Cbx4 regulates the proliferation of thymic epithelial cells and thymus function

Bo Liu1,2,*, Yuan-Feng Liu1,*, Ya-Rui Du2, Andrei N. Mardaryev4, Wei Yang3, Hui Chen2, Zhi-Mei Xu2, Chen-Qi Xu3, Xiao-Ren Zhang5, Vladimir A. Botchkarev4,6, Yu Zhang1,† and Guo-Liang Xu2,†

SUMMARY
Thymic epithelial cells (TECs) are the main component of the thymic stroma, which supports T-cell proliferation and repertoire selection. Here, we demonstrate that Cbx4, a Polycomb protein that is highly expressed in the thymic epithelium, has an essential and non-redundant role in thymic organogenesis. Targeted disruption of Cbx4 causes severe hypoplasia of the fetal thymus as a result of reduced thymocyte proliferation. Cell-specific deletion of Cbx4 shows that the compromised thymopoiesis is rooted in a defective epithelial compartment. Cbx4-deficient TECs exhibit impaired proliferative capacity, and the limited thymic epithelial architecture quickly deteriorates in postnatal mutant mice, leading to an almost complete blockade of T-cell development shortly after birth and markedly reduced peripheral T-cell populations in adult mice. Furthermore, we show that Cbx4 physically interacts and functionally correlates with p63, which is a transcriptional regulator that is proposed to be important for the maintenance of the stemness of epithelial progenitors. Together, these data establish Cbx4 as a crucial regulator for the generation and maintenance of the thymic epithelium and, hence, for thymocyte development.

KEY WORDS: Thymic epithelial cell, Cbx4, Thymic hypoplasia, T-lymphopoiesis, p63 (Trp63), Polycomb, Epithelial progenitor

INTRODUCTION
The thymus is the site of T lymphopoiesis. The unique role of the thymus in the establishment and maintenance of the T-cell arm of the immune system is intimately linked to specialized functions of thymic stromal cells and the thymus architecture (Rodewald, 2008; Heng et al., 2010). The major component of the thymic stroma is epithelial cells (TECs), which have a crucial role in T lymphopoiesis by generating unique microenvironmental niches that support T-cell proliferation, differentiation and repertoire selection (Petrie and Zúñiga-Pflücker, 2007; Gordon and Manley, 2011; Koch and Radtke, 2011). Both the number and proliferative status of TECs are crucial for thymopoiesis. The active proliferation of TECs, such as in young animals or CyclinD1 transgenic mice, is accompanied by enhanced thymocyte production (Klug et al., 2000), whereas the reduced proliferation of TECs in aged mice results in decreased thymic output (Gray et al., 2006). Although epithelial cellularity is closely related to thymic function, the regulation of TEC proliferation is poorly understood.

Cbx4 (chromobox homolog 4) is a member of the Polycomb (PcG) family. The PcG proteins form two main multimeric protein complexes, termed Polycomb repressive complex (PRC) 1 and PRC2. By maintaining the repressed state of target genes through histone modification, these proteins have a crucial role in the epigenetic regulation of cell proliferation and differentiation (Liu et al., 2006; Schwartz and Pirrotta, 2008; Ezhkova et al., 2009). Cbx proteins, including Cbx2, Cbx4, Cbx6, Cbx7 and Cbx8, are important constituent of the PRC1 complex. Recent studies have suggested that different PRC1 subsets, as defined by specific Cbx components, may exert distinct cellular functions (Surface et al., 2010; Luis et al., 2011). However, the function of each Cbx protein in cell proliferation and differentiation remains unknown. Among the five Cbx proteins, we identified Cbx4 as the most abundant in the thymic epithelium (data not shown), which leads us to hypothesize that Cbx4 has a unique role in regulating TEC development.

Although several PcG proteins have been identified as epigenetic regulators of the immune system, most of them function during hematopoiesis or intrinsic to the developing thymocytes, rather than modulating the thymic stromal function. For example, Bmi1-deficient mice are born with a fully differentiated and functional hematopoietic system, but the hematopoiesis fails within 30 days owing to exhaustion of proliferative HSCs (Park et al., 2003). Reduced thymocyte production was reported in the absence of Cbx2, and the defect in the Cbx2+ thymus is rooted in the developing thymocytes themselves, as indicated by the poor reconstitution of T-cell development in the wild-type thymus with Cbx2+ fetal liver cells (Coré et al., 1997). In this article, we provide evidence that Cbx4 modulates T lymphopoiesis by regulating the proliferation of TECs and the maintenance of the thymic epithelium, thus demonstrating a novel regulatory mechanism for PcG proteins in the immune system.

MATERIALS AND METHODS
Gene targeting and mice
For the disruption of Cbx4 gene, the N-terminal region of the Cbx4 gene including the first two exons and a 0.9 kb upstream region was targeted. Targeted ES clones (MPI-II, 129Sv/Pas derived) were identified by Southern blotting, and C57BL/6J blastocytes were used for microinjection. The Neo cassette in the heterozygous was removed by crossed with Actin-
Flp mice. EIIa-Cre, Foxn1-Cre or Lck-Cre mice were used for global or conditional knockout, and the mice were bred on the C57BL/6J-129Sv genetic background. The conditional knockout p63-deficient mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The experimental procedures for the use and care of animals were approved by the Ethics Committees of Peking University Health Science Center and the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and by Home Office Project Licence (University of Bradford, UK).

Antibodies

The Cbx4 polyclonal antibody against amino acids 363-551 was raised in rabbits and affinity purified as described previously (Ge et al., 2004). The monoclonal antibody was prepared by immunizing mice with the recombinant protein encompassing amino acids 144-362. Injections, hybridoma production, screening, and antibody production and purification were carried out as described previously (Choi and Dreyfus, 1984). The monoclonal anti-Flag (F3165), anti-tubulin (T6199) and anti-HA (H9658) were from Sigma. Anti-Gapdh (KC-5G4) was from KangChen Biotech. Fluorochrome-labeled antibodies against CD4 (11-0041-85, 24-0041-81), CD8 (12-0081-83), B220 (11-0452), Ter119 (12-5921), Aire (51-5094-80) and CD3 (11-0031-82) were from eBioscience. Fluorochrome-labeled antibodies against CD44 (553133), CD25 (553866), anti-CD31 (553177), CD45 (559864), streptavidin (554061), biotin-labeled Ly51 (553159) and the antibody specific for CD31 (550274) were from BD Pharmingen. Anti-pan-CK (628601) anti-panBrdU (619002) and fluorochrome-labeled anti-EpCAM (118216), B220 (103235), CD44 (103028), CD25 (102027), CD45 (103105), Kit (105805) and CD24 (101813) were from Biolegend. Anti-cytokeratin 5 was from Covance. Anti-cytokeratin 8 and anti-Meca32 were from Developmental Studies Hybridoma Bank. Anti-CD205 (MCA949) was from AbD serotec, anti-p63 (H-137) was from Santa-Cruz Biotechnology, anti-claudin 1 (37-4900) was from Zymed, anti-Ki67 was from Novacasta and anti-Cre (69050-3) was from Novagen.

Histology, immunofluorescence microscopy and morphometry

Embryos were processed for histological analysis as described previously (Kaufman and Adams, 1954). Tissues for immunofluorescence were embedded in OCT and frozen immediately in liquid nitrogen. Sections were fixed for 10 minutes in 4% PFA, washed in PBS and blocked in 2% BSA, 10% goat serum, 0.1% Tween-20 in PBS. Subsequently, sections were sequentially incubated with primary and fluorochrome-labeled secondary antibody. After DAPI staining, sections were mounted with mounting gel (Invitrogen), and images of stained sections were captured using a confocal microscope. For flow cytometry analysis of the thymus and adjacent structures in E12.5-E15.5 embryos and were cultured in vitro for 5 days in the presence of 1.35 mM 2′-deoxyguanosine (Sigma). CD24+Kit+ hematopoietic progenitor cells (HPCs) were sorted from E13.5-E15.5 fetal livers using a BD FACS Aria flow cytometer, and the purity of the harvested cells was >97% upon reanalysis by flow cytometry. Each thymic lobe was mixed with 4000 HPCs and was cultured in a hanging drop in Terasaki plates for up to 2 days. After further culture on an Isopore membrane, thymic lobes were collected, and cells within each lobe were counted and analyzed using the BD FACSCalibur platform.

Statistical analysis

Pryism software (GraphPad) was used for all statistical analysis. Datasets were compared using a t-test, and error bars indicate s.e.m.

RESULTS

Loss of Cbx4 leads to thymic hypoplasia

We prepared antibodies specific for Cbx4, validated their specificity (supplementary material Fig. S1A), and then examined Cbx4 expression in mouse tissues. Western blot analysis demonstrated high levels of Cbx4 in the brain, lung and thymus of newborn mice (supplementary material Fig. S1B). To investigate the physiological function of Cbx4, we generated Cbx4-deficient mice by targeting the first two exons of the Cbx4 gene (supplementary material Fig. S2A). Homologous recombination was confirmed using Southern blot analysis (supplementary material Fig. S2B), and the null allele was gained upon Cre-loxP excision by crossing mice carrying the floxed allele with EIIa-Cre transgenic mice (supplementary material Fig. S2C,D).

Heterozygous mice were grossly normal in morphology and growth. The homozygous pups were born alive at an expected Mendelian ratio but died within 1 hour of birth. Notably, all mutant pups suffered from thymic hypogenesis (Fig. 1A). The numbers of both total thymic cells and TECs (CD45 EpCAMth) in Cbx4−/− mice at E17.5 were decreased by over 85% in comparison with wild-type littermates (Fig. 1B). The hypoplastic thymus did not appear to be the consequence of a general hematopoietic defect because the number of total splenocytes and bone marrow cells in the homozygous pups was comparable with that of the wild-type littermates. To explore the timing of the thymic developmental defect, we performed a histological assessment of the thymus and adjacent structures in E12.5-E15.5.
Cbx4 deficiency impairs the proliferation of fetal thymocytes

To elucidate the cellular basis of the thymic hypoplasia caused by Cbx4 deficiency, we examined whether there was any defect in the survival and proliferation of thymic cells. We performed the TUNEL assay using E17.5 thymic sections. The proportion of apoptotic cells to total thymic cells was low and comparable in the wild-type and mutant thymus (data not shown). Alternatively, flow cytometric analysis of propidium iodide (PI)-stained E17.5 thymocytes showed an increased proportion of cells in the G0/G1 phase but reduced cell numbers in the S and G2/M phases in the knockout mice (Fig. 1C). The proliferation of thymocytes in vivo during embryogenesis was further examined by in utero bromodeoxyuridine (BrdU) labeling. Although there were similar percentages of BrdU+ cells within the CD4+CD8+ double-negative (DN) cells in wild-type and knockout thymi, the BrdU+ fraction in the CD4+CD8+ double-positive (DP) thymocytes was reduced by 50% in the deficient animals (Fig. 1D). Because DP thymocytes constitute the majority of the thymic cell population in late embryonic development, impaired proliferation of DP cells might directly contribute to the thymic hypoplasia observed in Cbx4−/− mice.

Despite sharply reduced absolute cell numbers, the Cbx4−/− thymus displayed a normal profile of DN and DP, as well as CD4+ and CD8+ single-positive (SP) cells at E17.5 (Fig. 1E, left). Furthermore, the percentage of thymocytes in each of the four DN subsets defined by CD44 and CD25 expression in wild-type and Cbx4−/− thymus was comparable (Fig. 1E, middle). Moreover, the TCRγδ+ population in the Cbx4−/− thymus was intact (Fig. 1E, right). These results indicate that the progression of thymocyte differentiation during the perinatal stages is not affected by the loss of Cbx4.

Thymic hypocellularity results from a thymic epithelial cell defect

The thymic abnormality in Cbx4-deficient mice could be the consequence of either an intrinsic defect of the hematopoietic progenitors or the dysfunction of the thymic stroma. To distinguish between these two possibilities, we performed a detailed analysis of the thymic cells expressing Cbx4. Thymocytes, which make up the majority of thymic mass, expressed little Cbx4, as determined by western blot analysis of purified cells (data not shown). Instead, high levels of Cbx4 protein were detected in the thymic stroma, specifically in epithelial cells that were positive for either cytokeratin 5 (K5; Krt5 – Mouse Genome Informatics) or cytokeratin 8 (K8; Krt8 – Mouse Genome Informatics) (Fig. 2A). Other thymic components, including endothelial cells (supplementary material Fig. S4) and the mesenchyme ensheathing the developing thymus (data not shown), showed no Cbx4 expression.

To elucidate the contribution of TECs to the observed thymic defect in Cbx4−/− mice, reconstituted fetal thymic organ culture (RFTOC) was performed. Fetal thymi with differing genotypes were pretreated with 2′-deoxyguanosine to eliminate hematopoietic cells and were then reconstituted with hematopoietic progenitor cells (HPCs) purified from the E15.5 fetal livers of wild-type or Cbx4−/− embryos (supplementary material Fig. S5A). Similar numbers of HPCs were obtained, suggesting that early hematopoiesis was not affected by the absence of Cbx4 (supplementary material Fig. S5B). After 12 days of culture, comparable numbers of thymocytes were generated in the wild-type thymic lobes reconstituted with either wild-type or Cbx4-
Thymi were treated with 2/11032 hematopoietic cells and then reconstituted with CD24 low Kit+ cells Cbx4+/+ from Cbx4. Representative images of thymi from Cbx4 lox/+ (n in the deficient HPCs. By contrast, thymocytes were barely detectable in Cbx4 and thymic development sample and horizontal bars denote the mean (± S.E.M. (n≥ 5)).

**Fig. 2. Thymic hypoplasia results from a defective TEC compartment.** (A) Immunostaining of the thymus for Cbx4. Frozen sections of E17.5 fetal thymus were co-stained with antibodies against Cbx4 (green) and the medullary epithelial marker K5 or the cortical epithelial marker K8 (red). Scale bars: 10 μm. (B) Cell yield from fetal thymic organ cultures following reconstitution with hematopoietic progenitor cells. Cbx4+/+, Cbx4+/− or Cbx4−/− fetal thymi were treated with 2’-deoxyguanosine for 5 days to eliminate hematopoietic cells and then reconstituted with CD24+Kit+ cells from Cbx4+/+ or Cbx4−/− fetal livers. The numbers of viable cells recovered from 12-day culture are presented as mean ± S.E.M. (n≥ 3). (C) Gross appearance of thymus with the TEC-specific disruption of Cbx4. Representative images of thymus from [Cbx4+/+, Foxn1-Cre] and [Cbx4−/−, Foxn1-Cre] newborn mice are shown. (D) The total number of thymocytes in [Cbx4+/+, Foxn1-Cre] versus [Cbx4−/−, Foxn1-Cre] (right) newborn mice. Each dot represents an individual sample and horizontal bars denote the mean (n≥ 5).

deficient HPCs. By contrast, thymocytes were barely detectable in the Cbx4−/− lobes regardless of whether the lobes were reconstituted with wild-type or mutant HPCs (Fig. 2B). Therefore, Cbx4 deficiency primarily affects the thymic stromal compartment.

This conclusion was validated in studies using mice with cell-specific deletion of Cbx4. Cbx4-floxed mice were cross-bred with Lck-Cre mice (Pan et al., 2002) to specifically disrupt Cbx4 in the T-cell lineage. Such mice had a normal number of thymocytes (supplementary material Fig. S6). Furthermore, TEC-specific Cbx4 deletion was achieved by cross-breeding with Foxn1-Cre mice, in which Cre is expressed in the TECs from early in thymic organogenesis. Similar to the thymic phenotype of Cbx4 global knockout mouse, [Cbx4−/−, Foxn1-Cre] mice also suffered severe thymic atrophy (Fig. 2C,D). Taken together, these results suggest an essential role for Cbx4 in the proper functioning of TECs, which support the expansion of thymic cellularity during embryonic development.

**TEC proliferation and maturation is impaired by disruption of Cbx4**

Having attributed the defect in the Cbx4-deficient thymus to the epithelial cells, we focused our subsequent analysis on the TEC compartment. Similar percentages of K5+ and K8+ cells were detected among nucleated cells in the thymi of E18.5 [Cbx4+/+, Foxn1-Cre] and [Cbx4−/−, Foxn1-Cre] mice (Fig. 3C-F). This, as well as the result shown in Fig. 1B, indicated that the reduced total thymic cellularity was accompanied by a proportional decrease of the epithelial compartment. We next examined the proliferation status of the TEC populations. Immunostaining revealed equal percentages of Ki67+ cells in the p63+ (Trp63− Mouse Genome Informatics) population of control and mutant thymi at E18.5 (Fig. 3A,B), indicating that the proliferation of the putative TEC progenitors is independent of Cbx4. By contrast, the proportion of Ki67+ cells in either the K5+ or K8+ population in the mutant thymus was approximately half of that found in the control animals (Fig. 3C-F), which is suggestive of impaired proliferation of the developing epithelial cells.

In addition to cell proliferation, TEC differentiation was examined using the transcription factor p63 as a marker for putative epithelial progenitors and claudin 1 as a marker for terminally differentiated TECs (Langbein et al., 2003; Senoo et al., 2007). More than 40% of the nucleated cells were found to be positive for p63 in the fetal thymi of both [Cbx4+/+, Foxn1-Cre] and [Cbx4−/−, Foxn1-Cre] embryos at E13.5 (Fig. 4A). The p63+ population quickly dropped to 2-3% in the control thymus at E18.5, but was maintained at a relatively high level (7-9%) in the mutant thymus (Fig. 4B,C). In contrast to the relative increase of p63+ cells, the number of claudin 1+ cells in the mutant thymus was decreased by as much as 80% in comparison with the control thymus at E18.5 (Fig. 4B,C). Consistent with this, quantitative RT-PCR revealed a much elevated level of K14 mRNA, whereas CD80 and CD86 expression was found to be decreased (Fig. 4D). Furthermore, immunostaining demonstrated dramatically reduced numbers of Aire+, UEA-1+ and CD205+ cells, suggesting impaired maturation of both medullary and cortical TECs (mTECs and cTECs) (Fig. 4E-H). Together, these results demonstrate that Cbx4 is crucial both for the proliferation and maturation of TECs.

**Cbx4 is required for the maintenance of the thymic epithelium and T-cell differentiation in postnatal mice**

Despite the reduced thymic cellularity, the overall architecture of the thymic epithelium was preserved, and both medullary and cortical epithelial cells were present in the Cbx4-deficient thymus at E18.5 (supplementary material Fig. S3D), indicating that Cbx4 is not required for the generation of thymic epithelial progenitors and their subsequent bifurcation into the medullary and cortical lineages during embryonic development. This is in contrast to the defect in nude mice, which manifests as a developmental block of the epithelium at the initial stage (Blackburn et al., 1996).

Intriguingly, the limited, but morphologically intact, thymic epithelial architecture formed during embryogenesis quickly deteriorates in postnatal [Cbx4−/−, Foxn1-Cre] mice. Immunostaining showed that the discrete cortical and medullary structures were no longer observable in 3-week-old mutant mice.
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Fig. 3. Proliferation of TECs is impaired in the absence of Cbx4. (A) Proliferation of p63+ cells in E18.5 [Cbx4lox/lox, Foxn1-Cre] and [Cbx4lox/+, Foxn1-Cre] thymi. Thymic sections were stained for p63 and the proliferation marker Ki67. Scale bars: 5 μm. Arrowheads indicate the Ki67+p63+ cells. (B) Percentage of proliferative p63+ cells in E18.5 thymi. The experiments were performed at least three times and over 600 p63+ cells were counted for each genotype. Data are mean±s.e.m. (C) Proliferation of K8+ cells in E18.5 thymi. Thymic sections were stained for Ki67 and the cTEC marker K8 with counterstaining of the nuclei by DAPI. Asterisks indicate quiescent cTECs, whereas proliferating cTECs are indicated by arrowheads. (D) Percentage of total and proliferative cTECs in E18.5 thymi. The percentage of total cTECs is defined as the proportion of K8+ cells within DAPI-stained cells, and the proportion of proliferative cTECs is the ratio of Ki67+ cells within K8+ cells. The experiments were performed at least three times. Over 2000 total cells or 500 K8+ cells were counted for each genotype. **P<0.01; n=3. Data are mean±s.e.m. (E) Proliferation of K5+ cells in E18.5 thymi. Experiments were performed as in C using K5 as an mTEC marker. Scale bars: 5 μm. Arrows and arrowheads indicate quiescent and proliferating mTECs, respectively. (F) Percentage of total and proliferative mTECs in E18.5 thymi calculated as in D. Experiments were performed at least three times. Over 1500 total cells or 500 K5+ cells were counted for each genotype. **P<0.01; n=3. Data are mean±s.e.m.

Cbx4-deficiency is associated with dysregulated expression of key molecules in the thymic stroma

We next investigated the expression of multiple molecules that are crucially involved in the regulation of TEC development and function in E14.5 fetal thymi depleted of hematopoietic cells following treatment with 2′-deoxyguanosine. As shown in Fig. 6A, Cbx4 deficiency resulted in significantly reduced expression of Fg7 and Fg10, whereas FgR2-IIb mRNA levels were not altered. As far as the BMP pathway was concerned, Cbx4-deficient thymic stromal cells showed enhanced expression of Bmp4, whereas Bmp2, BMP receptors and regulators of BMP signaling, such as Tsg, Noggin and Chordin, were expressed at levels comparable with those in the wild-type mice (Fig. 6B). T-cell migration within the thymus, which is primarily driven by the coordinated expression of multiple chemokines, is essential for their differentiation. In the absence of Cbx4, the transcription of a number of chemokines, including Ccl19, Ccl25, Cxcl11, and Cxcl12, is repressed (Fig. 6C). These results indicate an overt dysregulation of the TEC compartment at the molecular level owing to Cbx4 disruption.

Cbx4 physically interacts and functionally correlates with the transcription factor p63

To explore the molecular mechanism underlying Cbx4 function in TECs, yeast two-hybrid assays were performed, leading to the identification of several candidate interacting partners for Cbx4. Among them, ΔNp63 was of particular interest because it is known to be crucial for the proliferation of epithelial stem cells (Fig. 7A). In fact, the thymic defect observed in Cbx4-deficient mice in many ways mimics the defect in p63−/− mice. To verify the interaction between Cbx4 and p63, we co-transfected Flag-tagged Cbx4 and
HA-tagged p63 into 293T cells and conducted co-immunoprecipitation assays. As shown in Fig. 7B,C, p63 was detected in the immune complex that was precipitated with anti-Cbx4, and vice versa. We next examined the expression patterns of these two molecules in the thymus by immunostaining. The Cbx4 signal was found to match perfectly with that of p63 in the thymus (Fig. 7D). This co-localization suggests that these two proteins may be functionally related. In support of this conclusion, the number of Cbx4high cells in E16.5 p63−/− thymi was reduced by ~80% compared with the wild-type thymus (Fig. 7E,F). This observation, in combination with the relative accumulation of p63+ cells in the Cbx4−/− thymi, suggests that Cbx4 is a downstream factor of p63 that regulates TEC development.

**DISCUSSION**

**Unique functions of Cbx proteins**

Several PcG proteins have been shown to participate in the regulation of hematopoiesis and T-cell differentiation (Sauvageau and Sauvageau, 2010). However, as shown in the present study, the underlying mechanism of Cbx4 function is different from that of other PcG proteins. Thymocyte development failed to be reconstituted in the Cbx4−/− fetal thymus with wild-type hematopoietic progenitors. Similarly, thymic hypocellularity was reproduced following the tissue-specific deletion of Cbx4 gene in the epithelium but not in the thymocyte. Therefore, Cbx4 deficiency primarily affects the epithelial compartment, and reduced thymocyte production is a secondary effect.

Previous studies have revealed that different Cbx-associated PRC1 complexes exert unique functions throughout development. Cbx2-deficient mice display homeotic transformations of the axial skeleton (Coré et al., 1997), and mice deficient for both the Cbx2 and Bmi1 show stronger homeotic phenotypes (Bel et al., 1998), indicating that Cbx2 might synergistically regulate mesodermal genes. In contrast to Cbx2, the present study reveals the potentially unique role of Cbx4 in epithelial development. This hypothesis is also supported by the results of another study in which Cbx4 was shown to regulate epidermal stem cell differentiation and senescence (Luis et al., 2011). One recent study alternatively suggests that the maintenance of embryonic stem cell pluripotency might primarily depend on Cbx7 (Morey et al., 2012).

**Functional correlations between Cbx4 and p63 in thymic development**

In many aspects, the thymic phenotype of the Cbx4−/− mice resembles that of the p63−/− mice (Candi et al., 2007; Senoo et al., 2007). First, both lines exhibit unusual thymic hypoplasia with greatly reduced production but normal maturation of fetal thymocytes. Second, the proliferation of developing epithelial cells is severely compromised in both cases. Last, and most importantly, the two mutant lines share a common defect in the maintenance of the thymic epithelium. In p63−/− mice, the failure has been suggested to be due to an impaired proliferative potential of the epithelial stem cells, which leads to their premature proliferative exhaustion (Senoo et al., 2007). Notably, Cbx4 colocalizes with p63 in a subset of TECs. Moreover, we demonstrated that there was a relative accumulation of p63− cells in the Cbx4-deficient thymus (Fig. 4C), whereas the p63-deficient thymus contained fewer Cbx4+ cells (Fig. 7F). The latter observation is consistent with a
recent report of decreased Cbx4 transcription in the skin of p63–/– mice (Fessing et al., 2011). These data support the theory Cbx4 and p63 may function in the same pathway to promote the proliferative and self-renewal activities of thymic epithelial stem cells. Specifically, Cbx4 may act downstream of p63. This speculation, however, seems to be at odds with the observed physical interaction between Cbx4 and p63. One explanation could be that Cbx4, once induced by p63, may be recruited into the p63 complex to regulate its own expression via a feedback mechanism. Alternatively, the complex formed between p63 and Cbx4 is required for the transcriptional regulation of downstream targets.

**Downstream molecular events of Cbx4**

The downstream molecular events leading to the thymic defect in Cbx4–/– mice remain to be further defined. The Ink4a/Arf (Cdkn2a – Mouse Genome Informatics) locus is one of the best-characterized targets of transcriptional repression mediated by PcG proteins (Sauvageau and Sauvageau, 2010). The Cbx4–/– thymus, however, showed no significant induction of p16Ink4a and p19Arf (data not shown). We also examined the expression of multiple molecules that are crucially involved in the regulation of TEC development and function. Candi et al. have identified FgfR2-IIIb as one of the downstream effectors of p63 (Candi et al., 2007). We found no significant changes in FgfR2-IIIb levels in the Cbx4–/– thymus, indicating that Cbx4 may function in parallel to p63-FGFR signaling. Alternatively, Fgf10, which is the primary ligand of FgfR2-IIIb in the thymus, was found to be markedly suppressed (Fig. 6A). Given the importance of FgfR2-IIIb and Fgf10 in thymus development (Revest et al., 2001), altered Fgf10 expression may be partly responsible for the thymic defect observed in Cbx4–/– mice. Besides, the Cbx4–/– thymus showed substantially elevated levels of Bmp4 (Fig. 6B), which is reported essential for thymic development (Nosaka et al., 2003; Tsai et al., 2003). The inhibitory effect of Bmp4 on thymus development suggests that the elevated level of Bmp4 in the Cbx4–/– thymus is relevant to the defect.

In addition to the signal molecules involved in the development of TECs itself, our expression analysis included those molecules that are crucial for the T-lymphopoiesis-supporting function of TECs (Ciofani and Zúñiga-Pflücker, 2007). As postnatal T-cell development was almost completely blocked in Cbx4-deficient mice (Fig. 5C), we examined the expression of molecules crucial for lineage commitment and the proliferation of T cell progenitors, such as Delta-like 4 (supplementary material Fig. S7E), stem cell factor and interleukin 7 (data not shown), but found no difference in their mRNA levels between the wild-type and mutant thymi. However, the mutant thymus showed reduced expression of a number of chemokines (Fig. 6C), which, we believe, may impair
the migration of early thymic progenitors into the thymus and the directional movement of developing thymocytes. We analyzed the thymic DN1 profiles of the newborn conditional knockout mice and littermate controls by staining for Kit and CD24 (Sambandam et al., 2005). As shown in supplementary material Fig. S8, the thymic DN1 profiles of the newborn conditional knockout mice and control mice are similar.

**Fig. 7. Cbx4 is regulated by p63.** (A) Interaction between Cbx4 and p63 as shown by a yeast two-hybrid system. Yeast co-transformed with GAL4BD-Cbx4 and GAL4AD-p63 was allowed to grow on 5D minimal media (SD-L-T-A-H) lacking leucine, tryptophan, adenine and histidine. The yeast co-transformed with GAL4BD-p63 and GAL4AD-Cbx4 was similarly cultured. A schematic representation of the transformants is shown on the right. (B,C) Co-immunoprecipitation of p63 and Cbx4 in transfected cells. HEK 293T cells were transfected with pcDNA3-HA-p63 and pCMV-Flag-Cbx4 alone or in combination, as indicated above the figure. (B) Immunoprecipitation with anti-Flag and probing with anti-HA or anti-Flag; (C) immunoprecipitation with anti-HA and probing with anti-Flag or anti-HA. (D) Colocalization of Cbx4 and p63 in the thymus in mice at different ages, as determined by immunofluorescent staining. Scale bars: 10 μm. (E) Cbx4 expression in p63+/+ thymi. Thymic sections were stained for Cbx4 (red) and CD45 (green) as a control. Representative images are shown. Cbx4high cells are indicated by arrows. Scale bars: 10 μm. (F) Quantification of Cbx4high cells in E16.5 thymi. The y axis shows the percentage of Cbx4high cells among DAPI-stained cells. Over 1500 p63+/+ and 1000 p63−/− thymic cells were counted. **P<0.01; n=3. Data are means±s.e.m.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.085035/-/DC1

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