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# Chromobox Protein Homolog 3 Is Essential for Stem Cell Differentiation to Smooth Muscles In Vitro and in Embryonic Arteriogenesis

Qingzhong Xiao, Gang Wang, Xiaoke Yin, Zhenling Luo, Andriani Margariti, Lingfang Zeng, Manuel Mayr, Shu Ye, Qingbo Xu

- *Objective*—Smooth muscle cell (SMC) differentiation is a critical process during cardiovascular formation and development, but the underlying molecular mechanism remains unclear.
- *Methods and Results*—Here we demonstrated that chromobox protein homolog 3 (Cbx3) is crucial for SMC differentiation from stem cells and that the chromodomain and chromoshadow domain of Cbx3 are responsible for Cbx3-induced SMC differentiation. Moreover, we identified that 4 amino acids (165 to 168) within the chromoshadow domain of Cbx3 are key elements for Cbx3 interaction with Dia-1- and Cbx3-induced SMC differentiation. Mechanistically, we found that Cbx3 mediates SMC differentiation through modulating serum response factor (SRF) recruitment to the promoters of SMC genes, in which the interaction between Cbx3 and Dia-1/SRF plays a crucial role in this process. Moreover, our in vivo study demonstrated that the misexpression of Cbx3 within neural crest cells of chick embryos resulted in the death of chick embryos at early stages because of the maldevelopment of branchial arch arteries.
- Conclusion—Our findings suggest that the interaction between Cbx3 and Dia-1/SRF is essential for SMC differentiation from stem cells and for the development of functional cardiovascular system. (Arterioscler Thromb Vasc Biol. 2011; 31:1842-1852.)

Key Words: chromobox protein homolog 3 ■ arteriogenesis ■ chick embryos ■ smooth muscle cells ■ stem cells

major achievement in the past decade has been made in A the field of cardiovascular research and stem cell biology, in which embryonic stem (ES) cells were reported to differentiate into vascular cells, including vascular smooth muscle cells (SMCs)<sup>1,2</sup> and endothelial cells,<sup>3–8</sup> representing a potential unlimited cell source for vascular tissue repair and constructing engineered vasculatures. Moreover, growing evidence suggests that vascular stem/progenitor cells play a major role in various cardiovascular diseases, including atherosclerosis and angioplasty-induced restenosis.9-12 Cell differentiation from stem/progenitor cells is a multistep process marked by progressive silencing of pluripotent gene expression and the activation of specific cell-lineage gene expression through mechanisms likely to involve heterochromatin and associated proteins. As a major heterochromatinassociated protein, the protein encoded by the chromobox protein homolog gene (Cbx) could be an ideal candidate for controlling terminal cell differentiation from stem cells.

Indeed, previous studies have suggested the involvement of Cbx3 (encoding the heterochromatin protein [HP]-1 $\gamma$  protein) and its close family members Cbx1 and Cbx5 (encoding

HP1 $\beta$  and  $\alpha$ , respectively) in cell lineage differentiation. By interacting with transcriptional intermediary factor  $1\beta$ , Cbx proteins have been reported to be involved in retinoic-acidinduced primitive endoderm differentiation.<sup>13,14</sup> Further study from the same research group suggested that although all 3 Cbx proteins are found in both eu- and heterochromatin, they display a clear specificity of interaction and localization when associated with specific partners. This strongly indicates that different Cbx isotypes play specific nonredundant functions during cell differentiation.<sup>15</sup> More specifically, Cbx3 has been documented to negatively regulate adipocyte differentiation from preadipocyte cells and is required for male germ cell survival and spermatogenesis.16 Another study also suggested Cbx3 plays an important role in the epigenetic regulation of both cell differentiation and cancer development.17 However, little is known about the functional involvement of Cbx3 protein in SMC differentiation from stem/progenitor cells and cardiovascular system development. In the present study, by combining a well-established in vitro SMC differentiation model and an in vivo chick embryo gene transfer system, we demonstrated for the first time that

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Cbx3 plays an important role in SMC differentiation and is crucial for the functional development of the cardiovascular system in vivo.

# **Materials and Methods**

### Cell Culture and SMC Differentiation

Detailed protocols for mouse ES cell (ES-D3 cell line CRL-1934, American Type Culture Collection, Manassas, VA) culture and SMC differentiation were described in our previous studies.<sup>2,18–21</sup> Briefly, for SMC differentiation, undifferentiated ES cells were seeded on mouse collagen IV (5  $\mu$ g/mL)–coated flasks or plates in differentiation medium (DM) (minimum essential medium [MEM]- $\alpha$  medium [Gibco] supplemented with 10% fetal bovine serum, 0.1 mmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin) for 3 to 7 days before further treatments. The medium was refreshed every other day. Detailed procedures for the isolation and culture of Sca-1 + SMC precursor cells from differentiating ES cells were described in our previous study.<sup>2</sup>

Nucleofection, small interfering RNA (siRNA) experiments, coimmunoprecipitation, indirect immunofluorescent staining for cells, and real-time reverse transcription–polymerase chain reaction (RT-PCR) were performed as previously described.<sup>2,18</sup> Chromatin immunoprecipitation assays were performed as previously described.<sup>20,21</sup>

#### Cbx3 and Its Deletion/Mutation Cloning

The mouse full-length Cbx3 gene was amplified using RT-PCR from day 3 of differentiating ES cells with the primer set as shown in Supplemental Table I (available online at http://atvb.ahajournals.org) and cloned into the KpnI/PstI sites of the pCMV5-HA expression vector, designated pCMV5-HA-Cbx3 (full length). Cbx3 chromodomain (CD) (amino acids [aa] 36 to 79), Hinge domain (aa 80 to 122), and chromoshadow domain (CSD) (aa 123 to 175) deletions and an aa 165 to 168 mutation were generated from pCMV5-HA-Cbx3 with specific primers shown in Supplemental Table I, designated pCMV-HA-Cbx3ΔCD (ΔCD), pCMV-HA-Cbx3ΔHinge (ΔHinge), pCMV-HA-Cbx3ΔCSD (ΔCSD), and pCMV-HA-Cbx3Δ165/8 (Δ165/8), respectively. Two restriction enzyme cutting sites were introduced into the mutated plasmids for easy and prompt identification of the resultant constructs. These vectors express truncated Cbx3 or the PxVxL pentapeptide binding site mutated Cbx3 with the N terminus tagged with HA epitope. Mouse Cbx3 CSD (aa 118 to 176) was cloned into pCAb-green fluorescent protein (GFP) and used as the Cbx3 dominant-negative form (DNF) (expression vector driven by chicken *B*-actin promoter [pCAb]-Cbx3 DNF-GFP) as described previously (a kind gift from Dr Andrea Streit, King's College London).22 All vectors were verified by DNA sequencing.

#### **Chick Embryo Cultivation and Staging**

Fertilized chick eggs were obtained from Joyce and Hill Farm and incubated at 38.5°C in a humidified incubator. Hamburger-Hamilton staging was applied throughout the study.<sup>23</sup>

#### Whole-Mount Immunohistochemistry

The chick embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight. After graded dehydration in methanol, the embryos were incubated with freshly prepared methanol:dimethyl sulfoxide: $H_2O_2$  (4:1:1) at room temperature for 5 hours. Then, the embryos were rehydrated in a series of 50% methanol/PBS and PBS and blocked with PBSMT (2% nonfat milk powder, 0.5% Triton X-100 in PBS). Subsequently, the embryos were incubated with mouse smooth muscle alpha-actin antibody (anti-SM $\alpha$ A) (1:2000, Sigma-Aldrich) or anti-Cbx3 (1:500, Santa Cruz Biotechnology) in PBSMT at 4°C overnight. Mouse IgG was used at same concentration as control (data not shown). After horseradish peroxidase–conjugated secondary antibody and 3,3'-diaminobenzidine staining, the embryos were fixed in 4% paraformaldehyde for 10 minutes, rinsed in 25% and 50% methanol/PBS, and mounted with 75% glycerol/PBS for observation.

#### In Vivo Electroporation on Chick Embryos

In vivo electroporation carried out on chick embryos was performed as previously described but with some modifications.<sup>24</sup> Eggs were incubated in a humidified incubator at 38°C for around 36 hours. A window was opened at the top of the eggshell using a blade, and embryos at Hamburger-Hamilton stages 10/10–/10+ were electroporated with pCAb-Cbx3 DNF-GFP (1  $\mu g/\mu L$ ) or pCAb-GFP (1  $\mu g/\mu L$ ) as control. The electroporator was set at 7.5 V, 4 pulses, length of 50 milliseconds, and interval of 1000 milliseconds. Constructs were mixed with 60% sucrose and Fast Green for better visualization before electroporation. After electroporation, the eggs were well sealed and placed back into the incubator, window side up, and incubated until they reached the required developmental stages.

#### **Statistical Analysis**

Data were expressed as mean $\pm$ SEM and analyzed using 2-tailed Student *t* test for 2-group comparison or 1-way ANOVA for comparing different groups.  $\chi^2$  analysis was performed using SPSS 17.0 software to compare the percentage of embryonic maldevelopment in 2 groups. A value of *P*<0.05 was considered statistically significant.

For detailed Materials and Methods, see supplemental information.

#### **Results**

# Nuclear Protein Cbx3 Is Upregulated During SMC Differentiation

Nuclear proteins are involved in many fundamental cellular processes, such as signal transduction, gene regulation, and DNA/RNA synthesis. Therefore, we hypothesized that certain nuclear proteins are critical for SMC differentiation from stem cells. To confirm this hypothesis, ES cells were seeded on mouse collagen IV-coated flasks and cultured in the absence of leukemia inhibitory factor for 1 to 7 days to allow SMC differentiation as described previously.<sup>2,18–21</sup> As shown in Figure 1A, gene expression levels of SMC differentiationspecific markers, such as SM $\alpha$ A and smooth muscle myosin heavy chain (SM-MHC), were significantly induced in our stem cell differentiation model. Interestingly, the expression levels of nuclear protein Cbx3, which we have previously identified as 1 of the several nuclear proteins upregulated during early SMC differentiation in our nuclear proteomics analysis (data not shown), were transiently upregulated. The levels of Cbx3 expression peaked at day 3 and displayed a sustained signal until day 7 (Figure 1B and 1C). This suggests that Cbx3 may have a role in SMC differentiation. As expected, immunofluorescence staining with specific antibody against Cbx3 further confirmed its nuclear localization in differentiating SMCs (Figure 1D).

#### Cbx3 Is Crucial for SMC Differentiation

To investigate whether Cbx3 expression was essential for SMC differentiation, siRNA knockdown experiments were performed in differentiating ES cells using Cbx3 or control siRNA. Our data revealed that Cbx3 knockdown inhibited expression of smooth muscle differentiation–specific markers (SM $\alpha$ A, SM22 $\alpha$ , h1-Calponin, SM-MHC) and muscle related transcription factors (serum response factor [SRF], myocardin, MEF2c) at both RNA (Figure 2A) and protein levels (Figure 2B). To further test whether Cbx3 is sufficient for SMC differentiation, Cbx3 expression plasmids were successfully generated, and overexpression studies were con-



Figure 1. Cbx3 is upregulated during SMC differentiation. A, Induction of SMC differentiation markers during stem cell differentiation. Undifferentiated ES cells were plated onto dishes coated with 5 µg/mL collagen IV and cultured in DM. Total RNA from undifferentiated ES cells (d0) or differentiating ES cells at days 3 (d3), 5 (d5), and 7 (d7) were harvested and subjected to real-time PCR analysis with specific primer pairs for SM $\alpha$ A and SM-MHC. B, mRNA induction of Cbx3 is upregulated during SMC differentiation. C, Nuclear protein from undifferentiated ES cells (d0) or differentiating ES cells at the indicated time points was harvested and subjected to Western blot analysis. KD indicates kilodaltons. D, Nuclear localization of Cbx3 in differentiating ES cells. Immunofluorescence staining was conducted on day 7 differentiating ES cells with antibodies against Cbx3. DAPI indicates 4'.6-diamidino-2-phenylindole. The data presented here are representative or an average of 3 independent experiments. \*P<0.05 compared with control.

ducted in the differentiating ES cells. As expected, enforced Cbx3 expression significantly induced expression of all 4 SMC-specific genes (SM $\alpha$ A, SM22 $\alpha$ , h1-Calponin, and SM-MHC) and muscle transcription factors in a dose-dependent manner (Figure 2C). Similarly, Western blot analysis revealed the increased protein production of these genes in the cells overexpressing Cbx3 (Figure 2D). The increase of mRNA and protein level induced by Cbx3 overexpression could result from an increase of the expression in a single cell, the increase of the number of positive cells, or both. To distinguish this, flow cytometry analysis was conducted to detect SM $\alpha$ A expression in the differentiated cells. As shown in Figure 2E to 2G, both SM $\alpha$ A-positive cell number in the differentiated cells (Figure 2I) and SM $\alpha$ A protein expression levels in the SM $\alpha$ A-positive cells (Figure 2G) were significantly increased by overexpressing Cbx3. This suggests that enforced Cbx3 expression in the ES cells not only induced more SMC differentiation from stem cells but also enhanced SMC gene expression in individual differentiated SMC within the cell culture. These data imply that Cbx3 is not only necessary but also sufficient for SMC differentiation from stem cells. It is well known that SMC-specific actin dynamics/organization, including the polymerization of actin monomers into filaments or depolymerization process, plays a key



Figure 2. Cbx3 is crucial for SMC differentiation from ES cells. A and B, Cbx3 knockdown downregulated SMC differentiation gene expression. Cbx3-specific siRNA and random siRNA control were transfected into day 3 differentiating ES cells; after an additional 48 or 72 hours of culture, total RNA and protein were harvested and subjected to quantitative real-time RT-PCR (A) and Western blot analysis (B), respectively. KD indicates kilodaltons. C and D, Enforced Cbx3 expression promotes SMC differentiation. Undifferentiated ES cells were nucleofected by nucleofector II with different amounts of Cbx3 expression plasmid pCMV5-Cbx3. Nucleofected cells were plated in dishes coated with 5  $\mu$ g/mL collagen IV and cultured for 3 to 4 days in DM. Total RNA and protein were harvested and subjected to realtime PCR analysis for gene expression (C) and Western blot analysis for protein levels (D), respectively. Appropriate amounts of empty vector pCMV5 were included as plasmid amount compensation. *a*-Tubulin was included as internal control. The data presented here are representative or an average of 3 independent experiments. E to G, Flow cytometry analysis of the SMaA expression pattern in the differentiated SMCs. Undifferentiated ES cells were nucleofected using nucleofector II with control (pCMV5) or Cbx3 expression plasmid pCMV5-Cbx3 (1  $\mu\text{g}/10^6$ cells) and cultured for 3 to 4 days in DM to allow SMC differentiation. Differentiated cells were harvested and subjected to flow cytometry analysis with SMaA-specific antibody. Representative flow cytometry analysis images (E), the percentage of SMαApositive cells in the differentiated cells (F), and mean fluorescence intensity (MFI) of SMaA-positive cells (G) from 3 independent experiments are presented here. \*P<0.05 compared with control.

role in SMC cellular functions such as cell motility, contractility, and phagocytosis. We further wondered whether Cbx3 was necessary and sufficient for SMC cytoskeletal organization. To determine this, day 4 differentiating ES cells were transfected with Cbx3 siRNA or control random siRNA, and undifferentiated ES cells were transfected with Cbx3 overexpression plasmids (pCbx3) or control plasmids (pCMV5). Cells were cultured for a further 48 or 72 hours and subjected to immunofluorescent analysis with antibodies against SMCspecific myofilament proteins, such as SM $\alpha$ A and SM-MHC. Data shown in Supplemental Figure I clearly revealed that knockdown of Cbx3 disrupted SMC-specific myofilament networks (Supplemental Figure IA), whereas overexpression of Cbx3 enhanced cytoskeletal organization (Supplemental Figure IB). It is noteworthy that although day 3 differentiating cells have started to express SM $\alpha$ A and SM-MHC protein, no clear filament networks could be observed within these cells (pCMV5, Supplemental Figure IB). These data clearly suggest that Cbx3 is critical in the regulation of SMC-specific myofilament organization. To further validate the role of Cbx3 in SMC differentiation from stem cells, stem cell antigen 1-positive (Sca-1<sup>+</sup>) cells, previously reported as SMC precursor cells capable of differentiating into SMCs with high purity (>95%),<sup>2</sup> were transfected with control (pCMV5) or Cbx3 expression plasmid pCMV5-Cbx3 (1  $\mu g/10^6$  cells) and induced to become SMCs. As shown in Supplemental Figure II, overexpression of Cbx3 in Sca-1<sup>+</sup> cells significantly upregulated SMC gene expression at both mRNA and protein levels, indicating that Cbx3 plays a direct role in SMC differentiation from purified SMC precursor cells.

To further examine whether the expression of other genes associated with smooth muscle lineages of the heart, including cardiac and endothelial genes, was affected by Cbx3 expression, Cbx3 gene overexpression and knockdown experiments in differentiating ES cells were performed. The cell lineage markers were chosen based on our previous real-time RT-PCR analysis and were upregulated during ES cell differentiation on collagen IV.<sup>18</sup> Data showed that some cardiac-specific genes ( $\alpha$ -actin, cardiac muscle-1 [actc-1] and troponin C type 1 [Tnnc-1]) but none of the endothelialspecific genes were affected by Cbx3 expression levels (Supplemental Figure III), suggesting that Cbx3 may also play a role in regulation of the expression of these 2 genes.

#### CD and CSD but Not Hinge Domain of Cbx3 Were Responsible for Cbx3-Induced SMC Differentiation

To determine whether the molecular domains of Cbx3 are required for its specific physiological functions, a series of Cbx3 gene deletions, including Cbx3 CD (aa 36 to 79) deletion, Hinge domain (aa 80 to 122) deletion, and CSD (aa 123 to 175) deletion, was constructed as illustrated in Figure 3A. Meanwhile, the Cbx3 aa 165 to 168 mutation, which was previously demonstrated to be critical for the recognition of HP1 proteins to the PxVxL motif,25,26 was also generated from full-length Cbx3 gene expression plasmids. All the plasmids were transfected into 293 cells, and their protein expression levels could be detected by HA antibody as shown in Figure 3A. This shows that the resultant vectors for all 3 Cbx3 deletions and aa 165 to 168 mutation could be expressed efficiently and accurately in cultured cells. To distinguish their functional significance in SMC differentiation, undifferentiated ES cells were transfected with full-length Cbx3 pCMV5-HA-Cbx3 or truncated/mutated forms of Cbx3 expression plasmids ( $\Delta$ CD,  $\Delta$ Hinge,  $\Delta$ CSD, and  $\Delta$ 165/8) (1



Figure 3. The CD and CSD but not the Hinge component of Cbx3 are responsible for Cbx3-induced SMC differentiation. A, Schematic illustration of Cbx3 gene structure, Cbx3 domain deletion, and PxVxL motif potential binding site mutation.  $\Delta CD$ indicates CD deletion;  $\Delta$ Hinge, Hinge domain deletion;  $\Delta$ CSD, CSD deletion;  $\Delta 165/8$ , as 165 to 168 mutation; KD, kilodaltons. B and C, The CD and CSD but not Hinge of Cbx3 were responsible for its potential to promote SMC differentiation. Undifferentiated ES cells were nucleofected using nucleofector II with fulllength Cbx3 pCMV5-HA-Cbx3 or truncated/mutated forms of Cbx3 expression plasmids ( $\Delta$ CD,  $\Delta$ Hinge,  $\Delta$ CSD, and  $\Delta$ 165/8) (1  $\mu$ g per 10<sup>6</sup> cells). Nucleofected cells were plated in dishes coated with 5 µg/mL collagen IV and cultured for 3 to 4 days in DM. Total RNA and protein were harvested and subjected to real-time PCR analysis for gene expression (B) and Western blot analysis for protein levels (C), respectively.  $\alpha$ -Tubulin was included as internal control. D, Bar graphs represent mean±SEM of densitometric analysis (n=3) of the relative protein levels of SM $\alpha$ A and SM-MHC. \*P<0.05 (full-length vs pCMV5), #P<0.05 (truncated/mutated forms of Cbx3 vs fulllength Cbx3).

 $\mu$ g per 10<sup>6</sup> cells) and cultured in SMC DM for 3 to 4 days to allow SMC differentiation. Data from real-time PCR analysis (Figure 3B) and Western blot analysis (Figure 3C and 3D) clearly revealed that deleting CD and CSD and mutating aa 165 to 168 almost abolished Cbx3-induced upregulation of SMC differentiation genes and proteins. However, deletion of the Hinge domain had no such effects, suggesting that the CD and CSD but not the Hinge domain of Cbx3 are essential for Cbx3-induced SMC differentiation. It was noteworthy to point out that the effect of mutation deletion of aa 165 to 168 on SM $\alpha$ A and SM-MHC protein expression is more pronounced than the effect of deletion of CSD (Figure 3C and 3D), suggesting that some regulatory elements that have inhibitory effects on SM $\alpha$ A and SM-MHC protein stabilization exist within CSD of Cbx3.

#### Interaction of Cbx3 with Dia-1 Is Important for Cbx3-Mediated SMC Differentiation, and aa 165 to 168 Within the CSD Are Critical for Such Interaction

Mutated aa 165 to 168 within the CSD failed to induce SMC marker gene upregulation as opposed to the full-length Cbx3 shown in our system (Figure 3B to 3D). This prompted us to speculate that 1 or more Cbx3 partner proteins containing the PxVxL motif would be crucial for Cbx3-mediated SMC marker gene expression. Moreover, growing evidence suggest that protein diaphanous homolog 1 (Dia-1 or mDia-1), which is a mammalian homolog of Drosophila diaphanous and works as an effector of the small GTPase Rho,27 is also involved in actin polymerization28 and SMC differentiation.29 Therefore, we wondered whether Dia-1 could be a direct partner for such a function in our system. The knockdown of Dia-1 with specific siRNA significantly abrogated Cbx3 overexpression-induced SMC gene expression at both the RNA and protein levels (Figure 4A), suggesting that Dia-1 is crucial for Cbx3-mediating SMC differentiation. Unexpectedly, Dia-1 protein levels were not affected by Cbx3 overexpression (Figure 4B). However, by looking at the protein sequence of Dia-1, 1 HP1 box or PxVxL motif (pevql) can be found within the C terminus of Dia-1 (Figure 4C), which strongly indicates that Dia-1 is an ideal candidate partner protein for Cbx3 interaction. Indeed, Cbx3 can directly bind to Dia-1, and importantly, Dia-1 can also directly interact with another SMC-specific transcription factor, SRF, as demonstrated by coimmunoprecipitation (Figure 4D), indicating that Cbx3 can recruit SRF through its interaction with Dia-1. To further determine the molecular domains and the importance of aa 165 to 168 of Cbx3 required for the interactions between Cbx3 and Dia-1, full-length Cbx3 pCMV5-HA-Cbx3 or truncated/mutated forms of Cbx3 expression plasmids ( $\Delta$ CD,  $\Delta$ Hinge,  $\Delta$ CSD, and  $\Delta$ 165/8) (1  $\mu$ g per 10<sup>6</sup> cells) were transfected into undifferentiated ES cells and cultured in SMC DM for 3 to 4 days to allow SMC differentiation. Coimmunoprecipitation analysis was performed to pull down all the protein complexes interacted with full-length or different truncated/mutated forms of Cbx3. It was later discovered that deleting the CSD, particularly the mutated aa 165 to 168, but not CD and Hinge domain significantly inhibited the interactions between Cbx3 and Dia-1 (Figure 4E). These results imply that aa 165 to 168 within the CSD are critical for Cbx3 interaction with Dia-1 and that this interaction is crucial for Cbx3-mediated SMC differentiation.

### **Cbx3 Mediates SMC Differentiation Gene Expression Through Modulating Nuclear Translocalization of Dia-1 and SRF Recruitment** to the Promoter of SMC-Specific Genes

To further explore the molecular mechanisms of Cbx3mediated SMC differentiation, control plasmids (pCMV5), pCbx3-FL-HA, or pCbx3-ΔCSD-HA was introduced into differentiating cells. Double immunostaining with antibodies against Dia-1 and HA (exogenous Cbx3) was used to detect the cellular localization of Dia-1 and exogenous Cbx3 and their interaction during cell differentiation. Data shown in



Figure 4. Cbx3 mediates SMC differentiation gene expression through interaction with Dia-1. A. Dia-1 knockdown abolished Cbx3-induced SMC differentiation. Differentiating ES cells were cotransfected with pCMV5- or pCMV5-Cbx3 and random control siRNA or Dia-1-specific siRNA. Total RNA and protein were harvested and subjected to real-time PCR analysis (left) and Western blot analysis (right), respectively. *α*-Tubulin was included as internal control. \*P<0.05 (control siRNA/pCMV5-Cbx3 vs control siRNA/pCMV5), &P<0.05 (Dia-1 siRNA/pCMV5-Cbx3 vs control siRNA/pCMV5-Cbx3). KD indicates kilodaltons. B, Dia-1 protein levels were not changed by Cbx3 overexpression. C, One PxVxL motif within the Dia-1 protein. D, Coimmunoprecipitation assay showed the interactions of Cbx3/Dia-1/ SRF. The data presented in A, B, and D are representative or an average of 3 independent experiments. E, Coimmunoprecipitation assay revealed that aa 165 to 168 of Cbx3 were responsible for the interaction between Cbx3 and Dia-1. Left, representative data. Right, mean ± SEM of densitometric analysis (n=3), \*P<0.05 (ACSD or A165/8 vs full-length). Relative level of Dia-1 binding was defined as the ratio of the densitometric value of Dia-1 bands in the HA-IP lanes vs that of the related input lanes, with that of the full length set as 1.0. IP indicates immunoprecipitation; IB, immunoblot; HA-IP, IP with antihemagglutinin in antibody.

Figure 5A revealed that Dia-1 was expressed mainly in the cytoplasm in cells transfected with pCMV5 but was translocated into the nucleus when cells expressed high levels of full-length Cbx3. Importantly, after shifting into the nucleus, Dia-1 was colocalized with Cbx3 (white arrows). However, no such phenomenon was observed in cells transfected with CSD-deleted Cbx3 (pCbx3- $\Delta$ CSD-HA). To further confirm this finding, cytosol and nuclear fraction were harvested from the above transfected cells, and Western blot analysis was used to examine the cellular localization and protein levels of Dia-1. We observed that the level of Dia-1 in the nucleus was significantly upregulated by overexpressing full-length Cbx3 (pCbx3-FL-HA), whereas the accumulation of Dia-1 in the nucleus of cells treated with pCbx3- $\Delta$ CSD-HA was dramat-



Figure 5. Cbx3 promotes Dia-1 nuclear translocalization and SRF recruitment to the promoter of SMC specific genes. A and B, Overexpression of full-length Cbx3 (pCbx3-FL-HA) but not CSD-deleted Cbx3 (pCbx3- $\Delta$ CSD-HA) promoted Dia-1 nuclear translocalization. ES cells were nucleofected using nucleofector II with control plasmids (pCMV5), pCbx3-FL-HA, or pCbx3-ΔCSD-HA (1 μg per 10<sup>6</sup> cells). Nucleofected cells were plated onto dishes or cover slips coated with 5 µg/mL collagen IV and cultured for 4 days in DM. Differentiated cells were subjected to immunofluorescence staining with HA and Dia-1 antibodies (A). The intracellular localization of Dia-1 was examined by confocal microscopy. Representative images were taken and are presented here (A, white arrows indicate the colocalization of Dia-1 with Cbx3). Cytosol and nuclear fraction were harvested and subjected to Western blot analysis to examine the cellular localization and protein levels of Dia-1 (B). a-Tubulin and histone 4 were included as internal control for cytosol and nuclear fraction, respectively. B, Left, Representative data. B, Right, Mean±SEM of densitometric analysis (n=3). \*P<0.05 (pCbx3-FL-HA vs pCMV5), #P<0.05 (pCbx3-∆CSD-HA vs pCbx3-FL-HA). C, Dia-1 knockdown abrogated the recruitment of SRF to SMC-specific gene promoter induced by Cbx3. Day 3 to 4 differentiating ES cells were cotransfected with pCMV5- or pCMV5-Cbx3 and random control siRNA or Dia-1-specific siRNA and then cultured for a further 2 days. Chromatin immunoprecipitation assays were performed using antibody against SRF. Rabbit IgG was used as negative control. Aliquots of chromatin before immunoprecipitation served as an input control. Precipitated chromatin DNA was used to amplify the promoter regions of SM $\alpha$ A and SM22 $\alpha$  gene using PCR with specific primers. Left, Representative data. Right, Mean±SEM of densitometric analysis (n=3). \*P<0.05 (pCMV5-Cbx3 vs pCMV5 in the presence of control siRNA), #P<0.05 (Dia-1 siRNA vs control siRNA in the presence of pCMV5-Cbx3).

ically less than that of pCbx3-FL-HA treated cells (Figure 5B). These data strongly suggest that full-length Cbx3 but not CSD-deleted Cbx3 promotes Dia-1 nuclear translocalization and interacts with Dia-1 in the nucleus.

SRF regulates SMC differentiation gene expression through the binding of SRF/myocardin complex to CArG boxes in SMC-specific gene promoters and thus activates SMC-specific gene transcription and expression. Because Dia-1 was demonstrated to directly interact with SRF (Figure 4D), we wondered whether Cbx3 mediates SMC differentiation through modulating the binding capacity of SRF/myocardin complex to SMC specific gene promoters. To test whether Cbx3 overexpression affected the recruitment of SRF/myocardin complex to SMC gene promoter regions and as such whether the interruption of interactions between Cbx3, Dia-1, and SRF plays a role, chromatin immunoprecipitation assays were performed using SRF-specific antibodies and primers specific for SM $\alpha$ A and SM22 $\alpha$  gene promoters containing CArG box. The experiments showed that Cbx3 overexpression significantly increased SRF binding to the SM $\alpha$ A and SM22 $\alpha$  promoters (Figure 5C). Strikingly, the induction of SRF binding to SM $\alpha$ A and SM22 $\alpha$  gene promoters was significantly downregulated by Dia-1 knockdown (Figure 5C), indicating that Cbx3 modulates SRF/ myocardin complex binding to SMC-specific gene promoters through interaction with Dia-1. This is consistent with and further confirmed the above findings that the CSD of Cbx3 is critical for Cbx3 interaction with Dia-1 and that such interaction is crucial for Cbx3-mediated SMC-specific gene expression.

## Disruption of Cbx3 in Neural Crest Cells Resulted in Death of Chick Embryos

Elucidating the in vivo physiological or pathological functions of any gene is critical for evaluating its importance. To investigate the involvement of Cbx3 during cardiovascular development, immunohistochemical staining for Cbx3 antibody was performed in transverse sections of chick embryos (Hamburger-Hamilton stage 20). Cbx3 was strongly expressed in the neural tube, dorsal aorta, and somite (Supplemental Figure IVA). Importantly, Cbx3 expression colocalized with SMC-specific marker SM $\alpha$ A in dorsal aorta as demonstrated by triple-immunofluorescent staining (Supplemental Figure IVB), suggesting that Cbx3 might be involved in vascular smooth muscle development in chick embryo. It was previously demonstrated that neural crest cells (NCCs) are the only cell source to contribute to SMCs of the branchial arch arteries, which form from solid angioblastic cords and canalize in situ at an early stage of chick embryo development.<sup>30–35</sup> Therefore, NCCs can be chosen as an ideal target to manipulate the branchial arch artery smooth muscle development and are an excellent in vivo model for investigating its physiological functions of any genes in SMC differentiation and migration and the development of the vascular system. To examine the involvement of Cbx3 in the differentiation of NCCs into SMCs, the dominant-negative form of Cbx3 was introduced into the right side neural crest by electroporation with pCAb-Cbx3 DNF-GFP or pCAb-GFP (Supplemental Figure VA). The efficiency of gene transfection was checked after 6 hours (Supplemental Figure VB) and 24 hours (Supplemental Figure VC). Setting the electroporator at 7.5 V and 4 pulses has achieved good transfection efficiency, which is similar in both pCAb-Cbx3 DNF-GFP and pCAb-GFP groups (data not shown). Twenty-four hours after transfection, the embryos were checked, and only those with the correct fluorescence labeling (pCAb-Cbx3 DNF-GFP versus pCAb-GFP, n=16 and 13, respectively) were included for further evaluation. Generally, it was observed that chick embryos electroporated with pCAb-Cbx3 DNF-GFP grew slowly, and most of them were smaller than embryos in the pCAb-GFP control group. In particular, 48 hours after electroporation, the branchial arch arteries were not functioning properly, which showed either invisible or reduced blood circulation. Strikingly, at the same day or the following day (72 hours after electroporation), 14 of 16 embryos in the pCAb-Cbx3 DNF-GFP group died. This could be due to the maldevelopment of branchial arches (Figure 6A, right panel), as evidenced by hematoxylin/eosin staining (Figure 6A, right, inset, the broken branchial arch artery indicated by the red arrow). However, only 2 of the 13 embryos in the pCAb-GFP group developed abnormally, with a slowly beating heart, and died at a later stage, whereas all other embryos (n=11) developed normally even at a much later stage (5 days after electroporation) (Figure 6A, left panel).  $\chi^2$  tests showed that the percentage of embryos with maldevelopment in pCAb-Cbx3 DNF-GFP group was dramatically higher than that of pCAb-GFP group (P < 0.00001, Figure 6B).

### Disruption of Cbx3 in NCCs Led to Developmental Defects of the Branchial Arch Arteries

To investigate the cause of death and maldevelopment of chick embryos electroporated with pCAb-Cbx3 DNF-GFP, 5 embryos were chosen randomly for whole-mount immunohistochemistry analysis to determine SM $\alpha$ A expression from both groups. Compared with control (the left side of pCAb-Cbx3 DNF-GFP served as self control, and both sides of pCAb-GFP served as expression vector transfection control), the branchial arch arteries on the right side of embryos electroporated with pCAb-Cbx3 DNF-GFP displayed a much weaker signal in SM $\alpha$ A staining. These branchial arch arteries also showed disrupted structures (Figure 6C), suggesting that SMC differentiation or migration to the branchial arch artery in the embryos electroporated with pCAb-Cbx3 DNF-GFP was either delayed or inhibited. To further elucidate the underlying mechanism of causing embryo death or maldevelopment, another 5 embryos from each group were randomly selected for double immunofluorescent staining for  $SM\alpha A$  and GFP to trace NCC migration and differentiation in the developing chick embryos. Our data revealed that the branchial arch arteries on the right side of embryos in pCAb-Cbx3 DNF-GFP group were not fully developed (Figure 6D, Da-i, R, white arrows), whereas the branchial arch arteries of the left side (Figure 6D, Da-i, L) and both sides of embryos in pCAb-GFP group (Figure 6D, Db-i, L and R) developed normally. More importantly, the migration of NCCs transfected with pCAb-Cbx3 DNF-GFP toward the branchial arch was either delayed or disrupted severely (Figure 6D, Da-i, R, red arrows, and Figure 6E, E2, R, green arrows). Although some cells transfected with pCAb-Cbx3

DNF-GFP could occasionally migrate to branchial arch artery wall, they could not further differentiate into SMCs, which is crucial for vessel solidification and function (Figure 6E, E2, R, white arrows). However, the NCCs transfected with pCAb-GFP not only could migrate into vessel wall but also could differentiate into SM $\alpha$ A-positive cells (Figure 6D, Db-i, R, white arrows). Taken together, these data strongly suggest that disruption of Cbx3 in NCCs results in developmental defects of branchial arch arteries of chick embryos, which lead to early embryonic death.

#### Discussion

In the present study, we have successfully identified nuclear protein Cbx3 as an important SMC differentiation regulator. Crucially, we also demonstrated for the first time that CD and CSD but not Hinge domain of Cbx3 were responsible for Cbx3-induced SMC differentiation. We further identified 4 aa (165 to 168) within the CSD of Cbx3 that are responsible for Cbx3 interaction with Dia-1 and Cbx3-induced SMC differentiation. We also found that Cbx3 mediates SMC-specific gene expression through modulating SRF recruitment to the promoters of specific SMC genes, and the interaction between Cbx3, Dia-1, and SRF plays a crucial role in this process. In the evaluation of Cbx3 function, we found that the disruption of Cbx3 gene expression within NCCs results in maldevelopment and death of chick embryos at an early stage because of the developmental defects of branchial arch arteries of chick embryos. Taken together, our findings indicated that Cbx3 plays an important role in SMC differentiation and is crucial for the functional development of cardiovascular system.

# Functional Domains of Cbx3 and Stem Cell Differentiation

As mentioned above, the structure of the HP1-like proteins comprises a conserved CD and CSD separated by a less conserved hinge region; each of the domains seems to play specific roles in exerting HP1 gene functions. In the present study, by constructing various deletions for each of the 3 domains of the Cbx3 gene and introducing them into differentiating ES cells, we clearly demonstrated that the CD and CSD but not the Hinge domain of Cbx3 are essential for Cbx3-induced SMC differentiation. This indicates that the binding of Cbx3 to methylated Lys9 of histone H336,37 or the histone fold motif of histone H3<sup>38</sup> through CD, the homo-/ heterodimerization of HP1 isoforms,39,40 or the interactions of Cbx3 with its partner proteins<sup>25,26,41</sup> via the CSD is critical for Cbx3-mediating SMC differentiation. Importantly, our data further revealed that mutating aa 165 to 168 within the CSD almost completely abolished SMC differentiation gene upregulation mediated by Cbx3 in our system (Figure 3B and 3C). This implies that the interaction of Cbx3 with 1 or more of partner proteins containing the PxVxL motif is crucial for SMC differentiation from stem cells.

It was reported that Dia-1 (or mDia-1) is highly expressed in a number of SMC-containing organs, including aorta, bladder, lung, and esophagus, and that activation of Dia-1 signaling by RhoA stimulates SMC-specific promoter activity in multiple cell types, including primary aortic SMCs, whereas Dia-1 inhibition significantly decreases SMC-

#### pCAb-GFP pCAb-Cbx3 DNF-GFP B Α









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# Effects of pCAb-Cbx3 DNF-GFP on chick embryonic development

Groups	Chick embryos		Total	Pearson Chi-Square	
	Normal	Maldevelopment	1	Value	P-value
pCAb-GFP	11	2	13	15.082	<0.00001
pCAb-Cbx3 DNF-GFP	2	14	16	]	
Total	13	16	29	1	

SMA/GFP/DAPI pCAb-Cbx3 DNF-GFP pCAb-GFP In situ In situ SMal CbR Ch D GFP/SMaA/DAPI GFP/SMaA/DAPI Da Da-i Db Db-i T îİ

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Figure 6. Disruption of Cbx3 in NCCs results in the maldevelopment of 3rd and 4th of branchial arch artery and death of chick embryos. A, Embryos were electroporated with pCAb-Cbx3 DNF-GFP or pCAb-GFP and observed on the 2nd and 3rd days under a dissection microscope. In the right panels, note the abnormal structure of the branchial arch artery (black arrows in green boxes) and at the hemorrhage site (green arrow). Green arrow in the bottom right panel indicates the broken 4th branchial arch artery, which was sectioned and stained by hematoxylin/eosin (inset). Scale bars=1 mm. B,  $\chi^2$  analysis was performed using SPSS 17.0 software on the both groups, and pCAb-Cbx3 DNF was found to significantly affect chick embryonic development (P<0.00001). C, Defect in branchial arch artery caused by Cbx3 misexpression in chick embryos. Ca and Cb, Representative chick embryos electroporated with pCAb-Cbx3 DNF-GFP (Ca) or pCAb-GFP (Cb). Images were taken before harvest. Notably, embryos from pCAb-Cbx3 DNF-GFP group showed invisible blood circulation of branchial arch

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specific transcription in SMCs.<sup>29</sup> Consist with these findings, data from our cotransfection experiments with specific Dia-1 siRNA and Cbx3 expression plasmids revealed that Dia-1 is a mediator in Cbx3-induced SMC differentiation. Furthermore, by searching the protein sequence of Dia-1, 1 HP1 box or PxVxL motif (pevql) can be found within the C terminus of Dia-1 (Figure 4C). It is also noteworthy that the effects of Dia-1 on SMC-specific transcription require the presence of SRF<sup>29</sup> and that Dia-1 potentiates SRF activity through its effects on actin polymerization<sup>42</sup> and cooperation with lin11, isl-1, mec-3 (LIM) kinase.43 Therefore, we speculate that interactions among Cbx3, Dia-1, and SRF occur during stem cell differentiation and that such interactions, at least in part, contribute to the underlying molecular mechanism of Cbx3mediated SMC differentiation gene regulation. Data from our coimmunoprecipitation experiments strongly support this notion (Figure 4D and 4E). Importantly, data from our mutation experiments by substituting the sequence Thr-Arg-Lys-Leu for Ile-Ala-Phe-Tyr at aa 165 to 168 of Cbx3 further revealed that aa 165 to 168 within the CSD of the Cbx3 protein is critical for the interactions between Cbx3 and Dia-1 (Figure 4E). The interaction between Cbx3 and Dia-1 mediated by aa

Figure 6. (continued) arteries 3 and 4 (Ca, red square) on the right side, whereas the pCAb-GFP group displayed normal circulation (Cb, red square). The embryos were harvested for whole-mount immunohistochemical staining for SMαA. Strikingly, disrupted branchial arch arteries with relative weak SMαA staining (CaR, red square) could be observed in the right side of embryos with Cbx3 misexpression, whereas well-formed branchial arch arteries 3 and 4 with strong SMaA expression (CaL, CbL, and CbR, red squares) could be observed in the left side of embryos from both groups (CaL and CbL) and in the right side of embryos from control group (CbR). Representative images from 5 embryos in each group are presented here. Scale bars=400  $\mu$ m. iii and iv indicate 3rd and 4th branchial arches, respectively. D, Disruption of Cbx3 in NCCs led to developmental defects of 3rd and 4th of branchial arch arteries in chick embryos. Da, Embryos electroporated with pCAb-Cbx3 DNF-GFP showed significantly reduced blood circulation in branchial arches (green box). Da-i, Triple staining for 4',6-diamidino-2phenylindole (DAPI) (blue), SMaA (red), and GFP (green) with sections cut from embryos in Da. Note that GFP-labeled NCCs could not migrate into branchial arch arteries on the right side (Da-i, R, red arrows), leading to developmental defects of 3rd and 4th of branchial arches (Da-i, R, white arrows and green box). R, p-CAb-Cbr, DNF-GFP; L, salt control. Db, The cardiovascular system and blood circulation of embryos in control group developed normally (green box). Db-i, From sections cut from embryos shown in Db, the branchial arch arteries showed no difference or developmental defects on either side. White arrows indicate that GFP-labeled NCCs (red) were incorporated into the vessel wall and expressed SM $\alpha$ A (green). R, pCAb-GFP; L, salt control. Representative images from 5 embryos in each group are presented here. Scale bars=1 mm (Da, Db), 50  $\mu$ m (Da-i, Db-i). ii, iii, and iv indicate 2nd, 3rd and 4th branchial arches, respectively. E, The migratory ability and SMC differentiation potential of NCCs in branchial arch arteries were affected by Cbx3 misexpression. E1, The embryos were electroporated with pCAb-Cbx3 DNF-GFP and showed frail heart beating on the 2nd day, possibly because of blood loss. E2, Triple staining for DAPI (blue), SM $\alpha$ A (green), and GFP (red). Note that labeled NCCs could not migrate into 4th branchial arch artery (green arrow) or differentiate into SMCs (white arrows) in the right side (red box). R, pCAb-Cbx3 DNF-GFP; L, self control. Representative images from 5 embryos are presented here. Scale bars=1 mm (E1), 50  $\mu$ m (E2, L and R). iv indicates 4th branchial arch.

165 to 168 of Cbx3 and the PxVxL motif of Dia-1 involves hydrophobic binding because all 4 amino acids (Ile-Ala-Phe-Tyr) have hydrophobic side chains that facilitate their binding to the PxVxL motif (the amino acids Val and Leu in PxVxL motif also have hydrophobic side chains). However, this binding can be disrupted when Ile-Ala-Phe-Tyr at aa 165 to 168 of Cbx3 is replaced with Thr-Arg-Lys-Leu, because Arg and Lys have positive charges.

It is believed that SRF and its cofactor myocardin play crucial roles in SMC differentiation and embryonic cardiovascular system development.44-46 In the present study, we demonstrated that the expression levels of SMC-specific transcription factors (SRF, myocardin, and MEF2c) were regulated by Cbx3, although its underlying mechanisms remain to be elucidated. However, as we and others have previously shown that the binding of the SRF/myocardin complex to the CArG box in the promoter region of SMCspecific genes is critical for SMC differentiation from stem cells<sup>19,47</sup> and that SRF-CArG interaction is required for transcriptional activation of SMC genes,48 we speculate that Cbx3 mediates SMC differentiation gene expression through modulating SRF/myocardin complex binding to SMCspecific gene promoters. Indeed, data from a chromatin immunoprecipitation assay using SRF antibody have clearly shown that Cbx3 overexpression significantly increased SRF binding to SM $\alpha$ A and SM22 $\alpha$  gene promoters (Figure 5C). More importantly, the disruption of the interaction of Cbx3, Dia-1, and SRF within the promoter region of SMC genes by knocking down Dia-1 significantly abolished the increased binding capacity of SRF to SM $\alpha$ A and SM22 $\alpha$  gene promoters induced by Cbx3 overexpression. This suggests that Cbx3 mediates SMC differentiation gene expression through modulating SMC-specific transcription factor expression levels and the binding of SRF/myocardin complex to SMC-specific gene promoters. In addition, our unpublished data also showed that during stem cell differentiation, H3K9 methylation was dramatically enriched within SMC-specific gene promoter region such as SM $\alpha$ A. We therefore propose that during the early phases of stem cell differentiation, histone modifications such as H3K9 occur within or around the promoter region of SMC differentiation genes, which can be recognized specifically by Cbx3 through the CD. After binding, Cbx3 functions as a bridge/anchor protein to recruit or catch SMC-specific transcription factor or regulator, SRF, and Dia-1 to the chromosome. It will in turn facilitate SRF/Dia-1 binding to SRF-specific binding elements (CArG elements) or unknown Dia-1 binding elements within the promoter-enhancer regions of SMC-specific genes, which then regulates SMC differentiation from ES cells (Supplemental Figure VI).

### **Cbx3 and Cardiovascular Development**

SMC differentiation is a critical step in the formation of the cardiovascular system. During the process of arteriogenesis, the smooth muscle precursor cells are recruited to the primitive endothelial tubes and subsequently differentiate into SMCs. Any mechanistic findings about SMC differentiation are important for understanding the cardiovascular development and warrant further in vivo study to explore its

physiological functions. Chick embryos can be a suitable model to study the functions of Cbx3 gene in vivo, because it is easy to access and carry out micromanipulation in fertile eggs during chick development. With the establishment of molecules transfer system in vivo by electroporation using dominant-negative forms, morpholinos, or siRNAs, the contributions of the precise cell lineage to embryonic organ/ tissue development and the importance of specific molecular pathways in appropriate tissue/organ/system formation during embryonic development can be investigated extensively.49 It is known that NCCs are the only cell population that contributes to SMCs during early branchial arch arteriogenesis.<sup>30,31</sup> Moreover, NCCs residing at the level of rhombomeres 2, 4, 6, and 7 will migrate into branchial arches 1, 2, 3, and 4 and differentiate toward SMCs at days 3 and 4 in embryonic chick development.50-52 Therefore, these NCCs serve as a specific target for studying the differentiation of embryonic progenitors into SMCs. To verify the impact of Cbx3 in SMC differentiation during branchial arch arteriogenesis, a dominant-negative form of Cbx3 (pCAb-Cbx3 DNF-GFP) was used for electroporation. Because the vector also has a GFP coding domain, the efficiency of vector transfer can be easily monitored in vivo. As expected, the development of embryos in the pCAb-Cbx3 DNF-GFP group has been significantly retarded, whereby most of them grew smaller in size compared with the pCAb-GFP group during harvesting. This could be attributed to the deficiency of nutrient distribution in the chick embryo's blood circulation because weakness or absence of the blood circulation in the branchial arch arteries was consistently observed in the pCAb-Cbx3 DNF-GFP group (Figure 6A, right panel, and Figure 6C, Ca, and 6D, Da). The rate of maldevelopment in pCAb-Cbx3 DNF-GFP group was also significantly increased (Figure 6B). Moreover, embryos in the pCAb-Cbx3 DNF-GFP group had apparently disrupted/broken branchial arch arteries, as indicated by hematoxylin/eosin or immunofluorescence staining (Figure 6A, inset, and Figure 6D, Da-i, and 6E, E2). It is noteworthy that the different levels of severity of the branchial arch artery malformation and embryonic maldevelopment observed in the pCAb-Cbx3 DNF-GFP group may be due to the different levels of Cbx3 misexpression in NCCs or individual variations of chick embryos. Nevertheless, almost all embryos with branchial arch artery malformation died at a later stage. These results confirm the impact of Cbx3 on SMC formation during in vivo development.

In summary, Cbx3 upregulation during stem cell differentiation into SMCs has been demonstrated at both RNA and protein levels. Our data from gene gain and loss experiments in the differentiating ES cells indeed confirmed that Cbx3 is essential for SMC differentiation. We have also successfully established an in vivo neural crest targeting model and demonstrated that Cbx3 plays an important role in regulating NCC migration and the differentiation of NCCs toward SMCs during chick embryonic development. Our findings highlight the importance of Cbx3 for SMC differentiation from stem cells and branchial arch artery development, which may contribute significantly to the understanding of cardiovascular development.

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# SUPPLEMENT MATERIAL.

# Cbx3 is essential for stem cell differentiation to smooth muscles *in vitro* and in embryonic branchial arch arteriogenesis

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# **Detailed Methods & Materials**

**Materials.** Antibodies against Cbx3/HP1 $\gamma$  (goat, N-15, sc-10213), SRF (rabbit, G-20, sc-335), MEF2c (goat, C-17, sc-13268), and Dia-1 (goat, C-20, sc-10885) were purchased from Santa Cruz Biotech, USA. Antibody against Smooth Muscle Myosin Heavy Chain (SM-MHC) was from AbD Serotec (Rabbit, AHP1117). Antibodies against HA (H6908),  $\alpha$ -tubulin (mouse), histone 4 (Rabbit) and monoclonal anti- $\alpha$  smooth muscle actin (SM $\alpha$ A) (Clone 1A4, A5228) were from Sigma. All secondary antibodies were from Dako, Denmark. All other materials used in this study were purchased from Sigma except those indicated.

Cell culture and SMC differentiation. Detailed protocols for mouse ES cell (ES-D3 cell line, CRL-1934; ATCC, Manassas, USA) culture and SMC differentiation were described in our previous studies<sup>1-5</sup>. Briefly, for SMC differentiation, undifferentiated ES cells were seeded on mouse collagen IV ( $5\mu$ g/ml)-coated flasks or plates in differentiation medium [DM, MEM alpha medium (Gibco) supplemented with 10% FBS, 0.1mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin] for 3 to 7 days prior to further treatment. The medium was refreshed every other day. Detailed procedures for the isolation and culture of Sca-1+ SMC precursor cells from differentiating ES cells were described in our previous study<sup>1</sup>.

Immunoblotting. Cells were harvested and lysed in lysis buffer (50mM Tris-Cl pH 7.5, 150mM NaCl, 1 mM EDTA pH 8.0) supplemented with protease inhibitors and 0.5% Triton by sonication for whole cell lysate, or with Hypotonic buffer (10 mM HEPES-KOH pH 7.2, 1.5 mM MgCl2, 10 mM KCl) and High-salt buffer (20 mM HEPES-KOH pH 7.2, 25% Glycerol, 1.5 mM MgCl2, 420 mM KCl, 0.2 mM EDTA) supplemented with protease inhibitors and 0.5% NP-40 for nuclear and cytoplasmic fractions. Forty micrograms of protein was separated by SDS-PAGE with 4%~20% Tris-Glycine gel (Invitrogen, Carlsbad, CA, USA) and subjected to standard Western blot analysis.

**Co-immunoprecipitation.**The procedure was performed as described previously <sup>3</sup>. In brief, cell samples were lysed by rotation for 1 hour at 4°C. 1 mg whole lysate was subjected to a standard co-

immunoprecipitation procedure. Lysates were pre-cleared with normal IgG, then incubated with appropriate specific antibodies for 2 hours at 4°C and precipitated by incubation for a further 2 hours with protein-G-Sepharose beads. For un-conjugated antibody, 2µg antibody and 10µl protein-G beads (Sigma) were used for each immunoprecipitation assay. For agarose-conjugated antibody, 10µl such beads were directly used for each immunoprecipitation assay. Precipitated proteins were resolved by SDS gel electrophoresis and subsequently immunoblotted with related specific antibodies.

Indirect immunofluorescent staining for cells. Indirect immunofluorescent assay was performed as described before <sup>1-2</sup>. Briefly, Cells were labelled with isotype IgG control or antibodies against Cbx3/HP1 $\gamma$ , or Dia-1 and HA, and visualized with appropriate secondary antibodies conjugated with phycoerythrin (PE) or fluorescein isothiocyanate (FITC) (DAKO). Cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma). Images were examined using SP5 confocal microscope with Plan-NEOFLUAR 63x objective lenses and Leica TCS Sp5 software (Leica, Germany) at room temperature, and were processed with Photoshop software (Adobe).

**Real-time RT-PCR.** Real-time RT-PCR was performed as described before <sup>2</sup>. Briefly, total RNA was extracted from cells using RNeasy kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed using an Improm-II<sup>TM</sup> RT kit (Promega, Madison, WI, USA) with RNase inhibitor (Promega), and Random primers (Promega). Simultaneous RT reactions were performed without the addition of reverse transcriptase to control the possible transcription of contaminating genomic DNA. Primers were designed using Primer Express software (Applied Biosystems) and the published sequence for the mouse genes are available here (**Table S1**). Relative mRNA expression level was defined as the ratio of target gene expression level to 18S expression level with that of the control sample set as 1.0.

**Cbx3 and its deletion/mutation cloning**. Mouse full length of Cbx3 gene was amplified using RT-PCR from day 3 of differentiating ES cells with primer set as shown in **Table S1** and cloned into Kpn *VPst* I sites of the pCMV5-HA expression vector, designated as pCMV5-HA-Cbx3 (full legenth). Cbx3 CD (aa36-79), Hinge domain (aa80-122) and CSD (aa123-175) deletions and amino acid 165-168 mutation were generated from pCMV5-HA-Cbx3 with specific primers shown in **Table S1**, designated as pCMV-HA-Cbx3 $\Delta$ CD ( $\Delta$ CD), pCMV-HA-Cbx3 $\Delta$ Hinge ( $\Delta$ Hinge), pCMV-HA-Cbx3 $\Delta$ CSD ( $\Delta$ CSD) and pCMV-HA-Cbx3 $\Delta$ 165/8 ( $\Delta$ 165/8), respectively. Two restriction enzyme cutting sites were introduced into the mutated plasmids for easy and prompt identification of resultant constructs can be identified easily and promptly (**Figure 3A**). These vectors will express truncated Cbx3 or PxVxL pentapeptide binding site mutated Cbx3 with N-terminal tagged with HA epitope. Mouse Cbx3 chromoshadow domain (aa 118–176) were cloned into pCAb-GFP and used as Cbx3 dominant negative form (pCAb-Cbx3 DNF-GFP) as described previously (A kind gift from Dr. Andrea Streit, King's College London)<sup>6</sup>. All the vectors were verified by DNA sequencing.

**Nucleofection.** Nucleofection was performed as described before <sup>2</sup>. Briefly, for transient transfection, Cbx3 plasmids pCMV5-HA-Cbx3 were introduced into undifferentiated ES cells with mouse ES cell nucleofection kit (amaxa, VPH-1001) using nucleofector II (amaxa, Germany) according to the manufacturer's instructions. Transfected cells were plated in dishes coated with  $5\mu$ g/ml of collagen IV and cultured for 3-4 days in the DM to allow SMC differentiation.

siRNA experiments. The Cbx3 (or HP1 $\gamma$ ) siRNA (sc-35590), Dia-1 siRNA (sc-35191), and fluorescein conjugated non-targeting control siRNA-C (sc-44240) were purchased from Santa Cruz Biotech. ES cells were cultured on collagen IV-coated 6-well plates for 2-4 days, and 10 µl of 10µM siRNA was introduced with siIMPORTER transfection reagents (Millipore) according to the protocol provided. Cells were harvested at 48 or 72 hours after transfection and real-time RT-PCR and Western blot analysis were performed.

**Chromatin immunoprecipitation (ChIP).** The ChIP assays were performed as described previously<sup>4-5</sup>. Differentiating ES cells co-transfected with control siRNA or Dia-1 siRNA and

pCMV5 or pCMV5-Cbx3 were treated with 1% (v/v) formaldehyde at room temperature for 10 min and then quenched with glycine at room temperature. The medium was removed, and cells were harvested for sonication. The sheared samples were diluted into 1 ml immunoprecipitation buffer containing 25 mM Tris-HCl, pH 7.2, 0.1% NP-40, 150 mM NaCl, 1 mM EDTA, and immunoprecipitation was conducted with antibody raised against SRF, together with single-strand salmon sperm DNA saturated with protein-G-Sepharose beads. Normal IgG was used as a control. The immunoprecipitates were eluted from the beads using 100 µl elution buffer (50 mM NaHCO3, 1% SDS). A total of 200 µl proteinase K solution was added to a total elution volume of 300 µl and incubated at 60°C overnight. Immunoprecipitated DNA was extracted, purified, and then used to amplify target sequences by PCR. The primers used to amplify the promoter regions of SM $\alpha$ A and SM22 $\alpha$  were shown in **Table S1**. The data obtained from three independent experiments were quantified, using Image Quant TL (Amersham Bioscience). Fold of relative binding was defined as the ratio of band intensity in ChIP samples to that in the Input sample.

**Chick Embryo Cultivation and Staging**. Fertilized chick eggs were obtained from Joyce and Hill (Farm) and incubated at 38.5°C in a humidified incubator. Hamburger and Hamilton (HH) staging was applied throughout the study <sup>7-8</sup>.

Whole-mount immunohistochemistry. The chick embryos were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight. After graded dehydration in Methanol, the embryos were incubated with freshly prepared Methanol:DMSO:H2O2 (4:1:1) at room temperature for 5 hours. Then the embryos were rehydrated in a series of 50% Methanol/PBS, PBS, and blocked with PBSMT (2% non-fat milk powder, 0.5% Triton X-100 in PBS). Subsequently, the embryos were incubated with mouse anti-SM $\alpha$ A (1:2000, Sigma), anti-Cbx3 (1:500, Santa Cruze) in PBMST at 4°C overnight. Mouse IgG was used at same concentration as control (**data not shown**). Followed by HRP conjugated secondary antibody and DAB staining, the embryos were fixed in 4% PFA for 10 mins, rinsed in 25%, 50%, and mounted with 75% glycerol/PBS for observation.

*In vivo* electroporation on chick embryos. *In vivo* electroporation carried out on chick embryos was performed as previously described but with some modifications <sup>9-11</sup>. Eggs were incubated in a humidified incubator at 38°C for around 36 hours. A window was opened at the top of the egg shell using a blade, and embryos at HH 10/10<sup>-</sup>/10+ were electroperated with pCAb-Cbx3 DNF-GFP (1 $\mu$ g/ $\mu$ l) or pCAb-GFP (1 $\mu$ g/ $\mu$ l) as control. Electroperator was set at 7.5 volts, 4 pulses, length of 50ms and interval of 1000ms. Constructs were mixed with 60% sucrose and fast green for better visualization before electroporation. After electroporation, the eggs were well sealed and placed back into the incubator, window side up, and incubated until the required developmental stages.

Immunofluorescent staining for sections. Chick embryos were fixed in 4% formaldehyde (FA) in PBS for 1 hour, rinsed in PBS and dehydrated by 5% sucrose, 20% sucrose (Sigma) in PBS at 4°C overnight. Then the embryos were embedded in 7.5% gelatin (Sigma G2500, 300-Bloom) in 15% Sucrose/PBS). Sections were cut at 10 um, picked up on Superfrost Microscope Slide (Thermo Scientific), air dried for staining or stored in -80 °C. Prior to antibody staining, gelatin was removed in 42°C PBS for 10 mins. The sections were then rinsed in PBS and blocked with 10% Goat Serum in PBS (Dako) for 1 hr at room temperature in a humid chamber. The incubation with primary antibody diluted in blocking buffer was performed at cold room (4°C) overnight. Followed by incubation with Alexa Fluor®597/468 conjugated secondary antibodies. Sections were then incubated with DAPI (1:1000, Sigma) for 2 mins. The primary antibodies used are as follows: mouse anti-SM $\alpha$ A (1:500, Sigma); rabbit anti-SM $\alpha$ A (1:100, Abcam); mouse/rabbit anti-GFP (1:1000, Abcam). Mouse or Rabbit IgG was used at the same concentration as control (data not shown). Images were assessed with Axioplan 2 imaging microscope with Plan-NEOFLUAR 20×, NA 0.5, objective lenses, AxioCam camera, and Axiovision software (all Carl Zeiss MicroImaging, Inc.) at room temperature, and were processed with Photoshop software (Adobe).

**Statistical analysis**. Data were expressed as mean±SEM and analyzed using a two-tailed student's *t*-test for two-groups comparision or one-way ANOVA for comparing different groups. Chi-Square

analysis was performed using SPSS 17.0 software to compare the percentage of embryonic

maldevelopment in two groups. A value of P < 0.05 was considered statistically significant.

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Gene	<b>Forward (5'-3')</b>	<b>Reverse (5'-3')</b>	Application
names			11
18s	CCCAGTAAGTGCGGGTCATAA	CCGAGGGCCTCACTAAACC	Real-time RT-PCR
SMαA	TCCTGACGCTGAAGTATCCGAT	GGCCACACGAAGCTCGTTATAG	Real-time RT-PCR
SM22a	GAT ATG GCA GCA GTG CAG AG	AGT TGG CTG TCT GTG AAG TC	Real-time RT-PCR
h1-Calponin	GGT CCT GCC TAC GGC TTG TC	TCG CAA AGA ATG ATC CCG TC	Real-time RT-PCR
SM-MHC	AAG CAG CCA GCA TCA AGG AG	AGC TCT GCC ATG TCC TCC AC	Real-time RT-PCR
SRF	CCTACCAGGTGTCGGAATCTGA	TCTGGATTGTGGAGGTGGTACC	Real-time RT-PCR
Myocardin	TCAATGAGAAGATCGCTCTCCG	GTCATCCTCAAAGGCGAATGC	Real-time RT-PCR
MEF2C	AAGCCAAATCTCCTCCCCCTAT	TGATTCACTGATGGCATCGTGT	Real-time RT-PCR
Cbx3	GAACGAATAATCGGCGCCA	ATGTTCGCCTCCTTTGCCA	Real-time RT-PCR
SMaA-P	GGCAACACAGGCTGGTTAAT	AGCCCCTGTCAGGCTAGTCT	CHIP assay
SM22α -P	GCAGGTTCCTTTGTCGGGCCA	CTGCTTGGCTCACCACCCCG	CHIP assay
pCMV5-Cbx3	CAGATCGGTACCTTGGCCTCCAATAAAACTAC	TGCAACCTGCAGTTGTGCTTCATCTTCAGGAC	Cbx3 clone
ΔCD	CTTTCTCAAAAAGCTGGTAAAG	CTTTACTTTTTCTACCACAAA	CD deletion
ΔCSD	CTTTCTTGTCCTGAAGATGAAGC	CTTGTCGAGACCTCTGGCAAAGC	CSD deletion
Δhinge	CTTCCTGAACGAATAATCGG	CTTATTAAGAAATGCTTCAA	Hinge deletion
Δ165/8	AAGCTTGAGGAGCGGCTGACTTG	ACGCGTGACAATCTGAGGACACT	Aa165-168 mutation
pSPT18-Cbx3 (Gallus)	AGTCACCTGCAGATGGGAAAGAAACAAAATGGC	TGATGCGGTACCTTATTGTGCTTCATCTTCAG	Chick Cbx3 clone

# Supplementary table I: Primer sets used in the present study

# **Supplementary Figures**

# Figure I. Cbx3 plays a role in myofilament organization in differentiated SMCs.

Day 4 differentiating ES cells were transfected with Cbx3 siRNA or control random siRNA (A), and undifferentiated ES cells were transfected with Cbx3 overexpression plasmids (pCbx3) or control plasmids (pCMV5) (B), respectively. Cells were cultured for further 48 or 72 hours and subjected to immunofluorescent analysis with antibodies against SMC specific myofilament proteins such as SM $\alpha$ A and SM-MHC.

# Figure II. Cbx3 plays an important role in SMC differentiation from SMC precursors.

Sca-1<sup>+</sup> cells were transfected with control (pCMV5) or Cbx3 expression plasmids pCMV5-Cbx3  $(1\mu g/10^{6} \text{ cells})$  and differentiated towards SMCs as described in Figure S1. Total RNA and protein were harvested and subjected to real-time PCR analysis for gene expression (A) and western blot analysis for protein levels (B), respectively.  $\alpha$ -tubulin was included as internal control. The data presented here were representative or an average of three independent experiments. Significant difference from control, \*P<0.05.

## Figure III: Cbx3 may play a role in cardiomyocyte, but not endothelial cell gene regulation.

Undifferentiated ES cells were nucleofected using nucleofector II with pCMV5-Cbx3 and pCMV5  $(1\mu g/10^6 \text{ cells})$ . Nucleofected cells were plated in collagen IV-coated dishes and cultured for 3-4 days in the DM. Total RNA were harvested and subjected to real-time PCR analysis for endothelial (Flt-1, CD144) and cardiomyocyte (Tnnc 1, actc 1) cell lineage gene expression (**A**). The same RNA samples from Figure 2A were subjected to real-time PCR analysis for endothelial/cardiomyocyte cell lineage gene expression (**B**). The data presented here were an average of three independent experiments.

# Figure IV. Cbx3 expression pattern in the development of chick embryos.

(A) Immunohistochemical staining for Cbx3 with transverse sections of chick embryos (HH20) showed that Cbx3 is strongly expressed in the cells within neural tube (NT), dorsal aorta (DA) and

somite (SM). (B) Triple staining for DAPI (blue), SM $\alpha$ A (green) and Cbx3 (red) showed colocalization of Cbx3 and SM $\alpha$ A in dorsal aorta (HH20, transverse section). Representative images from 5 embryos are presented here. Scale bar represents 50 $\mu$ m.

# Figure V: In vivo electroporation on neural crest in chick embryos.

(A) A schematic illustration of *in vivo* electroporation on neural crest in chick embryos. (B) Expression plasmids harboring GFP DNA was injected into neural tube and driven to neural crest at the level of rhombomere 2-7 on the right-hand side in 7.5 volts electric field. Migrating neural crest cells labeled by GFP (red circle) can be observed after 6 hrs of electroporation. Neural tube was also shown within the white line. (C) GFP labeled neural crest cells migrated into branchial arches (red circle) after 24 hrs of electroporation.

# Figure VI. Proposal model of Cbx3 in SMC differentiation.

During the early phases of ESC differentiation, histone modifications such as H3K9 occur within the promoter region of SMC differentiation genes, which can be recognised specifically by Cbx3 through CD domain. After binding, Cbx3 functions as bridge/anchor protein to recruit/catch SMC specific transcription factor, SRF, through interaction with Dia-1, to chromosome, which in turn facilitates SRF binding to CArG elements within promoter-enhancer region of SMC-specific genes, therefore regulating SMC differentiation from ES cells. **Figure I** 



Figure II

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В

A



Figure V



# Figure VI

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