T-box genes coordinate regional rates of proliferation and regional specification during cardiogenesis

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Summary

Mutations in T-box genes are the cause of several congenital diseases and are implicated in cancer. *Tbx20*-null mice exhibit severely hypoplastic hearts and express Tbx2, which is normally restricted to outflow tract and atrioventricular canal, throughout the heart. *Tbx20* mutant hearts closely resemble those seen in mice overexpressing Tbx2 in myocardium, suggesting that upregulation of Tbx2 can largely account for the cardiac phenotype in *Tbx20*-null mice. We provide evidence that Tbx2 is a direct target for repression by Tbx20 in developing heart. We have also found that Tbx2 directly binds to the Nmyc1 promoter in developing heart, and can repress expression of the Nmyc1 promoter in transient transfection studies. Repression of Nmyc1 (N-myc) by aberrantly regulated Tbx2 can account in part for the observed cardiac hypoplasia in Tbx20 mutants. Nmyc1 is required for growth and development of multiple organs, including the heart, and overexpression of Nmyc1 is associated with childhood tumors. Despite its clinical relevance, the factors that regulate Nmyc1 expression during development are unknown. Our data present a paradigm by which T-box proteins regulate regional differences in Nmyc1 expression and proliferation to effect organ morphogenesis. We present a model whereby Tbx2 directly represses Nmyc1 in outflow tract and atrioventricular canal of the developing heart, resulting in relatively low proliferation. In chamber myocardium, Tbx20 represses Tbx2, preventing repression of Nmyc1 and resulting in relatively high proliferation. In addition to its role in regulating regional proliferation, we have found that Tbx20 regulates expression of a number of genes that specify regional identity within the heart, thereby coordinating these two important aspects of organ development.

Key words: T-box, Tbx20, Heart development, Proliferation, Nmyc1 (N-myc)

Introduction

T-box (Tbx) transcription factors are highly conserved across species, expressed in a wide variety of tissue types, often in an overlapping manner, and are required for development of diverse organs and tissues (Kiefer, 2004). T-box genes regulate patterning and cell fate, cell survival and/or proliferation, and are also of great clinical relevance. Mutations in T-box genes are the cause of a number of human disorders (Packham and Brook, 2003), including ulnar-mammary syndrome (*TBX3*), Holt-Oram syndrome (*TBX5*), isolated adrenocorticotrophin deficiency (*TBX19*) and cleft palate with ankyloglossia (*TBX22*). Mutation of *TBX1* is likely to contribute to DiGeorge syndrome. Expression of *TBX2* and *TBX3* is amplified in breast, ovarian and pancreatic cancers, and both can contribute to oncogenic transformation (Fan et al., 2004; Rowley et al., 2004).

Tbx20 is a T-box transcription factor that is expressed throughout the early cardiac crescent, and later in both myocardium and endocardium (Iio et al., 2001; Kraus et al., 2001). Expression of Tbx20 or its homologues in cardiac structures has been conserved from *Drosophila* to mammals (Plageman and Yutzey, 2005). In zebrafish and *Xenopus*, loss- or gain-of-function of Tbx20 causes abnormal cardiogenesis (Brown et al., 2005; Stennard et al., 2003; Szeto et al., 2002). Injection of antisense morpholinos to Tbx20 in zebrafish prevent heart looping, and result in defects in chamber morphology, aberrant expression of ventricular-specific myosin heavy chain in atrium and upregulation of Tbx5 (Szeto et al., 2002). In *Xenopus*, the cardiac mass of Tbx20 morphants is reduced, although no downstream targets of Tbx20 have been identified, and Tbx5 expression is normal (Brown et al., 2005). These results demonstrate a crucial role for Tbx20 in cardiac morphogenesis, but do not provide mechanistic insight into how Tbx20 is required for normal heart formation.

To investigate the role of Tbx20 in mammalian heart, we have generated mice that are homozygous null for *Tbx20*. Null mutants arrest development in utero with arrested cardiac morphogenesis and hypoplastic hearts. Analysis of these mutants has revealed that *Tbx20* is a key component in a genetic network controlling regional differences in

proliferation and regulating morphogenesis within the developing heart. We have identified Tbx2 as a crucial direct target for repression by Tbx20 and have discovered that Tbx2 itself directly represses Nmyc1 (also known as N-myc) activity. Nmyc1 is required for early myocardial proliferation, as demonstrated by severe cardiac hypoplasia in mice that are homozygous null for *Nmyc1* (Davis and Bradley, 1993).

Regional differences in proliferation rates within early looping heart have been determined and found to be consistent in chick, rat and mouse (Sedmera et al., 2003). Relatively low proliferation is observed in the sinoatrial region, the atrioventricular region, the outflow tract and within forming trabeculae of the ventricular myocardium. Differential proliferation has both morphogenetic and functional consequences. In forming ventricles, trabecular myocardium is relatively more differentiated, providing contractile force and allowing for proliferation of less-differentiated compact zone – the future thick-walled working myocardium (Rumyantsev, 1991). Other regions of low proliferative activity in early looping heart correlate with slow conducting myocardium, which acts as sphincters prior to valve development (de Jong et al., 1992).

Aberrant proliferation may underlie some adverse phenotypic consequences of T-box gene mutations in some human disorders and in cancer. *TBX1* is required for proliferation of cardiogenic progenitors that will contribute to the outflow tract, a region which does not form normally in individuals with di George syndrome (Xu et al., 2004). Tbx5 has been shown to suppress proliferation of cardiomyocytes (Hatcher et al., 2001). A missense mutation of *TBX5*, which is causative for Holt Oram syndrome, lacks antiproliferative activity – a characteristic that can be blocked by treatment with wild-type *TBX5*. Mechanisms by which either Tbx1 or Tbx5 affect proliferation are unknown. Tbx2 and Tbx3 have each been identified in senescence bypass screens, and either Tbx2 or Tbx3 can immortalize mouse embryo fibroblasts and cooperate with the oncogenes Myc or Ras to result in transformation (Brummelkamp et al., 2002; Carlson et al., 2001; Carlson et al., 2002; Jacobs et al., 2000). Tbx2 or Tbx3 can promote immortalization by the direct repression of the tumor suppressor cyclin-dependent kinase 2a, Cdkn2a (Brummelkamp et al., 2002; Jacobs et al., 2000; Lingbeek et al., 2002). Cells that lack human *Cdkn2a* fail to senesce in culture and can be propagated indefinitely (Kamijo et al., 1997). *Cdkn2a* promotes stabilization of the tumor suppressor p53 (Sherr and Weber, 2000). Tbx2 may also regulate proliferation/survival through direct repression of p21, a cyclin dependent kinase inhibitor implicated in senescence (Prince et al., 2004). Thus, Tbx2 and Tbx3 play crucial roles in cell cycle control via suppression of senescence genes.

In addition to its role in regulating regional proliferation, we have found that Tbx20 regulates expression of a number of genes that specify regional identity within the heart, thereby coordinating these two important aspects of organ development.

Materials and methods

Targeted disruption of murine *Tbx20*

Tbx20 genomic clones were isolated by screening a mouse 129/sv genomic library (Stratagene). The targeting vector was constructed in a plasmid containing PGKNeo and HSV-TK cassettes flanked by two LoxP sites. To generate a floxed allele targeting construct, a *Sma*I-*Sma*I 1 kb genomic DNA fragment containing the second exon was cloned into a site flanked by two LoxP sites (Fig. 1). A 2.6 kb *Sma*I-*Sma*I fragment within intron 1 and a 4.4 kb *Sma*I-*Not*I fragment were cloned into the vector as the 5′ arm or 3′ arm, respectively. Targeting vector was linearized with *Not*I and electroporated into SM-1 ES cells derived from 129/sv mice. After G418 selection, homologous recombinants were identified by digesting genomic DNA with *Nco*I and hybridizing with a 176 bp *Nco*I-*Sma*I 5′ probe. Recombinant ES cells were then transfected with a Cre plasmid in order to remove PGKNeo, HSV-TK cassettes (Fig. 1). ES cells with a floxed Tbx20 allele were injected into C57BL/6 blastocysts. Chimeras were mated with C57BL/6 females and offspring were genotyped by PCR and Southern blot analysis of tail DNA. *Tbx20*-null mice were obtained by crossing mice with *Tbx20* floxed allele to protamine-Cre mice (O'Gorman et al., 1997). Genotypes were determined by PCR with primers: P-810, 5′-AGTGCTACCCTCTGCAGCTGCAAA-3′; P-1120, 5′-AGTAGGAAGGAGCTGGGAAGAGTA-3′; and P-2320, 5′-CAGAAAATGACACGCGGATGGTGG-3′. The wild-type allele band was 310 bp and the mutant allele band was 650 bp (Fig. 1).

Whole-mount RNA in situ hybridization and histological analyses

Whole-mount RNA in situ hybridization was carried out according to Wilkinson's protocol (Wilkinson, 1992). For sectioning, mouse embryos were fixed in 4% paraformaldehyde, dehydrated in ethanol and embedded in paraffin wax. Transverse sections were cut and stained with Hematoxylin-Eosin according to a standard methods.

Chromatin immunoprecipitation (ChIP) assay

For in vivo ChIP experiments, extracts were prepared from 20 E8.75-9.5 wild-type mouse hearts. Embryos were dissected in ice-cold PBS. Following gentle pipetting, tissue was crosslinked with 2% formaldehyde for 2 hours at room temperature. Chromatin extraction and immunoprecipitations were performed using a ChIP assay kit (Upstate, 17-295) according to manufacturer's protocol. Protein-DNA crosslinking was reversed by overnight incubation at 65°C. A PCR purification kit (QIAGEN, 28106) was used to recover DNA in 50 μl. The following PCR primers against the 5′ Tbx2 promoter region were used: P-813 (5′-CTCCCTCTGAAGTGCATGGAC-3′) and P-573 (5′-AGCGCAGAGGACCGATCTGAC-3′). As control, primers against an unrelated region of Tbx2 promoter region were used: primer E (5′-CTCTGGTTCCTAGGCAGGACTCGG-3′) and primer F (5′-TCC-TCTGCAGTCTGCCTGTCTGTG-3′).

The following PCR primers against the Nmyc1 intron 1 promoter region were used: P-4030 (5′-CCAGGCAGAGAAATAGCTTTA-GCG-3′) and P-4330 (5′-CCTTTCCATTCCCCTTCCTTCAGA-3′); P4630 (5′-CCAGGCAGTGCCTTGTGTGAAT-3′) and P-4930 (5′-GCCAACCTCCAACTCTACAACC-3′). As control, primers against an unrelated region of Nmyc1 promoter region were used: primer G (5′-GAGGCTATGTGGCTTCTAGGAGAG-3′) and primer H (5′-GGATGTTAGATGTCCAGTCTCACC-3′).

The following PCR primers against the Isl1 5′ promoter region were used: P-842 (5′-CGGGAGGAAAGGAACCAACCT-3′) and P-581 (5′-CCGGAGTAGGACGGTTAGACC-3′). As controls, primers against an unrelated region of Isl1 promoter region were used: primer I (5′-CTCTGGTTCCTAGGCAGGACTCGG-3′) and primer J (5′-GCGGTCTGCTGCTCGGCTCTCAGC-3′).

Tbx20 polyclonal antibody was obtained from Orbigen (PAB-11248) and Tbx2 polyclonal antibody was obtained from Upstate (07-318).

Promoter cloning and luciferase transfection assay

A 1 kb genomic DNA fragment upstream of Tbx2 ATG was amplified with high fidelity DNA polymerase (Novagen, 71086-3) and cloned into pGL3-basic vector (Promega, E1751). Primers were: 5′ primer

5′-CATCAGGGTTCTGCCATGGCTC-3′, 3′ primer 5′-GGCTCTC-TCATCGGGACATCC-3′. A full-length 1.3 kb Nmyc1 intron 1 promoter fragment was cloned into pGL3-basic vector following PCR. Primers were: 5′ primer 5′-AGCGGTACTTGCGAAGCTTC-GA-3′, 3′ primer 5′-CGCCTCTCTTTTAATATCTCCGCT-3′.

A 1.5 kb genomic DNA fragment upstream of Isl1 ATG was amplified and cloned into pGL3-basic vector. Primers were: 5′ primer 5′-GAATTCTGTGTGTCCCCTAATAAC-3′, 3′ primer 5′-AGAGGG-AGTAATGTCCACAGTGAA-3′.

Transfections were carried out in HEK 293 cells according to standard techniques by FUGENE6 (Roche). Cells were lysed 48 hours after transfection, and luciferase and β-galactosidase activities were measured on a Luminoskan Ascent luminometer (Thermo Labsystems). For luciferase reporters, CMV-β-galactosidase was used to control for transfection efficiency. Normalized luciferase activities were compared with a pGL3 control to calculate relative repression. Results are from one representative experiment carried out in triplicates and expressed as mean±s.d. At least three independent transfection experiments were performed.

Site-directed mutagenesis

The QuickChange sited-directed mutagenesis kit (Stratagene, 200518) was used to make point mutations in T-box binding sites in the promoter region according to the manufacture's protocol.

Whole-mount cell death (TUNEL) assay

Whole-mount TUNEL staining was performed with In Situ Cell Death Detection Kit (Roche, 1 684 817) followed a modified protocol (Chi et al., 2003; Hensey and Gautier, 1998; Yamamoto and Henderson, 1999).

Whole-mount proliferation assay

The whole-mount mouse embryo cell proliferation assay was performed as described (Nagy et al., 2003). Anti-phospho-Histone H3 (Ser10) (1:100 dilution) was obtained from Upstate (06-570).

Results

Tbx20-null mice are embryonic lethal and exhibit unlooped severely hypoplastic hearts

To investigate the role of Tbx20 in mammalian heart development, we generated *Tbx20* knockout mice. Homozygous null mice arrested development at E9.0 and died at E10.5. Hearts of Tbx20 mutants exhibited an unusual cardiac phenotype, somewhat resembling an 'hourglass', and were unlooped and severely hypoplastic (Fig. 1E-L).

Myocardial differentiation and anteroposterior patterning occur in Tbx20 mutant hearts

Tbx20 is expressed in throughout early differentiating myocardium, suggesting that it might play a role in differentiation. To investigate this, we performed whole-mount in situ hybridization to examine RNAs encoding myofibrillar proteins myosin light chain 2a (MLC2a) and β myosin heavy chain (β-MHC), both expressed throughout myocardium, myosin light chain 2v (MLC2v), restricted to ventricle and atrioventricular canal, and α myosin heavy chain (α-MHC), restricted to the atrioventricular canal and forming atrium at early stages. Expression of MLC2a, MLC2v and myosin heavy chain was intact in Tbx20 mutants, demonstrating that myocardial differentiation had occurred. Expression of α-MHC, however, was greatly reduced relative to wild-type controls, suggesting that some aspects of specification or differentiation may be perturbed in *Tbx20*-null mice (Fig. 2A-H).

To determine whether individual cardiac segments were present, we performed whole-mount RNA in situ analysis for markers of distinct cardiac segments and anterior posterior polarity: Tbx5 (left ventricle and atria), Wnt11 (outflow tract, right ventricle and atrioventricular canal) and GATA4 (expressed throughout the heart in an anterior posterior gradient) (Fig. 2I-N). Results demonstrated that overall anteroposterior patterning had occurred in *Tbx20* mutants, although Wnt11 expression in a putative atrioventricular canal region was absent in Tbx20 mutants. This could indicate regional downregulation of Wnt11 or an absence of atrioventricular canal identity.

Tbx2 is upregulated in Tbx20 mutants and is directly repressed by Tbx20

Hearts in *Tbx20*-null mice closely resembled those described for transgenic mice overexpressing Tbx2 in myocardium (Christoffels et al., 2004). Indeed, we found that Tbx2 is highly upregulated in Tbx20 null mice, beginning at early cardiac crescent stages (Fig. 3A-F). These data suggested that upregulation of Tbx2 could account for the heart phenotype in Tbx20 mutants. Transgenic mice overexpressing Tbx2 in myocardium exhibit decreased expression of several genes, including atrial natriuretic peptide (*Nppa*), the muscle-specific gene chisel (*Smpx* – Mouse Genome Informatics) and the transcriptional co-activator *Cited1*. Expression of each of these genes is greatly reduced or absent in *Tbx20* mutants (Fig. 3G-L).

To investigate a potential feedback loop between Tbx20 and Tbx2, we examined expression of *Tbx20* mRNA in *Tbx20* mutants. Although no Tbx20 protein is present in homozygous null mice, *Tbx20* mRNA can still be detected. We observed no differences between Tbx20 expression in wild-type and null mice, demonstrating that the observed regulatory interaction between Tbx20 and Tbx2 is unidirectional, and suggesting lack of autoregulation of Tbx20 at the transcriptional level (Fig. 3M-N).

Tbx2 is most closely related to Tbx3, and the two genes are expressed in an overlapping pattern in developing heart (Hoogaars et al., 2004), suggesting that they may, in some instances, be regulated coordinately. However, Tbx3 expression was unaltered in *Tbx20* mutants (Fig. 3O-P).

We investigated whether upregulation of Tbx2 in Tbx20 mutants reflected direct repression by Tbx20 in wild-type hearts. Analysis revealed two conserved T-box recognition sites between 677-688 bp upstream of a putative transcription start site of the Tbx2 gene. ChIP analysis was performed on extracts from E8.75-E9.5 hearts and demonstrated that Tbx20 protein was recruited to this region, but not to an unrelated 5′ genomic region of Tbx2 (Fig. 3Q). Co-transfection assays with a luciferase reporter driven by a 1 kb Tbx2 promoter and a Tbx20 expression vector demonstrated a fourfold repression of the Tbx2 promoter by Tbx20. This repression was relieved by mutation of the conserved T-box sites (Fig. 3R). These data demonstrated that Tbx20 directly represses Tbx2 within developing heart.

Proliferation, but not apoptosis, is affected in Tbx20 mutants

Reduced heart size in *Tbx20*-null mutants suggested an increase in cell death or decrease in cell proliferation, or both.

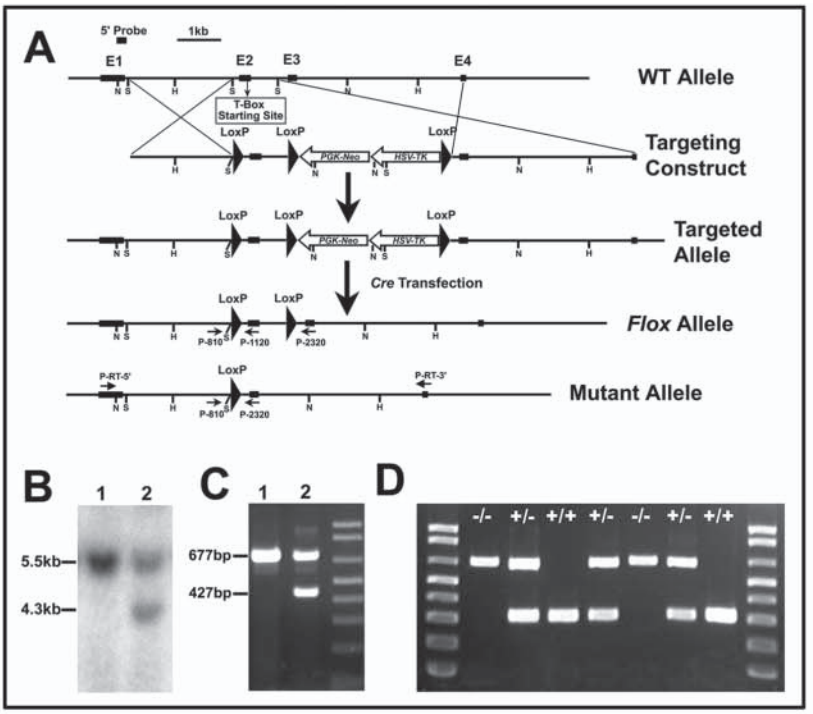
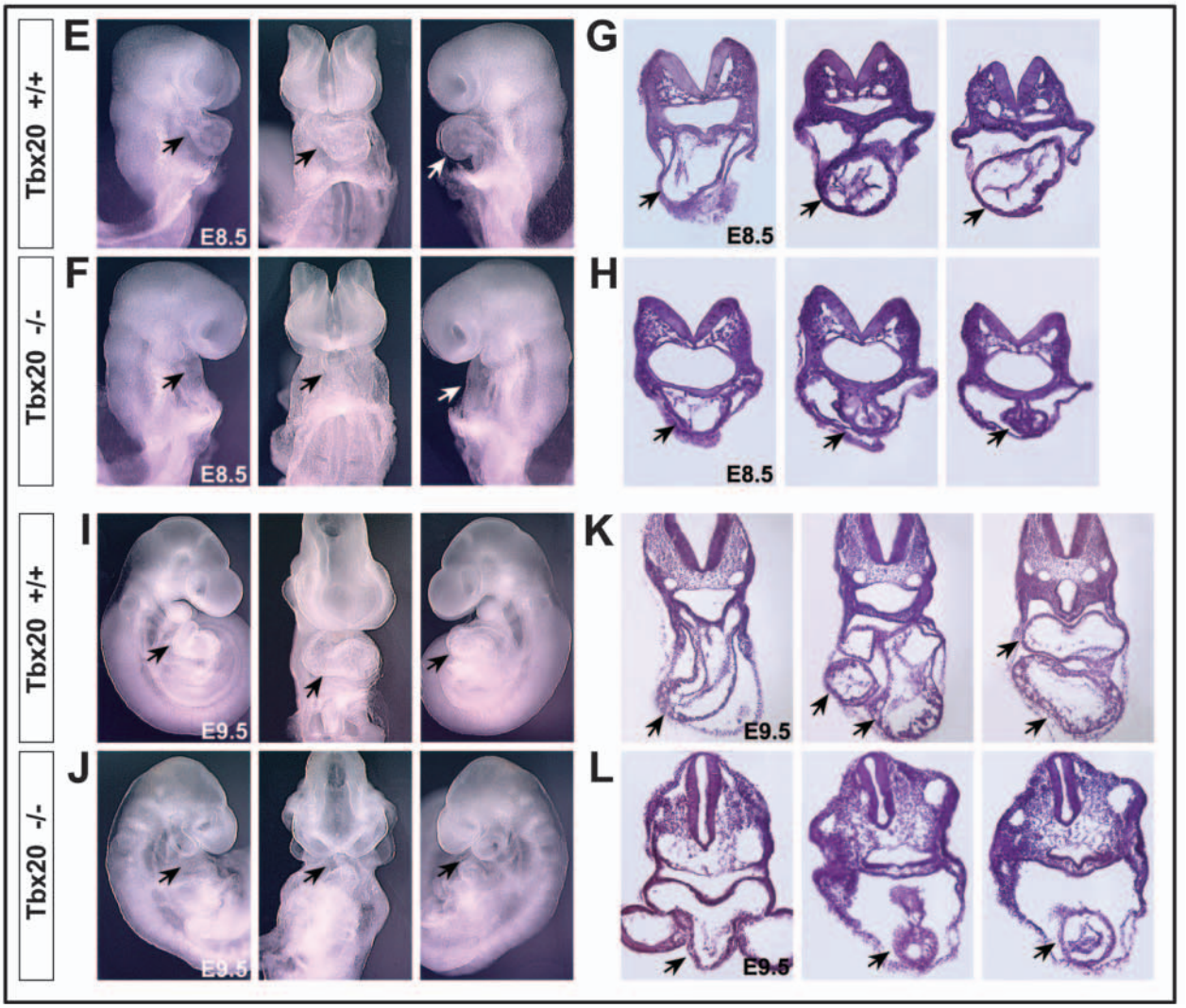


Fig. 1. (A-D) Generation of Tbx20 targeted allele. (A) Two LoxP sites were induced into Tbx20 exon 2 where the T-box domain starts. (B) Southern blot of ES cell DNA digested with *Nco*I and hybridized with a genomic fragment external to the targeting construct with wild-type band 5.5 kb and recombinant band 4.3 kb. Recombinant ES cells were then transfected with a Cre plasmid in order to remove PGKNeo, HSV-TK cassettes. *Tbx20*-null mice were obtained by crossing mice with *Tbx20* floxed allele to protamine-Cre mice. (C) RT-PCR using whole heart RNA obtained from adult wild-type (lane 1) and heterozygous mice (lane 2) with primers located in exon 1 (P-RT-5′) and exon 3 (P-RT-3′) showed the wild-type band 677 bp and mutant band 427 bp. Excision of exon 2 of Tbx20 created a new immediate stop codon within exon 3. (D) Genotypes determined by PCR of one littermate embryos from heterozygous cross. The wild-type allele band was 310 bp and the mutant allele band was 650 bp. (E-L) Whole-mount (E,F,I,J) and histological (G,H,K,L) views of *Tbx20*-null mice and littermate controls at E8.5 and E9.5. The left, middle and right columns give right, frontal and left views, respectively. Hearts of *Tbx20*-null mice are severely hypoplastic relative to control littermates at both stages. Arrows indicate the heart region.



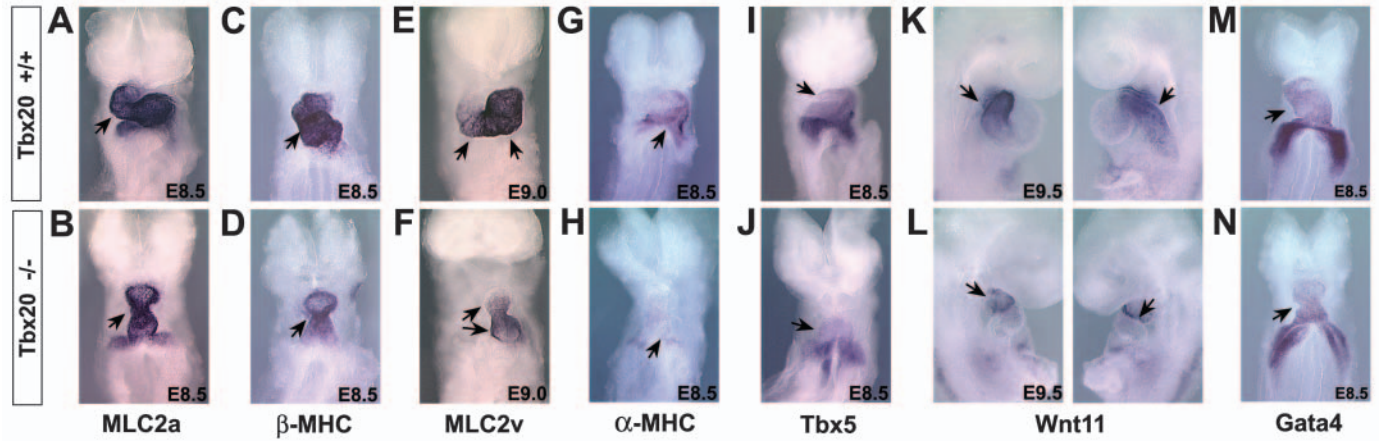


Fig. 2. Whole-mount in situ analysis of *Tbx20*-null mutants and control littermates. (A-H) Differentiation markers MLC2a, β-MHC and MLC2v are normally expressed in *Tbx20* mutants; α-MHC, however, is downregulated. (I,J) Tbx5 is expressed in left ventricular, atrioventricular and atrial progenitors in an anteroposterior gradient. This expression profile is maintained in *Tbx20* mutants. (K,L) Wnt11 is expressed in outflow tract and atrioventricular canal. In *Tbx20* mutants, outflow tract expression is present, but atrioventricular canal expression is lacking. (M,N) GATA4 is expressed throughout the heart in an anteroposterior gradient that is maintained in *Tbx20* mutants. Arrows indicate the corresponding heart regions in wild type and mutant embryos.

TUNEL assays demonstrated no differences in apoptosis between wild-type or mutant embryos at E7.5 or E8.5 (Fig. 4A-D). Whole-mount immunostaining with antibody to phosphorylated histone H3, however, demonstrated decreased proliferation in *Tbx20* mutant hearts relative to hearts of wild-type littermates at E8.0 and E8.5 (Fig. 4E-L). Proliferation rates were assessed by examination of sections. At E8.0, the number of phosphorylated histone H3 positive nuclei within myocardium of *Tbx20* mutants was reduced from 3.5% in wild type to 1.4% in mutants. At E8.5, the number of positive nuclei was reduced from 3.7% in wild type to 1.0% in mutants, indicating significant reduction in proliferation rate in myocardium of *Tbx20* mutants. To ensure that the proliferative decrease in heart was specific, we assessed proliferation rates in neural folds, which do not express Tbx20 at this stage, and found no significant difference between wild-type and mutant embryos (5.9% and 5.7%, respectively).

Nmyc1 and cyclin A2 are downregulated in Tbx20 mutant hearts

Decreased proliferation in Tbx20 mutant hearts suggested downregulation of genes important for cell cycle regulation in cardiomyocytes. Nmyc1 is required for early myocardial proliferation (Davis and Bradley, 1993). Cyclin A2 is required for proliferation in early embryos, and is implicated in myocyte proliferation (Chaudhry et al., 2004; Murphy et al., 1997). We found expression of both genes was severely downregulated in *Tbx20* mutant hearts, consistent with observed defects in proliferation (Fig. 5A-H).

Regional variation in expression of Nmyc1, cyclin A2 and phosphorylated histone H3 correlates with Tbx2 expression

During our analysis, we observed local differences in expression of cyclin A2 and Nmyc1 within wild-type heart (Fig. 5A,E,C,G). Regions that had relatively low levels of expression were similar for cyclin A2 and Nmyc1, and included the outflow tract and atrioventricular canal. These regions are those in which Tbx2 is expressed, and suggested that one of these genes might be a direct target of Tbx2. Bioinformatic analysis of 2 kb upstream of the translation start codon or 2 kb downstream, including intron 1, of cyclin A2 did not reveal any conserved T-box sites between human and mouse. However, similar analysis of sequences within the *Nmyc1* gene revealed two clusters of conserved T-box sites within intron 1 (Fig. 5M). These sites are within regions previously defined to be required for expression of Nmyc1 both in vitro and in vivo (Strieder and Lutz, 2002).

Tbx2 directly represses *Nmyc1*

ChIP analysis with E8.75-E9.5 embryonic heart extracts revealed that Tbx2 was recruited to both clusters of T-box sites within intron1 of Nmyc1 (Fig. 5M-1,M-2). Control sites further upstream were negative (Fig. 5M-3). Co-transfection of an Nmyc1 intron 1-luciferase reporter with a Tbx2 expression vector in HEK293 cells demonstrated significant downregulation of the Nmyc1 promoter fragment by Tbx2 (Fig. 5O). By contrast, similar studies with Tbx20 demonstrated in vivo binding, and in vitro activation of the Nmyc1 promoter fragment by Tbx20, demonstrating specificity of repression by Tbx2 (Fig. 5N). Repression by Tbx2 of the Nmyc1 promoter was dose dependent. These data demonstrate that Tbx2 directly binds to T-box consensus sites within intron 1 of the *Nmyc1* gene to repress transcriptional activity of Nmyc1.

Tbx20 is required for expression of Bmp2 and Bmp5

Our data demonstrated downregulation of *Nmyc1* in *Tbx20* mutants. Cardiac hypoplasia in *Tbx20* mutants, or myocardial-*Tbx2* transgenics (Christoffels et al., 2004), however, appears to be more severe than observed in *Nmyc1*-null mice (Charron et al., 1992; Moens et al., 1993; Sawai et al., 1993), suggesting that perturbation of genes in addition to *Nmyc1* might account for the severity of the growth phenotype in *Tbx20*-null mice. Accordingly, we examined expression of bone morphogenetic

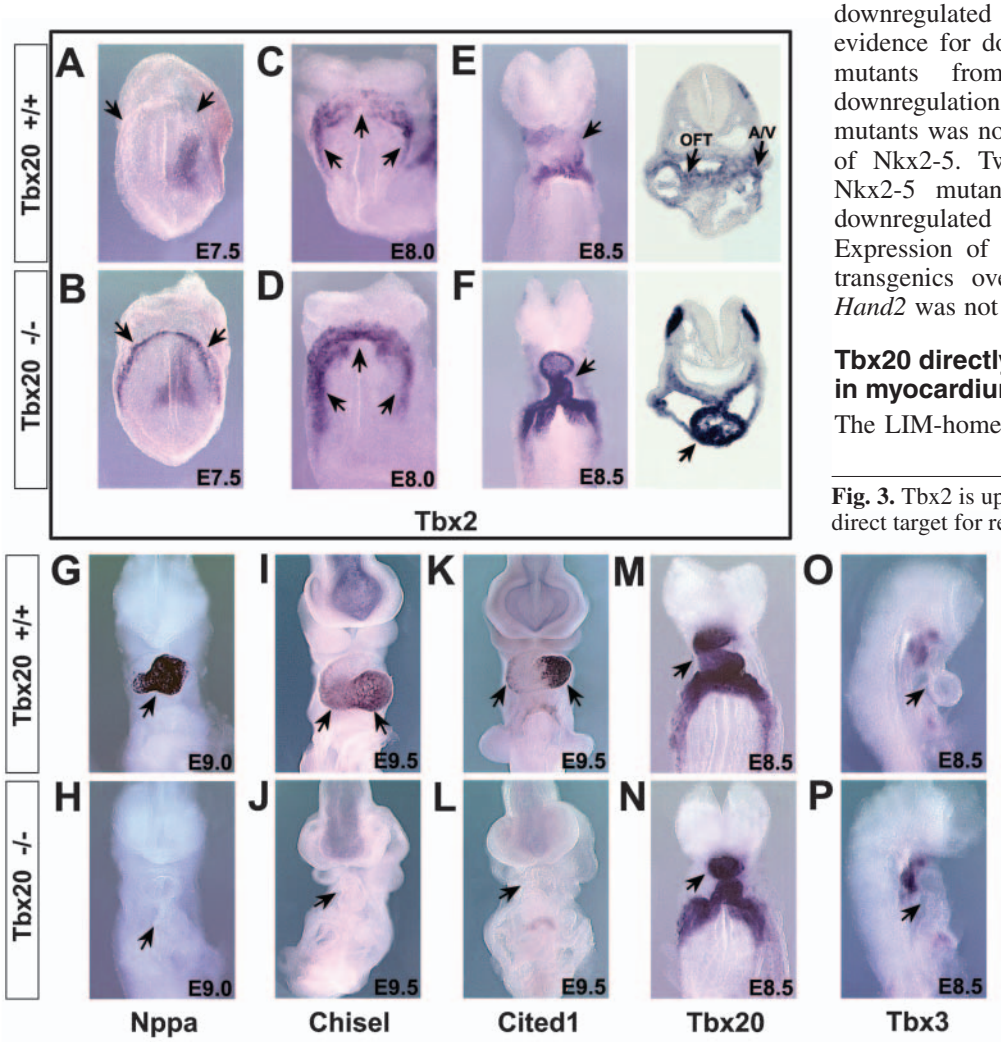
proteins, which have been demonstrated to play a role in early myocardial growth, often in a redundant fashion (Kim et al., 2001; Liu et al., 2004; Solloway and Robertson, 1999; Zhang and Bradley, 1996). Results demonstrated that expression of Bmp4 and Bmp7 was not downregulated in *Tbx20*-null mice, whereas expression of Bmp2 and Bmp5 was severely downregulated (Fig. 6).

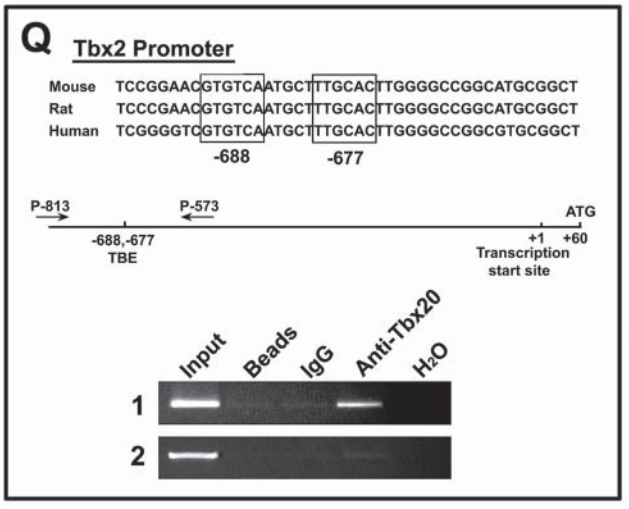
Tbx20 is required for expression of genes that also require Nkx2-5 expression

Several genes downregulated in transgenic mice overexpressing Tbx2 in myocardium (Christoffels et al., 2004), and in *Tbx20* knockouts, are also downregulated in *Nkx2-5* knockout mice (Harvey, 2002), including *Nppa*, chisel and *Cited1*. Accordingly, we investigated whether Nkx2-5 was downregulated in *Tbx20* mutants. We found no evidence for downregulation of Nkx2-5 in *Tbx20* mutants from E8.5-E9.25, suggesting that downregulation of *Nppa*, chisel and *Cited1* in *Tbx20* mutants was not a consequence of downregulation of Nkx2-5. Two other genes downregulated in Nkx2-5 mutants, *Hand1* and *Irx4*, were also downregulated in *Tbx20* mutants (Fig. 7). Expression of these genes was not examined in transgenics overexpressing *Tbx2*. Expression of *Hand2* was not affected in *Tbx20* mutants.

Tbx20 directly represses expression of Isl1 in myocardium

The LIM-homeodomain transcription factor Isl1 is





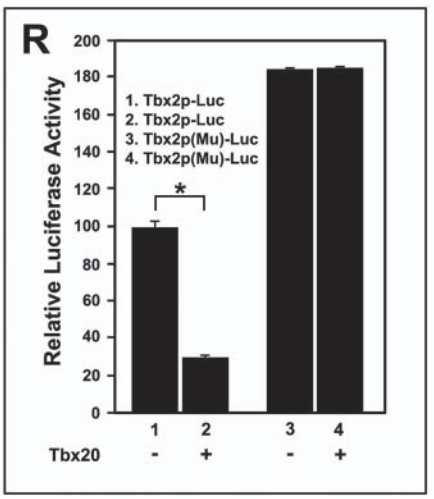


Fig. 3. Tbx2 is upregulated in Tbx20 mutants and is a direct target for repression by Tbx20. (A-F) Whole-mount RNA in situ and section analysis for Tbx2 expression in *Tbx20*-null embryos and wild-type littermates. Arrows indicate the corresponding heart regions in wild type and mutant embryos. OFT, outflow tract; A/V, atrioventricular canal. (G-L) Whole-mount RNA in situ demonstrates downregulation of Nppa, chisel and cited 1 in *Tbx20* mutants. (M-P) Expression of Tbx20 and Tbx3 is unaffected. Arrows indicate the corresponding heart regions in wild type and mutant embryos. (Q) ChIP analysis with embryonic heart extracts revealed binding of Tbx20 to region containing conserved T-box sites within Tbx2 promoter (lane 1, primer P-813, P-573). ChIP analysis with primers against an unrelated promoter region revealed no Tbx20 recruitment (lane 2) (see Materials and methods for primers). No recruitment was found with beads, IgG or H_2O. (R) Co-transfection of Tbx20 expression vector (500 ng) with Tbx2 promoter-luciferase constructs (100 ng) into HEK293 cells demonstrates repression by Tbx20, which was abrogated by mutation (Mu) of conserved T-box elements within the Tbx2 promoter. *P<0.05, paired t-test.

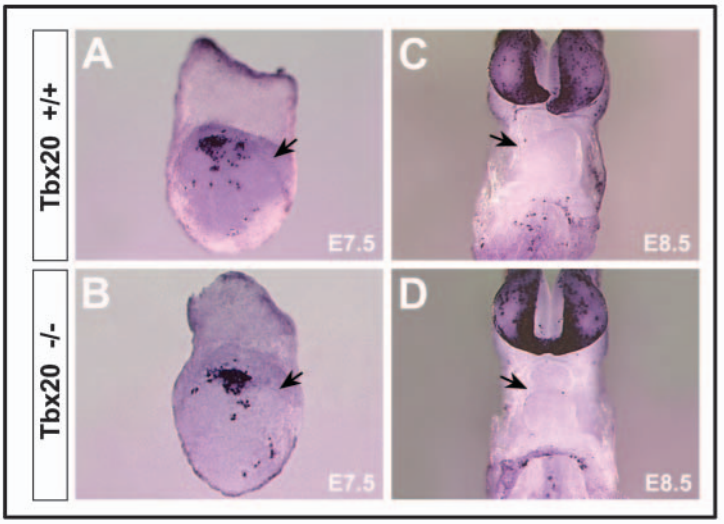
required for proliferation, survival and migration of a subset of undifferentiated cardiac progenitors, and is downregulated as they enter the heart and differentiate (Cai et al., 2003). We observed that Isl1 is upregulated throughout the heart of Tbx20 mutants (Fig. 8A-D) and subsequently demonstrated by ChIP analysis and transfection studies that Tbx20 directly binds and represses conserved T-box sites within Isl1 promoter sequences (1.5 kb upstream of ATG) (Fig. 8E-F). Functional consequences of this upregulation are not yet clear, but it is unlikely that Isl1 upregulation contributes substantially to the *Tbx20*-null phenotype, as hearts of transgenic mice expressing Isl1 at comparable levels throughout myocardium appear relatively normal at E10.5 (Fig. 8G-J).

Discussion

In these studies, we have identified two direct targets for repression by Tbx20, including Tbx2 and Isl1. Interactions between Tbx20 and Tbx2 are required for regional proliferation, morphogenesis and aspects of specification in early heart, while Isl1 is required for proliferation, survival and migration of a subset of undifferentiated cardiac progenitors (Cai et al., 2003). As these progenitors enter the heart and differentiate, Isl1 is downregulated. Factors regulating its expression in this context have not been defined previously. Although functional consequences of Isl1 expression throughout myocardium remain to be explored, evidence from transgenic mice expressing Isl1 throughout myocardium (Fig. 8G-J) have demonstrated that upregulation of Isl1 does not account for the cardiac phenotype in *Tbx20* mutants.

Our studies suggest that T-box genes regulate the *Nmyc1* promoter with consequences for organ morphogenesis and implications for human congenital disease and cancer. Tbx2, which is normally expressed in outflow tract and atrioventricular canal, is upregulated throughout the heart in *Tbx20* mutants. The cardiac phenotype of *Tbx20* mutants mimics that of mice overexpressing Tbx2 in myocardium (Christoffels et al., 2004), suggesting that upregulation of Tbx2 could account for the observed cardiac phenotype of *Tbx20*-null mice. We have provided evidence that *Tbx2* is a direct target of Tbx20 in developing heart. In vivo ChIP analysis performed on embryonic heart extracts has demonstrated direct binding of Tbx20 to a region of the *Tbx2* promoter containing conserved T-box consensus sites. Transfection studies demonstrated that activity of this promoter was repressed by co-transfection with a *Tbx2* expression vector, in a manner dependent on presence of conserved T-box sites within the promoter. It should be noted that Tbx2 and Tbx20 are co-expressed in outflow tract and atrioventricular canal, suggesting that repression of *Tbx2* by Tbx20 is context dependent.

To define targets of Tbx2 that could explain defects in proliferation, we examined two cell cycle genes previously implicated in cardiomyocyte proliferation, cyclin A2 and Nmyc1 (Chaudhry et al., 2004; Davis and Bradley, 1993; Murphy et al., 1997), and found that both were downregulated in *Tbx20* mutant hearts. During our analysis, we observed similar regional differences in expression of both these genes in wild-type heart. Regions of relatively low expression coincided with regions expressing Tbx2, consistent with the idea that Tbx2 might be suppressing proliferation by acting directly on either cyclin A2 or Nmyc1. No consensus T-box sites were identified in putative promoter regions of cyclin A2. However, a cluster of conserved T-box sites was identified within intron 1 of Nmyc1. This intron is within a human



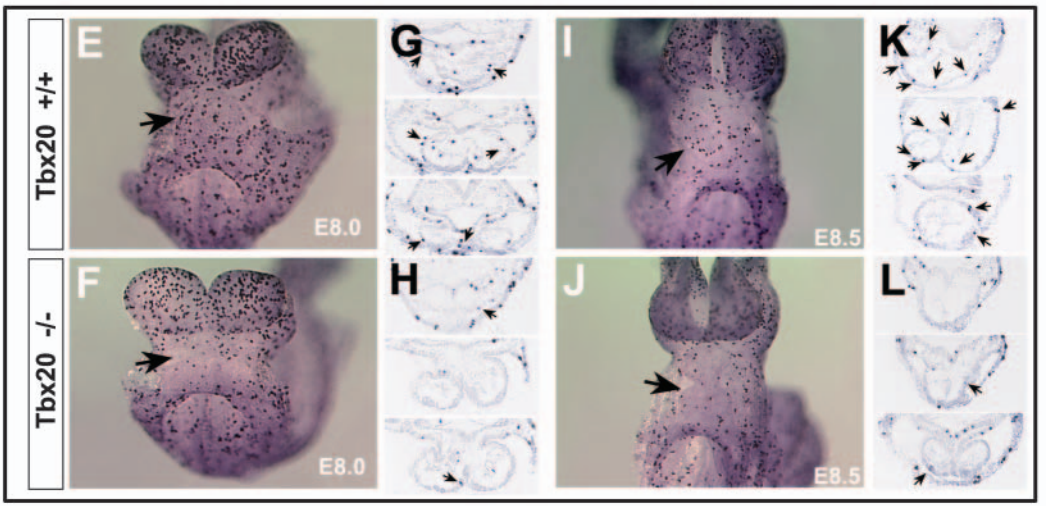


Fig. 4. Apoptosis and proliferation assays in *Tbx20*-null mutants and control littermates. (A-D) TUNEL analysis revealed no increase in apoptosis in *Tbx20*-null embryos relative to control littermates. Arrows indicate cardiac crescent (A,B) and heart tube (C,D). (E-L) Antibody staining (whole mount and sections) for phosphorylated histone H3 reveals decreased proliferation in *Tbx20*-null mutants relative to control littermates. Arrows indicate positive phosphorylated histone H3 staining in cardiomyocytes.

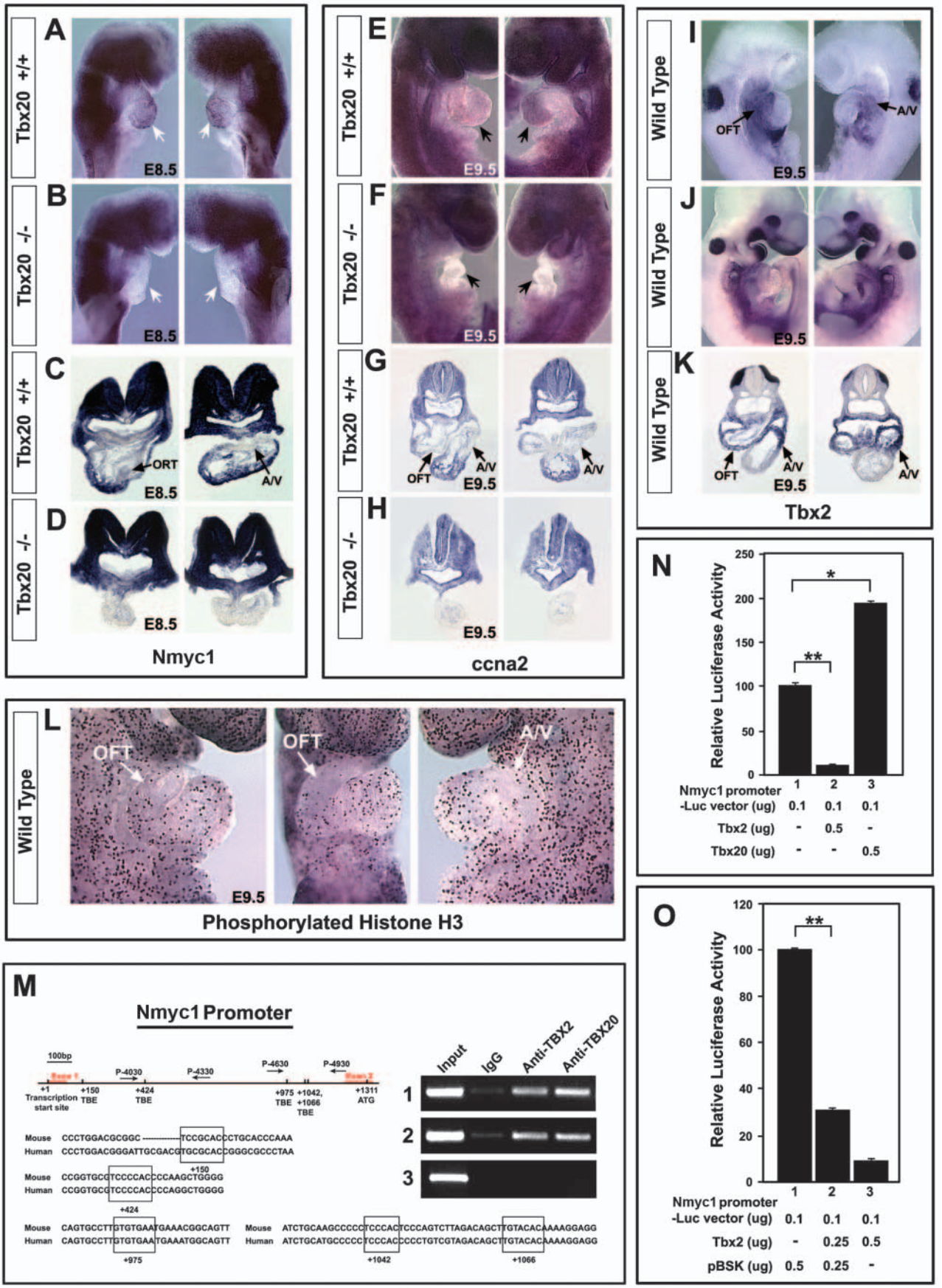


Fig. 5. See next page for legend.

Fig. 5. Tbx2 directly binds *Nmyc1* and represses its expression in regions of relatively low proliferation within the heart. (A-H) Regions of relatively low proliferation; outflow tract (OFT) and atrioventricular canal (A/V) are indicated by arrows. Corresponding sections (C,D; G,H; K) are shown progressively from anterior to posterior, respectively. Expression of Nmyc1 (A-D) and cyclin A2 (E-H) is downregulated in *Tbx20*-null mutants. Section analysis of wild-type littermates reveals regional differences in expression of Nmyc1 (C) and cyclin A2 (Ccna2) (G), with relatively low levels in OFT and A/V. (I-K) Wild-type Tbx2 expression is complementary to that of Nmyc1 and cyclinA2. (L) Expression of phosphorylated histone H3 in wild-type embryos revealed regions of low proliferation within developing heart. The left, middle and right panels show right, frontal and left side views, respectively. (M) ChIP analysis with embryonic heart extracts demonstrated recruitment of Tbx2 and Tbx20 to regions containing T-box consensus sites within intron 1 of the *Nmyc1* gene (lane 1, primer P-4030, P-4330; lane 2, primer P-4630, P-4930). ChIP analysis with primers against an unrelated promoter region revealed no Tbx2 recruitment (lane 3) (see Materials and methods for primers). No recruitment was found with IgG. (N) Co-transfections of Tbx2 or Tbx20 expression vectors with Nmyc1 intron 1-luciferase reporter into HEK293 cells demonstrated repression or activation, respectively. $^{**}P<0.005$, paired *t*-test; $^{*}P<0.05$, paired *t*-test. (O) Co-transfections of Tbx2 expression vector with Nmyc1 intron 1-luciferase reporter into HEK293 cells demonstrated dose-dependent repression by Tbx2. $^{**}P<0.005$, paired *t*-test.

NMYC1 transgene that recapitulates expression pattern of the endogenous Nmyc1 gene in newborn mice (Zimmerman et al., 1990). Sequences within exon1 and/or intron 1 of Nmyc1 direct tissue-specific expression in cancer cell lines, and contain both positive and negative regulatory elements, some acting at a post-transcriptional level (Strieder and Lutz, 2002).

Nmyc1 is expressed in early myocardial cells, and is required for normal proliferative growth of the heart (Charron et al., 1992; Moens et al., 1993; Sawai et al., 1993). Mice that are homozygous null for *Nmyc1* arrest their development at ~E9.5, and die between E10.5 and E11.5, with severely hypoplastic hearts, undivided thin-walled ventricles and few trabeculae. Factors that regulate *Nmyc1* expression during embryogenesis have not been defined previously. We have found that Tbx2 directly binds to Nmyc1 enhancer elements with conserved T-box sites in early developing heart, and can repress Nmyc1 promoter activity in transfection studies. Tbx20 is also able to bind to this Nmyc1 enhancer, but does not repress its activity in our in vitro assay system. This observation suggests the possibility that Tbx20 may block Tbx2 repression of Nmyc1 by competitive binding. Although both *Tbx20* and *Nmyc1* mutants have hypoplastic hearts, the cardiac hypoplasia in *Tbx20* mutants is more severe than that of *Nmyc1* mutant mice. Close resemblance of the Tbx20 cardiac phenotype to that of β-MHC-Tbx2 transgenic mice suggests that aberrant regulation of additional downstream targets of Tbx2 may work in concert with Nmyc1 repression to contribute to the severely hypoplastic phenotype. In this regard, we observed downregulation of two BMP growth factor genes, *Bmp2* and *Bmp5*, in *Tbx20* null mice. Previous studies have demonstrated that ablation of these genes can affect early myocardial development (Solloway and Robertson, 1999; Zhang and Bradley, 1996). Intriguingly, studies in chick embryos have demonstrated that Bmp2 can induce expression of Tbx2 in heart (Yamada et al., 2000). Here, we observe downregulation of *Bmp2* in a situation where Tbx2 is overexpressed in the heart, suggesting that there may be a negative feedback loop between *Bmp2* and Tbx2. Interactions between Tbx2, Tbx20 and BMPs will be a subject for future investigation.

Our results suggest a model in which regional expression of Tbx2 in outflow tract and atrioventricular canal suppresses Nmyc1 expression to result in relatively low rates of proliferation. By contrast, in chamber myocardium, Tbx20 represses *Tbx2*, preventing its repression of *Nmyc1* and allowing for higher rates of proliferation. Regulation of *Nmyc1* by Tbx2 was dose dependent, suggesting that differential proliferation rates can be regulated by the amount of Tbx2 present.

Our data have demonstrated a role for Tbx20 in control of regional proliferation at the early heart tube stage. Mutations in other genetic pathways have demonstrated a role in later growth of ventricular myocardium, resulting in mid-gestational embryonic lethality. These include neuregulin/erbB signaling from endocardium to myocardium (Carraway, 1996; Negro et al., 2004), Fgf/Fgfr signaling from endocardium and epicardium to myocardium (Lavine et al., 2005), and Bmp10 signaling within myocardium, negatively regulated by Nkx2-5 (Chen et al., 2004; Pashmforoush et al., 2004). As Tbx20 is

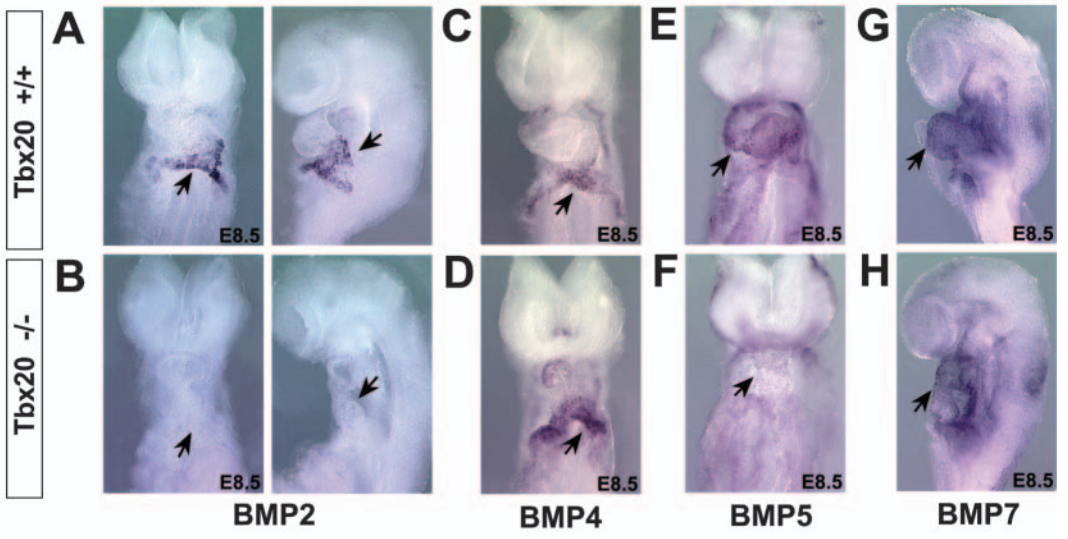


Fig. 6. Tbx20 regulates expression of a subset of BMP genes. Expression of Bmp4 (C,D) and Bmp7 (G,H) are not downregulated in *Tbx20* mutants, whereas expression of Bmp2 (A,B) and Bmp5 (E,F) is severely downregulated specifically in the heart, as indicated by arrows. The left and right images in A and B show frontal and left views, respectively.

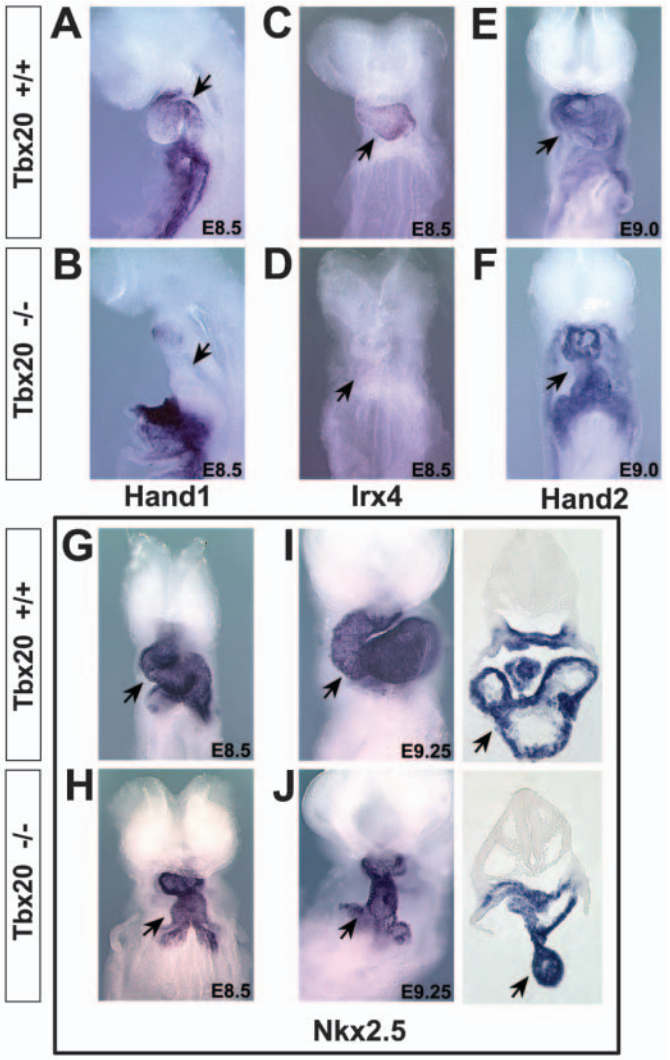


Fig. 7. Tbx20 and Nkx2-5 independently regulate common downstream targets. (A-D) Expression of Hand1 and Irx4 is downregulated in *Tbx20* mutants. (E,F) Hand2 and (G-J) Nkx2-5 expression are unaffected in *Tbx20* mutants. Images in A,B show left side views and images in C-J show frontal views. Arrows indicate the corresponding heart regions in wild type and mutant embryos.

expressed in ventricular myocardium during midgestation, it may also be working in concert with these pathways to control later cardiac growth.

Evidence suggests that Tbx2 may regulate *Nmyc1* in other contexts. Downregulation of *Nmyc1* is an early response in retinoic acid-induced differentiation of neuroblastoma cells; Tbx2 is expressed in neuroblastoma cells, as demonstrated by microarray analysis (Schulte et al., 2005; Thiele et al., 1985). It will be of great interest to investigate interactions between *Nmyc1* and Tbx2 in neuroblastoma. In melanocytes and melanoma cells, *Tbx2* is a direct target of Mitf, a basic helix-loop-helix leucine zipper transcription factor that is central to pathways controlling proliferation and differentiation of melanoblasts and melanocytes, and is likely to be a negative regulator of cell cycle progression (Vance and Goding, 2004). B16 melanoma cells differentiate in response to retinoic acid and Tbx2 is an immediate-early target (Niles, 2003). These data suggest that Tbx2 plays a key role in cell cycle regulation in both melanocytes and melanoma cells, and that *Nmyc1* may be a target of Tbx2 in this context.

A role for Tbx2 in cancer is postulated from the observation that overexpression of Tbx2 allowed bypass of senescence in cells predisposed to senesce; Tbx2 is amplified in breast, ovarian and pancreatic cancer cells (Rowley et al., 2004). Tbx2 can bypass senescence by direct repression of senescence genes. If Tbx2 represses *Nmyc1* in cancer cells, consequences of this repression may depend on relative expression levels of Tbx2 and Nmyc1. For example, high levels of Nmyc1 can trigger senescence. Therefore moderate downregulation of *Nmyc1* by Tbx2 in this context could bypass senescence, promoting immortalization. However, severe downregulation of *Nmyc1* by Tbx2 could prevent proliferation, promoting differentiation and rendering transformation less likely. Bypass of senescence is a property of stem cells (Park et al., 2004). The ability of Tbx2 to bypass senescence, either by targeting of senescence genes or downregulation of *Nmyc1* suggests that Tbx2 might play a role in stem cell maintenance.

The T-box genes Tbx2, Tbx3, Tbx4 and Tbx5 are closely related, and may have arisen from an ancient duplication that gave rise to the precursor genes Tbx2/3 and Tbx4/5 (Agulnik et al., 1996). Tbx2 and Tbx3 are expressed in a partially overlapping manner during development, particularly in the heart. *Tbx2* knockout mice are embryonic lethal between E11.5 and E14.5, and exhibit morphological defects in outflow tract and in atrioventricular canal (Harrelson et al., 2004). No proliferative differences were observed in the heart of *Tbx2*-null mice and wild-type littermates, despite previous data demonstrating a role for Tbx2 in cell cycle control. One potential reason for this is functional redundancy between Tbx2 and Tbx3, as both have been shown to repress the same senescence genes, are amplified in breast cancer and cooperate with oncogenes to transform cells (Rowley et al., 2004). These observations suggest that Tbx3 may also target *Nmyc1*. Tbx3 is highly expressed in the developing central conduction system of the heart, a region characterized by low rates of proliferation (Hoogaars et al., 2004; Sedmera et al., 2003). Tbx3 is mutated in ulnar mammary syndrome, where phenotypic defects may result from proliferative abnormalities (Bamshad et al., 1999); it will be of interest to examine the role of Tbx3 and Nmyc1 in this regard. Recent microarray analysis has demonstrated high levels of Tbx3 expression in an Acth (adrenal corticotropic hormone; Pomc1 – Mouse Genome Informatics) -producing small cell lung carcinoma (Turney et al., 2004). Small cell lung carcinomas are associated with Nmyc1 amplification. Investigating potential interactions between Tbx3 and Nmyc1 in this context is warranted.

Tbx5 has been shown to exhibit antiproliferative activity in cardiomyocytes (Hatcher et al., 2001). A missense mutation that causes Holt-Oram syndrome can act in a dominant-negative fashion to counteract the antiproliferative activity of the wild-type gene. The close relationship of Tbx2 and Tbx5 suggest that perhaps Tbx5 also can target the conserved T-box elements within the Nmyc1 promoter. Tbx5 is highly expressed in the atrioventricular canal and in the developing cardiac conduction system, regions of relatively low proliferative activity (Hoogaars et al., 2004).

In addition to their role in proliferation, Tbx2 and Tbx20 regulate genes that specify regional identity within the heart.

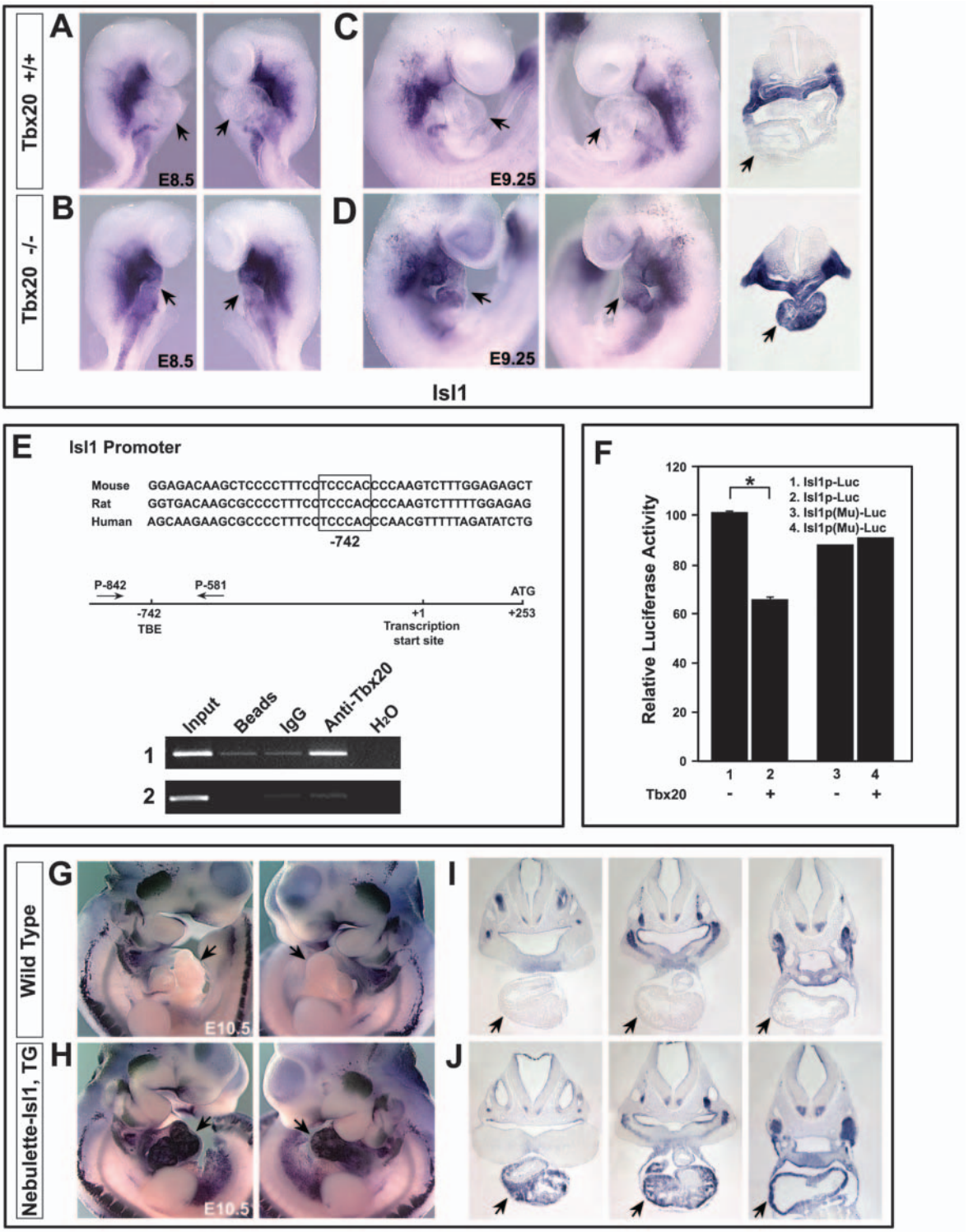


Fig. 8. Tbx20 directly binds and represses Isl1 in myocardium. (A-D) Isl1 is expressed throughout myocardium in *Tbx20* mutants. (E) ChIP analysis revealed Tbx20 recruitment to region of Isl1 promoter with conserved T-box site (lane 1, primer P-842, P-581). ChIP analysis with primers against an unrelated promoter region revealed no Tbx20 recruitment (lane 2) (see Materials an methods for primers). No recruitment was found with beads, IgG or H_2O. (F) Co-transfection of Tbx20 expression vector (500 ng) with Isl1 promoter-luciferase reporter (100 ng) demonstrated repression of *Isl1* by Tbx20. Repression was abrogated by mutation of the consensus T-box element within the *Isl1* promoter. **P*<0.05, paired *t*-test. (G-J) The nebulette promoter, expressed exclusively in myocardium during early embryogenesis (J.C., unpublished) was used to drive expression of Isl1 in transient transgenic mice. Whole-mount in situ hybridization (G,H) revealed expression of Isl1 throughout myocardium in transgenic mice (H). Hearts of transgenics appeared normal at E10.5. The left and right images in A-D,G,H show right and left side views. Corresponding sections are shown progressively from anterior to posterior (C,D; I,J). Arrows indicate the corresponding heart regions in wild type and mutant embryos.

Loss-of-function studies have demonstrated that Tbx2 is required in atrioventricular canal to repress expression of chamber-specific genes *Cited1*, chisel and *Nppa* (Harrelson et al., 2004). These chamber-specific genes are downregulated in β-MHC-Tbx2 transgenics (Christoffels et al., 2004) and in Tbx20 mutants, in which Tbx2 is similarly upregulated throughout myocardium. Tbx20, however, may also be required to activate expression of chamber specific genes, as Tbx20 and Nkx2-5 can cooperatively activate the Nppa promoter in some cell contexts (Stennard et al., 2003). We have found two other regionally specific genes that appear to be regulated by Tbx20 independently of Tbx2. Expression of α-MHC is downregulated in *Tbx20* mutants, but is not affected in β-MHC-Tbx2 transgenics; Hand1 is downregulated in *Tbx20* mutants, but not affected in Tbx2 knockouts. Another chamber-specific gene, Irx4, which is expressed in ventricular chamber myocardium, is downregulated in *Tbx20*-null mice but was not examined in β-MHC-Tbx2 transgenics. Several genes that are downregulated in *Tbx20* mutants are downregulated in Nkx2-5 (*Nppa*, chisel, *Cited1*, *Hand1*, *Irx4*) and/or *Tbx5* mutants (*Nppa*, *Irx4*) (Harvey, 2002), suggesting that Tbx20, Nkx2-5 and Tbx5 may cooperatively regulate a subset of downstream targets. Mutations in *Nkx2-5* and *Tbx5* cause congenital disease in humans (Seidman and Seidman, 2002), opening the possibility that causative mutations may also be found in *Tbx20*.

In addition to *Nmyc1*, two other downstream targets of Tbx20, *Hand1* and *Irx4*, give cardiac phenotypes in null mice, although their cardiac phenotypes are distinct from those of Tbx20 mutants (Bruneau et al., 2000; Olson, 2004; Srivastava, 1999). These observations demonstrate that, in addition to the control of regional proliferation detailed here, Tbx20 is required for other crucial aspects of heart development. Although both *Tbx20* and *Nmyc1* mutants have severely hypoplastic hearts, the cardiac phenotype in *Tbx20*-null mice is slightly more severe than that of *Nmyc1* mutant mice, suggesting that factors in addition to *Nmyc1* downregulation contribute to the phenotype.

Phenotypes observed following *Tbx20* morpholino injection into zebrafish or *Xenopus* embryos (Brown et al., 2005; Stennard et al., 2003; Szeto et al., 2002) support a conserved role for *Tbx20* in chamber morphogenesis and specification, although downstream targets may be species specific. In zebrafish, morphant hearts exhibit no distinction between chambers, and aberrantly express ventricular myosin heavy chain in atria. In contrast to our results with *Tbx20*-null mice, Tbx5 is strongly upregulated in zebrafish *tbx20* morphants. Expression of *tbx5* in zebrafish differs from that in mouse, becoming restricted to ventricle, not atrium, suggesting species specific differences in the regulation of these genes. *Tbx20* morphant frogs also exhibit severe reduction in heart size. No downstream targets of *Tbx20* were identified in the frog experiments, and *XNppa* expression is unaffected in *Tbx20* morphants. Although *Tbx5* was not a target of *Tbx20* knockdown, combined injection of morpholinos against *Tbx5* and *Tbx20* synergistically affected heart development, suggesting concerted activity of these two transcription factors in cardiogenesis.

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