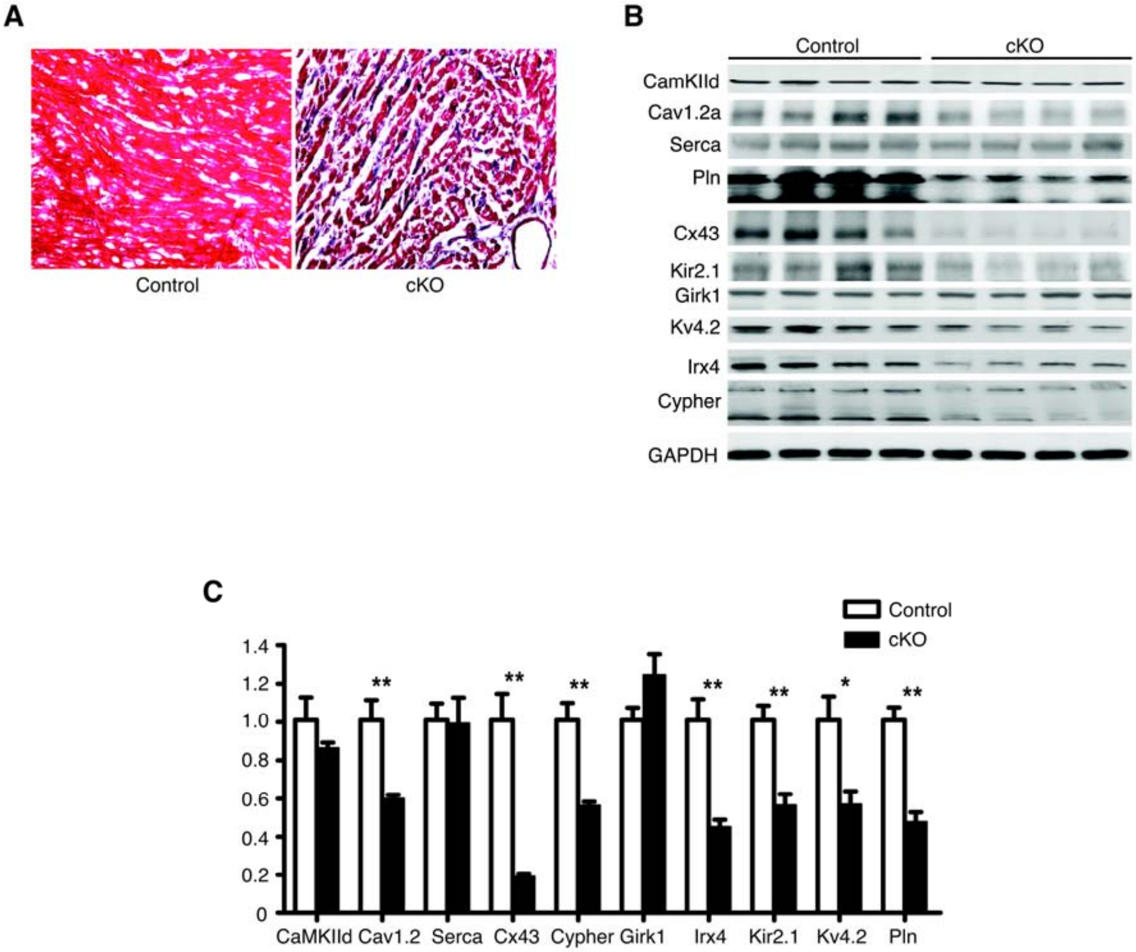


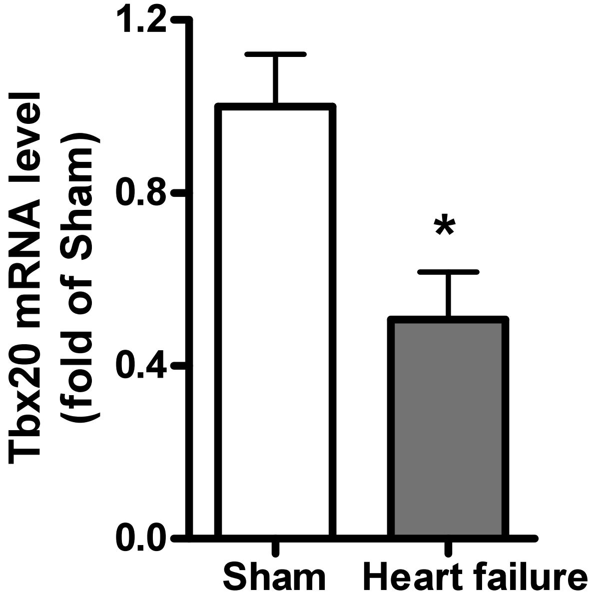
Supplemental Figure 1

Tbx20 cKO mice exhibit myocardial fibrosis and dysregulation of protein expression. (**A**) Masson's trichrome staining of ventricular myocardium. (**B**) Western blot analysis of selected proteins encoded by mRNAs significantly dysregulated in Tbx20 conditional knockouts. Cardiomyocytes from mice were harvested 5 days after initiation of tamoxifen induction. (**C**) Quantification of (**B**).

* $p < 0.05$; ** $p < 0.01$

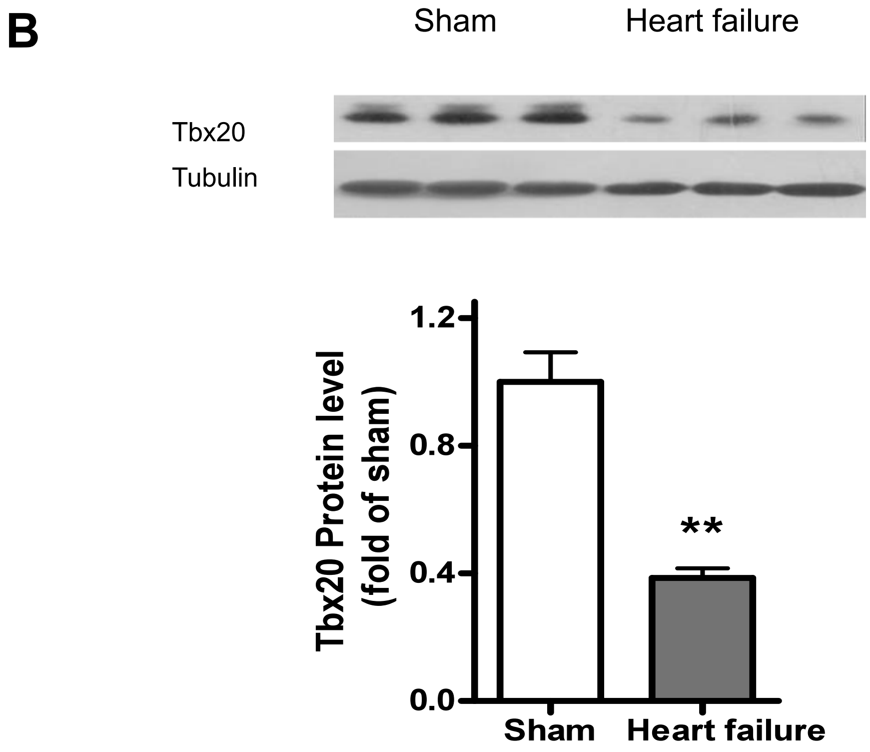
Mouse Myocardial infarction induced heart failure

A



N=5-6

Isoproterenol induced rat heart failure

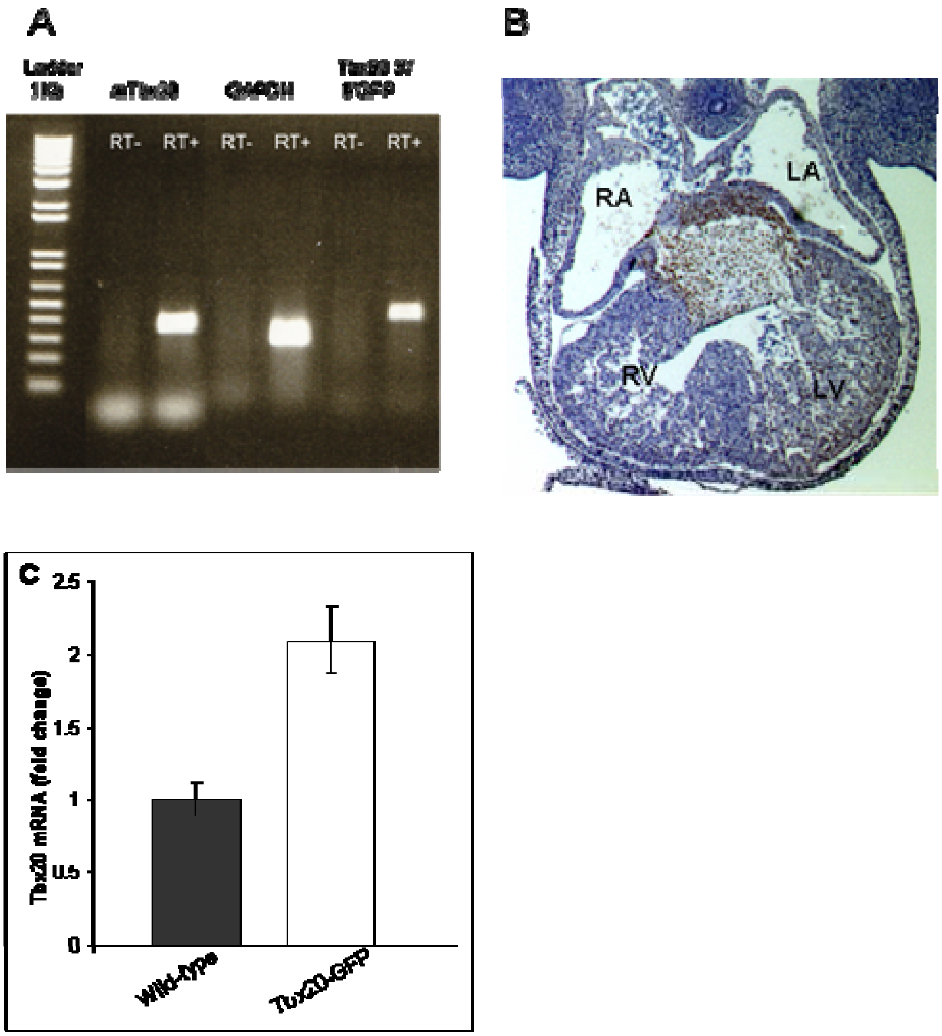


N=4-8 Sham N=4 Heart failure N=8

Supplemental Figure 2

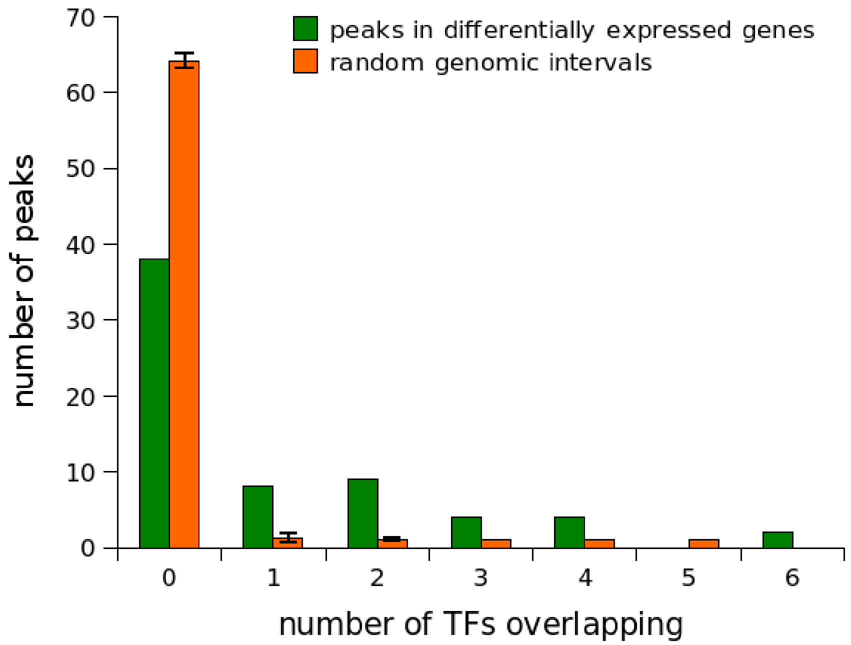
(**A**)Tbx20 mRNA expression in non-infarcted myocardium in a mouse heart failure model after myocardial infarction.*$p < 0.05$, sham n=5, infarction n=6.

(**B)** Tbx20 protein levels in hearts in a rat model of isoprenaline-induced heart failure. Sham n=4, isoprenaline n=8.



Supplemental Figure 3

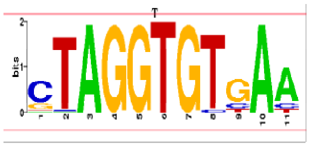
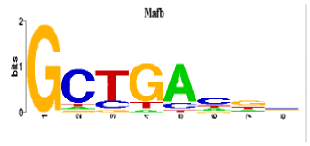
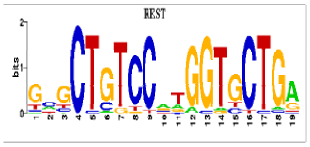
Characterization of *Tbx20-GFP* BAC transgenic Mice. (A) RT-PCR to detect expression of Tbx20-GFP fusion protein in cDNA derived from E11.5 mouse embryo hearts. Primers were designed in different exons to distinguish amplification from mRNA to genomic DNA in the presence (RT+) or absence (RT-) of reverse transcriptase. Tbx20 (NM_194263.1) 5'TGAGCTGACGAGTCTGGAT3' / 5'ATGACCGGTGGTAGGCATAG3' ; GAPDH (NM_008084.2) 5'AATGTGTCCGTCGTGGATCT3'; / 5'CCCTGTTGCTGTAGCCGTAT3' ; TBX20/GFP- 5'ATGTTCTGGGGGAGGAGAGT3'/ 5'TTAGCAGCAGCGGTTTCTTT3' (B) Immunohistochemical detection of GFP protein in a transverse section of an E11.5 embryo heart. The fused Tbx20-GFP protein recapitulates the endogenous cardiac expression pattern of *Tbx20*. *GFP antibody AB290.* (C) *Tbx20* mRNA in whole hearts of 6-week old adult BAC Tbx20-GFP transgenic mice and wild-type littermates. BAC transgenic mice express *Tbx20* at 2.08 fold higher levels than wild-type animals, indicating that approximately half of it comprises the *Tbx20-GFP* fusion transcript.



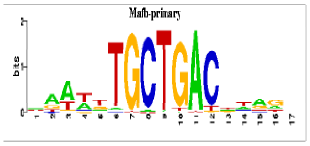
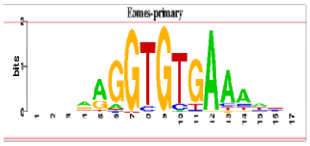
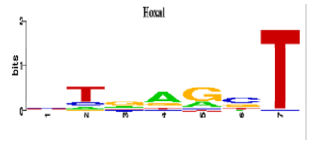
Supplemental Figure 4
Overlap of Tbx20 ChIP peaks (near genes for which we measured mRNA expression) with peaks for NKX2-5, GATA4, SRF, TBX5, MEF2A and p300 from He et al (2011). A significantly higher proportion of Tbx20 ChIP peaks overlap with ChIP peaks for these other factors than randomly sampled genomic intervals. P-value for random overlapping with 1 or more factors is zero (1,000 simulations).

He A, Kong SW, Ma Q, Pu WT. (2011) Co-occupancy by multiple cardiac transcription factors identifies transcriptional enhancers active in heart. PNAS 108(14):5632-7.

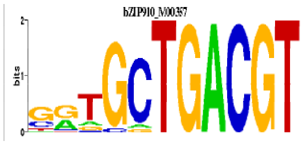
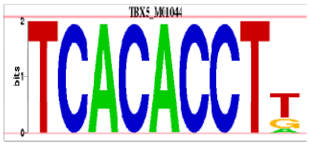
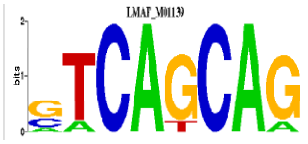
(A)

T	2.0285e-04	GTCANCACYN TTCA-CACCT	
Mafb	2.4642e-04	GNTGACAG GCTGAN-N	
REST	4.0977e-03	TCANCACY-N TCAGCACCWN	

(B)

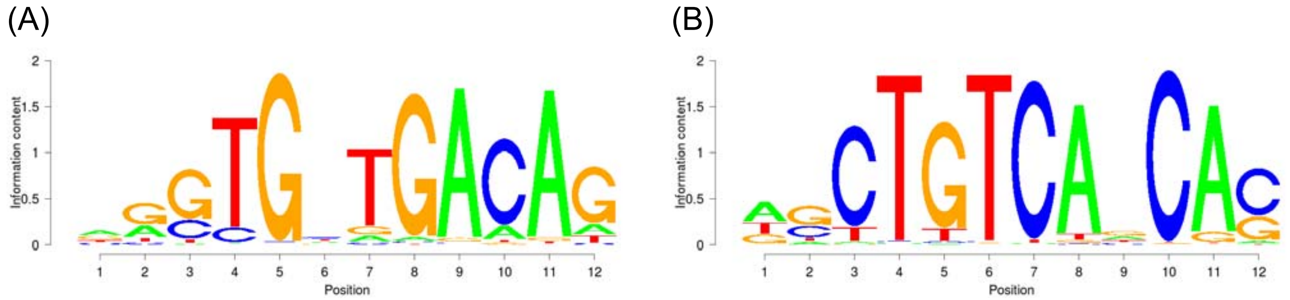
Mafb-primary	9.8641e-04	NR-GTGNTGAC--AG NAWNTGCTGACNNNN	
Eomes-primary	1.6137e-03	TGTCANCACYN TTTCA-CACCT	
Hoxa1	3.5539e-03	GNTGACAG NYT--NAN	

(C)

bZIP910_M00357	1.0851e-06	TGTCANCACY CGTCAGCAYS	
TBX5_M01044	5.6533e-05	NRGTGNTGA AGGTG-TGA	
LMAF_M01139	7.7623e-05	TGNTGAC TGCTGAS	

Supplemental Figure 5

Screenshots of top STAMP matches from (A) Jaspar, (B) PBM and (C) Transfac motifs to the Tbx20 de novo motif identified by MEME.



1.6k peaks, FDR 2%

4k peaks, FDR 9%

(C)

A	C	G	T	
0.395334	0.120842	0.299274	0.184550	A
0.305138	0.053195	**0.544372**	0.097295	G
0.009804	0.259804	**0.679412**	0.050980	G
0.003922	0.120588	0.001961	**0.873529**	T
0.002941	0.002941	**0.993137**	0.000980	G
0.209804	**0.345098**	0.161765	0.283333	C
0.067883	0.022803	0.091430	**0.817883**	T
0.024982	0.010312	**0.959331**	0.005375	G
0.967139	0.007371	0.024037	0.001453	A
0.132825	**0.830900**	0.017175	0.019100	C
0.964198	0.001488	0.021096	0.013218	A
0.141885	0.016448	**0.745860**	0.095807	G

(D)

A	C	G	T	
0.490204	0.004114	0.230124	0.275557	A
0.077325	0.322296	**0.523054**	0.077325	G
0.014194	**0.862700**	0.004114	0.118992	C
0.002830	0.007902	0.000326	**0.988941**	T
0.007881	0.010427	**0.880377**	0.101315	G
0.001568	0.005377	0.002852	**0.990204**	T
0.001568	**0.978862**	0.000326	0.019244	C
0.934343	0.007576	0.018939	0.039141	A
0.271465	0.140152	**0.414141**	0.174242	?
0.000305	**0.996539**	0.000326	0.002830	C
0.918234	0.001589	0.076084	0.004093	A
0.035658	**0.640478**	0.317246	0.006618	C

Supplemental Figure 6

Top motif recovered by MEME in the set of (A) 1.6k and (B) 4 peaks. Note that the 4k motif is the reverse complement of (A). (C) Position frequency matrix for (A), obtained by MEME (D) Position frequency matrix description for (B).

Supplementary Movies 1 and 2

Echocardiographic parasternal long axis view of control (Suppl. movie 1) and conditional Tbx20 knockout mouse (Suppl. movie 2) 4 days after initiation of tamoxifen induction.

Supplemental Table 1. Echocardiography findings by genotype six days after tamoxifen induction. ** p<0.01 for cKO compared to Tbx20f/f;MHC Cre-; ## p<0.01 for cKO compared to Tbx20+/+;MHC Cre+

	Tbx20 f/f; α-MHC-mER-Cre-mER- n=6	**Tbx20 +/+; α-MHC-mER-Cre+ n=6**	**Tbx20 f/f; α-MHC-mER-Cre+ n=13**
Body Weight (g)	26.00	28.17	25.23
IVSd(mm)	0.57	0.64	0.54
LVIDd(mm)	3.43	3.48	4.39 ** ##
LVPWd(mm)	0.57	0.67	0.56
IVSs(mm)	1.03	1.10	0.77 ** ##
LVIDs(mm)	1.77	1.88	3.81 ** ##
LVPWs(mm)	1.11	1.22	0.80 ** ##
Ao-ET(ms)	48.33	44.50	48.15
%FS	48.63	46.15	13.25 ** ##
EDD/PWD	6.06	5.26	7.85 ** ##
LVDd/BW	0.13	0.12	0.18 ** ##
LVM (d)(mg)	58.27	73.27	87.15 * ##
LV/BW Index (%)	0.22	0.26	0.35 ** ##

Supplemental Table 2. Overlap between TBX20 and cardiac TFs. In yellow, overlap between actual peaks; in cyan, overlap between randomly generated sets and HL1 ChIP peaks. Fold-enrichment was calculated by dividing the number of Tbx20 peaks overlapping another peak and the number of random peaks overlapping another peak.

random overlaps for 100 iterations	**Total # of peaks**	**# peaks overlapping**	**% of peaks overlapping**	Fold enrichment
tbx20	4012	634	**0.16**	25.69481118
gata4	16979	637	0.04	
random intervals	4012	24.67424242	0.01	
gata4	16979	25.64282828	0.00	
tbx20	4012	294	**0.07**	14.28417189
nkx2.5	20707	297	0.01	
random intervals	4012	20.58222222	0.01	
nkx2.5	20707	21.57363636	0.00	
tbx20	4012	159	**0.04**	85.10488754
p300	1504	159	0.11	
tbx20	4012	1.868282828	0.00	
random intervals	1504	1.868282828	0.00	
tbx20	4012	91	**0.02**	67.15116279
mef2a	1339	91	0.07	
tbx20	4012	1.355151515	0.00	
random intervals	1339	1.355151515	0.00	
tbx20	4012	579	**0.14**	24.62919359
Srf	24091	588	0.02	
random intervals	4012	23.50868687	0.01	
Srf	24091	24.59232323	0.00	
tbx20	4012	1078	**0.27**	14.52452533
tbx5	56352	1105	0.02	
random intervals	4012	74.21929293	0.02	
tbx5	56352	79.87919192	0.00	

Supplemental Table 3. Number of Tbx20 ChIP peaks containing Tbx20/Eomes motif (≥1 instance)

motif	**3rd order Markov**			**5th order Markov**		
	4k Tbx20 ChIP peaks (1)	random (2)	fold-enrichment (1)/(2)	4k Tbx20 ChIP peaks (3)	random (4)	fold-enrichment (3)/(4)
Eomes	1,122	1,171 ± 26	0.96	1,195	1,143 ± 17	1.04
Tbx20 de novo	2,036	1,158 ± 27	1.76	1,946	1,303 ± 30	1.49
Tbx20 SELEX/JMB	154	98 ± 10	1.57	144	103 ± 12	1.4
Tbx20_core SELEX/JMB	1,353	1,408 ± 38	0.96	1,396	1,365 ± 33	1.02

100 random sets with the same number of peaks and with the same length of the 4k set were generated and scanned with MotifLocator (>80% similarity)

Supplemental Table 4. Putative enhancer regions tested using a Zebrafish transgenic assay. tss = ±6kb of the transcription start site; gb = gene body; igr = intergenic region). All tested regions are direct ChIP-Seq genomic targets of Tbx20.
"-> gene" name indicates genes that were not the nearest to the peak, but were assigned for this experiment due to lack of any other closer peak

# internal ID	**genomic coordinate (mm9)**	**enhancer activity in zebrafish**	**nearby gene/assigned gene**
AA001	chr6:119143401-119144289	+	Cacna1c (tss)
AA002	chr6:119146295-119147508	+	Cacna1c (tss)
AA003	chr4:150514528-150516482	-	Camta1 (gb)
AA004	chr10:56699303-56701451	+	Gja1 (igr), Hsf2 (igr)
AA005	chr13:72402000-72404776	-	D430050G20 (igr), Irx1 (igr)
AA006	chr13:73473874-73475176	+	Mrpl36 (tss) -> Irx4
AA007	chr6:21215891-21218802	+	Kcnd2 (gb)
AA008	chr10:53154633-53155700	+	Mcm9 (igr), Pln (igr)
AA009	chr9:119377992-119379466	-	Exog (igr), Scn5a (igr)
AA010	chr5:120100951-120102659	+	Med13l (igr), Tbx3 (igr)
AA011	chr5:107443496-107444208	+	Cdc7 (igr), Tgfbr3 (igr)
AA012	chr11:94312011-94314399	-	Cacna1g (gb)
AA013	chr4:150528196-150529068	-	Camta1 (gb)
AA014	chr13:12224736-12226215	+	Mtr (igr), Ryr2 (igr)
AA016	chr5:122970459-122971537	+	Atp2a2 (igr), Ift81 (igr)
AA018	chr5:122974082-122975281	-	Atp2a2 (igr), Ift81 (igr)
AA021	chr14:63829075-63829644	+	Gata4 (gb)
AA022	chr14:63864145-63864467	+	Gata4 (tss)
AA024	chr13:83675138-83676985	-	Mef2c (gb)
AA025	chr2:166972171-166973977	-	Kcnb1 (gb)
AA026	chr5:23858118-23859194	-	Kcnh2 (tss)
AA027	chr5:23835283-23836035	-	Kcnh2 (gb)
AA028	chr11:110925898-110926942	-	Kcnj2 (tss)
AA029	chr5:15512527-15513371	+	Cacna2d1 (gb)
AA030	chr5:15382852-15383853	+	Cacna2d1 (igr), Speer7-ps1 (igr)
AA031	chr6:90456448-90458428	+	Aldh1l1 (igr), Klf15 (igr)
AA032	chr14:35386200-35387892	-	Ldb3 (gb)
AA033	chr14:35398580-35399692	+	Ldb3 (tss)
AA034	chr14:35361906-35363226	-	Ldb3 (gb)
AA035	chr17:46023137-46024188	+	Mrpl14 (igr), Vegfa (igr)
AA036	chr1:68078511-68079780	+	Cps1 (igr), Erbb4 (igr)
AA039	chr8:94879018-94879620	+	Irx5 (tss)
AA040	chr3:105323985-105324774	-	Kcnd3 (gb)
AA041	chr16:92345328-92345927	+	4930563D23Rik (igr), Kcne1 (igr)
AA042	chr19:45928809-45930562	-	9130011E15Rik (gb) -> Kcnip2
AA043	chr10:56436760-56437634	+	Gja1 (igr), Hsf2 (igr)
AA044	chr19:45925423-45926836	-	9130011E15Rik (gb) -> Kcnip2
AA045	chr19:45900391-45900999	-	9130011E15Rik (gb) -> Kcnip2
AA046	chr15:99532290-99532730	-	Smarcd1 (tss)
AA048	chr8:94511664-94513121	+	Irx3 (igr), Irx5 (igr)
AA049	chr11:110925976-110926938	+	Kcnj2 (tss)

Supplemental Table 5. Number of genes associated with all combinations of MEF2A, CREB1, Esrra, TEAD1 and Tbx20 de novo motifs and the number of enriched GO terms.

TFBS combination					**# of genes**	**# of ion transport/ contraction GO terms**	**# of heart/circulatory system morphogenesis GO term**	**Total # of GO terms**		
						A	**B**	**C**	**A / C**	**B / C**
Creb1					2230	0	0	0	0	0
Mef2a					1099	0	4	11	0	0.36
Tead1					1211	7	3	25	0.28	0.12
Esrra					2141	0	0	0	0	0
Tbx20					2212	0	0	0	0	0
Creb1	Mef2a				603	4	4	9	0.44	0.44
Creb1	Tead1				746	10	9	36	0.27	0.25
Creb1	Esrra				1338	4	3	30	0.13	0.1
Creb1	Tbx20				1319	6	2	15	0.4	0.13
Mef2a	Tead1				327	3	2	12	0.25	0.16
Mef2a	Esrra				592	4	3	8	0.5	0.37
Mef2a	Tbx20				683	6	14	35	0.17	0.4
Tead1	Esrra				695	10	7	41	0.24	0.17
Tead1	Tbx20				696	5	8	16	0.31	0.5
Esrra	Tbx20				1267	2	1	8	0.25	0.12
Creb1	Mef2a	Tead1			192	2	4	7	0.28	0.57
Creb1	Mef2a	Esrra			365	3	5	8	0.37	0.62
Creb1	Mef2a	Tbx20			385	5	8	17	0.29	0.47
Creb1	Tead1	Esrra			464	14	20	61	0.22	0.32
Creb1	Tead1	Tbx20			450	9	5	19	0.47	0.26
Creb1	Esrra	Tbx20			826	5	2	21	0.23	0.09
Mef2a	Tead1	Esrra			190	10	3	13	0.76	0.23
Mef2a	Tead1	Tbx20			218	2	2	8	0.25	0.25
Mef2a	Esrra	Tbx20			396	12	3	16	0.75	0.18
Tead1	Esrra	Tbx20			425	8	6	27	0.29	0.22
Creb1	Mef2a	Tead1	Esrra		122	7	6	16	0.43	0.37
Creb1	Mef2a	Tead1	Tbx20		128	0	5	5	0	1
Creb1	Mef2a	Esrra	Tbx20		251	6	11	19	0.31	0.57
Creb1	Tead1	Esrra	Tbx20		297	8	8	44	0.18	0.18
Mef2a	Tead1	Esrra	Tbx20		142	7	2	9	0.77	0.22
Creb1	Mef2a	Tead1	Esrra	Tbx20	89	5	5	11	0.45	0.45

Supplemental Table 6. MEF2A, TEAD1, Tbx20 mutagenesis causes loss of enhancer function

Peak coordinate	Targeted TFBS	Motif position within peak	Motif mutated	Mutated to	# zebrafish with heart expression	% zebrafish with heart expression
chr6: 119,146,295-119,147,508	none (original peak)	NA	none	none	59/134	(44%)
chr6: 119,146,646-119,146,655	None (negative control)	352	AGCCTCTTGT	AGCAGTCGAC	29/66	(43%)
	Esrra	186	TGGTCAAGGTGG	TGGTTCTCCCAT	15/67	(22%)
	Creb1	93	TGACGAAC	AATTAAAC	14/58	(24%)
	Creb2	220	CGACTCGA	AATTAAGA	1/34	(0%)
	Mef2a	276	CTATTTTAAG	CATCGCGCCG	2/88	(2%)
	Tbx20	19	ATGCGGAGACAA	ATAGAAACCTTA	4/81	(5%)
	Tead1	227	CACATTCCAGCT	CAAAGGTTGGCT	10/123	(8%)
chr13: 73,473,874-73,475,176	none (original peak)		none	none	34/81	(42%)
	Esrra	42	CCGTGAAGGTTA	CCGTATCGCGCC	25/61	(41%)
	Mef2a	348	CTATAAATAA	CGGCGCGATA	5/51	(10%)
	Tbx20-1	176	GGGTGCTGACAG	GGAGAAACCTTG	0/36	(0%)
	Tbx20-2	193	TGGTGGTGACAG	TGTCTTTGGAAG	27/68	(40%)
	Tbx20 (double mutant)	176/ 193	GGGTGCTGACAG + TGGTGGTGACAG	GGAGAAACCTTG TGTCTTTGGAAG	0/81	(0%)
	Tead1	80	TCCATTCAAGCG	TAAAGGTTGGCG	31/61	(51%)

NA- not applicable

Supplemental Table 7. qPCR primers for qRT-PCR analyses

	Forward	*Reverse*
Tbx20	gcagcagagaacaccatcaa	tgtgcacagagaggatgagg
Mef2c	ccattggactcaccagacct	agcacacacacacactgcaa
Tbx5	agccgtacatggagacatcc	cactgaggtctggtgctgaa
Irx1	gcaacgaagacgaagaggac	gggcgaatcttgagacttga
Irx4	agggctatggcaactacgtg	tccttggactcgaagctgtt
Irx5	tcttacgtgggctctcccta	ggccttgagagtggctgtag
Gata4	gcagcagcagtgaagagatg	gcgatgtctgagtgacagga
Kcnd2	gggagtgcaaatgcctacat	ctatggcttcccgacacatt
Kcnd3	tgaatgcatctctgcctacg	gcatggactcctggttgttt
Kcnj2	ggagtcccagagagcacaag	tagaggtacgcttgcctggt
Kcnj3	accctggtggatctcaagtg	gggagtgtagttgccgacat
Kcnip2	ggagactccagcaactacgc	acaaaccagccacaaagtcc
Gja1	aggtggactgcttcctctca	gagcgagagacaccaaggac
Cacna1c	tgctgctcctcttcctcttc	cggtcaggatctgaaacaca
Atp2a2	tgagacgctcaagtttgtgg	ctccagtattgcgggttgtt
Ryr2	agcttgaaagacaccgagga	tagagagccatctgccacct
Camk2d	ctggcacacctgggtatctt	atcccagaagggtgggtatc
Ldb3	ctatctcccggatcactcca	tagctggccgacttgatctt
Des	cctgctcaatgtgaagatgg	gttgttgctgtgtagcctcg
Nppa	gatagatgaaggcaggaagccg	aggattggagcccagagtggactagg

Supplemental Table 8. Mutagenesis primers

Chr6: 119,146,295 – 119,147,508	
Tead1	F: ACAGACGACTCGAGCCAACCTTTGTACTTCCG R: GCTCGAGTCGTCTGTGTCGTGGGCC
Mef2a	F: CTGCTGATCTACAAAACATCGCGCCGGAGAGAAAG R: GTTTTGTAGATCAGCAGCACCGTGAATC
Tbx20	F: CTGTGGTCAGACCTATAGAAACCTTAGCCCTTCCCC R: ATAGGTCTGACCACAGCAGCAAAGTCAG
Creb1.1	F: ATCAAGCCTTCAGGAGGAATTAAACTGACAGG R: CCTCCTGAAGGCTTGATGAGCTCTGGTC
Creb1.2	F: GTACACATTCCAGCTCTTAATTTCTGTGTCG R: GAGCTGGAATGTGTACTTCCGTGGATTC
Esrra	F: GGGTGATAGGCCTGGTTCTCCCATGGGCCTGGGC R: ACCAGGCCTATCACCCTTCCACACAAGA
Chr13: 73,473,874 – 73,475,176	
Tead1	F: AGGGACCATATTAGCCGCCAACCTTTAATTCATTAG R: GCGGCTAATATGGTCCCTCGGTGTTATG
Mef2a	F: ACCTGTCACCACCATATCGCGCCGGTGAGTGTGTCAGC R: ATGGTGGTGACAGGTCCCCTGTCAGCAC
Tbx20.1	F: GCCGTCCATCAGCGGAGAAACCTTGGGGACCTGT R: CCGCTGATGGACGGCTCCTTCTCCTGCCAT
Tbx20.2	F: GCTGACAGGGGACCTTCCAAAGACATTATTTATAGG R: AGGTCCCCTGTCAGCACCCGCTGATGGA
Esrra	F: TAATGCAATGATCCGTATCGCGCCCCATAACACC R: ACGGATCATTGCATTATGTAAATAAATT

GO enrichment of genes near peaks containing Mef2a, Esrr, Tead1, Creb1 and Tbx20 motifs

To verify whether these TFs act as Tbx20 co-factors in ion transport regulation, we selected peaks containing combinations of Tbx20 with these motifs and the genes to which these peaks were assigned. We then performed GO enrichment analysis of these genes against the set of 3,799 genes with at least 1 assigned Tbx20 peak and verified if they were enriched for ion transport terms. The rationale of this analysis was to verify whether specific combinations of these motifs in Tbx20 peaks are associated with particular genes and GO functions, indicating that these TFs co-regulate the expression of these genes. We highlight that by using genes with at least on Tbx20 peak as background, and not the entire set of ~20k genes, any GO enrichment observed corresponds to an even more specific subgroup of genes putatively regulated by Tbx20, for example, ion transport genes.

Whereas many motif combinations did not result in any enrichment or resulted in enrichment for only a few terms (**Supplementary File 3 and Supplementary Table 5**), certain combinations clearly yielded GO enrichment for heart development/morphogenesis and ion transport/contraction-related terms such as “heart morphogenesis”, “calcium ion homeostasis”, “muscle contraction”, including the combination of all 5 motifs.

We found that the enrichment observed for the 5 motifs is not common in a similar setting, only 0.4% of 10,000 random groups of 5 motifs occurring in similar frequencies of the test motifs yielded comparable results (≥45% of enriched GO terms being ion transport/contraction).