J. Biochem. 2012;152(3):241–249 doi:10.1093/jb/mvs065

JB THE JOURNAL OF BIOCHEMISTRY

Mediator CDK subunits are platforms for interactions with various chromatin regulatory complexes

Received March 20, 2012; accepted May 10, 2012; published online June 4, 2012

Rikiya Fukasawa, Taiki Tsutsui, Yutaka Hirose, Aki Tanaka and Yoshiaki Ohkuma*

Laboratory of Gene Regulation, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

*Yoshiaki Ohkuma, Laboratory of Gene Regulation, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. Tel: +81-76-434-7540, Fax: 81-76-434-5171, email: ohkumay@pha.u-toyama.ac.jp

The Mediator complex consists of more than 20 subunits. This is composed of four modules: head, middle, tail and CDK/Cyclin. Importantly, Mediator complex is known to play pivotal roles in transcriptional regulation, but its molecular mechanisms are still elusive. Many studies, including our own, have revealed that CDK8, a kinase subunit of the CDK/Cyclin module, is one of the key subunits involved in these roles. Additionally, we previously demonstrated that a novel CDK component, CDK19, played similar roles. It is assumed that various factors that directly affect transcriptional regulation target these two CDKs; thus, we conducted yeast two-hybrid screenings to isolate the CDK19-interacting proteins. From a screening of 40 million colonies, we obtained 287 clones that provided positive results encoded mRNAs, and it turned out that 59 clones of them encoded nuclear proteins. We checked the reading frames of the candidate clones and obtained three positive clones, all of which encoded the transcriptional cofactors, Brahma-related gene 1, B-cell CLL/lymphoma 6 and suppressor of zeste 12 homolog. Intriguingly, these three cofactors are also related to chromatin regulation. Further studies demonstrated that those could bind not only to CDK19 but also to CDK8. These results help elucidate the functional mechanism for the mutual regulations between transcription and chromatin.

Keywords: CDK-interacting proteins/chromatin regulation/Mediator/transcriptional cofactors/transcriptional regulation.

Abbreviations: BCL6, B-cell CLL/lymphoma 6; BRG1, Brahma-related gene 1; CBB, Coomassie brilliant blue; CDK, cyclin-dependent kinase; DRB, 5,6-dichlorobenzimidazole riboside; GST, glutathione-S-transferase; hCDK8, human CDK8; PMSF, phenyl methyl sulphonyl fluoride; Pol II, RNA polymerase II; SWI/SNF, switching/sucrose non-fermenting.

In eukaryotes, transcription is elaborately tuned in conjunction with chromatin regulation (*1*, *2*). Precise spatio-temporal regulation of protein-coding gene transcription is conducted by RNA polymerase II (Pol II) together with five general transcription factors (TFIIB, TFIID, TFIIE, TFIIF and TFIIH) for the execution of sophisticated gene expression programmes in vertebrates in response to various signals (*3*). The Mediator complex (Mediator) is one of the key players in this regulation. This complex consists of more than 20 subunits, most of which are conserved among eukaryotes. Mediator has been shown to be involved in both activation and repression of transcription (*4*, *5*). However, the molecular mechanisms of its pivotal roles in transcription are still elusive.

On the basis of biochemical and genetic studies, Mediator has been widely recognized as a target for multiple transcriptional regulators, including coactivators and co-repressors (*6*, *7*). These studies revealed that Mediator consists of multiple functionally distinct forms (*6*, *8*). Recent progresses have further demonstrated that, in addition to transcription factors, various protein factors that function other than transcription also targets the Mediator complexes (*1*, *5*). These protein factors include the chromatin remodelling complex switching/sucrose non-fermenting (SWI/SNF); Pol II-associated factor (PAF); tumour suppressor protein p53 and various transcription elongation factors, such as TFIIS, positive transcription elongation factor b (P-TEFb), DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF). These results clearly indicate that the Mediator complexes function not only in pre-initiation regulatory steps but also in post-initiation steps (*9*).

The canonical Mediator consists of four modules: head, middle, tail and CDK/Cyclin (*6*, *7*). The CDK/Cyclin module differs from the others because of its kinase activity, whereby it phosphorylates the C-terminal domain of the largest subunit of Pol II, and because of its propensity to dissociate on receiving stimuli from several transcriptional regulators. This module is reported to be composed of four Mediator subunits, namely, CDK8 (formerly known as Srb10), Cyclin C (formerly Srb11), MED12 and MED13 (*5*, *10*). CDK8 is a serine/threonine kinase that was believed to function as a negative regulatory component in Mediator, and various repressive functions have been demonstrated to date (*11–13*). However, we demonstrated for the first time that human CDK8 (hCDK8) plays a positive role in transcriptional regulation by Mediator (*8*). Recently, human CDK19 (hCDK19, formerly called as CDK11 or

CDC2L6) was discovered in the human Mediator fractions by Conaway and colleagues (*14*). Our previous studies of hCDK19 demonstrated that two CDKs, hCDK8 and hCDK19, were mutually exclusive in the Mediator, and each CDK formed a holo-Mediator complex (*4*, *15*). Intriguingly, reporter assays demonstrated that hCDK8 and hCDK19 play opposite roles in viral protein VP16-mediated transcriptional activation. Specifically, hCDK8 supports activation, whereas hCDK19 represses it. Since then, there have been many reports concerning various functions of CDK8 in transcriptional regulation and the physiological consequences of these functions (*16*, *17*). In addition, recent studies have revealed that CDK8 also phosphorylates histone H3 at serine 10 in conjunction with acetylation (*18*). Collectively, these data have allowed us to identify CDK-interacting proteins that might collaboratively regulate transcriptional activation with Mediator complexes.

To isolate hCDK19-interacting proteins, we performed a yeast two-hybrid screening using hCDK19 as a bait (*19*). Consequently, three positive clones that encode proteins, that is, BRG1 (Brahma-related gene 1), BCL6 (B-cell CLL/lymphoma 6) and SUZ12 (suppressor of zeste 12 homolog) known to regulate both transcription and chromatin (*20*–*22*), were obtained. *In vitro* biochemical studies demonstrated that three protein fragments identified in positive clones bound not only to hCDK19 but also to hCDK8 via their N-terminal serine/threonine kinase domains (S/T kinase). This clearly indicates that further clues are necessary to elucidate the functional mechanisms of these two CDKs, CDK8 and CDK19. Our present results might be a clue for the functional approaches by showing the mutual regulation between transcription and chromatin status through the Mediator kinases.

Experimental Procedures

DNA constructs

For bacterial expression, hCDK8 and hCDK19 were subcloned between the NdeI and XhoI sites of the pGEX-6P-2L(+) vector (the multicloning sites NcoI, NdeI, HindIII, AflI, BglII and BamHI were inserted in pGEX-6P (GE Healthcare) between the BamHI and EcoRI sites) and 6HT-pET11d (Novagen). Deletion mutants of each CDKs were generated by PCR and subcloned between the NdeI and XhoI sites of pGEX-6P-2L(+) and 6HT-pET11d. The yeast two-hybrid positive clones in the prey vector pACT2 were digested with NdeI and XhoI and subcloned between the NdeI and XhoI sites of the bacterial expression vectors 6HT-pET11d and pGEX-6P-2L(+) (*15*). All the subcloned regions were confirmed by DNA sequencing.

Yeast two-hybrid screening

The *Saccharomyces cerevisiae* strain Mavχ (*MAT*α, *ura3-52*, *leu2-3,112*, *trp1-901*, *his3-200*, *gal4*Δ *gal80*Δ, *SPAL10::URA3*, *LYS2::KanMX-LexAop-His*, *GAL1::lacZ*) was used for this yeast two-hybrid screening (*23*, *24*). This screening was performed using a human fetal brain cDNA library in pACT2 with a selectable marker Leu and a GAL4-activation domain (Clontech). To prepare the bait constructs for the yeast two-hybrid assay, the coding sequences of full-length hCDK19 was subcloned into the pHLZ vector containing a LexA binding domain (LexA) (*19*). The vector was subcloned by two steps. First, hCDK19 cDNA was amplified by PCR such that the PCR product contained an XbaI site at the 5′-end and an MfeI site at the 3′-end. This flagment was subcloned into the pBluescript SK(-) vector (Agilent Technologies) between SmaI sites to proof read its sequence. Next, the amplified region was cut out with XbaI and MfeI restriction enzymes and subcloned into the corresponding sites of the pHLZ vector. The bait construct pHLZ-hCDK19, containing the full-length hCDK19 and zeocin as a selection marker, were transformed according to one-step transformation protocol. After confirming the expression of the LexA-hCDK19 proteins by western blotting using anti-LexA (Bio Academia), yeast cells were further transformed with the prey vector pACT2 encoding a human fetal brain cDNA library (Life Technologies, ProQuest Two-Hybrid cDNA Library) using the standard lithium acetate transformation protocol. The cells were plated on selective medium SC-Leu–His to screen the interacting partners. His^+ colonies appeared from 3 to 5 days after plating and were restreaked. These positive colonies were picked up, and direct colony check PCR was performed; subsequently, PCR products were sequenced. A BLAST search was carried out to identify the putative binding partners isolated in this screening. Based on PCR and sequence results, the plasmids containing putative CDK19 interacting polypeptides were recovered from yeast. The screening result was reconfirmed individually by transforming recovered plasmids into Mavχ expressing LexA-CDK19.

Expression of recombinant proteins

Escherichia coli BL21(DE3) pLysS cells were transformed with bacterial expression vectors containing the cDNAs for N-terminally hexa histidine (6H)-tagged BRG1 and BCL6, N-terminally glutathione-S-transferase (GST)-tagged SUZ12 deletion mutants, CDK8, CDK19 and their CDK deletion mutants. The cells were grown in TBGM9 medium with carbenicillin as described previously (*25*). When the cell culture reached a density of $A_{595} = 0.3$–0.5, the expression of the 6H-tagged fusion proteins was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside for 3 h at 30°C. Harvested bacterial pellets were sonicated in buffer B [20 mM Tris–HCl (pH 7.9 at 4°C), 10% (v/v) glycerol, 0.2 mM PMSF (phenylmethylsulphonyl fluoride), 0.1% (v/v) NP40 and 1 × protease inhibitor (PI)] containing 500 mM NaCl (BB500). The extracts were cleared by centrifugation at 15,000 rpm for 20 min and incubated with Ni-NTA agarose (QIAGEN). The expression and the solubility of the recombinant proteins were determined by SDS–PAGE followed by staining with Coomassie blue.

GST pull down and immunoblotting

GST-fusion proteins were used for protein–protein interaction assays. A 500 ng sample of each GST-tagged protein to be tested or GST alone was mixed with 500 ng of each purified 6H-tagged protein, and incubated with 10 µl of Glutathione-Sepharose™ 4B (GE Healthcare) for 4 h at 4°C in 400 µl of buffer C [20 mM Tris–HCl (pH 7.9 at 4°C), 10% (v/v) glycerol, 0.2 mM PMSF, 0.05% (v/v) NP40, 0.5 mM EDTA (pH 8.0) and 1 × PI)] containing 100 mM KCl [BC(0.1)]. Samples were washed twice with BC(0.1) and then incubated with equal amounts of purified 6H-tagged BCL6 or BRG1 overnight at 4°C in BC(0.1). The resin was washed with BC(0.2) twice and BC(0.1) one time to remove non-specifically bound proteins, and then boiled in SDS sample buffer. The proteins eluted from the beads were separated by SDS–PAGE. The proteins were transferred to an Immobilon P membrane (Millipore) using a semi-dry transfer system at 15 V for 30 min and incubated with an anti-His-tag antibody (MBL). Chemiluminescent signals were detected using the ECL western blotting signal detection kit (GE Healthcare) and LAS-4000 mini (Fuji film).

Results

Identification of human CDK19-interacting proteins

Two human CDK subunits (hCDK8 and hCDK19) have been identified in the Mediator complexes and shown to play roles in transcription (*4*, *10*, *14*, *15*). These CDKs are kinase subunits and are highly similar to each other. Recently, several reports concerning the functions of CDK8 have been published, whereas the functions of CDK19 remain to be elucidated. Although CDK8 is widely conserved among eukaryotes, CDK19 exists only in vertebrates. Therefore, we planned to isolate the specific hCDK19-interacting

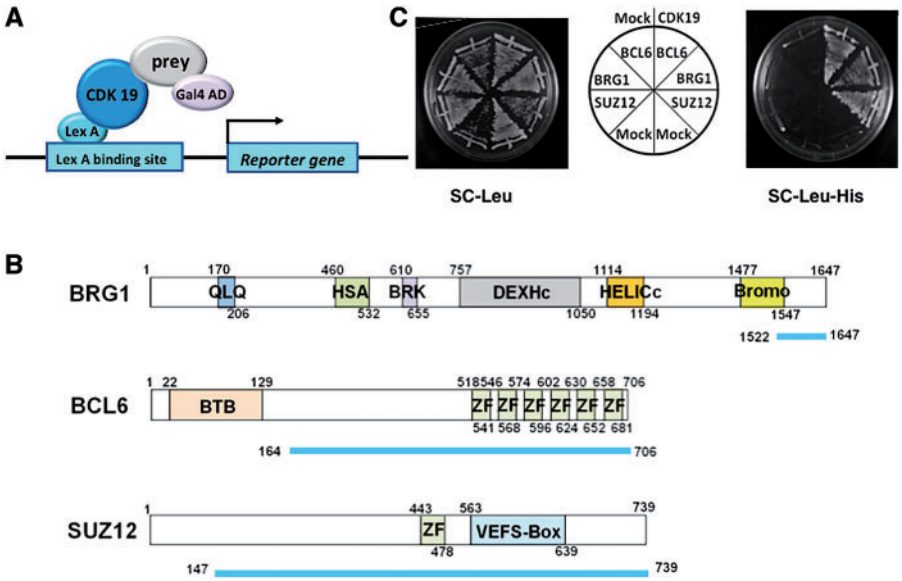

Fig. 1 Identification of the hCDK19-interacting proteins. (**A**) A scheme of the yeast two-hybrid screening. Once a protein with Gal4-activation domain at the C-terminus binds to hCDK19, the marker gene, HIS3, is expressed. Histidine is required for yeast cell growth and, therefore, the activator protein binding to hCDK19 is essential. (**B**) Schematic representation of the protein motifs and domains of BRG1, BCL6 and SUZ12. BRG1 contains a glutamine–leucine–glutamine motif (QLQ), a helicase/SANT-associated domain, a BRK domain, an ATP-dependent helicase domain DEXHc, an RNA helicase domain near the C terminus (HELICc) and an acetylated lysine recognition domain (Bromo). BCL6 contains a BTB domain (BTB) at the N-terminus and six stretch of zinc finger motifs at the C-terminus. SUZ12 contains a zinc finger and a VRN2-EMF2-FIS2-SUZ12 box (VEFS-Box). Positive clone-coding regions were indicated by light blue lines. (**C**) Confirmation yeast two-hybrid assay. To confirm that three isolated protein fragments possess hCDK19 interacting abilities, yeast two-hybrid assay was carried out.

proteins to study the functions of hCDK19 in the nuclear signaling pathways. To elucidate the hCDK19-interacting proteins so that we could distinguish the roles of these two CDKs, we performed a yeast two-hybrid screening system using a human fetal brain cDNA library (Fig. 1).

From 40 million yeast colonies tested, 538 clones were obtained as putative positive clones; thereafter, all the 538 clones were sequenced. As a result, 287 clones possessed mRNA sequences. Among them, it was found that 59 clones encoded mRNA sequences of nuclear protein genes. After confirmation of the proper reading frames, three clones were finally identified as confirmed positive-interacting proteins, and their proteins were partial sequences of BRG1, BCL6 and SUZ12 (Fig. 1B). These interactions were confirmed by using three partial sequences as preys for yeast two-hybrid assay system (Fig. 1C). BRG1 is a common ATPase subunit of various chromatin remodelling complexes (*20*, *26*). BCL6 plays essential roles in T and B lymphocyte differentiation and, therefore, B cell lymphoma, it has also been reported to be involved in transcriptional repression and chromatin regulation (*21*, *27*, *28*). SUZ12 is a subunit of PRC2 (Polycomb repressive complex 2) known to repress transcription through tri-methylation of histone H3 Lys27 (H3K27me3) (*22*, *29*).

Interactions of three candidate proteins with hCDK8 and hCDK19

To test the interactions of these three factors identified earlier with the human CDK subunits, we first tried to express isolated regions of BRG1 and BCL6. BRG1 (residues 1522–1647) and BCL6 (residues 164–706) were N-terminally 6H tagged, expressed in bacteria and purified with Ni NTA-agarose (Qiagen) (Fig. 2A, lanes 2 and 3, respectively). Then, the GST pull-down assays of BRG1 and BCL6 were conducted using bacterially expressed isolated regions of these proteins (Fig. 2B). Our data clearly demonstrated that both BRG1 and BCL6 could bind to both hCDK8 and hCDK19 (Fig. 2B, lanes 3 and 4).

We then studied on SUZ12. Since the identified region of SUZ12 could not be expressed in bacteria, four deletion mutant expression clones (D1–D4) with N-terminal GST tag were constructed (Fig. 3A), expressed in bacteria and purified with glutathione-Sepharose (GE Healthcare) (Fig. 3B). Then, the GST pull-down assays of 6H-hCDK8 and 6H-hCDK19 were conducted with these deletion mutant proteins (Fig. 3C). It turned out that SUZ12 also bound to both hCDK8 and hCDK19, especially at its N-terminal half (residues 1–447) (Fig. 3C, lanes 3 and 4). These results indicate that the interactions with hCDK19 were properly identified during this screening.

BRG1, BCL6 and SUZ12 bind to the N-terminal Ser/Thr kinase domains of both CDKs

To map the regions where BRG1 and BCL6 bind, GST-tagged deletion mutants of both CDKs were constructed as shown in Fig. 4A. Since the N-terminal 335 amino acids of both CDKs are similar (>90% identical), two mutants of each CDK were designed; one set of mutants (hCDK8K and hCDK19K) consists of these 335 amino acids, which contain a S/T kinase (residues 20–335), and the other mutants (hCDK8C and hCDK19C) consist of the remainder of the

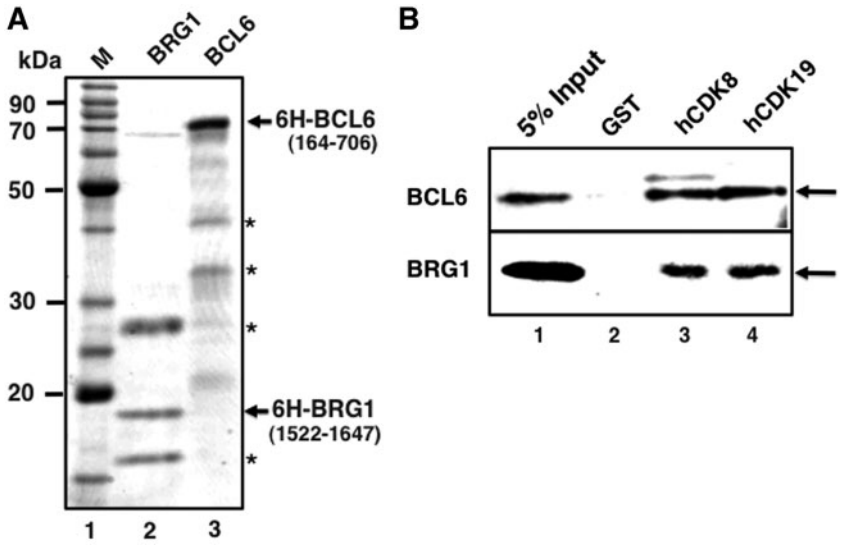

Fig. 2 Binding studies of the CDK-interacting fragments of BRG1 and BCL6. (**A**) Purification of partial fragments of BRG1 and BCL6. The partial cDNA regions of two-hybrid positive BRG1 and BCL6 were subcloned into the bacterial expression vector 6HT-pET11d, and were transformed and expressed in bacteria. N-terminally hexa histidine (6H)-tagged each protein was purified through Ni-NTA agarose (Qiagen) and was subjected to SDS–PAGE (12.5% acrylamide) followed by Coomassie brilliant blue (CBB) staining. Lane 1, 10 kDa-ladder protein marker (BioRad); lane 2, 6H-BRG1 (1522–1647) fraction; lane 3, 6H-BCL6 (164–706) fraction. Arrows indicate the positions of purified 6H-BRG1 and 6H-BCL6. Asterisks indicate non-specific bands. (**B**) GST pull-down assay of Mediator CDKs with BRG1 and BCL6 fragments. Also, 400 ng of each fragment was incubated with 300 ng of GST-tagged hCDK8 or hCDK19 and was pulled down with glutathione-Sepharose (GE Healthcare). Bound BRG1 and BCL6 were separated by SDS–PAGE (12.5 and 7.5% acrylamide, respectively) and were detected by anti-penta histidine antibody (Qiagen). Lane 1, 5% input; lane 2, GST alone; lane 3, GST-hCDK8 and lane 4, GST-hCDK19.

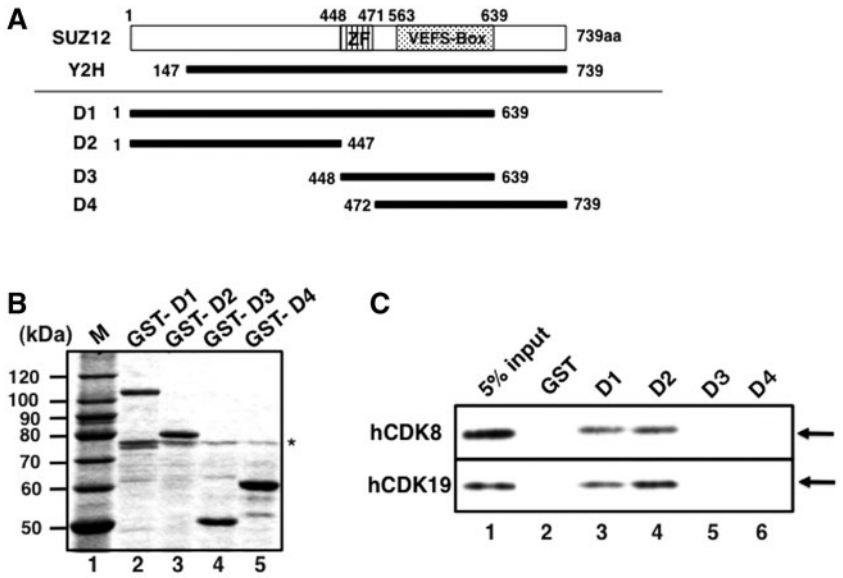

Fig. 3 Binding study of the CDK-interacting fragments of SUZ12. (**A**) Schematic representation of SUZ12 deletion mutants. Since full length SUZ12 and its yeast two-hybrid positive sequence (Y2H) were difficult to express in bacteria, four deletion constructs were designed. D1: SUZ12 (1–639), D2: SUZ12 (1–447), D3: SUZ12 (448–639) and D4: (472–739). (**B**) Purification of GST-tagged SUZ12 deletion mutants. The SUZ12 deletion cDNA regions were subcloned into the bacterial expression vector 6HT-pET11d, and were transformed and expressed in bacteria. N-terminally GST-tagged each protein was purified through glutathione-Sepharose (GE Healthcare) and was subjected to SDS–PAGE (10% acrylamide) followed by CBB staining. Lane 1, 10 kDa-ladder protein marker (BioRad); lane 2, GST-D1; lane 3, GST-D2; lane 4, GST-D3 and lane 5, GST-D4. An asterisk indicates a non-specific band. (**C**) GST pull-down assay of SUZ12 deletion mutants with either 6H-hCDK8 or 6H-hCDK19. Also, 400 ng of each CDK was incubated with 300 ng of GST-tagged SUZ12 deletion mutants and pulled down with glutathione-Sepharose (GE Healthcare). Bound CDKs were separated by SDS–PAGE and were detected by anti-hexa histidine antibody (MBL). Lane 1, 5% input; lane 2, GST alone; lane 3, GST-D1; lane 4, GST-D2; lane 5, GST-D3 and lane 6, GST-D4.

C-terminal region. All CDK constructs, including the full-length wild-type, were bacterially expressed and purified with glutathione-Sepharose (GE Healthcare) (Fig. 4B). The interaction domains of BRG1 and BCL6 were then mapped for both CDKs using a GST pull-down assay. As shown in Fig. 5A, both BRG1 and BCL6 specifically bound to the GST-tagged S/T kinase domain-containing N-terminal

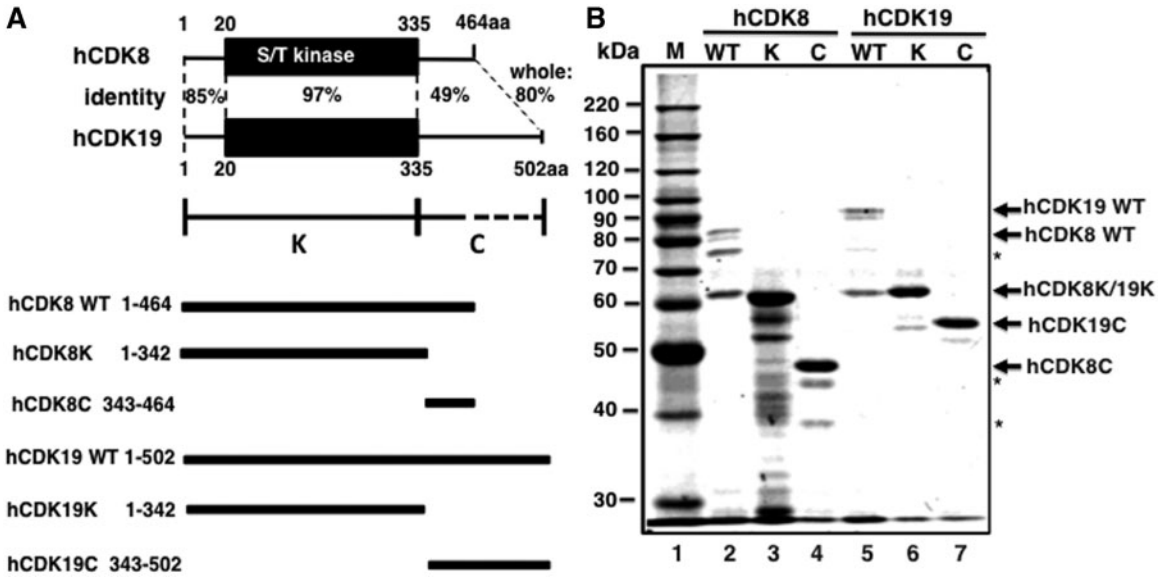

Fig. 4 Deletion mutants of two Mediator CDKs. (**A**) Schematic representation of deletion mutants. Since hCDK8 and hCDK19 have higher identity in the N-terminal 335 residues that contain the S/T Kinase, two mutants of this N-terminal region (hCDK8K and hCDK19K) and two mutants of the C-terminal rest (hCDK8C and hCDK19C) were constructed. WT means full-length wild-type. (**B**) Purification of CDK mutants. GST-tagged mutants were bacterially expressed, purified through Glutathione-Sepharose, and each purified proteins were subjected to SDS–PAGE (10% acrylamide) and detected by CBB staining. Arrows indicate the positions of purified GST-tagged hCDK8 and hCDK19 proteins. Asterisks indicate nonspecific bands.

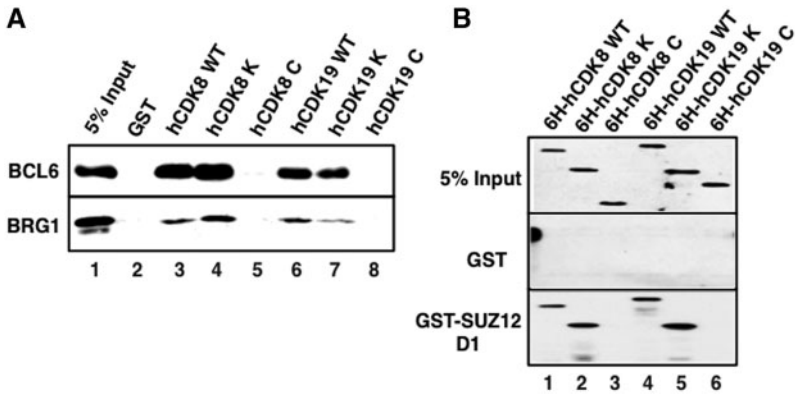

Fig. 5 Binding study of CDK mutants with three interacting proteins. (**A**) Binding study of CDK mutants with BRG1 and BCL6. GST pull-down assay was performed for CDK mutants and bound 6H-BRG1 or 6H-BCL6 fragment was detected by western blotting after SDS–PAGE. Also, 400 ng of each fragment was incubated with 300 ng of GST-tagged CDK mutants. After blotting, each bound fragment was detected by anti-hexa histidine antibody (MBL). Lane 1, 5% input; lane 2, GST alone; lane 3, GST-hCDK8 wild type (WT); lane 4, GST-hCDK8 K; lane 5, GST-hCDK8 C; lane 6, GST-hCDK19 wild-type (WT); lane 7, GST-hCDK19 K and lane 8, GST-hCDK19 C. (**B**) Binding study of CDK mutants with SUZ12. GST pull-down assay was performed for CDK mutants, and bound 6H-CDK proteins were detected by western blotting after SDS–PAGE. Also, 400 ng of each 6H-CDK mutant was incubated with 300 ng of GST-tagged SUZ12 D1. After blotting, each bound mutant was detected by anti-hexa histidine antibody (MBL). Lane 1, 6H-hCDK8 WT; lane 2, 6H-hCDK8 K; lane 3, 6H-hCDK8 C; lane 4, 6H-hCDK19 WT; lane 5, 6H-hCDK19 K and lane 6, 6H-hCDK19 C.

regions (hCDK8K and hCDK19K) (Fig. 5A, lanes 4 and 7). In Fig. 5B, the binding specificities of SUZ12 D1 (residues 1–639) to the two CDK deletion mutants were studied. Similarly to other two proteins, SUZ12 D1 also bound to the N-terminal kinase regions of both two CDKs (Fig. 5B, lanes 2 and 5). These results clearly explain why the yeast two-hybrid positive proteins, BRG1, BCL6 and SUZ12, bound equally well to both CDKs.

BRG1, BCL6 and SUZ12 bind to CDK-containing intact Mediator complexes

Because the binding of BRG1, BCL6 and SUZ12 to two different recombinant CDKs was observed, the interactions of these three proteins with the intact Mediator complexes were also studied (Fig. 6). A 4 mg sample of HeLa nuclear extract was mixed with 500 ng of either GST-tagged hCDK8 or hCDK19 and pulled down using glutathione-Sepharose (GE Healthcare). After washing with buffer C containing 200 mM KCl, column-bound proteins were separated by SDS–PAGE and detected by western blotting. As shown in Fig. 6, BRG1, BCL6 and SUZ12 bound to the intact Mediator complexes (Fig. 6 all 4 columns, lanes 3, 4 and 5). It has become more and more apparent that transcription is tightly regulated in combination with chromatin regulation and is intimately related to epigenetic regulation. From results presented

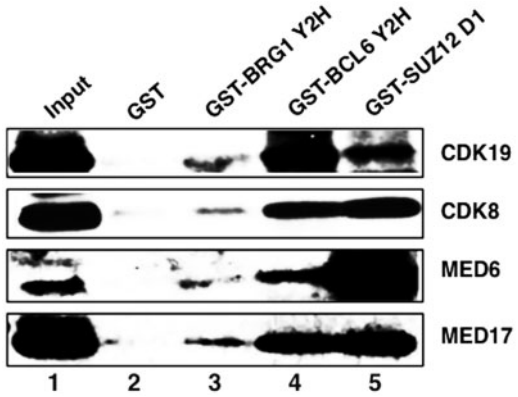

Fig. 6 Binding study of three protein fragments with intact Mediator complexes. GST pull-down assay was performed for GST-tagged CDK-binding fragments of BRG1 (GST-BRG1 Y2H), BCL6 (GST-BCL6 Y2H) and SUZ12 (GST-SUZ12 D1) with Mediator complexes. Also, 30 mg of HeLa nuclear extracts were incubated each GST-tagged protein. After incubation at 4°C for 4 h, bound proteins were detected by western blotting after SDS–PAGE.

herein concerning interactions between Mediator and both SWI/SNF and histone deacetylase (HDAC) co-repressor complexes, direct relationships between the aforementioned types of regulations were suggested (Fig. 7). These results further elucidate the mechanisms of both transcriptional activation and repression via the Mediator complexes.

Discussion

The manner in which transcriptional regulation switches between activation, and repression has remained unknown for some time (*3*, *29*). Previously, we used short inhibitory RNA treatment to study hCDK8 and hCDK19 (formerly hCDK11 or CDC2L6) and demonstrated that these two CDKs are mutually exclusive, individually form the Mediator complexes, and distinctly regulate transcription (*4*, *10*, *15*). Using the luciferase reporter assay under a Gal4-VP16-dependent transcription activation condition in combination with siRNA treatment, hCDK8 was observed to have a positive effect on transcriptional activation, whereas hCDK19 was observed to have a negative effect (*10*). These results suggest that CDKs are the molecular switches for transcriptional regulation and, subsequently, many studies have confirmed our results, especially those for hCDK8 (*5*, *16*, *17*, *30*, *31*). Although the hCDK8-containing Mediator has been widely recognized as a negative effector in transcriptional activation; the roles and its functional mechanisms of the newly identified Mediator kinase subunit, hCDK19, are yet to be elucidated. Therefore, in this study, we have attempted to identify hCDK19-specific interaction proteins by using a yeast two-hybrid system.

Mediators recruit co-factors via the kinase domain of two CDK subunits

In the end of our two-hybrid assay, three transcriptional co-factor proteins, BRG1, BCL6 and SUZ12, were identified to bind directly to both CDKs (Figs 2B and 3C). When the amino acid sequences of both CDKs were compared, their N-terminal regions of 335 amino acids containing the S/T Kinase are >90% identical, but the C-terminal regions are diverged with 49% identity (Fig. 4A). Further biochemical analysis using CDK deletion mutants lacking their N-terminal regions (hCDK8C and hCDK19C) and C-terminal regions (hCDK8K and hCDK19K) clearly demonstrated that they both bound to the N-terminal regions (Fig. 5). Judging from the sequence identity, it can be judged that the biochemical binding results are reasonable. It would be less possible for them to bind to the diverged C-terminal sequences. Finally, those co-factor binding to the intact Mediator complexes were studied (Fig. 6). Further precise studies for their interactions with CDKs are inevitable for elucidation of the switching mechanisms of transcriptional regulations. However, from these data, it was suggested that the S/T kinase and its surrounding region of Mediator CDK subunits, hCDK8 and hCDK19, might be important as platforms for communication of transcriptional regulation with chromatin regulations and their intimately related epigenetic events.

Functional meaning of transcriptional co-factor/chromatin regulator binding to Mediators

BRG1, BCL6 and SUZ12 are transcriptional co-factors and, at the same time, chromatin regulators as well. The name of BRG1 comes from Brahma-related gene 1, and therefore this homologue Brm (Brahma) and BRG1 are two of the ATP-dependent catalytic subunits for chromatin remodelling. They are now widely known to be mutually exclusive to form three different SWI/SNF-type ATP-dependent chromatin re-modelling complexes; BRG1/BAF, Brm/BAF and PBAF complexes and target distinct genes (*20*, *32*). Among them, BRG1 is a component of BRG1/BAF and PBAF complexes. These two complexes share most of the subunits but differ in the association of BAF180, BAF200 and BAF250. In addition, BRG1 is also known to form subset complexes with HDACs (*33*). Although their precise roles are yet to be elucidated, they definitely play important roles in transcriptional regulation both positively and negatively in conjunction with chromatin regulation.

In contrast, BCL6 is highly expressed in the germinal centre B cells undergoing affinity maturation of immunoglobulin (*34*, *35*). BCL6 is a sequence-specific transcriptional repressor with six zinc finger motifs at the C-terminus and a BTB domain at the N-terminus (Fig. 2B) (*21*, *36*). The transcriptional co-repressors NCoR, SMRT and BCOR were reported to bind competitively to the BTB domain of BCL6 (*36*, *37*). In the context of these binding, BCL6 also recruits HDACs. In this case, Mediators will work negatively in transcription together with histone deacetylation. It is also intriguing that the binding amounts of BCL6 and Brg1 to hCDK8 and hCDK19 were almost equal, but those to MED17 and MED6 were not (Fig. 6). It is possible that different subtypes of Mediators (some

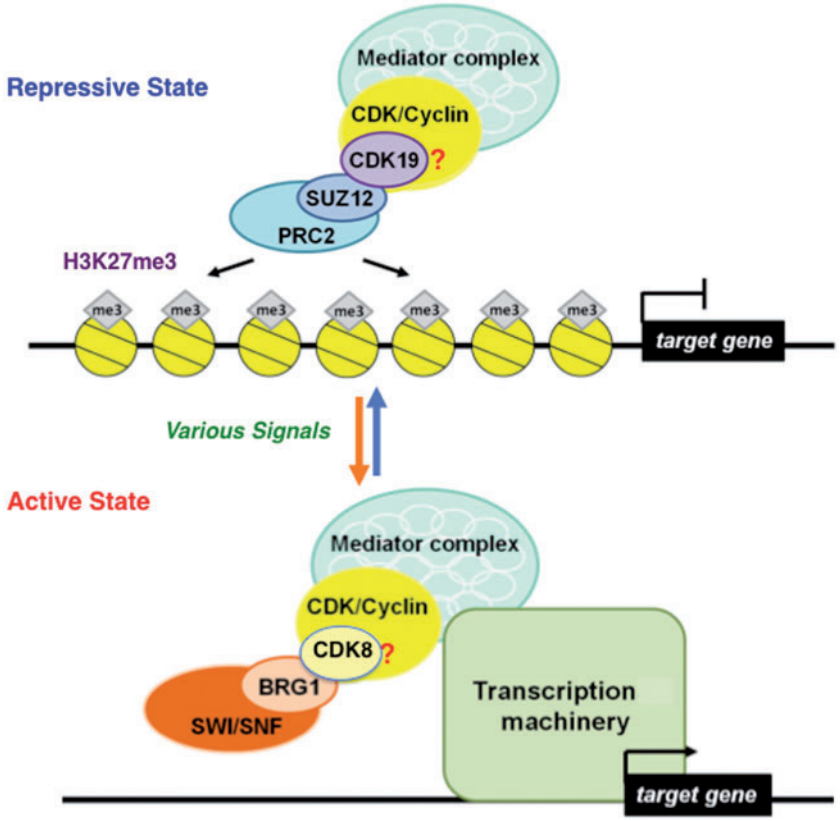

Fig. 7 Transition model of Mediator complex between repressive state and active state. The Mediator may serve a repressive state when CDK (possibly CDK19) associates with SUZ12, which forms the PRC2 complex that contributes to tri-methylate histone H3K27 (H3K27me3). This state may reversibly change to an active state by various signals when CDK (possibly CDK8), in turn, associates with BRG1, which forms the SWI/SNF complex that remodels chromatin to an active form.

Mediators may lack head and/or middle submodules) play different roles by binding to BCL6 and BRG1.

The other CDK-interacting protein SUZ12 is known to be a component of the PRC2 (*22*, *38*). PRC2 is thought to inhibit transcription by tri-methylation of histone H3 at lysine 27 through either EZH1 or EZH2 subunit. Therefore, it is easily speculated that CDKs, when bind to SUZ12, play negative roles in transcription.

As mentioned earlier in the text, the trials to identify the Mediator CDK target genes by using siRNA knockdown, and DNA microarray studies unveiled that the regulatory mechanisms of transcription by Mediators are not such simple but possibly are context dependent (*15*). It is most likely that Mediators regulate transcription by interacting with co-activators or co-repressors in tight association with chromatin regulatory factors (chromatin remodellers and histone modifiers) dependent on their context. Our present results suggested the direct relationships between transcription and chromatin regulation and may become clues to elucidate the molecular mechanisms of transcriptional regulation (Fig. 7). Here, we suggest a new concept on the transcriptional regulation by the Mediator via kinase subunit, which offer as 'transition model'. We previously showed that hCDK8 and hCDK19 function oppositely, hCDK8 is involved in activation of transcription and hCDK19 is involved in repression of that (*4*). At the repressive state, co-repressor PRC2 associates with one of the Mediator CDK subunit (possibly CDK19) via SUZ12, trimethylates histone H3K27 and finally causes to repress transcription (upper panel). Also, at the active state, SWI/SNF complex associates with the other Mediator CDK (possibly CDK8) via BRG1, helps to remodel chromatin in collaboration with transcription machinery and cause to stimulate transcription (lower panel). These two states may be reversibly changed on various signals. We are currently studying their biological activities of isolated Mediator fractions in chromatin remodelling and histone modification and are trying to identify their bound locus on the human whole genome by isolating DNA fragments using chromatin immunoprecipitation (ChIP) followed by nucleotide sequencing (ChIP-seq).

Acknowledgements

We thank Dr Fumitaka Mizuki for technical advices of yeast studies, and our colleagues at the University of Toyama for helpful advice and discussions.

Funding

This work was supported in part by Grant-in-Aid for Scientific Research (Grant # 17054022 to Y.O. and Grant # 21790066 to A.T.) and by Targeted Proteins Research Programme (to Y.O.) from the Ministry of Education, Science, and Culture of Japan, and by Grant-in-Aid for JSPS fellows (to T.T.).

Conflict of interest

None declared.

References

1. Malik, S. and Roeder, R.G. (2010) The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. *Nat. Rev. Genet.* **11**, 761–772
2. Li, G. and Reinberg, D. (2011) Chromatin higher-order structures and gene regulation. *Curr. Opin. Genet. Dev.* **21**, 175–186
3. Roeder, R.G. (2005) Transcriptional regulation and the role of diverse coactivators in animal cells. *FEBS Lett.* **579**, 909–915
4. Tsutsui, T., Umemura, H., Tanaka, A., Mizuki, F., Hirose, Y., and Ohkuma, Y. (2008) Human Mediator kinase subunit CDK11 plays a negative role in viral activator VP16-dependent transcription. *Genes Cells* **13**, 817–826
5. Knuesel, M.T., Meyer, K.D., Bernecky, C., and Taatjes, D.J. (2009) The human CDK8 subcomplex is a molecular switch that controls Mediator activator function. *Genes Dev.* **23**, 439–451
6. Malik, S. and Roeder, R.G. (2005) Dynamic regulation of pol II transcription by the mammalian Mediator complex. *Trends Biochem. Sci.* **30**, 256–263
7. Conaway, R.C., Sato, S., Tomomori-Sato, C., Yao, T., and Conaway, J.W. (2005) The mammalian Mediator complex and its role in transcriptional regulation. *Trends Biochem. Sci.* **30**, 250–255
8. Liu, Y., Ranish, J.A., Aebersold, R., and Hahn, S. (2001) Yeast nuclear extract contains two major forms of RNA polymerase II mediator complexes. *J. Biol. Chem.* **276**, 7169–7175
9. Takahashi, H., Parmely, T.J., Sato, S., Tomomori-Sato, C., Banks, C.A., Kong, S.E., Szutorisz, H., Swanson, S.K., Martin-Brown, S., Washburn, M.P., Florens, L., Seidel, C.W., Lin, C., Smith, E.R., Shilatifard, A., Conaway, R.C., and Conaway, J.W. (2011) Human mediator subunit MED26 functions as a docking site for transcription elongation factors. *Cell* **146**, 92–104
10. Furumoto, T., Tanaka, A., Ito, M., Malik, S., Hirose, Y., Hanaoka, F., and Ohkuma, Y. (2007) A kinase subunit of the human Mediator complex, CDK8, positively regulates transcriptional activation. *Genes Cells* **12**, 119–132
11. Hengartner, C.J., Myer, V.E., Liao, S.M., Wilson, C.J., Koh, S.S., and Young, R.A. (1998) Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases. *Mol. Cell* **2**, 43–53
12. Akoulitchev, S., Chuikov, S., and Reinberg, D. (2000) TFIIH is negatively regulated by cdk8-containing mediator complexes. *Nature* **407**, 102–106
13. Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2001) Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes Dev.* **15**, 1078–1092
14. Sato, S., Tomomori-Sato, C., Parmely, T.J., Florens, L., Zybailov, B., Swanson, S.K., Banks, C.A., Jin, J., Cai, Y., Washburn, M.P., Conaway, J.W., and Conaway, R.C. (2004) A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. *Mol. Cell* **14**, 685–691
15. Tsutsui, T., Fukasawa, R., Tanaka, A., Hirose, Y., and Ohkuma, Y. (2011) Identification of target genes for the CDK subunits of the Mediator complex. *Genes Cells* **15**, in press
16. Galbraith, M.D., Donner, A.J., and Espinosa, J.M. (2010) CDK8: a positive regulator of transcription. *Transcription* **1**, 4–12
17. Conaway, R.C. and Conaway, J.W. (2011) Funation and regulation of the Mediator complex. *Curr. Opin. Genet. Dev.* **21**, 225–230
18. Meyer, K.D., Donner, A.J., Knuesel, M.T., York, A.G., Espinosa, J.M., and Taatjes, D.J. (2008) Cooperative activity of cdk8 and GCN5L within Mediator directs tandem phosphoacetylation of histone H3. *EMBO J.* **27**, 1447–1457
19. Chakraborty, T., Martin, J., and Olson, E.N. (1992) Analysis of the oligomerization of myogenin and E2A products in vivo usinf a two-hybrid assay system. *J. Biol. Chem.* **267**, 17496–17501
20. Khavari, P.A., Peterson, C.L., Tamkun, J.W., Mendel, D.B., and Crabtree, G.R. (1993) BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. *Nature* **366**, 170–174
21. Dhordain, P., Lin, R.J., Quief, S., Lantoine, D., Kerckaert, J.P., Evans, R.M., and Albagli, O. (1998) The LAZ3 (BCL-6) oncoprotein recruits a SMRT/mSIN3A/histone deacetylase containing complex to mediate transcriptional repression. *Nucl. Acids Res.* **26**, 2899–2907
22. Cao, R. and Zhang, Y. (2004) SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol. Cell* **15**, 57–67
23. Ito, T., Nakamura, R., Sumimoto, H., Takeshige, K., and Sakaki, Y. (1996) An SH3 domain-mediated interaction between the phagocyte NADPH oxidase factors p40phox and p47phox. *FEBS Lett.* **385**, 229–232
24. Kubota, H., Ota, K., Sakaki, Y., and Ito, T. (2001) Budding yeast GCN1 binds the GI domain to activate the eIF2alpha kinase GCN2. *J. Biol. Chem.* **276**, 17591–17596
25. Okamoto, T., Yamamoto, S., Watanabe, Y., Ohta, T., Hanaoka, F., Roeder, R.G., and Ohkuma, Y. (1998) Analysis of the role of TFIIE in transcriptional regulation through structure-function studies of the TFIIEbeta subunit. *J. Biol. Chem.* **273**, 19866–19876
26. Trotter, K.W. and Archer, T.K. (2008) The BRG1 transcriptional coregulator. *Nucl. Recept. Signal.* **6**, e004
27. Linggi, B.E., Brandt, S.J., Sun, Z.W., and Hiebert, S.W. (2005) Translating the histone code into leukemia. *J. Cell. Biochem.* **96**, 938–950
28. Ochiai, K., Muto, A., Tanaka, H., Takahashi, S., and Igarashi, K. (2008) Regulation of the plasma cell transcription factor Blimp-1 gene by Bach2 and Bcl6. *Int. Immunol.* **20**, 453–460
29. Levine, M. and Tjian, R. (2003) Transcription regulation and animal diversity. *Nature* **424**, 147–151
30. Donner, A.J., Szostek, S., Hoover, J.M., and Espinosa, J.M. (2007) CDK8 is a stimulus-specific positive coregulator of p53 target genes. *Mol. Cell* **27**, 121–133
31. Belakavadi, M. and Fondell, J.D. (2010) Cyclin-dependent kinase 8 positively cooperates with Mediator to promote thyroid hormone receptor-dependent transcriptional activation. *Mol. Cell. Biol.* **30**, 2437–2448
32. Reisman, D., Glaros, S., and Thompson, E.A. (2009) The SWI/SNF complex and cancer. *Oncogene* **28**, 1653–1668
33. Kuzmichev, A., Zhang, Y., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002) Role of the

Sin3-histone deacetylase complex in growth regulation by the candidate tumor suppressor p33 (ING1). *Mol. Cell. Biol.* **22**, 835–848

34. Igarashi, K., Ochiai, K., and Muto, A. (2007) Architecture and dynamics of the transcription factor network that regulates B-to-plasma cell differentiation. *J. Biochem.* **141**, 783–789
35. Martins, G. and Calame, K. (2008) Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu. Rev. Immunol.* **26**, 133–169
36. Ghetu, A.F., Corcoran, C.M., Cerchietti, L., Bardwell, V.J., Melnick, A., and Privé, G.G. (2008) Structure of a BCOR corepressor peptide in complex with the BCL6 BTB domain dimer. *Mol. Cell* **29**, 384–391
37. Ahmad, K.F., Melnick, A., Lax, S., Bouchard, D., Liu, J., Kiang, C.L., Mayer, S., Takahashi, S., Licht, J.D., and Privé, G.G. (2003) Mechanism of SMRT corepressor recruitment by the BCL6 BTB domain. *Mol. Cell* **12**, 1551–1564
38. Margueron, R. and Reinberg, D. (2011) The Polycomb complex PRC2 and its mark in life. *Nature* **469**, 343–349