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# Caldesmon is essential for cardiac morphogenesis and function: *In vivo* study using a zebrafish model

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ABSTRACT

The zebrafish homologue of caldesmon is similar to the mammalian low molecular weight caldesmon (*l*-CaD). In this study, we explored the effects of caldesmon knockdown on vertebrate heart development *in vivo*. In a zebrafish model caldesmon was knocked down resulting in defective cardiac morphogenesis, muscularization and function. The data provide the first functional assessment of the role of caldesmon in cardiac development *in vivo*, and indicate that caldesmon is essential for proper cardiac organogenesis and function. Because caldesmon expression remarkably influences cardiac muscularization, the findings are relevant for designing future therapeutic strategies in the regeneration of cardiac damage.



Cardiac development is susceptible to anomalies because of the precisely coordinated cellular proliferation, migration, differentiation and the complex morphogenetic interactions implicated. Caldesmon (CaD) is an actin-binding protein implicated in the cytoskeletal organization and various signaling pathways [1]. In previous studies we found that low-molecular weight caldesmon (*l*-CaD) is associated with the development of tumor vasculature [2–7]. Because of the essential role of this protein in neoplastic angiogenesis and vasculogenesis, we set out to explore the *in vivo* effects of caldesmon on the normal development of the cardiovascular system. The zebrafish homologue is similar to mammalian low-molecular weight caldesmon (*l*-CaD) as reported at the website: *Caldesmon: Orthologs: UCSD-Nature Molecule Pages*. In the present study CaD was knocked down in zebrafish and the effects on cardiovascular development were explored at the anatomical and functional level. The zebrafish model is advantageous for the study of abnormal cardiovascular development because zebrafish embryos can survive for several days without a proper functioning heart. The analysis of morphants with compromised or absent cardiac function is, therefore, possible for a significant period of time [8]. In contrast, a similar phenotype in a mouse model would result in early lethality and embryo reabsorption. In addition, the molecular mechanisms steering cardiac patterning of zebrafish are similar to those of the more complex, higher vertebrates [9].

All vertebrate hearts, including that of zebrafish, develop from a simple tube [8,10], which bends and twists (loops) rightward to create the basic plan of the mature heart [11]. The zebrafish heart consists of only one atrium and one ventricle. Two additional smaller compartments are the sinus venosus (SV) and the bulbus arteriosus (BA). By 36 hpf the looping of the zebrafish heart is completed and each compartment displays a distinct contractile pulsing. By 5 dpf, the zebrafish heart is essentially in the adult teleost configuration. The four compartments are now separated from one another by three sets of fully functional valves [10]. The most distinct and functionally important compartments are the atrium and ventricle [8]. At this stage, the ventricle has developed an extensive trabeculation. In each compartment, the myocardium is about one cell layer thick except for the ventricle, which consists of two to three cell layers. The heart is enclosed in the pericardial cavity with paired pericardial muscles running in caudo-cranial direction and is covered by an epicardial membrane.

In this report, we characterized the aberrant phenotype of the developing heart in the CaD-knockdown morphants (CaD-MOs).

## Material and methods

*Animal model, morpholino injections and the verification of the specificity.* The use of the zebrafish embryos was approved by the institutional review board for experimental animals. The caldesmon anti-sense morpholino oligonucleotides (MO1) 5-AGTAA AGTCTCTTATTCTTCAACGC-3 and (MO2) 5-TAAGAGTTCATCCTGT AGAGTGATG-3, designed to inhibit translation of the caldesmon

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RNA (Gene: ENSDARG00000032052; Transcript: ENSDART00000067366; Translation: ENSDARP00000067365), and a 5-base mismatch control 5-AGAAAACTCTCTTATTGTTGAAGGC-3 were purchased from Gene Tools (Philomath, OR). First, in a titration experiment, the MOs were injected into the yolk sac of zebrafish embryos between one- and two-cell stages at different concentrations (2, 4 and 8 ng/embryo) and the fishes were raised at 28.5 °C until analysis under standard laboratory conditions. The concentration (4 ng/embryo) was used in all of the subsequent experiments, because survival of the embryos was satisfactory (>86%).

For translation blocking morpholinos (MO), there are several approaches to determine whether a phenotype is the result of knocking down for a gene-of-interest: (1) quantification of the target protein by an antibody (check if the translation is blocked); (2) RNA rescue experiments; (3) a control morpholino with 5-base mismatch (see above); (4) designing a second MO (see above). In this study, we verified the specificity of the MO phenotype by all approaches mentioned (Supplemental material).

*Whole-mount immunofluorescence and phalloidin staining.* Briefly, embryos were fixed in 4% PFA at RT for minimal 3 h following standard procedures. Embryos were treated with 1 M $NH_4Cl$ at RT for 3 h to quench autofluorescence, permealized, blocked by 5% goat serum in PBST for 1 h at RT and incubated with anti-tropomyosin (Lab vision) at dilution 1: 100 for 2–3 overnights at 4 °C. Double labelling of phalloidin-TRITC (Sigma–Aldrich) at 50 μg/ml and anti-actinin (Sigma–Aldrich) at 1: 200 was performed and pretreated as above. After post-incubation washing, embryos were incubated with rhodamine-conjugated goat-anti-mouse or FITC-conjugated goat-anti-mouse (Jackson ImmunoResearch Laboratories, Inc.) at dilution 1:100 for 2 overnights at 4 °C. After thoroughly washing, fluorescent images were recorded by fluorescence microscopy and/or confocal laser scanning microscopy. Embryos at identical developmental stages, processed without primary antibody, were used as controls for each experiment. 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining.

*Parameters for assessment of the cardiac structure and function.* The parameters for cardiac structure include the anatomy of the atrioventricular valve (AVV), looping of the cardiac tube, chamber formation and array of the cardiomyocytes in fixed embryos at whole mount and section level. The parameters for assessing the cardiac function involve contractility, rhythm and blood regurgitation in living embryos.

## Results and discussion

### *Confirmation of the specificity of CaD-knockdown*

Whole mount immunostaining, whole mount ELISA and dot blot assay indicated that the targeted translation is blocked. The insignificant penetrance (~5%) of the 5-base mismatch control, and the same effects derived from the second morpholino are additional evidences for the specificity. The specificity was further confirmed by the RNA rescue experiments. Co-injection of the ATG MOs with the RNA resulted in a significant rescue (89%, $n = 66$, $p < 0.001$). The morpholino phenotype was remarkably improved (Fig. 1B3).

### *CaD-knockdown affecting cardiac morphogenesis*

The most frequently encountered cardiac phenotypes of the CaD-MOs included defective cardiac looping, ill-defined AV boundaries and impaired chamber formation (Table 1). Looping is a vital event during heart development as even relatively minor looping perturbations can lead to serious structural defects [11]. Cardiac looping in CaD-MOs was impaired as reflected by elongation of the ventricles; elongation or enlargement of atria resulting in significant longer SV-BA distances (Fig. 1B2, F1 and H1) and loss of the normal orientation of the heart tube. Enlarged fluid-filled pericardia (pericardial edema) and severely thinned cardiac walls were often seen (Fig. 1F1 and H1). The phenotypes observed in early stages were still present in 5 dpf CaD-MOs.

Growth of the heart is caused by hypertrophy of individual cells and multiplication (hyperplasia) of cardiomyocytes. Hence, hypoplasia of the cardiac chamber walls in CaD-MOs is indicative of defective proliferation (number of cells) and/or underdevelopment of the cardiomyocytes. Visualization of the cardiomyocytes was performed by whole mount immunohistochemistry of tropomyosin (TM) [10], actinin [12] and phalloidin (for visualization of actin filament). The expression of TM (Fig. 1F2), actin (Fig. 1D2) and actinin (Fig. 1D3) was significantly reduced or absent in the hearts of the CaD-MOs, compatible with underdevelopment and/or a reduced number of cardiomyocytes. In the human *CALD1* gene there are two major functional domains: N-terminal myosin-binding domains and C-terminal calmodulin/tropomyosin/actin-binding domains, which are conserved in each isoform [13–15]. The functional domains are highly conserved within the zebrafish homologue (Supplemental Table). Each cardiomyocyte contains a bundle of myofibrils divided into contractile units/apparatus, or sarcomeres, consisting of several contractile proteins in their skeleton which provide the contractile force [16,17]. Actin and tropomyosin are essential components of the thin filaments [12,18]. Alpha-actinin is an actin-binding protein and one of the major structural elements of the Z-disc [19], which interacts with tropomyosin [20]. These various proteins are mutually binding and form complexes. Reduced expression of one of them causes instability or degradation of the others, resulting in disassembly of the complex of α-actin, α-actinin and tropomyosin (impaired contractile apparatus). The intactness of the protein complex is important to provide structural stability required for proper alignment of thin filaments and for force transmission during contraction. In the CaD-MOs unstable and short myofilaments (disassembly of sarcomeres) were evidenced by reduced or absent expression of these proteins (Fig. 1D2, D3 and F2), underlying the inability of effective contraction of the morphant heart. Generally, the cardiac development up to the stage of chamber specification is still retained in the CaD-knockdown, suggesting that CaD plays a role in cardiac development at a later stage.

### *CaD-knockdown causing cardiac dysfunction*

The functional defects included reduced contractility (wave-like swaying with limited ventricular contraction), abnormal rhythm and blood regurgitation (Table 1). The heartbeat/min of 5 dpf WT is 206 ± 5 and 3 dpf WT 190 ± 6 at room temperature (22 °C). The heartbeat/min in the corresponding CaD-MOs had decreased dramatically (Table 1). The abnormal rhythm manifests as cardiac tremor or chaotic cardiac contraction (fibrillation), resulting in defective circulation. In many morphants a retrograde flow with simultaneously diminished forward flow was noticed. At the molecular level the functional deficiency of the cardiac contractility observed in CaD-MOs was associated with disturbances of the sarcomere assembly. Backflow of the bloodstream may be caused by perturbed development of the valve leaflets in CaD-MOs. The atrioventricular valve (AVV) boundary appeared to be missing or was ill-defined in the CaD-MOs, indicative of defective formation of the valve (Fig. 1A2). The enlargement or dilatation of the chambers observed in many animals can also be taken responsible for the blood backflow.

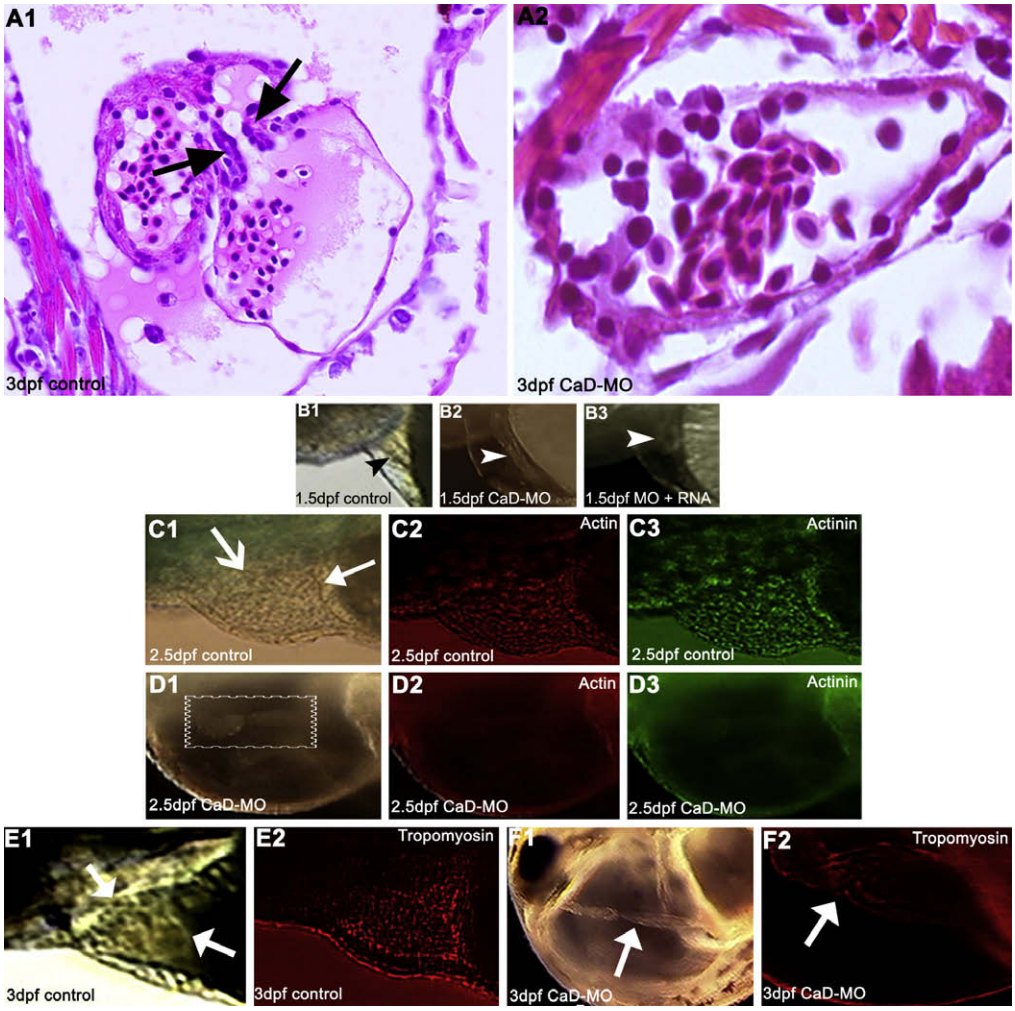


**Fig. 1.** Defective AVV, cardiac looping and muscularization of CaD-knockdown. (A1) The AVV as revealed (arrows) in an HE stained section of a 3dpf control embryo. (A2) The AVV is missing in a CaD-MO. (B1) A normal heart is shown in 1.5dpf control (arrowhead). (B2) A long strip heart (arrohead) is shown without looping and with severe pericardial edema in a CaD-MO. (B3) The morpholino phenotype of heart as observed in B2 is significantly improved (arrowhead) by the RNA rescue experiment. (C1 to C3) A normal heart in a 2.5 dpf control in C1 (arrows), which is double labelled with phalloidin (actin) and actinin. (D1 to D3) A morphant heart in D1 (square), which is double labelled as C2 and C3. Loss of the signals of actin and actinin with severe pericardial edema is seen. (E1) A normal heart in a 3 dpf control (arrows). (E2) The expression of tropomyosin (TM) outlines the normal heart. (F1) A heart without looping (arrow) with severe pericardial edema is noticed in 3 dpf CaD-MO. (F2) Loss of TM signal of the hypoplastic heart with severe pericardial edema (arrow). B1 to F2: whole mount embryos, anterior to left and dorsal is up. dpf = day post-fertilization.

**Table 1**
CaD-knockdown cause cardiac dysfunction and affect morphogenesis.

| Functional phenotypes | Frequency (%) | Structural phenotypes | Frequency (%) |
|---|---|---|---|
| Reduced contractivity | | Defective AVV | 62 |
| Wave-like swaying/ fibrillation | 86 | | |
| Bradycardia | | Defective looping | 86 |
| Slow: 60–160 beats/min | 62 | | |
| Barely: <60 beats/min | 33 | Defective/thin chambers | 78 |
| Regurgitation | 68 | | |
| Total embryos = 106 | | | |

In conclusion, CaD is essential for normal vertebrate cardiac looping, chamber formation, muscularization and proper cardial function. Our findings add novel information to the CaD functions. The obvious contribution of CaD to cardiac muscularization offers clues for future therapeutic strategies in regeneration of cardiomyocytes.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.10.165.

## References

[1] P.A. Huber, Caldesmon, Int. J. Biochem. Cell Biol. 29 (1997) 1047–1051.
[2] P.P. Zheng, T.M. Luider, R. Pieters, C.J. Avezaat, M.J. van den Bent, P.A. Sillevis Smitt, J.M. Kros, Identification of tumor-related proteins by proteomic analysis of cerebrospinal fluid from patients with primary brain tumors, J. Neuropathol. Exp. Neurol. 62 (2003) 855–862.
[3] P.P. Zheng, A.M. Sieuwerts, T.M. Luider, M. van der Weiden, P.A. Sillevis-Smitt, J.M. Kros, Differential expression of splicing variants of the human caldesmon gene (CALD1) in glioma neovascularization versus normal brain microvasculature, Am. J. Pathol. 164 (2004) 2217–2228.
[4] P.P. Zheng, M. van der Weiden, J.M. Kros, Differential expression of Hela-type caldesmon in tumour neovascularization: a new marker of angiogenic endothelial cells, J. Pathol. 205 (2005) 408–414.
[5] P.P. Zheng, W.C. Hop, P.A. Sillevis Smitt, M.J. van den Bent, C.J. Avezaat, T.M. Luider, J.M. Kros, Low-molecular weight caldesmon as a potential serum marker for glioma, Clin. Cancer Res. 11 (2005) 4388–4392.
[6] P.P. Zheng, M. van der Weiden, J.M. Kros, Hela l-CaD is implicated in the migration of endothelial cells/endothelial progenitor cells in human neoplasms, Cell Adhes. Migration 1 (2007) 84–91.

[7] P.P. Zheng, M. Weiden, P.A. Sillevis Smitt, T.M. Luider, J.M. Kros, Hela /-CaD undergoes a DNA replication-associated switch in localization from the cytoplasm to the nuclei of endothelial cells/endothelial progenitor cells in human tumor vasculature, Cancer Biol. Ther. 6 (2007) 886–890.
[8] D.Y. Stainier, M.C. Fishman, Patterning the zebrafish heart tube: acquisition of anteroposterior polarity, Dev. Biol. 153 (1992) 91–101.
[9] B.M. Weinstein, M.C. Fishman, Cardiovascular morphogenesis in zebrafish, Cardiovasc. Res. 31 (Spec No) (1996) E17–24.
[10] D.Y. Stainier, R.K. Lee, M.C. Fishman, Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation, Development 119 (1993) 31–40.
[11] L.A. Taber, Biophysical mechanisms of cardiac looping, Int. J. Dev. Biol. 50 (2006) 323–332.
[12] M. Seeley, W. Huang, Z. Chen, W.O. Wolff, X. Lin, X. Xu, Depletion of zebrafish titin reduces cardiac contractility by disrupting the assembly of Z-discs and A-bands, Circ. Res. 100 (2007) 238–245.
[13] K. Sobue, J.R. Sellers, Caldesmon, a novel regulatory protein in smooth muscle and nonmuscle actomyosin systems, J. Biol. Chem. 266 (1991) 12115–12118.
[14] K. Hayashi, Y. Fujio, I. Kato, K. Sobue, Structural and functional relationships between h- and l-caldesmons, J. Biol. Chem. 266 (1991) 355–361.
[15] C.L. Wang, J.M. Chalovich, P. Graceffa, R.C. Lu, K. Mabuchi, W.F. Stafford, A long helix from the central region of smooth muscle caldesmon, J. Biol. Chem. 266 (1991) 13958–13963.
[16] S.D. Bird, P.A. Doevendans, M.A. van Rooijen, A. Brutel de la Riviere, R.J. Hassink, R. Passier, C.L. Mummery, The human adult cardiomyocyte phenotype, Cardiovasc. Res. 58 (2003) 423–434.
[17] S. Kostin, S. Hein, E. Arnon, D. Scholz, J. Schaper, The cytoskeleton and related proteins in the human failing heart, Heart Fail. Rev. 5 (2000) 271–280.
[18] R.W. Zajdel, M.D. McLean, S.L. Lemanski, M. Muthuchamy, D.F. Wieczorek, L.F. Lemanski, D.K. Dube, Ectopic expression of tropomyosin promotes myofibrillogenesis in mutant axolotl hearts, Dev. Dyn. 213 (1998) 412–420.
[19] Y. Chan, H.Q. Tong, A.H. Beggs, L.M. Kunkel, Human skeletal muscle-specific alpha-actinin-2 and -3 isoforms form homodimers and heterodimers in vitro and in vivo, Biochem. Biophys. Res. Commun. 248 (1998) 134–139.
[20] S. Puszkin, E. Puszkin, J. Maimon, C. Rouault, W. Schook, C. Ores, S. Kochwa, R. Rosenfield, alpha-Actinin and tropomyosin interactions with a hybrid complex of erythrocyte-actin and muscle-myosin, J. Biol. Chem. 252 (1977) 5529–5537.