Supplemental Material

Cai et al.,

A Nodal to TGFβ Cascade Exerts Biphasic Control of Cardiopoiesis

Contents:

Extended methods

mESCs differentiation

Mouse ESCs were differentiated either in 10% serum containing Iscove's Modified Dulbecco Media (IMDM) or serum free IMDM as embryoid bodies (EBs), hanging drops or as monolayer. In serum conditions, IMDM was supplemented with 10% FBS, 2mmol/L glutamine, $4.5x10^{-4}$ mol/L monothioglycerol, 0.5 mmol/L ascorbic acid, 200 μg/mL transferrin (Roche), 5% protein-free hybridoma media (PFHM-II; Invitrogen) and Penicillin/Streptomycin; Serum free IMDM was supplemented with 25% DMEM/Ham's F-12, 2 mmol/L glutamine, $4.5x10^{-4}$ mol/L monothioglycerol, 0.5 mmol/L ascorbic acid, B27 supplement without Vitamin A (Gibco), N2 supplement (Gibco) and Penicillin/Streptomycin.

Cell mixing assays

R1 Cripto$^{-/-}$ ESCs engineered to express GFP were mixed with Cripto$^{-/-}$ caACVR1b ESCs at a ratio of 5:1 in hanging drops. Hanging drops were also generated from pure populations of Cripto$^{-/-}$ ESCs and Cripto$^{-/-}$ caACVR1b ESCs as controls. Cells were maintained as hanging drops for two days, then as EBs in suspension for remaining days. EBs were then subjected to either FACS analysis at differentiation day 5 for Kdr expression or qRT-PCR analysis on day 9 for the expression of cardiac markers. In some cases, FACS isolated single cells were re-aggregated for subsequent culture. , Dr. Malcolm Whitman (Harvard School of Dental Medicine) provided the caACVR1b-HA cDNA and Dr. Eileen Adamson (Sanford-Burnham Medical Research Institute) provided Cripto$^{-/-}$ ESCs.

Flow cytometric analysis and cell sorting

Day 5 EBs were dissociated into single cells by 0.25% Trypsin (Gibco, Invitrogen), stained with phycoerythrin (PE)–conjugated anti-mouse Kdr (1:100, eBioscience), and then immediately analyzed with a FACSCanto (BD Biosciences). Total events of 100,000 were analyzed in each sample. Cell sorting was performed with FACS Vantage-Diva sorter (BD Bioscience) for GFP^{+} and GFP^{-} fractions or GFP^{+} /PE^{+} and GFP^{+} /PE^{-} fractions as indicated in figures and text. Dead cells were identified by staining of 0.1 μg/mL propidium iodide (PI) (Sigma-Aldrich) and excluded from analysis.

RNA extraction and qRT-PCR

Total RNA was extracted with TRIzol (Invitrogen) and reverse transcribed to cDNA with QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

cDNA samples synthesized from 500ng of total RNA by Quantitect RT kit (Qiagen) were subjected to qRT-PCR with LightCycler 480 SYBR Green I Master kit (Roche) performed with LightCycler 480 Real-Time PCR System (Roche). Primer sequences are listed in Supplementary Table 1.

siRNAs transfection

Pre-designed siRNAs against *Tgfbr1* and *Tgfbr2* (Ambion) and validated siRNA control (Ambion) were transfected at 100nmol/L final concentration into Cripto$^{-/-}$, R1 or CGR8 cells by Lipofectamine RNAiMAX (Invitrogen), as per manufacturer's instruction.

Western blotting

Cell pellets were lysed with RIPA buffer supplemented with protease and phosphatase inhibitors (Sigma) on ice and were mixed with 2X sample buffer (Invitrogen). Protein samples were then run on 10% SDS-tris glycine pre-cast gels (Invitrogen) and transferred onto a 45 μm PVDF membrane. Antibodies anti phospho-Smad2/3 (1:500, Cell Signaling), total Smad2/3 (1:500, Cell Signaling), Mef2c (1:1000, Aviva), Tbx5 (1:1000, Santa Cruz) and β-actin (1:5000, Sigma) were applied on blots and detected by ECL Plus detection kit (Abcam).

Data analysis and statistics

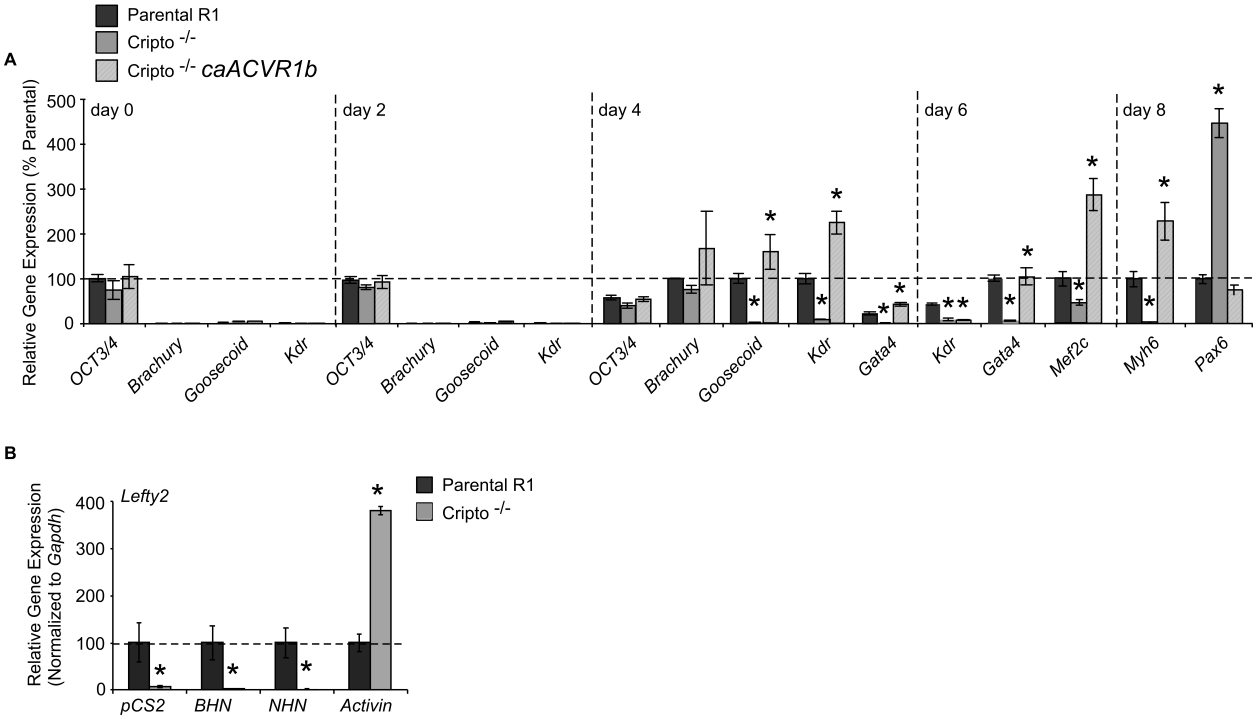
In qRT-PCR data analysis, values are expressed either as 2^DeltaDeltaCt, with DeltaDeltaCt defined as the difference in crossing threshold (Ct) values between experimental and control samples, using *Gapdh* as a control gene.

Each experiment was repeated at least two times using a minimum of three biological replicates per condition. Statistical analysis was performed with unpaired Student's T-test, Asterisk in figures represents $P<0.05$. Error bars indicate the S.E.M.

Online Table I. qRT-PCR Primer Sequences

GENE (Accession No.)	FORWARD PRIMER	REVERSE PRIMER
Oct3/4 (NM_013633)	tcagcttgggctagagaagg	tgacgggaacagagggaaag
T/Brachyury (NM_009309)	agcttcgtgacggctgacaa	cgagtctgggtggatgtag
Goosecoid (NM_010351)	accatcttcaccgatgagcagc	cttggctcggcggttcttaaac
Kdr (NM_010612)	tgccggcatggtcttctg	aaatcaagccccacatt
Mesp1 (BC012689)	aatgcaacggatgattgt	agcgtgtaccctattgg
Gata4 (NM_008092)	catcaaatcgcagcct	aagcaagctagagtcct
Mef2c (NM_025282)	agatacccacaacacaccacgcgcc	cattatccttcagagagtcgcatgcgctt
Myh6 (M76601)	catgccaatgacgacct	cctacactcctgtactgcc
Pax6 (NM_013627)	gacctcctcatactcgtg	gtgcttctaaccgcca
GFP (YP_002302326)	ctgctgcccgacaacca	tgtgatcgcgcttctcgtt
Gapdh (K0_1458)	aatggatacggctacagc	gtgcagcgaactttattg
Nodal (NM_013611)	ccagacagaagccaact	aagcatgctcagtggct
Lefty1 (NM_010094)	ctcgatcaaccgcca	ccattccgaacactagc
Lefty2 (NM_177099)	aggttcgcatctgag	cttgtctagggctaaggt
Inhibina (NM_010564)	ccaggctatccttttcccagc	gggatggccggaatacataag
Inhibinb (NM_008381)	atagaggacgacattggcagg	cacgctccactactgacagg
Cerberus1 (NM_009887)	gcagacctatgtgtgga	atgagacatgatcgcttt
Tgfb1 (NM_011577)	atcctgtccaaactaaggctcg	acctctttagcatagtagtccgc
Tgfb2 (NM_009367)	tcgacatggatcagtttatgcg	ccctggtactgttgtagatgga
Tgfb3 (NM_009368)	cctggccctgctgaacttg	gacgtgggtcatcaccgat
Pecam1/CD31(NM_008816)	tgcacccatcacttaccacc	cttcatccaccggggctatc
Myh11(NM_013607)	aagctgcggctagaggtca	agctctctttggaagtccttcat
Aplnr (NM_011784.3)	ggttacaactactatggggctga	agctgagcgtctcttttcgc
Cdh5 (NM_009868)	tgccctcattgtggacaagaa	tggcacagatgcgttgaatac
Tagln (NM_013607)	agggatcgaagccagtgaag	actgctgccatatccttacct
Acta2 (NM_007392)	gcatccacgaaaccacctat	tccacatctgctggaaggta
Mixl1 (NM_013729)	acgcagtgctttccaaacc	cccgcaagtggatgtctgg

Supplementary Figures

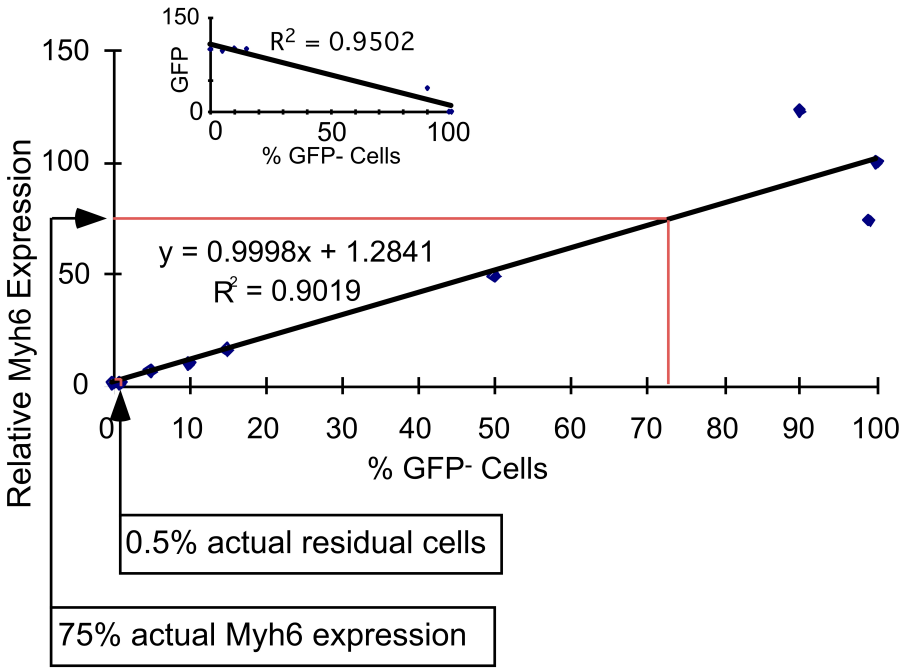


Online Fig. I. Constitutively active, ligand independent Nodal signaling restores cardiogenesis in *Cripto*$^{-/-}$ ESC.

A) Expression of mesoderm and cardiac markers is restored in Cripto$^{-/-}$ ESCs stably transduced with caACVR1b. qRT-PCR analysis for each gene marker was performed at developmentally relevant time points during mESC differentiation for markers of stem state (*Oct3/4*); mesoderm (*T*/*Brachyury, Kdr, Goosecoid*); cardiogenic mesoderm (*Gata4, Mef2c*); cardiomyocytes (*Myh6*) and neural tissue (*Pax6*).

B) Relative mRNA expression of *Lefty2* in R1 and *Cripto*$^{-/-}$ ESCs, which were transiently transfected with either control empty plasmid vector (pCS2+) or plasmids directing expression of Activin A, Nodal (NHN), or a Nodal fusion protein (BHN) that contains the BMP2/4 pro-region in place of the native pro-region to facilitate processing. Note that Nodal (BHN, NHN) is insufficient to induce *Lefty2* expression in Cripto$^{-/-}$ ESCs whereas Activin A,which signals independently of Cripto, restored downstream genes expression to levels.

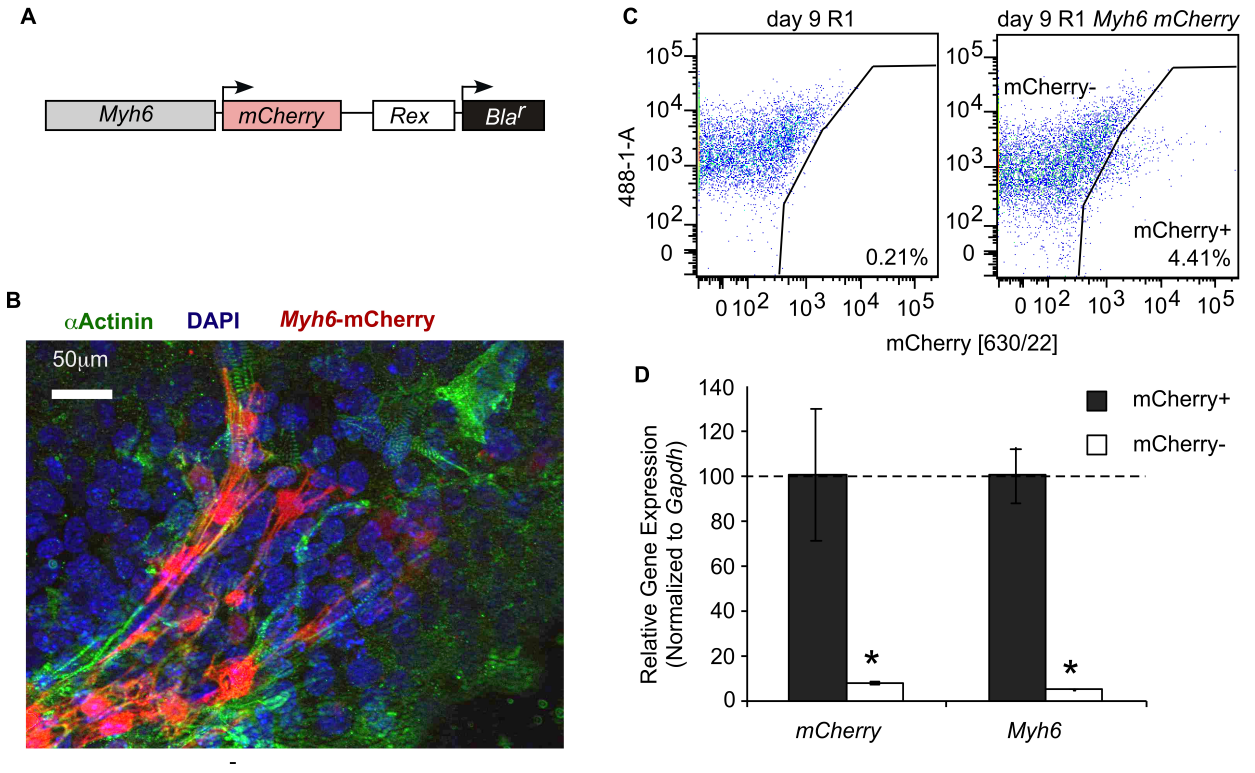
**P< 0.05*, unpaired Student's T-test. Error bars indicate the S. E. M.; n=3.



Chi-squared Test	Expected Value	Actual Value
% GFP⁻ Cells	73.7	0.5
Relative Myh6 Expression	75.0	75.0
P Value	1.5×10^{-17}	

Online Fig. II. Residual inducer cells are insufficient to account for cardiac *Myh6* gene expression in FACS-isolated responder population.

GFP$^-$ inducer and GFP$^+$ responder cells were combined without co-culture and tested for gene expression by qRT-PCR, yielding a positive linear correlation for *Myh6* mRNA and negative for GFP mRNA (inset) relative to the GFP$^-$ inducer cells. The actual *Myh6* expression in the co-culture experiment (Fig. 1F) would require 73.9% percent contamination. This value differs from the 0.5% actual residual value derived from re-sorting and results in P= ~10^{-17} for the experiment from χ-squared distribution (Table inset).



Online Fig. III. Validation of *Myh6*-mCherry fluorescent reporter ESC line.

A) Schematic representation of the cardiac-specific *Myh6* promoter driven mCherry reporter.

B) Coincident α-actinin immunostaining (green) with *Myh6*-mCherry expression, validating reporter fidelity.

C) Flow cytometry profiles of parental R1 and *Myh6*-mCherry reporter cells at day 9 in EB culture yielding 4.41% mCherry^{+} cells in the reporter line.

D) qRT-PCR analysis of *mCherry* and *Myh6* in day 9 FACS sorted mCherry^{+} and mCherry^{-} cells (**C**) showed *Myh6* mRNA expression localized primarily in the mCherry^{+} cells.

*$P< 0.05$, unpaired Student's T-test. Error bars indicate the S. E. M.; n=3.

Movie Descriptions

Online Movie I. Cell non-autonomous rescue of beating cardiomyocytes in Cripto$^{-/-}$ ESCs.

Cripto$^{-/-}$ ESCs with the cardiac specific *Myh6*-mCherry reporter (responder) were mixed with GFP^{+} caACVR1b Cripto$^{-/-}$ ESCs (inducer) and imaged at day 10 of differentiation.

Online Movie II. 3-dimensional reconstruction of confocal images shows cll non-autonomous rescue of cardiogenesis in Cripto$^{-/-}$ ESCs.

Cripto$^{-/-}$ ESCs with the cardiac specific *Myh6*-mCherry reporter (responder) were mixed with GFP^{+} caACVR1b Cripto$^{-/-}$ ESCs (inducer) and analyzed by confocal microscopy at day 9 of differentiation. Confocal imaging demonstrated that the mCherry^{+} cardiomyocytes induced in the Cripto$^{-/-}$ population were distinct from GFP^{+} caACVR1b inducer cells.