Zebrafish *cul4a*, but not *cul4b*, modulates cardiac and forelimb development by upregulating *tbx5a* expression

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CUL4A and CUL4B are closely related cullin family members and can each assemble a Cullin-RING E3 ligase complex (CRL) and participate in a variety of biological processes. While the CRLs formed by the two cullin members may have common targets, the two appeared to have very different consequences when mutated or disrupted in mammals. We here investigated the roles of *cul4a* and *cul4b* during zebrafish embryogenesis by using the morpholino knockdown approach. We found that *cul4a* is essential for cardiac development as well as for pectoral fin development. Whereas *cul4a* morphants appeared to be unperturbed in chamber specification, they failed to undergo heart looping. The failures in heart looping and pectoral fin formation in *cul4a* morphants were accompanied by greatly reduced proliferation of cardiac cells and pectoral fin-forming cells. We demonstrated that *tbx5a*, a transcription factor essential for heart and limb development, is transcriptionally upregulated by *cul4a* and mediates the function of *cul4a* in cardiac and pectoral fin development. In contrast to the critical importance of *cul4a*, *cul4b* appeared to be dispensable for zebrafish development and was incapable of compensating for the loss of *cul4a*. This work provides the first demonstration of an essential role of *cul4a*, but not *cul4b*, in cardiac development and in the regulation of *tbx5a* in zebrafish. These findings justify exploring the functional role of CUL4A in human cardiac development.

INTRODUCTION

Cullin-RING E3 ligase (CRLs) complexes, which contain cullin, RING protein and substrate-recognition subunit as the core components, represent the largest known class of ubiquitin ligases and participate in a broad variety of physiologically and developmentally controlled processes such as cell cycle progression, transcription and signal transduction (1–3). Among cullin family members, CUL4A and CUL4B have the highest degree of homology, with ~80% identity in protein sequences, and are believed to be derived from one common ancestor Cul4 (4). There is only one ortholog, Cul4, in lower organisms. Acting as the scaffold component, CUL4 assembles with ROC1 at its C terminus and with damaged DNA-binding protein 1 (DDB1) at its N terminus to form CRL4 complex (1–4).

As CUL4A and CUL4B share a high degree of homology and utilize the same adapter protein DDB1 in assembling CRL4 complexes, they can potentially target the same substrates and function redundantly in some cellular functions (5). However, when mutated or disrupted, those two genes appeared to have very different consequences. Mutations in human *CUL4B* have been found to be a common cause of X-linked mental retardation (XLMR) (6,7). To date, at least 12 families of XLMR have been reported to be attributable to the loss-of-function mutations in *CUL4B* (6–10). In addition to being mentally retarded, patients carrying *CUL4B* mutation also manifest short stature, abnormal gait, impaired speech and other abnormalities (6–9,11). These phenotypes underscore the functional importance of CUL4B. Furthermore, in peripheral blood of human *CUL4B* heterozygotes, cells in which the mutant *CUL4B* resides on the active X chromosome were strongly selected against (6,10).

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Accordingly, *Cul4b* null mice are embryonic lethal and *Cul4b* nonfunctional cells are selected against in *Cul4b* heterozygous mice (12). Recent reports showed that *Cul4b* is particularly critical for the development of extraembryonic tissues (12,13). *Cul4A* has also been shown to promote tumorigenesis and to be highly expressed in a broad variety of cancer (14,15). Recently, we showed that CRL4B can also catalyze H2AK119 monoubiquitination and, by coordinating with PRC2, promote H3K27 trimethylation, leading to the transcriptional repression of tumor suppressor genes and enhanced tumorigenesis (14).

On the other hand, no germline mutations in the human *CUL4A* gene have been documented, although *CUL4A* is found to be overexpressed in breast cancers, hepatocellular carcinomas, squamous cell carcinomas, adenocortical carcinomas and childhood medulloblastoma (16–21). In addition, *Cul4a* knockout mice were viable and displayed no apparent developmental phenotype except that the knockout males were sterile due to impaired spermatogenesis (22–24). These results suggest that the two *CUL4A* genes are not entirely redundant in mammals.

Zebrafish have been extensively used as a model for the study of embryogenesis. A recent study showed that cereblon (CRBN)-mediated CRL4A E3 ligase activity is critical for several important developmental processes such as limb outgrowth and otic vesicle development in zebrafish (25). Knockdown of *crbn* or *cul4a* caused specific defects in pectoral fin and otic vesicle development. Interestingly, as the substrate receptor for CRL4A, CRBN is also targeted by thalidomide, a well-known teratogen. Therefore, new functions of *CUL4A* and *CUL4B* remain to be identified.

In this study, we examined the roles of *cul4a* and *cul4b* during zebrafish embryogenesis and found that *cul4a*, but not *cul4b*, plays an essential role during heart and limb development. We demonstrated that *cul4a* is required for the expression of *tbx5a*, an essential transcription factor in heart and limb development. This study provides the first demonstration of a crucial role for *cul4a* in cardiovascular development.

**RESULTS**

**Knockdown of *cul4a*, but not *cul4b*, results in developmental defects in zebrafish**

As in mammals, there are two *cul4* members, *cul4a* and *cul4b*, in zebrafish. The two have a high degree of homology with their respective mammalian counterparts *CUL4A* and *CUL4B* (Fig. 1A). Zebrafish *cul4a*, which resides on chromosome 1 and encodes 635 amino acids, displays 80% amino acid identity to human *CUL4A*, and *cul4b*, on chromosome 14 and encoding 864 amino acids, has 84% amino acid identity to human *CUL4B*. As expected, the protein sequences of zebrafish *cul4a* and *cul4b* also show a high degree of homology (78%) (Fig. 1A).

To determine the physiological roles of *cul4a* and *cul4b* in vivo, we designed *cul4a*- and *cul4b*-specific MOs, designated as *cul4a*-MO1 and *cul4b*-MO1, respectively, to block the splice donor junction of exon 2 (Fig. 1B). The MOs were predicted to skip exon 2 in the final mRNA transcripts, thereby changing the reading frame and introducing a stop codon. Splice-blocking MOs are advantageous as the knockdown efficiency can be evaluated using RT-PCR. To validate the efficacy of *cul4a*-MO1 and *cul4b*-MO1, primers flanking exon 2 were employed for RT-PCR amplification of *cul4a* or *cul4b* in wild-type and MO-injected embryos at 24 h post fertilization (hpf). As shown in Figure 1C, injection of 4 ng of *cul4a*-MO1 or *cul4b*-MO1 resulted in complete exclusion of exon 2 from the *cul4a* or *cul4b* mRNA, which was confirmed by cDNA sequencing (Fig. 1D). The deletion in *cul4a* would result in a frameshift consisting of eight missense codons followed by a stop codon. The mutant allele is thus predicted to generate a peptide of merely 44 amino acids, if it were to be expressed. There was no detectable inhibition of *cul4a* splicing in embryos injected with control morpholino (*cul4a*-CMO), which has five mismatches with *cul4a*-MO1. Similarly, injection of 4 ng *cul4b*-MO1 resulted in the complete exclusion of exon 2 from the *cul4b* mRNA, as shown by the absence of the 571 bp product representing wild-type *cul4b* message and the presence of a smaller 455 bp product (Fig. 1C, bottom). These data indicate that these MOs were efficient in disrupting the expression of their target genes in zebrafish embryos.

We determined the dose of the MOs required for knocking down the target genes by injecting 1–4 ng of CMO, *cul4a*-MO1 or *cul4b*-MO1 into one-cell stage zebrafish embryos and allowed them to develop for 3 days post fertilization (dpf). Unexpectedly, the *cul4b*-MO1-injected embryos (*cul4b* morphants) were phenotypically indistinguishable from *cul4b*-CMO-injected embryos (Fig. 2A). To confirm this observation, a separate morpholino, *cul4b*-MO2, was designed to target the 5′-UTR of *cul4b* to inhibit translation. Experiments with *cul4b*-MO2 produced similar results (Fig. 2A), suggesting that lack of *cul4b* is compatible with normal zebrafish development.

In contrast to the lack of abnormal phenotypes in *cul4b* morphants, *cul4a*-MO1-injected embryos (*cul4a* morphants) displayed an array of profound morphological defects, including tail curling, hindbrain edema, stunted or completely absent pectoral fins and pericardial edema that was accompanied by heart looping defects (Fig. 2B–E). To exclude possible off-target effects of *cul4a*-MO1, a second morpholino, *cul4a*-MO2, was designed to inhibit *cul4a* translation by targeting its 5′-UTR. Embryos injected with *cul4a*-MO2 exhibited similar phenotypes (Fig. 2B–E; Supplementary Material, Fig. S1), confirming an important role of *cul4a* in zebrafish development.

We next performed rescue experiment in *cul4a* morphants by injecting a mixture of capped zebrafish *cul4a* mRNA and *cul4a*-MO1 into one-cell embryos. While only 15.2% (16 of 105) embryos appeared normal when *cul4a*-MO1 alone was injected, co-injection of *cul4a* mRNA and *cul4a*-MO1 increased the percentage of normal embryos to 79.3% (130/164) at 72 hpf, which was comparable with that in the *cul4a*-CMO-injected group, indicating that the abnormalities caused by *cul4a*-MO1 can be rescued by co-injection of zebrafish *cul4a* mRNA (Fig. 2F). Interestingly, the defects of *cul4a* morphants could also be rescued by co-injection of human *CUL4A* mRNA, indicating a functional conservation of this protein between zebrafish and humans (Fig. 2F). Strikingly, we also observed that human *CUL4A* mRNA was as effective as *CUL4A* mRNA in rescuing the developmental defects in *cul4a* morphants (Fig. 2F). Zebrafish *cul4b* mRNA, on the other hand, was ineffective. Furthermore, we also examined the gross morphology of *cul4a* and *cul4b* double knockdown zebrafish (Fig. 2G) and found that 19.1% (33/173) of the *cul4a/cul4b* double knockdown embryos appeared normal, which was comparable with *cul4a*
single knockdown embryos. These results indicate that cul4b is dispensable for zebrafish development and is incapable of compensating for the loss of cul4a.

Knockdown of cul4a led to heart looping failure

To further define the cardiac abnormality in cul4a morphants, we injected cul4a-CMO or cul4a-MO1 into zebrafish embryos of the transgenic line *Tg(cmlc:gfp)* in which enhanced green fluorescent protein is expressed in all cardiac cells (26). Although the initial heart tubes in cul4a morphants were indistinguishable from those in controls at 24–33 hpf (Fig. 3A and B), abnormalities were noted at later developmental stage. At 48 hpf, the heart tubes in cul4a-MO1 morphants remained relatively straight, whereas they appeared to loop to the right in CMO-injected embryos (Fig. 3C and D). At 60 hpf, the hearts in cul4a-MO1
Figure 2. Morphological defects of cul4a knockdown zebrafish. Knockdown of cul4a led to an array of profound morphological defects, including tail curling, hindbrain edema, stunted or completely absent pectoral fins and pericardial edema accompanied with heart looping defects. (A) Phenotype profile from zebrafish cul4b knockdown experiments. The embryos were classified as having cardiac defect, truncated or missing pectoral fins, and normal. (B) Morphological features of zebrafish at 72 hpf from cul4a knockdown experiments with tail curling dorsally. (C) The hindbrain of embryo injected with cul4a MO1 showed prominent edema (arrow). (D) The dorsal view of zebrafish embryos injected with cul4a MO1, MO2 or CMO. (E) Zebrafish embryos injected with cul4a MO1, MO2 or CMO. A large pericardial edema was evident in cul4a MO1 and MO2. (F) Summary of phenotypes in zebrafish rescue experiments. The embryos were classified as having cardiac defect, truncated or missing pectoral fins, and normal. (G) Summary of phenotypes in zebrafish cul4a and cul4b double knockdown experiments. The embryos were classified as having cardiac defect, truncated or missing pectoral fins, and normal. *PF defect: stunted or completely absent pectoral fins. Scale bar: 200 μm. *P < 0.05, **P < 0.01, ***P < 0.001.
morphants remained configured as cylinders, though there was no obvious perturbation in cmlc-driven GFP expression when compared with the controls (Fig. 3E and F). Cul4a morphants also showed significantly slower heart rhythm than the controls (Fig. 3G). At 60 hpf, MO1 and CMO morphants differed by 26 beats/min, 123 ± 10 beats/min (n = 30) versus 149 ± 5 beats/min (n = 29), P < 0.001. At 72 hpf, the difference increased to 42 beats/min, 170 ± 20 beats/min (n = 38) versus 212 ± 13 beats/min (n = 25), P < 0.001. Together, these data indicate that the cardiac development in cul4a morphants was structurally and functionally impaired.

To determine the developmental stage when cardiac development was first perturbed in cul4a morphants, we injected embryos with cul4a-CMO or cul4a-MO1 and processed them for in situ hybridization with various cardiac markers. Examination of cardiac myosin light chain 2 (cmlc2), a pan-cardiac marker (27), revealed no difference between the two groups during the early stage of the cardiac development, from the time when cardiac cone appears, through the period of tube formation, to the stage of cardiac jogging (Fig. 4A, top), indicating that the myocardial specification occurred normally in cul4a morphants. By 48 hpf, however, when the hearts in cul4a-CMO-injected embryos were observed to bend rightward in the ventricular region (D-loop), this landmark of cardiac development was not noted in cul4a morphants (Fig. 4A, bottom). Thus, the cardiac defect caused by cul4a deficiency was not evident until the cardiac looping stage.

To determine whether the chamber identity was altered in cul4a morphants, we examined cardiac chamber development by using monoclonal antibodies against pan cardiac as well as chamber-specific myosin markers (27,28). Immunostaining of MF20, which is expressed in all cardiomyocytes, revealed no notable difference between control and cul4a morphants at 48 hpf (Fig. 4B). The expression pattern of atrial marker S46 also appeared to be similar between cul4a morphants and control embryos (Fig. 4C). We also compared the distributions of ventricular myosin heavy chain (vmhc) (27). At 48 hpf, the expression pattern of vmhc in cul4a morphants was again indistinguishable from that in control embryos, indicating that the ventricular specification proceeded normally in cul4a morphants (Fig. 4D). Altogether, these data indicate that chamber specification and initial differentiation were unperturbed in cul4a knockdown embryos.

Increased apoptosis and decreased proliferation of myocardial cells in the cul4a morphants

Cell proliferation, cellular organization and cell shape changes have been suggested to contribute to cardiac looping (29–31). To investigate the mechanism underlying the failure in heart looping in cul4a morphants, we investigated whether the number of cardiomyocytes was decreased in cul4a morphants. Examination of histological sections of cul4a-MO1-injected embryos at 3 dpf showed that the walls of ventricle and atrium were markedly thinner than those in age-matched controls, and the cardiomyocytes were more scarcely distributed along the ventricular and atrial walls in cul4a morphants (Fig. 5A).

The observed paucity of cardiac cells in cul4a morphants prompted us to characterize the levels of apoptosis and proliferation during heart looping. Apoptosis was examined using TUNEL assay (Fig. 5B). While no obvious apoptosis was observed in the atria or ventricle of cul4a-CMO-injected embryos, a remarkable number of apoptotic cells were detected in the heart of cul4a knockdown embryos (Fig. 5B). This result indicates that the cardiac cells in cul4a morphants were impaired in their survival.

We examined cell proliferation using a polyclonal antibody against proliferating cell nuclear antigen (PCNA). Immunostaining of PCNA indicated that the number of dividing cardiac cells was lower in cul4a morphants than in cul4a-CMO embryos (Fig. 5C). To validate this result, we injected bromodeoxyuridine (BrdU) into the pericardiac cavity at 48 hpf and processed the embryos for BrdU staining 2 h later (30). As shown in Figure 5D, the number of BrdU-positive cells was significantly decreased in the heart of cul4a-MO1-injected embryos, compared with that in control embryos (cul4a-CMO: 29.5 ± 1.5, n = 4; cul4a-MO1: 13.3 ± 4.7, n = 3). These results indicate that the cardiac cells in cul4a morphants were impaired in their proliferation.

In addition to having a low cell number, the cardiac cells in the cul4a morphants also appeared to be disorganized when
compared with the controls. Whereas the cardiomyocytes were cuboidal and well compacted in \textit{cul4a}-CMO-injected \textit{Tg(cmlc2:gfp)} embryos, they were irregularly shaped and were distributed in disarray in \textit{cul4a}-MO1-injected embryos (Fig. 5E). Thus, there is an insufficient supply of cardiomyocytes in \textit{cul4a} morphants, which may have led to irregularity in cell shape and compaction, and consequently the failure in heart looping.

**Impaired pectoral fin development in \textit{cul4a} knockdown zebrafish**

Injection of \textit{cul4a}-MO1 also led to the truncation or absence of pectoral fins in zebrafish. The length of pectoral fins was \(73.73 \pm 69.87\, \mu m\) \((n = 16)\) in average in \textit{cul4a} morphants, compared with \(285.84 \pm 38.89\, \mu m\) \((n = 19)\) in controls (Fig. 6A). Alcian Blue staining, which marks cartilage and bone structures in the developing embryos, also revealed shortened or absent pectoral fins in \textit{cul4a} morphants (Fig. 6B). One-hour pulse of BrdU at 33 hpf revealed fewer proliferating (BrdU-positive) cells in the buds of pectoral fins in \textit{cul4a} morphants than in control embryos (Fig. 6C). These results suggest that, as for the failure of heart looping in \textit{cul4a} morphants, proliferation defect might also be responsible for the failure in the pectoral fin development.

**Tbx5a was downregulated in \textit{cul4a} morphants**

We noted that the phenotypes displayed by \textit{cul4a} morphants were reminiscent of those in \textit{tbx5a} mutants or \textit{tbx5a} knockdown morphants (32,33). Both \textit{tbx5a} and \textit{cul4a} morphants exhibited structural defects in heart and pectoral fin. The common phenotypes shared between \textit{cul4a} and \textit{tbx5a} loss-of-function morphants prompted us to test whether the two lie in the same pathway. We first examined the expression of \textit{cul4a} in heart and pectoral fins and found that \textit{cul4a} was highly expressed in those organs (Fig. 7A). This expression pattern was similar to that of \textit{tbx5a} previously reported (32,33). We then determined the levels of \textit{tbx5a} mRNA in control and \textit{cul4a}-MO1-treated zebrafish embryos at 32 hpf by quantitative RT-PCR. The \textit{tbx5a} expression was remarkably reduced in \textit{cul4a}-MO1-injected embryos compared with that in embryos injected with \textit{cul4a}-CMO (Fig. 7B). The downregulation of \textit{tbx5a} in \textit{cul4a} morphants was confirmed at protein level (Fig. 7C). Whole-mount \textit{in situ} hybridization using \textit{tbx5a} probes also indicated a reduction in \textit{tbx5a} expression in the heart and in the buds of pectoral fins in \textit{cul4a} morphants (Fig. 7D), which corresponded to the lack of \textit{cul4a} expressions in those regions. Because \textit{tbx5a} functions as a transcription factor, we thus also measured the expression levels of its transcriptional targets \textit{fgf10} and \textit{hand2}. 858 Human Molecular Genetics, 2015, Vol. 24, No. 3
As expected, the target genes were significantly downregulated (Fig. 7B).

We next determined whether the phenotypes caused by cul4a knockdown were mediated by the downregulation of tbx5a by co-injecting tbx5a mRNA and cul4a-MO1 into zebrafish embryos. We found that co-injection of tbx5a mRNA significantly rescued the developmental defects in cul4a morphants, with the percentage of embryos with malformed heart and pectoral fin significantly decreased (Fig. 7E). These results indicated that the phenotypes of cul4a morphants were mediated by the downregulation of tbx5a.

The finding that cul4a can positively regulate tbx5a expression during zebrafish development is analogous to the report that CUL4A contributes to transcriptional activation of p16 in human tumor cells by coordinating with H3K4 methyltransferase MLL1 complex (34). Thus, we next tested whether CUL4A could activate Tbx5a expression in H9C2, a line of rat myocardial cells. As expected, knockdown of Cul4a resulted in a reduction of Tbx5a expression at both mRNA and protein levels (Fig. 7F and G), suggesting that Cul4a can positively regulate the expression of Tbx5a. To further support this notion, we used chromatin immunoprecipitation (ChIP) to examine whether Cul4a directly binds to the promoter of Tbx5a gene. As shown in Figure 7H, Cul4a is directly bound to the –908 to –511 bp region of Tbx5a promoter. Notably, H3K4me3, a histone mark associated with transcriptional activation, was also enriched in the same region (Fig. 7H). Furthermore, knockdown of Cul4a led to a significant decrease in the enrichment of H3K4me3 at the Tbx5a promoter.
Cul4a and H3K4me3 on the Tbx5a promoter. These results suggest that Cul4a can directly bind to the promoter of Tbx5a gene and promotes H3K4 trimethylation, leading to the transcriptional activation of Tbx5a.

DISCUSSION

By assembling various CRL4 E3 ubiquitin ligase complexes, CUL4 participates in the regulation of a broad spectrum of biological processes. Studies done on CUL4A and CUL4B, the two mammalian CUL4 paralogs, showed that CUL4A and CUL4B may each form their own ubiquitin ligase complexes that target different substrates for proteosomal degradation or for protein modification (14,35,36). Loss-of-function mutations in CUL4B lead to impaired cognitive development, absence of speech, short stature and other abnormalities in humans. However, while constitutive Cul4b knockout mice are embryonic lethal (12,13), conditional Cul4b KO mice in which Cul4b function is only retained in extraembryonic tissues exhibit relatively mild phenotypes (13,37). Cul4a knockout mice are even less affected (22–24). To determine the respective roles of cul4a and cul4b in zebrafish development, we knocked down cul4a and cul4b, respectively, in zebrafish by the morpholino and characterized the phenotypes of the cul4a and cul4b knockdown morphants. We found, to our surprise, that the phenotypes of cul4a morphants are drastically different from those of cul4b morphants. While cul4a morphants were severely impaired in their cardiac development and pectoral fin morphogenesis, cul4b morphants exhibited no obvious phenotypes, indicating that cul4a is functionally more important than cul4b in zebrafish. Interestingly, we found that the phenotypes of cul4a morphants could be rescued by human CUL4B mRNA, but not by zebrafish cul4b mRNA. Together, these findings suggest that the functions of cul4 paralogs are varied in different species.

We showed that while the chamber specification and initial differentiation were unperturbed, the cul4a morphants failed to undergo heart looping. The cardiac cells appeared to be scarcer along the ventricular and atrial walls of cul4a morphants than in the control. The scarcity of cardiac cells is probably due to their reduced rate of proliferation. The cul4a morphants were also affected in their pectoral fin development. As in the developing hearts of cul4a morphants in which the proliferation of myocar- dial cells was reduced, the regions that would give rise to the buds of pectoral fins in cul4a morphants contained much fewer proliferating cells. Thus, both the cardiac and the forelimb defects are likely to be caused by an insufficient supply of cells that would constitute the organs.

Transcription factor Tbx5a has been well documented to play a critical role in cardiac development and limb morphogenesis (33). Mutations in TBX5a cause Holt-Oram syndrome that is characterized by morphological defects in heart and forelimb, and progressive cardiac conduction disease (38). Tbx5a deficiency in mammalian and zebrafish models recapitulates the cardiac and limb phenotypes of Holt-Oram syndrome (32,33,39). Tbx5a gene depletion could block cell cycle progression and result in reduced cardiac cell number (40,41). Interestingly, hyperactivity of Tbx5a also results in atrioventricular canal malformation and heart looping failure (42). These findings suggest that a tight regulation of Tbx5a activity is required for the proper formation of atrioventricular canal. We herein identified cul4a as a novel positive regulator of tbx5a. Because the cardiac and forelimb phenotypes of cul4a morphants could be rescued by co-injection of tbx5a mRNA, we conclude that cul4a contributes to atrioventricular canal formation by upregulating tbx5a.

The absence or shortening of pectoral fins in cul4a morphants is reminiscent of the limb phenotypes caused by thalidomide (25). Knockdown of Crbn, which functions as a substrate receptor for CRL4A and is targeted by thalidomide, results in similar phenotypes (25). The observations we made with cul4a morphants suggest that thalidomide may also cause cardiac defects in zebrafish. It is possible that thalidomide may exert its teratogenic effect on heart and forelimb via disruption of the cul4a–tbx5a pathway.
How *cul4a* upregulates the expression of *tbx5a* remains to be further investigated. We previously demonstrated that CRL4B complexes can catalyze the monoubiquitination of H2AK119 and coordinate with PRC2 to repress various target genes (14). In contrast, CRL4A was shown to promote the expression of *p16* in mammalian cells (34). Consistent with the reported
positive regulation of gene expression by CRL4A, we found that Cul4a directly binds to the promoter of Tbx5a gene and promotes H3K4me3, a histone mark of active transcription, in rat myocar-
dial cells. Development of new tools and agents suitable for study of epigenetic regulation of zebrafish genes may help shed light on the mechanistic link between cul4a and tbx5a. The important function of cul4a in cardiovascular development in zebrafish as demonstrated here also suggests that efforts should be made to explore whether germline mutations in CUL4A are associated with human congenital heart diseases.

MATERIALS AND METHODS

Zebrafish maintenance and morpholino oligonucleotide injections

Zebrafish (Danio rerio) embryos were obtained by natural spawning of wild-type TU strain. Embryos were raised and maintained at 28.5°C in system water. Morpholinos (Gene Tools LLC, OR, USA) were designed to target the splicing site of cul4a ( cul4a-MO1: 5′-CTTGGGATCTGCTGTAACACAC AGA-3′) and cul4b ( cul4b-MO1: 5′-CTAGTTTTATTATAGCA CGCACCTGA-3′). A second cul4a morpholino, cul4a-MO2: 5′-GCTGGGTGCTGAACATCTTCTGCCAT-3′, was designed to target the 5′-UTR of cul4a and thus to block its translation. Morpholino cul4b-MO2: 5′-TCGCTAATCTACTAATGCTAC-3′, similarly targets the 5′-UTR of cul4b. The control MO (cul4a-CMO: 5′-CTAGCGTCTCTCTCTACACACACA-3′; cul4b-CMO: 5′-TCCTTTACTACATTCTACCTAGT-3′) contains five mismatched bases with cul4a-MO1 and cul4b-MO1, respectively. A BLAST search indicated that the morpholino-targeted sequences are unique in the zebrafish genome. All morpholinos were diluted to 0.0625 mM in sterile water. Fertilized targeted sequences are unique in the zebrafish genome. All mor-

Quantitative real-time PCR

The total RNA was extracted from 32 hpf embryos or H9C2 cells using the Trizol reagent (Invitrogen Technologies, USA) according to the manufacturer’s protocol. First-strand cDNA was reverse transcribed using random primer and RevertAID Reverse transcriptase (Fermentas). The cDNA was analyzed immediately or stored at −20°C. Quantitative-PCR was performed by Roche 480 real-time PCR system. The sequence-specific primers were designed using Primer 5 software. The SYBR green (Roche) method was used to quantify cDNA. GAPDH was used as control to confirm similar amount of starting cDNA from all stages tested. The relative expression levels of other genes were computed with respect to the amount of GAPDH in each sample by dividing the amount of other genes. Specificity of each reaction was controlled by melting curve analysis. Primers: zfcul4a forward: TAGCGTTTGGTG TATCGCTATTAT, reverse: TGTCAGCTTGGGTCTGTCT TTA; zfbtx5a forward: ACACCTTGGCTCCTCAG GAAACT, reverse: TTTGTCCACAGCTCTCGCT, zfbmp2a forward: CACTCAGTGTGTTTTTGCGG, reverse: CTGAGCTGGA CGATCCTC; zhtdforward: GCAAAGAAGAGAGG GAAA, reverse: GCCGTATGCTGTGTTGT; ztfgf10 forward: CTCTACGTGCTGCGTGGT, reverse: GTCAATGC CGAAATCCCTC; zbeta-actin forward: CGAAGTTGCTT TCCACAGCAGCT, reverse: GAAGAGGTGGCTGTG CGT; ztbx5a forward: AGCAGGAGCGCCCTAA, reverse: CCGCTGGGCTAGCCTGTG.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed with digoxigenin-labeled RNA probes essentially as described (45). At least 10 embryos were used in each assay. All in situ hybridizations were repeated at least three times. The cardiac myosin light chain 2 (cmlc2) riboprobe was a gift from Dr. David Kimelman (University of Washington), and ventricular myosin heavy chain (vmhc) riboprobe was a gift from Dr Deborah Yelon (New York University School of Medicine). The antisense probes for tbx5a and cul4a were obtained by RT-PCR, the primers are: zfcul4a probe forward: GCGGGGCCTCCCG GGCTTTTTATT; reverse: GCGCTGACTGCTGTA TGAGTGTTG; zfbtx5a probe forward: GGGCCCGAGCA CCGGGAGAAACC, reverse: CGCGAATTCGTCTCCAC CCTTCTCTGCT.

Histology and immunohistochemistry

Histological staining (Haematoxylin and eosin, H&E) on paraf-
fin (4 μm) sections was performed as previously described (43). Whole-mount immunohistochemistry of zebrafish heart was carried out using S46 and MF20 antibodies (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA) as described (44). Sections of zebrafish heart were dewaxed, rehy-
drated and stained with antibodies against PCNA (Santa Cruz).
Western blotting

Embryos were homogenized on ice in lysis buffer. Western blotting was performed as described (4). The primary antibodies used included anti-β-actin antibody (Sigma), anti-thb5a antibody (AVIVA), anti-Cul4a antibody (Abcam) and anti-Gapdh antibody (Sigma).

RNA interference

Small interfering RNA (siRNA) duplexes were used to knock down Cul4a in H9C2 cells. The Cul4a and negative control siRNA duplexes were purchased from GenePharma, and the oligonucleotide sequences were as follows: cul4a, 5'-GAAGUCAUCUUCATT-3'; negative control, 5'-UUCUCGCGAGAACGU-3'.

ChIP assay

ChIPs were performed as described previously (14). Briefly, cells were mechanically dissociated by pipetting and 1 × 10^7 cells were cross-linked with 1% formaldehyde, sonicated, pre-cleared and incubated with 5 μg of antibody per reaction. Complexes were washed with low and high salt buffers, and the DNA was extracted and precipitated. The enrichment of the DNA template was analyzed by PCR using primers specific to Tbx5a promoter. The sources of the antibodies were: anti-Cul4a (Abcam) and anti-H3K4me3 (Millipore). Protein A/G Sepharose CL-4B beads were from Amersham Biosciences, and protease inhibitor mixture cocktail was from Roche Applied Science. The primers used were: rat Tbx5a promoter fragment a forward: CCCTACTAACCCCCCTCTTG, reverse: GCTGGCTACTCAAAACTTGC; fragment b forward: ATCTGGGACTGATTTAGGC, reverse: CGGAGAATAAAGGATAGTAAT; fragment c forward: GC TGGGAAGAGGAGAATAAAGGATAGTAAT; fragment d forward: TTTCTACCCGATGGCTTCC, reverse: GTTTGACCCCTCACTCCA.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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