

MATERIALS AND METHODS

Database Searching and Cloning of CIP

We screened for novel cardiac-specific genes *in silico* by performing a BLAST search with ESTs from mouse embryonic heart cDNA libraries in the database as described previously¹. One of the cDNA sequences identified in this screen (Access number AA919489) corresponded to the 3' untranslated region (3'-UTR) of the CIP transcript and was found in a cDNA library of E13.5 mouse embryonic hearts. This short cDNA fragment (273 nt) was used as a probe to screen a mouse embryonic heart cDNA library. Several cDNA clones were identified. Among them, the longest clone is 1336 nt in length. Polymerase chain reaction (PCR)-based cloning was further used to identify additional cDNA isoforms.

CIP Reporter Mice

The CIP reporter line with a retroviral gene trap cassette inserted into mouse genome chromosome 9 (between chr9: 77,046,001 and 77,046,002) was generated and obtained from Texas A&M Institute for Genomic Medicine (TIGM). The gene trap cassette containing the selectable marker β -geo, a functional fusion between the β -galactosidase and neomycin resistance gene, was inserted into the putative third intron of the CIP gene (Online Figure III).

In Situ Hybridization and Northern Analysis

Whole-mount and section *in situ* hybridization and Northern analyses were performed as described^{1,2}. The 273 nt cDNA fragment corresponding to the 3'-UTR of the CIP gene was used as a probe to perform both whole-mount and tissue section *in situ* hybridization on staged mouse embryos.

For whole-mount *in situ* hybridization, E9.5 mouse embryos were dissected in PBS buffer and fixed in M buffer (100mM MOPS pH 7.4, 2mM EGTA, 1mM MgSO₄, and 3.7% formaldehyde) at 4°C overnight. After bleaching in M buffer with 6% hydrogen peroxide for 48 hr at room temperature, embryos were dehydrated through an ascending methanol series in PBS (25, 50, 75 and 100% methanol) and stored in 100% methanol at -20°C until needed. Embryos were rehydrated through a descending methanol series in PBT (PBS plus 1% Tween 20) and PBT, permeabilized in PBT containing proteinase K (10 μ g/ml in PBT) for 30 minutes at room temperature. After post-fixing in 4% paraformaldehyde in PBT, embryos were washed three times in PBT, rinsed once each in 0.1M triethanolamine (pH 8) and in 0.1M triethanolamine plus acetic anhydride (2.5 μ l/ml). After incubation with prehybridization solution (0.3M NaCl, 20mM Tris pH 8.0, 1mM EDTA, 0.1M DTT, 1X Denharts, 10% dextran sulfate, 50% formamide, 250 μ g/ml yeast tRNA, and 100 μ g/ml salmon sperm DNA) for 2 hr at 60°C, embryos were hybridized with DIG-labeled riboprobes in hybridization solution (prehybridization solution plus 0.5-1 μ g/ml riboprobe) at 60°C overnight. After hybridization, embryos were washed two times (30 min each) with prehybridization solution at the hybridization temperature, followed by washing two times (30 min each)

with 50% formamide, 2X SSC at the same temperature. Embryos were then treated with RNase A (1 μ g/ml) and RNase T1 (1 unit/ml) for 30 min at 37°C. After a final wash with 0.2X SSC, 1% Tween 20 at 60°C, embryos were incubated with PBT plus 10% heat inactivated sheep serum (Sigma, St. Louis, MO) for 2 hr at room temperature and then with alkaline phosphatase conjugated Fab anti-DIG solution (1 to 2000 dilution in PBT plus 10% sheep serum) overnight at 4°C. Embryos were washed at least 8 times with PBT (1 hour each) and left in PBT overnight at 4°C to completely wash out excess amounts of alkaline phosphatase conjugated Fab. Color reaction was performed by washing embryos three times for 10 min each with buffer A containing 100 mM Tris pH 9.5, 50mM MgCl₂, 100mM NaCl, 1% Tween 20, and 2mM levamisole, followed by incubating with detection solution (buffer A plus 4.5 μ l/ml nitroblue tetrazolium and 3.5 μ l/ml 5-bromo-4-chloro-3-indolyl-phosphate toluidinium) for various times at room temperature. When the color reaction was complete, embryos were washed twice with buffer A, once with PBT, and then post-fixed in 4% paraformaldehyde in PBT.

For Northern blot analysis, adult mouse multiple tissue northern blot purchased from Clontech (Palo Alto, CA) was used. A DNA probe containing 3'-UTR of the CIP gene was labeled with α -³²P-dATP using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The hybridization was performed at 68°C for 1 hour in an ExpressHyb hybridization solution (Clontech, Palo Alto, CA). The filter was then washed twice in 2 \times SSC, 0.05% SDS at room temperature for 30 minutes each, followed by 30 minutes at 50°C in 0.1 \times SSC, 0.1% SDS. The filter was then exposed to X-ray film at -70°C with the presence of intensifying screens.

Constructs, Cell Culture, and Luciferase Reporter Assays

Hela, COS7 and HEK293T cells were cultured in DMEM supplemented with 10% FBS in a 5% CO₂ atmosphere at 37°C. Luciferase reporter constructs fused with the MEF2C enhancer were as reported and is a generous gift of Dr. Brian Black (University of California, San Francisco)³. Transfections were performed with either FuGENE6 (Roche) or Lipofectamine (Invitrogen) reagents according to manufacturers instruction. Unless otherwise indicated, 100 ng of reporter plasmid and 100 ng of each activator plasmid were applied. 48 hours after transfection, cell extracts were prepared and luciferase activity was determined. For luciferase assay, normalized luciferase expression from triplicate samples in 12-well plates relative to LacZ expression was calculated, and the results are expressed as fold activation over the value relative to the control (Luciferase reporter and empty pcDNA). The CIP adenoviral expression construct (Ad-CIP) was constructed as previously described⁴. The CIP adenoviral expression construct (Ad-CIP) contained a cDNA encoding amino acids 1-309 of mouse CIP. Ad-CIP adenoviral constructs were N-terminal FLAG tagged.

Tissue Dissociation and Cell Sorting of Mouse Embryos

E10.5 embryonic hearts were isolated by microdissection and dissociated to single cells by collagenase digestion as previously described⁵. Isolated cells were FACS sorted into GFP positive and GFP negative populations. Sorted cells were collected into Trizol

(Invitrogen) and frozen at -20°C for RNA isolation.

Immunocytochemistry and β -Gal Staining

Staged mouse embryos were dissected out, collected and fixed in 4% paraformaldehyde at 4°C for 4 hours. After washing in PBS, embryos were treated in 15% and 30% sucrose for 2 hours each and embedded in OCT. About 5–8 μ m cryostat sections were collected on positively charged slides. Sections were washed in PBS, blocked in 5% serum/PBS, and subjected to immunostaining. Antibody sources were as follows: GFP (Invitrogen); PECAM (BD Biosciences); Nkx2-5 (Santa Cruz); Wt1 (Santa Cruz); Cardiac Troponin T (Tnnt2) (generous gift of Dr. Jim Lin, University of Iowa); Cardiac Troponin I (Tnni3) (Santa Cruz); Actn2 (Sigma); Isl1 (DSHB, University of Iowa); β -galactosidase (MP Biomedical). Alexa-488 and 594 secondary antibodies (Invitrogen). Fluorescently stained slides were counterstained with DAPI and imaged with an FV1000 confocal microscope (Olympus). For β -gal staining, samples were stained with a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal substrate at 37°C for 12 hours after fixation.

Quantitative RT-PCR and Western blot analyses

Total RNAs were isolated using Trizol Reagent (Invitrogen) from cells and tissue samples. For Quantitative RT-PCR, 2.0 μ g RNA samples were reverse-transcribed to cDNA by using random hexamers and MMLV reverse transcriptase (Invitrogen) in 20 μ l reaction system. In each analysis, 0.1 μ l cDNA pool was used for quantitative PCR. For Western blot analyses, cell extractions were cleared by 10,000 \times g centrifugation for 10 min at 4°C. Samples were subsequently separated by SDS/PAGE and transferred to PVDF membranes that were incubated with 5% milk and Anti-FLAG (1:1,000, Sigma); Anti-Myc (1:1,000, Santa Cruz); β -tubulin (1:10,000, Sigma) overnight at 4°C and then washed three times with TBST buffer before adding secondary antibody. Polyclonal antibodies against the CIP protein were generated by immunizing rabbits with CIP proteins which were expressed and purified as GST-CIP fusion proteins in bacterial. Specific protein bands were visualized by using ECL (Invitrogen) reagents.

Coimmunoprecipitation Assays

COS7 cells were transiently transfected with plasmids encoding FLAG-tagged CIP and Myc-tagged Isl1 proteins with FuGENE6 (Roche). Cells were harvested 48 h after transfection in lysis buffer composed of PBS containing 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF, and complete protease inhibitors (Roche). After a brief sonication and removal of cellular debris by 10,000 \times g centrifugation for 10 min at 4°C, FLAG-tagged CIP proteins were precipitated with anti-FLAG antibodies and protein A/G beads and associated proteins analyzed by Western blotting with anti-Myc antibodies.

***In vitro* GST Protein-Binding Assays**

Plasmids encoding a GST fusion with CIP were transformed into BL21 plus cells

(Stratagene). The cells were grown at 37°C in 2×YT medium to an optical density of 1.0. Isopropyl-β-D-thiogalactopyranoside (50 μM) was then added to the culture to induce protein expression. After being shaken at room temperature for 4 h, the cells were harvested and the GST protein was purified with glutathione beads. Isl1 proteins translated *in vitro* were labeled with ³⁵S methionine by using a TNT T7 reticulocyte lysate system (Promega). Glutathione beads conjugated with GST fusion protein were incubated with 10 μl of TNT product at 4°C for 2 h in 500 μl of GST-binding buffer (20 mM Tris, pH 7.3/150 mM NaCl/0.5% Nonidet P-40/protease inhibitor/1 mM phenylmethylsulfonyl fluoride). The beads were washed three times with GST binding buffer. 50 μl of SDS loading buffer was then added to the beads. After boiling, 20 μl was loaded onto an SDS/PAGE gel and analyzed by autoradiography.

Cardiomyocyte Culture

Neonatal rat cardiomyocytes were prepared as previously described⁴. Briefly, Neonatal rat cardiomyocytes were isolated by enzymatic disassociation of 1- to 2-day old neonatal rat hearts with the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp.). Cardiomyocytes were plated differentially for 2 hours to remove fibroblasts. Cells were plated on 1% gelatin-coated plates in medium containing 10% horse serum and 5% fetal calf serum (FCS). 18 hours after plating, cells were changed into serum-free medium and infected with adenovirus (Ad-LacZ for control and Ad-CIP) at a multiplicity of infection (m.o.i.) of 25. 24 hours later, cells were treated with hypertrophic agent, phenylephrine (PE), by changing to the PE-contained serum-free medium. Cells were harvested 24 hours after PE treatment for RNA isolation or 48 hours after PE treatment for immunochemistry.

REFERENCES:

1. Wang D, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E, Krieg PA, Olson EN. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell*. 2001;105:851-862.
2. Wang DZ, Reiter RS, Lin JL, Wang Q, Williams HS, Krob SL, Schultheiss TM, Evans S, Lin JJ. Requirement of a novel gene, *Xin*, in cardiac morphogenesis. *Development*. 1999;126:1281-1294.
3. Dodou E, Verzi MP, Anderson JP, Xu SM, Black BL. *Mef2c* is a direct transcriptional target of *ISL1* and *GATA* factors in the anterior heart field during mouse embryonic development. *Development*. 2004;131:3931-3942.
4. Xing W, Zhang TC, Cao D, Wang Z, Antos CL, Li S, Wang Y, Olson EN, Wang DZ. Myocardin induces cardiomyocyte hypertrophy. *Circ Res*. 2006;98:1089-1097.
5. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A,

Ikeda S, Chien KR, Pu WT. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature*. 2008;454:109-113.

Supplemental Material

Online Figure I

QUERY	PRAA	RET	KY	ANL
O43167/1-13	KRKR	RP	KKV	NTL
CPD1 DROME/1-13	IKKR	RP	PAKN	KGS
O45912/1-13	KKGR	RP	PAKN	PSA
CPD1 DROME 6/1-13	TKGR	RP	PKSS	GGA
CAB40849 3/1-13	SRGA	RP	PKA	KSP
Q38778_1/1-13	GRPR	RP	PKA	KDP
AAC68776/1-13	KKPR	RP	PKKY	EDS
O59212/1-13	EKGR	RP	PKKY	STR
Q43877_1/1-13	PRPR	RP	PKD	PNA
ORC1 SCHPO/1-13	SRGR	RP	PKY	PLP
O15030 1/1-13	ARAR	RP	PKT	KPG
AAD21618_3/1-13	KRGR	RP	PLH	RSE
CAB42096 2/1-13	KRRP	RP	RKH	KPE
Q22173/1-13	KKTR	RP	PKD	RSQ
P92954/1-13	KRGR	RP	PKQ	KTQ
Q43877 3/1-13	GRPR	RP	PKKI	ART
CPD1 DROME 1/1-13	VKKR	RP	SKA	SVG
O49276/1-13	PRKR	RP	PKS	MED
O49694 1/1-13	KRNR	RP	PGS	GGT
O49694/1-13	KKKR	RP	PKY	AAD
Q22381/1-13	QKR	RP	PKT	DA
Q40725 12/1-13	KRGA	RP	PKK	RPL
AAD21618 6/1-13	KRKR	RP	PLN	KPK
Q40725_3/1-13	GRGR	RP	PKK	ASS
CPD1 DROME 9/1-13	TKPR	RP	PAKN	IDD
P91155/1-13	PRAR	RP	PKA	TNL
O45912_7/1-13	VLKR	RP	SVKQ	PKD
CPD1 DROME 8/1-13	GRGL	RP	PKR	AVE
O15030 2/1-13	PKRR	RP	PSK	FFK
O65795/1-13	AKPR	RP	PKA	AKT
Q50887/1-13	PKKR	RP	PKA	KTE
O49276 1/1-13	GRAR	RP	PGV	KNG
CPD1_DROME_2/1-13	KRKA	RP	PKH	QPS
O43095/1-13	TRKR	RP	PKT	IAS
O82964/1-13	KRGR	RP	PKA	HAM
Q06339/1-13	AKKR	RP	PKS	VVA
JH0797/1-13	ARKR	RP	PKK	IOL
CPD1 DROME 5/1-13	PKKR	RP	PSLA	AGK
HMGC HUMAN 1/1-13	KRPR	RP	PKW	POQ
Q01086/1-13	KRGR	RP	PKRS	VTD
AAC2 DICDI/1-13	KRKR	RP	PKM	DEE
O49276 2/1-13	RKKR	RP	PKKF	DRI
P76546/1-13	PKKR	RP	PKY	NEK
O15026 1/1-13	KRRR	RP	PKA	RDL
Q40725 4/1-13	KRGV	RP	PKN	ATP
HMGC HUMAN 2/1-13	KRPR	RP	PKGS	KNK
O23142 1/1-13	EKKR	RP	PKGS	SSK
YN06 CAEEL/1-13	KRRR	RP	PKD	EEA
PRH PETCR/1-13	KRSR	RP	PKV	ONS
O00536 1/1-13	TGKR	RP	RNT	EKA
CPD1_DROME_7/1-13	GGQR	RP	PKA	SKI
Consensus/60%	.+.RGRP	.Ks	.s.	

Online Figure I. Sequence alignment between the putative AT_hook DNA binding domain in CIP and other known AT_hook DNA binding domain.

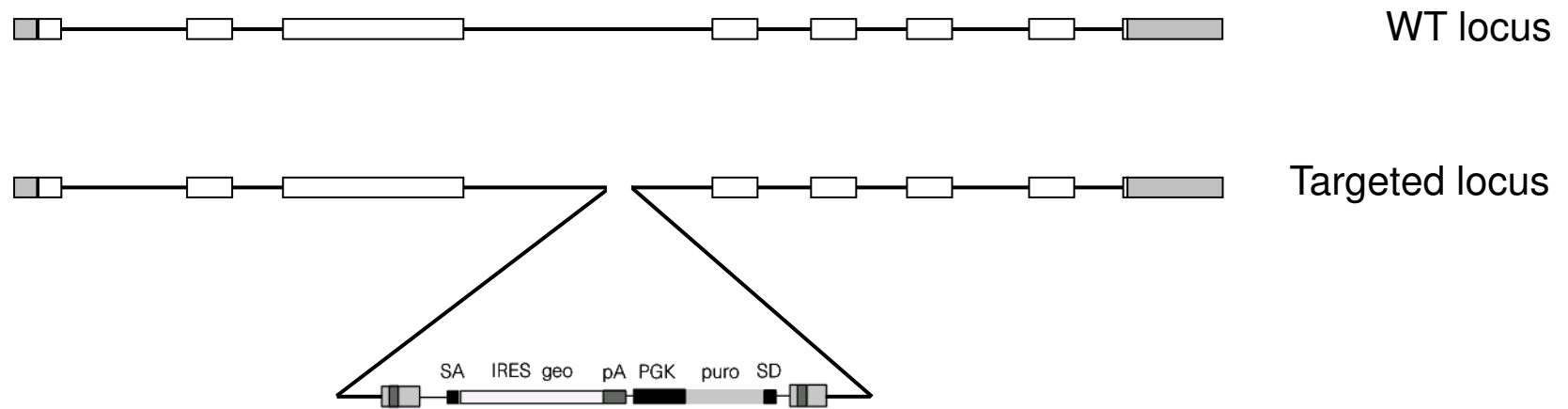
Online Figure II

Human	MELEKREK--RSL-----NKNLE-EKLTVSAGGSEAKPLIFTFVPTVRRPLPHTQLADTSKFLVKI---PEES	63
Mouse	MEFGKHEP--GSSLKR-----NKNLE-EGVTVSPGDPEAKPLIFTFVPTLRRPLPHTQLADTSKFLVKI---PEEP	65
Rat	MEFEKHEQ--GNALKK-----NEKLE-ERVTF-----	24
Dog	MEFEKHEK--GSL-----NKNLE-EKLTVSAGGSEAKPLIFTFVPTVRRPLPHTSKLADTSKFLVKI---PEEP	63
Cow	MDFEKHGK--GSL-----NENLE-EKLT-----	22
Horse	MEFEKHEK--GSL-----HKNLE-EKLTVSAGGSEAKPLIFTFVPTVRRPLPHTQLADTSKFLVKI---PEEP	63
Opossum	MEFERHGKDLGSSL-----ASNLL-TS-----	21
Chicken	MELGKHESK--G-----RAALE-EKQMVTSQESEAKPLTFTFVPSIGQLPTHFEVVDVSKFLVIT---PEEP	61
Xenopus	MVLNKSSSEK--REVWADGSKHADSLTIRKQKISSIAPGTQPLNFTFIPITLGTLP SQVLVKGHDHNYTKVYVSCRKEE	74
Zebrafish	MALEQPHRTL-----G-KVTTVF-----ATKLKSFPPVFLKRLPAENRVRKVF-F-----	44
	* : :	
Human	SDK-SPETVNRSK-SNDYLT--NAGSQQERDQAKLTCPSEVSGTILQEREFEANKLQGMQSQDLFKAEYVIVDS	135
Mouse	TDK-SPETVNRFE-YSDHMTF--SSESKQERVQRILDYPSEVSGRNSQQKEFNTKEPQGMQKGDLFKAEYVIVDS	137
Rat	-----E-YSDHMTF--SCEskeERDQRILDYPSEVSGKNSQRKEFNTKEPQGMQKGDLFKAEYVIVDS	85
Dog	SDK-NPETVNRSE-SNEYLT--NAGSQPERNQGLIYPSEVSEKISQERGFKAELQGMQSQDLFKAEYVIVDS	135
Cow	-----D-SSEYFTL--NVGRQQRERGAALTCPSEAEDKASQGRESKEKPKQGMQSQDLFKAEYVIVDS	83
Horse	SDK-NPETVNRSN-SNEYLT--NAGSQQERDQGLTYPSEVSGKISQERGFKANEPQGMQSQDLFKAEYVIVDS	135
Opossum	-----NSNE-FLDCVTL--KSGIQEESDIQTYVCSTETSPKIQGRGLKLNQPEKMQSQDLFKAEYVIVDS	85
Chicken	KDLSNQEIKNKAVLSDELNL--NSGQQQDRVPAIST--DSTQTVLQSLV--SNSKGMQENDLFAEFILITDS	131
Xenopus	MSA-KPEGASVTEK-NKNTTSCIPASYDR-----SSAEELFPQQGVSASHTQDLQRNDFIAEFLVMDS	138
Zebrafish	-----QTNR--A-KSSGEPGDCEKSMEEGVYKAEVVFQDA	78
	. . . : * : * :	
Human	E-GEDEAASRK-VEQG-----PPGGIGTAAVRPKSLA SSSLVSDVVRPKTQGTDLKTSHPPEMLHGMAPQQ	200
Mouse	D-GEDEATCRQ-GEQG-----PPGGPGNIIATRPKSLA SSSLASDVVRPKVRGADLKTSSHPPIPHG APQQ	202
Rat	D-GEDEATCRQ-GEQG-----PPGATGNIATRPKSLA SSSLASDVVRPKVRGVDVYVSSHPPIPHG APQQ	150
Dog	E-GEDEATS GK-DEQG-----PPGGLGTASRPKSLA SSSLVSDVVRPKTRGTDLQAPSHSEMPHG APQQ	200
Cow	E-GEDEVPRGK-GDQG-----PPVGTGPPAARPA SSSLASDAVRPKTRGADLQAPSHPERPQAMASQQ	148
Horse	E-GEDEATSRK-SEQG-----PPGGMGTATRPKSLA SSSLSDVVRPKTRGTDLQAPSHPEMLGMAPQQ	200
Opossum	E-GEDEATGRK-EDKQ-----PTVGNHGLARPKSLALAPG ITLQKPHQ--GDFQGPQSDLPQDAASPQ	148
Chicken	G-DEDEAGAASINAQR-----PSNGFGPISAQL--LA--TSHVSPGMESRKPSPDGHLP--ATLHSTADQQ	192
Xenopus	DEGEDEVMNK-TNKALIFEESYKTSQAQLQVVEKPI-ASLSEVTVNEIC PEAQP-----NELSQDVNCEE	203
Zebrafish	E-DGQIGEMRKN-T-----	90
	. :	
Human	KHGQQYKTKSSYKAFAAIPTNTLLLEQKALDEPAKT-ESVS---KDNTLEPPVELYFPAQLRQQTEELCATIDK	270
Mouse	KHG-----	205
Rat	KHGQQYKTKSSYKAFAAIPTNTLLLEQKALDEPART-ESNS---KASVSDLVPELCFPAQLRQQTEELCATIDK	220
Dog	KHGQQYKTKSSYKAFAAIPTNTLLLEQKALDEPAKT-EGVF---KDNALDLPELCFPAQLRQQTEELCATIDK	270
Cow	RHGQ-----ALDEPAKT-DSIS---KDSLDPPL-----	174
Horse	KHGQQYKTKSSYKAFAAIPTNTLLLEQKALDEPAKT-DSVF---KDKTLDPPLEFCSPAQLRQQTEELCATIDK	270
Opossum	KQI-----QALDEPKS-ERIT---KDSLDSHLE-----	174
Chicken	N-QQQYKIKTSYKAFAAIPTNTLLMEQKALEEPTKA-ADVT---EGSILDTHEMCPAQLRQQTEELCAVIDQ	261
Xenopus	KDLELYR KPNYKVFAAIPTNKLLLDQKALDEPETN-EENP--Q-FDETTETRESEVSPALLRQQTEEVCAVIDE	274
Zebrafish	TQNETYKIKSTYKALAAIPTNTLLLEQQAIDEEVSKKEALLNPADNYSWEDPHAEMCSPAQLRQQTAELYATIDE	165
	. :	
Human	VLQDSL SMHSSDSPSRSPK--TLLGSDTVK--TPTTLPRAAAGRETKYANLSSPSSTVSESQTKPGVIRPVVKS	341
Mouse	-----L--TPTTHPRAAAGRETKYANLSSSSSTASESQTTPGVI RPVVKVKS	249
Rat	VLQDSL SMHSSDSPSRSPQ--TMLGSETIK--TPTTHPRAAAGRETKYANLSSSSSTSESQTKPGVIRPVVKS	291
Dog	VLQDSL SMHSSDSPSSAQ--TLLGSDMIK--MPTTLPRAAAGRETKYANLSSPSSTVSESQTKPGVIRPVVKS	341
Cow	-----MPTTLPRAAAGRETKYANLSSPSSTVPESQTKPGVIRPVVKS	217
Horse	VLQDSL SMHSSDSPSSSLQ--TLLGSDTIK--TPTTLPRAAAGRETKYANLSSPSSTVSESQTKPGVIRPVVKS	341
Opossum	-----MPVTVPRAAAGRETKYANLSPSSTMAVSQTKPGVIRPVVKS	217
Chicken	VLQDPLTMRCESSPSFLH--MNTESDVGK--VSTTLQRAAGRETRYANLYKSTPVVAESQMTKPGVIRPVVKS	332
Xenopus	VLHDPLPLHCDSSRSTKARWDSKRFNESSQ--MPKSSGSAGRETKYAFLLQSRKNAV DCQETKPGVIRPLASKV	347
Zebrafish	VLEDTIQTRQSNHVNNAML--KSLAAEALRQQTSSPSFKLLGRETRYASSPFQPSVTTEKMTKPGVIRPVI TS	238
	****.*	*****
Human	RILLKKE-EEVYEPNPF SKYLEDNSDLFSEQDVT-----VPPKPVSLHPLYQTKLYPPAKSLLHPQ	401
Mouse	KLLLRKD-EEVYEPNPF SKYLEDNSGLFSEQAPD-----	282
Rat	KLFLKKE-EEVYEPNPF SKYLEDSSGLFSE-----	320
Dog	KILLKKE-EEVYEPNPF SKYLEDNSDFFSEQDVT-----VPPKPLSLHPLYQTRLCPAKSLLPPQ	401
Cow	KILLKKE-EEVYEPNPF SKYLEDNSDFFSEQDVP-----APPKPVSLHPLYQTRLHPPAKSLLRPQ	277
Horse	KILLKKE-EEIYEPNPF SKYLEDNSDLFSEQDVT-----VPHKPVSLHPLYQTRL YPPAKSLLHPQ	401
Opossum	KILLKKEEETIYEPNPF SKYLEDTSGLFAGQDVT-----SLHDATPLHPLYKTKFPFPAKSLLYPQ	278
Chicken	RIAQK-E-ELYQPNPFKYLEEISDQDVEQ-----P-----	362
Xenopus	TYTEKK-DD-DCSSNPF SHFTSSRTDFDARSK-----	378
Zebrafish	RFTEdq-D-EEFHPNPFKILHGNKSNRYQKVFSGGLCAEAPADAPADSDPQPSERQEDYKSLA-----	301

	. :	
Human	TLSHADCLAPGPF SHLS-FLSDEQENSHTLLSHNA-CNKL SHPMVA PEHEALDSK-----EQ	458
Mouse	-----HKLGI FS-----VKLGHPMVT PEHDTLDS-----KE	309
Rat	-----	Q
Dog	TLsqADCLTPGPF SHLSSFLSDEQNSHTLFSGNT-YNKL SHPMVA PEHEALDSK-----EQ	459
Cow	TRPHADGLTPGPF SHLSSFLSDEQNSHTLFSHNA-YN-----KQ	312
Horse	TLSHADCLTPGPF SHLSSFLSDEQNSHTLFSHNA-YNKL SHPMVA PEHEALDSK-----EQ	459
Opossum	NLSYTDCLTPGPF SHLSSVSLGDGHVNSPTFSHNRLYN-----KV	319
Chicken	-----SHP VP PENETLSSKEVTNERGSV-----	387
Xenopus	-----KRTHQHTDL-----	387
Zebrafish	-----STQD-----VPSA-----VSTHETH-----	317

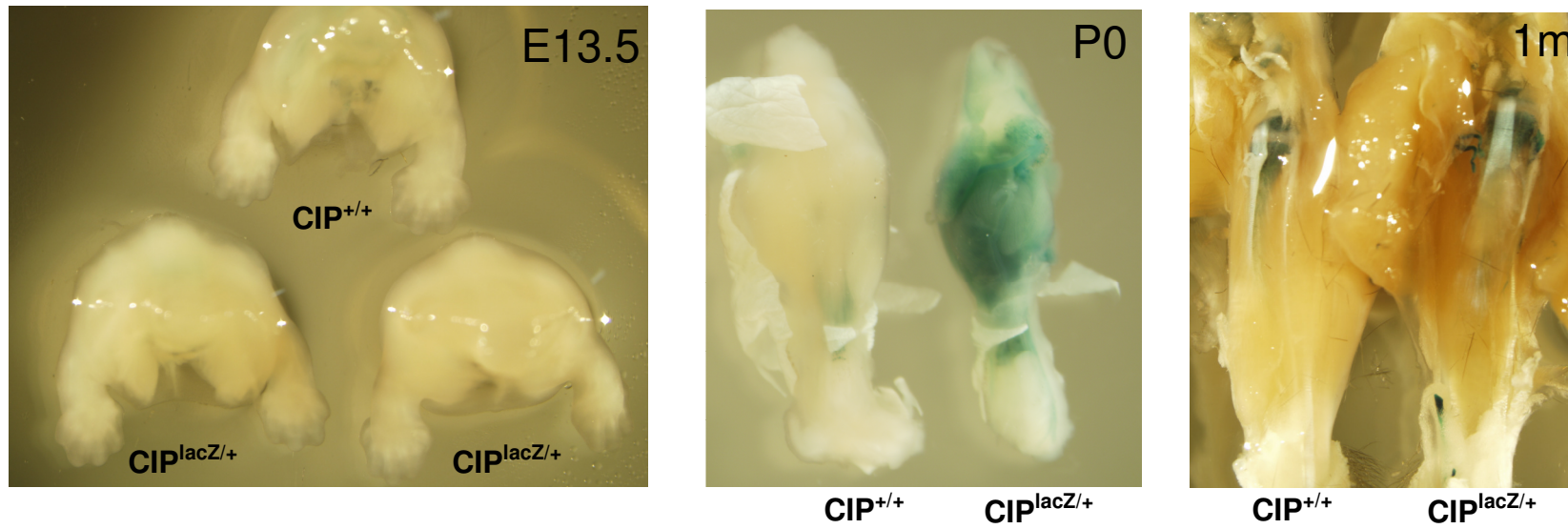
Online Figure II. Amino acid sequence alignment of CIP orthologs in multiple species. The AT hook DNA binding domain (in box) is highly conserved during evolution.

Online Figure III



Online Figure III. Targeting strategy of CIP reporter line. The expression of β -gal-Neo (geo) fusion gene is driven by endogenous CIP promoter. An internal ribosome entry site (IRES) allows for translation initiation of β -gal-Neo fusion gene.

Online Figure IV



Online Figure IV. β -gal staining of forelimb/hindlimb in multiple stages of CIP reporter line showing the transient expression of CIP in skeletal muscle.