Supporting Information

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SI Materials and Methods

Mouse Mutant Lines. Lines *Tbx1*^{lacZ} (here referred to as *Tbx1*$^{-}$) (1), *Tbx1*^{neo2} (2), and *Trp53*$^{-}$ mutants (3) were maintained in our SPF colony in a mixed C57Bl6/129SvEv background and genotyped according to the original reports. For timed crosses, developmental stage was evaluated by considering the morning of vaginal plug as embryonic day (E) 0.5. Pifithrin-α (Enzo Life Sciences, BML-GR325) was diluted in PBS and injected at 2.2 mg/kg body weight. Injections were carried out intraperitoneally at E7.5, E8.5, and E9.5. Controls were injected with the same amount of carrier, but without drug.

All animal handling and experimentations were performed in accordance with the regulations of the Italian Ministry of Health.

Quantitative Real-Time PCR, RNA Extraction. RNA was isolated from whole E8.5 WT, *Tbx1*$^{+/-}$, *Trp53*$^{+/-}$, and *Tbx1*$^{+/-}$;*Trp53*$^{+/-}$ embryos, from C2C12 cells or from P19CL6 cells (4) using TRIzol reagent (Invitrogen, 15596018). For cDNA synthesis we started from 300 ng of RNA from E8.5 embryos and 1 μg of RNA from cultured cells using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, 4368814). mRNA expression was evaluated by quantitative real-time PCR carried out using FastStart Universal SYBR Green (Roche, 04913850001), on a 7900HT Fast Real-Time PCR System (Applied Biosystem). Primer sequences are listed in Table S2.

Ink Injection and Histology. Ink injection was performed on E10.5 embryos, which were collected and injected intracardially with India ink, and fixed in 4% (wt/vol) paraformaldehyde. Embryos were then dehydrated and cleared in 1:1 benzyl benzoate:methyl salicylate.

Hearts collected from E18.5 embryos were fixed in 4% paraformaldehyde overnight at 4 °C, embedded in paraffin and cut into 10-μm sections. Histological staining was performed using eosin and counterstaining was performed using Nuclear Fast Red (Vector Labs, H-3403).

For proliferation studies, E9.5 embryos were fixed in 4% paraformaldehyde overnight at 4 °C, embedded in paraffin and cut into 7-μm sections. Double immunofluorescence was performed on sagittal sections using a polyclonal anti–phospho-Histone H3 (Ser10) antibody (Millipore, 06–570), 1:200 and a monoclonal anti-Isl1 antibody (Developmental Studies Hybridoma Bank), 1:50. Secondary antibodies used were goat anti-rabbit Alexa Fluor 488 (Invitrogen, A11034) and goat anti-mouse Alexa Fluor 594 (Invitrogen, A11032), 1:400. We counted Isl1+ and Isl1+;P-H3+ cells in the second heart field (SHF; at least 2,000 cells per embryo) of three somite-matched embryos per genotype.

To test apoptosis, we used an anti–Cleaved Caspase-3 antibody (D175) (Cell signaling, 9661S), 1:100, detected with an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:400).

Coimmunoprecipitation. P19CL6 cells were grown in MEM Eagle Alpha Modification (Sigma Aldrich, M4526) with 10% (vol/vol) FBS, and 2×10^5 cells were plated on a 100-mm culture dish. The next day, cells were transfected with a *Trp53* expression vector or with empty vector. Cells were collected after 48 h, and 200 μg of nuclear extracts were immunoprecipitated with 2 μg of an anti-TBX1 antibody (Abcam, ab18530) or with 2 μg of rabbit IgG (Santa Cruz Biotechnology, 2027) as a negative control and then incubated with 20 μL of Protein A/G PLUS Agarose (Santa Cruz Biotechnology, sc-2003) at 4 °C overnight. The samples were washed seven times with IPP150 buffer (10 mmol/L Tris·HCl, pH 8.0, 150 mmol/L NaCl, 0.1% Nonidet P-40) and resuspended in SDS sample buffer. Ten percent of input and immunoprecipitated samples were used for Western blot analysis. We used an anti-p53 antibody (Santa Cruz Biotechnology, sc-6243) and an anti-Smarcd1 antibody (BD Bioscience, 611728). For p53 immunoprecipitation, 10 μg of the p53 antibody or 10 μg of Control Rabbit IgG were conjugated with Dynabeads Protein G (Novex by Life Technologies, 10004D) and cross-linked using 20 mmol/L Dimethyl pimelimidate dihydrochloride (Sigma Aldrich, D8388). Approximately 150 μg of nuclear extracts were immunoprecipitated at 4 °C overnight. Samples were then washed six times with cold PBS-0.01% Tween-20 and protein complexes were eluted in 30 μL of 0.1 mol/L Glycine, pH 2.6, at room temperature, for a total of three elutions. Protein denaturation was done at 95 °C for 5 min in SDS sample buffer. Twenty percent of input and immunoprecipitated samples were subjected to Western blot analysis. We used an anti-Ezh2 antibody (BD Biosciences, 612666) 1:1,000 and an anti-p53 antibody (Cell signaling, 2524), 1:1,000.

Chromatin Immunoprecipitation (ChIP) and Western Blotting. To induce differentiation, we used a published protocol (4). Briefly, 3×10^6 P19CL6 cells were plated on a 100-mm tissue culture dish. The next day (day 0 of differentiation), cells were treated with 10 μmol/L 5-Azacytidine (Sigma Aldrich, A2385) for 24 h, and subsequently, cells were incubated with 1% (vol/vol) DMSO. For ChIP assay, P19CL6 cells were collected at day 2 of differentiation. Cells were fixed with 1% (vol/vol) formaldehyde diluted in PBS and Buffer A (Transcription Factor ChIP Kit reagent, Diagenode, Kch-redTBP-012) at room temperature for 10 min. The cross-linking reaction was stopped using 0.125 mol/L glycine at room temperature for 5 min. Cells were collected by scraping with cold PBS, cells were then lysed and chromatin was sonicated to obtain 150- to 350-bp fragments using S2 Covaris System (Duty Cycle: 5%, Intensity: 3, Cycles/Burst: 200, Cycles: 5, Cycles time: 60 s, Temperature: 4 °C). Sonicated chromatin (6 μg) was immunoprecipitated with 5 μg of a TBX1 antibody (Abcam, ab18530), 5 μg of a p53 antibody (Santa Cruz Biotechnology, sc-6243), 5 μg of a Trimethyl-Histone H3 (Lys27) antibody (Millipore, 17–622), or 5 μg of Rabbit Control IgG (Abcam, ab46540). Samples were then incubated with 30 μL of Preblocked protein A/G coated beads at 4 °C overnight. After incubation, the samples were washed and DNA–protein–antibody complexes were eluted in Buffer F of the above-mentioned kit, at room temperature for 25 min. To reverse cross-linking, samples were incubated at 65 °C overnight. Phenol/chloroform-purified and ethanol-precipitated DNA was resuspended in 200 μL of H_2O and equal DNA amounts of input and immunoprecipitated DNA were subjected to quantitative PCR amplification. ChIP signals were normalized to those of an internal amplimer selected from an ORF-free region of mouse chromosome 14, as described (5). For ChIP–WB, sonicated chromatin (obtained as described above) was immunoprecipitated with 10 μg of the TBX1 antibody, 10 μg of the p53 antibody, or 10 μg of Control Rabbit IgG; all antibodies were conjugated with Dynabeads Protein G as described above. Approximately 50 μg of sonicated chromatin was immunoprecipitated at 4 °C overnight. Samples were then washed six times with cold PBS–0.01% Tween-20 and DNA–protein complexes were eluted in 50 μL of 0.1 mol/L Glycine, pH 2.6 at room temperature, for a total of three elutions. Reverse cross-linking was done at 95 °C for 30 min in SDS sample buffer. Ten percent of input and

immunoprecipitated samples were detected using Western blot analysis.

Silencing of *Trp53* by siRNA. For *Trp53* silencing, P19CL6 cells were differentiated as described above. At day 0 of differentiation, cells were transfected with anti-p53 siRNA (Sigma, target sequence shown on Table S2) to knockdown p53 expression, using Lipofectamine RNAiMAX Reagent (Invitrogen, 13778–150) following the standard protocol. Nontargeting siRNA was used for control transfections. Twenty-four hours after transfection, cells were transfected again at the same conditions and collected 24 h after the second transfection (day 2 of differentiation). mRNA expression was evaluated by quantitative real-time PCR (72% silencing) and protein expression by Western blotting.

Statistical Analyses. To evaluate the statistical significance of haploinsufficiency rescue, we used the χ^2 test.

To evaluate cell proliferation in *Tbx1*$^{-/neo2}$;*Trp53*$^{+/+}$ Vs. *Tbx1*$^{-/neo2}$;*Trp53*$^{+/-}$ SHF cells, we counted Isl1+ and Isl1+;P-H3+ cells in the SHF of 3 embryos per genotype. Data were subjected to normalizing transformation and evaluated using ANOVA (*t* test).

Quantitative PCR analyses were performed using internal controls and the delta–delta–cycle threshold method for fold change calculations with the StepOne v2.3 software (Applied Biosystems). Statistical significance of differences was tested using the *t* test.

1. Lindsay EA, et al. (2001) Tbx1 haploinsufficieny in the DiGeorge syndrome region causes aortic arch defects in mice. *Nature* 410(6824):97–101.
2. Zhang Z, Huynh T, Baldini A (2006) Mesodermal expression of Tbx1 is necessary and sufficient for pharyngeal arch and cardiac outflow tract development. *Development* 133(18):3587–3595.
3. Jacks T, et al. (1994) Tumor spectrum analysis in p53-mutant mice. *Curr Biol* 4(1):1–7.
4. Mueller I, Kobayashi R, Nakajima T, Ishii M, Ogawa K (2010) Effective and steady differentiation of a clonal derivative of P19CL6 embryonal carcinoma cell line into beating cardiomyocytes. *J Biomed Biotechnol* 2010:380561.
5. Chen L, et al. (2012) Transcriptional control in cardiac progenitors: Tbx1 interacts with the BAF chromatin remodeling complex and regulates Wnt5a. *PLoS Genet* 8(3): e1002571.

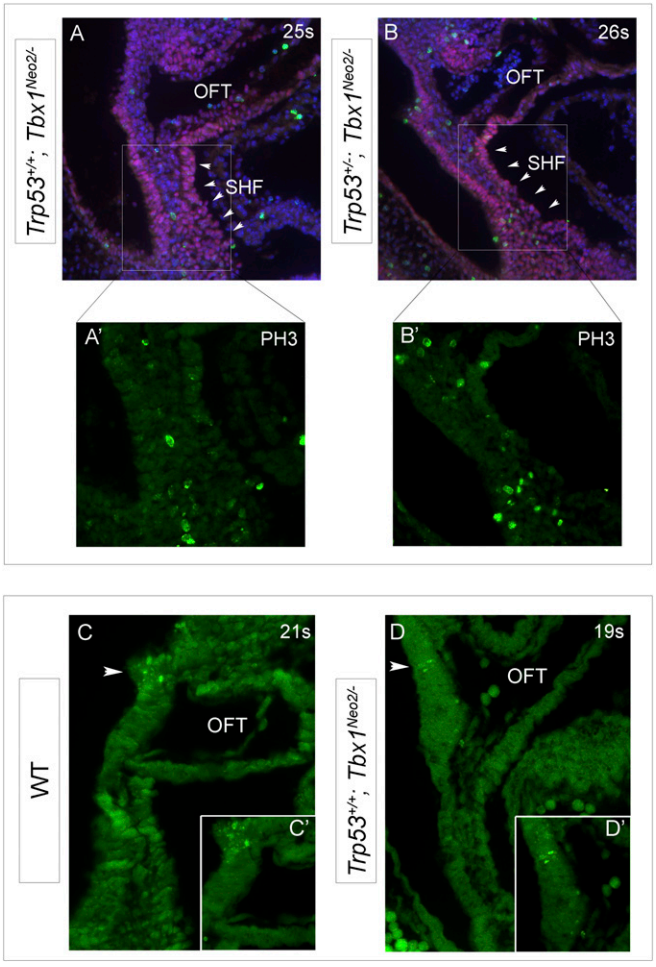

Fig. S1. Examples of immunofluorescence staining of sagittal sections of E9.5 embryos with the genotype indicated. (*A* and *B*) Merged images of DAPI staining (blue) PH3 staining (green) and Isl1 staining (red). (*A*′ and *B*′) PH3 images of the same sections. Counts of Isl1+ and PH3+ cells were performed directly under the microscope using a dual wavelength filter. (*C* and *D*) Cleaved Caspase 3 immunofluorescence evidentiated no apoptosis in the SHF. For reference, the arrowheads and the *C*′ and *D*′ *Insets* indicate regions of physiological apoptosis in the thyroid primordium region of the pharyngeal endoderm. OFT, Cardiac outflow tract; SHF, second heart field.

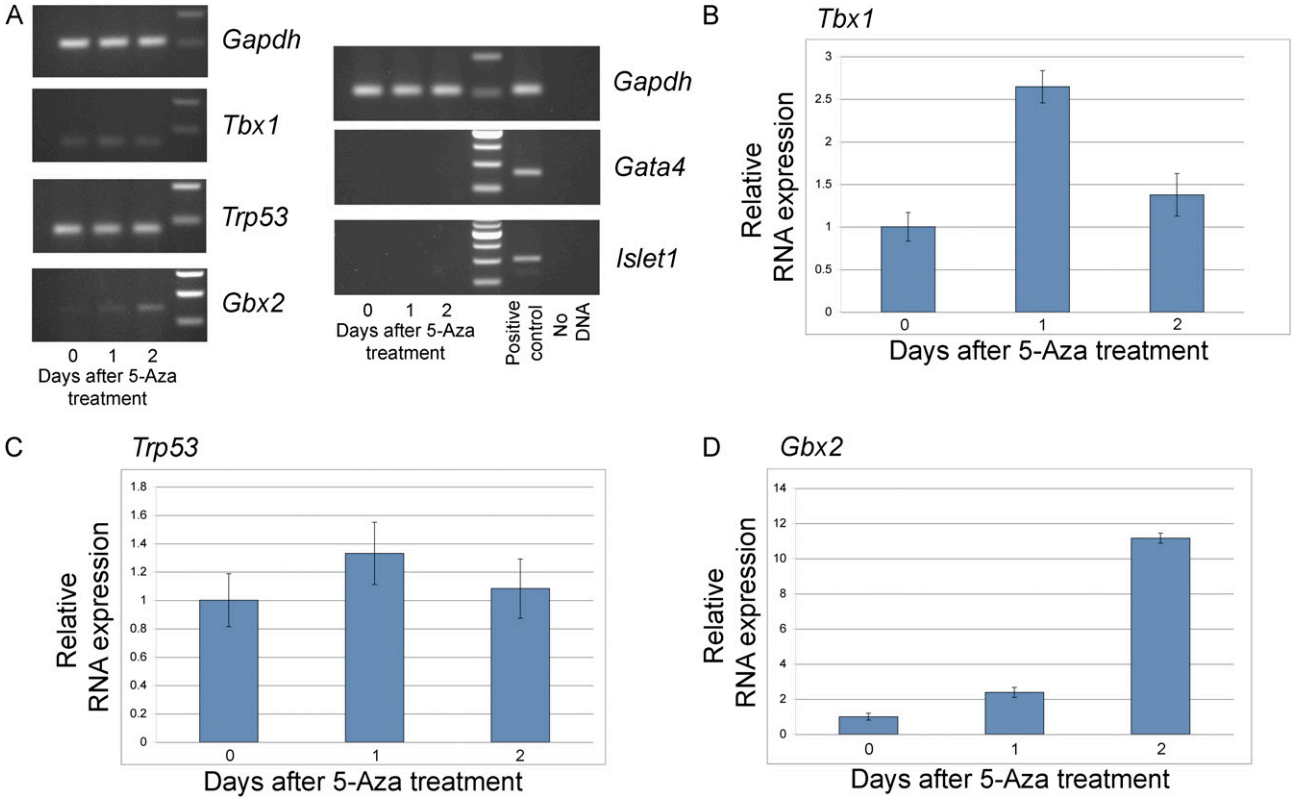

Fig. S2. Gene expression analyses of RNA from 5-Azacytidine/DMSO induced differentiation of P19Cl6 cells at 0, 1, and 2 d of induction. (*A*) RT-PCR expression assays of the genes indicated. Note the absence of cardiac progenitor markers Gata4 and Islet1. (*B–D*) Quantitative real-time PCR assay of expression of the genes indicated.

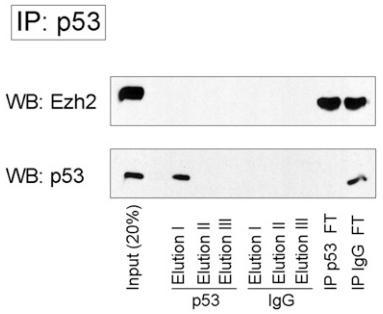

Fig. S3. Coimmunoprecipitation assay showing lack of interaction between p53 and Ezh2. The immunoprecipitation was performed using an antibody against p53. Western blotting analysis was performed using anti-Ezh2 and anti-p53 (control) antibodies.

Table S1. Trp53 deletion modifies the hypomorphic but not the null Tbx1 mutant phenotype

Genotype		E18.5	
Tbx1	*Trp53*	Normal	CV defects
+/+	+/+	13	0
+/+	+/–	9	0
+/–	+/+	5	11[†]
+/–	+/–	19*	3[†]
–/–	+/+	0	10[‡]
–/–	+/–	0	6[‡]

Cardiovascular (CV) phenotype of E18.5 embryos. *$P = 0.0005$ compared with $Tbx1^{+/-};Trp53^{+/+}$ embryos.
[†]Fourth PAA defects.
[‡]Truncus arteriosus communis.

Table S2. Sequence of oligonucleotides used in this work

Primer name	Sequence 5′–3′
Gapdh-F:	TGCACCACCAACTGCTTAGC
Gapdh-R:	TCTTCTGGGTGGCAGTGATG
Tbx1-F:	CTGACCAATAACCTGCTGGATGA
Tbx1-R:	GGCTGATATCTGTGCATGGAGTT
Smad7-F:	AACGAGAGTCAGCACTGCCA
Smad7-R:	GAAGGTGGTGCCCACTTTCA
Wnt5a-F:	CTCCTTCGCCCAGGTTGTTATAG
Wnt5a-R:	TGTCTTCGCACCTTCTCCAATG
Gata4-F:	CTGTCATCTCACTATGGGCA
Gata4-R:	CCAAGTCCGAGCAGGAATTT
Gbx2-F:	GCTGCTCGCTTTCTCTGC
Gbx2-R:	GCTGTAATCCACATCGCTCTC
Isl-F:	GCCTCAGTCCCAGAGTCATC
Isl-R:	AGAGCCTGGTCCTCCTTCTG
*Gbx2*TBE1-F:	CTAGGAGGTGCCTCTGAGTTCT
*Gbx2*TBE1-R:	ACCACCATGGAGAGGCCCTAA
*Gbx2*TBE2-F:	TCACAGCACTAAAGTTAGAGAGG
*Gbx2*TBE2-R:	GCCTTGGAAGAGCGACCTGTA
*Gbx2*TBE3-F:	GAGAGCGTTGAGTTCTGAGTGC
*Gbx2*TBE3-R:	GGACCGTGAAAAGAGTCCAGC
*Gbx2*TBE4-F:	CAGGGAGGACGGGATGGAATAA
*Gbx2*TBE4-R:	GCTGATTAATGAGACCCACGAG
*Gbx2*p53BE1-F:	ATTGCGGCGAAAGGAAAGT
*Gbx2*p53BE1-R:	CCACGAAAGGATCCCATTTT
*Gbx2*p53BE2-F:	GTCCTGCAGTCCGTCGTC
*Gbx2*p53BE2-R:	GAGGAGCGCGATTTAAAGGT
*Gbx2*p53BE3-F:	TCGGGGATACGCAGTCTTAG
*Gbx2*p53BE3-R:	TTTAGTGCTGTGACCGAAGC
*Gbx2*p53BE4-F:	GCACCCATGCATTCCATC
*Gbx2*p53BE4-R:	GCCGATGCCGAAAATTCTA
Control siRNA	GUCGUAACGUCGAUUAUAG
Anti-p53 siRNA	CCACUUGAUGGAGAGUAUU