Chick *Pcl2* regulates the left-right asymmetry by repressing *Shh* expression in Hensen's node

Shusheng Wang[1], Xueyan Yu[1], Tao Zhang[1], Xiaoyun Zhang[1], Zunyi Zhang[1] and YiPing Chen[1,2,*]

[1]Division of Developmental Biology, Department of Cell and Molecular Biology and Center for Bioenvironmental Research, Tulane University, New Orleans, LA 70118, USA
[2]College of Bioengineering, Fujian Normal University, Fuzhou, Fujian Province, 350007, P. R. China
*Author for correspondence (e-mail: ychen@tulane.edu)

Accepted 18 May 2004

Development 131, 4381-4391
Published by The Company of Biologists 2004
doi:10.1242/dev.01269

Summary

Asymmetric expression of *sonic hedgehog* (*Shh*) in the left side of Hensen's node, a crucial step for specifying the left-right (LR) axis in the chick embryo, is established by the repression of *Shh* expression in the right side of the node. The transcriptional regulator that mediates this repression has not been identified. We report the isolation and characterization of a novel chick *Polycomblike 2* gene, chick *Pcl2*, which encodes a transcription repressor and displays an asymmetric expression, downstream from *Activin-βB* and *Bmp4*, in the right side of Hensen's node in the developing embryo. In vitro mapping studies define the transcription repression activity to the PHD finger domain of the chick Pcl2 protein. Repression of chick *Pcl2* expression in the early embryo results in randomized heart looping direction, which is accompanied by the ectopic expression of *Shh* in the right side of the node and *Shh* downstream genes in the right lateral plate mesoderm (LPM), while overexpression of chick *Pcl2* represses *Shh* expression in the node. The repression of *Shh* by chick Pcl2 was also supported by studies in which chick *Pcl2* was overexpressed in the developing chick limb bud and feather bud. Similarly, transgenic overexpression of chick *Pcl2* in the developing mouse limb inhibits *Shh* expression in the ZPA. In vitro pull-down assays demonstrated a direct interaction of the chick Pcl2 PHD finger with EZH2, a component of the ESC/E(Z) repressive complex. Taken together with the fact that chick *Pcl2* was found to directly repress *Shh* promoter activity in vitro, our results demonstrate a crucial role for chick *Pcl2* in regulating LR axis patterning in the chick by silencing *Shh* in the right side of the node.

Key words: *Polycomblike 2* gene, Transcriptional repressor, Left-right asymmetry, *Shh*, Chick embryo

Introduction

The left-right (LR) asymmetry of an embryo, which is characterized by asymmetric structures and/or asymmetric placement of internal organs, is a vital feature of vertebrate embryogenesis. The rightward looping of the developing heart represents the first morphological indication of LR asymmetry. A failure in the normal development of the LR axis is associated with laterality defects, including isomerism, heterotaxia and situs inversus. Recent studies demonstrate that the initial positional information that specifies the LR axis originates in/around the node, a disc-shaped structure at the anterior end of the primitive streak in developing chick and mouse embryos. In the chick, the asymmetric expression of *Activin-βB* and *ActRIIA* in the right side of Hensen's node induces the expression of *Bmp4*, via chick Mid1, which then induces right-sided *Fgf8* expression (Levin et al., 1995; Levin et al., 1997; Monsoro-Burg and Le Douarin, 2001; Granata and Quaderi, 2003). The asymmetrically expressed BMP4 inhibits *Shh* expression in the right side of the node, thereby restricting *Shh* expression to the left side of the node (Monsoro-Burg and Le Douarin, 2001). This asymmetric pattern of gene expression in the node is translated into asymmetric gene expression in the lateral plate mesoderm (LPM), which further regulates the LR axis development. The asymmetrically expressed *Shh* is responsible for the asymmetric expression of *Nodal* in the left LPM. *Caronte* (*Car*), a member of *Cerberus*/*Dan* gene family, was shown to mediate *Nodal* induction in the left LPM by antagonizing symmetrically expressed BMP activity (Rodriguez-Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). However, it has recently been demonstrated that BMPs are also involved in the activation of *Nodal* in the left LPM through the activation of CFC, a member of the EGF-CFC family, and ActRIIA (Schlange et al., 2001; Schlange et al., 2002; Piedra and Ros, 2002). Meanwhile, *Nodal* expression around the node is also independently regulated by the *Wnt*/*β-catenin* signaling pathway, a protein kinase A-dependent pathway and Notch signaling pathway (Garcia-Castro et al., 2000; Kawakami and Nakanishi, 2001; Rodriguez-Esteban et al., 2001; Raya et al., 2004). Studies in other vertebrate species, including the mouse and *Xenopus*, indicate that the asymmetrical expression of *Nodal* in the left LPM is conserved across species and is crucial for establishing initial LR asymmetry (Collignon et al., 1996; Hyatt et al., 1996; Lowe et al., 1996; Sampath et al., 1997). Downstream from this left side signaling cascade reside *Nkx3.2* and *Pitx2* (Campione et al., 1999; Logan and Tabin, 1998; Piedra et al., 1998; Ryan et al., 1998; St Amand et al., 1998;

Yoshioka et al., 1998; Schneider et al., 1999). Pitx2, a homeodomain transcription factor, is possibly involved in the morphogenetic execution of the LR asymmetry. In the right side of Hensen's node, FGF8 is required to upregulate *SnR* expression in the right LPM and to prevent the left-sided pathway from becoming inappropriately activated in the right LPM (Boettger et al., 1999; Isaac et al., 1997). *Lefty1*, a downstream gene from *Shh*, is expressed in the prospective floor plate and functions as a midline barrier to prevent induction of *Nodal* and *Pitx2* on the right side (Tsukui et al., 1999). Although significant progress has been made towards the understanding the molecular mechanism of LR axis development in vertebrates (Capdevila et al., 2000), the precise mechanism of how the LR axis is specified and established remains largely elusive. A central puzzle in the existing pathways that regulate LR axis development is how the initial symmetric expression of *Shh* in Hensen's node later becomes restricted to the left side of the node.

During embryonic development, once cell fate is specified, cell identity is maintained by epigenetic functions. The *Trithorax*-group (Trx-G) and *Polycomb* group (PcG) genes are part of the widely conserved cell memory system that maintains both the active and silenced states of transcription patterns (Kennison, 1995). The PcG proteins are encoded by about 40 genes in *Drosophila*, which include *Polycomb*, *Polyhomeotic*, *Polycomblike* (*Pcl*) and *posterior sex comb*. PcG mutants exhibit posterior homeotic transformation because of the ectopic expression of the *HOM-C* genes. Structural homologs of the *Drosophila* PcG proteins have been identified in mammals, and the mechanisms by which these proteins silence target genes and stabilize developmental decisions are likely to be conserved between *Drosophila* and vertebrates. Similar to *Drosophila*, mutations in different mammalian PcG genes cause posterior axial transformations of the mouse skeleton and anterior shifts of Hox expression boundaries (Akasaka et al., 1996; Schumacher et al., 1996; Coré et al., 1997; Takihara et al., 1997; Suzuki et al., 2002). In addition to the regulation of *Hox* gene expression, PcG gene members have been shown recently to regulate developmentally important genes, such as *hedgehog* (Maurange and Paro, 2002), and cell cycle regulation genes, such as *Rb* (Dahiya et al., 2001). In *Drosophila*, *Polycomblike* (*Pcl*), a member of the PcG, plays an important and perhaps central role in PcG function (Landecker et al., 1994). The Pcl protein contains two Cys_4-His-Cys_3 motifs, known as the plant homeodomain (PHD) type zinc finger (Aasland et al., 1995). The PHD motif, also called the leukemia-associated protein domain, is found in more than 400 eukaryotic proteins. Most of the PHD domain proteins are thought to be involved in transcription regulation, possibly acting through chromatin remodeling and histone acetylation (Aasland et al., 1995; Yochum and Ayer, 2001; Kalkhoven et al., 2002). More specifically the PHD finger appears to act as a protein-protein interaction domain to mediate the regulation of gene expression (Jacobson and Pillus, 1999). Although the function of the Pcl genes in vertebrate development remains unclear, studies in *Xenopus* indicate that they negatively regulate, or repress, gene expression in the developing anterior central nervous system (Yoshitake et al., 1999; Kitaguchi et al., 2001).

In this report, we show that a novel chick *Polycomblike* gene, chick *Pcl2*, encodes a transcription repressor and exhibits asymmetric expression in the right side of Hensen's node. Protein-soaked bead implantation studies indicate that chick *Plc2* resides downstream of *Activin-βB* and *Bmp4* in the node. Inhibition of chick *Pcl2* expression in the early embryos led to randomization of cardiac looping direction. Using gain-of-function and loss-of-function approaches, we demonstrate that chick *Pcl2* is both necessary and sufficient for the repression of *Shh* expression in the node. The repression of *Shh* expression by chick *Pcl2* seems to be conserved in other developing organs and even across species. Overexpression of chick *Pcl2* by RCAS retroviral infection in the developing chick limb bud and feather bud inhibited *Shh* expression in the ZPA of the limb bud and the epithelia of the feather bud. Transgenic overexpression of chick *Pcl2* in the mouse limb bud also inhibited *Shh* expression in the ZPA. We further demonstrated that chick *Pcl2* can repress the activities of the mouse *Shh* promoter in cell culture assays. Pull-down assays indicate that chick Pcl2 might function as a repressor by recruiting EZH2 via its PHD finger domain. These results indicate that chick *Pcl2* plays an essential role in the LR axis specification by silencing *Shh* expression in the node.

Materials and methods

Isolation of chick *polycomblike-2* gene chick *Pcl2*

A differential gene expression screen was performed using the PCR-Select cDNA subtraction kit from Clontech (Palo Alto, CA), according to the manufacturer's protocol, using mRNAs extracted from the left and right halves of about 600 stage 5-9 chick embryos (Hamburger and Hamilton, 1951). A 280 bp chick *Pcl2* cDNA fragment was obtained and labeled with ^{32}P by random priming using Red Primer II labeling system (Amersham Pharmacia Biotech) to screen a stage 23 chick embryonic cDNA library at high stringency as described previously (St Amand et al., 1998). Two independent clones were obtained and sequenced. One contained a 2299 bp open reading frame encoding a putative 595 amino acid residue protein and was named chick *Pcl2*. The sequence was deposited into Genbank (Accession Number AY251284).

Probes and in situ hybridization

For the detection of chick *Pcl2* expression, the full-length chick *Pcl2* containing plasmid was linearized with *Xho*I. Non-radioactive RNA probes were generated and labeled with digoxigenin (DIG) using T3 RNA Polymerase. Other cDNAs used for gene expression studies include: a 1.1 kb cDNAs of the chick *Nodal* (Levin et al., 1995), a 0.5 kb *Shh* cDNA (Ogura et al., 1996), a 0.9 kb *Caronte* cDNA (Rodriguez-Esteban et al., 1999), a 1.8 kb *Pitx2* cDNA (St Amand et al., 1998), a 1.9 kb *SnR* cDNA (Isaac et al., 1997), a 0.8 kb *Fgf8* cDNA (Meyers and Martin, 1999), a 1.4 kb *Lefty1* cDNA (Schlange et al., 2001) and a 0.6 kb mouse *Shh* cDNA (Echelard et al., 1993). DIG-labeled riboprobes were generated according to the manufacturer's instruction (Boehringer Mannheim, Indianapolis, IN). Whole-mount and section in situ hybridization analyses were performed as described previously (St Amand et al., 1998; St Amand et al., 2000). Briefly, samples were fixed in freshly made 4% paraformaldehyde/PBS at 4°C overnight. For whole-mount in situ hybridization, samples were bleached with 6% H_2O_2 prior to dehydration through a graded methanol series. For section in situ hybridization, samples were dehydrated through a graded ethanol series, embedded in paraffin wax and sectioned at 10 μm. Hybridization signals were visualized using the BM purple AP substrate at 4°C.

Bead implantation and oligonucleotide treatment

Bead implantation experiments were performed on stage 4 chick

embryos explanted in New cultures (New, 1955). Briefly, Affigel-Blue beads (BioRad) were soaked in Activin-A (500 ng/μl in PBS), Follistatin (500 ng/μl in PBS), BMP4 (1 μg/μl in PBS) or 1% BSA in PBS (as a control), and were implanted on the left or right side of Hensen's node. All proteins were purchased from R&D Systems (Minneapolis, MN). Embryos were cultured to desired stages and collected for whole-mount in situ hybridization analyses. Oligonucleotide treatment was performed on stage 4-6 chick embryos explanted in New culture as described previously (Isaac et al., 1997; Srivastava et al., 1995; Yu et al., 2001). The sequence for the 20 base antisense oligonuleotide that targets the first PHD domain of chick *Pcl2* is 5′-CTCCTCCTGACATATTGTAC-3′. The sequence for the random control oligonucleotide is 5′-GACTATCTAGATAGCTACGT-3′. The oligonuleotides were synthesized as phosphorothioate derivatives and were purified by HPLC (IDT, Corralville, IA). About 10 μl oligonucleotide mixed with lipofectAMINE (GibcoBRL) at a concentration of 40 μM was applied onto cultured embryos constrained by a plastic ring in New cultures. Embryos were collected at appropriate stages for analysis of gene expression and for scoring the direction of cardiac looping.

Expression vectors and microelectroporation

We used microelectroporation to transfer plasmid DNA into early chick embryonic tissues. To generate expression plasmids, the coding region of chick *Pcl2* (amino acids 1-595) was amplified from the full-length chick *Pcl2* cDNA plasmid using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and was cloned into the pMES expression vector in front of *IRES-Gfp*-coding sequence. The resulting plasmid, pMES-*Pcl2*, expresses both the transgene and *Gfp* simultaneously under the control of the chick *β-actin* promoter. To perform microelectroporation, a gold-plated cathode was fixed to the bottom of a 60 cm dish with a thin layer of Ringer's saline. Stage 4 chick embryos were prepared for New culture and embryo constrained by a ring was placed onto the cathode. About 1 μl of expression plasmids (1 μg/μl), mixed with fast green to visualize DNA, was injected to the target site between the blastoderm and the vitelline membrane using a glass capillary. A platinum anode (0.5 mm in diameter) was then placed on the hypoblast of the node. The distance between the two electrodes was maintained within 2 mm. Electroporation was performed using BTX electroporator (Electro Square Porator™ ECM 830 Model, BTX, San Diego, CA) with five pulses of 5 V for duration of 25 mseconds and intervals of 454 mseconds. Embryos were then placed onto the agar media in New cultures and were incubated at 38°C to the appropriate stages. *Gfp* expression in targeted tissues was monitored before embryos were harvested for gene expression analysis.

Retroviral construction and infection

RCAS retroviral construction and infection was performed as described previously (Yu et al., 2001). To make the RCAS-*Pcl2* construct, the coding region of chick *Pcl2* (amino acids 1-595) was amplified from chick *Pcl2* cDNA plasmid and cloned into the *Cla*12 vector. The insert was then released by *Cla*I digestion and cloned into the RCASBP retroviral vector. Chicken embryonic fibroblast (CEF) cells expressing RCAS-*Pcl2* or RCAS-*Gfp* were pelleted according to a protocol described previously (Logan and Francis-West, 1999). To infect the chick developing limb bud, virus-free chick eggs (CBT farms, Chestertown, MD) were incubated to approximately stage 12. RCAS-*Pcl2*-expressing CEF cells were centrifuged briefly and incubated as a pellet for 2 hours at 37°C to allow tight cell aggregates to form. Cell pellets were cut into small pieces and were implanted into the right LPM of the prospective forelimb-forming region of chick embryos as described previously (Logan and Tabin, 1998). The infected embryos were then cultured in ovo and harvested from stage 19 to stage 24 for morphological and gene expression analyses. For infection of feather buds, dorsal skin tissues from stage 31 chick embryos were dissected in PBS and transferred to Trowell type organ cultures in DMEM supplemented with 10% fetal calf serum. About 10 μl RCAS retroviruses were injected with a microcapillary needle at multiple sites in the dorsal skin explants. The infected explants were incubated at 37°C in a 5% CO_2 atmosphere for 4 days, and were then collected for gene expression assays.

Transgenic construct and pronucleus injection

Construction of the chick *Pcl2* transgenic construct and pronuclear injection were performed as described previously (Zhang et al., 2000). Briefly, the chick *Pcl2* full-length cDNA was cloned into the PCI mammalian expression vector (Promega, Madison, WI). The chick *Pcl2* full-length cDNA flanked by a chimeric intron and the SV40 late Poly(A) sequence was released by *Pst*I/*Bam*HI, This fragment was cloned downstream of the 3.6 kb *Hoxb6* promoter (Schughart et al., 1991). The orientation of the insert was confirmed by restriction digestion and sequencing. Preparation of DNA fragments for injection, collection of zygotes and pronuclear injection was carried out as described previously (Hogan et al., 1994; Zhang et al., 2000). Embryos were collected at E10.75 for analysis of transgene expression and *Shh* expression. The integration of the *Hoxb6-Pcl2* transgene was determined by PCR using genomic DNA from the head of each embryo. The PCR primers used are as follows: 5′-TTTTGGTGCAGCAGGTAGAATAGC-3′ (upper primer) and 5′-CTCCCCCTGAACCTGAAACATAAA-3′ (lower primer).

In vitro CAT assays

To map the repressive domain of chick Pcl2 protein, the DNA fragments encoding the N terminus (amino acids 1-329), the C terminus (amino acids 331-595) and the PHD fingers domain (amino acids 103-238) of chick *Pcl2* were amplified from the full-length chick *Pcl2* cDNA plasmid and cloned into the pBXG1 vector that contains the GAL4 DNA-binding domain under the control of SV40 enhancer/promoter (Lillie and Green, 1989). The derived constructs, named *GAL4/Pcl2-N*, *GAL4/Pcl2-C* and *GAL4/PHD*, were confirmed by sequencing. The reporter plasmid, *pG5tkCAT*, contains the chloramphenicol acetyltransferase (CAT) reporter gene directed by the herpes simplex virus thymidine kinase (TK) promoter with five GAL4 binding sites upstream of the TATA-box. Transfection and CAT assays were carried out in cultured P19 cells, as described previously (Yu et al., 2001). A CMV-β-gal plasmid was included as an internal control for transfection efficiency. Transfected cells were cultured for 36 hours and then CAT activities were determined by thin layer chromatography (TLC) and scintillation counting. Each experiment was repeated at least three times to ensure consistent results.

A 1 kb and a 3 kb mouse *Shh* upstream regions, were amplified using EXL™ Taq (Stratagene, La Jolla, CA) from the mouse genomic clone (RPCI RP23 429M20), and cloned as *Bam*HI-*Xho*I fragments to replace the TK promoter in *pG5tkCAT* vector, respectively. The 1 kb *Shh* upstream region did not give reasonable promoter activities (data not shown). Only the plasmid containing the 3 kb *Shh* upstream region was used in this study. The pShh-CAT plasmids were co-transfected with pMES-*Pcl2*, pMES-*PHD* or *pMES* (as a control) into P19 cells. Transfection efficiency was monitored by inclusion of CMV-β-gal plasmid. Transfected cells were cultured for 36 hours prior to CAT assays by both TLC and scintillation counting, which were normalized by protein concentrations. Again, each experiment was carried at least three times.

Immunoprecipitation and protein blotting

To make the constructs for co-immunoprecipitation, the chick *Pcl2* full-length cDNA and chick *Pcl2* PHD finger domains (amino acids 103-238) were cloned in frame into the pIRES-hrGFP-1α vector (Stratagene, La Jolla, CA), while the mouse EZH2 sequence was amplified by PCR from the mouse EZH2 cDNA and cloned into the pCMV-Tag 3A vector (Stratagene). The resultant expression plasmids, pFLAG-Pcl2, pFLAG-PHD and pMyc-EZH2, were transiently transfected or co-transfected into 293T cells with Lipofectamine™

2000 (Invitrogen, Carlsbad, CA). Cells were harvested 48 hours after transfection in a lysis buffer consisting of 0.05 M HEPES, 1% Triton X-100, 0.15 M NaCl, 10% glycerol, 1 mM EDTA, 1.5 mM $MgCl_2$•$6H_2O$, Trypsin inhibitor 0.01 mg/ml, aprotinin 0.01 mg/ml, phenylmethanesufonyl fluoride 1 mM, leupeptin 0.02 mg/ml, sodium orthovanadate 1.25 mM and sodium fluoride 0.1 mM. Lysates were incubated with either a monoclonal anti-Myc antibody (Abcam, Cambridge, MA) or an anti-FLAG M2 antibody (Stratagene), and then with Protein A Sepharose beads (Amersham Pharmacia, Piscataway, NJ). After extensive washing, the eluted proteins were resolved on PAGE gels, and immunoblotted with an anti-FLAG M2 antibody or a monoclonal anti-Myc antibody, and the binding was detected using the ECL™ plus Western Blotting Detection system (Amersham Biosciences).

Results

Isolation and characterization of a novel *Polycomblike* gene, chick *Pcl2*, in the chick

In an effort to identify new components in the genetic pathways regulating LR axis development, a PCR-select cDNA subtraction hybridization screen was performed using mRNAs extracted from the left and right halves of stage 5-9 chick embryos (Hamburger and Hamilton, 1951). Thirty-four independent cDNA fragments varying from 200-800 bp in length were isolated. Nineteen of these clones exhibited a certain level of homology (higher than 60%) to known genes, while 15 clones showed no homology to known genes. A second round of screening by whole-mount in situ hybridization was then conducted to examine the expression pattern of these clones. Among 10 clones checked, two clones show asymmetric expression in the Hensen's node (data not shown). We focused on one clone that exhibited a high level of homology to the human *Polycomblike* (*PCL*) gene. A stage 23, chick whole embryo cDNA library was screened with this chick *Pcl* fragment as described previously (St Amand et al., 1998). A 2299 bp nucleotide acid cDNA encoding a putative protein of 595 amino acid residue was obtained. The putative amino acid sequence of the chick Pcl is most homologous to the *Xenopus* Pcl2 when compared with other Pcl family members. It was therefore named chick Pcl2 (chick Pcl2). Similar to other Pcl2 proteins, the predicted amino acid sequence of chick Pcl2 contains two PHD fingers, a well-characterized feature of the PcG proteins (Aasland et al., 1995) and a Tudor domain (Ponting, 1997) (Fig. 1A). The amino acid sequence of chick Pcl2 exhibits 86% and 87% identity to the human and mouse putative Pcl2 (M96) proteins, respectively (ID, XP_002013.5; ID, XP_132195.1); and 74% identity with the *Xenopus* Pcl2 protein (ID, BAB43943.1). Phylogenetic tree analysis (Gap penalty, 10; Gap length penalty, 10) clearly groups chick Pcl2 into a cluster distinct from the Pcl1 proteins (Fig. 1B). This analysis indicates that rather than falling into the Pcl1 group, the *Drosophila* Pcl protein which contains two PHD fingers more resembles Pcl2 subfamily. Pcl2 proteins are evolved earlier and are more conserved than Pcl1 proteins. We propose a conserved function for Pcl2 among different species.

Expression and regulation of chick *Pcl2* during LR development

Using the full-length chick *Pcl2* cDNA as a probe, the expression of chick *Pcl2* was examined during early embryonic development by whole-mount in situ hybridization. At HH stage 4, chick *Pcl2* expression was found mainly in the posterior one-third of the primitive streak, and was not expressed in Hensen's node (Fig. 2A). Beginning at HH stage 5, chick *Pcl2* expression was found to shift the anterior region of the primitive streak, and was present asymmetrically in the ectoderm of the right side of Hensen's node (Fig. 2B,C). This asymmetric pattern of chick *Pcl2* expression is complementary to that of *Shh*, which initially appears symmetrically in the Hensen's node, but becomes restricted to the left side of Hensen's node beginning at stage 5 (Levin et al., 1995). Chick *Pcl2* expression remained restricted in the right side of Hensen's node until HH stage 8 (Fig. 2D). Activin signaling, which is mediated by Activin-βB, has been known to set up the asymmetric pattern of *Shh* in the node, by repressing *Shh* on the right side of the node (Levin et al., 1997). Because of the PcG family of proteins act as transcriptional repressors, it is possible that chick Pcl2 could act downstream of Activin signals to regulate gene expression in the node. To begin to test this hypothesis, we asked whether Activin-βB regulates chick *Pcl2* expression in the right side of Hensen's node. Activin-A-soaked beads (500 ng/μl) were implanted on the left side of Hensen's node at stage 4. Six hours after bead implantation, embryos were processed for in situ hybridization. The activity of the Activin-A in these assays was confirmed by the repression of *Shh* in the left side of Hensen's node (data not shown). Activin-A beads induced ectopic chick *Pcl2* expression in the left side of Hensen's node (7/12; Fig. 2E).

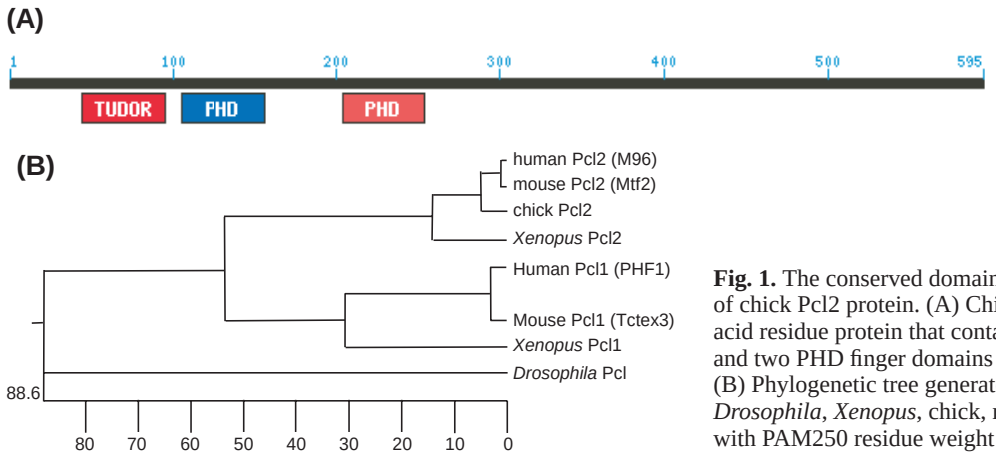

Fig. 1. The conserved domains and position on the phylogenetic tree of chick Pcl2 protein. (A) Chick *Pcl2* encodes a putative 595 amino acid residue protein that contains one Tudor domain (AA:45-100) and two PHD finger domains (AA:105-147; AA:204-238). (B) Phylogenetic tree generated from known Pcl proteins from *Drosophila*, *Xenopus*, chick, mouse and human using Clustal method with PAM250 residue weight table.

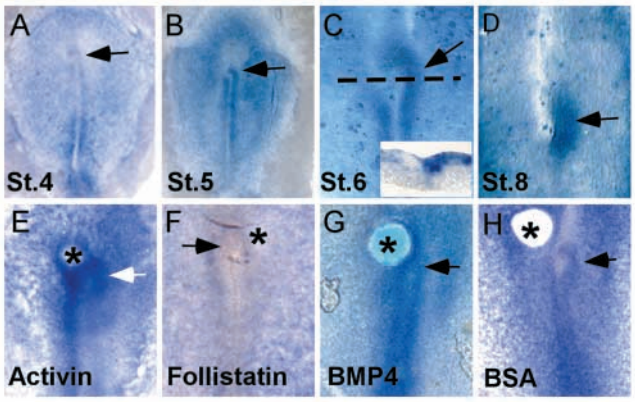

Fig. 2. Expression and regulation of chick *Pcl2* in Hensen's node. (A-D) Whole-mount in situ hybridization analyses reveal chick *Pcl2* expression in early chick embryos at stages 4 (A), 5 (B), 6 (C) and 8 (D). Asymmetric chick *Pcl2* expression in the node appears from stage 5, and is restricted in the ectoderm (insert in C). (E-H) Ectopic chick *Pcl2* expression is induced by Activin-A (E) and BMP4 (G) around the node, while Follistatin represses chick *Pcl2* expression (F). BSA control bead does not alter chick *Pcl2* expression (H). All embryos are dorsal views. Arrows indicate the node; asterisks indicate bead or bead position.

Control beads (soaked in BSA) had no effect on the expression of either chick *Pcl2* (Fig. 2H) or *Shh* (data not shown). To test whether an Activin-like signal is necessary for the restriction of chick *Pcl2* expression in the right side of Hensen's node, beads soaked with Follistatin, an antagonist of Activin and BMP signaling (Hemmati-Brivanlou et al., 1994; Yamashita et al., 1995; Iemura et al., 1998), were implanted into the right side of Hensen's node at stage 4. chick *Pcl2* expression was repressed not only on the right side of node (6/11) but also in the primitive streak (3/10) following Follistatin treatment (Fig. 2F). The effect of BMP4 signaling on chick *Pcl2* expression was also examined. Similar to Activin-A, BMP4-soaked beads were able to induce ectopic chick *Pcl2* expression in the left side of Hensen's node and the primitive streak (6/7; Fig. 2G). Taken together, the data indicate that chick *Pcl2* is asymmetrically expressed in Hensen's node during developmental stages that are crucial for LR axis development, and both Activin and BMP4 can positively regulate chick *Pcl2* expression.

Overexpression of chick *Pcl2* left of Hensen's node represses *Shh* and its downstream genes

To study the effect of a gain-of-function of chick *Pcl2*, we used microelectroporation to deliver this gene to chick embryos in New cultures. Although retrovirally mediated gene delivery has been routinely used to ectopically express target genes in chick embryos, the delay of target gene expression after viral infection makes it inappropriate for use in this study. Electroporation is a rapid and efficient strategy to introduce ectopic gene expression (Yasuda et al., 2000; Uchikawa et al., 2003). We adopted and modified a microelectroporation method (Momose et al., 1999) to introduce ectopic gene expression in early chick embryos in New cultures. By optimizing the electrode size, DNA concentration and injection method, as well as electroporation parameters, we detected strong expression of the target gene within 6 hours after electroporation with minimal side effects to the embryos (Fig. 3A,B). In these assays, pMES-*Pcl2* or pMES-*Gfp* control vectors were targeted to Hensen's node, and embryos were placed in New cultures until the desired stages of development were reached. Whole-mount in situ hybridization was performed using riboprobes for *Shh*, *Nodal*, chick *SnR*, *Pitx2* and *Lefty1* genes that are known to be involved in LR axis determination. Ectopic chick *Pcl2* expression to the left side of Hensen's node significantly repressed *Shh* expression in the node (10/12, Fig. 3D), expression of its downstream genes *Nodal* (5/9, Fig. 3F) and *Pitx2* (7/16, Fig. 3H) in the left LPM, and expression of *Lefty1* (5/6, Fig. 3J) in the left prospective floor plate. However, chick *SnR*, a gene known to be negatively regulated by Nodal (Patel et al., 1999), was ectopically

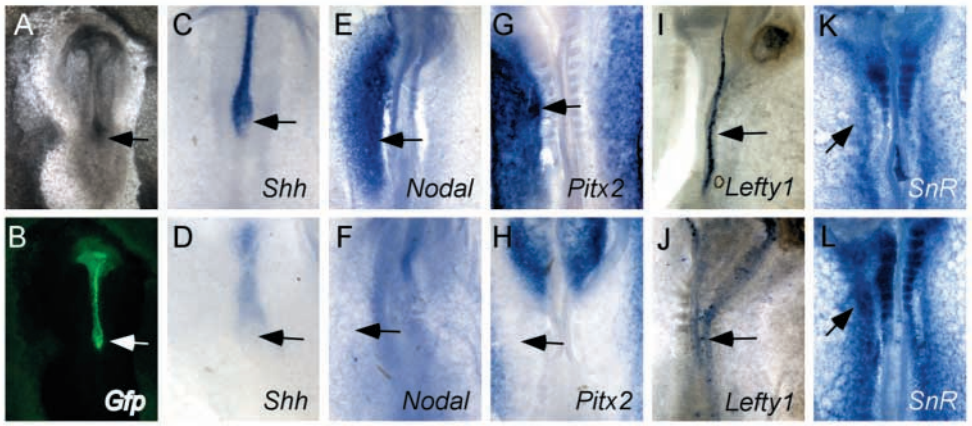

Fig. 3. Overexpression chick *Pcl2* represses *Shh* expression in the node and alters *Shh* downstream genes in the LPM. (A,B) A stage 7 chick embryo shows *Gfp* expression in the node and its derivatives after pMES-*Gfp* expression vector was targeted to Hensen's node at stage 4. (C,E,G,I) Control embryos transfected with pMES-*Gfp* vectors show unaltered expression of *Shh* (C) in the node and notochord, and of *Nodal* (E) and *Pitx2* (G) in the left LPM, and of *Lefty1* (I) in the prospective floor plate. (D,F,H,J) Embryos overexpressing chick *Pcl2* show downregulation of *Shh* (D) in the node and notochord, of *Nodal* (F) and *Pitx2* (H) in the left LPM, and of *Lefty1* (J) in the prospective floor plate. (K,L) *SnR* is ectopically activated in the left PLM of embryo overexpressing chick *Pcl2* (L), but not in the control embryo (K). Arrows in A-D indicate the node; arrows in E-H,K,L indicate the left LPM, and arrows in I,J indicate the midline. Images are all dorsal views.

expressed in the left LPM (4/10, Fig. 3L). In the control embryos targeted with the pMES-*Gfp* vector, the expression patterns of these genes were unaltered (Fig. 3C,E,G,I,K). These results indicate that chick *Pcl2* expression is sufficient to repress *Shh* in the node. The repression of *Shh* by chick *Pcl2* ectopic expression was also seen in the notochord (Fig. 3D), suggesting that chick *Pcl2* could repress *Shh* in other developing organs and tissues.

Overexpression of chick *Pcl2* in chick limb and feather buds and transgenic expression of chick *Pcl2* in the mouse limb inhibits *Shh* expression

To evaluate the possibility that chick *Pcl2* might repress *Shh* expression in other tissues, we first analyzed chick *Pcl2* expression in several developing chick organs where the expression of *Shh* has been well characterized. In these assays that focused on the limb and feather buds, chick *Pcl2* showed a dynamic expression patterns. chick *Pcl2* was found expressed in the medial limb mesenchyme at HH stage 18 (Fig. 4A), when *Shh* is barely detectable (Pearse et al., 2001). Chick *Pcl2* expression remained in the medial mesenchyme at the time when *Shh* expression is initiated in the posterior mesenchyme to mediate the polarizing activity of the ZPA (Riddle et al., 1993). By HH stage 24, the chick *Pcl2* expression domain split into two regions in the dorsal and ventral limb mesenchyme,

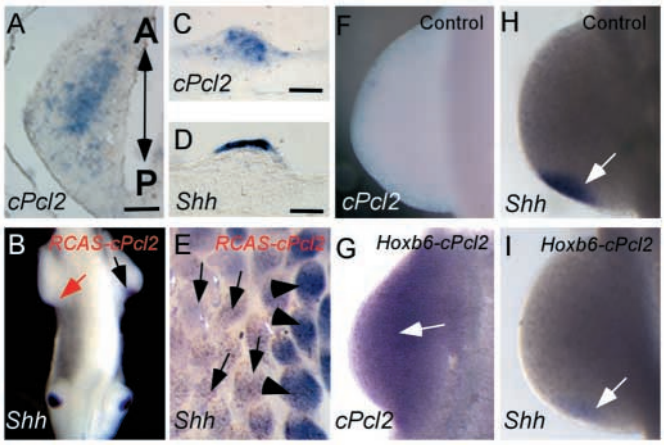

Fig. 4. Ectopic expression of chick *Pcl2* suppresses *Shh* expression in the ZPA of the chick and mouse limb buds and in the chick feather buds. (A) Chick *Pcl2* transcripts are detected in the medial mesenchyme of a stage 18 chick wing bud. The anteroposterior orientation is indicated. (B) A stage 22 chick embryo shows complete repression of *Shh* expression in the ZPA (red arrow) of the right wing infected with RCAS-*Pcl2* and normal *Shh* expression in the control wing (black arrow). (C,D) Chick *Pcl2* expression is detected in the mesenchyme of a feather bud from a stage 31 chick embryo (C), while *Shh* expression is restricted to the epithelium of a feather bud from the same stage (D), exhibiting a complementary expression pattern to that of chick *Pcl2*. (E) *Shh* expression is inhibited in feather buds (arrows) infected with RCAS-chick *Pcl2*. There is normal *Shh* expression (arrowheads) in adjacent uninfected feather buds. (F,G) Chick *Pcl2* transgene expression (arrow) is detected in a hindlimb of an E10.5 transgenic embryo (G), but not in a wild-type limb (F). (H,I) Transgenic expression of chick *Pcl2* in the mesenchyme of mouse developing hindlimb suppresses *Shh* expression in the ZPA (arrow in I), when compared with a control hindlimb from E10.5 nontransgenic littermate (H). Scale bars: 100 μm in A; and 50 μm in C,D.

and overlapped with regions of muscle mass (data not shown). These results indicated that, although chick *Pcl2* expression is not completely complementary to *Shh*, it does not overlap with the *Shh*-expressing domain in the developing limb bud. In developing feathers, *Shh* expression was first detected at the center of the epithelia placode (Fig. 4D). In the long feather bud, *Shh* expression is known to shift to the distal and posterior side of the feather epithelia (Ting-Berreth and Chuong, 1996). Interestingly, chick *Pcl2* shows a complementary expression pattern to that of *Shh* in the feather bud. Chick *Pcl2* expression was first detected in the feather placode mesenchyme in the middle region, and then became more strongly expressed in the posterior mesenchyme in the feather bud (Fig. 4C). To test the effect of ectopic chick *Pcl2* on *Shh* expression in these organs, we proceeded to misexpress chick *Pcl2* to the developing limb bud and feather bud using RCAS-*Pcl2* retroviruses. Infection efficiency was determined by whole-mount in situ hybridization using a probe against RCAS *gag* RNA sequences (data not shown). Overexpression of chick *Pcl2* in these assays caused an unambiguous downregulation of *Shh* expression in the ZPA of infected limb buds (Fig. 4B), and in feather buds (Fig. 4E). In controls, infection of either limb or feather buds with the RCAS-*Gfp* control retroviruses did not alter *Shh* expression (data not shown). These results indicate that ectopic expression of chick *Pcl2* indeed is able to repress *Shh* expression in the limb and feather buds.

We further asked whether repression of *Shh* by chick *Pcl2* is also conserved across different species. A transgenic construct containing the full-length chick *Pcl2* cDNA under the control of the mouse *Hoxb6* promoter was generated. The 3.6 kb mouse *Hoxb6* promoter drives transgene expression specifically to the posterior mesenchyme of the forelimb and in all mesenchymal tissue in the hindlimb of mouse embryos at E9.5 and E10.5 when *Shh* is expressed in the ZPA (Schughart et al., 1991; Zhang et al., 2000). *Hoxb6-Pcl2* transgenic mice were generated by pronuclear injection. Out of 28 embryos collected on E10.5-10.75, five were positive for transgenicity. Mid-sagittal cut was made to separate these embryos into left and right half embryos that were processed for whole-mount in situ hybridization with chick *Pcl2* probe for the transgene expression and the mouse *Shh* probe for *Shh* expression in the ZPA, respectively. Two embryos that showed the transgene expression in a correct pattern (Fig. 4G) exhibited strong repression of *Shh* expression in the ZPA of both the forelimb and hindlimb (Fig. 4I). Non-transgenic littermates showed normal *Shh* expression (Fig. 4H).

Inhibition of chick *Pcl2* expression in the early chick embryo results in ectopic *Shh* expression in the node and randomized heart looping direction.

Our results established that chick *Pcl2* is sufficient for *Shh* repression in the node and in other developing organs. We further examined the function of chick *Pcl2* in the development of chick LR axis by a loss-of-function approach using antisense oliogonucleotides (Srivastava et al., 1995; Yu et al., 2001). A 20 base antisense oligonucleotide targeting to the first PHD domain of chick *Pcl2* and random control oligonucleotides were synthesized as phosphorothioate derivatives and were purified by HPLC. Stage 4 chick embryos were treated with oligonucleotides in New cultures as described previously (Yu et al., 2001), and were allowed to develop to stage 11 for an

examination of heart looping direction. In the studies, 46% (12/26) of embryos treated with antisense oligonucleotides to chick *Pcl2* at stage 4 exhibited reversed cardiac looping (Fig. 5B), while only 8% (1/12) of embryos treated with control oligonucleotides exhibited a reversed cardiac looping (Fig. 5A). However, when antisense oligonucleotides were applied at stage 5, only 39% (7/18) of embryos showed reverse cardiac looping; and when applied at stage 6, only 14% (1/7) showed that phenotype. This stage-dependent effect of antisense oligonucleotides treatment is consistent with the timing of the asymmetrical chick *Pcl2* expression in the node. Our results indicate that chick *Pcl2* plays a crucial role in the LR axis development in the chick.

We next asked whether the inhibition of chick *Pcl2* expression led to randomization of heart looping by causing ectopic *Shh* expression in the node and its downstream genes in the right LPM. Embryos treated with oligonucleotides at stage 4 were allowed to develop to stage 6 for the examination of *Shh*, *Fgf8* and chick *Pcl2*, and to stage 8-10 for other genes. chick *Pcl2* expression was found significantly reduced in the chick *Pcl2* antisense oligonucleotide treated embryos (Fig. 5C), indicating the efficiency of antisense treatment. In antisense-treated embryos, *Shh* expression was seen in the right side of the node in about 30% (4/14) of embryos (Fig. 5G). Furthermore, ectopic expression of *Caronte* (2/8, Fig. 5I), *Nodal* (3/11, Fig. 5K) and *Pitx2* (3/9, Fig. 5M) was also observed in the right LPM, possibly resulting from ectopic *Shh* expression in the right side of the node. However, *Fgf8* (8/9) expression was repressed in the right side of the node following antisense treatment (Fig. 5D,E). In control embryos treated with random oligonucleotides, the expression of all these genes appeared normal (Fig. 5F,H,J,L). These results established a crucial role for chick *Pcl2* in the LR axis development by repressing *Shh* in the right side of the node.

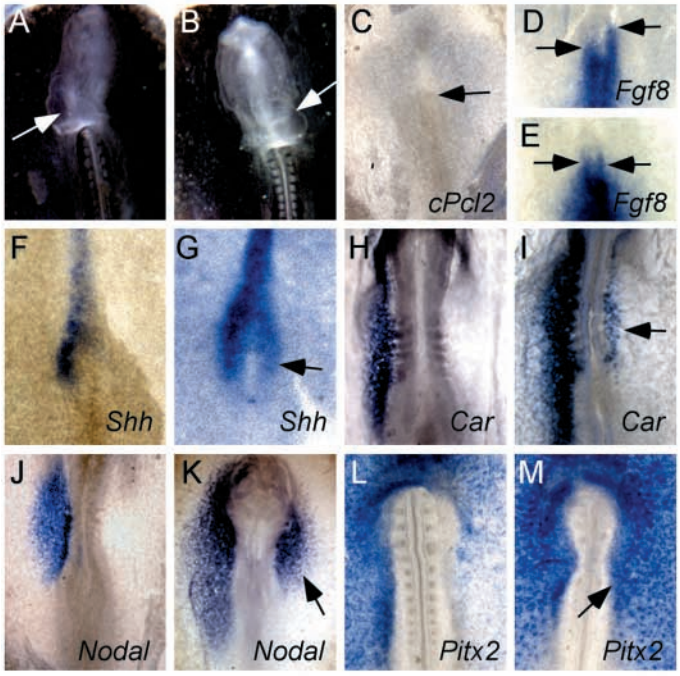

Fig. 5. Knock-down of chick *Pcl2* randomizes cardiac looping direction and alters gene expression. (A-C) Ventral view of stage 11 embryo treated with control oligonucleotide shows normal rightwards heart looping (A), while embryo treated with antisense oligonucleotide specific to chick *Pcl2*, which abolished chick *Pcl2* expression as detected by whole-mount in situ hybridization (C), exhibits reversed cardiac looping direction (B). (D,E) *Fgf8* expression is unaltered (arrows) in embryos treated with control oligonucleotide (E), but is repressed in the right side of the node (arrows) of embryos treated with antisense oligonucleotide to chick *Pcl2* (D). (F-M) Embryos treated with control oligonucleotide show normal expression of *Shh* (F) in the node, of *Caronte* (H), *Nodal* (J) and *Pitx2* (L) in the left LPM. By contrast, embryos treated with antisense oligonucleotide show ectopic expression of *Shh* in the right side (arrow) of the node (G), and ectopic expression of *Caronte* (I), *Nodal* (K) and *Pitx2* (M) in the left LPM (arrows). Embryos in C-M are shown dorsal views.

Chick *Pcl2* encodes a transcription repressor that negatively regulates the activity of the mouse *Shh* promoter

Products of PcG family genes in *Drosophila* are required for the epigenetic repression of homeotic genes and other key developmental regulatory genes such as *hedgehog*, through direct transcription effects (Kennison, 1995; Maschat et al., 1998; Maurange and Paro, 2002). In addition, overexpression of the Pcl subfamily members has been shown to repress target gene expression in vertebrates (Yoshitake et al., 1999; Kitaguchi et al., 2001). We next performed in vitro assays to map the repression domain of the chick Pcl2 protein. Constructs containing DNA fragments encoding the chick *Pcl2* N terminus (amino acids 1-329), C terminus (amino acids 331-595), and the region encompassing the two PHD finger domains (amino acids 103-238) which mediates the association of *Drosophila* Pcl with ESC/E(Z) repression complexes (O'Connell et al., 2001), fused in frame to the 147 amino acid yeast GAL4 DNA-binding domain were generated. A construct expressing the N terminus of the chick Pitx2a transcription activator, which was previously shown to have no effect on reporter gene expression (Yu et al., 2001), was included as control. The transcription activity of the fusion proteins was assessed on the GAL4-responsive *pG5tkCAT* reporter. These assays showed that the fused protein containing the PHD finger domains (GAL4-Pcl2$^{aa103-238}$) significantly repressed reporter gene expression (Fig. 6A). GAL4-Pcl2N which contains the PHD domains also exhibited a repressive effect on reporter gene expression. The GAL4-Pcl2C had no repressive effect and neither did the control vector GAL4-Pitx2aN (Fig. 6A). It was therefore concluded that chick *Pcl2* indeed encodes for a transcription repressor and the repression is mediated by PHD finger domain.

We further asked whether chick *Pcl2* represses *Shh* expression by acting directly on the *Shh* promoter. As transgenic expression of chick *Pcl2* in the mouse developing limb bud repressed *Shh* expression and because of the availability of mouse genome information, we decided to use the mouse *Shh* upstream region in this study. It has been shown previously that a 1 kb mouse *Shh* upstream region was able to drive reporter gene expression in cell culture (Knezevic et al., 1997). We cloned this 1 kb mouse *Shh* upstream region and found that this genomic fragment could only drive weak reporter gene expression in P19 cells and in the MPLB-2, a mouse embryonic limb bud cell line (Trevino et al., 1993), making it inappropriate for this study. A bioinformatic

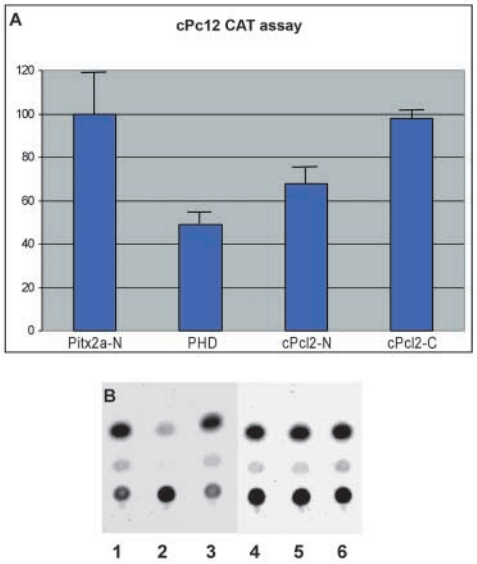

Fig. 6. Mapping of repression domain of chick Pcl2 and inhibition of the mouse *Shh* promoter activities by chick Pcl2. (A) P19 cells were transfected with the reporter plasmid, *pG5tkCAT*, and the constructs indicated, and CAT (chloramphenicol acetyltransferase) activities were assayed. The N-domain of chick Pcl2, which covers the PHD fingers, represses about a third of the promoter activities, while the PHD domain suppresses the promoter activities to a half. C-terminal domain of chick Pcl2 exhibits similar effect to the controls on the promoter activities. (B) The 3 kb mouse *Shh* upstream region drives the expression of the CAT reporter gene in P19 cells (lane 1). The promoter activities are suppressed by co-expression of chick *Pcl2* (lane 2) but not the PHD fingers (lane 3). Lanes 4-6 show the TK promoter activities that are not suppressed by co-expression of either chick Pcl2 (lane 5) or the PHD domain (lane 6), when compared with controls (lane 4). Data shown here represent at least three independent experiments.

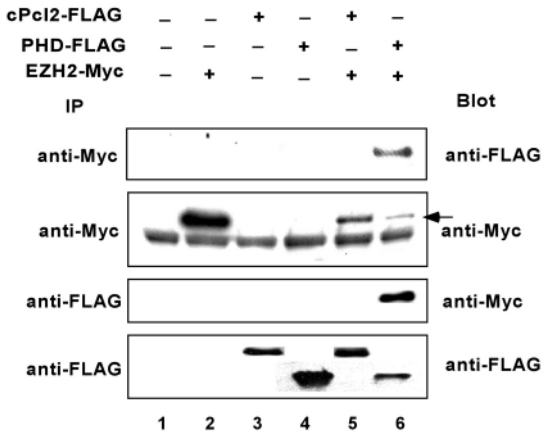

Fig. 7. Interaction of the PHD domain of chick Pcl2 with EZH2. 293T cells were transfected with expression vectors expressing the FLAG-tagged chick Pcl2, the FLAG-tagged PHD fingers (AA103-238) and the Myc-tagged mouse EZH2. Cell extracts were immunoprecipitated and blotted with antibodies against FLAG or Myc, reciprocally. Lane 1, negative control; lane 2, positive control for Myc pull-down and blot (arrow indicates the blotted band); lanes 3 and 4, positive control for FLAG pull-down and blot; lane 5, pull-down and blot of chick Pcl2-FLAG and EZH2-Myc reciprocally; lane 6, pull-down and blot of PHD-FLAG and EZH2-Myc reciprocally. Data shown are representatives of at least three independent experiments.

approach using rVISTA software was subsequently performed to search for potential PcG protein-binding sites in the 10 kb upstream sequences of the mouse *Shh* gene (Loots et al., 2002), multiple YY1 (the vertebrate homolog of *Drosophila* PcG *pleiohomeotic* gene) binding sites were found within 3 kb upstream of the mouse *Shh* gene. Accordingly, this 3 kb mouse *Shh* upstream region was PCR amplified and linked with the CAT reporter gene, generating the *Shh*-CAT construct. CAT assays revealed that this 3 kb *Shh* upstream region drove strong reporter gene expression in P19 cells (Fig. 6B). P19 cells were co-tranfected with expression vectors expressing the full-length chick Pcl2 or the PHD domain construct and the *Shh*-CAT construct. *Shh* promoter activity was significantly reduced (2.4-fold) by the full-length chick Pcl2 construct, but was not affected by the PHD domain construct, when compared with controls (Fig. 6B). Neither the chick Pcl2 nor the PHD domain construct repressed activity of the TK promoter (Fig. 6B). Therefore, we conclude that chick Pcl2 can specifically repress *Shh* promoter activity, but this repression requires chick Pcl2 protein integrity.

Chick Pcl2 interacts with EZH2 via its PHD fingers

Generally, PcG proteins function by forming protein complexes with other proteins. It was shown that the PHD fingers from the *Drosophila* Pcl protein interact directly with E(Z) (Enhancer of Zeste), a component of the ESC/E(Z) repressive complex (O'Connell et al., 2001). This interaction between Pcl and E(Z) is also conserved in their human homologs (O'Connell et al., 2001). To test whether chick Pcl2 might also interact with EZH2, the vertebrate homolog of the E(Z) protein, co-immunoprecipitation studies were performed. FLAG-tagged chick Pcl2 (FLAG-Pcl2) or PHD fingers (amino acids 103-238; FLAG-PHD) and Myc-tagged mouse EZH2 (Myc-EZH2) were co-expressed in the 293T cells. As shown in Fig. 7, the PHD fingers and EZH2 proteins were steadily pulled down by antibodies against FLAG or Myc reciprocally, confirming an interaction between EZH2 and the PHD fingers of chick Pcl2. By contrast, chick Pcl2 proteins failed to pull down EZH2 reciprocally using the same approach (Fig. 7). This also happened to the *Drosophila* Pcl fusion protein which was unable to but its PHD finger domain could interact with E(Z) (O'Connell et al., 2001). This could be explained by the mask of the PHD finger domain in chick Pcl2 fusion protein or instability of chick Pcl2-EZH2 association.

Discussion

Left-sided *Shh* expression in Hensen's node is crucial for setting up LR asymmetry in the chick (Levin et al., 1995; Pagan-Westphal and Tabin, 1998). This is achieved by the repressive action of the asymmetrically expressed *Activin-βB* and *Bmp4* in the right side of the node, which restricts *Shh* to the left side of the node. However, little is known about the transcriptional repressor that mediates *Shh* inactivation in the right side of the node. In this study, we have provided evidence that a novel chick *Polycomblike* gene, chick *Pcl2*, which exhibits right-sided expression in the node, is an excellent

candidate for this repression. Our data demonstrate that chick *Pcl2* encodes a transcription repressor that is both sufficient and necessary for the repression of *Shh* in the right side of Hensen's node and that the repression of *Shh* by chick *Pcl2* is conserved in different organs and even across species. Furthermore, chick *Pcl2* can repress the mouse *Shh* promoter activity and probably interacts with EZH2 via the PHD fingers. Based on these results, we propose that chick *Pcl2* plays a crucial role in regulating LR axis patterning by silencing *Shh* in the right side of the node.

Chick *Pcl2* is both sufficient and necessary for the repression of *Shh* in the node

Hensen's node, the chick organizer, has been a focus in the study of the molecular mechanism that establishes LR asymmetry. The chick organizer shows striking asymmetries well before the detection of any overt morphological signs of asymmetry. For example, the morphological asymmetry is apparent at the chick node as early as stage 4 (Cooke, 1995; Dathe et al., 2002). At the molecular level, a number of genes have been found to be expressed in or near the chick node in an asymmetric fashion. These include *Activin βB* and its receptor *cAct-RIIa* (Levin et al., 1995; Levin et al., 1997), *Bmp4* (Monsoro-Burq and Le Douarin, 2001), *Fgf8* (Boettger et al., 1999), *N-Cadherin* (Garcia-Castro et al., 2000) and chick *Mid1* (Granata and Quaderi, 2003) in the right side of the node, and *Shh* (Levin et al., 1995) and *Nodal* (Levin et al., 1995) in or near the left side of the node. *Shh* is initially expressed symmetrically in the node and becomes restricted to the left side of the node at stage 5 by an inferred Activin-like signal, possibly Activin-βB. This asymmetrical *Shh* in Hensen's node is both necessary and sufficient for the left-sided expression of *Nodal* in the LPM which is conserved in vertebrates (Levin et al., 1995; Pagan-Westphal and Tabin, 1998). Interestingly, asymmetric expression of *Shh* in the node is not observed in mice, zebrafish or rabbit (Levin et al., 1995; Fischer et al., 2002), arguing against the conserved mechanism of the initial symmetry-breaking events. However, *Shh* mutant mice do display a variety of laterality defects (Tsukui et al., 1999). It is known that the antagonistic interactions between *Shh* and *Bmp4* maintain the restricted *Shh* expression in the left side of Hensen's node (Monsoro-Burg et al., 2001). Recently, chick Mid1, a microtubule-associated ubiquitin ligase, was shown to act upstream of *Bmp4* to mediate the antagonistic interaction (Granata et al., 2003). It is reasonable to suspect that a putative transcription repressor is expressed in the right side of node to mediate the repression of *Shh* by the Activin-like signals. We identified such repressor, a novel chick *Polycomblike* gene, chick *Pcl2*, which acts downstream of *Activin* and *Bmp4* and is expressed in the right side of Hensen's node. In the node region, chick *Pcl2* is expressed in the ectoderm as is Shh but in a complementary pattern. Chick *Pcl2* encodes a transcription repressor which specifically represses the activity of the *Shh* promoter. These studies establish chick *Pcl2* as a candidate for the putative transcriptional repressor of *Shh* in the node. In support of this hypothesis, ectopic expression of chick *Pcl2* in the left side of Hensen's node abolished *Shh* expression in the node and blocked the expression of its downstream genes in the LPM and the midline. Ablating chick *Pcl2* expression by an antisense oligonucleotide approach caused ectopic *Shh* expression in the right side of Hensen's node, which in turn randomized heart looping. These data indicate that chick *Pcl2* is both sufficient and necessary for the repression of *Shh* in the right side of the node. In the chick, the LR identity is liable at stage 4, but becomes fixed at stage 5, concurrent with the right-sided expression of chick *Pcl2* in the node. Chick *Pcl2* seems to participate in stabilizing developmental decision that establish the LR asymmetry.

Repression of *Shh* by chick *Pcl2* is a conserved feature in different organs and species

Shh, when acting as a morphogen, is essential for crucial pathways that regulate the differentiation and patterning of a number of tissues. In our studies, overexpression of chick *Pcl2* by electroporation to the node of stage 4 chick embryos repressed *Shh* expression in the node and also in the notochord, a node-derived tissue. Retrovirus-mediated ectopic expression of chick *Pcl2* also repressed *Shh* expression in the developing chick limb bud and feather bud. Moreover, transgenic overexpression of chick *Pcl2* in the mouse limb bud similarly downregulated *Shh* expression. These consistent results indicate that the repression of *Shh* by chick *Pcl2* may represent a general regulatory mechanism for controlling *Shh* expression in different organs and tissues in the chick and even across species. The repression of *Shh* by chick *Pcl2* seems to be a specific rather than a general repressive effect, because chick *Pcl2* represses the mouse *Shh* promoter activity but not the TK promoter in vitro.

The repressive effect of chick Pcl2 on *Shh* expression suggests that the products of PcG genes not only maintain the silenced status of gene expression involved in long-term developmental decisions, but also function to regulate/silence the expression of active gene during embryonic development. This hypothesis is supported by the finding that in *Drosophila* the product of *Polyhomeotic* (*ph*) exerts negative transcriptional control on active genes (Randsholt et al., 2000). Furthermore, the *Xenopus* Pcl genes were also shown to repress gene expression in the developing central nervous system (Yoshitake et al., 1999; Kitaguchi et al., 2001). It was recently shown that the antagonistic functions of the Polycomb group complex and Trithorax complex on a Polycomb response element can govern the transition between the repressed and active status of gene expression (Poux et al., 2002).

Mechanism of chick Pcl2 function

Our results demonstrate that chick *Pcl2* encodes a transcription repressor, and its repression domain was mapped to the PHD fingers. We demonstrated that the PHD fingers of chick Pcl2 can interact directly with EZH2, a finding that is consistent with previous results showing that PHD fingers from both *Drosophila* and human Pcl bind with high specificity with EZ in ESC/E(Z) complexes (O'Connell et al., 2001). *Drosophila* Pcl was also shown to be a component of ESC/E(Z) complexes, which contains histone deacetylase and histone methyltransferase activities (Tie et al., 2003; Kuzmichev et al., 2002; Muller et al., 2002). These enzymatic activities contribute to chromatin remodeling and transcriptional repression (Zhang and Reinberg, 2001). Based on our evidence showing that chick Pcl2 can specifically repress *Shh* promoter activity in vitro and *Shh* expression in developing organs, we propose that chick Pcl2 may repress *Shh* by recruiting the EED/EHZ2 complex [the mammalian homolog of ESC/E(Z)]

through the conserved interaction between the PHD fingers and EZH2. The potential DNA-binding domain in chick *Pcl2* and chick *Pcl2* response element remain to be identified.

We thank Drs Judith Venuti and Michael Lan of Louisiana State University Health Sciences Center for reagent, and members of the Chen laboratory for discussion and technical support. We are also indebted to Dr Carol Bursdal of Tulane University for her critical reading of the manuscript. This work was supported by an Established Investigator Award (0340166N) from the American Heart Association and partially by grants from the NIH to Y.P.C.

References

Aasland, R., Gibson, T. J. and Stewart, A. F. (1995). The PHD finger, implication for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.* **20**, 56-59.

Akasaka, T., Kanno, M., Balling, R., Mieza, M. A., Taniguchi, M. and Koseki, H. (1996). A role for mel-18, a Polycomb group-related vertebrate gene, during the anterioposterior specification of the axial skeleton. *Development* **122**, 1513-1522.

Boettger, T., Wittler, L. and Kessel, M. (1999). FGF8 functions in the specification of the right body side of the chick. *Curr. Biol.* **9**, 277-280.

Campione, M., Steinbeisser, H., Schweickert, A., Deissler, K., van Bebber, F., Lowe, L. A., Nowotschin, S., Viebahn, C., Haffter, P., Kuehn, M. R. et al. (1999). The homeobox gene Pitx2, mediator of asymmetric left-right signaling in vertebrate heart and gut looping. *Development* **126**, 1225-1234.

Capdevila, J., Vogan, K. J., Tabin, C. J. and Izpisúa-Belmonte, J. C. (2000). Mechanisms of left-right determination in vertebrates. *Cell* **101**, 9-21.

Collignon, J., Varlet, I. and Robertson, E. J. (1996). Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature* **381**, 155-158.

Cooke, J. (1995). Vertebrate embryo handedness. *Nature* **374**, 681.

Coré, N., Bel, S., Gaunt, S. J., Aurrand-Lions, M., Pearce, J., Fisher, A. and Djabali, M. (1997). Altered cellular proliferation and mesoderm patterning in Polycomb-M33-deficient mice. *Development* **124**, 721-729.

Dahiya, A., Wong, S., Gonzalo, S., Gavin, M. and Dean, D. C. (2001). Linking the Rb and polycomb pathways. *Mol. Cell.* **8**, 557-569.

Dathe, V., Gamel, A., Manner, J., Brand-Saberi, B. and Christ, B. (2002). Morphological left-right asymmetry of Hensen's node precedes the asymmetric expression of Shh and Fgf8 in the chick embryo. *Anat. Embryol. (Berl).* **205**, 343-354.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Fischer, A., Viebahn, C. and Blum, M. (2002). FGF8 acts as a right determinant during establishment of the left-right axis in the rabbit. *Curr. Biol.* **12**, 1807-1816.

Garcia-Castro, M. I., Vielmetter, E. and Bronner-Fraser, M. (2000). N-Cadherin, a cell adhesion molecule involved in establishment of embryonic left-right asymmetry. *Science* **288**, 1047-1051.

Granata, A. and Quaderi, N. A. (2003). The Opitz syndrome gene MID1 is essential for establishing asymmetric gene expression in Hensen's node. *Dev. Biol.* **258**, 397-405.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-295.

Hogan, B., Beddington, R., Constantini, F. and Lacy, E. (1994). *Manupulating the Mouse Embryo, A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hyatt, B. A., Lohr, J. L. and Yost, H. J. (1996). Initiation of vertebrate left-right axis formation by maternal Vg1. *Nature* **384**, 62-65.

Iemura, S., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H. and Ueno, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9337-9342.

Isaac, A., Sargent, M. G. and Cooke, J. (1997). Conntrol of vertebrate left-right asymmetry by a snail-related zinc finger gene. *Science* **275**, 1301-1304.

Jacobson, S. and Pillus, L. (1999). Modifying chromatin and concepts of cancer. *Curr. Opin. Genet. Dev.* **9**, 175-184.

Kalkhoven, E., Teunissen, H., Howweling, A., Verrijzer, C. P. and Zantema, A. (2002). The PHD type zinc finger is an integral part of the CBP acetyltransferase domain. *Mol. Cell. Biol.* **22**, 1961-1970.

Kawakami, M. and Nakanishi, N. (2001). The role of an endogenous PKA inhibitor, PKI-alpha, in organizing left-right axis formation. *Development* **128**, 2509-2515.

Kennison, J. A. (1995). The Polycomb and trithorax group proteins of Drosophila, trans-regulators of homeotic gene function. *Annu. Rev. Genet.* **29**, 289-303.

Kitaguchi, T., Nakata, K., Nagai, T., Aruga, J. and Mikoshiba, K. (2001). Xenopus Polycomblike 2 (XPxl2) controls anterior to posterior patterning of the neural tissue. *Dev. Genes Evol*. **211**, 309-314.

Knezevic, V., de Santo, R., Schughart, K., Huffstadt, U., Chiang, C., Mahon, K. A. and Mackem, S. (1997). Hoxd-12 differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates Sonic hedgehog in a positive feedback loop. *Development* **124**, 4523-4536.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P. and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev*. **16**, 2893-2905.

Landecker, H. L., Sinclair, D. A. and Brock, H. W. (1994). Screen for enhancers of Polycomb and Polycomblike in Drosophila melanogaster. *Dev. Genet.* **15**, 425-434.

Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. and Tabin, C. J. (1995). A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82**, 803-814.

Levin, M., Pagan, S., Roberts, D. J., Cooke, J., Kuehn, M. R. and Tabin, C. J. (1997). Left/right patterning signals and the independent regulation of different aspects of situs in the chick embryo. *Dev. Biol.* **189**, 57-67.

Lillie, J. W. and Green, M. R. (1989). Transcription activation by the adenovirus E1a protein. *Nature* **338**, 39-44.

Logan, C. and Francis-West, P. (1999). Gene transfer in avian embryos using replication-competent retroviruses. In *Methods Mol. Biol.* (ed. P. T. Sharp and I. Mason), Vol. 97. Totowa, NJ: Humana Press.

Logan, M. and Tabin, C. J. (1998). Targeted gene misexpression in chick limb buds using avian replication-competent retroviruses. *Methods* **14**, 407-420.

Logan, M., Pagán-Westphal, S. M., Smith, D. M., Paganessi, L. and Tabin, C. J. (1998). The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell* **94**, 307-317.

Loots, G., Ovcharenko, I., Pachter, L., Dubchak, I. and Rubin, E. (2002). rVISTA for comparative sequence-based discovery of functional transcription factor binding sites. *Genome. Res.* **12**, 832-839.

Lowe, L. A., Supp, D. M., Sampath, K., Yokoyama, T., Wright, C. V. E., Potter, S. S., Overbeek, P. and Kuehn, M. R. (1996). Conserved left-right asymmetry of nodal expression and alterations in murine situs inversus. *Nature* **381**, 158-161.

Maschat, F., Serrano, N., Randsholt, N. B. and Geraud, G. (1998). Engrailed and polyhomeotic interactions are required to maintain the A/P boundary of the Drosophila developing wing. *Development* **125**, 2771-2780.

Maurange, C. and Paro, R. (2002). A cellular memory module conveys epigenetic inheritance of hedgehog expression during Drosophila wing imaginal disc development. *Genes Dev.* **16**, 2672-2683.

Meyers, E. N. and Martin, G. R. (1999). Differences in left-right axis pathways in mouse and chick, Function of FGF8 and SHH. *Science* **285**, 403-406.

Momose, T., Tonegawa, A., Takeuchi, J., Ogawa, H., Umesono, K. and Yasuda, K. (1999). Efficient targeting of gene expression in chick embryo by microelectroporation. *Develop. Growth Differ*. **41**, 335-344.

Monsoro-Burg, A. and Le Douarin, N. M. (2001). BMP4 plays a key role in left-right patterning in chick embryos by maintaining Sonic hedgehog asymmetry. *Mol. Cell* **7**, 789-799.

Muller, J., Hart, C. M., Francis, N. J., Vargas, M. L., Sengupta, A., Wild, B., Miller, E. L., O'Connor, M. B., Kingston, R. E. and Simon, J. A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. *Cell* **111**, 197-208.

New, D. A. T. (1955). A new technique for the cultivation of the chick embryo in vitro. *J. Embryol. Exp. Morphol.* **3**, 326-331.

O'Connell, S., Wang, L., Robert, S., Jones, C. A., Saint, R. and Jones, R. S. (2001). Polycomblike PHD fingers mediate conserved interaction with Enhancer of Zeste protein. *J. Biol. Chem*. **276**, 43065-43073.

Ogura, T., Alvarez, I. S., Vogel, A., Rodriguez, C., Evans, R. M. and

Izpisua Belmonte, J. C. (1996). Evidence that Shh cooperates with a retinoic acid inducible co-factor to establish ZPA-like activity. *Development* **122**, 537-542.

Pagan-Westphal, S. M. and Tabin, C. J. (1998). The transfer of left-right positional information during chick embryogenesis. *Cell* **93**, 25-35.

Patel, K., Isaac, A. and Cooke, J. (1999). Nodal signalling and the roles of the transcription factors SnR and Pitx2 in vertebrate left-right asymmetry. *Curr. Biol.* **9**, 609-612.

Pearse, R. V., II, Vogan, K. J. and Tabin, C. J. (2001). Ptc1 and Ptc2 transcripts provide distinct readouts of hedgehog signaling activity during chick embryogenesis. *Dev. Biol.* **239**, 15-29.

Piedra, M. E. and Ros, M. A. (2002). BMP signaling positively regulates Nodal expression during left right specification in the chick embryo. *Development* **129**, 3431-3440.

Piedra, M. E., Icardo, J. M., Albajar, M., Rodriguez-Rey, J.-C. and Ros, M. A. (1998). Pitx2 participates in the late phase of the pathway controlling left-right asymmetry. *Cell* **94**, 319-324.

Ponting, C. P. (1997). Tudor domains in proteins that interact with RNA. *Trends Biochem. Sci.* **22**, 51-52.

Poux, S., Horrad, B., Sigrist, C. J. and Pirrotta, V. (2002). The *Drosophila* trithorax protein is a coactivator required to prevent re-establishment of polycomb silencing. *Development* **129**, 2483-2493.

Randsholt, N. B., Maschat, F. and Santamaria, P. (2000). Polyhomeotic controls engrailed expression and the hedgehog signaling pathway in imaginal discs. *Mech. Dev.* **95**, 89-99.

Raya, A., Kawakami, Y., Rodrıguez-Esteban, C., Ibanes, M., Rasskin-Gutman, D., Rodrıguez-Leo, J., Buscher, D., Feijo, J. and Izpisúa-Belmonte, J. C. (2004). Notch activity acts as a sensor for extracellular calcium during vertebrate left–right determination. *Nature* **427**, 121-128.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabon, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

Rodriguez-Esteban, C., Capdevila, J., Economides, A. N., Pascual, J., Ortiz, A. and Izpisúa-Belmonte, J. C. (1999). The novel Cer-like protein Caronte mediates the establishment of embryonic left-right asymmetry. *Nature* **401**, 243-251.

Rodriguez-Esteban, C., Capdevila, J., Kawakami, Y. and Izpisúa-Belmonte, J. C. (2001). Wnt signaling and PKA control Nodal expression and left-right determination in the chick embryo. *Development* **128**, 3189-3195.

Ryan, A. K., Blumberg, B., Rodriguez-Esteban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., de la Pena, J., Sabbagh, W., Greenwald, J., Choe, S. et al. (1998). Pitx2 determines left-right asymmetry of internal organs in vertebrates. *Nature* **394**, 545-551.

Sampath, K., Cheng, A. M. S., Frisch, A. and Wright, C. V. E. (1997). Functional differences among Xenopus nodal-related genes in left-right axis determination. *Development* **124**, 3293-3302.

Schlange, T., Schnipkoweit, I., Andree, B., Ebert, A., Zile, M. H., Arnold, H. H. and Brand, T. (2001). Chick cfc controls lefty1 expression in the embryonic midline and nodal expression in the lateral plate. *Dev. Biol.* **234**, 376-389.

Schlange, T., Arnold, H. H. and Brand, T. (2002). BMP2 is a positive regulator of Nodal signaling during left-right axis formation in the chick embryo. *Development* **129**, 3421-3429.

Schneider, A., Mijalski, T., Schlange, T., Dai, W., Overbeek, P., Arnold, H. H. and Brand, T. (1999). The homeobox gene Nkx3.2 is a target of left-right signaling and is expressed on opposite sides in chick and mouse embryos. *Curr. Biol.* **9**, 911-914.

Schumacher, A., Faust, C. and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. *Nature* **383**, 250-253.

Schughart, K., Bieberich, C. J., Eid, R. and Ruddle, F. H. (1991). A regulatory region from the mouse *Hox-2.2* promoter directs gene expression into developing limbs. *Development* **112**, 807-811.

Srivastava, D., Cserjesi, P. and Olson, E. N. (1995). A subclass of bHLH proteins required for cardiac morphogenesis. *Science* **270**, 1995-1999.

St Amand, T. R., Ra, J., Zhang, Y., Hu, Y., Baber, S., Qiu, M. S. and Chen, Y. P. (1998). Cloning and expression pattern of chicken Pitx2, a new component in the SHH signaling pathway controlling embryonic heart looping. *Biochem. Biophys. Res. Commun.* **247**, 100-105.

St Amand, T. R., Zhang, Y., Semina, E. V., Zhao, X., Hu, Y., Nguyen, L., Murray, J. C. and Chen, Y. P. (2000). Antagonistic signals between BMP4 and FGF8 define the expression of *Pitx1* and *Pitx2* in mouse tooth-forming anlage. *Dev. Biol.* **217**, 323-332.

Suzuki, M., Mizutani-Koseki, Y., Fujimura, Y., Miyagishima, H., Kaneko, T., Takada, Y., Akasaka, T., Tanzawa, H., Takihara, Y., Nakano, M. et al. (2002). Involvement of the Polycomb-group gene Ring1B in the specification of the anterior-posterior axis in mice. *Development* **129**, 4171-4183.

Takihara, Y., Tomotsune, D., Shirai, M., Katoh-Fukui, Y., Nishii, K., Motaleb, M. A., Nomura, M., Tsuchiya, R., Fujita, Y., Shibata, Y. et al. (1997). Targeted disruption of the mouse homolog of the Drosophila polyhomeotic gene leads to altered anteroposterior patterning and neural crest defects. *Development* **124**, 3673-3682.

Tie, F., Prasad-Sinha, J., Birve, A., Rasmuson-Lestander, A. and Harte, P. J. (2003). A 1-megadalton ESC/E(Z) complex from Drosophila that contains polycomblike and RPD3. *Mol. Cell Biol.* **23**, 3352-3362.

Ting-Berreth, S. A. and Chuong, C. M. (1996). Sonic Hedgehog in feather morphogenesis, induction of mesenchymal condensation and association with cell death. *Dev. Dyn.* **207**, 157-170.

Trevino, C., Anderson, R., Landry, M., Konig, G., Tonthat, B., Shi, C. and Muneoka, K. (1993). MPLB-2, a posterior signaling cell line derived from the mouse limb bud. *Prog. Clin. Biol. Res.* **383**, 295-304.

Tsukui, T., Capdevila, J., Tamura, K., Ruiz-Lozano, P., Rodriguez-Esteban, C., Yonei-Tamura, S., Magallon, J., Chandraratna, R. A., Chien, K., Blumberg, B. et al. (1999). Multiple left-right asymmetry defects in *Shh* (–/–) mutant mice unveil a convergence of the Shh and retinoic acid pathways in the control of Lefty-1. *Proc. Natl. Acad. Sci. USA* **96**, 11376-11381.

Uchikawa, M., Ishida, Y., Takemoto, T., Kamachi, Y. and Kondoh, H. (2003). Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals. *Dev. Cell* **4**, 509-519.

Yamashita, H., Dijke, P., Huylebroeck, D., Sampath, T., Andries, M., Smith, J., Heldin, C. and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J. Cell Biol.* **130**, 217-226.

Yasuda, K., Momose, T. and Takahashi, Y. (2000). Application of microelectroporation for studies of chick embryogenesis. *Dev. Growth Differ.* **42**, 203-206.

Yochum, G. and Ayer, D. (2001). Pf1, a novel PHD zinc finger protein that links the TLE corepressor to the mSin3A-histone deacetylase complex. *Mol. Cell. Biol.* **21**, 4110-4118.

Yokouchi, Y., Vogan, K. J., Pearse, R. V., II and Tabin, C. J. (1999). Antagonistic signaling by Caronte, a novel Cerberus-related gene, establishes left-right asymmetric gene expression. *Cell* **98**, 573-583.

Yoshioka, H., Meno, C., Koshiba, K., Sugihara, M., Itoh, H., Ishimaru, Y., Inoue, T., Ohuchi, H., Semina, E. V., Murray, J. C. et al. (1998). Pitx2. a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. *Cell* **94**, 299-305.

Yoshitake, Y., Howard, T., Christian, J. L. and Hollenberg, S. M. (1999). Misexpression of Polycomb-group proteins in Xenopus alters anterior neural development and represses neural target genes. *Dev. Biol.* **215**, 375-387.

Yu, X. Y., St Amand, T. R., Wang, S. S., Li, G., Zhang, Y. D., Hu, Y. P., Nguyen, L., Qiu, M. S. and Chen, Y. P. (2001). Differential expression and functional analysis of Pitx2 isoforms in regulation of heart looping in the chick. *Development* **128**, 1005-1013.

Zhang, Y. and Reinberg, D. (2001). Transcription regulation by histone methylation, interplay between different covalent modifications of the core histone tails. *Genes Dev.* **15**, 2343-2360.

Zhang, Z. Y., Yu, X., Zhang, Y. D., Geronimo, B., Lovlie, A., Fromm, S. H. and Chen, Y. P. (2000). Targeted misexpression of constitutively active BMP receptor-IB causes bifurcation and duplication and posterior transformation of digit in mouse limb. *Dev. Biol.* **220**, 154-167.

Zhu, L., Marvin, M. J., Gardiner, A., Lassar, A., Mercola, M., Stern, C. D. and Levin, M. (1999). Cerberus regulates left-right asymmetry of the embryonic head and heart. *Curr. Biol.* **9**, 931-938.