CXXC finger protein 1 is critical for T-cell intrathymic development through regulating H3K4 trimethylation

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T-cell development in the thymus is largely controlled by an epigenetic program, involving in both DNA methylation and histone modifications. Previous studies have identified Cxxc1 as a regulator of both cytosine methylation and histone 3 lysine 4 trimethylation (H3K4me3). However, it is unknown whether Cxxc1 plays a role in thymocyte development. Here we show that T-cell development in the thymus is severely impaired in Cxxc1-deficient mice. Furthermore, we identify genome-wide Cxxc1-binding sites and H3K4me3 modification sites in wild-type and Cxxc1-deficient thymocytes. Our results demonstrate that Cxxc1 directly controls the expression of key genes important for thymocyte survival such as RORγt and for T-cell receptor signalling including Zap70 and CD8, through maintaining the appropriate H3K4me3 on their promoters. Importantly, we show that RORγt, a direct target of Cxxc1, can rescue the survival defects in Cxxc1-deficient thymocytes. Our data strongly support a critical role of Cxxc1 in thymocyte development.
lymphocyte development in the thymus is tightly regulated by a network controlled by several key transcription factors. Acquirement and expression of T-cell receptor (TCR) also play essential roles in generation of mature CD4+ or CD8+ T cells in the thymus. CD4 and CD8 double-negative T cells (DN) harbouring productive rearranged Tcrb genes start to express both CD4 and CD8 and progress to double-positive stage (DP), which composes the majority of thymocytes. DP thymocytes rearrange the Tcrb locus and proceed to positive and negative selection based on their affinity to major histocompatibility complex (MHC) class I and MHC class II molecules. Thymocytes expressing mature β2TcRs and having appropriate binding affinity to MHC molecules can develop into CD4−CD8+ single-positive (CD4+ SP) helper or CD4−CD8+ single-positive (CD8+ SP) cytotoxic thymocytes.

Recent studies clarified that epigenetic modifications such as DNA methylation and histone acetylations/methylations are crucial in controlling the commitment and maintenance of CD4 and CD8 lineages in the thymus. DNA methylation, which generally suppresses gene expression, establishes heritable epigenetic silencing of both CD4 and CD8 loci only in lineage-committed thymocytes, while H3K9,14ac and H3K4me2, which often mark transcriptionally active promoters or enhancers, promote stage-specific gene activation during DN–DP–SP step-wise development, with the most marked histone modification changes between DN and DP transition.

In mammal cells, DNA methyltransferase 1 (DNMT1) maintains heritable DNA methylation patterns through every cellular DNA replication cycle. A huge catalogue of histone modifications in various organisms (including the lysine methylation/acetylation/ubiquitination/sumoylation, arginine methylation/citrulination and serine/threonine/tyrosine phosphorylation) are maintained by numerous epigenetic enzyme complexes. These cellular machineries responsible for epigenetic regulation also contribute to the regulation of thymocyte development. Histone deacetylases (HDACs) are required for positive and negative selection or survival of thymocytes. Histone acetylation by a subunit of the Hbo1 histone acetyltransferase complex Brd1 is also crucial in activating CD8 expression in thymocytes. In addition, Manna et al. show that H3K27me3 demethylase Jmjd3 and Utx are required for CD4+ T-cell differentiation in the thymus through directly regulating the expression of sphingosine-phosphate receptor S1pr1. These studies suggest that epigenetic enzymes are important to coordinate thymocyte development. However, it is unclear how many of these epigenetic modifications direct the early T-cell differentiation.

CXXC finger protein 1 (Cxxc1) is defined as an unmethylated CpG-binding protein regulating DNA methylation through its DNA-binding domain. It is also a component of the H3K4 methyltransferase Setd1 complex and targets Setd1 and H3K4me3 to most CpG islands (CpGI) through its Setd1-interacting domain. Deficiency of the Cxxc1 in mice leads to peri-implantation lethality. Its deletion in embryonic stem (ES) cells and human myeloid cell lines blocks their differentiation in vitro. Moreover, its deletion in haematopoietic cells fails the haematopoiesis in vivo. These data all suggest a pivotal role of Cxxc1 in development. Carlone et al. show that loss of Cxxc1 in ES cells reduces heterochromatin by decreasing DNMT1 activity and global cytosine methylation, in addition to inducing global H3K4me2 and H3K4me3 (ref. 15). In contrast, Clouaire et al. shows that Cxxc1 is required for H3K4me3 and deficiency of Cxxc1 in ES cells leads to marked loss of H3K4me3 at expressed CpG-associated genes, with minimal consequences for transcription.

Furthermore, using chromatin immunoprecipitation sequencing (ChIP-seq) technology, Thomson et al. identify genome-wide Cxxc1-binding sites in a mixture of mouse brain tissues and find a notable concordance of Cxxc1 binding with H3K4me3 and non-methylated CpGI. Taken together, Cxxc1 may directly regulate gene expression through coordinating DNA methylation and H3K4me3 in an environment- and cell-type-specific manner.

To understand the role of Cxxc1 in intrathymic T-cell development, we generated Cxxc1 conditional knockout mouse strains and found that the intrathymic T-cell development was blocked severely in Cxxc1-deficient mice, due to impaired survival and TCR signalling in DP thymocytes. Using ChIP-seq technology, we identified genome-wide Cxxc1-binding sites and H3K4me3 modification changes in Cxxc1-deficient thymocytes. And we found that Cxxc1 directly regulated several key molecules in DP thymocytes, through introducing H3K4me3 modification on these genes’ promoter regions. Moreover, Cxxc1’s direct target RORγt could rescue the survival defects in Cxxc1-deficient thymocytes. Our data strongly support that Cxxc1-mediated epigenetic program is required for thymocyte development.

**Results**

**Cxxc1 is required for T-cell development.** Cxxc1 is a key epigenetic regulator and essential to embryogenesis. We also found that Cxxc1 was highly expressed in T-cell precursors in the thymus, especially at DN3 stage (Supplementary Fig. 1a,b). Therefore, we hypothesize that it may also be important for the development of T-cell thymic development. Since the Cxxc1-null mouse is embryonic lethal, we generated the conditional Cxxc1 allele (Cxxc1fl/fl), in which the exons 4 and 5 were flanked by loxp sites (Supplementary Fig. 2a). Intercrossing between Cxxc1+/− mice (generated by Cxxc1fl/fl crossing with PGKCre, see Methods) did not generate the Cxxc1 deleted offspring, which verified the lethality of Cxxc1-null mouse (Supplementary Fig. 2b). To understand its role in thymocyte development, the Cxxc1fl/fl mice (wild-type (WT) mice) were crossed with hCD2cre mice, leading to the deletion of functional CXXC1 protein in early stage of developing thymocytes, as well as other immune cells. To tract Cxxc1 deletion during T-cell development in the thymus, genomic DNA from sorted DN1, DN2, DN3, DN4 and DP subpopulations in WT and Cxxc1-deficient mice was tested for the expression of floxed and deleted Cxxc1 alleles. We found that the deleted band appeared at DN1 and the floxed band disappeared at DP stage (Supplementary Fig. 2c). Consistent with the deletion of DNA, the Cxxc1 messenger RNA (mRNA) was detectable in DN3 cells at the level of ~20% of its control, and was almost undetectable in DP cells in Cxxc1-deficient mice (Supplementary Fig. 2d). The deletion of CXXC1 protein in DP cells was further confirmed by western blot (Supplementary Fig. 2e). Taken together, these data illustrate that the complete deletion of Cxxc1 occurred at DP stage in our Cxxc1-deficient mice.

The size of thymus in Cxxc1-deficient mice was significantly reduced as compared with WT mice. Consistently, the thymic cellularity in Cxxc1-deficient mice was as low as ~1% of its counterpart in WT mice, although the total number of DN cells was not significantly changed (Fig. 1a). The majority of thymocyte loss came from the drastic decrease in the numbers of DP, CD4 SP and CD8 SP thymocytes (Fig. 1a). In fact, most of thymocytes in Cxxc1-deficient mice were either DP (33%) or DN (59.2%) cells, while both CD4 and CD8 SP cells were almost completely abolished (Fig. 1b). Further analysis of the DN subpopulations showed an increase of CD25+ DN3 cells and a decrease of DN4 cells in Cxxc1-deficient mice comparing with the WT mice (Fig. 1c), despite an incomplete deletion of Cxxc1 at DN3 stage (Supplementary Fig. 2c,d), suggesting that the transition from DN3 to DN4 might be compromised due to partial reduction of Cxxc1 expression. Subsequently, the total
number and composition of peripheral T cells were also remarkably changed in Cxxc1-deficient mice. CD4⁺ and CD8⁺ splenocytes were mostly depleted (Fig. 1d,e) and the a few survived CD4⁺ cells acquired effector/memory phenotype with elevated expression of CD44 and decline expression of CD62L (Fig. 1f), reflecting the developmental defect caused by Cxxc1 deficiency in the thymus.

To further confirm the function of Cxxc1 in thymocyte development, we crossed the Cxxc1fl/fl with LckCre mice, in which the deletion of Cxxc1 started at DN3 stage. LckCre-mediated deletion of Cxxc1 led to the similar thymocyte and peripheral T cell developing defects as seen in Cxxc1fl/fl/LckCre mice (Supplementary Fig. 3a–e), although less severe defects were found in Cxxc1fl/fl/LckCre due to the late expression of Cre recombinase and possible leaky deletion of the Cxxc1 gene by LckCre mice. On the other hand, existence of the substantial CD4⁺ and CD8⁺ SP populations in Cxxc1fl/fl/LckCre might also be due to an incomplete deletion of Cxxc1 gene (Supplementary Fig. 3f,g). Furthermore, we also used the Cxxc1fl/fl/Crest2+ and OP9-DL1 cell system to test the transition of DN to DP stage in vitro. The DN3 cells were sorted from Cxxc1fl/fl/Crest2+ and co-cultured with OP9-DL1 cells in presence or absence of 4-hydroxytamoxifen (4-HT; which initiated the access of Cre recombinase in nucleus and led to the deletion of Cxxc1 in vitro, shown in Supplementary Fig. 3h,i) for 5 days. As shown in Supplementary Fig. 3j,k, Cxxc1 deletion in vitro also resulted in the reduction of DN–DP transition and depletion of SP cells. Taken together, our data indicate that Cxxc1 is required for T-cell development. Given that the complete deletion of Cxxc1 occurred at DP stage and a severe disruption of DP thymocyte development was found in the Cxxc1-deficient mice, we therefore focused our further study on DP thymocytes.

Cxxc1 regulates T-cell development with its H3K4me3 function. Previous reports have demonstrated that Cxxc1 is a component of Set1 complex. We also found that Cxxc1 indeed interacted with SETD1 in DP thymocytes (Supplementary Fig. 4a). Cxxc1 interacts with unmethylated CpG DNA through its CXXC domain (N terminal). Three regions of Cxxc1 between amino acid 169 and 493 are found to mediate its interaction with DNMT1 and stabilize DNMT1 protein for regulating DNA methylation. A point mutation (C169A) interrupting DNA-binding activity of Cxxc1 ablates its DNA-binding ability in ES cells. On the other hand, Cxxc1 interacts with Setd1 H3K4 methyltransferase complex through SID domain (C terminal) to regulate histone methylation. A point mutation (C375A) interrupting the Setd1-interacting activity of Cxxc1 ablates its function in H3K4 methylation regulation in ES cells, but not its interaction with DNMT1 (refs 17,27). Using the in vitro T-cell development system and different vectors expressing mutated Cxxc1 proteins in Cxxc1-deficient thymocytes, we tried to test that functional domains within Cxxc1 were necessary for its role in T-cell development. We first generated vectors expressing the full-length Cxxc1 gene (Cxxc1-IRSE-GFP, with Cxxc1 1–656 aa), N-terminal fragment of Cxxc1 (Cxxc1-1–367-IRSE-GFP) that has DNA-binding activity, C-terminal fragment of Cxxc1 (361–656-IRSE-GFP) that interacts with the Setd1 complex, full-length Cxxc1 with the point mutation C169A (C169A-IRSE-GFP) in which the DNA-binding activity was abolished and full-length Cxxc1 with the point mutation C375A (C375A-IRSE-GFP) in which the Setd1-interacting activity was abolished, respectively (Supplementary Fig. 4b). Importantly, expression of Cxxc1 by the two vectors with point mutations was comparable to the one without point mutation in DP thymocytes (Supplementary Fig. 4c), which was consistent with the previous report in ES cells.

Ectopic expression of full-length Cxxc1 in WT DN cells had trivial effects on thymocyte development (Supplementary Fig. 4d), but it could fully rescue the T-cell developmental defects in Cxxc1-deficient cells (Fig. 2a). Intriguingly, the C-terminal fragment of Cxxc1 gene (361–656-IRSE-GFP) rather than the N-terminal fragment of Cxxc1 gene (1–367-IRSE-GFP) was able...
to rescue T-cell development in Cxxc1-deficient thymocytes (Fig. 2a). Similarly, the C375A muted full-length protein (C375A-IRES-GFP) with defect in interacting with Setd1 was unable to rescue impaired T-cell development in Cxxc1-deficient thymocytes, while the C169A muted full-length protein (C169A-IRES-GFP) was still able to convert DN to DP cells (Fig. 2a). These data strongly indicated that the Setd1 interacting, but not the DNA-binding domain in Cxxc1 is crucial for thymocyte development.

Cxxc1 forms a complex with Setd1 to promote H3K4 methylation and transcription activation. Therefore, we next examined whether H3K4 trimethylation was affected in Cxxc1-deficient thymus. The crude histone extracted from WT or Cxxc1-deficient DP thymocytes were subjected to detection of total H3K4me3 levels by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 2b,c, the total H3K4me3 level and SETD1 expression in Cxxc1-deficient cells were remarkably lower than that in WT DP thymocytes, suggesting an important role of Cxxc1 in maintaining the H3K4me3 level in DP thymocytes. However, we did not find any changes in overall DNA methylation (Fig. 2d) or DNMT1 protein levels (Supplementary Fig. 4e) in Cxxc1-deficient DP thymocytes. These data demonstrated that Cxxc1 may function through regulating H3K4me3 rather than DNA methylation in DP thymocytes.

**Figure 2** | Setd1-interacting domain of Cxxc1 is required for intrathymic T-cell development. (a) DN3 cells from Cxxc1-deficient mice were sorted and transfected with indicated retrovirus. GFP⁺ thymocytes were analysed by fluorescence-activated cell sorting after co-culturing with OP9-DL1 for 5 days. Data comes from four independently sorted DN3 samples (each from three mice). (b) Detection of total H3K4me3. Crude histone protein were extracted from DP thymocytes sorted from control or Cxxc1-deficient mice using acid extraction, the protein was subjected to H3K4me3 ELISA analysis. Data comes from three separately sorted DP cell sets (each from two mice). The statistical significance was calculated by unpaired t-test (two tailed). **P<0.01. Error bars indicate s.d. (c) Detection of Setd1 protein level. DP thymocytes from Cxxc1-deficient and control mice were used for western blot analysis. Number under lanes indicate densitometry of Setd1 (relative to Actb at same lane, below). Data comes from three separately sorted DP cell sets (each from three mice). (d) Dot blotting showed total 5meC levels in sequentially diluted genomic DNA from indicated DP thymocytes. Data are representative of three separate experiments (each from two mice).
coordinated the transcriptional regulation of Cxxc1 targets (Supplementary Fig. 5a).

We found that Cxxc1-dependent DNA methylation regulation might be dispensable during thymocyte differentiation (Fig. 2a,d). We here examined again whether Cxxc1-binding sites were associated with CpGII in DP thymocytes. As shown in Fig. 3c, Cxxc1 binding was enriched at the centre of CpGII. However, only very few Cxxc1-binding sites were found colocalized with CpGII in DP thymocytes. As shown in Fig. 3c, only 6% of Cxxc1-binding sites were found colocalized with CpGII in DP cells (Fig. 3d). This is significantly different with previous finding that 93% of Cxxc1-binding sites overlap with CpGII in brain tissues (Supplementary Fig. 5b), indicating a tissue-specific binding profile of Cxxc1 may decide its primary function in a cell-type-dependent manner.

Our results above showed that Cxxc1 was crucial for the maintenance of overall H3K4me3 levels in DP thymocytes. We therefore carried out ChIP-seq analysis to map the genome-wide H3K4me3 modifications in DP cells. We identified 2,083 genes at which loci the Cxxc1 directly bound within extended gene bodies (up to ~10 kb from TSS). Among these genes, 387 genes had at least twofold expression changes in Cxxc1-deficient DP cells as compared with WT cells (Fig. 3h; Supplementary Dataset 1), and the expression of 85% of them was downregulated due to the deficiency of Cxxc1 and the reduction of H3K4me3 on their promoters (Supplementary Fig. 5c). A gene ontology analysis of these 387 genes using DAVID revealed that the genes responsible for chromatin modification and regulation of thymic T-cell selection and differentiation, such as Cd8a, Lck, Zap70 and Rorc, were the major targets of Cxxc1 in DP cells (Supplementary Table 2). These data indicate that Cxxc1 controls T-cell development through epigenetically regulating the expression of key genes involved in intrathymic T-cell differentiation.

**Cxxc1 epigenetically regulates key genes in T-cell development.**

TCR signalling is vital for T-cell selection in the thymus and survival signal is also required for T cells to pass the positive and negative selection stage of DP cells to become mature SP T cells. ChIP-seq data showed that Cxxc1 bound to upstream or gene body enhancers of Cd8a, Zap70, Lck and Rorc gene loci, which was associated with a significant decrease in the H3K4me3 modifications on these genes’ promoter regions in Cxxc1-deficient DP cells (Fig. 4a). Direct binding of Cxxc1 in WT cells and reduction in H3K4me3 in Cxxc1-deficient cells on these gene loci were the majority of genes showed reductions in their expression in Cxxc1-deficient DP thymocytes (Fig. 3g). We identified 2,083 genes at which loci the Cxxc1 directly bound within extended gene bodies (up to ~10 kb from TSS). Among these genes, 387 genes had at least twofold expression changes in Cxxc1-deficient DP cells as compared with WT cells (Fig. 3h; Supplementary Dataset 1), and the expression of 85% of them was downregulated due to the deficiency of Cxxc1 and the reduction of H3K4me3 on their promoters (Supplementary Fig. 5c). A gene ontology analysis of these 387 genes using DAVID revealed that the genes responsible for chromatin modification and regulation of thymic T-cell selection and differentiation, such as Cd8a, Lck, Zap70 and Rorc, were the major targets of Cxxc1 in DP cells (Supplementary Table 2). These data indicate that Cxxc1 controls T-cell development through epigenetically regulating the expression of key genes involved in intrathymic T-cell differentiation.

**Figure 3 | Genome-wide maps of Cxxc1 binding and H3K4me3 modification in DP thymocytes.** (a) Distribution of Cxxc1-binding peaks in DP thymocytes, and distribution of the genetic features across the whole-mouse genome (mm9). (b) Distribution of Cxxc1-binding peaks across extended gene bodies. The tag density of Cxxc1 binding was calculated on gene bodies (between transcription start site, TSS and transcription termination site, TTS), as well as 3-kb upstream of TSS and 3-kb downstream of TTS regions of all RefSeq (mm9) genes. Uniquely, mapped tags were summarized in 100-bp windows for promoter regions and in 5% of gene body sequences. All window tag counts were normalized by the total number of bases in the windows and the total read number of the given sample. (c) Enrichment of Cxxc1-binding peaks on CpG islands (CpGII). The tag density of Cxxc1 binding was calculated on CpG islands and 5-kb flanking regions. (d) The number of genomic regions where CpGII and Cxxc1-binding sites were colocalized. (e) Distribution of H3K4me3 modification around 5-kb regions flanking TSS of all RefSeq (mm9) genes in WT and Cxxc1-deficient DP thymocytes. (f) Overlapped regions between Cxxc1-binding sites and H3K4me3 sites in WT and Cxxc1-deficient DP thymocytes. (g) Scatter plot of expression of all genes in WT and Cxxc1-deficient DP thymocytes, detected by RNA-seq analysis. (h) The number of genes with both Cxxc1-binding sites on their extended gene bodies (10-kb upstream of TSS plus gene body) and gene expression changes (over twofolds) between WT and Cxxc1-deficient DP thymocytes.
confirmed by ChIP–PCR assays (Fig. 4b,d). Importantly, we also found that the binding of SETD1 and CXXC1 were colocalized to the same promoter regions on Cd8a, Zap70, Lck and Rorc gene loci in WT DP thymocytes, which is significantly reduced in CXXC1-deficient DP thymocytes (Fig. 4b,c). In addition, we found that both H3K9ac and H3K27ac (two histone modifications associated with active chromosome), but not H3K27me3 and H3K9me2 (two histone modifications associated with inactivated chromosome) associated with these four gene loci (Fig. 4e,f; Supplementary Fig. 6a,b). Importantly, these active histone modifications on the four gene loci were all found lost in CXXC1-deficient DP thymocytes (Fig. 4d–f), while on the loci that CXXC1 and SETD1 are recruited to, the histone modifications were unchanged (Supplementary Fig. 6a–f).

Our RNA-seq data showed that deletion of CXXC1 in DP cells led to 6.9-, 2.8-, 4.2- and 3.7-fold reduction in the expression of Cd8a, Zap70, Lck and Rorc, respectively (Supplementary Dataset 1). These expression changes were verified by real-time quantitative PCR (qPCR) assays on biological replicated samples (Fig. 4g). Importantly, we were able to detect significant reduction of CD8α, CD8β, Zap70, and RORγt proteins in CXXC1-deficient DP cells (Supplementary Fig. 6g). In addition, we have performed qPCR, methylated DNA immunoprecipitation (MeDIP)–qPCR and ChIP experiments across different thymocyte developmental stages to examine the gene expression, methylation status, H3K4me3 modification, as well as CXXC1- and SETD1-binding status on the promoters of several target genes, respectively. We found a significant association between gradual acquisition of gene expression and reduction in DNA methylation, increase in H4K4me3 level, and binding of CXXC1 and SETD1 on gene promoters (Supplementary Fig. 7a–e). These results suggested that CXXC1-dependent epigenetic regulatory program may control genes important for DP survival and TCR signalling during thymocyte development.

CXXC1 is required for TCR signalling in DP thymocytes. The finding that CXXC1 controls the expression of key players involved in TCR activation led us to hypothesize that the developmental defects found in CXXC1-deficient thymocytes might be partially due to impaired TCR signalling. In addition to the reduction of CD8 and Zap70 expression, we found that the expression of other TCR activation markers such as CD69, TCRβ and CD5 was significantly reduced in CXXC1-deficient DP thymocytes (Fig. 5a). Furthermore, TCR activation-induced phosphorylation of both ERK and JNK proteins was also attenuated in CXXC1-deficient DP thymocytes (Fig. 5a). Despite its RNA reduction in CXXC1-deficient DP thymocytes (Fig. 4h), the protein level of lymphocyte protein tyrosine kinase (LCK) detected by western blot was not changed in spite of its RNA reduction in CXXC1-deficient DP cells (Supplementary Fig. 6g). In addition, we have performed qPCR, methylated DNA immunoprecipitation (MeDIP)–qPCR and ChIP experiments across different thymocyte developmental stages to examine the gene expression, methylation status, H3K4me3 modification, as well as CXXC1- and SETD1-binding status on the promoters of several target genes, respectively. We found a significant association between gradual acquisition of gene expression and reduction in DNA methylation, increase in H4K4me3 level, and binding of CXXC1 and SETD1 on gene promoters (Supplementary Fig. 7a–e). These results suggested that CXXC1-dependent epigenetic regulatory program may control genes important for DP survival and TCR signalling during thymocyte development.
together, our data demonstrated that Cxxc1 directly or indirectly controls the expression and activation of molecules involved in TCR signalling in DP thymocytes.

To investigate whether Cxxc1 regulates the positive selection of thymic T cells, we isolated DP thymocytes from WT- and Cxxc1-deficient mice, and stimulated them with anti-CD2 and anti-TCRβ for 20 h, followed by a recovery phase of 44 h. The WT DP thymocytes downregulated their CD4 and CD8 expression, and passed through CD4+CD8dim transitional stage (Fig. 5b). However, the Cxxc1-deficient DP thymocytes failed to generate the CD4+CD8dim population (Fig. 5b). Our results demonstrated that deficiency of Cxxc1 led to defects in positive selection of thymic T cells.

The low expression of TCRβ in Cxxc1-deficient DP thymocytes raised a possibility that Cxxc1 might regulate TCR expression during T-cell development, which may account for the impaired TCR signalling. Hence, we crossed MHC class I-restricted or MHC class II-restricted TCR transgenic (TG) mice (OTI or OTII) with Cxxc1-deficient mice to test whether exogenously expressed TCR would rescue the TCR signalling defects found in Cxxc1-deficient mice. As shown in Fig. 5c,d, the frequency of DP and SP cells, as well as the total numbers of thymocytes and CD8 SP cells in OTII-TG-Cxxc1fl/fl hCD2cre mice were still remarkably lower than their WT counterparts; the frequency of Vβ5high thymocytes in OTI-TG-Cxxc1fl/fl hCD2cre or Vβ5+ thymocytes in OTII-TG-Cxxc1fl/fl hCD2cre mice was still significantly lower than their WT counterparts, indicating that productive TCR expression is not able to rescue the TCR signalling defects in Cxxc1-deficient mice. In summary, our results indicated that Cxxc1 is required for TCR signalling transduction through regulating many key molecules mediating TCR activation in DP thymocytes.

Cxxc1 controls DP thymocytes’ survival by regulating RORγt. Rorc, another important Cxxc1 target, encoding the RORγt protein in T cells, functions to maintain DP thymocyte survival through regulating anti-apoptotic gene Bcl-xL32,33. Our data showed a significant decline in the protein levels of RORγt (Fig. 4h) and BCL-XL (Fig. 6a) in Cxxc1-deficient thymocytes. Thus, we speculated that the impaired survival of Cxxc1-deficient DP thymocytes was likely to account for the markedly reduced thymic cellularity. Indeed, the survival of Cxxc1-deficient DP thymocytes was severely diminished (Fig. 6b), exemplified by their compromised ability to survive from X-ray irradiation (Fig. 6c) and ionomycin stimulation (Fig. 6d). Although Bcl2 and
Bcl-xl have reciprocal patterns of expression in developing thymocytes, both of them can inhibit thymocyte death.3,4 We found that the reduced cellularity in Cxxc1-deficient thymus could be partially rescued by the ectopic expression of BCL2 in the hBcl2 transgenic mice (Supplementary Fig. 8b). In addition, the loss of DP and CD8 SP, as well as the survival of DP thymocytes could be reversed by ectopic expression of BCL2 (Supplementary Fig. 8c–e). The remarkable increase of Cxxc1-deficient DP thymocyte apoptosis, indicated by annexin V staining, could also be corrected by the ectopic expressed BCL2 (Supplementary Fig. 8f). These data suggest that the survival defects of Cxxc1-deficient DP thymocytes can be partially rescued by anti-apoptotic genes such as Bcl2.

Then, we asked whether RORγt could reverse the survival defects in Cxxc1-deficient thymocytes using the Cxxc1fl/fl CreERT2+ and OP9-DL1 cell co-culture system. Ectopic expression of RORγt in Cxxc1-sufficient cells (−4-HT) had no significant impact on DP differentiation (Fig. 6e). In contrast, ectopic expression of RORγt remarkably improved the number and frequency of Cxxc1-deficient DP cells (+4-HT) (Fig. 6e,f). Moreover, RORγt reversed the Cxxc1-deletion-induced apoptosis of DP cells (Fig. 6g) and significantly elevated the expression of Bcl-xl in Cxxc1-deficient thymocytes (Fig. 6h). These data strongly support that Cxxc1 controls DP thymocyte survival through regulating RORγt.

Discussion

Epigenetic regulation is an essential mechanism to coordinate intrathymic T-cell development. Here we reported that Cxxc1, an important component of the H3K4 methyltransferase complex Setd1, directly regulated the promoter-associated H3K4me3 and expression of genes that are essential for thymocyte survival and TCR signalling. Deficiency of Cxxc1 led to an almost complete block of transition from DN to DP stage in the thymus, suggesting that Cxxc1 is required for thymocyte development.

Our understanding of how epigenetic enzymes program thymocyte development has been significantly advanced in the past decade. Lee et al.5 has shown that LckCre-mediated deletion of Dmnt1 in the thymus leads to 85% reduction of total thymocytes and over 90% reduction of DP and CD4 SP thymocytes. On the other hand, deletion of the histone methyltransferase Ezh2 and G9a in T cells has minimal impact on thymocyte development.35,36 In addition, deletion of H3K27me3 demethylases Jmd3 and Utx by CD4Cre alone leads to minor changes in thymocyte subpopulations,35 while deletion of a component of Hbo1 histone acetyltransferase Brd1 does not change the total number of thymocytes, but significantly decreases CD8 expression in thymocytes.12 Deletion of Hdac1, Hdac2 or Hdac7 alone has no impact on thymocyte development, whereas only double deletion of both Hdac1 and Hdac2 leads to fivefold reduction in thymocyte cellularity.8,10 In addition, LekCre-mediated deletion of mixed-lineage leukemia 1 (MLL1), the key member of MLL histone methyltransferase family, does not interrupt normal T-cell development in the thymus.37 However, our data showed that the loss of Cxxc1 led to 99% reduction in the thymic cellularity, with very few DP left and almost 100% depletion of SP cells (Fig. 1a). The peripheral CD4+ and CD8+ T cells were also abolished due to inability of thymocyte development (Fig. 1d). These data suggest that Cxxc1 plays a non-redundant indispensable role during T-cell development in the thymus.
Cxxc1 functions through regulating of DNA methylation and/or H3K4me3 in ES cells. It is vital to clarify whether it also plays dual roles in thymocyte development. Our data demonstrated that Cxxc1 controlled T-cell differentiation by modifying the promoter-associated H3K4me3 levels (Figs 2a,b; 3c). However, its role in regulating DNA methylation was not essential for the appropriate thymocyte development (Fig. 2a,d; Supplementary Fig. 4e). Consistently, a recent report also suggests that Cxxc1 deletion has no effect on DNA methylation in haematopoietic stem cells. Therefore, unlike Cxxc1’s role in ES cells, it may only function as an H3K4me3 regulator in haematopoietic cells such as T cells.

Previous study has identified a significant overlap among genome-wide Cxxc1-binding sites, H3K4me3 marks and CpGIs in brain tissues. Considering the mixed nature of multiple cell types in brain tissues, the Cxxc1 binding and H3K4me3 profiles can be treated as a collection of all possible DNA loci associated with Cxxc1 or H3K4me3 modification disregarding the cell type and tissue specificity. However, it is unclear whether Cxxc1 colocalizes with H3K4me3 and/or CpGIs in a single type of cells. Our data indicated that ~6% of Cxxc1-binding sites were colocalized with H3K4me3 and CpGIs in DP thymocytes (Fig. 3d,f). In addition, only 12% of the Cxxc1-binding sites in DP thymocytes were also identified in brain tissues (Supplementary Fig. 5b). These data suggest a profound difference in Cxxc1-mediated epigenetic program between neuronal tissue and immune cells. Similarly, previous studies have also reported the diversity of Cxxc1 functions in multiple types of cells. Therefore, our data illustrates that a cell-type-specific binding profile of Cxxc1 may decide its primary function and targeting Cxxc1 for future epigenetic therapy that may achieve cell-type-specific drug effects with limited complications.

RORγt promotes survival of thymocyte through maintaining the expression of Bcl-xL. In our study, we found Cxxc1 directly bound to Rorc gene locus (Fig. 4a,b) and coordinated histone modifications (including H3K4me3, H3K9ac, and H3K27ac; Fig. 4d–f) to maintain active expression of RORγt in thymocytes (Fig. 4g,h). Deficiency of Cxxc1 led to the loss of Rorc gene expression as well as profound DP thymocyte survival defects, accompanying the reduction in BCL-XL expression (Fig. 6a). Importantly, our data also showed that RORγt partially rescued the survival defects caused by Cxxc1 deficiency, suggesting that Cxxc1 is required for thymocyte survival through regulating RORγt expression (Fig. 6e–g). In DP thymocytes, Wnt–β-catenin–TCF-1 pathway and HeLa E-box-binding factor (HEB) are responsible for maintaining RORγt expression and T-cell survival. However, it is yet to be defined how the Cxxc1 and Wnt signalling pathway work in concert to regulate RORγt expression in DP thymocytes.

Methods. Mice. Mice carrying the floxed Cxxc1 allele were generated by homologous recombination-mediated gene targeting in ES cells of strain 129 at the Shanghai Research Center for Model Organisms. Cxxc1-targeted (Cxxc1) mice were generated by homologous recombination-mediated gene targeting in ES cells of strain 129 (ref. 45), strategy was shown in Supplementary Fig. 2a, and the Cxxc1T mice were crossed with FLPeR mice to delete the Neo cassette and gain the Cxxc1loxP mice. The Cxxc1loxP mice were then backcrossed onto the C57BL/6 background for five generations. Exons 4 and 5 in Cxxc1 were deleted by Cre recombinase and a new stop code was generated at the downstream of exon 3 in Cxxc1 gene. To obtain the Cxxc1loxP mouse, the Cxxc1loxP mice were crossed with the PGKCre mice, which resulted in widespread deletion of Cxxc1 (ref. 46). The Cxxc1loxP is normal and fertile; however, no Cxxc1loxP mice were born from Cxxc1loxP/loxP crossing (Supplementary Fig. 2b). The primers for Cxxc1 detecting the homologous recombination, lox site and deleted band in Cxxc1 locus are listed in Supplementary Table 3.

The FLPeR (JAX: 003946), PGKCre (JAX: 020811), LckCre (JAX: 068889), hCD2Cre (JAX: 080520) mice, the OTI (JAX: 083381) and OTII (JAX: 001419) TCR transgenic mice were from Jackson Laboratory. The CreERT2 mice were gifts from Dr Y.W. He (Duke University Medical Center). The Bcl2 transgenic mice were gifts from Dr J. Adams (The Walter and Eliza Hall Institute of Medical Research). All experiments were performed with 3–4-week-old mice unless specified. All mice were kept in the Zhejiang University Laboratory Animal Center and all animal experimental procedures were approved by the Animal Review Committee at Zhejiang University School of Medicine.

Cell culture and retrovirus transfection. OP9 bone marrow stromal cells expressing the Notochord ligand DL-1 (OP-DL1), provided kindly by Dr J.C. Zúñiga-Pflucker (University of Toronto, Toronto, Canada), were cultured and maintained in α-MEM (Gibco) medium with 10% foetal bovine serum (Gibco). For DN3 cells co-cultures, the sorted DN3 cells were plated onto confluent OP9-DL1 monolayers (70–80% confluent) and cultured with 10 ng ml−1 recombinant murine interleukin-7 (Peprotech) and 5 ng ml−1 Flt3L (Peprotech).

Retrovirus preparation was performed in Plat-E cells. Plat-E cells were transfected with pMX-IRES-GFP containing indicated genes, the medium were replaced with fresh medium after 10 h, and retrovirus supernatant was collected after additional 72 h.

Expression of ectopic proteins in DN3 cells was performed as previously described. In brief, RetroNectin (Takara) coating and washing were carried out according to the manufacturer’s instruction. The retrovirus was added to wells coated with RetroNectin, followed by 4 h incubation at room temperature and removal of the retrovirus. After 24 h co-culture, DN3 cells were directly placed on plate-bound anti-RORγt with RetroNectin and retrovirus. And trypticin OP9-DL1 cells were added to the DN3 cells.

Assay of DP development. Purified thymocytes were cultured in RPMI-1640 medium overnight with plate-bound anti-TCRβ (H57; Biolegend, 10 ng ml−1) and anti-CD2 (RM2-5; BD Biosciences, 10 ng ml−1) antibodies as previously described. Then cells were washed and analysed immediately by flow cytometry (stimulatory culture) or cultured further for 24 h in the same medium (recovery culture).

Apoptosis assay. Purified thymocytes were resuspended in DMEM medium with 10% foetal bovine serum, and cultured under indicated conditions. At the indicated time points, cells were collected and analysed by flow cytometry for viability using annexin fluorescein isothiocyanate (V-FITC) apoptosis kit (Biolegend, 600914).

Flow cytometry and antibodies. The following eBiosciences antibodies were used in our experiments: CD4 (RM4-5, 1 µg ml−1), CD8a (53-6.7, 2.5 µg ml−1), CD8b (H57-17.2, 2.5 µg ml−1), CD25 (PC61, 1 µg ml−1), CD44 (IM7, 1 µg ml−1), CD73 (H57-597, 1 µg ml−1), NK1.1 (PK136, 1 µg ml−1), CD24 (M1/69, 1 µg ml−1), CD44v (GL3, 1 µg ml−1), CD5 (CD5-73.3, 1 µg ml−1), CD69 (H.12F3, 1 µg ml−1), CD62L (MEL-14, 1 µg ml−1), V2z (B20.1, 1 µg ml−1), Vβ5 (MR9-4, 1 µg ml−1), CD11b (M1/70, 1 µg ml−1), CD11c (N418, 1 µg ml−1), CD122 (BM-1, 1 µg ml−1), Gr-1 (RB6-8C5, 1 µg ml−1), Ter119 (TER-119, 1 µg ml−1), CD19 (eB01D3, 1 µg ml−1), Zap70 (E7.2, 2.5 µg ml−1) and RORγt (BD2, 2.5 µg ml−1). Allophycocyanin (APC) annexin V (1:20 dilution) and Brilliant Violet 421 anti-Rabbit IgG (Poly4064, 1:50 dilution) were from BioLegend. Flow cytometry was performed with FACSCalibur and FACSaria II machines (BD Biosciences). Data were analysed by FlowJo software (Tree Star, Inc.). Intracellular staining was processed using IC fixation buffer (eBioscience), Foxp3/transcription factor staining buffer set (eBioscience) was used for RORγt and DNMT1 staining. Cells were sorted by a FACSaria II flow cytometer. DN cells were sorted and analysed by staining thymocyte by the following antibodies, PE-NK1.1, PE-CD11c, PE-CD11b, PE-TER119, PE-TCRβ6, PE-CD122, PE-CD19 and PE-Gr-1 to exclude non-DN cells. And dead cells were excluded by 4,6-diamidino-2-phenylindole staining. DN3 cells (0.3 million) were used for each co-culture with OP9-DL1.

Anti-Bcl-xL (2H12, 1:1,000 dilution), anti-IC3k (ab3885, 1:1,000 dilution), anti-DNMT1 (ab13537, 1:1,000 dilution) and anti-Setd1 (ab70378, 1:1,000 dilution) were gifts from Dr. Guo (Harvard). 4 µg for each IP and ChIP reaction, anti-p-JNK (Thr183/Tyr185; C9, 1:1,000 dilution) were from Cell Signaling Technology. Anti-CXXC1 (H-120, 1:1,200 dilution for western blot; 4 µg for each IP and ChIP reaction), anti-ERK (C-9, 1:1,000 dilution) and anti-p-ERK (E-4, 1:1,000 dilution) were from Santa Cruz Biotechnology.

ChIP and data analysis. ChIP assays were performed according to the manufacturers’ instructions with modifications using the ChIP-IT kit (Active Motif, Carlsbad). The crosslinked chromatin was sonicated in a 4 °C water bath using Bioruptor UCD-200 sonicator to obtain DNA fragments sized between 100 and 500 bp. Chromatin from 2 × 10⁶ cells was used for each ChIP experiment. Antibodies against Cxxc1, H3K4me3 (Active Motif, 4 µg for each ChIP reaction), H3K27me3, H3K9ac and H3K27ac (Abcam, 4 µg for each ChIP reaction) were used. The ChIP qPCR primers were listed in Supplementary Table 4. The immunoprecipitated DNA was purified and subjected to sequencing library preparation using
the KAPA HTP Library Preparation Kit (Kapa Biosystems, USA) according to the manufacturer’s protocol. The DNA libraries were then sequenced with Illumina HiSeq 2500 at the Zhongshan Ophthalmic Center core facility. Sequenced reads of 50-bp were obtained using the CASAVA 1.8.2 package (Illumina). All reads were mapped to the mouse genome mm9 and those uniquely mapped reads were subjected to further peak identification process. SICER, V1.1 was used to identify significant peaks (FDR = 10%). All reads were mapped to the mouse genome mm9 and those uniquely mapped reads were subjected to Partek Genome Suite 6.6 (Partek, USA).

**RNA-seq and data analysis.** Total RNA was extracted from WT and Cxxc1-deficient DP thymocytes with TRIzol reagent (Invitrogen, USA). mRNA-seq libraries were prepared using NEBNext mRNA Library Prep Master Mix Set for Illumina according to the manufacturer’s protocol (NEB, USA). All libraries were then sequenced with Illumina HiSeq 2500 at the Zhongshan Ophthalmic Center core facility. Sequenced reads of 50 bp were obtained using the CASAVA 1.8.2 package (Illumina). All reads were mapped to the mouse genome mm9 and those uniquely mapped reads were subjected to RNA-seq data analysis using Partek Genome Suite 6.6 (Partek, USA).

**Histone H3 methylated Lys ELISA.** The ELISA was performed with the H3K4me3 detection kit from Active Motif (53101). The crude histone protein was prepared using acid extract method. The extracted crude histone protein was subjected to ELISA according to the manufacturer’s instruction.

**Total 5mc detection by dot blotting of genomic DNA.** Genomic DNA was extracted from DP thymocytes (adjusted to the same concentration), then denatured at 99°C for 10 min, followed by immediately incubation on ice. The single-strand DNA was bound to a nitrocellulose membrane, air dried and crosslinked with ultraviolet light. The membrane was subjected to immunoblotting using anti-5mc antibodies (Calbiochem, NA81, 1:1,000 dilution) followed by horseradish peroxidase-conjugated secondary antibodies staining.

**RNA isolation and real-time qPCR.** TRIzol reagent (Invitrogen) was used for total RNA extraction. Real-time qPCR was performed using SYBR Premix Ex TaqTM II on LC480II real-time PCR system (Roche, USA). Results were normalized to GAPDH expression. Primers for qPCR are listed in Supplementary Table 5.

**Plasmid construction.** Recombinant vector encoding murine RORCtet and the constructs with full-length and truncated/mutated Cxxc1 genes was constructed by PCR-based amplification and subcloning into the pMX IRES-GFP vector.

**Methylation DNA immunoprecipitation.** MeDIP assays were performed according to the manufacturer’s instruction (Active Motif 55009). In brief, DNA extracted from indicated populations of WT mice (Zymo Research D3024) was sonicated in a 4°C water bath using Bioruptor UCD-200 sonicator to obtain DNA fragments sized between 100 and 500 bp. Then sonicated DNA was denatured at 95°C for 10 min, followed by incubation on ice. And the IP reaction was performed using anti-5mc antibody. The immunoprecipitated DNA was purified and subjected to qPCR analysis. The primers were listed in Supplementary Table 6.

**Immunoprecipitation and western blot.** A measure of 50 μl protein G beads (Invitrogen) were pre-incubated with antibodies for 4 h. Cells were lysed in Triton X-100 lysis buffer containing 50 mM Tris (pH 7.4), 300 mM NaCl, 0.5% Triton X-100, phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor ‘cocktail’ (Roche). Cell lysates were immunoprecipitated overnight at 4°C with the antibody-conjugated beads. After being washed six times, samples were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) gels (10%) and blotted. For immunoblot analysis, sorted cells were lysed in complete lysis-M buffer (Roche; cat. no. 04719956001), and total protein was subjected to SDS–PAGE, transferred onto nitrocellulose membrane and hybrid-blotted. Densitometry was determined by Quantity One software. Images have been cropped for presentation. The full-size images are presented in the Supplementary Fig. 9.

**Deletion of Cxxc1 in Cxxc1fl/fl/CreERT2 cells.** Sorted DN3 cells from Cxxc1fl/fl CreERT2 mice thymocytes were co-cultured with OP9-DL1 monolayers at presence of 200 nm 4-hydroxamidoxion treatment.

**Statistical analysis.** Statistical analyses were performed using Excel except for RNA-seq and ChIPseq data. Bars in graphs indicate s.d. except where otherwise indicated, comparisons were performed by two-tailed unpaired Student’s t-test. Significance levels (P values) are presented on figures.

**Data availability.** The ChIP-seq and RNA-seq data sets were deposited in gene expression omnibus (GEO), with an accession number of GSE79976, are available via the repository’s data access request procedures.

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