Small RNA-directed epigenetic programming of embryonic stem cell cardiac differentiation

Hossein Ghanbarian1, Nicole Wagner2,3, Jean-François Michiels3,4, François Cuzin1, Kay-Dietrich Wagner2,3 & Minoo Rassoulzadegan3

Microinjection of small noncoding RNAs in one-cell embryos was reported in several instances to result in transcriptional activation of target genes. To determine the molecular mechanisms involved and to explore whether such epigenetic regulations could play a role in early development, we used a cell culture system as close as possible to the embryonic state. We report efficient cardiac differentiation of embryonic stem (ES) cells induced by small non-coding RNAs with sequences of Cdk9, a key player in cardiomyocyte differentiation. Transfer of oligoribonucleotides representing parts of the Cdk9 mRNA into ES and mouse embryo fibroblast cultures resulted in upregulation of transcription. Dependency on Argonaute proteins and endogenous antisense transcripts indicated that the inducer oligoribonucleotides were processed by the RNAi machinery. Upregulation of Cdk9 expression resulted in increased efficiency of cardiac differentiation suggesting a potential tool for stem cell-based regenerative medicine.

We reported earlier that microinjection of small non-coding RNAs is associated with epigenetic modifications and results in transcriptional activation of specific target genes1-3. Whether epigenetic mechanisms are involved in the initial determination of gene expression in the early embryo is an important question. Differentiation of cardiomyocytes is an early event during embryogenesis in vivo, which can be monitored by the appearance of beating cells in cultures in vitro4. To promote cardiac differentiation of ES cells, we attempted to modulate expression of Cdk9, one of the main actors of cardiac differentiation in vivo. We reported previously that transfer of small noncoding RNAs homologous to the Cdk9 transcript or of a cognate microRNA into one-cell embryos led to transcriptional activation of Cdk9 and hypertrophic growth of cardiomyocytes2. As a first step toward understanding how the epigenetic change is initiated in early embryonic cells upon transfer of the sequence-homologous oligonucleotides, we tested whether the effect could be mimicked in cell culture. For comparison to the developmental changes induced by injection in early embryos, we chose murine ES cells with a full potential of derivation into differentiated cell types. As for the basic mechanisms, we confirmed that changes in transcriptional activity were induced by homologous small RNAs in ES cells as in the early embryo and as in established cell lines5-7. We evidenced a role of antisense transcripts of the locus and a requirement for Argonaute proteins. We further observed a significant biological consequence with an increased potential of the reprogrammed ES cells to differentiate into cardiomyocytes.

Results

Cdk9 induction following transfer of transcript fragment (TF) in different cell systems. RNA-mediated transcriptional upregulation of Cdk9 and other loci had been reported previously upon microinjection of small size RNAs in fertilized eggs1-3. To investigate whether Cdk9 target mRNAs induce transcriptional variation in other cell types, mouse embryonic stem cells were analysed after electroporation of a 22-nt oligoribonucleotide with a nucleotide sequence identical to that of the Cdk9 mRNA in exon 7 (Cdk9-f, Table 1). Extracts prepared 48 hours after electroporation showed the same average increase in Cdk9 expression (approximately 2-fold) as observed previously in embryos after microinjection (Fig. 1a,b). During further growth in culture, the elevated RNA levels were maintained until they returned to control levels after an estimated number of 15 cell divisions.

1Biotechnology Department, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. 2Université Côte d’Azur, CNRS, INSERM, IRCAN, 06107, Nice, France. 3Université Côte d’Azur, CNRS, INSERM, iBV, 06107 Nice, France. 4Department of Pathology, CHU Nice, Nice, France. Correspondence and requests for materials should be addressed to K.-D.W. (email: kwagner@unice.fr) or M.R. (email: minoo@unice.fr)
notion that antisense RNAs are the targets of the sense oligoribonucleotides. Electroporation of the Cdk9 in which no antisense transcripts were detectable. Electroporation of these oligoribonucleotides did not modify and induced expression of the region antisense transcript reduced expression of the complementary 3′ region. As shown in Fig. 3b, oligonucleotides with either an intronic sequence of Cdk9 of antisense transcripts. As shown in Fig. 3b, oligonucleotides from the different regions of Sox9 (Fig. 3c). Single stranded DNA oligonucleotide (Table 1), whose electroporation induced upregulation of the locus, short single-stranded Cdk9 antisense transcripts in the keratinocyte cell line (Suppl. Fig. 2) and antisense transcripts covering the complete Sox9 locus in mouse ES cells (Suppl. Fig. 3).

To investigate a potential regulatory role of antisense transcripts in the Cdk9 locus, short single-stranded RNA oligonucleotides (antagoNATs) derived from the Cdk9 sense strand were examined to inhibit the activity of antisense transcripts. As shown in Fig. 3b, oligonucleotides with either an intronic sequence of Cdk9 (Cdk9-a, Table 1) or the exonic sequence in the 3′ region (Cdk9-f, see Fig. 3a) induced the transcriptional activation of Cdk9. Both correspond to regions in which antisense transcripts are detected. This was also the case for the Sox9-a oligonucleotide (Table 1), whose electroperation induced upregulation of Sox9 (Fig. 3c). Single stranded DNA oligonucleotides from the different regions of Cdk9 (Table 1) did not induce expression of the gene (Fig. 3d). Since antisense transcripts were not detected in the central region of Cdk9, we were able to test the possibility that antisense RNA could be dispensable for the establishment of the epigenetic change. Sequences of the oligonucleotide Cdk9-b in intron 2, Cdk9-c in exon 3, Cdk9-d in exon 4, and Cdk9-e in exon 5 (Fig. 3a) are from regions in which no antisense transcripts were detectable. Electroperation of these oligoribonucleotides did not modify expression of Cdk9 (Fig. 3b). Although other variables may have to be considered, this result clearly supports the notion that antisense RNAs are the targets of the sense oligoribonucleotides. Electroperation of the Cdk9-f oligoribonucleotide reduced expression of the complementary 3′ region antisense transcript while electroperation of the other Cdk9 oligoribonucleotides had no effect on the expression of this antisense transcript (Fig. 3e). A siRNA directed against the antisense transcript reduced expression of the complementary 3′ region antisense transcript and induced expression of Cdk9 (Suppl. Fig. 4).

Table 1. Oligoribonucleotides in ES electroporation experiments.

| Designation | Gene and accession number | Position in the gene (nt) | Sequence (5′-3′) |
|-------------|--------------------------|--------------------------|-----------------|
| miR-1       | mmu-miR-1 (MIMAT0000123) | Intron 1 (10165433–10165457) | UGGAAUGUAAGAGAAGUAUA |
| miR-16      | mmu-miR-16 (MIMAT0000527) | Intron 1 (10165433–10165457) | UAGGACGCGAGGAAUUGGCC |
| miR-19      | mmu-miR-19 (MIMAT0000651) | Intron 1 (10165433–10165457) | UGGUCAACUUAAGAAGAAAG |
| Cdk9-a      | Cdk9 (NT_039206) | Intron 1 (10165433–10165457) | GAGUAACGAGGGGGCAGAGAG |
| Cdk9-b      | Intron 2 (10167062–10167084) | CUCUGUGACGCCGUGCGACC |
| Cdk9-c      | Exon 3 (10167231–10167253) | GAACCUAAUUGAGAUGUGGCC |
| Cdk9-d      | Exon 3 (10167231–10167253) | GAACCTATGAGATTTGTCG |
| Cdk9-e      | Exon 4 (10167604–10167626) | UAGUCAAGUACGCGUAGUGA |
| Cdk9-f      | Exon 4 (10167604–10167626) | TAGTCAAGTTACGGTTCGTGA |
| Sox9        | (NT_165773.2) | Exon 3 (11454144–11454166) | GGUUGUUGAGAUCAGUAGUAG |
| Aca2        | (NT_039674) | Exon 2 (7179229–7179249) | GGCAGAAGUGUGGCUCGCC |
| LamC1       | (NT_078297) | Exon 20 (6778735–67787366) | GAGAUGCCGUCACCAAGGAC |
| Sox8        | (NT_039649) | Exon 2 (11915752–11915774) | GGCAACCCAGAGUAGUAGAG |
| Igf1        | (NT_039500) | Exon 6 (29658914–29658936) | UGCUUGCUAACCUCACCAAG |

Increased transcription of the Cdk9 locus. Run-on measurements of elongation rates pointed to an enhanced transcriptional activity as the cause for the increase in Cdk9 expression (Fig. 2a). This modification might result from changes in the chromatin structure involving the locus itself. Chromatin immunoprecipitation using an antibody directed against RNA polymerase II followed by quantitative PCR revealed higher enrichment in Cdk9-f-electroporated ES cells compared to controls in the P1 promoter region and the 3′ UTR, but not in the P2 promoter (Fig. 2b). The RNA-mediated transcriptional activation process indicated by our results reminds of RNA activation and suppression effects reported in human cells for p21, E-cadherin, and progesterone receptor genes, a finding subsequently extended to other mammalian species, including the mouse.

Antisense transcription at the Cdk9 and Sox9 loci. Specificity of transcriptional induction by transcript fragments implies a sequence recognition mechanism. The most obvious mechanism for specific locus recognition is hybridization with sequences complementary to the inducing RNAs. Antisense transcripts, a frequent feature of the mammalian genome, would provide a possible explanation. Strand-specific RT-PCR assays (Fig. 3a) confirmed the presence of transcripts complementary to the most 3′ region of the mRNA and identified antisense RNAs transcribed from a 5′ region corresponding to the promoter, the first exon, and the first intron. A schematic representation of the Cdk9 genomic locus including the antisense transcripts and the miR-1 binding site is provided in Supplemental Fig. 1. Similar analyses showed the presence of human Cdk9 antisense transcripts in the keratinocyte cell line (Suppl. Fig. 2) and antisense transcripts covering the complete Sox9 locus in mouse ES cells (Suppl. Fig. 3).
Argonaute proteins are required for reversible transcriptional activation by noncoding RNAs.

If locus-specificity were assured by interaction of the inducer oligoribonucleotides with antisense RNAs of the locus, one would expect a role of Argonaute complexes, which can be tested in cell culture systems by using Ago-negative mutants. As shown in Fig. 4a, when electroporated into ES cells deficient for Ago1, 3, and 4 and hypomorphic for Ago2, the transcript fragment did not induce an increase in Cdk9 expression and similarly, Sox9 transcriptional induction was not observed following electroporation of the Sox9 oligoribonucleotide in Ago-deficient ES cells (data not shown).

The observation that transcriptional activation was not detected by run-on assays in Ago-deficient cells (Fig. 4b) confirmed a causal relationship between transcriptional activation and the Ago-dependent state that we assume involves the sense oligoribonucleotides and reverse transcripts.

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Figure 1. Increased expression of Cdk9 RNA in cell systems after transfer of short transcript fragments (TF). (a) Increased Cdk9 expression in ES cells was analyzed by quantitative Real-Time PCR. RNA homologous to Cdk9 exon 7 (Cdk9-f, Table 1) was transferred by electroporation in mass cultures of the cells and quantitative RT-PCR determination of Cdk9 RNA was performed after 2 days (n = 5). (b) Northern blot analysis shows increased Cdk9 RNA level following TF electroporation in mass culture of ES cells. (c) Increased Cdk9 expression is maintained during growth of TF-electroporated cells in culture. Average values of total cell number (straight line) and of Cdk9 RNA measurements (bars) at successive times during cell growth and duplications. Changes in RNA levels were maintained until they returned to control levels after an estimated number of 15 duplications (n = 3). (d) Cdk9 protein level in ES cells was examined by Western blot analysis 2 days after small sense RNA fragment electroporation. Increased Cdk9 expression was also detected in Mouse Embryonic Fibroblasts (e) and a human keratinocyte cell line (f) following TF electroporation (n = 8 each). Data are mean ± S.E.M. *p < 0.05, ***p < 0.001.
Locus specificity of RNA-mediated gene activation. To evaluate whether the observed RNA-mediated gene activation is specific, we electroporated ES cells with the Cdk9-f oligoribonucleotide and measured expression of several genes including Cdk9, Sox9, Igf1, Acca2, Lamc1, C-myc, and Sox8 by quantitative real time PCR. Only Cdk9 expression increased upon electroporation of the Cdk9-f oligoribonucleotide (Fig. 5a). The same was evident for Sox9. Sox9 expression increased exclusively upon electroporation of the respective oligoribonucleotide (Fig. 5b). In further electroporation experiments, we confirmed that the transcriptional activation of Cdk9 and Sox9 is exclusively induced by the respective sense-strand oligoribonucleotides. Neither antisense nor double-stranded oligoribonucleotides or unrelated micro RNAs induced Cdk9 or Sox9 expression (Fig. 5c–e). To investigate whether RNA-mediated gene activation is a unique property of the Cdk9 and Sox9 loci, quantitative real time PCR assays were performed following electroporation of 22 bp sense transcript fragments of three other genes Acca2, Lamc1, and Igf1. No significant increase in the corresponding RNAs was evidenced (Fig. 5f–h). No antisense transcript was detected for these three genes (data not shown). Our results indicate that under our experimental conditions, not all genes are susceptible to transcriptional activation by non-coding RNAs.

RNA-mediated programming of ES cell differentiation. The morphology and growth characteristics of the RNA-modified ES cells were still those of the original ES line. They did not exhibit any morphological signs of differentiation, neither towards cardiac muscle nor other tissue-specific cell types. To confirm this observation, we investigated expression of two characteristic embryonic stem cell markers, Nanog and Oct3/4. Furthermore, we evaluated that transcripts characteristic for cardiac differentiation were not detectable (Table S1). However, these cells differentiated faster and more efficiently into cardiac muscle cells than the original ES line ("RNA-mediated programming"). According to well-established procedures, embryoid bodies were generated from sense Cdk9-f-electroporated and control ES cells by culture in hanging drops. Cells were plated back on gelatin-coated plates 3 days later. Cardiac differentiation monitored by the appearance of beating cells progressed at a faster rate in the progeny of Cdk9-induced cells than in the original ES cells (Fig. 6a). Accordingly, quantitative RT-PCR determination of cardiac marker genes showed higher values than in control cultures. The level of miR-1, undetectable in freshly electroporated cells as well as in the original ES cells, was increased during differentiation as reported previously. Interestingly, expression of Cdk9 in differentiating subcultured cells had returned to control values by day 6 (Fig. 6b), but remained elevated after electroporation for 15 days in cells without subculturing (data not shown).

Injection of miR-1 or Cdk9-f-electroporated ES cells into blastocysts resulted in increased expression of Cdk9 in embryonic hearts at E18.5 (Suppl. Fig. 5a). Furthermore, injection of Cdk9-f-electroporated ES cells into blastocysts increased heart-to-body weight ratios (Suppl. Fig. 5b) and higher ventricular dimensions (Suppl. Fig. 5c) in embryos at E18.5. Electroporation of a pIRESneo-EGFP DNA/miR-1 construct in ES cells and subsequent transfer into blastocysts confirmed a specific higher GFP expression in embryonic hearts (Suppl. Fig. 6a), but not in liver, kidney, and lungs (Suppl. Fig. 6a,b). In these embryos, heart weights were higher, while kidney and lung
Figure 3. Antisense transcription at the Cdk9 locus. (a) Regions analyzed for the presence of antisense transcripts throughout the Cdk9 gene. Top: schematic representation of the locus with exons shown as closed bars. The positions of the regions analyzed are indicated. Bottom: detection of antisense RNA transcripts by strand-specific RT-PCR reactions using primers listed in Table S2. All assays were performed in duplicate, “RT−”: reverse transcriptase omitted. The same analyses applied to the human Cdk9 and the murine Sox9 locus are shown in Figs S2 and S3, respectively. (b) Levels of Cdk9 expression following electroporation of oligoribonucleotides with exonic and intronic sequences in a region transcribed on both strands (and f regions) and sequence of regions with only sense transcription (b, c, d, and e regions). (c) Levels of Sox9 expression following electroporation of an oligoribonucleotide with exonic sequence. (d) Levels of Cdk9 expression following electroporation of the Cdk9-f oligoribonucleotide and several single stranded DNA oligonucleotides. (e) Cdk9 3′ antisense RNA levels in ES cells were analyzed by quantitative Real-Time PCR. The indicated RNA oligoribonucleotides (Table 1) were transferred by electroporation in mass cultures of the cells and quantitative RT-PCR determination of Cdk9 antisense RNA was performed after 2 days (n = 4). Data are mean ± S.E.M. *p < 0.1, ***p < 0.001.
weights were comparable to controls (Suppl. Fig. 6c). Histological analysis confirmed the increase in heart size (Suppl. Fig. 6d), which was due to cardiac hyperplasia as indicated by the increased nuclei count (Suppl. Fig. 6e) indicating that RNA programmed ES cells also contribute to the heart in vivo.

Discussion

While our previous results indicated the possibility of RNA-mediated induction of gene expression in embryos, they did not allow full investigation regarding the mechanisms leading to augmentation of transcriptional activity. The scarcity of one-cell embryos made the search for molecular genetic mechanisms technically demanding or impossible because of lethality or sterility of animals. To overcome this problem, we developed cell culture systems that could be more amenable to molecular analyses. Because of a relatively high level of expression in ES cells and the availability of well-established protocols for cardiac differentiation in vitro, induction of Cdk9 by a transcript fragment provides a convenient experimental system. The single-stranded sense transcript fragment homologous to the carboxy-terminal Cdk9 exon induced transcription of the gene in ES cells significantly. This effect was not unique to ES cells, since a comparable increase in expression was evidenced upon electroporation of the same transcript fragment in embryonic fibroblasts and a human keratinocyte cell line.

Furthermore, we examined transcriptional levels following electroporation of ES cells with several transcript fragments corresponding to different regions of Cdk9. Interestingly, Cdk9 induction was not observed upon electroporation of the oligoribonucleotides with a nucleotide sequence identical to that of the mRNA in the middle region of Cdk9 (Cdk9-b-e, Table 1) and of single stranded DNA oligonucleotides (Table 1). This convenient experimental system enabled us to outline the sequence of molecular events that result in augmentation of transcriptional activity. The requirement of Argonaute proteins for transcriptional activation indicates that Ago proteins charge the incoming small RNA fragments, and the RNAi machinery perceives the initial small RNA signal. Interestingly, natural antisense transcripts (NATs) complementary to the most 3′ and 5′ regions of the mRNA were detected and locus-specific induction of Cdk9 expression identified following targeting of their antisense transcripts by short single stranded cognate transcript fragments. Indeed, hybridization of the electroporated oligoribonucleotide with antisense transcripts provides the signal for transcriptional activation. However, it is unlikely that simply the anti-sense transcript impedes progression of the transcription machinery as targeting of the 3′ antisense transcript resulted in increased RNA pol II binding to DNA in the promoter and 3′ UTR region. Thus, it seems likely that increased transcription is due to modification of the genomic locus.

Similar to a recent study where specific induction of the BDNF locus was investigated, single stranded sense transcripts targeting natural antisense transcripts are sufficient to initiate an induction of Cdk9 mRNA expression. In case of the mentioned study, additional significant changes in histone marks have been identified. Aside from the presence of endogenous antisense transcripts, the RNAi machinery requires Argonaute proteins. The observation that transcriptional activation was not detected in Ago-deficient cells indicates that RNAi was linked to transcriptional activation. Although numerous studies have reported that RNAi gene silencing acts by targeting sense transcripts, our study shows that RNAi gene activation operates by targeting antisense transcripts. This is in agreement with the report by Zhang et al. showing in Hela cells with a CMV-EGFP reporter gene system that antisense RNAs and Ago2 are involved in transcriptional activation. We extend this finding to endogenous genes and provide evidence that not only long non-coding RNAs, but also small RNA fragments can induce gene activation.
Figure 5. Not all genes are susceptible to RNA mediated gene activation. (a,b) Analysis of several genes by quantitative real time PCR assay revealed that the increased expression levels of mRNA following TF electroporation were specific to Cdk9 and Sox9 (n = 4). (c) Levels of Cdk9 expression following electroporation of Cdk9 derived single (sense and antisense) and double-stranded transcript fragments in mass cultures of the ES cells (n = 4). (d,e) Among the several electroporated oligoribonucleotides only Cdk9 and Sox9 derived transcript fragments could induce the corresponding RNAs, respectively. (f–h) Transcription induction was not observed in Acaa2, Lamc1, and Igf1 loci upon electroporation of the transcript fragment corresponding to the respective genes. Quantitative RT-PCR determination was performed after 2 days. Control: mock-electroporated cultures (n = 4). Data are mean ± S.E.M. ***p < 0.001.
Mechanistically, Cdk9 antisense transcripts might form RNA-RNA duplexes with Cdk9 mRNA as it has been described for BDNF. Electroporation of sense oligoribonucleotides favors duplex formation between the sense oligoribonucleotide and the antisense transcript. This complex is processed by the RNAi machinery as indicated by the requirement of Ago proteins. Activation of the RNAi machinery may then result in changes in chromatin marks at the locus as described for several genes, leading to increased transcription of the Cdk9 locus (Fig. 2). Reduced expression of the antisense transcript in response to electroporation of the sense oligoribonucleotide (Fig. 3e) indicating destruction of the antisense transcript could represent a secondary phenomenon of the activation of the RNAi machinery. Although it is widely assumed that the RNAi machinery functions mainly in the cytoplasm, specific and potent activity has been demonstrated also in the nucleus. Whether the RNAi machinery gains access to genomic DNA during cell division when the nuclear membrane is disrupted or whether a fraction of RNA-protein complexes is actively transported into the nucleus remains an open question.

As a functional consequence of the Cdk9 locus activation, we observed an increased cardiac differentiation potential of the sense oligoribonucleotide electroporated ES cells. The modified ES cells overexpressing Cdk9 maintained a pluripotent state when propagated in LIF containing medium. They expressed Nanog and Oct4 and did not express cardiac-specific genes (Table 1). When cardiac differentiation was started, however, they exhibited a greater differentiation potential than the original ES cells. During this process, Cdk9 expression returned to normal values, which might be related to the limited stability of the sense RNA fragments.

Interestingly, levels of miR-1 have been shown to increase during normal heart development and upon spontaneous myocardial differentiation of ES cells in 2-D culture. Also in our experimental system of increased...
cardiac differentiation, miR-1 expression was increased. We show that a short pulse of sense transcript fragment induced over-expression is sufficient to create a “memory” for cardiac differentiation in ES cells. Also in vivo, reprogrammed ES cells injected in wild-type blastocysts contributed to heart development, which suggests that cells transiently exposed to small RNAs might become a novel tool for ES cell re-programming for regenerative medicine.

Our data are in agreement with the emerging view that short RNA derived from longer messages might not just be short degradation products, but could have specific functions as well16–18. A short t-RNA like structure has been shown to arise specifically from the long non-coding MALAT1 RNA16. Later, an important number of small and large RNAs generated from post-transcriptional processing of mature mRNAs have been identified17. Most recently, Pircher et al. showed that an mRNA-derived small RNA is able to bind and regulate ribosomes18. Thus, our identification of the requirement of endogenous antisense transcripts and Ago proteins to induce transcriptional activation by small sense RNAs contributes to the complexity of the picture.

Conclusion

Cdk9 transcript-derived oligoribonucleotides are capable to induce Cdk9 expression in different cell systems. Requirements for Argonaute proteins and for endogenous antisense transcripts at the locus indicate that the inducer oligoribonucleotides are processed by the RNAi machinery. Induction of Cdk9 resulted in efficient cardiac differentiation of ES cells in vitro. Injection of Cdk9-f-electroporated ES cells into blastocysts induced cardiac growth indicating that RNA-programmed ES cells contribute specifically to the heart in vivo.

Methods

Cell culture and RNA electroporation. Mouse AB1 ES cells were grown on mouse embryonic fibroblast (MEFs) feeders in standard ES culture medium. Briefly, ESCs were cultured on a feeder layer of Mitomycin C treated MEFs on 0.2% gelatin-coated cell culture dishes. Culture medium consisted of Dulbecco’s modified Eagle’s medium, 15% ES-grade fetal calf serum (FCS, Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Sigma), 100 U/ml penicillin and 0.1 mg/ml streptomycin and 1000 U/ml leukemia inhibitory factor (LIF). Cardiac differentiation of ESCs was induced according to established procedures4. Mouse embryonic fibroblasts (MEFs) and human keratinocyte cell lines were cultured in DMEM containing 10% FCS. Ago1−/−, Ago2−/−, 4−/− ES cells were kindly provided by X. Wang (Department of Biochemistry, Northwestern University, Evanston, USA). Electroporation was performed 1–2 days after plating (5 × 10^6 cells per plate) with 5 μg RNA using the Bio-Rad Gene Pulser apparatus (400 V, 250 F).

Quantitative RT-PCR RNA analysis. RNA was extracted using the Trizol Reagent (Invitrogen). 0.5 μg RNA samples were reverse transcribed to cDNA using random hexamer primers and MLV reverse transcriptase (Invitrogen). q-PCR was performed using the ‘Platinum® SYBR® Green qPCR SuperMix-UDG’ kit (Invitrogen). Oligoribonucleotides and their FITC- and Cy3-labelled derivatives were obtained from SIGMA-PROLABO. The average threshold (Ct) was determined for each gene and normalized to Gapdh mRNA level as internal normalization control. Sequences of deoxyribo-nucleotide primers are provided in Table S2.

Strand-specific RT-PCR was performed to detect potential locus-specific antisense transcripts. Specific 18–20 single stranded oligonucleotides complimentary to potential antisense transcripts were designed to target only antisense RNA strands. Thus, in the reverse transcription step, complementary DNA (cDNA) was generated from antisense templates. The reverse transcriptase was omitted for negative controls.

Run-on assay of transcriptional activity. Assays were performed according to Patrone et al.19 by q-PCR monitoring the incorporation of biotin-labeled triphosphate (biotin-16-UTP, 11388908910, Roche Applied Science) into RNA isolated on Streptavidin Magnetic Particles (11641778001, Roche Applied Science).

Western blot analysis. Total lysates from cell cultures were prepared, electrophoresed, and blotted as described20. The following antibodies were used for immunodetection: polyclonal anti-CDK9 antibody from rabbit (H-169, sc-8338, Santa Cruz Biotechnology) in a 1:500 dilution in PBS, 2.5% Blotto, 0.05% Tween-20, polyclonal anti-GAPDH, polyclonal anti-Ago1, polyclonal anti-Ago2, and polyclonal anti-Ago3 in 5% Blotto. Detection was performed using mouse monoclonal anti-gapdh (T6199, Sigma) 1:2000, peroxidase-coupled goat anti-rabbit secondary antibody (H-169, sc-8338, Santa Cruz Biotechnology) in a 1:500 dilution in PBS, 2.5% Blotto, 0.05% Tween-20, 0.1 mg/ml streptomycin, and 0.1 mg/ml penicillin and 100 U/ml streptomycin. Blots were developed using a chemiluminescent substrate (Bio-Rad, Hercules, CA, USA). Membranes were blocked with PBS containing 5% non-fat milk powder for 1 h at room temperature and washed with PBS containing 0.1% Tween-20. The indicated primary antibodies were then added and left to react at 4 °C overnight. Membranes were washed and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Signals were detected using an ECL Kit (GE Healthcare, Piscataway, NJ, USA).

Chromatin immunoprecipitation ( CHIP ) assay. Chromatin immunoprecipitation (CHIP) assay was carried out according to the protocol of the ChIP Assay Kit (Millipore cat. 17–295). Briefly, at least 1 × 10^6 cells were growth in 100 mm dishes, and were cross-linked by adding formaldehyde to a final concentration of 1% and incubated at room temperature for 10 minutes. Cells were washed twice, using ice cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1 μg/ml pepstatin. The scraped cell suspension was transferred to a conical tube and centrifuged 4 min at 2000 rpm at 4 °C. Cells were washed with PBS and re-suspended in ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0) with added

Cdk9-f-electroporated ES cells were cultured on a feeder layer of Mitomycin C treated MEFs on 0.2% gelatin-coated cell culture dishes. Culture medium consisted of Dulbecco’s modified Eagle’s medium, 15% ES-grade fetal calf serum (FCS, Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Sigma), 100 U/ml penicillin and 0.1 mg/ml streptomycin and 1000 U/ml leukemia inhibitory factor (LIF). Cardiac differentiation of ESCs was induced according to established procedures4. Mouse embryonic fibroblasts (MEFs) and human keratinocyte cell lines were cultured in DMEM containing 10% FCS. Ago1−/−, Ago2−/−, 4−/− ES cells were kindly provided by X. Wang (Department of Biochemistry, Northwestern University, Evanston, USA). Electroporation was performed 1–2 days after plating (5 × 10^6 cells per plate) with 5 μg RNA using the Bio-Rad Gene Pulser apparatus (400 V, 250 F).

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Northern blot analysis. Northern blot analysis was performed according to standard methods. Briefly, 6 μg total RNA extracted from cell cultures was loaded onto a 12% denaturing polyacrylamide gel and electrophoresed until the bromophenol blue marker reached the bottom of the gel. The separated RNA was electrotransferred to a Hybond N+ membrane (Amersham). Hybridization was carried out in the presence of 32P-end-labeled DNA probes. 440 and 594 nucleotide probes for Cdk9 and Gapdh, respectively, were generated by PCR using the following oligonucleotides: Cdk9 forward, 5′-TGAACCCTGGCAGATTTTGGG-3′; Cdk9 reverse, 5′-GCATCGTCACTGTCAATCCG-3′; Gapdh forward, 5′-TGAAGCTGGCAGATTTTGGG-3′; Gapdh reverse, 5′-TTCCTATAAATACCGGACTGAC-3′.

Chromatin immunoprecipitation ( CHIP ) assay. Chromatin immunoprecipitation (CHIP) assay was carried out according to the protocol of the ChIP Assay Kit (Millipore cat. 17–295). Briefly, at least 1 × 10^6 cells were growth in 100 mm dishes, and were cross-linked by adding formaldehyde to a final concentration of 1% and incubated at room temperature for 10 minutes. Cells were washed twice, using ice cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1 μg/ml pepstatin. The scraped cell suspension was transferred to a conical tube and centrifuged 4 min at 2000 rpm at 4 °C. Cells were washed with PBS and re-suspended in ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0) with added
protease inhibitors (inhibitors: 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). After 10 minutes incubation on ice, cells were sonicated to shear DNA to lengths between 200 and 1000 base pairs. Preparation of chromatin fragments, immunoprecipitation, and DNA recovery were performed as described in the manufacturer instructions. The antibody directed against RNA polymerase II was obtained from Santa Cruz Biotechnology (N-20, sc-899, Santa Cruz Biotechnology). The following primers were used: Cdk9 P2 promoters, Cdk9-P2F: 5′-ATGACGGCGGAGCCACCG-3′ and Cdk9-P2R: 5′-GGGAGCGCGAGCTGCAGAGG-3′; Cdk9 P1 promoters, Cdk9-P1F: 5′-GGGACTACAAGTCCAGGG-3′ and Cdk9-P1R: 5′-CACTCCAGGCCCTCCCGGCGG-3′; Cdk9 3′ UTR primers, UTR-F: 5′-TTAGAGATTTTCTCTCCAGTAC-3′ and UTR-R: 5′-TTGAGATTTTCTCTCCAGTAC-3′.

ES cells injection of blastocysts. According to standard methods, ES cells were injected in 3.5 days blastocysts after electroporation of Cdk9 sense transcript fragment (Cdk9-f: 5′-GAUUCUCCUCCUCCAGAUCAAU-3′), or microRNA-1 (miR-1: 5′-UGGAUGUAAGAAGUAUGUAU-3′), or a pIREsneo-EGFP DNA/miR-1 construct. As controls, blastocysts were microinjected with mock-electroporated ESCs. For studies of embryos during gestation, the embryos were re-implanted in the uterine horns of the foster mother. Investigations were conducted in accordance with French and European rules for the care and use of laboratory animals and approved by the local ethical committee (Ciepal). Embryos were dissected at embryonic day 18.5, organ weighted and hearts prepared for histological analysis as described.

Statistical analysis. Data are expressed as means ± S.E.M. Differences between two groups were tested using the Mann-Whitney test for non-parametric samples. A p value less than 0.05 was considered statistically significant.

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Author Contributions
H.G., K.D.W., and N.W. performed experiments and data analysis. M.R., F.C., J.F.M., K.D.W., and N.W. designed experiments. H.G., K.D.W., and N.W. prepared the manuscript. H.G., K.D.W. and F.C. wrote the paper.

Additional Information
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