

The *Polycomb*-group gene *Rae28* sustains *Nkx2.5/Csx* expression and is essential for cardiac morphogenesis

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The *Polycomb*-group (*PcG*) gene *Rae28* is a mammalian homologue of the *Drosophila* gene *polyhomeotic*. *PcG* genes are known to maintain transcription states, once initiated, probably by regulating chromatin structure. Since homozygous *Rae28*-deficient (*Rae28*^{-/-}) mice displayed cardiac anomalies similar to congenital heart diseases in humans, we examined the role of *Rae28* in cardiac morphogenesis at the molecular level. In *Rae28*^{-/-} embryos, expression of the cardiac selector gene *Nkx2.5/Csx* (*Nkx2.5*) was initiated properly but was not sufficiently sustained later in development. This impaired expression of *Nkx2.5* in the maintenance phase proved to have a crucial effect on cardiac morphogenesis, as demonstrated by the results of a genetic complementation experiment in which the cardiac anomalies were suppressed by overexpression of human *NKX2.5/CSX1* in *Rae28*^{-/-} embryos. Ubiquitous expression of exogenous *Rae28* likewise restored the impaired *Nkx2.5* expression in *Rae28*^{-/-} embryos, further supporting the notion that *Rae28* sustains *Nkx2.5* expression in cardiomyocytes. Thus, our data show that a mammalian *PcG* gene can play a key role in organogenesis by helping to maintain the expression of a selector gene.

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Introduction

During development, differential gene expression governs generation of the cellular specificity and diversity required for the establishment of patterning and organogenesis. In *Drosophila*, the initial pattern of homeotic gene expression, which governs cellular specificity, is established by maternal effect and segmentation gene products. Since expression of segmentation gene products is transient, the expression patterns of homeotic genes must be relayed by *Polycomb*-group (*PcG*) and *trithorax*-group genes, which are known to maintain repressed and active chromatin structures, respectively (1).

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Nonstandard abbreviations used: *Polycomb*-group (*PcG*); tetralogy of Fallot (TOF); double outlet of the right ventricle (DORV); *Nkx2.5/Csx* (*Nkx2.5*); *NKX2.5/CSX1* (*NKX2.5*); atrial natriuretic factor (ANF); myosin light chain 2v (*MLC2v*); atrioventricular (AV); days postcoitus (dpc); *Nkx2.5/Csx* (*Nkx2.5*).

Until recently, less has been known about the maintenance phase of the developmental regulation of transcription in mammals. Isolation of mammalian homologues of *Drosophila PcG* genes yielded insights into its mechanisms and biological functions. There exist at least two classes of *PcG* complexes, namely *PcG* complexes 1 and 2 (2). *PcG* complex 1 may be a homologue to *Drosophila Polycomb* repressive complex 1, which may competitively antagonize the ATP-dependent nucleosome remodeling ability of SWI/SNF-family complexes (3) and may also directly interact with the general transcription machinery (4, 5). As we previously reported, the *Rae28* gene (*Rae28*) is a mammalian homologue of the *Drosophila polyhomeotic* gene, a member of *PcG* genes, and encodes a constituent of *PcG* complex 1 (2).

Targeted replacement has been used to create mutant mice lacking each member of *PcG* complex 1 (6–11). All these mutations were found to cause abnormal antero-posterior patterning, similar to *PcG* mutations in flies, which resulted from altered *Hox* expression. We generated *Rae28*-deficient (*Rae28*^{-/-}) mice and clearly demonstrated that mammalian *PcG* genes have a highly conserved role in the maintenance of *Hox* expression but not in its initiation; i.e., *Hoxb3* expression was not affected in the initiation phase but its expression domain became progressively enhanced along the

anteroposterior axis in the later maintenance phase in the rhombomeres of *Rae28*^{-/-} embryos (10, 12).

Interestingly, these PcG gene-deficient mice further revealed that mammalian PcG genes have additional roles in hematopoiesis, sex determination, and cerebellar and heart development (6–10). Since *Rae28*^{-/-} embryos revealed cardiac anomalies compatible with tetralogy of Fallot (TOF) and double outlet of the right ventricle (DORV) (10), *Rae28*^{-/-} mice provided a clue to clarifying the role of PcG genes in heart development.

The cardiac precursors are generated in the anterior lateral plate mesoderm and migrate ventromedially to form the linear heart tube, which possesses a highly regionalized structure (13). Complex remodeling of the heart tube and immigration of the neural crest cells occur during looping to complete morphogenesis of the heart. The genetic pathways regulating regionalization and these complex processes were recently identified (13). The cardiac homeobox gene *Nkx2.5/Csx* (*Nkx2.5*), a mammalian homologue of the *Drosophila tinman* (14, 15), is presumed to be a cardiac selector gene essential for either cardiogenesis or cardiac morphogenesis (16). This hypothesis is based on the fact that *Nkx2.5*-deficient embryos display defects in looping morphogenesis of the heart and cardiac gene expression of *Hand1*, *atrial natriuretic factor* (*ANF*), and *myosin light chain 2v* (*MLC2v*) (17–19), and that exogenous expression of dominant negative *Nkx2.5* into early *Xenopus* blastomeres abrogates heart formation (20, 21). Several mutations in *NKX2.5/CSX1* (*NKX2.5*) have been reported in patients with a variety of cardiac anomalies, including atrioventricular (AV) conduction delays, atrial septal defects, TOF, and DORV (22–24), further underscoring the importance of *Nkx2.5* in cardiac development.

Studies have recently been reported on the *cis*-regulatory elements required for initiation of *Nkx2.5* expression. These elements, however, were unable to sustain the expression later in the development of the heart in the transgenic animals (25, 26). Thus, very little is known about the maintenance phase of cardiac *Nkx2.5* expression in mammals.

In the present study, we performed a detailed examination of *Rae28*^{-/-} embryos to determine the role of *Rae28* in the genetic programs governing heart development. First, we propose the presence of a maintenance phase in *Nkx2.5* expression in cardiomyocytes, which is sustained by *Rae28*. Secondly, the maintenance phase of cardiac *Nkx2.5* expression plays a crucial role in maturing cardiac morphogenesis. Based on this evidence, we argue the case for biological implication of the PcG gene-mediated transcription maintenance system in mammalian heart development.

Methods

Generation of Rae28^{-/-} embryos and those complemented by *NKX2.5* overexpression or exogenous *Rae28* expression. Mice deficient in *Rae28* were generated and maintained by heterozygous intercrossing as described previously (10). Transgenic mouse lines overexpressing human

NKX2.5/CSX1 cDNA were generated by using pCAGGS vector (27), and those overexpressing the full-length *Rae28* cDNA (*Rae2819*) (28) by using either chicken *β-actin* promoter with Rous sarcoma-virus enhancer (*RSV-Rae28*) or *β-myosin heavy chain* promoter (*βMHC-Rae28*) (29). These transgenic mice were backcrossed to C57BL/6 to minimize the effects of genetic background before being used in this study. The resulting progeny were mated with heterozygous *Rae28*-deficient mice (*Rae28*^{+/-}), and their genotypes were examined by means of PCR. Embryos obtained by cesarean section were examined histologically and subjected to *in situ* hybridization analyses.

Whole-mount and section in situ hybridization. Whole-mount *in situ* hybridization was performed essentially as previously described (12). Briefly, embryos were fixed in PBS containing 4% paraformaldehyde, bleached, and treated with 10 μg/ml proteinase K (Sigma-Aldrich, St. Louis, Missouri, USA). After additional fixation in 0.2% glutaraldehyde and 4% paraformaldehyde in PBS, the embryos were soaked in prewarmed prehybridization buffer for 1 hour at 70°C, and hybridized overnight with a digoxigenin-labeled riboprobe. Hybridization was detected by treatment of embryos with preabsorbed alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche Diagnostics GmbH, Mannheim, Germany), followed by reaction with 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Roche Diagnostics GmbH). Section *in situ* hybridization with *αMHC*, *cardiac α-actin*, and *cardiac Ca²⁺-dependent ATPase* probes was done as described previously (10).

RT-PCR assay. Total RNA was prepared from the hearts, which were dissected from embryos at 9.5 days postcoitus (dpc), by means of ISOGENE (Nippon Gene, Tokyo, Japan), treated with RNase-free DNase I (Roche Diagnostics GmbH), and suspended in 20 μl of distilled water. The first-strand cDNA synthesis was performed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, California, USA) and an oligo(dT)₂₀ primer according to the manufacturer's instructions. PCR amplification was performed with a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, California, USA) using the previously described oligonucleotide primers specific for *MLC2a*, *MLC2v*, *αMHC*, *ANF*, *Hand1*, *Nkx2.5*, and *β-actin* (17, 30–33). PCR cycles were as follows: 95°C for 5 minutes, followed by 25–50 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide.

Results

Morphology of the embryonic hearts. Wild-type and *Rae28*^{-/-} hearts were microscopically indistinguishable at the linear heart tube stage (8.0 dpc). At 8.5 dpc, the heart tube initiated dextro-looping, which orients the atrial and ventricular chambers and aligns the outflow tracts with the vasculature. At 9.5 dpc, 17 out of 20 *Rae28*^{-/-} hearts showed apparent positional

immaturity and/or deformation, as demonstrated by incomplete looping, deformation of the wall, and/or dilatation of the heart tube (Figure 1). The right frontal view shows that interventricular sulcus formation was incomplete and that the bulbus cordis and truncus arteriosus were extremely deformed (Figure 1c). Angulation between the ventricles, and between the bulbus cordis and truncus arteriosus, was reduced in comparison with that of the wild-type (Figure 1c). The left frontal view shows that the AV canal was neither narrowed nor elongated sufficiently (Figure 1d). This may be the result of impaired development of the AV cushion, which may be also responsible for positional immaturity and mislooping in the mutant heart. Because cardiac looping is an important morphologic event that is critical for the proper alignment and orientation of the atrial, ventricular, and vascular compartments of the heart (13), it is probable that positional and morphological changes in the looping heart caused the abnormal cardiac morphogenesis in *Rae28*^{-/-} embryos.

In situ hybridization analysis of gene expression. To examine the molecular effect of *Rae28* mutation on cardiac segments, expression of the cardiac-specific genes in 9.5-dpc *Rae28*^{-/-} embryos (13, 30, 34) was observed with the aid of whole-mount in situ hybridization. The domain of *ANF* expression in the presumptive right ventricle had almost disappeared in *Rae28*^{-/-} embryos (Figure 2b). Although *MLC2v* expression is normally restricted to the ventricles of the developing heart (30), *MLC2v* expression in *Rae28*^{-/-} embryos had extended to part of the atrium beyond the AV canal (Figure 2d). Although there are wild-type embryos in which *MLC2v* expression extends to the atrium to some extent, the extension was more pronounced in *Rae28*^{-/-} embryos. These altered expression domains of *ANF* and *MLC2v* suggest that segmental specification is abnormal in the mutant hearts. Other cardiac-specific genes, including *MLC2a* (Figure 2f), *αMHC*, *cardiac α-actin*, and *cardiac Ca²⁺-dependent ATPase*, were expressed normally in the mutant hearts (35, 36) (Table 1), suggesting that the *Rae28* mutation does not have an overall effect on the expression of heart-specific genes.

Recently, several genes have been implicated in cardiac segmentation and chamber specification. The basic helix-loop-helix genes *Hand1* and *Hand2* are expressed in complementary patterns in the developing heart and are involved in cardiac looping and chamber specification (37–41). Expression of *Hand1* was markedly reduced in the hearts of 9.5-dpc *Rae28*^{-/-} embryos (Figure 2, h, k, and l) but was unaffected in other tissues in these embryos, such as the pharyngeal arches and lateral mesoderm (Figure 2h). This finding indicates that misregulation of this gene in the mutant embryos is heart-specific. However, *Hand2* expression was not altered in the mutant embryos (Figure 2p), which is consistent with the specific, rather than general, misregulation of genes required for heart development.

The genes affected by the *Rae28* mutation, *ANF*, *MLC2v*, and *Hand1*, are downstream targets of *Nkx2.5* (17–19), which is essential for cardiac looping and morphogenesis (17–19). We therefore examined the expression of *Nkx2.5* in 9.5-dpc *Rae28*^{-/-} embryos. *Nkx2.5* expression had almost disappeared in the ventricles but was detectable at a very low level in the common atrium (Figure 2r). Interestingly, however, we could not find any significant difference in expression of *Nkx2.5* in the initial stage of cardiac looping (8.5 dpc) (Figure 2t); this was also confirmed by the finding that expression of *Hand1*, a downstream target of *Nkx2.5*, was normally initiated (Figure 2n). This finding is consistent with what has been observed in PcG regulation of homeotic genes (1).

The data from in situ hybridization analyses of *Rae28*^{-/-} embryos are summarized in Table 1. Representative findings are shown in the figures as described above. Although the severity of misexpression somehow varied in each of the embryos, all *Rae28*^{-/-} embryos showed apparent misexpression of *MLC2v*, *Hand1*, and *Nkx2.5*. *ANF* expression domain was altered in two out of three *Rae28*^{-/-} embryos as shown in Figure 2b, but not in the third.

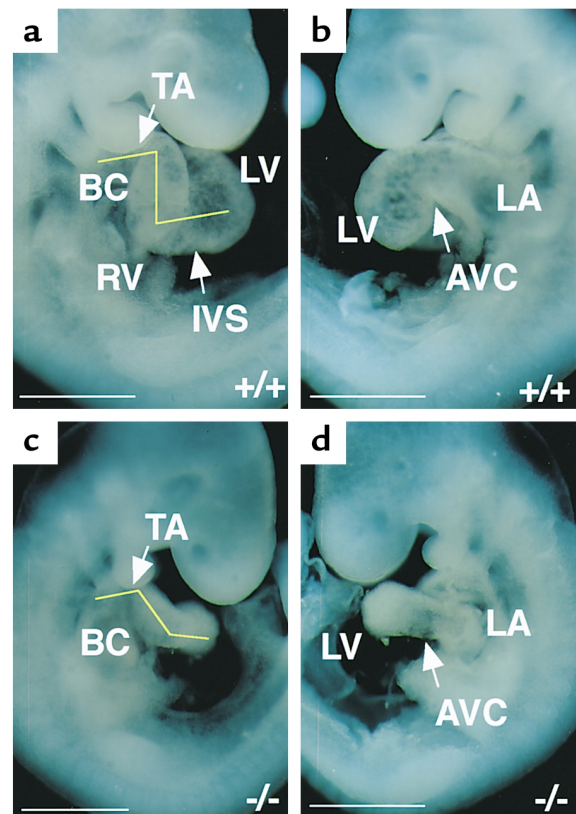


Figure 1 Cardiac looping morphogenesis in 9.5-dpc embryos. (a and c) Right and (b and d) left frontal views of whole embryos are shown. Cardiac looping was morphologically affected in the 9.5-dpc *Rae28*^{-/-} embryo (c and d). IVS, interventricular sulcus; BC, bulbus cordis; TA, truncus arteriosus; AVC, AV canal; LV, left ventricle; LA, left atrium; RV, right ventricle. +/+, a wild-type embryo; -/-, a *Rae28*^{-/-} embryo. Scale bar: 500 μm.

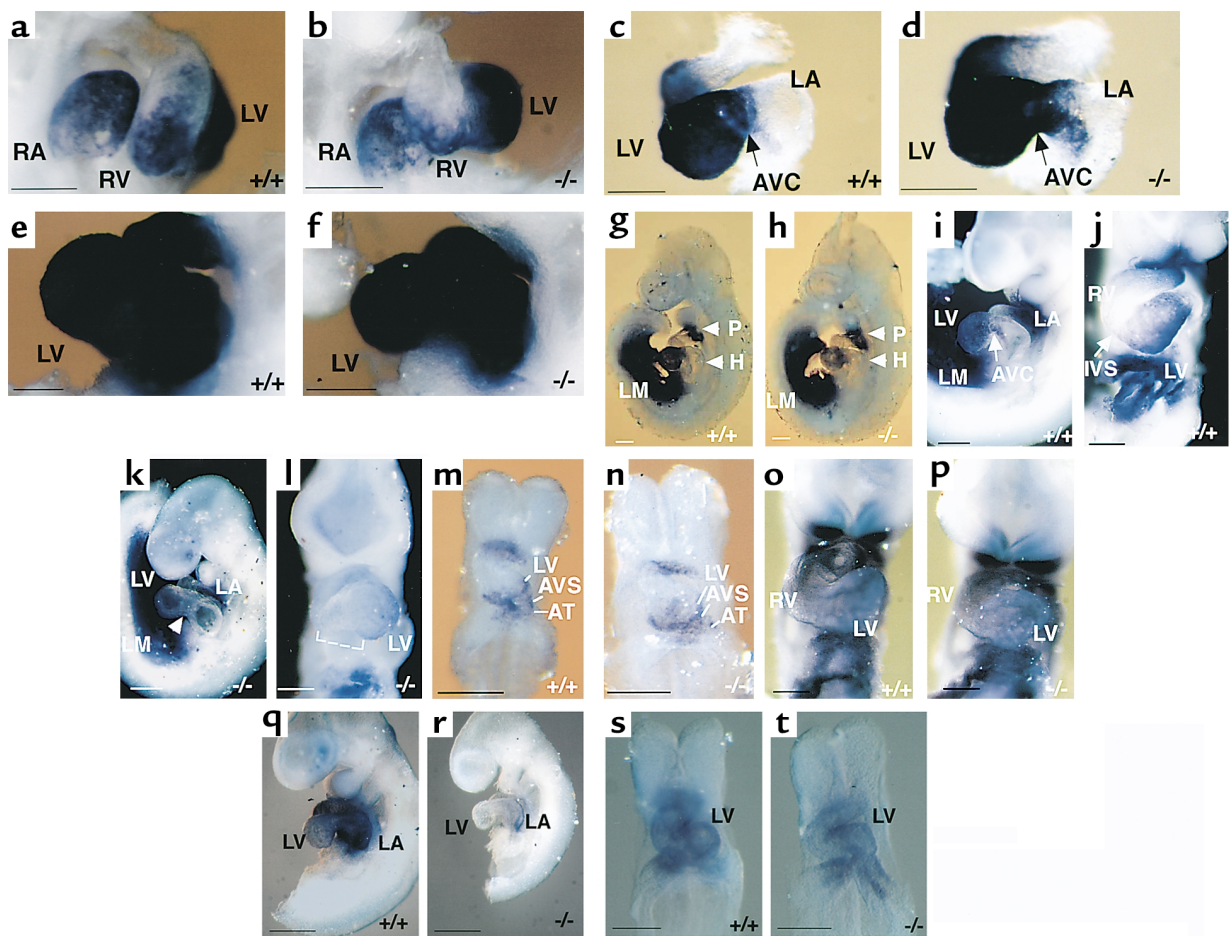


Figure 2

Whole-mount in situ hybridization. Expression of *ANF* (a and b), *MLC2v* (c and d), *MLC2a* (e and f), *Hand1* (g–n), *Hand2* (o and p), and *Nkx2.5* (q–t) was detected. (a–l and o–r) 9.5 dpc; (m, n, s, and t) 8.5 dpc; (a and b) Right lateral views; (c–i, k, q, and r) left lateral views; (j, l–p, s, and t) frontal views. *ANF* expression in the right ventricle is reduced in the *Rae28*^{-/-} embryo (b), while *MLC2v* expression extends into the right atrium (d). Expression of *Hand1* was markedly reduced in the hearts of the *Rae28*^{-/-} embryos (h, k, and l). Since AVC and IVS formations were incomplete in the *Rae28*^{-/-} embryos, the portions corresponding to AVC and IVS are indicated by an arrowhead and a dotted line, respectively (k and l). Note that *Nkx2.5* and *Hand1* expressions were markedly reduced in the *Rae28*^{-/-} embryos at 9.5 dpc (k, l, and r) but were unaffected at 8.5 dpc (n and t). RA, right atrium; LM, lateral mesoderm; AVS, AV sulcus; AT, atrium; P, pharyngeal arch; H, heart. Scale bar: 200 μ m.

RT-PCR analysis of gene expression. Cardiac gene expression was further examined by semiquantitative RT-PCR analysis. cDNA was generated from total cellular RNAs extracted from the hearts, which were derived from five stage-matched *Rae28*^{-/-} or wild-type embryos to minimize interembryo variability. A quantitative RT-PCR assay (17) confirmed that the transcripts of *Nkx2.5* and *Hand1* were downregulated by approximately a factor of four in the mutant hearts, and those of *MLC2v* by a factor of two (Figure 3). Although the expression domain of *ANF* in the right ventricle had almost disappeared in two out of three *Rae28*^{-/-} embryos (Table 1 and Figure 2b), the RT-PCR assay could not detect any significant change in the amount of *ANF* mRNA in the mutant hearts (Figure 3), probably because *ANF* expression had not necessarily been altered in all the mutant embryos and because a larger amount of *ANF* mRNA present in the left ventricle may overcome the difference. The consistency of

the changes was confirmed in RNAs extracted from another five embryonic hearts with each of the genotypes (data not shown). The specificity of these bands was also confirmed by Southern blot analysis with radiolabeled gene-specific probes (data not shown).

*Complementation analysis of *Rae28*^{-/-} embryos by exogenous human *NKX2.5*.* We next wanted to provide functional evidence of the diminished expression of *Nkx2.5* in *Rae28*^{-/-} embryos, and to determine in vivo to what extent downregulation of *Nkx2.5* is responsible for the cardiac phenotype. A genetic complementation experiment was performed by crossing *Rae28*-deficient animals with transgenic mice overexpressing *NKX2.5/CSX1* (*NKX2.5*) (27) (Figure 4a). Although the pCAGGS vector, including the chicken β -actin promoter with a cytomegalovirus enhancer, was used to generate the transgenic mice, *NKX2.5* expression was not ubiquitous but detected mainly in the heart and somites, as reported previously (Figure 4a). Northern blot analysis was

Table 1
Expression of cardiac-specific genes in *Rae28*^{-/-} embryos

Gene	Age (dpc)	Examined ^A	Affected ^B
<i>ANF</i>	9.5	3	2 ^C
<i>MLC2a</i>	9.5	3	0
<i>MLC2v</i>	9.5	4	4
<i>αMHC</i>	10.5	4	0
<i>α-actin</i>	10.5	4	0
<i>ATPase</i>	10.5	3	0
<i>Hand1</i>	8.5	2	0
	9.5	4	4
<i>Hand2</i>	9.5	5	0
<i>Nkx2.5</i>	8.5	2	0
	9.5	5	5

^AThe number of *Rae28*^{-/-} embryos examined by in situ hybridization analysis.

^BThe number of *Rae28*^{-/-} embryos with affected gene expression. Representative embryos with affected gene expression are shown in the figures. ^C*ANF* expression was affected in two *Rae28*^{-/-} embryos as shown in Figure 2b but was not in the other. Note that all the affected genes, *ANF*, *MLC2v*, and *Hand1*, are downstream targets of *Nkx2.5*. *α-actin*, cardiac *α-actin*; *ATPase*, cardiac *Ca*²⁺-dependent *ATPase*.

used to confirm the persistent expression of the *NKX2.5* transgene even in the neonatal stage (data not shown). We examined 12 *Rae28*^{-/-} neonates including eight carrying the *NKX2.5* transgene (*Rae28*^{-/-}/*NKX2.5*). Four *Rae28*^{-/-} embryos without the *NKX2.5* transgene showed cardiac anomalies, including a large membranous ventricular septal defect straddled on the aorta (Figure 4c), which is compatible with TOF, and a large atrial septal defect with bizarre tricuspid valves (Figure 4d). These abnormal morphological findings were essentially similar to those reported previously in *Rae28*^{-/-} mice (10). On the other hand, complete reversion of the cardiac phenotype at the morphologic level was found in each of the seven *Rae28*^{-/-}/*NKX2.5* embryos (Figure 4, e and f) except for one, which displayed a muscular-type ventricular septal defect (data not shown). Thus, overexpression of *NKX2.5* suppressed the majority of the heart phenotype observed in *Rae28*^{-/-} mice. Not only *Hand1* (Figure 4, i and j), *ANF*, and *MLC2v* expressions (data not shown) but also cardiac looping improved remarkably in 9.5-dpc *Rae28*^{-/-}/*NKX2.5* embryos (Figure 4, i and j). Although weak *NKX2.5* expression was detected in the regions outside the heart in the *NKX2.5* transgenic embryos (Figure 4a), no significant effect of the exogenous *NKX2.5* expression was detected on the mutant phenotypes outside the heart, such as abnormal facies with ophthalmic hypoplasia, cleft palate, and parathyroid and thymic hypoplasia (data not shown).

Effect of exogenous *Rae28* expression on *Nkx2.5* expression.
To address the question of how *Rae28* regulates *Nkx2.5* expression in cardiomyocytes, we generated transgenic mice overexpressing *Rae28*, using either the chicken *β-actin* promoter with a Rous sarcoma-virus enhancer (*RSV-Rae28*) or the *β-myosin heavy chain* promoter (*βMHC-Rae28*) (29). Further, we used genetic intercrossing to generate *Rae28*^{-/-} embryos carrying either *RSV-Rae28* (*Rae28*^{-/-}/*RSV-Rae28*) or *βMHC-Rae28*

transgene (*Rae28*^{-/-}/*βMHC-Rae28*). Exogenous *Rae28* expression was detected at 8.5 dpc (Figure 5, b and c). The *Rae28* expression was ubiquitous in *Rae28*^{-/-}/*RSV-Rae28* embryos but was cardiomyocyte-specific in *Rae28*^{-/-}/*βMHC-Rae28* embryos (Figure 5, b and c). The similar expression patterns were also found at 9.5 dpc (29). Thus the exogenous *Rae28* expression was considered to occur prior to or during the time that *Nkx2.5* expression was altered in *Rae28*^{-/-} embryos. The expression of exogenous *Rae28* protein was confirmed in both of these transgenic hearts using Western blot analysis (29). All the hearts from *Rae28*^{-/-}/*RSV-Rae28* embryos displayed complete reversion of cardiac anomalies, while those from *Rae28*^{-/-}/*βMHC-Rae28* embryos showed no alteration in the cardiac phenotype (29). Thus, whole-mount in situ hybridization was used to examine *Nkx2.5* expression in *Rae28*^{-/-}/*RSV-Rae28* or *Rae28*^{-/-}/*βMHC-Rae28* embryos at 9.5 dpc. Reduced *Nkx2.5* expression in *Rae28*^{-/-} had reverted to the wild-type level in *Rae28*^{-/-}/*RSV-Rae28* embryos (Figure 5e), but not in *Rae28*^{-/-}/*βMHC-Rae28* embryos (Figure 5g); this was highly compatible with phenotypic alterations in these mutant embryos, as mentioned above (29). Thus, *βMHC* promoter-mediated cardiomyocyte-specific *Rae28* expression was unable to revert the reduced *Nkx2.5* expression in the *Rae28*^{-/-} hearts. We further confirmed that overexpression of *Rae28* in cardiomyocytes neither induced nor reduced *Nkx2.5* expression either at 9.5 dpc (Figure 5f) or in the neonatal period (data not shown).

Discussion

In general, little has been known about the molecular mechanisms that maintain gene expression after the initial transcriptional decision has been made during mammalian development. Recently, more than 14

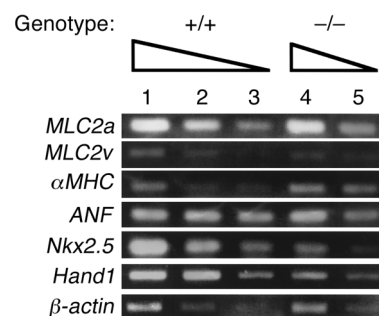


Figure 3

Quantitative RT-PCR analysis of gene expression in the hearts. Total cellular RNAs were extracted from the hearts dissected from five wild-type and five *Rae28*^{-/-} embryos at 9.5 dpc. As indicated by the wedges, twofold serial dilutions of cDNAs were used as templates for PCR amplification with primers specific for *MLC2a*, *MLC2v*, *αMHC*, *ANF*, *Nkx2.5*, *Hand1*, and *β-actin*. Signals for *β-actin* mRNA were used as controls to adjust the amount of cDNAs. Equal amounts of cDNAs were subjected to the PCR assays in lanes 1 and 4 as well as in lanes 2 and 5. Although the expression domain of *ANF* in RV was affected in *Rae28*^{-/-} (Figure 2b), RT-PCR analysis did not detect a significant difference in the amount of *ANF* mRNA.

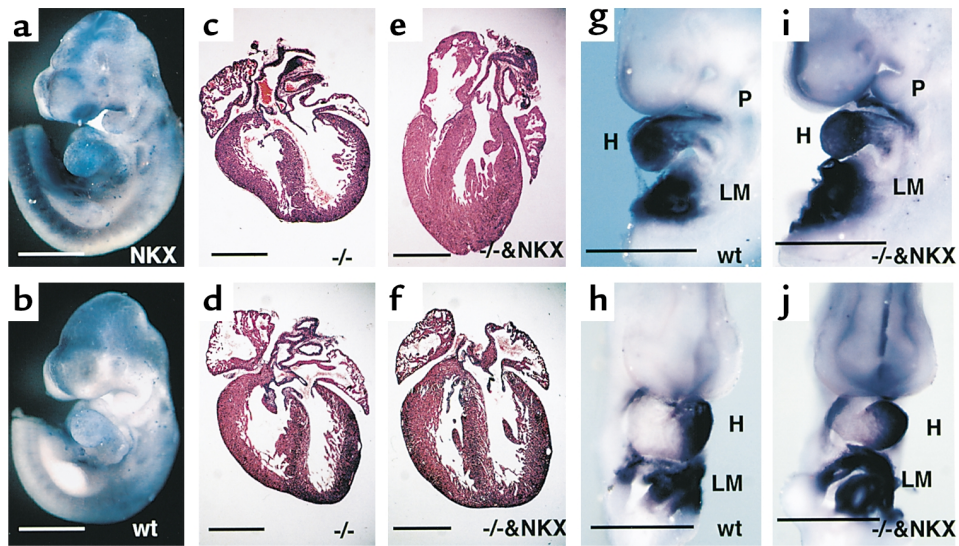


Figure 4

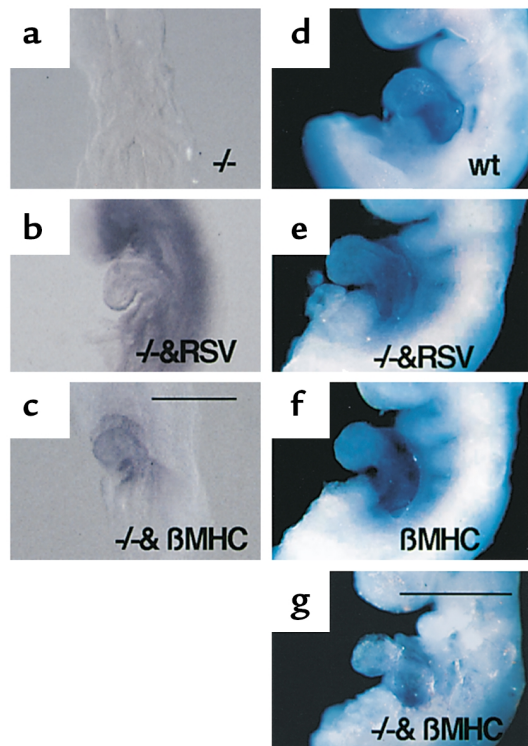
Suppression of cardiac phenotypes in *Rae28*^{-/-} embryos by overexpression of *NKX2.5*. (a and b) Whole-mount in situ hybridization of 9.5-dpc embryos with a *NKX2.5* probe. Since we used a human *NKX2.5* probe, the signal in the wild-type embryo was weaker, while that from the transgene was detected clearly. (c-f) Histological sections of the mutant hearts. The offspring were generated by intercrossing of *Rae28*^{+/-} mice carrying the *NKX2.5* transgene, and the hearts derived from *Rae28*^{-/-} embryos with or without the transgene were examined histologically. (g-j) Whole-mount in situ hybridization of 9.5-dpc embryos with a *Hand1* probe. The findings were reproducible in the other embryo with the same genotype (data not shown). WT, wild-type embryos; -/-, *Rae28*^{-/-} embryos; NKX, *NKX2.5* transgenic embryos; -/-&NKX, *Rae28*^{-/-} embryos carrying the *NKX2.5* transgene; LM, lateral mesoderm. Scale bar: 500 μm.

mammalian homologues of *Drosophila* PcG genes have been identified, and genetic evidence is accumulating that mammalian PcG genes are involved in wider biological functions than *Drosophila* PcG genes (2). *Rae28*^{-/-} mice not only showed affected anteroposterior patterning in the paraxial mesoderm and rhombomeres but also displayed abnormal cardiac morphogenesis (10). In the study reported here, we revealed that expression of *Nkx2.5* and its downstream targets was systematically affected during the later stage of heart development in *Rae28*^{-/-} embryos. The impaired expression of *Nkx2.5* proved to be mainly responsible for cardiac dysmorphogenesis, because complementary expression of human *NKX2.5* effectively reverted cardiac anomalies in *Rae28*^{-/-} embryos. Interestingly, we could not

find any significant reduction in *Nkx2.5* expression and that of its downstream target *Hand1* in the initial stage of cardiac looping at 8.5 dpc, while these expressions were affected in the later stage (9.5 dpc). This finding is consistent with what has been observed for PcG regulation of homeotic genes in *Drosophila* and in mammals

Figure 5

Effect of exogenous *Rae28* on *Nkx2.5* expression in the heart. Expression of the exogenous *Rae28* genes and their effect on *Nkx2.5* expression were examined by whole-mount in situ hybridization with *Rae28* (a-c) and *Nkx2.5* probes (d-g). Ubiquitous and cardiomyocyte-specific *Rae28* expression was detected at 8.5 dpc in *Rae28*^{-/-}/*RSV-Rae28* and *Rae28*^{-/-}/*βMHC-Rae28* embryos, respectively (b and c), either of which possessed a genetic background of *Rae28*^{-/-} where *Rae28* expression was undetectable (a). Ubiquitous *Rae28* expression efficiently reverted reduced expression of *Nkx2.5* in the *Rae28*^{-/-} embryo (e), while cardiomyocyte-specific expression of *Rae28* neither augmented nor reduced *Nkx2.5* expression in the wild-type heart (f) and did not exert any effect on reduced expression of *Nkx2.5* in the *Rae28*^{-/-} embryo (g). We further confirmed these findings in other embryos (data not shown). WT, wild-type embryo; βMHC, βMHC-*Rae28*; -/-&RSV, *Rae28*^{-/-}/*RSV-Rae28*; -/-&βMHC, *Rae28*^{-/-}/*βMHC-Rae28*. Scale bar: 200 μm (a-c), 500 μm (d-g).



(1, 10, 12). Normal initiation but alteration in *Nkx2.5* and *Hand1* expression in the maintenance phase may be the reason why cardiac development was not completely eliminated in the looping stage as observed in *Nkx2.5*- and *Hand1*-deficient mice (17–19, 39, 42). Instead, these alterations may have generated cardiac anomalies such as TOF and/or DORV, indicating the important role of the maintenance phase of *Nkx2.5* expression in maturing cardiac morphogenesis. Ubiquitous expression of exogenous *Rae28* reverted the impaired *Nkx2.5* expression in *Rae28*^{-/-} embryos, further confirming that *Rae28* is required for sustaining *Nkx2.5* expression in cardiomyocytes.

Since *Nkx2.5* is highly conserved and plays an essential role in heart development (43), analysis of the transcription regulation is crucial for understanding the molecular mechanisms of heart development and the pathogenesis of congenital heart diseases in humans. *Nkx2.5* transcription was shown to be regulated by complex modular *cis*-regulatory elements with distinct regional specificity (16), suggesting that the heart is presumed to evolve as a modular organ in higher organisms (43). Although these elements recapitulate the initial expression pattern of *Nkx2.5* relatively well, they were unable to sustain *Nkx2.5* expression in the maintenance phase after the initiation (25, 26). In *Drosophila*, it has been suggested that tinman sustains its own transcription through a positive autoregulatory feedback loop in combination with decapentaplegic and wingless signals (44, 45). In mammals, however, it remains controversial whether *Nkx2.5* forms a positive feedback loop for sustaining the proper expression. A combination of *Nkx2.5* and GATA4 was reported to show a transactivation effect on the *Nkx2.5* promoter (25), while a negative feedback loop of *Nkx2.5* was also suggested by transgenic experiments (26). Thus, molecular mechanisms for sustaining *Nkx2.5* expression had been largely unknown. Since we demonstrated in this study that *Rae28* is required for sustaining *Nkx2.5* expression in the entire cardiac region later in heart development, it is possible to speculate that *Rae28* has a role in systematically governing each module for maturing cardiac morphogenesis. This system might also help to sustain proper expression of the selector gene, once initiated, even in the presence of various internal and external agents affecting the transcription, because a *PcG* gene is implicated in negatively defining accessibility to transcriptional regulatory elements such as retinoic acid response elements (8).

An interesting aspect of these results is that, whereas *PcG* genes maintain genes in a repressed state (1), *Rae28* is required for sustaining *Nkx2.5* expression in cardiomyocytes. A need for *PcG* genes in the activation of gene activity has been observed in *Drosophila* (46–48), so activation of *Nkx2.5* by *Rae28* is not unprecedented. Recently it was reported that, in *Drosophila*, binding of the ATP-dependent chromatin remodeling factors SWI and SNF to the nucleosomal template is competed for

by Polycomb repressive complex 1, including Polycomb, Posterior sex combs, sex combs on midleg, and poly-homeotic, a *Drosophila* counterpart of *Rae28* (3). Furthermore, the SWI/SNF complexes can produce a nucleosome structure both conducive to transcription at some promoters and repressive at others (49). It is possible that *Rae28* positively regulates *Nkx2.5* expression through regulation of the SWI/SNF-mediated nucleosome remodeling, but notwithstanding the arguments presented above, it remains a possibility that *Rae28*-mediated regulation of *Nkx2.5* is indirect.

We then attempted to examine whether *Rae28* regulates *Nkx2.5* expression in cardiomyocytes directly or indirectly. Although ubiquitous expression of *Rae28* driven by the *RSV* enhancer reverted the reduced expression of *Nkx2.5* in *Rae28*^{-/-} embryos at 9.5 dpc, cardiomyocyte-specific expression of *Rae28* driven by the β MHC promoter was unable to sustain *Nkx2.5* expression. The possibility exists that *Rae28* could be required at an earlier developmental stage and that *Rae28* expression derived from the β MHC-*Rae28* transgene did not occur early enough to sustain proper *Nkx2.5* expression. However, we detected no significant difference in *Nkx2.5* expression in *Rae28*^{-/-} embryos as early as 8.5 dpc, at which stage the β MHC promoter had already started the transcription (Figure 5c). Thus, it is unlikely that the exogenous expression of *Rae28* did not occur early enough to correct reduced *Nkx2.5* expression in *Rae28*^{-/-} embryos. Furthermore, we detected *Rae28* protein in the hearts of β MHC-*Rae28* embryos, and β MHC-*Rae28* embryos carried the same full-length cDNA for *Rae28* as was used to generate *RSV-Rae28* mice, in which exogenous *Rae28* revealed a capacity to suppress all the phenotypes and also reduced *Nkx2.5* expression observed in *Rae28*^{-/-} mice (29). It is therefore also unlikely that the *Rae28* protein derived from the β MHC-*Rae28* transgene encoded the nonfunctional proteins in the cardiomyocytes. These findings support the hypothesis that *Rae28* has a role in sustaining *Nkx2.5* expression in cardiomyocytes through a non-cell-autonomous pathway. This assumption might be consistent with the fact that, in general, *PcG* genes are known to maintain repressed states of transcription (1). Further analysis is, however, required to elucidate the mechanisms by which *Rae28* regulates *Nkx2.5* expression in cardiomyocytes.

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