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Polycomb Repressive Complex 1 subunit Cbx4 positively regulates effector responses in CD8 T cells

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Abstract

Cytotoxic T lymphocyte (CTL) differentiation is controlled by the crosstalk of various transcription factors and epigenetic modulators. Uncovering this process is fundamental to improving immunotherapy and designing novel therapeutic approaches. Here, we show that

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Polycomb Repressive Complex (PRC)1 subunit Chromobox (Cbx)4 favors effector CTL differentiation in murine model. Cbx4 deficiency in CTLs induced transcriptional signature of memory cells and increase the memory CTL population during acute viral infection. It has been previously shown that besides binding to H3K27me3 through its chromodomain, Cbx4 function as a SUMO E3 ligase in a SUMO interacting motifs (SIM)-dependent way. Overexpression of Cbx4 mutants in distinct domains showed that this protein regulates CTL differentiation primarily in a SIM-dependent way and partially through its chromodomain. Our data suggest a novel role of a Polycomb group protein Cbx4 controlling CTL differentiation and indicates the SUMOylation as a key molecular mechanism connected to chromatin modification in this process.

Keywords

Epigenetics; Polycomb; Cbx4; PRC1; T cell differentiation; CD8 T cell

Introduction

Cytotoxic CD8 T lymphocytes (CTLs) are an essential component of adaptive immunity, working as key players in the elimination of intracellular pathogens and tumor cells (1). Upon activation, CTLs undergo intense expansion and differentiate into a heterogeneous population composed of terminal effector cells (TE), that are characterized by the expression of KLRG1 and low expression of IL-7Ra ($KLRG1^{+}CD127^{-}$), and memory precursor (MP) cells, that express the opposite pattern ($KLRG1^{-}CD127^{+}$). Complex and dynamically regulated transcriptional programs control the differentiation of these distinct CD8 T cell states and the identification of the key players in this process is critical for understanding these molecular events and for advancing novel therapeutic strategies (1, 2). Transcription factors cooperate with epigenetic regulators, including chromatin modifiers that add, remove or recognize post-translational modifications in histones, adding a new level of complexity to this mechanisms (3–5). The Polycomb Repressor Complex (PRC)2 plays an essential role in effector cell differentiation through the deposition of its repressive histone mark, H3K27me3, in memory-related genes during acute viral infection (4, 6, 7). Furthermore, H3K27me3 removal by lysine demethylase Kdm6 promotes effector differentiation through de-repression of effector genes (8–10), highlighting the importance of H3K27me3 methylation/demethylation dynamic regulation to CTL differentiation. Another Polycomb group (PcG) epigenetic complex with repressive function, the PRC1, is composed by several distinct subunits and the canonical configurations (cPRC1) can be recruited to genome sites by H3K27me3. This process is mediated by recognition and binding of Chromobox (Cbx) family proteins (Cbx2, Cbx4, Cbx6, Cbx7, and Cbx8) to H3K27me3 through its conserved chromodomain (11, 12). Additionally, Cbx4 is reported to function as a small ubiquitin-like modifier (SUMO) E3 ligase through its two SUMO interacting motifs (SIM) (13, 14) and Cbx4 E3 SUMO ligase activity has been reported to regulate essential transcriptional regulators, such as Dnmt3a and Hif-1α (15, 16). Here we report that Cbx4 deficiency promotes memory-associated phenotype and transcriptional profile in CTLs, and overexpression of Cbx4 mutants in distinct functional domains showed that this epigenetic regulator induces effector differentiation primarily through its SIM domain.

Materials and Methods

Mice

All mice were on a C57BL/6 background. Experimental mice were 6- to 8-wk-old and sex- and age-matched. P14 [lymphocytic choriomeningitis virus (LCMV) gp33–41-H2-Db–specific] Thy1.1 and Cd4-Cre mice have been previously described (17). $Cbx4^{fl/fl}$ mice (obtained from Dr. Guoliang Xu, Shanghai, Institute of Biochemistry and Cell Biology, China) were bred to create $Cbx4^{fl/fl}Cd4^{Cre}$ and P14 $Thy1.1^{+}$ $Cbx4^{fl/fl}Cd4^{Cre}$ mice. Mice were housed according to protocols approved by the Rosalind Franklin University of Medicine and Science Institutional Animal Care and Use Committee or according to CONCEA (Conselho Nacional de Experimentação Animal) rules and UFRJ CEUA (Comitê de Ética no Uso de Animais) approval (Protocols 054/20 and 003/23).

T cell in vitro culture and retroviral transduction

Naive $CD8^{+}$ T cells were purified and cultured to generate effector- and memory-like CD8 T cells *in vitro*. Cells were activated with anti-CD3 (1 μg/mL for effector-like cells; 50 ng/mL for memory-like cells) and anti-CD28 (1 μg/mL for both) and polarized to effector- or memory-like phenotypes through supplementation with 100 U/mL of recombinant human IL-2 (rhIL-2) or 10 U/mL of rhIL-2 and 10 ng/mL of rhIL-7 and rhIL-15, respectively (18, 19). Knockdown experiments used Ametrine-expressing murine retroviral vectors containing shRNAs targeting CD4 (shCD4) or Cbx4 (shCbx4) mRNA (20). Overexpression experiments used GFP-expressing murine retroviral vectors containing Cbx4 coding sequence (Cbx4-OE), Cbx4 mutants carrying two point mutations (F11A/W35L) at chromodomain (ΔChromo), deletion of the two SUMO interacting motifs (SIM) domains (ΔSIM1–2) or empty vector (Mock) kindly donated by Dr. Wang (21). Transduction was carried as previously described (9).

LCMV acute infection model and adoptive cell transfer

For adoptive transfer experiments, congenic C57BL/6 (Thy1.2) mice received i.v. 5×10^5 *in vitro*-transduced P14 Thy1.1 CTLs, were subsequently infected i.p. with 1.5×10^5 PFUs of LCMV clone13 and analyzed 8 days post-infection (dpi), as previously described to induce an acute infection response (20). $Cbx4^{fl/fl}Cd4^{Cre}$ mice were infected i.p. with 2×10^5 PFUs of LCMV Armstrong and analyzed on 7 dpi and 60 dpi. LCMV strains were initially provided by Dr. Shane Crotty, La Jolla Institute, CA and expanded with BHK cells as described before (22).

Flow Cytometry analysis

Cell surface, intracellular and LCMV tetramer (H2Db-gp33–41 (KAVYNFATC) Alexa647 or APC) staining were performed as previously described (9). Cytokine production was measured from *in vitro* cultured cells upon restimulation with 10 nM PMA and 1 μM Ionomycin for 4 h in the presence of brefeldin A. Samples were run on FACSAria IIu, LSRII or Fortessa (BD Biosciences), and data were analyzed with FlowJo (Versions 9.9.4 and 10.7.1).

Cytotoxicity assay

In vitro cultured and transduced P14 CD8 T cells were purified by FACS (Ametrine$^+$) and co-cultured at different ratios with GFP-expressing parental mammary carcinoma cell line EO771 or EO771 cells expressing LCMV-antigen gp33–41 (EO771-GP33), as previously described (9). After 12 h, live GFP-expressing EO771 cells were determined by flow cytometry.

RNA sequencing

FACS-purified cells from *in vivo* experiment were used for preparation of RNA-seq libraries using SMARTer Stranded RNA-Seq Kit (Clontech). Reads were analyzed as previously described (23). Differentially expressed genes (DEGs) were considered when the DESeq2 analysis resulted in p value < 0.05. GSEA (*Gene Set Enrichment Analysis*) was performed by comparing DEGs to published dataset (24).

Quantitative real-time RT-PCR

Total RNA was isolated from FACS-purified CD8 T cells using TRIzol (Invitrogen). cDNA synthesis and gene expression analysis were performed as previously described (9). Gene expression was normalized to *Rpl22*.

Statistics and analysis

Statistical analysis was performed in Prism 7 or 8 (GraphPad) using nonpaired one-way ANOVA followed by Tukey multiple comparisons, two-tailed paired or nonpaired Student t test, or two-way ANOVA followed by Tukey or Sidak multiple comparisons, as indicated.

Results and Discussion

Cbx4 deficiency skews CTL differentiation to a memory phenotype and impacts cytotoxic function

To assess the role of Cbx4 in CTL differentiation, we employed LCMV acute infection model using adoptive transfer of P14 CD8 T cells. Naïve CD8 T cells from P14 Thy1.1$^+$ mice were activated *in vitro* and transduced with retroviral vectors expressing shRNA targeting Cbx4 mRNA (shCbx4) or CD4 mRNA as a control (shCD4). Transduced cells were then adoptively transferred to WT Thy1.2$^+$ congenic mice that were infected on the same day with LCMV (20) (Fig. 1A). The efficiency of Cbx4 silencing was confirmed by RT-qPCR (Supplemental Fig. 1A).

The differentiation of P14 cells to effector or memory phenotype was analyzed 8 days post-infection by measuring KLRG1 and CD127 expression in transduced (Ametrine$^+$) and adoptively transferred (Thy1.1$^+$) P14 CD8 T cells recovered from the spleen (Fig. 1B–D; Supplemental Fig. 1B). Transferred LCMV-specific Cbx4-deficient CTLs (P14 shCbx4) had lower frequency and number of TEs (KLRG1$^+$CD127$^-$) and, accordingly, higher frequency and cell number of populations expressing the memory-associated marker CD127, both in MP (KLRG1$^-$CD127$^+$) and in KLRG1$^+$CD127$^+$ population. The frequency of transferred Thy1.1$^+$ P14 cells and transduced Ametrine$^+$ Thy1.1$^+$ P14 cells were lower in mice

receiving P14 shCbx4 cells in comparison to mice receiving control cells; however, no significant difference was observed in the cell numbers (Supplemental Fig. 1B–D).

To further investigate the contribution of Cbx4 to the regulation of CTL transcriptional profile, P14 CTLs from the LCMV acute infection model described above were sort-purified on d8 post-infection, and total RNA was extracted for RNA-Seq. Data analysis showed 836 differentially expressed genes (DEGs) when comparing P14 shCbx4 to P14 shCD4 cells (Fig. 1E). Among those genes, several surface markers that positively correlate with the memory cells were upregulated on Cbx4 deficient cells, including *Il7r* (CD127) and *Slamf6* (2, 25). In addition, genes encoding key transcription factors *Eomes* and *Tcf7* (Tcf-1), which promote the generation and persistence of central memory CD8 T cells, were also upregulated in comparison to control cells (26–29). Concordantly, genes positively correlated with the effector phenotype and function, such as *Klrg1*, *Prf1* and *Cx3cr1*, were downregulated in P14 shCbx4 compared to P14 shCD4 cells. These gene expression alterations, such as the upregulation of *Tcf7* and *Il7r*, were not just due to a higher frequency of memory cells in our Cbx4-deficient population, as sorted Cbx4-deficient KLRG1^{hi} CTLs also showed an enrichment of memory-associated genes (Supplemental Fig. 1E). To test whether these gene expression alterations lead to a global shift towards memory transcriptional programs, we performed Gene Set Enrichment Analysis (GSEA) using previously published memory precursor (KLRG1^{lo}CD127^{hi}) transcriptional signature from day 8 acute LCMV infection (24). We found that genes upregulated in P14 shCbx4 cells showed enrichment in memory precursor transcriptional signature (Fig. 1F), indicating that Cbx4 is involved in the regulation of memory-associated genes. It is also plausible that Cbx4 could indirectly impact the expression of transcription factors related to CTL differentiation by controlling other transcriptional regulators. For example, our RNA-Seq showed that *c-Myb*, a transcriptional activator of *Tcf7* (30), is upregulated in P14 shCbx4 cells. Similarly, Cbx4 was reported to activate the Wnt/β-catenin pathway, a pathway upstream of TCF-1 activation, in human lung adenocarcinoma cells (31).

As we observed the downregulation of genes related to effector function (i.e.: *Prf1*), we explored if Cbx4 deficiency could impact CD8 T cell cytotoxic function co-culturing activated and retrovirally transduced P14 cells with GP33-expressing GFP^{+} EO771 tumor cells (EO771) (Fig. 1G). We observed that P14 shCbx4 cells had diminished cytotoxicity compared to P14 shCD4, which further supports the observation of defective effector differentiation with concomitant skewing to memory phenotype in the absence of Cbx4 protein.

Collectively, our findings show that Cbx4 deficiency upregulates memory precursor transcriptional signature and expression of memory surface markers, and decreases effector cytotoxic function, revealing a skewing of CD8 T cells towards a memory phenotype.

Cbx4 deficiency leads to increased memory CTL formation

To confirm that Cbx4 could control memory CTL formation, we used the acute LCMV infection in T cell-specific Cbx4 deficient mice (Cbx4$^{fl/fl}$Cd4^{Cre}, herein referred to as Cbx4 T KO mice), analyzing polyclonal Cbx4 deficient CD8 T cells 7 or 60 dpi (Fig. 2A; Supplemental Fig. 2). Analysis of lymphocyte populations on thymus, lymph nodes

and spleens of Cbx4 T KO mice at steady state showed no alteration (data not shown). On day 60 post-infection, analysis of Cbx4 T KO mice spleen cells showed a slight increase in the frequency and number of CTLs, as well as higher number of LCMV-specific H2Db-gp33–41^{+} (GP33^{+}) cells (Fig. 2B,C). In addition, Cbx4 T KO mice had higher frequency of KLRG1^{+}CD127^{+} population in GP33^{+} CD8 T cells both at d7 and d60 pi (Fig. 2.D–F; Supplemental Fig. 2C, D). On d7, we observed increased frequency of KLRG1^{-} CD127^{+} memory precursor cells (Supplemental Fig. 2C, D). Similarly, at 60 dpi we observed higher number of memory KLRG1^{-}CD127^{+} population, although this was not reflected in population frequency. Accordingly, we found decreased frequency and cell number of terminal effector (KLRG1^{+}CD127^{-}) GP33^{+} CTLs (Fig. 2D–F). Validating the enrichment of memory-associated genes in Cbx4 deficient CTLs, we observed that T-bet/Eomes ratio was significantly lower in Cbx4 T KO mice, regardless of KLRG1 expression, in line with a global increased memory profile upon Cbx4 deficiency (Fig. 2G). Overall, our data demonstrates a CD8 T cell-intrinsic role of Cbx4 in controlling effector T cell differentiation. However, the results presented in the CD4^{Cre} mouse system cannot exclude a role of Cbx4 in CD4 T cells. Future studies will focus on the role of this protein in CD4 T cell biology.

To recapitulate this phenotype in an *in vitro* model system, we polarized CD8 T cells to either effector- or memory-like cells using a previously established protocol (19) (Supplemental Fig. 3A). Consistently, we observed increased levels of the memory markers CD127 and CD62L in Cbx4-deficient cells, regardless of the polarized phenotypes (Supplemental Fig. 3B,C). In addition, memory-like shCbx4 CD8 T cells revealed significant reduction of IFNγ^{+}TNF^{+} population frequency and IL-2 expression upon PMA/Ionomycin stimulation (Supplemental Fig. 3D–J). In summary, deficiency in Cbx4 leads to increased expression of memory-associated markers in CTLs and a concomitant reduction in effector function, both *in vivo* and *in vitro*.

Repression of memory phenotype by Cbx4 is primarily dependent on SIM1–2 domains

We next investigated the mechanism through which Cbx4 impacts CTL differentiation. As Cbx4 protein can mediate PcG-dependent repression and function in parallel as an E3 ligase enzyme (11–14), we enforced the expression, in either wild-type or Cbx4-deficient P14 cells, of wild type or mutant Cbx4 cDNAs lacking key functional domains: (1) ΔChromo, with an amino acid substitution at chromodomain that prevents its binding to H3K27me3; (2) ΔSIM1–2, that lacks both SUMO-interacting motifs (Fig. 3A).

Cbx4 KO CD8 T cells activated and differentiated *in vitro* to a memory-like phenotype displayed accentuated expression of CD62L and CD127 during cell culture, and complementation of Cbx4-deficient cells with wildtype Cbx4 counteracted this phenotype (Fig. 3B,C). Overexpression of ΔChromo mutant reproduces to a lesser degree the effect seen upon wild-type isoform overexpression, indicating that the Cbx4 H3K27me3 binding function has a partial contribution to its role in the commitment to effector cell differentiation. On the other hand, deletion of SIM sequences not only reversed wild-type isoform Cbx4 overexpression impact but also induced the opposite effect, promoting memory phenotype in both P14 WT and P14 Cbx4 T KO cells. Overexpression of ΔSIM1–

2 mutant in P14 WT cells raised the frequency of $CD62L^{+}CD127^{+}$ population to levels observed in Mock-transduced P14 Cbx4 KO cells, suggesting that overexpression of SIM-deficient Cbx4 isoform might be competing with endogenous Cbx4 function, and potentially acting as a dominant negative version of the protein (Fig. 3.C). The same patterns were observed for the expression of memory-related markers CD62L and CD127 at protein (Supplemental Fig. 4A,B) and transcriptional levels (Supplemental Fig. 4C,D). Expression of CD25, a marker related to the effector phenotype, corroborated these data, showing an increase upon wild-type Cbx4 overexpression while ΔChromo mutant overexpression partially reproduced wild type Cbx4 effect and ΔSIM1–2 mutant reversed the effect (Supplemental Fig. 4E). A similar pattern has been observed for CXCR3, consistent with the fact that its expression favors the development of short-lived effector cells (Supplemental Fig. 4F) (32).

The observation that both chromodomain and SIM domains are required for effector CTL differentiation is consistent with the fact that it has been shown in mouse embryonic fibroblast that conjugation of SUMO at the Cbx4 SIM domain is essential for recruitment of cPRC1 to H3K27me3 in genome *loci* and control of PRC1 repressive activity (33). Moreover, it was reported that Cbx4-mediated SUMOylation of Ezh2 promoted its recruitment and enhanced Ezh2 methyltransferase activity, demonstrating that Cbx4 can regulate PRC2 (34). Further studies investigating Cbx4 SUMOylation, Cbx4 SUMO E3 ligase function targets, and partnerships with other PcG proteins (especially Ezh2) in CTLs are needed to fully define the role of Cbx4 in CTL differentiation. In addition, examination of the potential interaction of Cbx4 with H3K27me3 residues through the chromodomain is needed to understand how this protein controls Polycomb-mediated repressive mechanisms during CTL differentiation.

Taken together, our data demonstrates that Cbx4, a Polycomb-group protein, participates in the control of CTL differentiation and both Cbx4 SIM1–2 domains and chromodomain are required for the repression of memory phenotype, however SIM1–2 might play a more dominant role in this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points:

(I) The PcG protein Cbx4 favors CTL differentiation to effector phenotype; (II) Cbx4 deficiency alters CTL differentiation, increasing memory formation; (III) Cbx4 drives effector formation mainly through its SIM motifs.

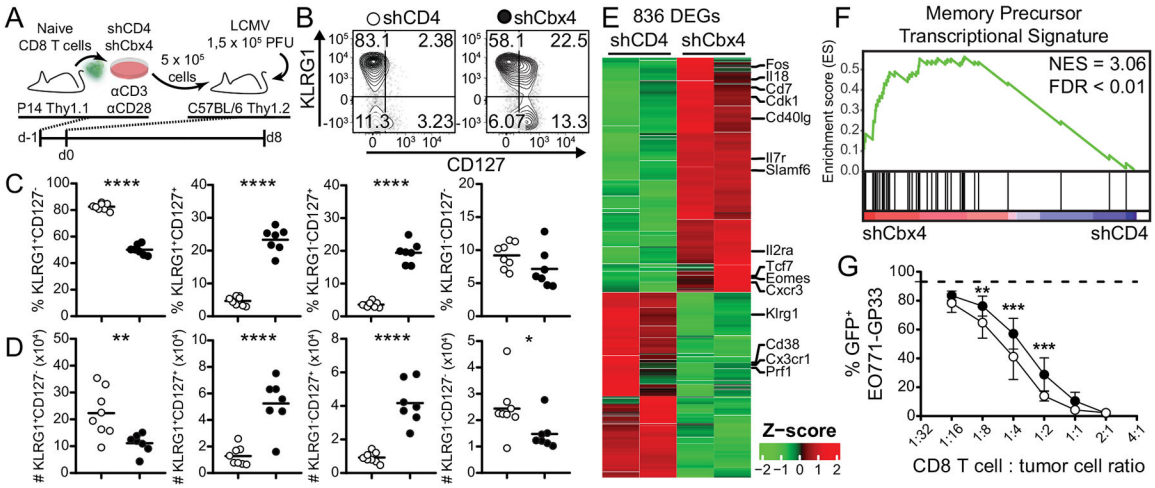

Figure 1. Cbx4 deficiency skews CTL differentiation to a memory phenotype.

(A) *In vitro*-activated P14 Thy1.1$^+$ cells transduced with retrovirus expressing control shRNA (shCD4, white) or shRNA targeting Cbx4 (shCbx4, black) were transferred to congenic receptor mice subsequently infected with LCMV and (B) mice spleen cells were analyzed by flow cytometry for KLRG1 and CD127 expression in Ametrine$^+$ Thy1.1$^+$ CD8$^+$ T cells at 8 dpi or Ametrine$^+$ were sorted for RNA-Seq. Summary of (C) frequency and (D) total cell numbers of populations analyzed in KLRG1 x CD127 gate. (E) RNA-Seq results showed 836 differentially expressed genes (DEGs) that were visualized in a heatmap of Z-score values clustered by hierarchical clustering. (F) DEGs were tested by GSEA for enrichment of transcription signature from memory precursor (KLRG1^{lo}CD127^{hi}) population from day 8 of acute LCMV infection (24). (G) P14 shCD4 and shCbx4 cells were also assessed for *in vitro* specific cytotoxicity (dashed line indicates baseline tumor cell survival). Representative contour plots for KLRG1 and CD127 expression are shown (B). Data are representative of two independent experiments (n ≥ 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by unpaired two-tailed Student *t* test (C, D) or two-way ANOVA with Sidak's test for multiple comparisons (G).

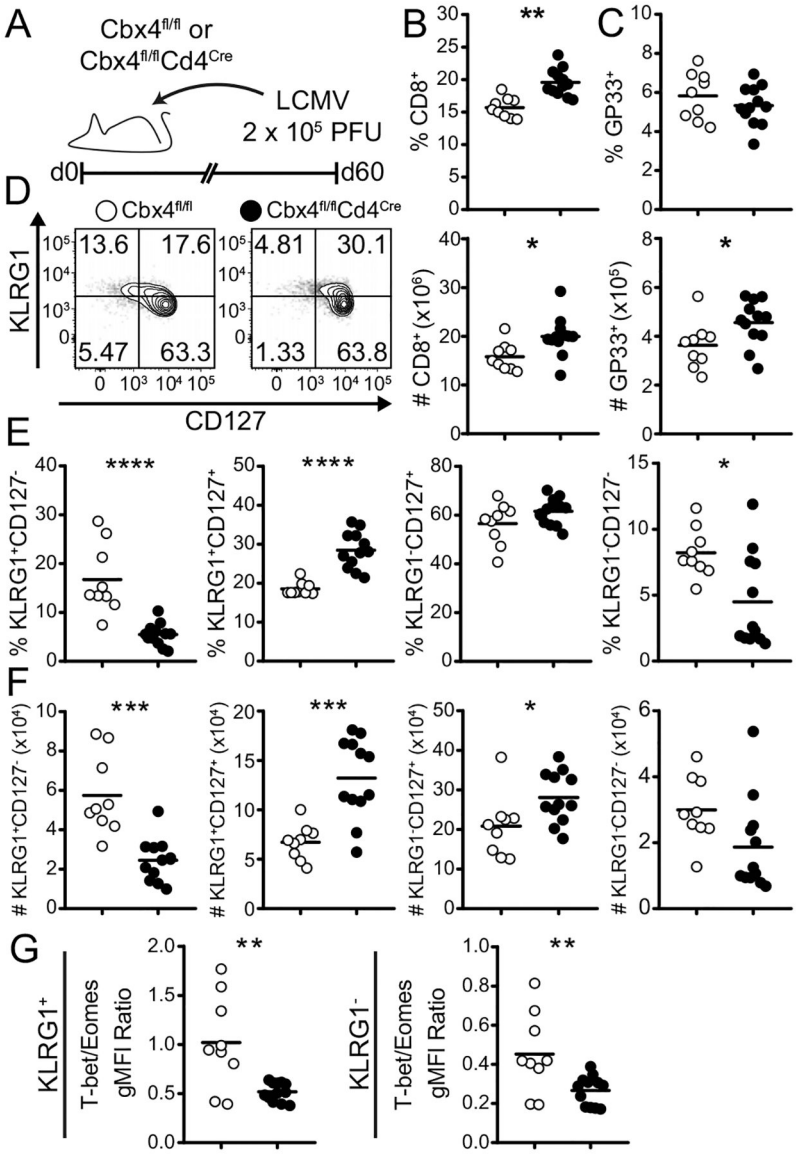

Figure 2. Cbx4 deficiency increases memory CTL formation.

(A) Polyclonal Cbx4$^{fl/fl}$ (white) or Cbx4$^{fl/fl}$Cd4^{Cre} (black) mice were infected with acute LCMV infection and their splenocytes were analyzed at 60 dpi. (B) Frequency and cell numbers of total CD8 T cell population or (C) LCMV-specific CD8 T cells were measured by flow cytometry. (D) The expression of KLRG1 and CD127 analyzed by flow cytometry at 60 dpi, and (E) frequency and (F) cell numbers of each subpopulation were calculated. (G) The expression of T-bet and Eomes was measured by gMFI in flow cytometry and T-bet/Eomes ratio was calculated using those values in both KLRG1^{+} and KLRG1^{-} populations. Representative contour plots for KLRG1 and CD127 expression are shown (D). Data are representative of two independent experiments (n ≥ 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by unpaired two-tailed Student *t* test (B, C, E-G).

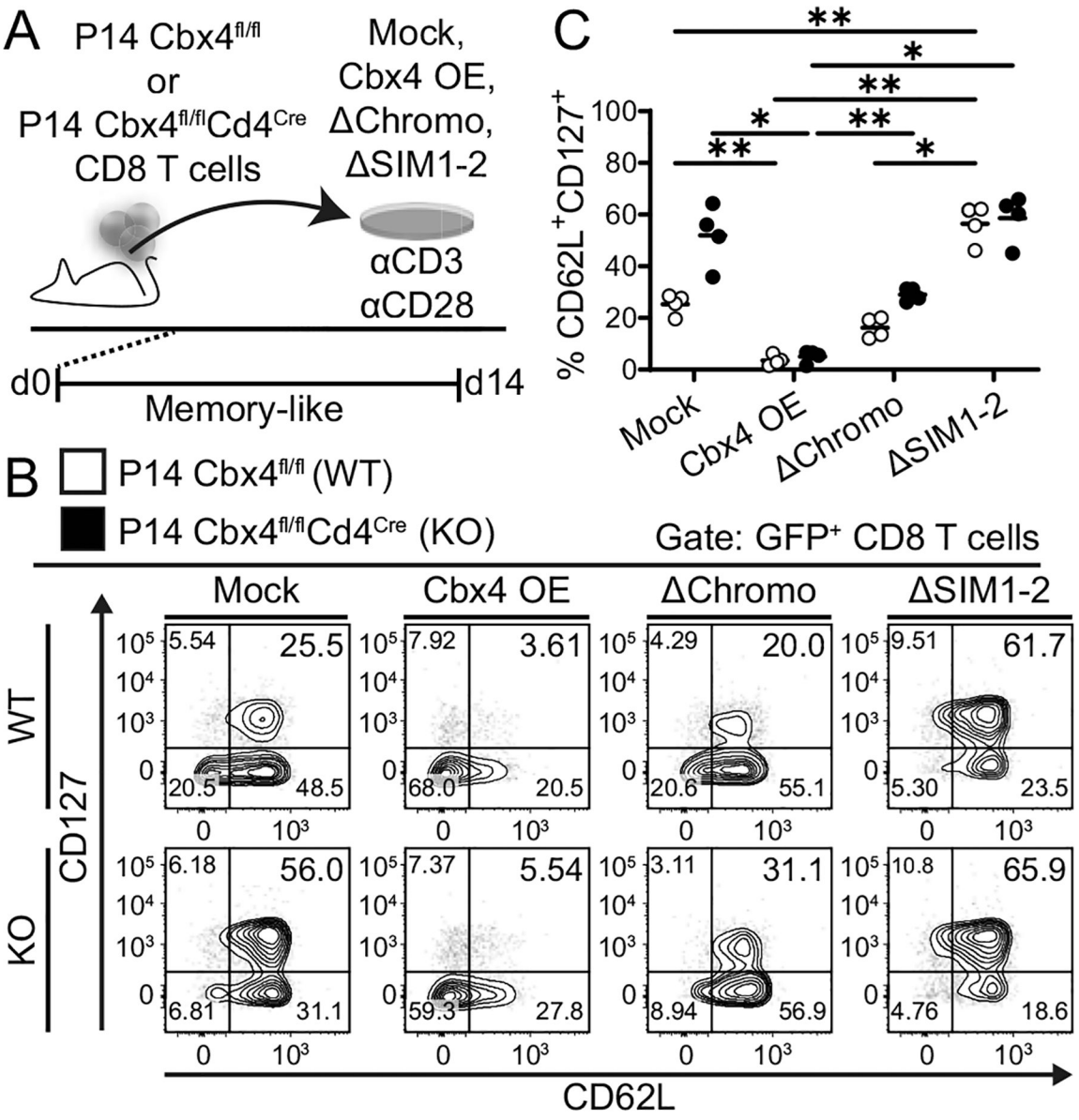

Figure 3. Differential requirement of Chromo and SIM1–2 Cbx4 domains for CTL differentiation.

(A) *In vitro*-activated P14 Thy1.1^{+} WT (white) or P14 Thy1.1^{+} Cbx4$^{fl/fl}$CD4^{Cre} (black) cells were transduced with either of Mock (control), wild type Cbx4 mRNA, Cbx4 ΔChromo mutant or Cbx4 ΔSIM1–2 mutant, and then polarized *in vitro* to memory-like phenotype (10U/mL of rhIL-2, 10 ng/mL of rhIL-7 and 10 ng/mL of rhIL-15) until day 14 of culture. (B, C) The frequency of CD8 T cells expressing both CD62L and CD127 was analyzed by flow cytometry. Representative contour plots for CD62L and CD127 cytometry are shown (B). Data are representative of four independent experiments. *$p < 0.05$, **$p < 0.01$ by two-way ANOVA with Tukey's test for multiple comparisons.