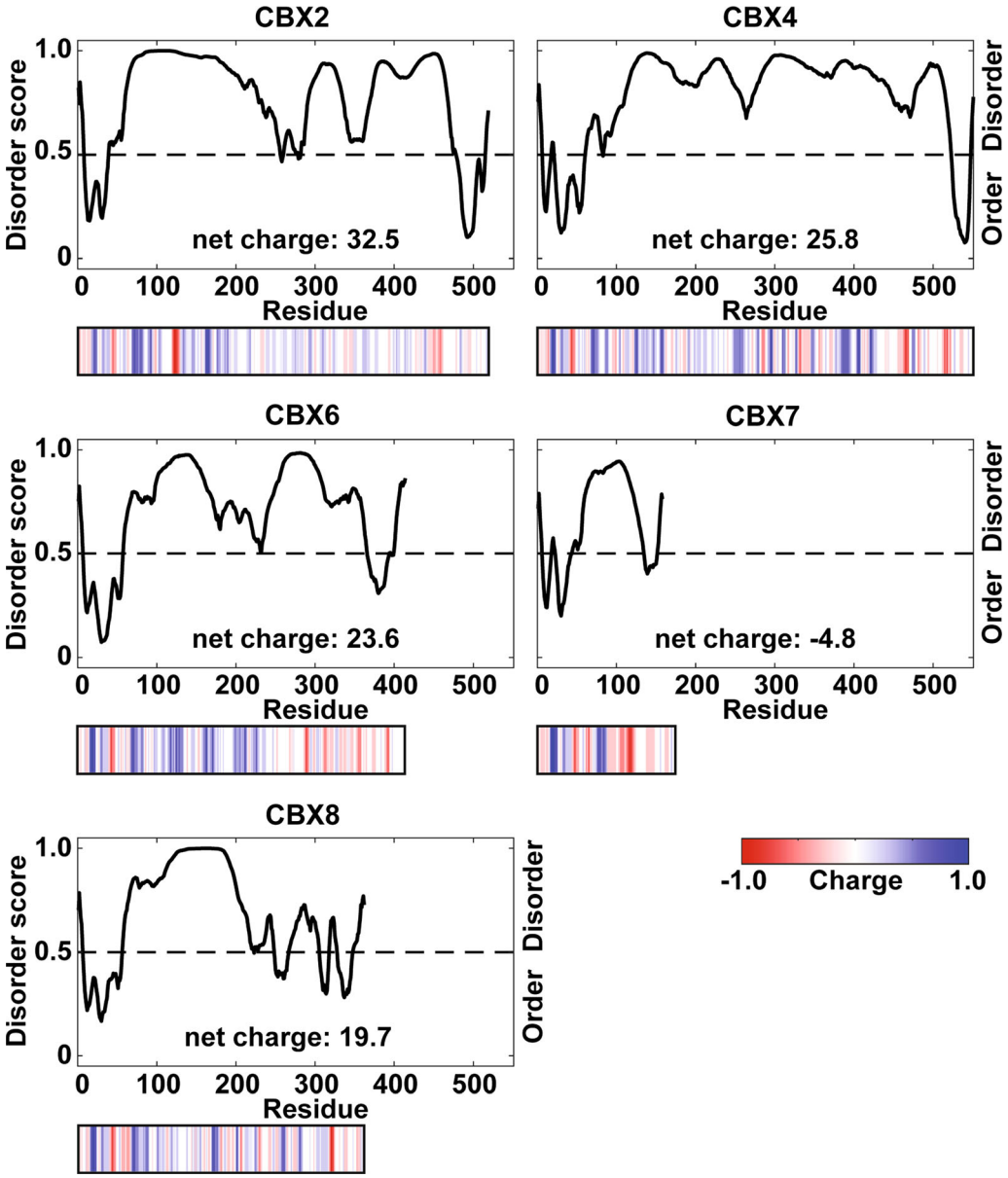


Figure 3. Disorder and charge plot of cPRC1-associated mouse CBX proteins. Disorder score was obtained using PONDR (Predictor of Natural Disordered Regions)'s VSL2 algorithm (Peng *et al.* 2006). Predicted net charge values are from Grau *et al.* 2011. Heatmaps represent protein charge distribution across each CBX proteins. Charge values were obtained using the EMBOSS Charge with a window size of five amino acids (Rice *et al.* 2000).

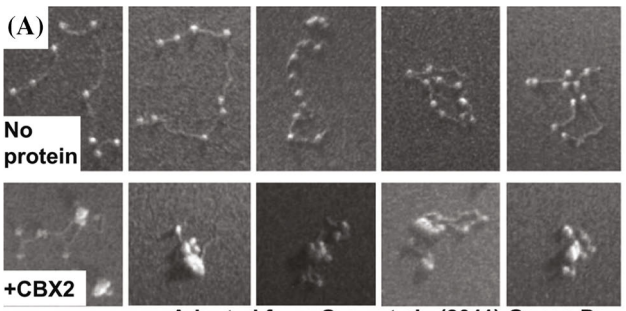
development (Katoh-Fukui *et al.* 2019). These phenotypes indicate CBX2 is expressed and functions in specific cell types such as embryonic somatic gonad and mesenchymal stromal cells. In addition, CBX4 also has been shown to have specific expression in thymic epithelial cells (Liu *et al.* 2013), epidermal stem cells (Luis *et al.* 2011) and brown fat (Chen *et al.* 2018). The role of CBX8 in normal physiology has not been extensively studied; however, several reports demonstrated that CBX8 plays critical roles in cancer, such as in leukemic (Tan *et al.* 2011) and mammary tumorigenesis (Chung *et al.* 2016). CBX4 and CBX8's tissue-specific functions might be independent from cPRC1; for example, CBX4 has SUMOylation activity (Kagey *et al.* 2003). Therefore, we will focus our discussion on the modification of chromatin structure by cPRC1 using CBX2 as a representative example.

3. The CaPS domain of CBX proteins

The central domain of the differentiation specific CBX proteins has been studied most thoroughly in the CBX2 protein. This domain is able to generate compaction of

nucleosomal arrays *in vitro* (Grau *et al.* 2011), is able to undergo liquid–liquid phase separation *in vitro*, and is able to drive formation of phase separated puncta in cells (Plys *et al.* 2019; Tatavosian *et al.* 2019). We, therefore, refer to it here as the Compaction and Phase Separation (CaPS) domain (figure 2).

The CaPS domain drives chromatin compaction. Compaction of nucleosomal templates is visualized *in vitro* by electron microscopy. In these experiments, a single cPRC1 complex was capable of organizing four nucleosomes into a compact globular structure (figure 4A) (Francis *et al.* 2004). The CaPS domain of CBX2 has a high net positive charge due to an abundance of lysine and arginine residues. Mutations to these residues diminish or eliminate the ability of CBX2 to compact nucleosomes *in vitro*. Specifically, mutation of 23 lysines and arginines within this domain essentially eliminates compaction, while mutation of 13 impairs compaction (Grau *et al.* 2011). Compaction is also observed as a property of Drosophila cPRC1. Interestingly, the charged and low complexity domain in flies is found in the Psc protein, not in the Pc protein that is homologous to the CBX family (Grau *et al.* 2011; Beh *et al.* 2012). In both, flies and mammals, mutations that disrupt compaction also disrupt full repression of genes (King *et al.* 2005; Lau *et al.* 2017).



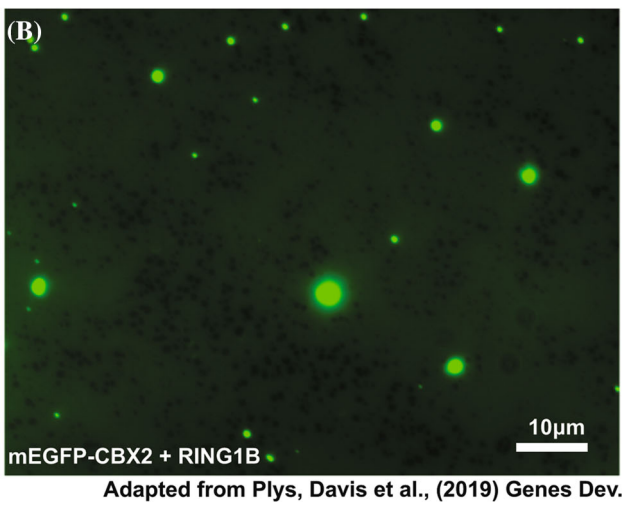


Figure 4. Nucleosome compaction and phase separation by CBX2. (**A**) Electron micrographs of nucleosome arrays alone (upper panels) and after incubation with CBX2 (lower panels) (Grau *et al.* 2011). (**B**) Fluorescence micrograph of purified mEGFP-CBX2 and RING1B heterodimer at 6.3 μM protein concentration in buffer with 20 mM HEPES (pH7.9), 100 mM KCl, and 1 mM $MgSO_4$ (Plys *et al.* 2019).

Phase separation by cPRC1 was first inferred based upon fluorescent microscopy studies performed in the late 1990s (Satijn *et al.* 1997; Saurin *et al.* 1998). These studies identified puncta that were called 'PcG bodies' and had characteristics of membrane-less organelles. One mechanism for formation of membrane-less organelles is phase separation (Zhu and Brangwynne, 2015). More recently, studies of CBX2 have shown that it has the ability to form liquid–liquid phase separated structures in solution (figure 4B) (Plys *et al.* 2019; Tatavosian *et al.* 2019). The CaPS domain was identified by sequence analysis as likely being responsible for this behavior, as it has low complexity and is predicted to be disordered – two characteristics normally seen in proteins that phase separate. Mutations in the CaPS domain, in fact, disrupt phase separation. Intriguingly, the same mutations that disrupt compaction also disrupt phase separation, and to similar extents, when the two distinct assays are compared. These mutations also disrupt the ability of CBX2 containing cPRC1 complexes to form puncta in cells. Thus, CBX2 appears to drive phase separation of cPRC1 in cells through the CaPS domain.

The CaPS domain is also important for appropriate axial patterning in mice. Due to the redundancies in the CBX family in mammals, none of the null mutations in the CBX genes show dramatic patterning defects, such as seen with Pc mutations in flies. CBX2 null mice show axial patterning defects in which several vertebrae show anterior-to-posterior transformation (Core *et al.* 1997). The point mutations in the lysines and arginines of the CaPS domain of CBX2 essentially phenocopy the null mutation of CBX2 in these axial patterning defects (Lau *et al.* 2017). Thus, the function of the CaPS domain is necessary for both appropriate repression of gene expression and for appropriate maintenance of cell type during development.

The CBX4 and CBX8 proteins also have central domains with similar characteristics to the CaPS domain of CBX2 in terms of charge, complexity, and predicted disorder (figure 3). These proteins also form puncta in cells (Dietrich *et al.* 2007; Ismail *et al.* 2012) indicating that they might be able to phase separate. They are also able to compact nucleosomal templates

in vitro (unpublished observations). Thus, while further analysis is needed to continue to compare the characteristics of these three members of the CBX family, it currently appears that all three have functional CaPS domains.

4. Repression and epigenetic memory

The presence of a single domain, that is responsible for both compaction and phase separation, raises the possibility that a mechanism that cPRC1 uses to repress transcription is intertwined with a mechanism used for memory of that repression during DNA replication and cell division.

Chromatin compaction has been related to gene repression in numerous studies and is a prominent mechanism for repression by the cPRC1 family. The model envisions that nucleosomes are packaged into a structure that is compact and that cannot be easily remodeled to allow access of transcriptional activating proteins to the packaged DNA. Thus, the compacted nucleosomes physically block the re-arrangements and binding needed to allow transcription resulting in a repressed chromatin state. The extent to which this state impacts transcription initiation and/or transcription elongation in cells is a current area of experimentation. Gathering further data that examine whether compaction directly interferes with specific steps in the transcription process is currently hampered by the lack of a clear-cut assay for the compacted chromatin state in cells.

Phase separation has been proposed as a mechanism for epigenetic memory, initially in studies on the heterochromatin protein HP1 (Larson *et al.* 2017; Strom *et al.* 2017). A compelling hypothesis is that phase separation concentrates proteins responsible for the repressed state, thus increasing the likelihood that these proteins will reform on the template following replication due to thermodynamic considerations. Quantification of the CBX2 protein indicates that its concentration is increased approximately one order of magnitude both in cells and *in vitro* when found in phase separated structures (Plys *et al.* 2019). In cells, CBX2 interacts with the remaining components of the cPRC1 complex, implying that all of these components would have increased local concentration. Nucleosomal arrays with H3K27me3 mimics can incorporate into phase separated cPRC1 structures *in vitro*, and in certain cases can increase the ability of unphosphorylated CBX2 protein to phase separate. This raises the possibility that the methylated PcG domain and cPRC1 are all in a phase separated compartment. It is intriguing to speculate that PRC2 might also be present in this structure in cells, through its known ability to bind to the H3K27me3 mark via the EED subunit (Margueron *et al.* 2009). Thus, the modified template, the complex that generates methylation, and the binding module that 'reads' the modification, might all be concentrated into the same membrane-less structure in the cell. If true, each of these components would be available at high local concentration to reset the repressed state following the generation of two daughter strands during replication.

The ability of compaction and phase separation to potentially contribute to two distinct aspects of cPRC1 function (repression and epigenetic memory) resonates with the finding that these two capabilities are encoded by the same protein domain. Strengthening that connection, to date all mutations that affect one of these functions also affect the other function. This might indicate that precisely the same specific contacts that generate compaction also generate phase separation. Or it might indicate that both processes require stretches of positively charged and disordered amino acids and that these charged residues form two distinct types of interactions to generate compaction and phase separation. In either case, there is either a functional or evolutionary link between these two activities.

5. The Polyhomeotic family of proteins

An important unanswered issue concerns how proteins in the Polyhomeotic family (PHC1, 2, 3 in mammals) function in PRC1 to generate networks of structure, and how those networks interact with compaction and phase separation. PHC proteins are the other unique core components of cPRC1, besides the CBX component, and are known to be able to generate long range interactions via their SAM domain (Isono *et al.* 2013). This results from the ability of the SAM domain to oligomerize (Kim *et al.* 2002). cPRC1 bound domains in both flies and mammals form large compacted structures when examined by either immunofluorescence or by super-resolution microscopy (Boettiger *et al.* 2016; Wani *et al.* 2016; Kundu *et al.* 2017). These domains are disrupted by mutation of PHC in mammals or mutation of Ph in flies. This level of organization, which can create domains up to 150 kb in size, is at a different scale than nucleosome compaction, which organizes four nucleosomes or about 1 kb.

This large-scale compaction created by PHC proteins presents a conundrum. The CBX proteins generate short-range interactions and also drive phase separation, which produces puncta that are large enough to contain megabases of DNA. How do the domains generated by action of PHC relate to these structures? Are these domains contained within phase separated puncta? Or are the puncta distinct structures from these large PHC domains? The large domains have been mainly described on HOX loci (Isono *et al.* 2013), which occupy more than 100 kb, so it is possible that PHC proteins are mainly involved in organizing HOX loci in mammals (or the ANT and BX complexes in flies). Other PcG targets on individual genes are much smaller and might be regulated differently than the large HOX clusters. Perhaps these smaller domains might rely upon CBX functions for repression and organization. Answering these questions will require a careful examination by visualizing each type of domain, separately, in the same cell.

6. Perspectives and unanswered issues

The above considerations highlight several areas for future studies. We briefly discuss two of these areas below; testing of hypotheses surrounding epigenetic memory and understanding the functional biology of the distinct CBX paralogs.

6.1 *Is physical organization of PcG targets part of the mechanism for epigenetic memory?*

The hypothesis that epigenetic memory of the repressed state is enhanced by physically isolating PcG repressed domains, either via phase separation or in compacted networks, is attractive at a theoretical level. Isolating repressed domains in structures containing high concentrations of PcG proteins and low concentrations of activating proteins would be expected to increase memory of repression. It is difficult to test this hypothesis because any perturbation of the ability of either CBX proteins or PHC proteins to impact organization also impacts gene repression. This makes it hard to tease apart mechanisms that remember the repressed state from mechanisms that establish repression. Newer technologies that can monitor chromatin states through cell division might be applied here, as might optical and chemical methods for manipulating phase separation and thereby testing whether gaining the ability to phase separate increases memory.

6.2 *Is it important for biological function that the CBX proteins all have distinct CaPS domains?*

Each of the four CBX proteins discussed here (CBX2, 4, 7, 8) have distinct CaPS domains. It seems likely that CBX7 is, in essence, lacking the CaPS domain for a mechanistic reason related to its expression in pluripotent cells that must maintain plasticity. The larger unanswered question concerns the other three CBX paralogs that are all expressed in differentiated lineages in various combinations. Do these proteins have distinct abilities to compact chromatin or to phase separate that are important for their function in each lineage? Or are other aspects of their expression and interactions important for their distinct roles in development?

The PcG system offers a good opportunity to examine how changes in the physical nature of the template and its solution characteristics contribute to regulation. The conservation of compaction and phase separation capabilities in the same domain of the CBX family suggests a link and leads to specific and testable hypotheses for how repression and epigenetic memory might work together.

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