Developmental Cell *14*

Supplemental Data

Vertebrate CASTOR Is Required for Differentiation of Cardiac Precursor Cells at the Ventral Midline

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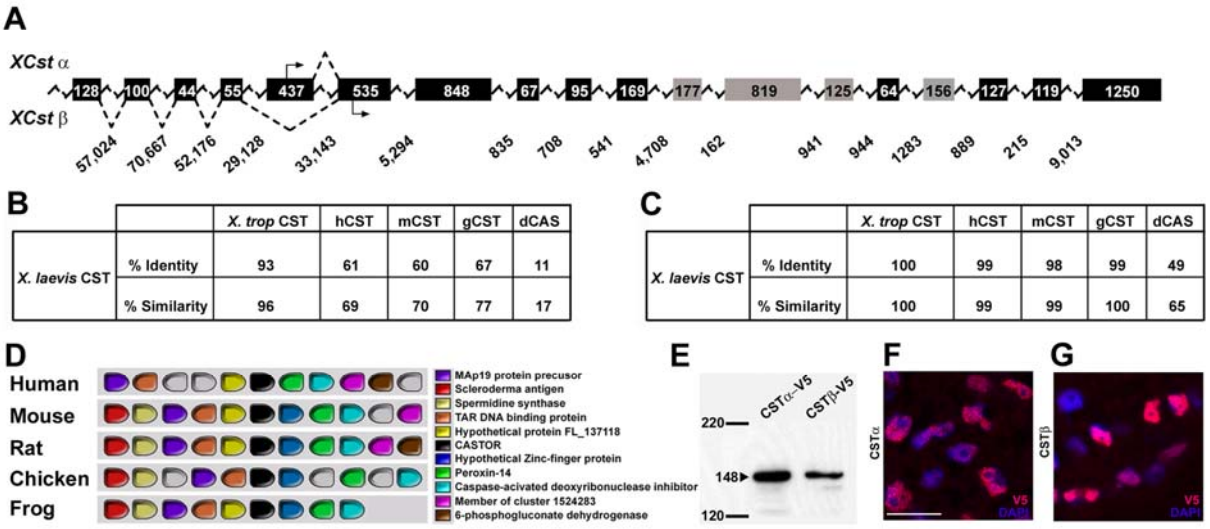

Figure S1. **Identification and Characterization of *Xenopus* CST**
(A) Predicted genomic locus structure of *Xenopus Cstα* and *Cstβ* (5' to 3', not to scale). Exons are shown in boxes with the corresponding size given in basepairs. Exons in gray depict those containing the zinc finger repeats, and the sizes of intervening introns are indicated beneath each intron. Alternative splicing of the 5' regions is also indicated. (B,C) Table showing the evolutionary conservation of the full length CST amino acid sequence (B) and the zinc finger repeats (repeats 1-4) (C). The percentage of identical amino acids (identity) and the percentage of conservative substitutions (similarity) are given for comparison between CST proteins of *X. tropicalis*, hCST (human), mCST (mouse), predicted gCST (chicken), and dCAS (*Drosophila*). (D) Syntenic relationship between vertebrate *Cst* genomic loci using Metazome blast analysis. (E) Western blot analysis of CSTα-V5 and CSTβ-V5 *in vitro* translation. Both CST proteins run at the predicted size of 148 kDa. (F,G) CSTα and CSTβ cellular localization. Transverse confocal images of CSTα-V5 and CSTβ-V5 injected Xenopus embryos at Stage 32. Histological sections were stained with a V5 antibody (Red) to visualize the CST proteins and with DAPI (Blue) to identify the nucleus. Scale bar indicates 10μm.

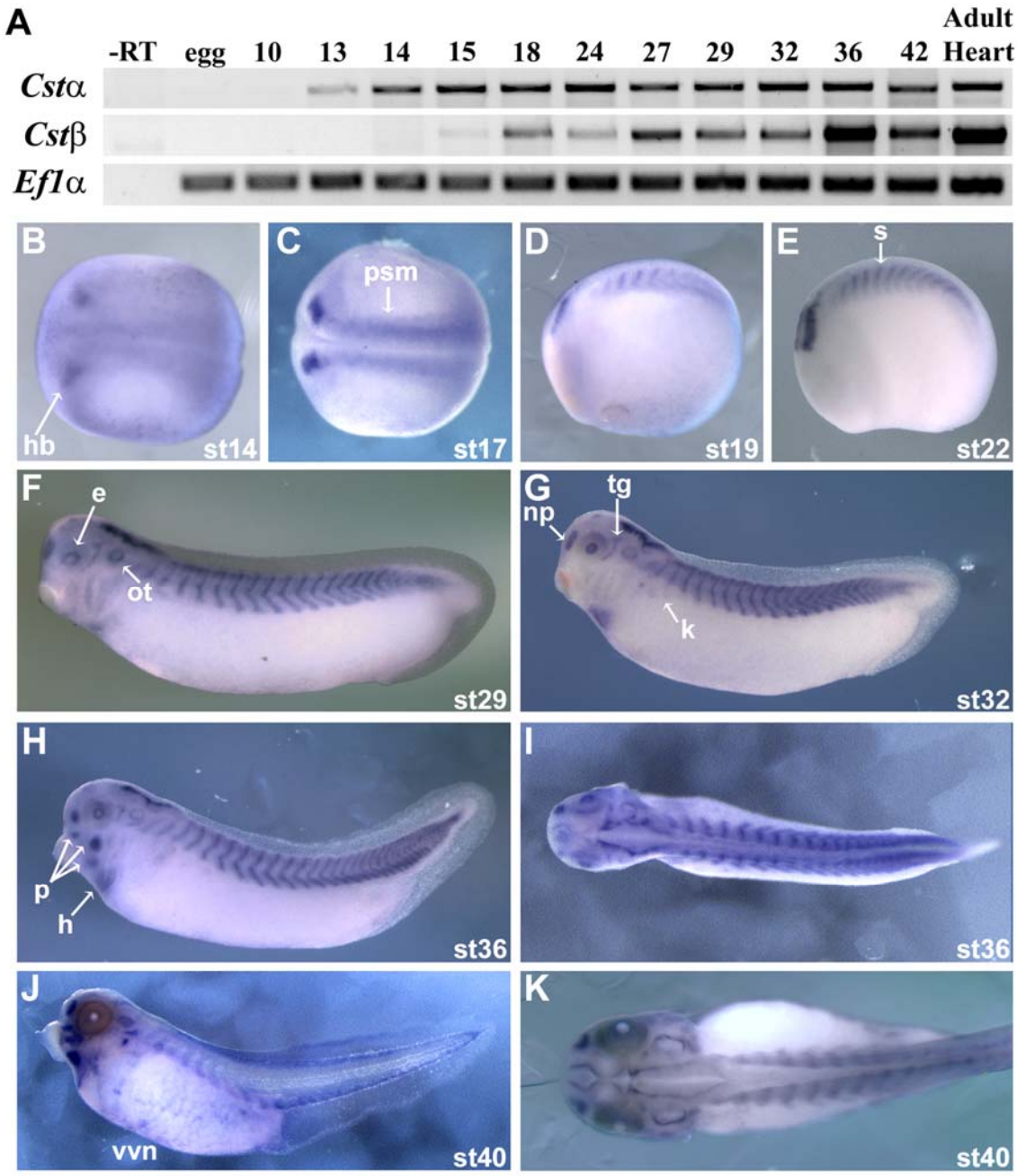

Figure S2. *Xenopus laevis Cst* Developmental and Spatial Expression
(A) Developmental time course of *X. laevis Cstα* and *Cstβ* expression by RT-PCR of egg lysate (maternal transcripts), whole embryo ranging from Stage 10 (gastrulation) to Stage 42 (late tadpole), and adult heart. *Ef1α* is used as a positive control. (B-K) Whole mount *in situ* hybridization of Stage 14 (neurula) to Stage 40 (tadpole) embryos using a *Cst*-specific probe common to both *Cstα* and *Cstβ*. B,C are dorsal views with anterior to the left. D-H, and J are lateral views with anterior to the left: hindbrain (hb), presomitic mesoderm (psm), somites (s), heart primordium (hp), eye (e), otic vesicle (ot), nasal placode (np), trigeminal ganglion (tg), facial placodes (p), heart (h), kidney (k), vascular vitelline network (vvn).

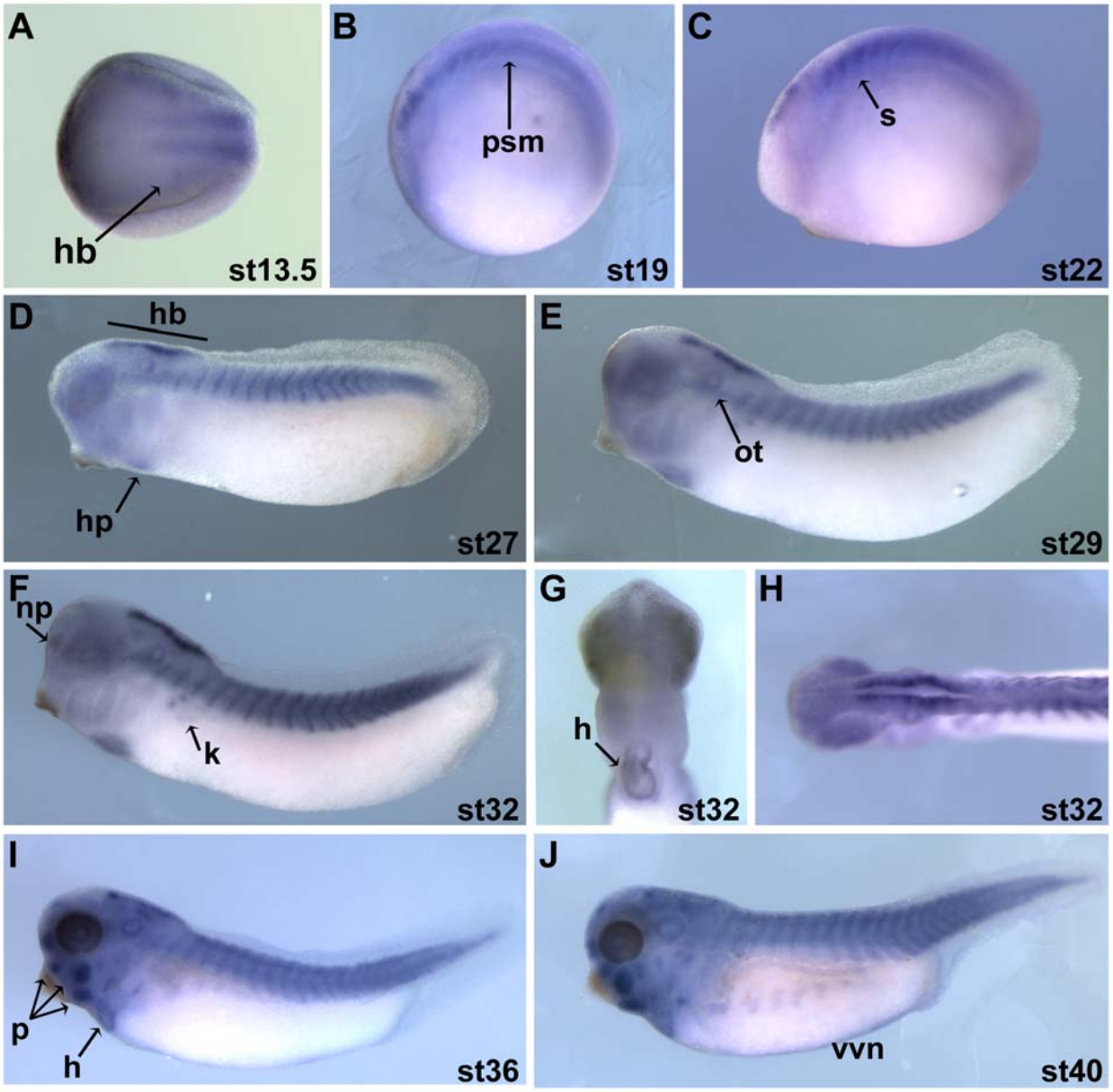

Figure S3. ***Xenopus tropicalis Cst*** **Spatial Expression**
Whole mount *in situ* hybridization of *Cst* in *X. tropicalis* of Stage 14 (neurula) to Stage 40 (tadpole) embryos using a Cst-specific probe common to both *Cstα* and *Cstβ*. A is an anterior view. B-F, I-J are lateral views with anterior to the left. G is a ventral view with anterior to the top. H is a dorsal view with anterior to the left. hindbrain (hb), presomitic mesoderm (psm), somites (s), heart primordium (hp), otic vesicle (ot), nasal placode (np), facial placodes (p), heart (h), kidney (k), vascular vitelline network (vvn).

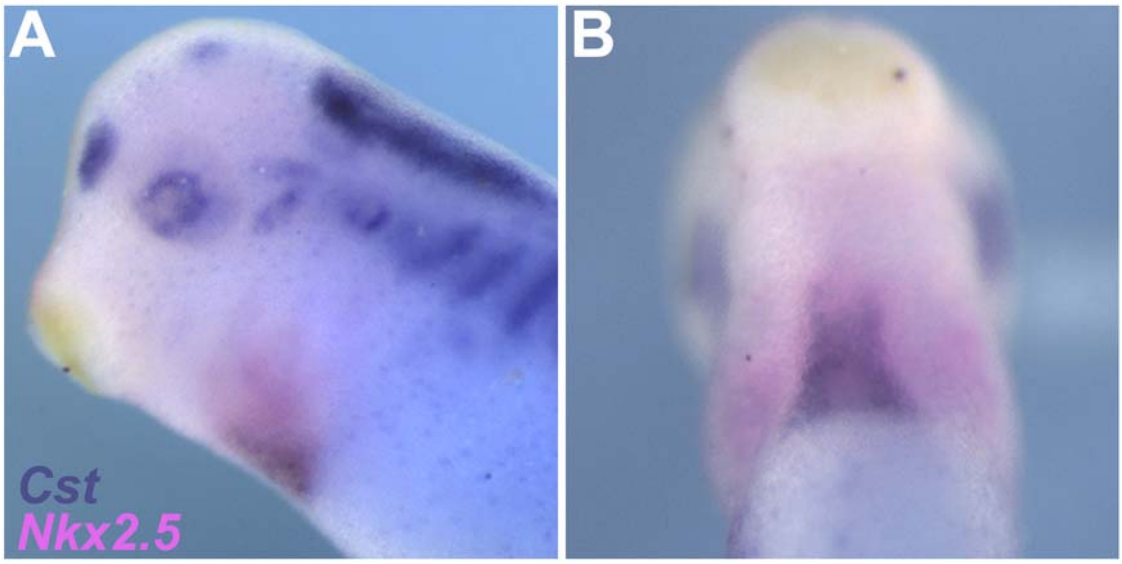

Figure S4. *Xenopus Cst* Is Expressed throughout the Linear Heart Tube
Whole mount double *in situ* hybridization of Stage 29 embryos using a *Nkx2.5*-specific probe (pink) to mark the cardiac field and a *Cst*-specific probe (blue). Left panel is a lateral view with anterior to the left. Right panel is a ventral view with anterior to the top.

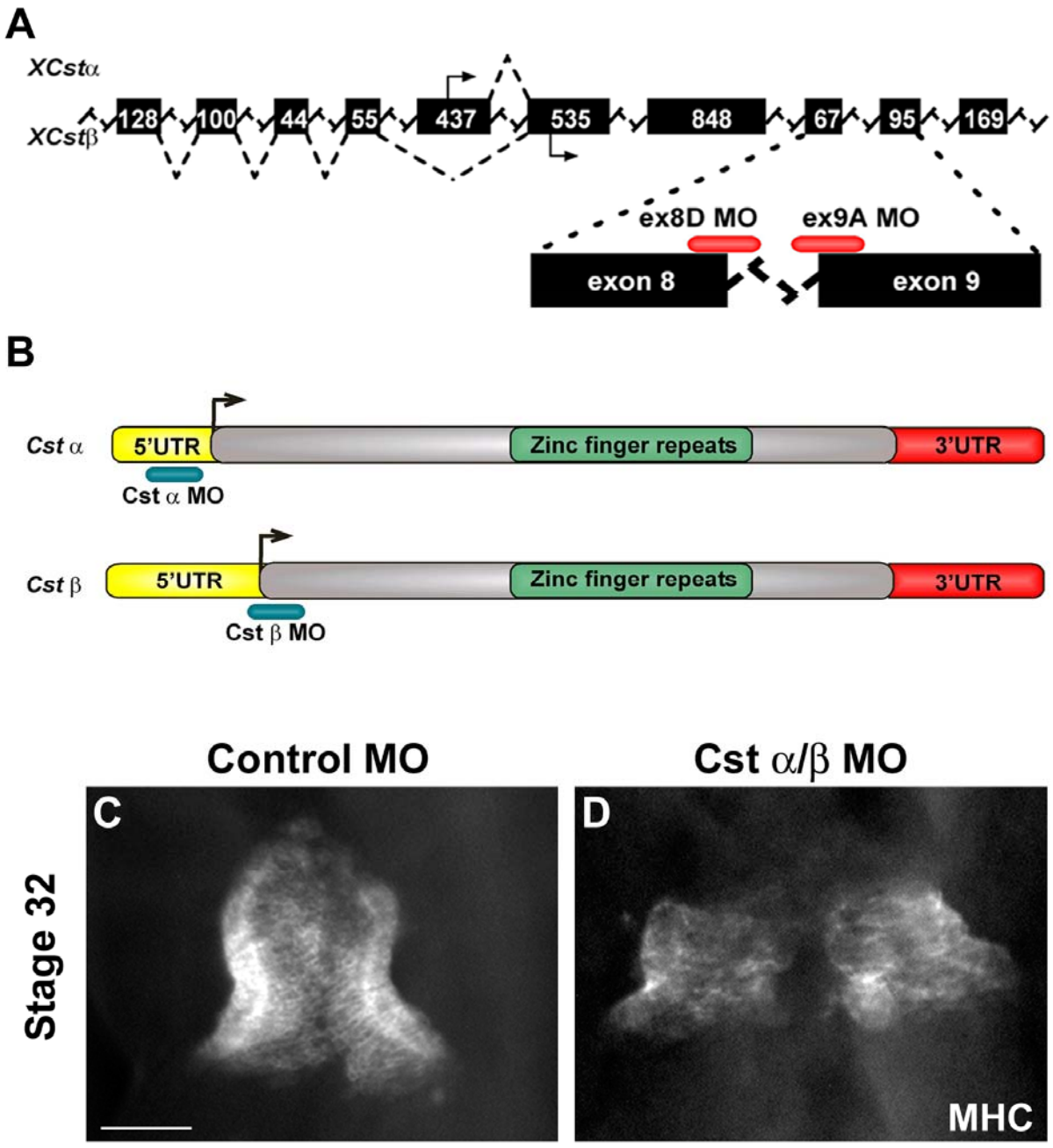

Figure S5. Morpholino Design and Phenotype
(A) Position of the *Cst* splice junction morpholinos relative to the pre-mRNA transcripts targeting the donor the exon 8 (ex8D MO) and the acceptor of exon 9 (ex9A MO), referred collectively as CstMO. (B) Position of the *Cst*-5′ UTR morpholinos (red) relative to the *Cstα* and *Cstβ* cDNA transcripts. (C-D) Whole mount antibody staining with anti-MHC of Stage 32 control MO (C) and Cstα/β MO (D) embryos (ventral view) indicating an identical cardia bifida phenotype is obtained with both the CstMO (splice MO) as the Cstα/β MO (5′ UTR MO). Scale bar: C = 100 μm.

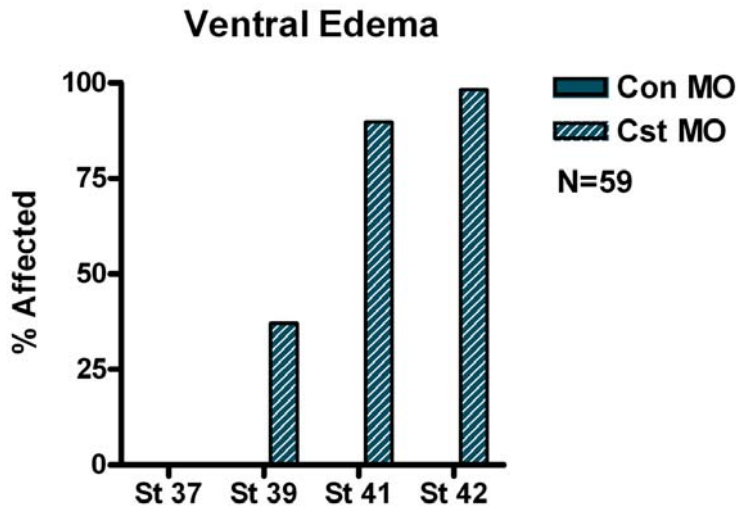

Figure S6. Statistics of Ventral Edema in CST-Depleted Embryos
Distribution of incidences of ventral edema in control and CST-depleted embryos from Stage 37 to Stage 42. Graph represents one batch of 56 embryos. Analysis was performed with two independent experiments.

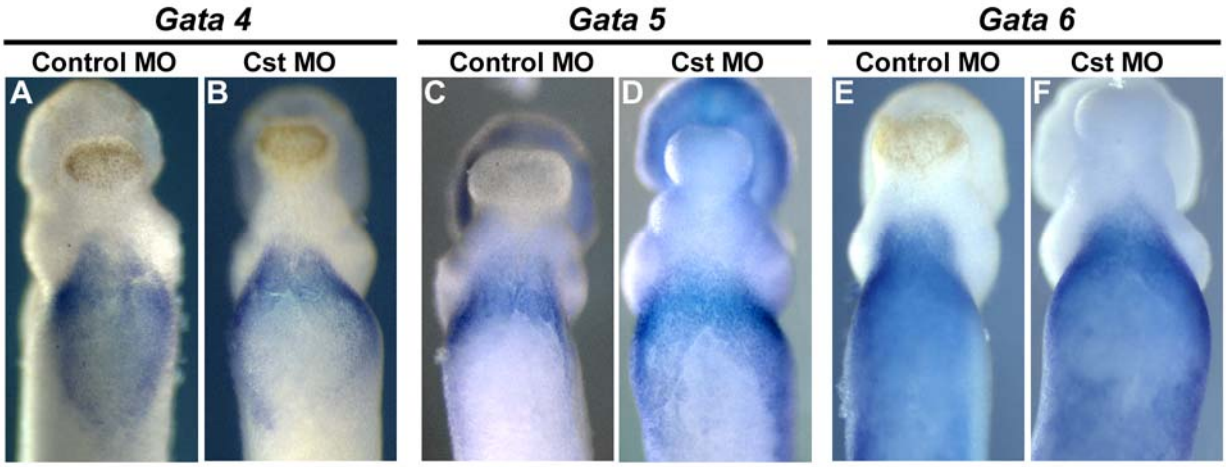

Figure S7. CST Is Not Required for Expression of *Gata4*, *Gata5*, or *Gata6*
Whole mount *in situ* hybridization of Stage 29 control MO and CST-depleted embryos demonstrating proper expression of (A,B) *Gata4*, (C,D) *Gata5*, and (E,F) *Gata6*. Ventral views with anterior to the top.

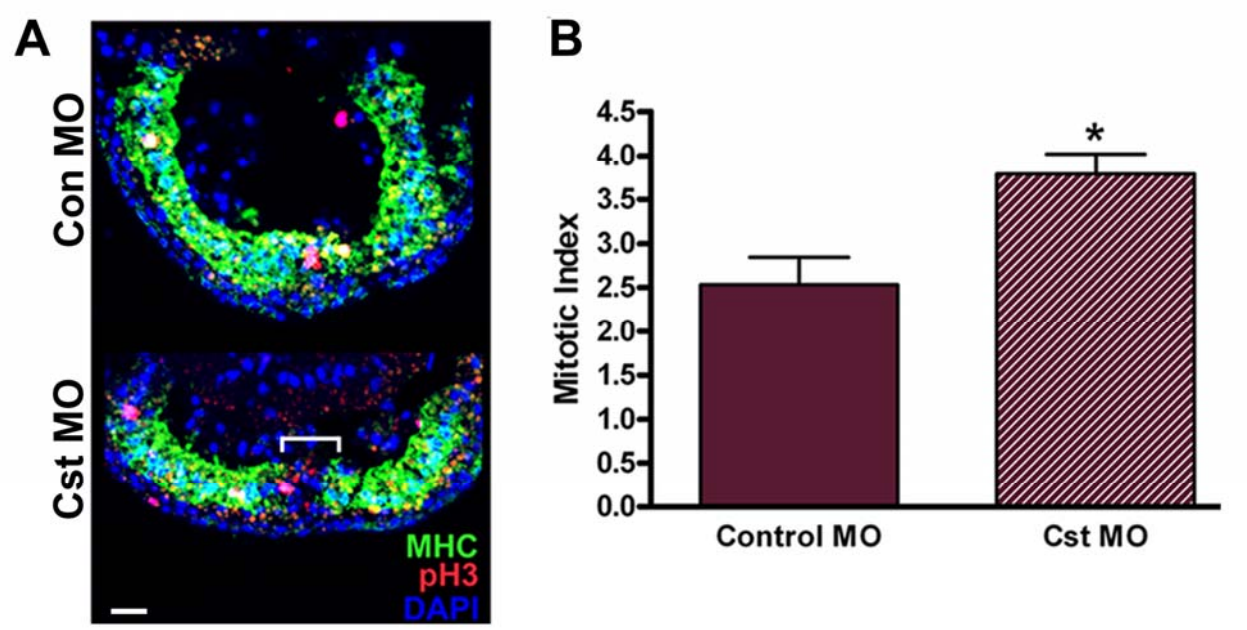

Figure S8. CST Is Required for Proper Cell Growth of Cardiomyocytes Dorsal to the Cardiac Ventral Midline

(A) Representative transverse sections of Stage 29 control MO (top) and CstMO (bottom) injected embryos stained with MHC antibody to mark differentiated cardiomyocytes, phospho-histone H3 (pH3) antibody to mark cardiac cells in the M-phase of the cell cycle, and DAPI. Bracket highlights undifferentiated cardiac ventral midline cells. (B) CST-depleted differentiated cardiomyocytes have an increased mitotic index at Stage 29. Quantification of the mitotic index was determined by calculating the percentage of pH3-positive differentiated cardiomyocytes. Bars represent the average of at least three embryos per condition +/- SEM. *, $p<0.01$; Scale bars: 200 μm.

Supplementary Experimental Procedures

Cst Identification and Characterization

RNA was extracted from embryos using Trizol (Invitrogen) and the 5' untranslated regions of *Cstα* and *Cstβ* identified by 5'RLM RACE (Ambion) according to manufacturer's instructions with a *Cst*-specific primer. Coding DNA regions were determined by RT-PCR using primers based on regions of homology of murine *Cst* (Acc# XM_112612) to *X. tropicalis* genome available through the Joint Genomic Institute (http://genome.jgi-psf.org). *Cst* 3'untranslated region was determined by RT-

PCR using primers designed from a *X. tropicalis* UniGene cluster (UniGene Str.46155) containing 3' untranslated region of *Cst*. Sequence conservation was analyzed using GeneDoc (www.psc.edu/biomed/genedoc) and synteny comparison by Metazome analysis (www.metazome.net).

Protein Expression and Cellular Localization

Cstα and *Cstβ* cDNAs were cloned into pcDNA 3.1-V5/His TOPO vector (Invitrogen) and *in vitro* translated using TnT Coupled Reticulocyte Lysate System (Promega). Tagged proteins were detected by western blot using mouse anti-V5 antibody (Invitrogen) and peroxidase-conjugated Affinity Pure donkey anti-mouse secondary antibody (Jackson Immunoresearch Laboratories). Cellular localization was determined by injecting 3 ng *Cstα*-V5 and *Cstβ*-V5 mRNA, prepared by mMessage *in vitro* Transcription System (Ambion). RNAs were injected at the one-cell stage. Embryos were cultured until Stage 32, fixed and sectioned as previously described (Goetz et al., 2006). Sections were stained with mouse anti-V5, Cy3-conjugated anti-mouse secondary antibody and DAPI (both Sigma) as described in Goetz et al, 2006. Sections were imaged on a Zeiss LSM410 confocal microscope.

Developmental Temporal Expression

RNA was extracted from homogenized embryos at indicated stages (10 embryos per stage) in lysis buffer (Goetz et al., 2006) followed by phenol:chloroform extraction. cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen). Transcript specific PCR reactions were performed with Taq Polymerase using 1 µl of cDNA using

the following PCR program: 94°- 3 min, 40 cycles of 94°- 30 sec, 55°- 30 sec, 72°- 2 min followed by 7 min of 72°. Forward primers were designed to unique regions of each *Cst* transcript and the reverse primer was designed to a common region of both transcripts spanning introns.

Developmental *In Situ* Hybridization

Whole mount *in situ* hybridization was carried out using antisense RNA probes of *Gata4*, *Gata5* and *Gata6*. *in situ* hybridization of sectioned *Xenopus* heart and endoderm tissue was performed on 20 µm frozen sections using DIG-labeled antisense RNA probes followed by detection according to manufacture's protocol.

Supplemental Reference

Goetz, S. C., Brown, D. D., and Conlon, F. L. (2006). TBX5 is required for embryonic cardiac cell cycle progression. Development *133*, 2575-2584.