Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
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Review:

Epigenetic regulation by polycomb group complexes: focus on roles of CBX proteins*

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Received Mar. 16, 2014; Revision accepted Apr. 13, 2014; Crosschecked Apr. 16, 2014

Abstract: Polycomb group (PcG) complexes are epigenetic regulatory complexes that conduct transcriptional repression of target genes via modifying the chromatin. The two best characterized forms of PcG complexes, polycomb repressive complexes 1 and 2 (PRC1 and PRC2), are required for maintaining the stemness of embryonic stem cells and many types of adult stem cells. The spectra of target genes for PRCs are dynamically changing with cell differentiation, which is essential for proper decisions on cell fate during developmental processes. Chromobox (CBX) family proteins are canonical components in PRC1, responsible for targeting PRC1 to the chromatin. Recent studies highlight the function specifications among CBX family members in undifferentiated and differentiated stem cells, which reveal the interplay between compositional diversity and functional specificity of PRC1. In this review, we summarize the current knowledge about targeting and functional mechanisms of PRCs, emphasizing the recent breakthroughs related to CBX proteins under a number of physiological and pathological conditions.

Key words: Polycomb, Polycomb repressive complex 1 (PRC1), Chromobox (CBX) protein, Epigenetic regulation, Cancer

doi:10.1631/jzus.B1400077 **Document code:** A **CLC number:** Q291; Q38

1 Compositions and functions of polycomb group (PcG) complexes

PcG proteins were originally identified as a set of genes controlling proper body segmentation in *Drosophila* via repressing *Hox* genes (Lewis, 1978; Struhl, 1981). PcG proteins are now widely recognized in all metazoans for their roles in the variety of biological processes, such as cell cycle control, X-inactivation, maintenance of pluripotency and self-renewal in embryonic stem cells (ESCs), cell fate decisions, and developmental controls (Fig. 1c) (Muller and Verrijzer, 2009; Richly *et al.*, 2011; Aloia *et al.*, 2013). PcG proteins are assembled into a variety of multi-protein complexes, and the best characterized are polycomb repressive complexes 1 and 2 (PRC1 and PRC2). The PRC2 core complex of *Drosophila* includes the enhancer of zeste [E(z)], the suppressor of zeste [Su(z)], and extra sex combs (Esc). The mammalian PRC2 core components comprise EZH1 or EZH2 [a homolog of E(z)], SUZ12 [a homolog of Su(z)], and an Esc homolog called EED. As the only PRC2 subunit with enzymatic activity, EZH2 is responsible for catalyzing the di- and tri-methylation of Lys27 on histone H3 (H3K27me$^{2\text{ or }3}$) (Margueron *et al.*, 2008). The other core PRC2 components are necessary for complex assembly and for proper enzymatic activity (Pasini *et al.*, 2004; Margueron *et al.*, 2009). In *Drosophila*, PRC1 core complex consists of polycomb (Pc), polyhomeotic (Ph), posterior sex combs (Psc), and sex combs extra (Sce) (also known as RING) (Fig. 1a), and mammals have several homologs to each subunit (Fig. 1b). There are five Pc

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* Project supported by the Fundamental Research Funds for the Central Universities from Lanzhou University (No. lzujbky-2014-87), China

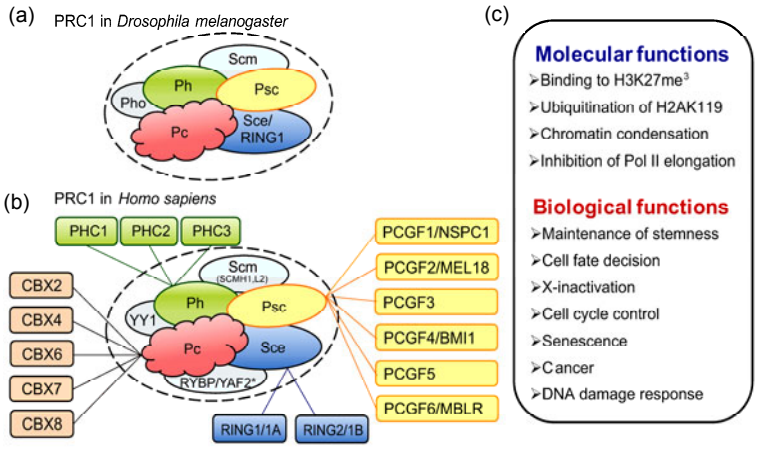

Fig. 1 Compositional diversity and functions of PRC1
(a) Main compositions of PRC1 in fruit flies; (b) Corresponding homologs of fly PRC1 subunits in humans; (c) Main functions known about PRC1. * The assembly of RYBP/YAF2 and CBX proteins in PRC1 may be mutually exclusive

(CBX2, CBX4, CBX6, CBX7, and CBX8), two Sce (RING1/RING1A and RING2/RING1B), three Ph (PHC1, PHC2, and PHC3), and six Psc known collectively as polycomb group ring fingers (PCGFs) (Morey and Helin, 2010).

For PRC1, recent advances indicate that the composition and patterns of assembly are far more biochemically diverse than originally estimated. Besides the canonical subunits listed above, there are many other PRC1 components and supporting factors, such as RING1/YY1-binding protein (RYBP) and its homolog YAF2, and the mammalian orthologs of the *Drosophila* sex comb on midleg (Scm). For details about the complexity and diversity of the PRC1 subcategories, please refer to some recent reports and reviews (Gao *et al.*, 2012; Schwartz and Pirrotta, 2013). The compositions among different mammalian PRC1 subcategories are largely divergent while all of these PRC1 complexes contain at least one of the two RING proteins, which are known as an E3 ubiquitin ligase responsible for ubiquitination of histone H2A (H2AK119ub) (Wang *et al.*, 2004). Several types of PRC1 have been shown to have none or low levels of chromobox (CBX) proteins. RYBP or YAF2 is known to stimulate the enzymatic activity of RING1B toward H2AK119ub, while CBX proteins may not exist simultaneously in this type of PRC1 (Gao *et al.*, 2012).

2 CBX proteins in PRC1

There are at least eight members of the CBX proteins in both mouse and human genomes, each of which contains a single N-terminal chromodomain (Wotton and Merrill, 2007). They are all involved in the regulation of heterochromatin, gene expression, and developmental programs. They are further divided into two groups: (1) CBX1, CBX3, and CBX5, also known as heterochromatin protein 1β (HP1β), HP1γ, and HP1α, respectively; (2) CBX2, CBX4, CBX6, CBX7, and CBX8, all having a C-terminal polycomb repressor box, serve as canonical components in PRC1 (Wotton and Merrill, 2007). In this review, we will mainly discuss the roles of these five CBX proteins in the context of PRC1.

The domain organization of CBX proteins is shown in Fig. 2. CBX proteins physically interact with $H3K27me^3$ via their chromodomains, which helps the recruitment and stabilization of PRC1 to specific regions of the chromatin (Bernstein B.E. *et al.*, 2006; Buchwald *et al.*, 2006). It was reported that mammalian CBX proteins exhibited differential bindings to methylated histone tails. CBX2 and CBX7 bind to both $H3K9Me^3$ and $H3K27Me^3$ whereas CBX4 shows stronger affinity for $H3K9Me^3$ (Bernstein E. *et al.*, 2006). The C-terminal polycomb repressor box of CBX proteins is involved in transcriptional silencing and binding to other PRC1 components such as RING1B (Muller *et al.*, 1995; Bezsonova *et al.*, 2009). Adjacent to chromodomain, all these CBX homologs have a DNA binding motif, AT-hook (in CBX2) or an AT-hook like motif (in the other four CBX proteins) (Senthilkumar and Mishra, 2009). The less conserved sequences in the middle of the CBX proteins may play a role in specifically directing each CBX family member to distinct regions of the chromatin (Vincenz and Kerppola, 2008).

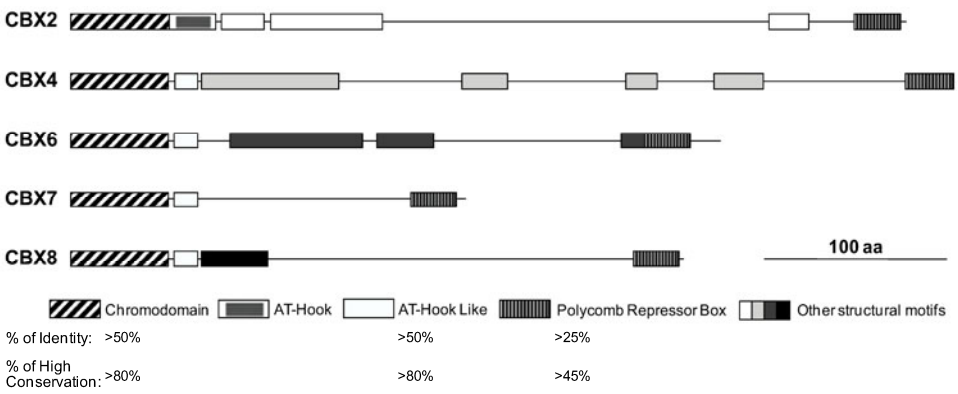

Fig. 2 Domain organization and conservation among CBX proteins

% of identity: ratio of the number of identical amino acid (aa) to the number of total aa; % of high conservation: ratio of the number of highly conserved aa to the number of total aa. This figure is modified from Senthilkumar and Mishra (2009)

3 Recruitment of PcG complexes

PcG complexes are involved in repressing over several thousand genes in mammalian genomes and the pool of their target genes is distinct in different cell types and dynamically changing in various cell states (Simon and Kingston, 2013). Components in PRC1 and PRC2 generally do not have DNA binding properties. The targeting mechanisms of the mammalian PcG complexes are diverse and extremely complicated. Many sources of the 'targeting command' are comprehensively integrated, including chromatin signatures (such as histone modifications, histone variants, DNA sequences, and CpG islands), varieties of non-coding RNA (ncRNA) species, transcription factors and cofactors, and possibly the status of RNA polymerase II (Pol II).

In *Drosophila*, specific DNA modules called polycomb response elements (PREs) are responsible for recruiting PcG complexes to their chromatin targeting regions (Simon *et al.*, 1993). This process is mediated by sequence-specific DNA binding proteins such as the zinc finger protein PHO. Recent studies found that *Drosophila* PREs consist of binding sites for a complex array of DNA binding factors and the interplay among these regulatory factors is important for specifying the function of the PREs in a cell or tissue-specific fashion (Oktaba *et al.*, 2008; Brown and Kassis, 2013). In mammals, it becomes much more difficult in searching and defining 'PRE'. Transcription repressor protein YY1 is the mammalian homolog of the *Drosophila* PHO, which works together with YAF2 and the transcription corepressor C-terminal binding protein (CtBP) in recruiting PcG complexes to many of their target genes, including *HOX* genes (Atchison *et al.*, 2003; Srinivasan and Atchison, 2004; Basu *et al.*, 2014). Several potential human PREs have been found in *HOXB*, *HOXC*, and *HOXD* clusters to facilitate the recruitment of both PRC2 and PRC1 components to a reporter gene, yet the DNA binding factors mediating these interactions are variable (Woo *et al.*, 2010; 2013). Another potential human PRE containing 25 repeats of YY1 binding motif was validated to be able to down-regulate reporter genes via PRC2 in both HEK cells and in *Drosophila* (Bengani *et al.*, 2013). Besides YY1, many other transcription factors have been reported to act as recruiters for PcG complexes, such as neuronal inhibitor REST (Ren and Kerppola, 2011).

In addition to potential PREs, chromatin regions enriched in CpG dinucleotides (CpG islands) may also facilitate targeting of PcG complexes in mammals. CpG islands that lack both 5-methylcytosine and activator-binding sites are largely overlapping with H3K27me^3, PRC2, and PRC1, and notably, constructs, containing CpG island-like sequences, are found to be capable of recruiting PRC2 upon integration into a mouse genome (Ku *et al.*, 2008; Mendenhall *et al.*, 2010). One PRC1 component Kdm2b/Fbxl10 was recently identified as a binding factor towards CpG islands (Wu X. *et al.*, 2013). Other signatures of the chromatin may also contribute to the recruitment of PcG complexes, such as histone variants H2AZ (Creyghton *et al.*, 2008) and MacroH2A (Buschbeck *et al.*, 2009).

The accumulating evidence has emphasized another scheme involved in ncRNA for the recruitment of PcG complexes. In the model of mammalian X chromosome inactivation, the long non-coding RNA (lncRNA) Xist (more specifically its A repeat region called RepA) has been implicated in PRC2 recruitment (Plath *et al.*, 2003; Zhao *et al.*, 2008). Both Suz12 and Ezh2 are known to have RNA binding capability in PRC2. The chromatin recruitment of canonical PRC1 complex is mainly mediated by CBX proteins. As mentioned above, the histone modification mark H3K27me^3 serves as a signal for CBX protein mediated PRC1 recruitment (Fig. 3a). CBX proteins are the only known RNA binding proteins in PRC1 and their chromodomains are required for RNA binding (Bernstein E. *et al.*, 2006). At the *Ink4a*/*ARF*/*Ink4b* locus, the anti-sence lncRNA ANRIL has been demonstrated to recruit both PRC1 and PRC2 in a *cis*-manner, and this guidance plays a key role in repressing these target genes by PcG complexes (Yap *et al.*, 2010; Kotake *et al.*, 2011) (Fig. 3b). Other similar examples including H19 ncRNA and Kcnqtlot1 ncRNA are described in more detail in other reviews (Brockdorff, 2013; Simon and Kingston, 2013). ncRNA may also recruit PcG complexes in a *trans*-manner. The only reported example is the human HOTAIR ncRNA, which is transcribed from the *HOXC* loci while having influence on genes located in the *HOXD* loci (Tsai *et al.*, 2010). However, deletion of the putative mouse HOTAIR did not exhibit any effects on PRC2 targeting (Schorderet and Duboule, 2011), and deleting various segments within the mouse *HoxD* loci did not disturb the overall patterns of PRC2 recruitment (Schorderet *et al.*, 2013). Besides lncRNA species, genome-wide studies have revealed that promoter-associated short RNAs transcribed from CpG island promoters and other types of RNA species also potentially facilitate the recruitment of PRC2 (Kanhere *et al.*, 2010; Zhao *et al.*, 2010).

In addition to the above mechanisms, the phosphorylation status of the serine residues within the carboxyl-terminal domain (CTD) of Pol II has also been shown to correlate with the occupancy of PcG complexes at certain groups of target genes. The phosphorylation at Ser5 normally occurs right after initiation (Ser5p$^+$) while the phosphorylation at Ser2 and Ser7 positions are generally associated with productive elongation. The new genome-wide study discovered that in mouse ESC, the development-related PRC targets were generally associated with poised Pol II (Ser5p$^+$, Ser2p$^-$, Ser7p$^-$) while PRC targets involved in metabolism were decorated with transcriptional active Pol II (Ser5p$^+$, Ser2p$^+$, Ser7p$^+$) (Brookes *et al.*, 2012). It remains highly debatable whether the chromatin-bound PcG complexes help establish the block of transcription elongation or the preset states of Pol II serve as signs for recruiting the PcG complex.

Although the targeting of PRC1 to chromatin is mainly dependent on PRC2, differential recruitment of mammalian PRC1 vs. PRC2 has been observed in several cases (Ren *et al.*, 2008; Vincenz and Kerppola, 2008; Tavares *et al.*, 2012; Wu X. *et al.*, 2013). The detailed mechanisms remain unclear. PRC1 may be

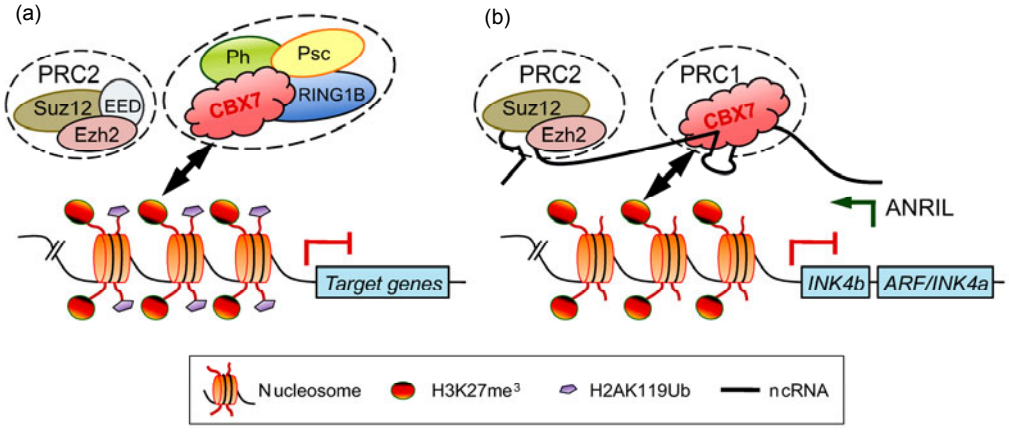

Fig. 3 Recruitment of PRC1 to chromatin through CBX proteins

Recruitment through binding to histone marks H3K27me^3 (a) and/or binding to ncRNA (b). Note that only RNA binding components in each complex are shown in (b)

initially recruited to chromatin regions distal to the target genes, searching for its targets; further recognition to H3K27me^3 with or without the guidance of ncRNA helps direct and stabilize the complex to final destinations.

4 Molecular functions of CBX proteins

4.1 Functions in common

The functions of CBX family proteins have been studied in many cell types. The *Ink4a*/*ARF*/*Ink4b* locus is one of the earliest identified and the most well-known targets for CBX-containing PRC1 complex (Gil *et al.*, 2004). The three tumor suppressors encoded by this locus play a central role in cell-cycle inhibition, senescence, and stress-induced apoptosis. Consistently, knockdown of *Cbx* genes is generally associated with reduced cell proliferation, and in contrast, overexpression of *Cbx* genes in some primary cells normally results in the extension of life span or even cell immortalization (Gil *et al.*, 2004) (Table 1).

4.2 Interplay among CBX proteins

In stem cells, the consequences upon changing expressions of various CBX family members are not very consistent because it seems that CBX/PRC1 can promote both self-renewal and differentiation. This apparent inconsistency can now be better explained by recent discoveries of an autoregulatory loop among CBX family members (Morey *et al.*, 2012; O'Loghlen *et al.*, 2012; Klauke *et al.*, 2013) (Fig. 4a). Studies indicated that CBX7 was the primary CBX protein assembled in PRC1 in the mouse ESC, and notably, it directly participated in transcription repression of the genes encoding other CBX family members (including *Cbx2*, *Cbx4*, and *Cbx8*) by PcG complexes. Interestingly, there was a dynamic switch of the identity of CBX proteins in PRC1 upon differentiation. During ESC differentiation, the expression of *Cbx7* was down-regulated and *Cbx2*, *Cbx4*, and *Cbx8* were up-regulated simultaneously. The mechanism that triggers the onset of this switch remains elusive. Depletion of CBX7 induced differentiation of ESC and mainly ectoderm-lineage-associated genes were derepressed, while the ectopic expression of *Cbx7* enhanced ESC self-renewal. In differentiating cells, CBX2 and CBX4 replaced the function of CBX7 in PRC1 targeting but they were found to have non-overlapping functions, repressing distinct target genes. The teratomas derived from CBX2- and CBX4-depleted ESC displayed abnormal differentiation phenotypes with increased number of cells expressing endodermal or mesodermal marker genes. More recent studies demonstrated that in mouse hematopoietic stem cells (HSCs), CBX7 was also required for their self-renewal, whereas CBX2, CBX4, and CBX8 were crucial for their proper differentiation (Klauke *et al.*, 2013). Ectopically expressed CBX2, CBX4, or CBX8 in HSC was able to compete with CBX7 in PRC1 assembly while they shifted the targets of PRC1 and resulted in the differentiation of HSC. However, in a human study, the depletion of CBX2 resulted in decreased cell proliferation and increasing levels of apoptosis, emphasizing the requirement of CBX2 in the maintenance of HSC (van den Boom *et al.*, 2013). Overall, in stem cells (including embryonic and adult stem cells), CBX proteins play a key role in maintenance of pluripotency or multipotency. The discrepant results in some of these studies suggest the specific roles each CBX protein might be variable in different species. Some data about manipulation of *Cbx* expression in cancer cell lines are also listed in Table 1, while the roles of CBX proteins in cancer will be mainly discussed later in this review.

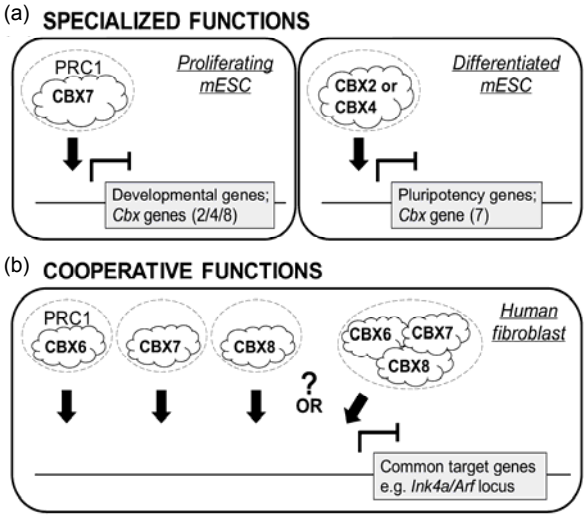

Fig. 4 Interplay among CBX proteins

(a) Auto-regulation among CBX proteins in proliferating vs. differentiated mouse ESCs (mESCs); (b) Co-function among CBX proteins in human fibroblasts

Table 1 Effects of knockdown or overexpression of *Cbx* genes

Gene	Cell type	Species	Phenotype	Reference
Knockdown				
Cbx2	HSC and progenitor cell	Human	Decreased proliferation and increased apoptosis; increased expression of the cyclin-dependent kinase inhibitor p21	van den Boom *et al.* (2013)
	HSC	Mouse	No effect on HSC self renewal	
Cbx4	Epidermal stem cell	Human	Reduced size and number of macroscopic colonies; terminal differentiation; increased size and granularity of differentiated cells	Luis *et al.* (2011)
	HCC	Human	Decreased cell proliferation and slower cell cycle progression	Wang *et al.* (2013)
Cbx7	Primary and cancerous prostate cell lines	Human	Impairment of cell growth via controlling *Ink4a*/*Arf* genes	Bernard *et al.* (2005)
	ESC	Mouse	Differentiation; de-repression of PRC1 target genes, particularly ectoderm lineage associated genes	Morey *et al.* (2012); O'Loghlen *et al.* (2012)
Cbx8	Intervertebral disc nucleus pulposus cell	Rat	Decreased proliferation	Zhou *et al.* (2013)
Overexpression				
Cbx2	Hematopoietic stem/progenitor cell	Mouse	Differentiation	Klauke *et al.* (2013)
Cbx4	Epidermal stem cell	Human	Colonies remain undifferentiated but smaller	Luis *et al.* (2011)
	HSC	Mouse	Differentiation; decreased proliferation; lower numbers of secondary colonies	Klauke *et al.* (2013)
Cbx6	GBM cell	Human	Inhibited proliferation	Li *et al.* (2013)
Cbx7	Keratinocytes (NHKs), fibroblast cell line	Human	Extended replicative capacity (no immortalization)	Gil *et al.* (2004)
	MEF	Mouse	Extended replicative capacity; immortalization	Gil *et al.* (2004)
	Prostate cancer cell line (e.g., LNCaP)	Human	Slight growth advantage in both androgen-dependent and independent conditions	Bernard *et al.* (2005)
	HSC or early progenitor cells	Mouse	Produced transgenic chimeric mice developed tumors within one year of adoptive transfer; enlarged thymus and splenomegaly; promoted T cell lymphomas genesis; cooperated with *c-myc* to produce highly aggressive B-cell lymphomas	Scott *et al.* (2007)
	Thyroid carcinoma cell lines (e.g., ARO)	Human	Lower growth potential; less colony formation	Pallante *et al.* (2008)
	EC (TERA-2)	Human	Growth advantage; resistance to retinoic acid-induced differentiation	Mohammad *et al.* (2009)
	CRC cell lines	Human	Decreased proliferation	Pallante *et al.* (2010)
	ESC	Mouse	Lower level of spontaneous differentiation	O'Loghlen *et al.* (2012)
Cbx8	HSC	Mouse	Differentiation; decreased proliferation; less secondary colonies	Klauke *et al.* (2013)

CRC: colorectal cancer; EC: embryonal carcinoma cell; ESC: embryonic stem cell; GBM: glioblastoma multiforme; HCC: hepatocellular carcinoma cell line; HSC: hematopoietic stem cell; MEF: mouse embryonic fibroblast; NHKs: normal human keratinocytes

Besides the mutually inhibitory mode described above, multiple CBX proteins can also be expressed and shown to be functioning in the same cell type. Through genome-wide analyses, Klauke *et al.* (2013) found that targets of CBX7 and CBX8 were largely overlapping in the mouse HSC. Meanwhile, they also identified about 200 genes that were differentially targeted. Interestingly, the genes uniquely occupied by CBX8 were actually highly expressed in HSC and later became repressed in progenitors, which was opposite to the expression pattern of the CBX7 targets. This suggests that CBX/PRC1 can be pre-deposited to the future targets and distinct CBX family members may help specify the 'actively repressed' vs. 'ready-to-be repressed' targets. A similar phenomenon was also observed by Pemberton *et al.* (2014). In this study, the genome-wide occupancy of several PRC1 components was analyzed and compared in human fibroblast cell lines. The results demonstrated that the target genes of CBX6, CBX7, and CBX8 were mostly overlapping, yet the detailed configuration of co-occupied PRC1 complexes remains unknown (Pemberton *et al*., 2014). Theoretically, PRC1 complexes containing each CBX protein respectively could be lined up or form oligomers at their common targets, or it is possible that various CBX proteins are integrated into a single unit of PRC1 complex (Fig. 4b). Notably, Pemberton *et al.* (2014) also identified that cell type-specific PRC1 binding sites and the occupancy of PRC1 at many loci did not correlate with an outcome of transcription repression.

4.3 Phenotypes of *Cbx* knockout (KO) mice

The roles of CBX proteins playing during embryogenesis have been investigated in KO mice or human patients with genetic mutations (Table 2). The observed phenotypes revealed the functional specification of these CBX family members. All known *Cbx* KO mice (*Cbx2*, *Cbx4*, and *Cbx7*) can survive the entire embryonic development. Homozygous mutants of *Cbx2* or *Cbx4* KO mice displayed postnatal lethality. The distinct features of *Cbx2* KO mice include male-to-female sex reversal and defects related to skeletal development were observed in two separate KO lines. Some of these functions of CBX2 are apparently conserved across species: a combination of two point mutations in *Cbx2* led to male-to-female sex reversal in humans without other obvious developmental defects. CBX2 plays a critical role in germ cell development, meiosis onset and homologous chromosome synapsis in the mammalian germ line (Baumann and de la Fuente, 2011). CBX4 has been recently found to play a key role in the development of the immune system: the proliferation of thymic epithelial cells and the maintenance of thymic epithelium were impaired in the KO mice. The *Cbx7* KO mice were grossly normal in morphology and growth although these mice attempted to develop liver and lung adenomas and carcinomas at adulthood. Considering the dominant role it plays in mouse ESC compared to other CBX proteins, alternative pathways have to be applied to compensate for the loss of CBX7 in these mice.

4.4 Unique roles of CBX proteins

In the five CBX proteins, CBX4 is the only one known to have an enzymatic activity. It acts as a small ubiquitin-like modifier (SUMO) E3 ligase and this activity is dependent on its chromodomain and a C-terminal substrate binding domain (Kagey *et al.*, 2003). SUMOylation has been implicated in the regulation of many cellular processes, including transcriptional repression, genome stability, chromatin organization, and DNA repair (Galanty *et al.*, 2009; Morris *et al.*, 2009; Dou *et al.*, 2011). It has been well known that SUMO is covalently conjugated to lysine residues on its substrates while there are also specific motifs identified in SUMO E3 ligases that mediate non-covalent interactions with SUMO (Song *et al.*, 2004). CBX4 indeed contains two such motifs that facilitate non-covalent SUMO binding and are required for full E3 ligase activity (Nacerddine *et al.*, 2005). The known substrates for CBX4 are listed in Table 3. The CBX4-mediated SUMOylations of CCCTC-binding factor (CTCF) and homeo-domain interacting protein kinase 2 (HIPK2) are associated with transcriptional repression. The SUMOylations of heterogeneous nuclear ribonucleoprotein K (HnRNP-K) and BMI1 are essential for their stabilization or site-specific targeting, respectively, in response to DNA damage.

Like other CBX proteins, CBX8 was found in diploid human and mouse fibroblasts to regulate premature senescence through controlling the transcription of the *Ink4a*/*Arf* locus (Dietrich *et al.*, 2007). Recently, Zhou *et al.* (2013) discovered that the DNA

Table 2 Phenotypes of *Cbx* knockout mice and human patient with point mutations

Gene	Species	Phenotypes of gene knockout mice or human patient with point mutations	Note	Reference
Cbx2	Mouse	50% −/− mutants died within a few hours after born; 90% mutants died within 4 weeks and the lifespan did not exceed 6 weeks; a malformation of the exoccipital bone and the deficiency of a cervical vertebrae and a vertebrosternal rib; aberrant T cell expansion; senescence of embryonic fibroblasts	Excision of exons 1–4 (aa 1–96)	Coré *et al.* (1997; 2004)
	Mouse	About 30% of $XY^{-/-}$ embryos displayed sex reversal; recovered germ cells exhibited premature meiosis onset; $XX^{-/-}$ had small ovaries with severe germ cell loss; abnormal chromosome synapsis and structural damage of chromosomes during meiosis	Excision of exons 1–4 (aa 1–96)	Baumann and de la Fuente (2011)
	Mouse	50% −/− mutants died within 2 weeks after born; male survivors showed male-to-female sex reversal, and female survivors exhibited two smaller ovaries or the absence of an ovary; transformation of the axial skeleton and hypoplastic gonad formation; adrenal and spleen hypoplasia; male-to-female sex reversal rescued by crossing them with transgenic mice displaying forced expression of Sry or Sox9; yet testes remained hypoplastic	Disruption within exon 5 (affected aa 159–519)	Katoh-Fukui *et al.* (1998; 2005; 2012)
	Human	Male-to-female sex reversal: a girl (with uterus and histologically normal ovaries) displays 46, XY karyotype; the girl had normal female internal and external genitalia and normal bilateral ovaries instead of dysgenetic gonads at histology; no obvious defects related to skeletal development, adrenal, or splenic growth	Two point mutations (P98L and R443P)	Biason-Lauber *et al.* (2009)
Cbx4	Mouse	+/− mice: grossly normal in morphology and growth; −/− mutant mice: born alive at an expected Mendelian ratio but died within 1 h of birth; severely retarded growth of the mutant thymus; decreased cell numbers of both total thymic cells and thymic epithelial cells; numbers of thymocytes (E17.5) increased in G0/G1 phase, and reduced in S and G2/M phases	Excision of exons 1–2 (aa 1–38)	Liu *et al.* (2013)
Cbx7	Mouse	Increased body length for both sexes; mutants tend to develop liver and lung adenomas and carcinomas at adulthood; MEF cells from KO mice grow faster than WT controls	Excision of exons 5–6 (aa 83–158)	Forzati *et al.* (2012a)

aa: amino acid; MEF: mouse embryo fibroblast; WT: wild type; −/−: homozygous knockout; +/−: heterozygous knockout

oxidative damage caused up-regulation of *Cbx8* expression and blocking this response by silencing *Cbx8* led to more serious DNA damage, indicating that CBX8 played an important role in DNA repair. Notably, some recent studies uncovered a novel inhibitory mechanism for PRC1 activity. CBX8 acts as an essential cofactor required for mixed lineage leukemia (MLL)-AF9 fusion or MLL-ENL fusion-induced transcriptional activation and leukemic transformation, which is contrary to its role as a transcriptional repressor in PRC1 (Tan *et al.*, 2011; Maethner *et al.*, 2013). The physical interaction between ENL and CBX8 eliminated the transcription repression activity of PRC1, which was required for efficient transformation of hematopoietic cells (Maethner *et al.*, 2013). Along with that, CBX8 competed with H3K79 methyltransferase disruptor of telomeric silencing 1-like (DOT1L) for binding to AF9, resulting in up-regulation of the *ENaCα* gene, a known target of AF9-DOT1L (Malik and Hemenway, 2013). In this case, it was known that CBX8 worked in the context of PRC1 since the other PRC1 components including RING1B and BIM1 were found together with CBX8. Although essential for MLL-AF9 induced leukemic transformation, analyses of the *Cbx8* conditional-depletion mice on hematopoietic steady-state conditions revealed that CBX8 is not required for steady-state hematopoiesis, long-term HSC maintenance, or stem and progenitor cell function (Tan *et al.*, 2011).

Table 3 SUMOlyation substrates of CBX4

Substrate	Site of Rxn	Molecular function	Reference
CtBP	K428	Make the SUMOylated CtBP preferentially associate with CBX4	Kagey *et al.* (2003)
SIP1	K391 and K866	Attenuate its transcriptional repression activity	Long *et al.* (2005)
HIPK2	K25	Enhance the ability of HIPK2 to mediate transcriptional repression	Roscic *et al.* (2006)
Dnmt3a	An extended PWWP region	Involved in the functional regulation of DNA methyltransferases by promoting their SUMO modification	Li *et al.* (2007)
CBS	Not known	Decrease CBS activity of catalyzing the first irreversible step in the transsulfuration pathway	Agrawal and Banerjee (2008)
CTCF	K74 and K698	Contribute to the repressive function of CTCF on the *c-myc* P2 promoter	MacPherson *et al.* (2009)
E2F1	K266	Required for the activation of cell-growth-control genes in response to serum	Yang *et al.* (2011)
α-Synuclein	Not known	Function as a cytoprotector by increasing α-synuclein aggregate formation within fibroblast cells	Oh *et al.* (2011)
HnRNP K	K422	Required for p53-mediated cell-cycle arrest in upon DNA damage	Lee *et al.* (2012); Pelisch *et al.* (2012)
ZNF131	K567	Potentiate the inhibitory effect of ZNF131 on estrogen signaling and attenuate estrogen-induced cell growth in MCF-7 cells	Oh and Chung (2012)
BMI1	K88	Essential for BMI1 targeting to the sites of DNA breaks	Ismail *et al.* (2012)

CBS: cystathionine-β-synthase; CTCF: CCCTC-binding factor; Dnmt3a: DNA methyltransferase 3a; HIPK2: homeodomain interacting protein kinase 2; HnRNP-K: heterogeneous nuclear ribonucleoprotein-K; SIP1: Smad-interacting protein 1; ZNF131: zinc finger protein 131

5 Regulations of CBX proteins

Besides the auto-regulation among CBX family members at the transcriptional level discussed above, CBX proteins are also subjected to various post-translational regulations. The reported information about identified post-translational modifications is listed in Table 4, which involves phosphorylation, SUMOylation/de-SUMOylation, and methylation/de-methylation. Although phosphorylation events have been detected in all five CBX proteins, it is not clear whether they share any common functional mechanisms. Many phosphorylation sites were detected in mass spectrometry analyses without further validation. The most recent discovery was that phosphorylation of CBX7 by mitogen-activated protein kinase (MAPK) helped strengthen its association with PRC1 upon mitogen stimulation. As a SUMO E3 ligase, CBX4 itself is subject to the reversible regulation of SUMOylation and de-SUMOylation, and SUMOylation at Lys492 facilitates its interaction with H3K27me^3 (Kang *et al.*, 2010). A recent work has demonstrated that the unmethylated and methylated forms of CBX4 specifically bind to distinct ncRNA named MALAT1/NEAT2 and TUG1, respectively (Yang *et al.*, 2011). The switch between these interactions correlates with the subnuclear relocation of growth-control genes in response to growth signals. The binding of NEAT2 to unmethylated CBX4 promotes E2F1 SUMOylation, resulting in activation of the growth-control genes. Some key points remain unclear, such as whether the enzymatic activity of CBX4 in this transcription activation event relies on the presence of PRC1 complex, and whether the outcome of transcription activation is achieved by somehow inhibiting the PRC1 function.

6 Misregulation of CBX proteins and cancer

Misregulation of PcG proteins has been associated with many cancer types. In regard to CBX proteins, there is increasing evidence that they play an important role in tumor initiation, progression, and development by blocking differentiation and promoting self-renewal of cancer stem cells. The reports about misregulation of CBX proteins in various cancers are listed in Table 5. The findings are categorized

Table 4 Post-translational modification of CBX proteins

Protein	Modification	Modifying enzyme	Site	Function of modification	Reference
CBX2	Phosphorylation	Casein kinase 2 (CK2)	S42	Reduction of binding to H3K9me^3 and increase of binding to H3K27me^3 (*in vitro*)	Hatano *et al.* (2010)
CBX4	SUMOylation	CBX4 or unknown	K492	Stimulation of its interaction to H3K27me^3	Kang *et al.* (2010)
	De-SUMOylation	SENP2	K492	Reduction of its binding to H3K27me^3	Kang *et al.* (2010)
	Phosphorylation	Akt1	S415, S434	Help integrate multiple enzymatic activities to target CtBP1 for degradation	Merrill *et al.* (2010)
		HIPK2	T495	Promote CBX4's ability to increase HIPK2 SUMOylation	Roscic *et al.* (2006)
		Unknown	Y205	Identified in proteomics study using lung cancer samples; mechanisms unknown	Rikova *et al.* (2007)
		Unknown	S347, T349	Identified in proteomics study of cell cycle related changes; mechanisms unknown	Dephoure *et al.* (2008)
		Unknown	S413	Identified in proteomics study of EGF-stimulated cell signaling networks; mechanisms unknown	Olsen *et al.* (2006)
		Unknown	T415, T435	Identified in proteomics study searching for cellular targets of rapamycin in cancer cells; mechanisms unknown	Chen *et al.* (2009)
		Unknown	S430	Identified in proteomics study of EGF-pathway; mechanisms unknown	Cantin *et al.* (2008)
	Methylation	Suv39h1	K191	Methylated CBX4 acts as an important anti-mitogenic signal	Yang *et al.* (2011)
	De-methylation	KDM4C	K191	Unmethylated CBX4 is essential for physiological growth-control gene expression and cell proliferation	Yang *et al.* (2011)
CBX6	Phosphorylation	Unknown	S107	Identified in proteomics study searching for cellular targets of rapamycin in cancer cells; mechanisms unknown	Chen *et al.* (2009)
		Unknown	S301, S303	Identified in proteomics study of EGF-pathway; mechanisms unknown	Cantin *et al.* (2008)
CBX7	Phosphorylation	MAPK	T118	Enhance its association with PRC1 upon mitogen stimulation. The specific molecular mechanisms unknown	Wu H.A. *et al.* (2013)
CBX8	Phosphorylation	Unknown	S110, S130, S265, S352, S354, T234	Identified in proteomics study of cell cycle related changes; mechanisms unknown	Daub *et al.* (2008); Dephoure *et al.* (2008); Chen *et al.* (2009)
		Unknown	S256	Identified in proteomics study searching for cellular targets of rapamycin in cancer cells; mechanisms unknown	Daub *et al.* (2008); Chen *et al.* (2009)
		Unknown	S332	Identified in proteomics study of EGF-pathway; mechanisms unknown	Cantin *et al.* (2008); Dephoure *et al.* (2008)

EGF: epidermal growth factor; SENP2: SUMO-specific protease 2; MAPK: mitogen-activated protein kinase

Table 5 Cancers associated with abberant expression of CBX proteins

Expression	Type of cancer	Cancer cell line or specific sample	Reference	Molecular characterization
Elevated				
Cbx4	Hepatocellular carcinoma	HCC cell lines (e.g., BEL-7042)	Wang *et al.* (2013)	Knockdown of *Cbx4* resulted in decrease of both proliferating cell nuclear antigen (PCNA), cyclin E2, and increase of *p16*
Cbx7	Prostate cancer	Prostate cancer cell lines (e.g., DU-145)	Bernard *et al.* (2005)	Repressed expression from *Ink4a*/*Arf* locus
		Preneoplastic PIN and PCa prostate cancer cells	Yap *et al.* (2010)	
	Lymphoma cancer	Germinal center-derived follicular lymphomas	Scott *et al.* (2007)	
	Gastric cancer	Gastric cancer cell lines (e.g., SGC-7901)	Zhang *et al.* (2010)	
Cbx8	GBM	GBM cell lines (e.g., T98G)	Li *et al.* (2013)	Slightly increased expression (<2 fold); mechanisms not clear
	Breast cancer	Breast cancer cell lines (e.g., MCF7)	Lee *et al.* (2013)	Cooperated with SIRT1 for suppressing *p53* acetylation; repressed the expression of *p21* by inhibiting *p53* binding to the promoter
Declined				
Cbx6	GBM	GBM cell lines (e.g., T98G)	Li *et al.* (2013)	Decreased proliferative capacity and growth arrest when over-expressed; mechanisms not clear
Cbx7	Thyroid cancer	Thyroid cancer cell lines (e.g., TPC-1)	Pallante *et al.* (2008)	Levels of CBX7 gradually decrease with the progression of cancer and correlate with the increase of *Ink4a*/*Arf* expression
	Lung cancer	MEF isolated from *Cbx7* knockout mice	Forzati *et al.* (2012a)	Up-regulated expression of *CCNE1* gene; competing with *CCNE1* activator HMGA1 for binding
	Colon cancer	Colorectal cancer cell lines (e.g., HT29)	Pallante *et al.* (2010)	Loss of CBX7 expression correlates with a poor outcome of colorectal cancer; mechanisms not clear
	Pancreatic cancer	Samples from pancreatic cancer patients	Karamitopoulou *et al.* (2010)	Levels of CBX7 gradually decrease with the progression of cancer and correlate with the loss of E-cadherin
	Urothelial carcinoma	Samples from bladder cancer patients	Hinz *et al.* (2008)	Mechanisms not clear
	Breast cancer	Breast cancer cell lines (e.g., MCF7)	Hannafon *et al.* (2011)	Loss of the tumor suppressor *miR-125b* and gain of the oncogenic miRNAs (*miR-182* and *-183*)
	GBM	GBM cell lines (e.g., T98G)	Li *et al.* (2013)	Mechanisms not clear
	Ovarian clear cell adeno-carcinoma	OCCA cell lines (e.g., TOV21G/KOC-7C)	Shinjo *et al.* (2013)	Up-regulated expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) upon CBX7 knockdown

GBM: glioblastoma multiforme; HMGA1: high mobility group A1; CCNE: encoding cyclin E

based on the direction of expression changes for these CBX proteins in a given cancer type. CBX7 is the most characterized CBX protein in cancer-associated studies. The level of CBX7 is elevated in prostate, lymphoma, and gastric cancer; conversely loss of CBX7 has been detected in a prevalence of other cancer types, such as thyroid, colon, pancreatic, and breast cancer. The apparent dual activities of CBX proteins as either oncogenes or tumor suppressors in distinct cancer types may mirror their specialized function during embryonic development and tissue specification. As the discovery about mouse HSC discussed above, CBX7 was the primary CBX protein in the maintenance of the stemness and elevated level of CBX7 promoted T cell lymphomas genesis. However, in the same system, over-expression of other 'non-dominant' CBX proteins including CBX2, CBX4, and CBX8 actually triggered cell differentiation and decreased cell proliferation (Klauke *et al.*, 2013).

Piles of evidence indicate that elevated expression of CBX proteins (CBX2, CBX7, and CBX8), together with other PRC1 subunits such as BMI1, directly causes transcription repression of the *Ink4a*/*Arf* locus. The locus encodes two independent tumor suppressors, INK4a and ARF, which activate the retinoblastoma (Rb) and *p53* tumor suppressor pathways, respectively. Regulation of the expression of this locus by CBX protein-containing PRC1 modulates the balance between cell proliferation and senescence (Bernard *et al.*, 2005; Scott *et al.*, 2007; Maertens *et al.*, 2009; Zhang *et al.*, 2010).

Loss of *Cbx7* expression may be strictly correlated with the acquisition of invasiveness accompanied in a process called the epithelial mesenchymal transition (EMT), which has been seen in several types of cancer, such as thyroid cancer, lung cancer, colon cancer, and pancreatic cancer. CBX7 was found to control the cell cycle and cell proliferation via regulation of the expression of the encoding cyclin E 1 (*CCNE1*). CBX7 directly competed with the transcription activator HMGA1 for binding to the promoter of CCNE1, therefore causing down-regulation of the CCNE1. Lack of the CBX7 protein in human lung cancer carcinomas correlates with over-expression of CCNE1 (Forzati *et al.*, 2012a; 2012b).

CBX7 and EZH2 are also known to physically associate with DNA methyltransferases (DNMTs), responsible for programming the altered DNA methylation profiles observed in multiple cancer types (Mohammad *et al.*, 2009). This interplay uncovered another mechanism by which multiple sources of epigenetic regulators were integrated to silence their common targets. CBX7 was also known to influence early breast cancer development by controlling microRNA expression (Hannafon *et al.*, 2011), while in breast cancer, CBX8 cooperated with SIRT1 for suppressing p53 acetylation induced by sirtinol and etoposide/TSA (Lee *et al.*, 2013). Upon ectopic expression, CBX8 or SIRT1 repressed the expression of p21 by inhibiting p53 binding to the promoter.

The most studied components of PcG complexes in cancer research are EZH2 and BMI1, and they both are often over-expressed in cancers (Mills, 2010). They have been shown to be essential in controlling proliferation and maintaining self-renewal of cancer stem cells in various models (Crea *et al.*, 2012). Some PRC2 inhibitors, such as DZNeP (a non-specific inhibitor of histone methylation), have been tested on cancer models and exhibited effectiveness in abolishing cancer stem cells self-renewal and tumorigenicity (Crea *et al.*, 2012). The association between CBX proteins in cancer cells and stem cell proliferation makes them become the focus of ongoing investigations in cancer research. The functional specification among CBX family members provides a reasonable hypothesis that each CBX protein may play very unique roles in various lineages and alter the behavior of normal or transformed stem cells in specific contexts.

7 Concluding remarks

Epigenetic regulation by PcG complexes plays a crucial role in maintaining stemness and determining stem cell fates. The diversity of the composition of PcG complexes, particularly PRC1 has been found to be far more complicated than initially estimated. Recent studies uncovered the function specification of the PRC1 components, such as CBX family members, in fine-tuning the cell fate of the different lineages. These discoveries may help explain the reason why both aberrant up- and down-regulation of PcG proteins have been detected in distinct types of cancer. Further exploration of the specified functions

of PcG proteins and the detailed molecular consequences triggered by abnormally acting PcG complexes would be extremely meaningful. At the same time, a lot more remains to be investigated and discovered relating to the interplay among PcG functions and other transcription regulatory mechanisms including other epigenetic regulators, ncRNAs, transcription machinery, and transcription factors. In addition, there is a plethora of post-translational modifications of PcG proteins identified in various cell types and tissues with little known mechanisms. Altogether, increasing knowledge about the roles of PcG proteins in stem cell biology and oncology can help to explore these epigenetic modifiers as potential biomarkers for cancer and further develop therapeutic strategies for early diagnosis, prognosis, and treatment of cancer.

Authors' contributions

Rong-gang MA, Yang ZHANG, and Ting-ting SUN collected all the references, made all the figures and tables, and wrote a draft. Bo CHENG wrote the manuscript.

Compliance with ethics guidelines

Rong-gang MA, Yang ZHANG, Ting-ting SUN, and Bo CHENG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要：

本文题目：多梳抑制复合体 PRC1 的核心组分 CBX 家族蛋白在表观遗传调控中的作用

Epigenetic regulation by polycomb group complexes: focus on roles of CBX proteins

研究目的：多梳蛋白家族（PcG）是一类染色质水平上通过表观遗传修饰调控靶基因的转录因子，其主要功能是使其靶基因转录受到抑制进而沉默。PcG 通常以多梳蛋白复合体（PRC）的形式存在，目前研究的最多的是 PRC1 和 PRC2。PRC1 在 PcG 对其靶基因进行转录抑制发挥着主要作用。本综述主要论述了哺乳动物中 PRC1 核心成员 CBX 蛋白在多梳蛋白调控基因转录过程中发挥的作用及其对胚胎发育、细胞记忆、细胞周期、细胞增殖和肿瘤形成等过程的影响。

创新要点：现已有大量有关 PcG 在表观遗传水平对其靶基因进行修饰转录机制的综述报道，且以 PRC1 和 PRC2 为整体来介绍表观遗传调控机制的文章也屡见不鲜。然而，关于 PRC1 核心成员 CBX 蛋白在哺乳动中的同源蛋白 CBX2、CBX4、CBX6、CBX7、CBX8 对哺乳动物个体发育调节及肿瘤发生过程的分子机制并没有系统的论述。本综述主要将这五种 CBX 蛋白在转录分子水平上的所发挥的功能进行相关的介绍，并且总结了 CBX2、CBX4、CBX6、CBX7、CBX8 各自最新的研究进展，体现出五种 CBX 蛋白的共同功能、各自独特的功能及彼此间的相互联系。

重要结论：总结了在哺乳动物中的五种 CBX 蛋白在胚胎发育和肿瘤形成等过程中独特的功能调节机制以及整体的相互作用，发现 CBX 作为 PRC1 的核心组分在基因表观遗传调控中发挥着极其重要的作用。

关键词组：多梳蛋白；多梳抑制复合体；CBX 蛋白；表观遗传调控；癌症