Xenopus furry contributes to release of microRNA gene silencing

Toshiyasu Goto[a,b,c], Akimasa Fukui[a,d], Hiroshi Shibuya[c], Ray Keller[b], and Makoto Asashima[a,e,1]

[a]Department of Life Sciences (Biology), Graduate School of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153-8902, Japan; [b]Department of Biology, University of Virginia, Charlottesville, VA 22903; [c]Department of Molecular Cell Biology, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8510, Japan; [d]Division of Biological Sciences, Graduate School of Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan; and [e]Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8562, Japan

Edited by John Gerhart, University of California, Berkeley, CA, and approved September 27, 2010 (received for review June 23, 2010)

A transcriptional corepressor, *Xenopus furry* (*Xfurry*), is expressed in the chordamesodermal region and induces secondary dorsal axes when overexpressed on the ventral side of the embryo. The N-terminal furry domain functions as a repressor, and the C-terminal leucine zipper (LZ) motifs /coiled-coil structure, found only in vertebrate homologs, contributes to the nuclear localization. The *engrailed repressor* (*enR*)+*LZ* repressor construct, which has properties similar to *Xfurry*, induced several chordamesodermal genes. In contrast, an antisense morpholino oligonucleotide, *Xfurry*-MO, and the activating construct, *herpes simplex virus protein* (*VP16*)+*LZ*, had effects opposite those of *Xfurry* overexpression. Because blocking protein synthesis with cycloheximide superinduced several *Xfurry* transcriptional targets, and because expression of *enR+LZ* induced such genes under cycloheximide treatment, we analyzed the role of an *Xfurry* transcriptional target, microRNA *miR-15*. Cycloheximide reduced the expression of *primary miR-15* (*pri-miR-15*), whereas *miR-15* reduced the expression of genes superinduced by cycloheximide treatment. These results show that *Xfurry* regulates chordamesodermal genes by contributing to repression of pretranscriptional gene silencing by *miR-15*.

axis formation | transcriptional corepressor | chordamesodermal genes | cycloheximide

In *Drosophila*, *furry* is an important gene for maintaining the integrity of cellular extensions during wing morphogenesis (1). Mutations in *furry* result in a strong multiple hair-cell phenotype characterized by clusters of epidermal hairs and branched hairs. Furry functions via interactions with a serine/threonine kinase, tricornered, through the furry domain (FD) (1, 2). *Tricornered* encodes the *Drosophila* nuclear DBF2-related (NDR) kinase, and mutations of this gene result in dramatic multiple wing hair, branched bristle, and lateral arista phenotypes (3). Tricornered/furry signaling also plays a key role in the development of sensory dendrites in *Drosophila* neurons (4). In fission yeast, Mor2/Furry and Orb6/Tricornered are essential for morphogenesis through regulation of the actin cytoskeleton (5), whereas in budding yeast transcriptional activator of OCH1 (Tao3)/Furry affects cell wall biosynthesis kinase (Cbk1)/tricornered kinase activity in polarized growth (6). Recently, it was reported that Furry and the human homolog of *Drosophila tricornered*, NDR1, attach to spindle microtubules and play a role in mitotic chromosome alignment in mammalian cultured cells (7). Thus, *furry* and its homologs function generally in morphogenesis and cell division through interactions with *tricornered* and its homologs.

Furry protein localizes to the cell membrane in fly wings (2) and to the cytoplasm and spindle microtubules in yeast and mammalian cultured cells (5, 7). However, Furry also is found in the nuclei of salivary gland and fat body cells in the fly, suggesting an additional function in gene regulation (2). Human *furry* also has shown transcriptional regulatory properties in acute myeloid leukemia patients (8). Together, these observations suggest that *furry* functions in the nucleus, possibly in transcriptional regulation, and also highlight that much is unknown about the function of furry, especially in vertebrate embryogenesis.

Axis formation is an important event in early vertebrate embryogenesis that involves complex regulation of gene expression. In *Xenopus*, signaling molecules such as *β-catenin* and *siamois* in the early canonical *wingless* (Wnt) pathway (9–12) induce complete secondary axes with anterior structures when expressed on the ventral side, whereas activin/nodal-like signaling molecules such as *derriere*, *Xenopus nordal related-1 gene* (*Xnr1*), *Xnr5*, and *Xnr6* (13–15) induce secondary axes without anterior structures. MicroRNAs also play important roles in axis formation and organizer induction, especially in the context of nodal signaling. MicroRNAs miR-15 and miR-16 inhibit nodal signaling at the translational level during axis formation (16), and depletion of miR-427 inhibits organizer formation (17). Thus, small RNAs are involved in novel aspects of major signaling pathways during embryogenesis. Small RNAs function at both the translational and transcriptional levels in vertebrates. The transcriptional gene silencing operates by the antisense strand of the siRNA targeting chromatin-remodeling complexes to the specific promoter regions (18, 19). In mammalian cultured cells, miR-320 contains the promoter-region sequence of the cell-cycle *gene polymerase (RNA) III (DNA directed) polypeptide D* (*POLR3D*) in the antisense orientation and plays a *cis*-regulatory role in transcriptional silencing of *POLR3D* expression (20). Moreover, transcriptional activation can be induced through RNA duplexes targeting several of the promoter regions in mammalian cells (21, 22). Taken together, this evidence suggests that microRNA-mediated transcriptional gene silencing is an important process in vertebrate embryogenesis.

The current study demonstrated that *Xenopus furry* (*Xfurry*) functions as a transcriptional corepressor during embryogenesis. Direct reduction of microRNA expression and indirect induction of dorsal genes by *Xfurry* implicate microRNAs in the regulation of gene silencing during embryogenesis.

Results

Expression of *Xfurry*. We isolated a *Xenopus* homolog of *furry* (*Xfurry*) encoding a conserved, N-terminal FD (Fig. S1) with high identity to *Drosophila* furry and other furry homologs (Fig. S1). The nonconserved region of Xfurry has low identity with invertebrate furry but high identity with vertebrate furry. Xfurry has two C-terminal leucine zipper (LZ) motifs and a coiled-coil structure that are conserved only in vertebrates, suggesting that the function of *Xfurry* differs from its invertebrate homologs. Transcripts of *Xfurry* were present maternally at the same levels

Author contributions: T.G. designed research; T.G. and A.F. performed research; H.S., R.K., and M.A. contributed new reagents/analytic tools; T.G. and R.K. analyzed data; and T.G. and R.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: asashi@bio.c.u-tokyo.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1008954107/-/DCSupplemental.

until the late blastula stage (stage 9), after which they decreased through gastrulation and then increased gradually again through the neurula and tailbud stages (Fig. 1*A*). Whole-mount in situ hybridization and RT-PCR revealed that *Xfurry* is expressed weakly in the dorsal mesodermal region (Fig. 1 *B* and *C*) at the early gastrula stage. The expression then increases gradually in the chordamesoderm during gastrulation (Fig. 1*D*) and is restricted to the notochord at late stages (Fig. 1 *E* and *F*). These expression patterns implicate *Xfurry* in axis formation (23–26).

***Xfurry* Induces Chordamesodermal Genes.** Overexpression of *Xfurry* mRNA in ventral blastomeres induced a secondary axis (Fig. 1*H*, arrowhead), whereas dorsal overexpression had no effect on axis formation (Fig. S2). Chordamesodermal marker genes were induced in animal caps dissected from embryos injected with *Xfurry* mRNA (Fig. 1*I*). In contrast, dorsal injection of *Xfurry* morpholino (*Xfurry*-MO) (Fig. S3) had effects opposite those of *Xfurry* mRNA overexpression, including interference with head formation, shortened dorsal axes (Fig. 1*K*), and reduced expression of chordamesodermal marker genes (Fig. 1*L*). The phenotype of *Xfurry*-MO–injected embryos was rescued by coinjection with *Xfurry* mRNA (Fig. S4*C*). These results indicate that *Xfurry* plays an important role in axis formation during *Xenopus* development by inducing expression of chordamesodermal genes.

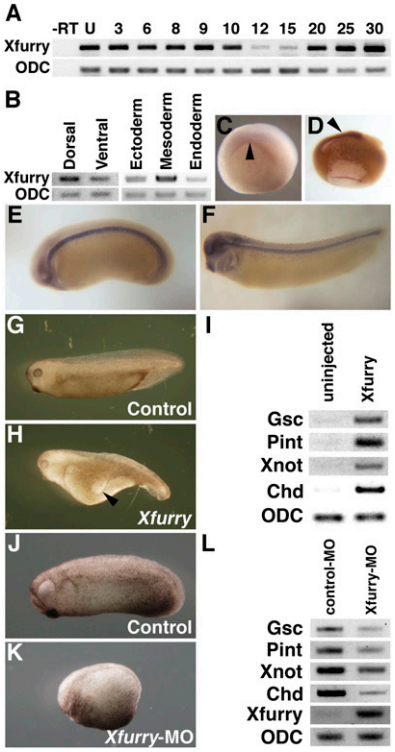

Fig. 1. Characterization of *Xfurry*. (*A*) Temporal expression of *Xfurry* detected by RT-PCR. Numbers indicate developmental stages. U, unfertilized eggs. (*B*) Regional RT-PCR of *Xfurry* at stage 10. (*C*–*F*) Whole-mount in situ hybridization. (*C*) Arrowhead indicates the dorsal lip (stage 10). (*D*) Arrowhead indicates the chordamesodermal region (stage 12). (*E* and *F*) Strong expression in the notochord was maintained through stage 25 (*E*) and stage 33 (*F*). (*G*) Control embryo (stage 33). (*H*) *Xfurry* (2 ng/blastomere) injected into ventral blastomeres of the four-cell embryo induced a secondary axis without anterior structures (arrowhead) in 63% of embryos (n = 60). (*I*) Expression of the chordamesodermal genes was induced in animal caps (stage 10) that were dissected from embryos injected with *Xfurry* (2 ng/blastomere). Gsc, goosecoid; Pint, pintallavis; Chd, chordin; ODC, Ornithine decarboxylase. (*J*) Control embryo (stage 26). (*K*) Injection of *Xfurry*-MO (5 ng/blastomere) into dorsal blastomeres of four-cell embryos shortened the dorsal axis in all embryos (n = 58). (*L*) Expression of chordamesodermal marker genes was reduced in *Xfurry*-MO–injected embryos, but transcripts of *Xfurry* were increased by *Xfurry*-MO (stage 10).

***Xfurry* Functions as a Transcriptional Corepressor.** To investigate the functional domains of Xfurry, we overexpressed various deletion constructs of *Xfurry*. A conjugate of the FD and the LZ/coiled-coil domain (*FD+LZ)* had an effect on secondary axis induction very similar to that of *Xfurry* mRNA (Fig. S5 and Table S1). These results suggest that the FD and LZ domains are essential and sufficient to induce a secondary axis.

Furry protein is expressed in a cell type-specific manner in *Drosophila*, notably in the cell membrane and cytoplasm of wing cells and in the nuclei of salivary gland and fat body cells (2). To investigate the function of Xfurry in *Xenopus* mesodermal cells during axis formation, we observed fluorescence expression of overexpressed full-length Xfurry conjugated with GFP at the C terminus (*Xfurry+GFP* cDNA) at gastrula stages. The fluorescent protein was detected throughout the nucleus and cytoplasm of mesodermal cells (Fig. 2*A*, *Top Row* and Fig. S5). Overexpressed FD+LZ+GFP, which has a function similar to that of Xfurry (Table S1), is localized in nuclei and cytoplasm (Fig. 2*A*, *Second Row* and Fig. S5). In contrast, LZ+GFP is localized mainly in nuclei and FD+GFP is localized in the cytoplasm (Fig. 2*A*, *Third and Bottom Rows*). We also confirmed the localization of GFP constructs by Western blotting of nuclear and cytoplasm extracts from the injected embryos (Fig. S6). The blot results suggest that Xfurry localizes to nuclei via the LZ structure and that the localization of Xfurry depends on the localization of partner proteins interacting via its FD and LZ domains. This evidence, together with the evidence that human *furry* has transcriptional properties in acute myeloid leukemia patients (8), supports the hypothesis that Xfurry enters the nucleus and functionally resembles a transcription factor. However, the LZ domain alone had no effect on axis formation (Table S1), indicating that another domain, the FD, is necessary for axis formation.

On the other hand, the FD fused to the Siamois homeodomain (FD+SiaHD) showed effects opposite to Siamois and the same as a fusion of the engrailed repressor domain and the homeodomain of Siamois (enR+SiaHD) (27). Dorsal expression of FD+SiaHD ventralized the injected embryos (Fig. 2*C*) and repressed expression of the organizer/head organizer genes *goosecoid* and *cerberus,* which are downstream of *Siamois* (27, 28) (Fig. 2*D*). The FD therefore seems to function as a transcriptional repressor, and these results suggest that Xfurry forms a complex with transcription factors having both DNA-binding and furry-interaction domains, including ones interacting with the LZ structure, and that this complex functions as a transcriptional repressor via the FD. Expression of the FD alone weakly induced secondary axes (Table S1), suggesting that it might interact weakly with other transcription factors in such a complex. The LZ motif and coiled-coil structure are found only in vertebrate *furry* homologs, and a transcriptional regulation function has not been reported for invertebrate *furry*.

Additionally, several reports in *Drosophila* indicate that *furry* and its homologs interact and function with *tricornered* and its homologs (2–7). To determine whether *Xenopus* homologs of *tricornered*, the NDR1 and NDR2 kinases, affect axis formation, we overexpressed these genes in ventral blastomeres at the four-cell stage. Neither *NDR1* nor *NDR2* affected axis formation in *Xenopus* (Fig. S7), suggesting that the function of *Xfurry* in axis formation is distinct from that shown in reports on other systems and does not involve Tricornered as a downstream component.

To investigate further the function of *Xfurry* as a transcriptional corepressor, we made cDNA constructs in which a repressor domain (*enR+LZ*) or an activator domain (*VP16+LZ)*, was fused to the C terminus of *Xfurry* (Fig. S5). Ventral expression of *enR+LZ* induced secondary axes similar to those induced by overexpression of

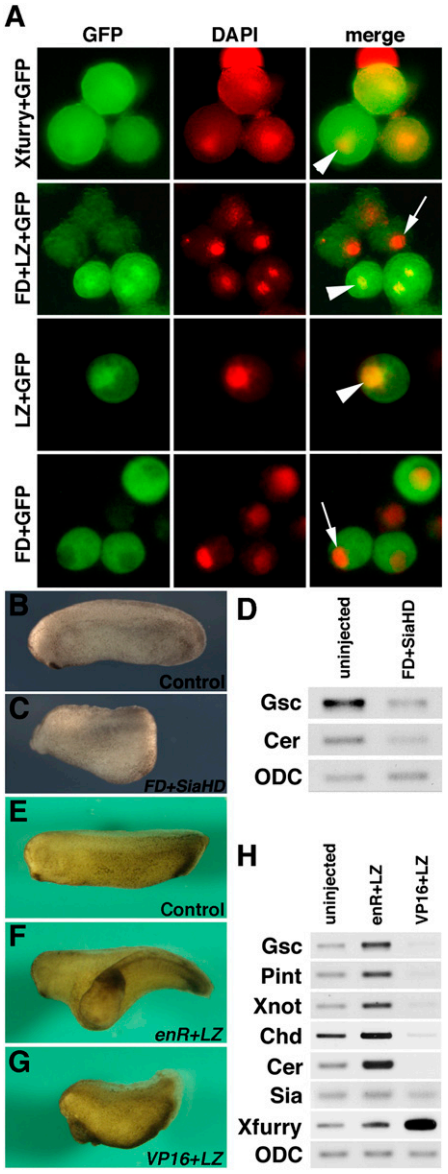

Fig. 2. Function of *Xfurry* as a transcriptional corepressor. (*A*) Fluorescent signals of Xfurry constructs in stage-10 mesodermal cells (Fig. S5). Red signals indicate cell nuclei stained with DAPI. (*Top Row*) Xfurry+GFP. (*Second Row*) FD+LZ+GFP construct. (*Third Row*) LZ+GFP. (*Bottom Row*) FD+GFP. Arrowheads show colocalization of GFP and DAPI signals. Arrows indicate noncolocalized signals. (*B*) Control embryo (stage 27). (*C*) Dorsal injection of *FD+SiaHD* interfered with head development. (*D*) Expression of *FD+SiaHD* reduced transcripts of *goosecoid* (Gsc) and *Cerberus* (Cer). (*E–G*) Phenotypes of injected stage-29 embryos. (*E*) Control embryo. (*F*) Ventral injection of *enR+LZ* (400 pg/blastomere) induced secondary axes in all embryos (n = 59). (*G*) Dorsal injection of *VP16+LZ* (400 pg/blastomere) shortened the dorsal axis in all embryos (n = 60). (*H*) Dorsal expression of *enR+LZ* induced chordamesodermal and head organizer genes, and expression of *VP16+LZ* reduced those genes (stage 10).

Xfurry mRNA (Fig. 2*F*). In contrast, dorsal expression of *VP16+LZ* had opposite effects, interfering with head formation and shortening the dorsal axis (Fig. 2*G*), similar to the phenotype generated by Xfurry depletion (Fig. 1*K*). Expression of several zygotic marker genes (23–28), including chordamesodermal genes, was increased by expression of *enR+LZ* and reduced by expression of *VP16+LZ* (Fig. 2*H*). Expression of *enR+LZ* rescued the phenotype of *Xfurry*-MO–injected embryos, and overexpression of *Xfurry* rescued the phenotype of *VP16+LZ*-injected embryos (Fig. S4 *D–F*). We thus proposed that *Xfurry* is a transcriptional corepressor that up-regulates chordamesodermal gene expression on the dorsal side of the embryo by downregulating an as yet unidentified general repressor of these chordamesodermal genes. We used constructs of *enR+LZ* and *VP16+LZ* to examine this hypothesis in the following experiments.

Overexpression of the early, dorsal-inducing genes *β-catenin* (12), *vegetal T-box transcription factor (VegT)* (29), and *Xnr5* (14) increased the levels of *Xfurry* transcript (Fig. 3*A*). Expression of *Xfurry* also was increased in *goosecoid*-injected embryos and in activin-treated animal caps (Fig. S8 *A* and *B*). These results suggest that *Xfurry* induction is a general response to dorsalization of the embryo. To identify potential repressors that are direct target genes of *Xfurry*, we performed a microarray assay using RNA extracted from pre-midblastula transition (MBT) cycloheximide-treated animal caps expressing *enR+LZ* or *VP16+LZ* (*SI Materials and Methods*). Unexpectedly, *enR+LZ* still induced early chordamesodermal marker genes in treated animal caps (Fig. 3*B*), suggesting that *Xfurry* functions as a transcriptional corepressor without zygotic protein synthesis.

Direct Target Gene of *Xfurry*. Cycloheximide treatment superinduces the transcription of several genes (30, 31) including some *Xfurry*-induced genes (Fig. 3*C*, *Left*). The other protein synthesis inhibitor, anisomycin, also induced such cycloheximide-induced genes (Fig. 3*C*, *Right*). These findings indicate that *Xfurry* could be involved in the superinduction of genes via protein synthesis inhibitors. It has been suggested that the superinduction could occur by inhibition of protein synthesis of transcriptional repressors, enzymes such as ribonucleases, and components of gene silencing. Here, the transcriptional corepressor *enR+LZ* induced early chordamesodermal marker genes under cycloheximide treatment (Fig. 3*B*); thus *Xfurry* would increase the transactivational effect of superinduction without zygotic protein synthesis. This suggests that *Xfurry* does not function by down-regulating proteins such as other transcriptional repressors and enzymes such as ribonucleases.

In further experiments, we found that blocking translation of specific cycloheximide-inducible genes with antisense morpholino oligonucleotides (MOs) also increased their transcripts. For example, injection of *pintallavis*-MO and *goosecoid*-MO in-

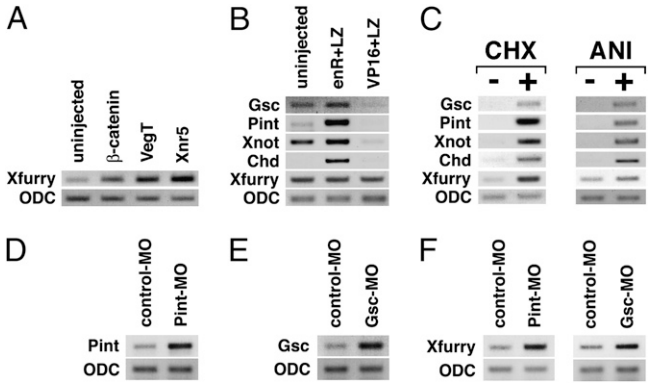

Fig. 3. Target genes of *Xfurry* and their superinduction. (*A*) Early inducers *β-catenin*, *VegT*, and *Xnr5* induced transcripts of *Xfurry* (stage 10). (*B*) *enR+LZ* induced chordamesodermal genes in the pre-MBT cycloheximide-treated animal caps, and *VP16+LZ* reduced or did not alter expression of those genes (stage 10). (*C*) Superinduction in the animal caps treated with both cycloheximide (CHX) (*Left*) and anisomycin (ANI) (*Right*) (stage 10). (*D*) Injection of *pintallavis*-MO induced expression of *pintallavis* (stage 10). (*E*) Injection of *goosecoid*-MO induced expression of *goosecoid* (stage 10). (*F*) Injection of both *pintallavis*-MO and *goosecoid*-MO induced expression of *Xfurry* (stage 10).

creased transcripts of pintallavis and goosecoid, respectively (Fig. 3 *D* and *E*). In the case of *goosecoid*, it is possible that the negative feedback loop, in which goosecoid protein works as a repressor of itself (32), is related to this transactivation. However, our data revealed that injection of *pintallavis*-MO and *goosecoid*-MO increased expression of a transcriptional corepressor, *Xfurry* (Fig. 3*F*). These results indicate that reduced levels of specific proteins are required for the increase of *Xfurry*. Moreover, cycloheximide and anisomycin treatment also induced *Xfurry* expression (Fig. 3*C*). Taken together, these findings suggest that *Xfurry* is necessary for repressing nontranslational (i.e., transcriptional) gene silencing to activate the transcription of early chordamesodermal genes.

We next isolated small (20- to 120-base) RNAs and injected them into dorsal blastomeres at the four-cell stage. Small RNAs from the fraction with 100–120 bases or less than 60 bases did not severely affect axis formation (Fig. 4 *B* and *D*), but small RNAs from the fraction with 60–100 bases, including premicroRNAs (around 70 bases), severely interfered with axis formation (Fig. 4*C*). Small RNAs, including premicroRNAs, strongly reduced early chordamesodermal gene expression (Fig. 4*E*). Because small RNAs from the fraction with less than 60 bases did not severely affect axis formation, we considered that appropriate processing would be necessary for function of matured small RNAs.

In yeast and mammalian cultured cells, microRNA forms an RNA-induced, transcriptional gene-silencing (RITS) complex that inhibits transcription via DNA methylation at the promoter region of its gene (16–22). However, the role of microRNA/RITS complexes in embryogenesis is still unexplained. To investigate whether small RNAs, including premicroRNAs, have RITS function in *Xenopus* embryogenesis, we injected small RNAs into four animal blastomeres of 32-cell embryos in a solution containing cycloheximide to prevent any translational inhibition by small RNAs. Because most of these embryos treated with cycloheximide died before the 32-cell stage, we began this experiment after the 32-cell stage. Small RNAs strongly reduced expression of superinduced genes in the injected animal cap cells and also slightly reduced expression of *Xfurry* (Fig. 4*F*). This suggests that small RNAs, including premicroRNAs, have RITS properties for the transcription of several genes. In *Xenopus*, *mir-15* inhibits nodal signaling at the translational level (16), and the phenotype of *Xfurry*-induced secondary axes lacking heads was similar to that produced when nodal signaling is activated (Fig. 1*H*) (33). Therefore we investigated whether *Xfurry* controls the expression of *mir-15* and whether *mir-15* functions via the RITS complex.

Expression of *enR+LZ* reduced transcripts of *primary miR-15 (pri-miR-15)* in cycloheximide-treated animal caps (Fig. 4*G*), whereas overexpression of *mir-15* reduced expression of *goosecoid*, *Xenopus not gene* (*Xnot*), and *pintallavis* and slightly reduced the expression of *chordin* with the cycloheximide treatment (Fig. 4*H*). Expression of *Xfurry* was not altered by *mir-15* expression (Fig. 4*H*). Moreover, expression of *pri-mir-15* was reduced by both cycloheximide and anisomycin treatments (Fig. 4*I* and Fig. S8*C*). Taken together, these results suggest that *Xfurry* functions to reduce expression of *mir-15*, which in turn, down-regulates both translational and transcriptional gene silencing for some early chordamesodermal genes and thus induces expression of these genes.

Discussion

The slight increase in *Xfurry* expression induced by *enR+LZ* without cycloheximide treatment in this study could be attributed to the complexity of related signal transduction events, including nodal/activin signaling (Figs. 2*H* and 3*A*). However, injection of *Xfurry*-MO or expression of *VP16+LZ* or small RNAs causes an increase in *Xfurry* transcripts (Figs. 1*L*, 2*H*, and 4*E*). The protein synthesis inhibitors also induced *Xfurry* transcripts (Fig. 3*C*), although *Xfurry* transcripts were not translated to protein. Therefore, inhibiting protein synthesis activates the corepressor function of *Xfurry* and other genes having a DNA-binding domain, and interaction with the FD would contribute to this activation. As stated, the requirements for increasing and activating *Xfurry*/Xfurry occur when the protein levels of *Xfurry* target genes are low. Expression of *enR+LZ* and *VP16+LZ* (Fig. 3*B*) and overexpression of *mir-15* (Fig. 4*H*) under the cycloheximide treatment did not alter *Xfurry* expression. These facts indicate that direct control of *Xfurry* expression is independent of *Xfurry* itself or of *mir-15*. Because small RNAs, including premicroRNAs, directly reduced expression of *Xfurry* (Fig. 4*F*), the release from transcriptional gene silencing by other microRNAs would increase *Xfurry* transcription under low-protein conditions such as that induced by the cycloheximide and anisomycin treatments.

Under low-protein conditions, both pretranscriptional and translational silencing by microRNAs should be inhibited for recovery to the normal protein levels. To decrease expression of microRNAs, some transcriptional repressors and related genes must be increased or activated, and one of those genes is *Xfurry*. We hypothesize that this requirement for repressor increase/activation would occur under the low-protein condition of one gene and might lead not only to the transcriptional activation of the low-protein gene but also to the silencing of other functionally related genes, indirectly increasing the transcription of the low-protein gene. In this study, injection of *pintallavis*-MO increased the expression of *pintallavis*, *Xfurry* (Fig. 3 *D* and *F*), *goosecoid,* and *Xnot* and decreased the expression of *pri-mir-15*, but the expression of *chordin* was not increased (Fig. 4*J*). Taken together, these observations indicate that the transcription of several genes might be regulated simultaneously by a single microRNA.

Normal development requires the precise spatial and temporal regulation of protein expression from various genes. When the amount of a specific protein is reduced and an increase is required, release of both translational and pretranscriptional gene silencing would occur more quickly than by the classical system involving an intermediate step of protein synthesis. Such a mechanism for the release of gene silencing would be suitable for the immediate and diverse pattern of gene regulation that characterizes embryogenesis. Translational gene silencing requires preexisting transcripts, such as maternal mRNAs, but transcriptional gene silencing does not require those transcripts. Our results show that cycloheximide treatment from the pre-MBT stage greatly reduced expression of *mir-15* (Fig. 4*I* and Fig. S8*C*) and, eventually, the up-regulation of a suite of chordamesodermal genes. This finding indicates that correct *mir-15* levels are necessary for correct levels of dorsal chordamesodermal proteins. We therefore propose that microRNA-mediated transcriptional gene silencing is necessary for regulation of zygotic protein levels. *Xfurry* does not function directly to induce chordamesodermal genes but instead regulates their expression to maintain their protein levels in a regulated band via a mechanism to repress microRNAs (Fig. 4*K*).

Because of the expression patterns of *Xfurry*, we posited that it functions in chordamesodermal gene regulation. However, weak expressions of *Xfurry* were observed in ventral, ectodermal, and endodermal regions (Fig. 1*B*). We therefore examined the expressions of several other marker genes by RT-PCR. Without protein synthesis inhibitors, expression of *enR+LZ* or *VP16+LZ* increased or decreased ectodermal (*keratin*), ventral (*bone morphogenetic protein 4*, *ventricular size 1*, and *ventricular size 2*), mesodermal [*Xenopus brachyury* (*Xbra*)], and endodermal (*cerberus* and *Siamois*) genes, respectively, whereas expression of *myogenic differentiation* (*MyoD*) was not affected (Fig. 2*H* and Fig. S8*D*). Although the cycloheximide and anisomycin treatments reduced expression of *keratin* in the animal caps, most of these genes were increased by such inhibition of protein synthesis (Fig. S8 *E*

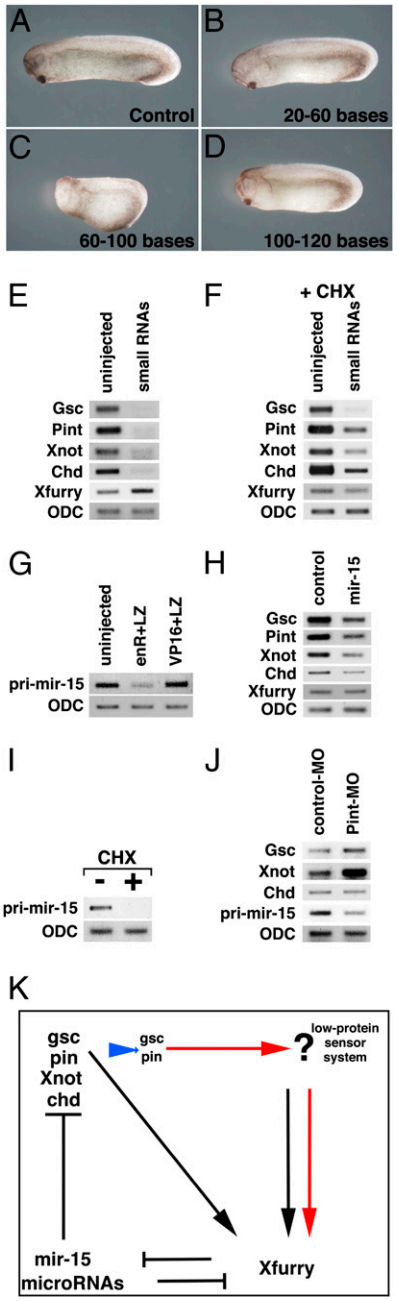

Fig. 4. The role of small RNAs in gene expression. (*A–D*) Phenotypes of small RNA-injected embryos (stage 30). (*A*) Control embryo. (*B–D*) Small RNAs in the range of 20–60 (*B*), 60–100 (*C*), and 100–120 (*D*) bases were injected into dorsal blastomeres of four-cell embryos. (*C*) Injection of small RNAs (60–100 bases) shortened the dorsal axis in all embryos (n = 56). (*E*) Injection of small RNAs (60–100 bases) into all blastomeres of four-cell embryos markedly reduced the expression of chordamesodermal genes but increased expression of *Xfurry* (stage 10). (*F*) Injection of small RNAs (60–100 bases) into four animal blastomeres of 32-cell embryos in solution containing cycloheximide decreased expression of chordamesodermal genes in the animal caps and slightly reduced the expression of *Xfurry* (stage 10). (*G*) Expression of *enR+LZ* reduced *pri-mir-15*, and expression of *VP16+LZ* slightly up-regulated *pri-mir-15* in the cycloheximide-treated animal caps (stage 10). (*H*) Overexpression of *mir-15* in the cycloheximide-treated animal caps reduced expression of chordamesodermal genes and did not alter expression of *Xfurry*. (*I*) Cycloheximide treatment from stage 7 reduced expression of *pri-mir-15* in the treated animal caps (stage 10). (*J*) Injection of *pintallavis*-MO (Pint-MO) into all blastomeres of four-cell embryos increased expression of *goosecoid* and *Xnot*, did not alter the expression of *chordin*, and reduced the expression of *pri-mir-15* (stage 10). (*K*) Model of the proposed *Xfurry* signaling pathway. *Xfurry* represses expression of *mir-15* and microRNAs. Several chordamesodermal genes are repressed by *mir-15* and microRNAs. *Xfurry* also is induced by *goosecoid* as a downstream gene. Then this feedback loop signaling pathway together with *Xfurry* regulates expression of chordamesodermal genes. Moreover, low protein levels of several genes activate a system that senses the low-protein condition, thereby inducing *Xfurry* expression and activating the function of *Xfurry* as a corepressor. Pin, *pintallavis*.

and *F*). We also found that *enR+LZ* induced those marker genes (except for *MyoD*) in the cycloheximide-treated animal caps (Fig. S8*G*). Expression of *Siamois* was not observed in animal caps (Fig. S8 *E–G*). Taken together, these findings suggest that the gene regulation by *Xfurry* is not specific for chordamesodermal genes. However, the regulations of *keratin* and *MyoD* expression were different from those of other marker genes, and induction was observed only with expression of *enR+LZ* (Fig. S8 *D* and *G*) or treatment with protein synthesis inhibitors (Fig. S8 *E* and *F*), suggesting that gene regulation in the chordamesodermal region by *Xfurry* is not conserved completely in other regions. The *Xfurry* transcripts gradually accumulated in the chordamesodermal region (Fig. 1), and the dorsal injection of *Xfurry* did not induce ventralization (Fig. S2). Based on this study, we propose that *Xfurry* functions mainly in chordamesodermal gene regulation and that other transcriptional factors operate in the gene regulation mechanisms of other tissues.

The release of microRNA-mediated transcriptional gene silencing could up-regulate transcripts of the targeted genes. However, small RNAs, including microRNA, also are involved in mRNA stabilization (34, 35); *mir-16* contains a UAAAUAUU sequence that is complementary to the adenine- and undine-rich elements and is required for mRNA degradation (34). Moreover, inactivation of miR-427 leads to stabilization of the mRNAs and might be involved in the turnover of maternal mRNAs (36). Our data revealed that *Xfurry* transcripts are maternally expressed and decrease temporally after the MBT (Fig. 1*A*). The *Xfurry*-mediated decrease in microRNAs therefore might prevent message degradation for several genes and at least temporarily control the levels of mRNAs.

This study could not reveal how cells recognize specific low-protein-level conditions. Defining the mechanisms of activation and isolation of *Xfurry*-related genes might be fruitful areas of investigation.

Materials and Methods

Cloning and Constructs of *Xfurry*. The *Xfurry* cDNA fragments (GenBank AB294244) were obtained by RT-PCR, including 5′- and 3′-RACE, with RNAs extracted from stage-10 embryos using degenerate primers. The *Xfurry* constructs were synthesized by PCR and were subcloned into a modified pCS2$^+$ vector. The primers used are described in *SI Materials and Methods*.

Antisense MOs and microRNA. We obtained the antisense MOs *Xfurry*-MO (5′-cgggcaggtaagaggctcacacact-3′), control-MO (5′-tgctgttgttttgatccatgttg-tg-3′), *pintallavis*-MO (37), and *goosecoid*-MO (38) from Gene Tools, LLC. We used the mature *mir-15* and control mutant *mir-15* (Invitrogen) previously described (16). Small RNAs were extracted from stage-10 embryos using a *mir*VanaTM miRNA Isolation Kit (Ambion) and were separated on 15% acrylamide gels. Small RNAs reextracted from gels were purified by Microcon-Y30 columns (Millipore). The developmental stages are according to Nieuwkoop and Faber (39). The microinjection and microsurgery methods used are described in *SI Materials and Methods*.

Whole-Mount in Situ Hybridization. Whole-mount in situ hybridization was performed as previously described (40).

Microarray Analysis. Total RNAs were extracted from animal caps of *enR+LZ*-injected and *VP16+LZ*-injected embryos (plus cycloheximide treatment) that were dissected at stage 8 and cultured until stage 10. The microarray assay was performed as previously described (41).

ACKNOWLEDGMENTS. We thank Paul Skoglund and Takuya Nakayama for helpful suggestions on the manuscript. This work was supported in part by grants from the Japan Science and Technology Agency, the Ministry of Health, Labour and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology (to M.A.) and by Grant R37 HD025594 from the National Institutes of Health (to R.K.).

1. Cong J, et al. (2001) The furry gene of *Drosophila* is important for maintaining the integrity of cellular extensions during morphogenesis. *Development* 128:2793–2802.
2. He Y, Fang X, Emoto K, Jan YN, Adler PN (2005) The tricornered Ser/Thr protein kinase is regulated by phosphorylation and interacts with furry during *Drosophila* wing hair development. *Mol Biol Cell* 16:689–700.
3. Geng W, He B, Wang M, Adler PN (2000) The tricornered gene, which is required for the integrity of epidermal cell extensions, encodes the *Drosophila* nuclear DBF2-related kinase. *Genetics* 156:1817–1828.
4. Emoto K, et al. (2004) Control of dendritic branching and tiling by the Tricornered-kinase/Furry signaling pathway in *Drosophila* sensory neurons. *Cell* 119:245–256.
5. Hirata D, et al. (2002) Fission yeast Mor2/Cps12, a protein similar to Drosophila Furry, is essential for cell morphogenesis and its mutation induces Wee1-dependent G(2) delay. *EMBO J* 21:4863–4874.
6. Nelson B, et al. (2003) RAM: A conserved signaling network that regulates Ace2p transcriptional activity and polarized morphogenesis. *Mol Biol Cell* 14:3782–3803.
7. Chiba S, Ikeda M, Katsunuma K, Ohashi K, Mizuno K (2009) MST2- and Furry-mediated activation of NDR1 kinase is critical for precise alignment of mitotic chromosomes. *Curr Biol* 19:1–7.
8. Hayette S, et al. (2005) AF4p12, a human homologue to the furry gene of Drosophila, as a novel MLL fusion partner. *Cancer Res* 65:6521–6525.
9. Heasman J, et al. (1994) Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell* 79: 791–803.
10. Lemaire P, Garrett N, Gurdon JB (1995) Expression cloning of Siamois, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* 81:85–94.
11. Wylie C, et al. (1996) Maternal beta-catenin establishes a 'dorsal signal' in early *Xenopus* embryos. *Development* 122:2987–2996.
12. Schohl A, Fagotto F (2003) A role for maternal beta-catenin in early mesoderm induction in *Xenopus*. *EMBO J* 22:3303–3313.
13. Lustig KD, et al. (1996) A *Xenopus* nodal-related gene that acts in synergy with noggin to induce complete secondary axis and notochord formation. *Development* 122:3275–3282.
14. Takahashi S, et al. (2000) Two novel nodal-related genes initiate early inductive events in *Xenopus* Nieuwkoop center. *Development* 127:5319–5329.
15. Onuma Y, Takahashi S, Yokota C, Asashima M (2002) Multiple nodal-related genes act coordinately in *Xenopus* embryogenesis. *Dev Biol* 241:94–105.
16. Martello G, et al. (2007) MicroRNA control of Nodal signalling. *Nature* 449:183–188.
17. Rosa A, Spagnoli FM, Brivanlou AH (2009) The miR-430/427/302 family controls mesendodermal fate specification via species-specific target selection. *Dev Cell* 16: 517–527.
18. Morris KV, Chan SW, Jacobsen SE, Looney DJ (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* 305:1289–1292.
19. Hawkins PG, Santoso S, Adams C, Anest V, Morris KV (2009) Promoter targeted small RNAs induce long-term transcriptional gene silencing in human cells. *Nucleic Acids Res* 37:2984–2995.
20. Kim DH, Saetrom P, Snøve O, Jr, Rossi JJ (2008) MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci USA* 105:16230–16235.
21. Morris KV (2009) RNA-directed transcriptional gene silencing and activation in human cells. *Oligonucleotides* 19:299–306.
22. Suzuki K, Kelleher AD (2009) Transcriptional regulation by promoter targeted RNAs. *Curr Top Med Chem* 9:1079–1087.
23. Cho KW, Blumberg B, Steinbeisser H, De Robertis EM (1991) Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene goosecoid. *Cell* 67: 1111–1120.
24. Ruiz i Altaba A, Cox C, Jessell TM, Klar A (1993) Ectopic neural expression of a floor plate marker in frog embryos injected with the midline transcription factor Pintallavis. *Proc Natl Acad Sci USA* 90:8268–8272.
25. von Dassow G, Schmidt JE, Kimelman D (1993) Induction of the *Xenopus* organizer: Expression and regulation of Xnot, a novel FGF and activin-regulated homeo box gene. *Genes Dev* 7:355–366.
26. Sasai Y, et al. (1994) *Xenopus* chordin: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* 79:779–790.
27. Fan MJ, Sokol SY (1997) A role for Siamois in Spemann organizer formation. *Development* 124:2581–2589.
28. Kessler DS (1997) Siamois is required for formation of Spemann's organizer. *Proc Natl Acad Sci USA* 94:13017–13022.
29. Zhang J, et al. (1998) The role of maternal VegT in establishing the primary germ layers in *Xenopus* embryos. *Cell* 94:515–524.
30. Dawid IB, Otani H, Curtiss P, Taira M (1993) Regulatory interactions during embryogenesis in *Xenopus laevis*. *C R Acad Sci III* 316:945–958.
31. Tadano T, Otani H, Taira M, Dawid IB (1993) Differential induction of regulatory genes during mesoderm formation in *Xenopus laevis* embryos. *Dev Genet* 14: 204–211.
32. Danilov V, Blum M, Schweickert A, Campione M, Steinbeisser H (1998) Negative autoregulation of the organizer-specific homeobox gene goosecoid. *J Biol Chem* 273: 627–635.
33. Piccolo S, et al. (1999) The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* 397:707–710.
34. Jing Q, et al. (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* 120:623–634.
35. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev* 20:515–524.
36. Lund E, Liu M, Hartley RS, Sheets MD, Dahlberg JE (2009) Deadenylation of maternal mRNAs mediated by miR-427 in *Xenopus laevis* embryos. *RNA* 15:2351–2363.
37. Martynova N, et al. (2004) Patterning the forebrain: FoxA4a/Pintallavis and Xvent2 determine the posterior limit of Xanf1 expression in the neural plate. *Development* 131:2329–2338.
38. Sander V, Reversade B, De Robertis EM (2007) The opposing homeobox genes Goosecoid and Vent1/2 self-regulate *Xenopus* patterning. *EMBO J* 26:2955–2965.
39. Nieuwkoop PD, Faber J (1967) *Normal Table of Xenopus laevis (Daudin)* (North Holland, Amsterdam).
40. Harland RM (1991) *In situ* hybridization: An improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol* 36:685–695.
41. Fukui A, Goto T, Kitamoto J, Homma M, Asashima M (2007) SDF-1 alpha regulates mesendodermal cell migration during frog gastrulation. *Biochem Biophys Res Commun* 354:472–477.