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Mutations in $Bcl9$ and $Pygo$ genes cause congenital heart defects by tissue-specific perturbation of Wnt/$\beta$-catenin signaling

Claudio Cantù$^{1,6*}$, Anastasia Felker$^{1,*}$, Dario Zimmerli$^{1,*}$, Karin D. Prummel$^1$, Elena M. Cabello$^1$, Elena Chiavacci$^{1,7}$, Kevin M. Méndez-Acevedo$^2$, Lucia Kirchgeorg$^1$, Sibylle Burger$^1$, Jorge Ripoll$^3$, Tomas Valenta$^1$, George Hausmann$^1$, Nathalie Vilain$^4$, Michel Aguët$^4$, Alexa Burger$^1$, Daniela Panáková$^{2,5}$, Konrad Basler$^{1,*}$, Christian Mosimann$^{1,*}$

$^1$ Institute of Molecular Life Sciences, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

$^2$ Electrochemical Signaling in Development and Disease, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, 13125 Berlin-Buch, Germany

$^3$ Department of Bioengineering and Aerospace Engineering, Universidad Carlos III de Madrid, 28911 Madrid, Spain

$^4$ Swiss Institute for Experimental Cancer Research (ISREC), École Polytechnique Fédérale de Lausanne (EPFL), School of Life Sciences, 1015 Lausanne, Switzerland

$^5$ DZHK (German Centre for Cardiovascular Research), partner site Berlin, 10115 Berlin, Germany

$^6$ Present address: Department of Clinical and Experimental Medicine (IKE), Faculty of Health Sciences, Wallenberg Center for Molecular Medicine (WCMM); Linköping University, S-58185, Linköping, Sweden

$^7$ Present address: ICGEB Trieste, Molecular Medicine group, 34149 Trieste, Italy

* contributed equally to this work

† contributed equally to this work

* correspondence to: christian.mosimann@imls.uzh.ch; kb@imls.uzh.ch
Abstract

Bcl9 and Pygopus are obligate Wnt/β-catenin cofactors in Drosophila, yet their contribution to Wnt signaling during vertebrate development remains unresolved. Combining zebrafish and mouse genetics, we document a conserved, β-catenin-associated function for BCL9 and Pygo proteins during vertebrate heart development. Disrupting the β-catenin-BCL9-Pygopus complex results in a broadly maintained canonical Wnt response, yet perturbs heart development and proper expression of key cardiac regulators. Our work highlights BCL9 and Pygo as selective β-catenin co-factors in a subset of canonical Wnt responses during vertebrate development. Moreover, our results implicate alterations in BCL9 and BCL9L in human congenital heart defects.

Introduction

The secreted factors of the Wnt family provide key morphogen activities during vertebrate development and tissue homeostasis (Nusse and Clevers 2017). Binding of the Wnt ligand to the transmembrane receptors of the Frizzled family and their LRP5/6 co-receptors triggers cytoplasmic events that culminate in stabilizing β-catenin that then moves into the nucleus to act as transcriptional regulator (Nusse and Clevers 2017). Nuclear β-catenin orchestrates canonical Wnt target gene expression by recruiting a host of co-factors to Wnt-responsive elements (WREs) occupied by TCF/LEF transcription factors (Mosimann et al. 2009). In Drosophila, the coupling of the histone code-reader plant homology domain (PHD) finger protein Pygopus (Pygo) via the adaptor protein Legless (Lgs) to the β-catenin N-terminal Armadillo repeats is necessary for virtually all canonical Wnt signaling-dependent responses (Kramps et al. 2002; van Tienen et al. 2017; Townsley et al. 2004a). Lgs was shown to simultaneously bind Pygo and β-catenin via two evolutionarily conserved domains, the homology domain 1 and 2 (referred to as HD1 and HD2) (Townsley et al. 2004b; Kramps et al. 2002). In Drosophila, the protein complex TCF/LEF>β-catenin>Lgs>Pygo, termed “chain of adaptors”, is required to efficiently activate Wnt target gene
expression (Städeli and Basler 2005). In support of their role in *Drosophila*, recent biochemical evidence further suggested that Pygo-BCL9 act as connecting factors of chromatin-engaged complexes for the assembly of a Wnt-specific enhanceosome (van Tienen et al. 2017).

Vertebrate genomes commonly harbor two paralogs of *lgs, Bcl9* and *Bcl9l (Bcl9/9l)*, and two of *pygo, Pygo1* and *Pygo2 (Pygo1/2)*. Morpholino antisense experiments and overexpression studies have suggested functions in β-catenin-associated axis formation during gastrulation in *Xenopus* and *zebrafish* (Brembeck et al. 2004; Kennedy et al. 2010). Nonetheless, genetic loss-of-function in the mouse has questioned their relevance for canonical Wnt signaling in mammals: neither the deletion of *Bcl9/9l* nor the deletion of *Pygo1/2* causes a general abrogation of canonical Wnt signaling in mouse development (Li et al. 2007; Cantù et al. 2013, 2014; Song et al. 2007). Mouse mutant analysis has further emphasized that BCL9 and Pygo proteins have evolved β-catenin-independent functions in mammals, including the genetic interaction with Pax6 during eye lens formation, a role in chromatin compaction during spermatogenesis, and cytoplasmic activity in enamel formation during tooth development (Cantù et al. 2017; Song et al. 2007; Cantù et al. 2014, 2013). Whether and to what extent BCL9/9L and Pygo1/2 contribute at all to β-catenin-dependent processes during mammalian development remains to be resolved.

Here, we provide genetic and molecular evidence that vertebrate BCL9 and Pygo proteins contribute as tissue-specific mediators of β-catenin in the development of specific structures and organs, in particular during heart formation. In zebrafish mutants for the *bcl9* and *pygo* genes, or upon selective chemical inhibition of the BCL9-β-catenin interaction, we uncovered that disrupting the β-catenin-BCL9-Pygo complex causes limited developmental phenotypes, including heart defects. In the mouse, both constitutive and heart-specific conditional loss of *Bcl9/9l* or *Pygo1/2*, or the simultaneous impairment of the BCL9/9L-β-catenin and BCL9/9L-PYGO2 interactions, leads to heart malformations which includes defects in chamber septation, outflow tract (OFT)
and valve formation. These data reveal that, in vertebrates, the Wnt-dependent function of the BCL9-Pygo module is restricted to select processes. Transcriptome analyses established that, in the developing heart and pharyngeal structures, the β-catenin-BCL9-PYGO complex regulates the expression of tissue-specific groups of genes. Genome-wide chromatin binding profiling, in addition, revealed that β-catenin and PYGO co-occupy putative cis-regulatory regions of cardiac regulatory factors. Collectively, our results provide functional and molecular evidence for a conserved tissue-specific contribution of the BCL9 and Pygo factors in canonical Wnt signaling, in two distant vertebrate species. In addition, our findings suggest a causative link between human CHDs and the reported copy number changes in BCL9 at 1q21.1 and mutations in BCL9L (Brunet et al. 2009; Christiansen et al. 2004; Dolcetti et al. 2013; Tomita-Mitchell et al. 2012).

**Results**

**BCL9 and Pygo perturbations cause developmental heart defects in zebrafish and mouse**

To investigate the contribution of BCL9/9L proteins to vertebrate heart development based on their repeated association with CHDs, we applied maximized CRISPR-Cas9-mediated mutagenesis in zebrafish embryos to generate bcl9 crispants (Burger et al. 2016) (Fig. 1A-C): we targeted both BCL9 family genes bcl9 and bcl9l with individual sgRNAs by injection of Cas9 ribonucleoprotein complexes into one-cell stage zebrafish embryos and observed highly penetrant cardiac phenotypes following somatic mutagenesis of bcl9 (Fig. 1B,C). We established mutant alleles for both bcl9 and bcl9l, featuring frameshift deletions in-between the coding sequences for HD1 and HD2 (Fig. 1D), the domains of BCL9 proteins that convey functional interaction with Pygo and β-catenin, respectively (Kramps et al. 2002; Cantù et al. 2014). We refer to these new alleles as bcl9Δ29 and bcl9lΔ4; if not null, their BCL9/9L protein products lost their HD2 and cannot bind β-catenin, but still interact with Pygo through the HD1 (Hoffmans et al. 2005; Kramps et al. 2002; Townsley et al. 2004b; Mosimann et al. 2009) (Fig. 1D,E Supplemental...
Fig. S1). Heterozygous \( bcl9^{Δ29} \), as well as homozygous \( bcl9^{Δ4} \) zebrafish and their maternally-zygotic mutant offspring (MZ\(bcl9^{Δ4}\)) were viable and fertile with no obvious phenotypes (observed for more than 4 generations) (Supplemental Fig. S1). Zygotic-mutant embryos homozygous for \( bcl9^{Δ29} \) displayed unaltered expression of early cardiac markers (\( nkx2.5,\) \( myh6/amhc,\) \( gata4\)) at 24 hours post-fertilization (hpf) (Supplemental Fig. S2) and developed seemingly normal until 48 hpf.

Between 56-72 hpf, we observed in homozygous \( bcl9^{Δ29} \) mutants a variable incidence and expressivity of pericardial edema (38% ± 12) and misexpression of the cardiac valve marker \( vcana \) (Supplemental Fig. S2). By 5 days post-fertilization (dpf), homozygous \( bcl9^{Δ29} \) embryos showed highly penetrant craniofacial and cardiac defects (n=181/879, 20.6% compared to Mendelian 25%, N=6) (Fig. 1F-H,I,N). Upon detailed inspection, mutants displayed perturbed heart looping with misaligned atrium and ventricle (Fig. 1I-R, Supplemental Fig. S3), and with more variable expressivity also an underdeveloped cardiopharyngeal vasculature (Fig. 1I-R, Supplemental Fig. S3). Detailed comparison of dissected sibling hearts versus homozygous \( bcl9^{Δ29} \) hearts at 5 dpf revealed slightly smaller ventricles and a significantly smaller smooth muscle-based bulbus arteriosus (BA) (Fig. 1S-V, Supplemental Fig. S3). Zygotic \( bcl9^{Δ29} \) mutants further developed a deformed pharyngeal skeleton, with abnormal Meckel’s and palatoquadrate cartilage development and fusion defects of the ceratohyal and ceratobranchial 1 cartilage (Supplemental Fig. S4). Homozygous \( bcl9^{Δ29} \)-mutant embryos failed to properly inflate their swim bladder (Fig. 1F-H, Supplemental Fig. 3) and invariantly died at 11-12 dpf. Notably, double-mutant zebrafish embryos homozygous for both \( bcl9^{Δ29} \) and \( bcl9^{Δ4} \) looked indistinguishable from homozygous \( bcl9^{Δ29} \) mutants (Fig. 1F-H), suggesting a non-essential function of \( bcl9l \) and that no significant genetic compensation (Rossi et al. 2015) by the paralog \( bcl9l \) occurs in \( bcl9^{Δ29} \) mutants.
To test for a potential role of the BCL9-interacting partner Pygo1/2 during zebrafish development, we also generated mutant *pygo1* and *pygo2* alleles. We retained strains that harbor alleles with frameshift mutations within the essential NH2-terminal homology domain (NHD) and result in a premature Stop codon before the BCL9-binding C-terminal PHD domain (Fig. 2A,B); we refer to these alleles as *pygo1Δ5* and *pygo2Δ1* (Fig. 2B-D). Embryos homozygous for *pygo2Δ1* and concomitantly hetero- or homozygous for *pygo1Δ5* displayed cardiac and a spectrum of craniofacial cartilage defects that, depending on expressivity, mimicked those observed in homozygous *bcl9Δ29* zebrafish mutants (Fig. 2E,F, Supplemental Fig. S4). In contrast, zebrafish embryos homozygous for *pygo1Δ5*, homozygous for *pygo2Δ4*, or homozygous *pygo1Δ5* embryos with one allele of *pygo2Δ4* were indistinguishable from wildtype siblings throughout development, and developed to fertile adults, including maternal-zygotic mutant *pygo1Δ5* animals (Supplemental Fig. S4). Taken together, these data reveal select post-gastrulation defects and sensitivity of cardiac development to functional BCL9 and Pygo levels in zebrafish.

We next sought to delineate the cardiac phenotypes caused by Pygo loss of function in the mouse. Combined loss-of-function of *Pygo1* and *Pygo2* (*Pygo1/2*) leads to well-established embryonic lethality at 13.5-14.5 days post-coitum (dpc) (Li et al. 2007; Cantù et al. 2013). Our re-analysis of these mutants revealed that *Pygo1/2*-mutant mouse embryos develop severe heart defects between 10.5 and 14.5 dpc: histology and 3D reconstruction of the heart documented hypoplastic ventricular myocardium, dilated atria, shorter and thinner atrio-ventricular valve leaflets, and aberrant chamber septation (Fig. 2G-J, Supplemental Videos 1,2, Supplemental Fig. S5). In addition, *Pygo1/2*-mutant embryos displayed prominent OFT anomalies such as transposition of the great arteries (TGA, penetrance of 80%, n=10; Fig. 2K,L) and hypoplastic aorta and pulmonary artery (penetrance of 90%, n=10; Fig. 2M,N).
To test the requirement of PYGO1/2 binding to BCL9/9L in heart formation, we used Bcl9/9l alleles carrying a deletion in the region encoding the PYGO1/2-binding HD1 (Bcl9/9l-ΔHD1) (Cantù et al. 2014). Bcl9/9l-ΔHD1-homozygous mouse embryos died at the same stage as Pygo1/2 mutants without apparent developmental delay (13.5-14.5 dpc), and histological sections revealed underdeveloped ventricular myocardium, dilated atria, defects in the atrio-ventricular valve leaflets, and perturbed septation (Fig. 2O,P; Supplemental Fig. S6). These results indicate that perturbing the cooperative action of BCL9/9L and PYGO1/2 is sufficient to trigger cardiac defects in the mouse. Taken together, analyses in mouse and zebrafish establish that perturbation of BCL9 and Pygo function causes a range of heart defects in two evolutionarily distant vertebrate species.

The BCL9-Pygo complex drives Wnt/β-catenin signaling in the developing heart

Due to the β-catenin-independent roles of BCL9 and Pygo proteins (Song et al. 2007; Cantù et al. 2013, 2014, 2017), we cannot formally link the phenotypes resulting from our mutants to defective canonical Wnt signaling. Since BCL9/9L can act as linker proteins connecting β-catenin and Pygo (Bienz 2006), we aimed at specifically testing the concurrent requirement of connecting these interaction partners.

To do so, we generated a trans-heterozygous allelic configuration for both Bcl9 and Bcl9l, in which one allele carries a deletion of the HD1 (essential for Pygo binding) while the other lacks the HD2 (essential for β-catenin binding) (Fig. 3A,B) (Cantù et al. 2014). Note that double-heterozygous mice for the HD1 deletion (Bcl9ΔHD1+/;Bcl9lΔHD1/+, referred to as Bcl9/9l-ΔHD1+/) or of the HD2 (Bcl9ΔHD2+/;Bcl9ΔHD2/+, referred to as Bcl9/9l-ΔHD2+/) are viable and fertile. Crossing Bcl9/9l-ΔHD1/+ and Bcl9/9l-ΔHD2/+ mice should lead to trans-heterozygous progeny (in 1/16 embryos) in which both domain deletions are present (Bcl9ΔHD1ΔHD2; Bcl9ΔHD1/ΔHD2, referred to as Bcl9/9l-ΔHD1/ΔHD2). The resulting protein products can form either BCL9-β-catenin or BCL9-
PYGO interactions, but not the full tripartite transcriptional module (Fig. 3B), allowing us to test the developmental requirement of the β-catenin-BCL9-PYGO complex.

From these crosses, we never recovered Bcl9/9l-ΔHD1/ΔHD2 pups (Fig. 3B, bottom table), indicating embryonic lethality. Even though at lower numbers than the expected Mendelian ratio, Bcl9/9l-ΔHD1/ΔHD2 embryos did reach the 13.5-14.5 dpc stage (Fig. 3B) and displayed heart defects including ventricular myocardium hypoplasia and smaller and thinner valve leaflets (Fig. 3C-F, Supplemental Fig. S6). This indicated that, for adequate development of these cardiac structures, BCL9/9L are required to physically connect PYGO proteins to β-catenin. Further relating these defects to perturbed canonical Wnt signaling was the reduced expression of the in vivo BATgal reporter (sensing nuclear β-catenin activity) in Bcl9/9l-ΔHD1/ΔHD2 mutants in cardiac valve progenitors, a region with active canonical Wnt signaling (Bosada et al. 2016a) (Fig. 3D,F). Moreover, single molecule mRNA in situ hybridization of Axin2, a prototypical pan-canonical Wnt target gene, confirmed that, at the time of analysis, canonical Wnt signaling activity was decreased in valve progenitors but not in other tissues with active Wnt signaling, such as in the skin (Zhu et al. 2014) (Fig. 3G-L). In addition to cardiac defects, we observed underdeveloped limbs (Fig. 3M-R), and mild skeletal malformations including shortened radius and ulna bones, incorrect specification of digit number and bifid ribs (Supplemental Fig. S7). Notably, while BATgal expression remained broadly unaffected in Bcl9/9l-ΔHD1/ΔHD2 embryos, its reduction occurred as for the cardiac valve progenitors mainly in the malformed tissues including the developing forelimbs (Fig. 3M-R). Correspondingly, in bcl9Δ29-mutant zebrafish at 5 dpf, the pattern and strength of the canonical Wnt signaling reporter Tg(7xTCF-Xla.Siam::nlsmCherry)ia5 (referred to as TCF-siam:Red) (Moro et al. 2012) remained globally unaffected, yet showed mild reduction or pattern changes in the atrio-ventricular valve region, in the OFT, and in craniofacial structures (Fig. 3S-V). Taken together, these data indicate that disrupting the β-catenin-BCL9-PYGO complex in vivo has minor impact on overall Wnt signaling, but selectively perturbs individual domains of active canonical Wnt signaling.
To independently disrupt the protein-protein interaction between BCL9 and β-catenin in zebrafish, we used the chemical compound LH-2-40 (compound 22) (Hoggard et al. 2015) that acts as uniquely selective inhibitor of the BCL9-HD2 interaction with the β-catenin Arm repeats 1-2 (Wisniewski et al. 2016; Hoggard et al. 2015). We treated wildtype zebrafish embryos with LH-2-40 at concentrations ranging from 1 to 50 µM at distinct developmental time-points: 4 cell-stage, shield stage, and 18 somite stages, respectively. Pre-gastrulation- and gastrulation-stage treatment with LH-2-40 did not induce any observable gastrulation defects (Fig. 4A,B); in contrast, at 5 dpf, treated embryos recapitulated the bcl9Δ29-mutant phenotypes in a dose-dependent manner (Fig. 4C-H). Consistent with our genetic observations, LH-2-40-treated embryos displayed altered TCF-siam:Red expression in the atrio-ventricular valve region and in craniofacial structures at 3 and 5 dpf (Fig. 4I-L), as well as phenotypes resembling those observed in bcl9Δ29 homozygous embryos (Supplemental Fig. S8).

While we cannot rule out influences by Wnt/β-catenin-independent functions of BCL9 and Pygo, our results are consistent with their joint requirement for β-catenin-dependent signaling, and emphasize that disconnecting β-catenin from the BCL9-Pygo module during both mouse and zebrafish development does not cause systemic perturbation of canonical Wnt signaling, yet has selective impact on isolated cell types, including heart formation.

**Different cardiac lineages are sensitive to mutations in BCL9 and Pygo**

β-catenin-dependent target gene control is required at different stages of vertebrate heart development, including the initiation of the cardiac program within the lateral plate mesoderm (LPM), chamber formation, and valve development (Armstrong and Bischoff 2004; Hurlstone et al. 2003; Gessert and Kühl 2010; Bosada et al. 2016b). Transcriptome analysis of anterior embryo tissues (including heart, pharyngeal arches, pectoral fins, and craniofacial structures, Fig. 5A) from bcl9Δ29-mutant zebrafish at 54 hpf compared to wildtype siblings, detected deregulation of 157 genes (83 genes down, FC <0.5; 74 genes up, FC>2.5) (Fig. 5B; Supplemental Fig. S9).
deregulated genes fall within different annotated gene expression categories, including heart, LPM, and neural crest (Supplemental Tables S1,S2).

In mammals and chick, cardiac neural crest (CNC) cells contributes to OFT development and septation (Bradshaw et al. 2009; Waldo et al. 2005). We tested the requirement of the PYGO/BCL9 complex in CNC cells by combining the Pygo-flox and the Bcl9-ΔHD1 strains with the NC-specific Wnt1-Cre driver. Pygo1\textsuperscript{flox/flox}; Pygo2\textsuperscript{flox/flox}; Wnt1-Cre\textsuperscript{Tg}\textsuperscript{+/−} (referred to as Wnt1-Cre;Pygo-flox) and Bcl9\textsuperscript{ΔHD1/flox}; Bcl9l\textsuperscript{ΔHD1/flox}; Wnt1-Cre\textsuperscript{Tg}\textsuperscript{+/−} (referred to as Wnt1-Cre;Bcl9/9l-ΔHD1/flox) displayed embryonic lethality at 13.5/14.5 dpc, and featured heart malformations that morphologically recapitulated the constitutive loss of Pygo1/2 (Fig. 5C-F). The expression of the CNC marker Pax3 (Bradshaw et al. 2009) was significantly decreased in migrating CNC cells at 10.5 dpc in Pygo1/2-KO embryos (Fig. 5G-J). These results indicate that BCL9 and PYGO loss in CNC is sufficient to perturb OFT, valves, and cardiac septation.

To test for the contribution of these proteins in LPM-derived heart progenitors, we combined the Bcl9/9l-flox conditional strain (Deka et al. 2010; Cantù et al. 2014) with Nkx2.5-Cre, in which Cre recombinase is active in the expression domain of the cardiac homeobox gene Nkx2.5 at early cardiac crescent stages (ca. 7.5 dpc) (Stanley et al. 2010). Nkx2.5-Cre-mediated Bcl9/9l recombination led to embryonic lethality, accompanied by cardiac malformations including thinner myocardium (Fig. 5K-N). The effect on the compact myocardium upon Bcl9/9l mutations is consistent with the role of canonical Wnt signaling in driving cardiomyocyte proliferation whilst preventing terminal maturation (Naito et al. 2006; Gessert and Kühl 2010).

Taken together, these results establish that the BCL9-PYGO complex connected to β-catenin is required both in migrating CNC cells and early mesodermal Nkx2.5-expressing cardiac progenitors, two key lineages whose interplay is crucial during mammalian heart development (Brade et al. 2013).

The tripartite Pygo-BCL9-β-catenin complex controls cardiac regulators
We next sought to gain insight into the transcriptional changes occurring in the developing cardiac lineages upon perturbation of the β-catenin-BCL9-PYGO module. RNA-seq of the developing heart tube and adjacent branchial arches 3-6 (through which CNC cells migrate toward the loping heart) from constitutive Pygo1/2-mutant mice and control siblings at 10.5 dpc (n=4, Fig. 6A) revealed a discrete set of 65 deregulated genes: 44 of which were downregulated and 21 upregulated (p-value<0.05, fold change <0.66 and >1.5) (Fig. 6B). GO analysis associated a broader group of the most deregulated genes with cardiac ventricle development, embryonic limb morphogenesis and skeletal system development, recapitulating the biological processes perturbed upon bcl9 mutation in zebrafish (Fig. 6C). Notably, the down-regulated genes comprised several encoding for mesodermal and NC transcription factors crucial for heart development, including Pitx2, Hand2, Msx1, and Prrx1 (Andersen et al. 2013; Ocaña et al. 2017; Chen et al. 2007; Kioussi et al. 2002) (Fig. 6B,C). We confirmed the downregulation of the most relevant genes by qRT-PCR and in situ hybridization (ISH) (Fig. 6D-F). RNA-seq of Pygo1/2-mutant hearts at 12.5 dpc when septation, valve formation, and myocardium proliferation and thickening have occurred (Gessert and Kühl 2010), showed more profound gene expression changes consistent with progressive deterioration of cardiac development (533 genes down, 1064 genes up, p-value<0.05, n=3, Supplementary Table 3).

To chart the genomic locations that are bound by the β-catenin-BCL9-Pygo complex, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq), in duplicate, for β-catenin and PYGO2 at 10.5 dpc from dissected pharyngeal arches 3-6 and the developing heart (Fig. 7A). ChIP-seq analysis assigned a total of 982 genes associated with genomic loci occupied by β-catenin, and 5252 by PYGO2 (Fig. 7B); the broader chromatin association of PYGO2 is in line with the known role of this factor as widely deployed chromatin code-reader (Fiedler et al. 2015; Bienz 2006) and its Wnt-independent functions (Cantù et al. 2013). Nonetheless, the genome-wide peak distribution was similar between β-catenin and PYGO2 (Fig. 7C), and their co-occupancy occurred at 59% of all β-catenin target genes (577/982, Fig. 7B,C), including at
those loci found in the vicinity of canonical Wnt targets such as *Axin2* and *Lef1* (Fig. 7D). These peaks correspond to previously described Wnt-regulatory-elements (WRE) (Jho et al. 2002; Hovanes et al. 2001) and displayed the consensus binding sequence of the TCF/LEF transcription factors as anticipated for β-catenin-responsive elements (Fig. 7E). Therefore, while systemic canonical Wnt target genes retain most of their activity in *Pygo* and *Bcl9* mutants, these data combined with the tissue-specific loss of *Axin2* expression in *Bcl9/9l* mutants (Fig. 3G-L) indicate a tissue-specific contribution from the β-catenin-BCL9-Pygo complex in branchial arches and heart cells. GO analysis of target genes that were associated to β-catenin/PYGO2 co-occupied regions [based on proximity to known transcriptional start sites (TSS)] revealed enrichment for genes involved in heart morphogenesis and valve formation, cardiac chamber septation, and skeletal development (Fig. 7F,G). Intersecting the data of the RNA-seq and the ChIP-seq experiments converged on a short list of heart-associated regulatory genes that are potentially controlled by the β-catenin-BCL9-Pygo complex, including *Msx1*, *Prrx1*, *Six1*, and *Six2* (Fig. 7H).

Collectively, our data support the notion that a subset of heart-specific transcriptional regulators is controlled by BCL9 and Pygo as a consequence of their context-dependent interaction with β-catenin.

**Discussion**

Numerous proteins have been shown to function with nuclear β-catenin, but only few are considered universal core components of the canonical Wnt pathway. Contrary to the mandatory requirement in *Drosophila* (Fiedler et al. 2015; van Tienen et al. 2017), genetic evidence in mammals has questioned the significance and involvement of BCL9-mediated tethering of the histone reader Pygo to β-catenin in Wnt target gene control (Cantù et al. 2017, 2014; Song et al. 2007). In parallel, *in vivo* evidence for BCL9 and Pygo function has suggested divergent functions for these proteins in different model organisms: while overexpression and morpholino-based
experiments in zebrafish and *Xenopus* have assigned β-catenin-dependent functions for BCL9 and Pygo proteins already during gastrulation, loss-of-function studies during mouse development have uncovered mainly Wnt-independent phenotypes (Brembeck et al. 2004; Kennedy et al. 2010; Cantù et al. 2014). To date, no clear consensus of their function during vertebrate development has emerged. Here, by combining genetic and chemical loss-of-function studies both in zebrafish and mouse, we revealed a conserved contribution of BCL9 and Pygo proteins as context-dependent regulators of Wnt/β-catenin signaling in vertebrates. The phenotypes resulting from abrogating their function imply that the Pygo-BCL9-β-catenin complex acts as context-dependent transcriptional regulator during tissue-specific processes, in particular during heart development. Our *in vivo* data clarify, consolidate, and extend previous work on BCL9 and Pygo protein function. Additionally, our results implicate perturbed BCL9/9L function as potentially causative for human CHDs, as it occurs in copy number variants of the chromosome region 1q21.1.

Previous work and our results confirm that various genetic loss-of-function permutations of BCL9 and PYGO proteins do not perturb gastrulation in the mouse (Cantù et al. 2014) (Fig. 2 and 3). In zebrafish, we generated mutants with a premature stop codon between the HD1 and HD2 coding sequence of *bcl9* and *bcl9l*; these mutations uncouple BCL9/9L-PYGO1/2 from engaging with β-catenin. We did not detect any gastrulation defects as previously reported for morpholino knockdown of *Xenopus* XBcl9 and zebrafish bcl9l (Kennedy et al. 2010; Brembeck et al. 2004) (Supplemental Fig. 1). Double-mutant bcl9/bcl9l zebrafish and specific chemical interruption of the BCL9/9L-β-catenin interaction by LH-4-20 (compound 22) (Hoggard et al. 2015) phenocopy the bcl9^∆29-mutant phenotype (Fig. 4, Supplemental Fig. 6). We cannot rule out that potentially high levels of maternal BCL9 are sufficient for gastrulation-stage functions; we further cannot rule out genetic compensation during gastrulation for bcl9 by bcl9l and *vice versa* (Rossi et al. 2015), despite absence of such an effect at later stages in the double-mutants (Fig. 1).
Both zebrafish and mouse embryos with perturbed BCL9 and Pygo maintain broad transcriptional activity of β-catenin (Fig. 3 and 4). Nonetheless, following our observation of cardiac defects in interaction-perturbing Bcl9 and Pygo mutants, transcriptome analysis (Fig. 6) and chromatin occupancy of β-catenin and PYGO (Fig. 7) (a challenging experiment in vivo for β-catenin, due to its indirect DNA binding) uncovered a collection of cardiac and pharyngeal genes sensitive to disrupting the β-catenin-BCL9-PYGO complex. Among these, we found common pan-β-catenin target genes, including Axin2 and Lef1. Importantly however, we predominantly uncover genes associated with tissue-specific functions (Fig. 7). Our data support a model of canonical Wnt target gene expression by context-dependent nuclear β-catenin interactions, and define BCL9 and Pygo proteins as selectively required β-catenin interactors in vertebrate heart development. Of note, cardiac defects in mice, such as AV valve malformations, lead to embryonic lethality between 13.5 and 16.5 dpc (Combs and Yutzey 2009; Ranger et al. 1998), suggesting that the cardiac malformations could indeed cause the lethality of Pygo1/2-mutant and Bcl9/9l-ΔHD1 and embryos. The limb and additional skeletal phenotypes observed in our mutants deserve detailed future analysis. Our findings further emphasize that BCL9 and Pygo cannot be strictly defined as core components of canonical Wnt pathway; if their mandatory contribution to canonical Wnt signaling in Drosophila represents an ancestral function or a specialization warrants further investigation.

Of note, in non-syndromic, sporadic cases of human CHD (with or without additional extra-cardiac phenotypes), copy number gains and losses have repeatedly been found in the genomic region 1q21.1 that includes the BCL9 locus (Mefford et al. 2008; Soemedi et al. 2012; Greenway et al. 2009; Dolcetti et al. 2013; Brunet et al. 2009; Christiansen et al. 2004; Tomita-Mitchell et al. 2012). CNVs that lead to either loss or gain of BCL9 copies could perturb the sensitive balance of the PYGO-interacting, β-catenin-coactivator function of BCL9 during human heart
development. Furthermore, mutations in \textit{BCL9L}, including changes in the HD1, have been genetically linked in a small pedigree to heterotaxia (HTX) with congenital cardiac malformations including ventricular and atrial septal defects (Saunders et al. 2012). Our genetic and functional data reveal a causative connection between varying Wnt/\(\beta\)-catenin signaling levels by mutations in \textit{Bcl9} and \textit{Pygo} with developmental heart defects in both zebrafish and in mice; therefore, our findings indicate that CNVs of, or mutations in, \textit{BCL9} and \textit{BCL9L} might contribute to the etiology of human CHDs through tissue-specific perturbation of canonical Wnt signaling.

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**Author contribution**

C.C., A.F. and D.Z. designed and performed the experiments, interpreted the data and wrote
the manuscript; E.C., S.B., L.K., and A.B. assisted with and coordinated zebrafish mutants generation and characterization; E.M.C. performed bioinformatics analyses; K.D.P. performed SPIM-based live imaging and data analyses; T.V. assisted with mouse breeding and genotyping; A.B. and G.H. helped with the design of the work and critically revised the article; J.R. performed and analyzed the mouse SPIM images; N.V. and M.A. performed the initial analyses on Pygo1/2-mutant mice; K.M.M-A. and D.P. performed and analyzed dissected zebrafish hearts experiments; K.B. and C.M. supervised and assisted the research teams, interpreted results, and wrote the manuscript.

**Competing interests**

The authors declare no competing interests.


**Methods**

**Zebrafish husbandry and transgenic strains**

All zebrafish embryos were raised and maintained in E3 medium at 28.5°C without light cycle essentially as previously described (Westerfield 2007). All experiments were performed on embryos up to 5 dpf and older larvae only kept for raising mating pairs in agreement with procedures mandated by the veterinary office of UZH and the Canton of Zürich. Embryo staging was done according to morphological characteristics corresponding to hours post fertilization (hpf) or days post fertilization (dpf) as described previously (Kimmel et al. 1995). Previously established transgenic zebrafish lines used for this study include Tg(-6.35drl:EGFP) (Mosimann et al. 2015), Tg(-6.35drl:mCherry) (Sánchez-Iranzo et al. 2018), Tg(7xTCF-Xla.Siam:nlsCherry)ia5 (Moro et al. 2012), and TgBAC(hand2:EGFP) (Yin et al. 2010).

**CRISPR-Cas9 mutagenesis in zebrafish**

CRISPR-Cas9 mutagenesis was essentially performed as described in Burger et al. 2016 (Burger et al. 2016). Oligo-based sgRNA templates (Bassett et al. 2013) were generated by PCR amplification using the invariant reverse primer 5’-AAAAGCACCAGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGC TT-3’ and forward primers of the sequence 5’-GAAATATTTAGGTGACACTATA-(N20-22)-GTTTTAGAGCTAGAAATAGC-3’ with N representing the 20 nucleotides of the sgRNA target sequence plus up to 2 Gs at the 5’-end for successful T7 in vitro transcription. sgRNA (plus added 5’Gs in brackets) sequences were: i) bcl9 5’-(G)GCCTGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGC TT-3’; ii) bcl9l 5’-(G)GATTAGGTGTGCCAATCGG-3’; iii) pygo1 5’-GGACTTCCCATGACAGCGCC-3’; iv) pygo2 5’-(G)GCCGATGGTGGACCACCTGG-3’.

Crispans were raised to adulthood and crossed to wildtypes to make F1 germline mutants. All analyses and experiments were taken out on F2 mutant generations and beyond. Genotyping
primers were designed to either amplify target regions of mutated alleles that were subsequently analyzed via sequencing (for pygo1∆1) or high-percentage gel electrophoresis (for bcl9∆29) or allele-specific primers were designed to bind to mutated vs. wildtype sequences specifically (for bcl9l∆4 and pygo1∆5). Primers used were: i) bcl9 5’-GGTGGAAAGCCCCAACTCC-3’ (fwd), 5’-CGTGTGCAAGTCTGCTGG-3’ (rev); ii) bcl9l 5’- CACTTGCAGGTGCTGATGG-3’ (fwd), 5’-CTTTGAGATTAGGTGTCGGG-3’ (rev); iii) pygo1 5’-CAGTCTTCTGACCCCCACAC-3’ (fwd), 5’-GGACTTCCAGTAGAGAGAGG-3’ (rev); iv) pygo2 5’- GCCCAGAGAGAAGAGG-3’ (fwd), 5’- GCTGCTCACTCCAGGTCC-3’ (rev). Genotyping results were analyzed and alleles visualized using CrispRVariantsLite (Lindsay et al. 2016).

**Chemical treatments**

Wildtypes, Tg(7xTCF-Xla.Siam:nlsCherry)ia5, and embryos obtained from bcl9∆29 heterozygous incrosses were treated with LH-2-40 to globally perturb Bcl9∆HD2-beta-catenin interaction at the respective developmental stages. LH-2-40, originally called compound 22 as referred to in Hoggard et al., 2015, was kindly provided by the laboratory of Dr. Haitao Ji (Moffitt Cancer Center, Tampa/FL, USA). Single-use LH-2-40 stocks were kept at a concentration of 100 mM in DMSO at -80°C and thawed and diluted in E3 to a working concentration indicated in individual experiments directly before administration to the embryos.

**Alcian Blue staining**

Wildtype, bcl9, and pygo1/2 mutant zebrafish embryos were fixed in 4% Paraformaldehyde (PFA) overnight at 4°C and after washing in 0.1% PBS-Tween (PBST) stained in Alcian blue staining solution (0.1 g Alcian blue, 70 mL ethanol, 30 mL glacial acetic acid) overnight at room temperature. Embryos were washed in Ethanol and transferred through an Ethanol series to PBST and subsequently bleached in hydrogen peroxide (3% H2O2 in 1% KOH in PBS) for 1 hour or until pigments of specimens became transparent.
**Zebrafish whole-mount in situ hybridization**

First-strand complementary DNA (cDNA) was generated from pooled zebrafish RNA isolated from different developmental stages using Superscript III First-Strand Synthesis kit (Invitrogen). DNA templates were generated using first-strand cDNA as PCR template and following primers:
- 5′-TTACGTATGCAGCCTTCTCG-3′ and 5′-GGTTCATGGGGTAACTGTGG-3′ (vcana),
- 5′-ACGGATCAAGTACGGAAGG-3′ and 5′-GTTCTCCAGTTGTTCAGTGTCCTGC-3′ (myh6),
- 5′-GAGCTGCGTCTTACGAGTCC-3′ and CAGACTGGCTCTCTTCTGC-3′ (gata4),
- 5′-ACTGATGAGGACGAGGAAGG-3′ and 5′-GACTCGGAATCCTCCTAGTGCG-3′ (spry4).

For *in vitro* transcription initiation, the T7 RNA polymerase promoter 5′-TAATACGACTCACTATAGGG-3′ was added to the 5′-end of reverse primers. The DNA template for *nkh2.5* was amplified using T3 and T7 primers. PCR reactions were performed under standard conditions using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific). RNA probes were generated via overnight incubation at 37°C using T7 RNA polymerase (20 U/µl) (Roche) and Digoxigenin (DIG)-labeled dNTPs (Roche). The resulting RNA was precipitated in lithium chloride and Ethanol.

Embryos were fixed in 4% PFA overnight at 4°C, transferred into 100% methanol and stored at -20°C. ISH of whole mount zebrafish embryos was performed essentially according to standard protocols (Thisse and Thisse 2008).

**Immunostaining on dissected zebrafish hearts**

Hearts from 120-hpf zebrafish embryos were dissected in Tyrode’s solution (136 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂ × 6H₂O, 5 mM D(+) glucose, 10 mM HEPES, 0.3 mM Na₂HPO₄ × 2H₂O, and 1.8 mM CaCl₂ × 2H₂O; pH 7.4) with 20 mg/mL BSA and fixed with Shandon™ Glyo-Fixx™ (Cat#9990920; Thermo Fisher Scientific™) for 20 min at room temperature. Hearts were blocked in blocking buffer containing 5% normal goat serum, 20 mg/mL BSA, 1% DMSO, 0.1% Tween-20 in PBS, followed by overnight incubation at 4 °C with primary antibodies diluted in blocking buffer.
Primary antibodies used were mouse anti-MHC (MF20 supernatant, DSHB, 1:50), rabbit anti-PKC-zeta (Cat.sc-216, Santa Cruz Biotechnology, 1:100). Hearts were washed three times in blocking buffer, then incubated with Alexa-conjugated secondary antibodies for 4 hours, and DAPI in PBS for 20 min at room temperature, then washed again three times with blocking buffer, and mounted in the ProLong Gold antifade reagent (Cat#P36934; ThermoFisher Scientific™).

Microscopy and data processing

Brightfield (BF), basic fluorescence, and ISH imaging was performed using a Leica M205FA equipped with a DFC450 C camera. Detailed fluorescent embryo imaging of reporter transgenics was performed by Single Plane Illumination Microscopy (SPIM) with a Zeiss Lightsheet Z.1 microscope with a Plan-Apochromat 20x/1.0 Water objective. Prior to imaging, embryos were embedded in a rod of 1% low melting agarose in E3 with 0.016% Ethyl 3-aminobenzoate methanesulfonate salt (Tricaine, Sigma) in a 50 µL glass capillary. Heart beat was viably stopped with 30 mM 2,3-Butanedione monoxime (BDM, Cat#B0753; Sigma). Embryos and their hearts were imaged from opposing sides and the views were combined into a single image. Imaging of dissected hearts was performed with a Leica SP8 confocal microscope using a Plan-Apochromat CS2 63x/1.3 Glycerol objective.

Image processing was done with Leica LAS, Zeiss Zen Black, ImageJ/Fiji, Adobe Photoshop and Illustrator CS6 according to image-preserving guidelines to ensure unbiased editing of the acquired image data. Quantitative data analysis was performed using GraphPad Prism 5.0. Data are presented as mean ± SEM, unpaired t-test with Welch correction. A lower case “n” denotes the number of embryos, while a capital “N” signifies the number of replicates. To perform SPIM on embryonic mouse heart, tissue samples were extracted at 13.5 dpc, embedded in low melting agarose, dehydrated, and cleared using Benzyl Alcohol/ Benzyl Benzoate (1:2). 3D optical sectioning of the anatomy of the samples was performed with a QIs-Scope lightsheet microscopy system (Planelight S.L., Madrid, Spain) by measuring autofluorescence with a 5x objective where
signal was optimal, in this case exciting at 532 nm and collecting emission at 590 +/- 40 nm. Samples were imaged from two opposing views and combined to form a single volumetric image with an isotropic voxel size of 2.6 μm.

**Mouse lines**

Knock-in mutants in *Bcl9* and *Bcl9l* were generated by standard techniques, as previously described (Cantù et al. 2014). Briefly, the targeting vector was electroporated into BA1 (*C57BL/6 x 129/SvEv*) hybrid embryonic stem cells. After selection with the antibiotic G418, surviving clones were expanded for PCR and Southern blotting analyses to confirm recombinant embryonic stem cell clones. Mouse embryonic stem cells harboring the knock-in allele were microinjected into *C57BL/6* blastocysts. Resulting chimeras were bred to wildtype *C57BL/6N* mice to generate F1 heterozygous offspring. Neo cassette excision was obtained by crossing heterozygous knock-in animals with mice expressing Flp recombinase. All mouse experiments were performed in accordance with Swiss guidelines and approved by the Veterinarian Office of the Kanton of Zurich, Switzerland.

**Histological analysis**

Embryos between day 9.5 and 14.5 dpc were fixed overnight in 4% PFA at 4°C, dehydrated and embedded in paraffin. Sections were stained with haematoxylin and eosin for histological analysis. The same paraffin embedded material was sectioned under RNase free conditions for mRNA *in situ* hybridization.

**Mouse whole-mount mRNA in situ hybridization**

Whole-mount *in situ* hybridization (ISH) was performed as described previously (Constam and Robertson 2000). The probes for *Pax3* and *Pitx2* were kindly provided by Dr. Joerg Huelsken and Dr. Daniel Constam, respectively. Digoxigenin-labeled probes (Roche) were detected by
enzymatic color reaction using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:1000, Roche) and BM purple alkaline phosphatase substrate (Roche). Digoxigenin-labeled RNA probes were detected with peroxidase-conjugated anti-digoxigenin Fab fragments (1:500, Roche) followed by fluorescence detection using Tyramide Signal Amplification (PerkinElmer). Mouse antisense RNA probes were as described in supplementary materials. Quantitative RNA in situ hybridization was performed using RNAscope 2.5 (ACD) according to manufacturers’ instruction. Mean intensity measurements were performed using ImageJ.

**Intracardiac injection of India ink**

For the analysis of the cardiovascular system, india ink was injected into the left ventricle of mouse embryos at 14.5 dpc, using a finely drawn glass pipette, right after embryonic dissection when the heart was still beating. The embryos were subsequently fixed in 4% PFA and hearts were dissected for the anatomic examination.

**Zebrafish RNA sequencing**

*Zebrafish samples (polyA selection):* Zebrafish embryos from bcl9^{Δ29/wt} crosses were dissected at 54 hpf: a cut was made between the anterior part of the embryo (containing the head, pharyngeal and cardiac structures) and the posterior part starting from the beginning of the yolk extension. The anterior tissues were directly snap-frozen in liquid nitrogen in single tubes of PCR 8-tube strips. The posterior trunks and tails were transferred in 50 mM NaOH and genomic DNA was extracted with alkaline lysis. Single embryos were genotypes using the target sequence primers listed above and PCR products were separated through high-percentage gel electrophoresis leading to a separation of the wildtype allele and mutant allele with the following outcome: i) a low running band in bcl9^{Δ29} mutants; ii) a high running band in wildtypes; iii) two bands in bcl9^{Δ29/wt} heterozygous mutants. All snap-frozen anterior parts of bcl9^{Δ29} mutants and of wildtypes were pooled in two separate tubes and RNA isolation performed with the RNeasy Plus
Mini Kit (Qiagen). The whole procedure was repeated for a total of three clutches making three independent replicates.

The TruSeq mRNA stranded kit from Illumina was used for the library preparation with 250 ng of total RNA as input. The libraries were 50-bases sequenced on an Illumina HiSeq 2500 sequencer. The quality control of the resulting reads was done with FastQC and the reads mapped to the UCSC *Danio rerio* danRer10 genome with the TopHat v.2.0.11 software. For differential expression analysis the gene features were counted with HTSeq v.0.6.1 (htseq-count) on the UCSC danRer10 gene annotation. The normalization and differential expression analysis were performed with R/Bioconductor package EdgeR v. 3.14. The p-values of the differentially expressed genes are corrected for multiple testing error with a 5% false discovery rate (FDR) using Benjamini-Hochberg (BH). The volcano plot was done selecting the genes that have > 10 counts per million (CPM) in at least 3 samples, p-value < 0.12 and absolute value of log fold change (logFC) above 1. STRING analysis was performed on differentially expressed genes implicated in cardiac development in the literature (see Supplemental Tables S1,S2). Permalink to analysis: [http://bit.ly/2wmba7z](http://bit.ly/2wmba7z).

**Mouse RNA sequencing**

Developing heart tube and adjacent branchial arches 3-6 were collected from 10.5 dpc mutant and control embryos (N=4). The Illumina TruSeq stranded Total RNA library Prep kit with RiboZero was used for the library preparation with 300 ng of total RNA as input. The libraries were 100-bases sequenced on an Illumina HiSeq 4000 sequencer. The quality control of the resulting reads was done with FastQC and the reads mapped to the UCSC *Mus musculus* mm10 genome with the TopHat v.2.0.11 software. For differential expression analysis the gene features were counted with HTSeq v.0.6.1 (htseq-count) on the UCSC mm10 gene annotation. The normalization and differential expression analysis were performed with R/Bioconductor package
EdgeR v. 3.14. The p-values of the differentially expressed genes are corrected for multiple testing error with a 5% false discovery rate (FDR) using Benjamini-Hochberg (BH).

**qRT-PCR**

Quantitative real-time SYBR Green-based RT-PCR reactions were performed in triplicate and monitored with the ABI Prism 7900HT system (Applied Biosystem). *Gapdh* expression was used as internal reference. Statistical significance was determined based on unpaired t-test. Primers sequences are available upon request.

**Chromatin Immunoprecipitation (ChIP)**

Ca. 200 branchial arches of E10.5 mouse embryos were dissected and dissociated to a single-cell suspension. Cells were cross-linked in 20 ml PBS for 40 min with the addition of 1.5 mM ethylene glycol-bis(succinimidyl succinate) (Thermo Scientific, Waltham, MA, USA), for protein-protein cross-linking, and 1% formaldehyde for the last 20 min of incubation, to preserve DNA-protein interactions. The reaction was blocked with glycine and the cells were subsequently lysed in 1 ml hepes buffer (0.3% SDS, 1% Triton-X 100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES). Chromatin was sheared using Covaris S2 (Covaris, Woburn, MA, USA) for 8 min with the following set up: duty cycle: max, intensity: max, cycles/burst: max, mode: Power Tracking. The sonicated chromatin was diluted to 0.15% SDS and incubated overnight at 4°C with anti IgG (Santa Cruz), anti-PYGO2 (Novus Biological, NBP1-46171) and anti-β-catenin (Santa cruz, sc-7199). The beads were washed at 4°C with wash buffer 1 (0.1% SDS, 0.1% deoxycholate, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES), wash buffer 2 (0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES), wash buffer 3 (0.25 M LiCl, 0.5% sodium deoxycholate, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES), and finally twice with Tris EDTA buffer. The chromatin was eluted with 1% SDS, 0.1 M NaHCO3, de-crosslinked by incubation at 65°C for 5
h with 200 mM NaCl, extracted with phenol-chloroform, and ethanol precipitated. The immunoprecipitated DNA was used as input material for DNA deep sequencing. The ChIP-seq experiment was performed in duplicate.

**Data analysis – peak calling:** All sequenced reads were mapped using the tool for fast and sensitive reads alignment, Bowtie 2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml), onto the UCSC mm10 reference mouse genome. The command "findPeaks" from the HOMER tool package (http://homer.salk.edu/homer/) was used to identify enriched regions in the beta-catenin immunoprecipitation samples using the "-style = factor" option (routinely used for transcription factors with the aim of identifying the precise location of DNA-protein contact). Input and IgG samples were used as enrichment-normalization controls. Peak calling parameters were adjusted as following: L = 4 (filtering based on local signal), F = 4 (fold-change in target experiment over input control). Annotation of peaks’ position (i.e. the association of individual peaks to nearby annotated genes) was obtained by the all-in-one program called "annotatePeaks.pl". Finally, the HOMER command "makeUCSCfile" was used to produce bedGraph formatted files that can be uploaded as custom tracks and visualized in the UCSC or IGV genome browsers (http://genome.ucsc.edu/; https://software.broadinstitute.org/software/igv/).

**Immunofluorescence**

FFPE sections were blocked with 5% heat-inactivated goat serum, % BSA and 0.1% Tween in PBS and incubated overnight at 4°C with the following antibodies: mouse anti p53 5E2 (NovusBio), rabbit anti Sox9 (Millipore), Troponin type2 (novus biologicals), Acta2, GFP (Aves). Slides were then incubated with a fluorescently labeled secondary antibody (Alexa 488 goat anti-mouse or Alexa 555 goat anti-rabbit, Alexa 594 goat anti-chicken; 1:500). Nuclei were stained with DAPI (1:1000; Sigma).
Accession codes

ChIP-seq and RNA-seq reads are being deposited in the Gene Expression Omnibus (GEO) repository: zebrafish mRNA-seq data as GSE108240, mouse mRNA-seq as GSE110782, and mouse ChIP-seq as GSE110781.

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Figure Legends

Figure 1: Mutations in *bcl9* lead to cardiac defects in zebrafish.
(A) A CRISPR-Cas9-mediated mutagenesis identified *bcl9* as a potential regulator of heart morphogenesis. (B, C) *bcl9* crispants have heart looping defects as visible in *drl:mCherry* transgenics leading to cardiac edema (asterisk); lateral view, anterior to the left. (D) Schematic representation of the *bcl9* gene locus and generation of the *bcl9*Δ29 germline allele. An sgRNA was designed to target the coding exon 6 between HD1 and HD2 of the zebrafish *bcl9* gene. The locus is represented as per annotation Zv10 with two isoforms that differ in the first coding exon and the untranslated regions (UTRs). The dotted box represents a zoomed region of the locus, the red line marks the location of the sgRNA to introduce the Cas9-mediated cut, which resulted in the indicated frameshift (red box) followed by two Stop codons in the isolated *bcl9*Δ29 allele; the isolated allele black boxes mark coding exons (CDS), white boxes mark UTRs, the blue boxes represent the CDS that contribute to HD1 and purple boxes to HD2. (E) CrispRVariants panel plot depiction of the *bcl9*Δ29 germline allele with a 29 bp deletion. Top shows genomic reference, *bcl9*Δ29 (*bcl9:g.176663_176691*) shown below. *bcl9*Δ29 features an out-of-frame deletion introducing a frameshift, followed by 157 novel amino acids terminated by two consecutive stop codons, thus disconnecting HD1 from HD2. The black box indicates the exact position of the sgRNA sequence, the grey shaded box the 5'-NGG-3' PAM sequence, and the black line the predicted Cas9-induced double-strand break position. (F-H) Brightfield images of 5 dpf homozygous *bcl9*Δ29 and *bcl9*Δ29;*bcl9*Δ4 embryos and their wildtype-looking siblings, lateral views, anterior to the left. Mutant embryos showed heart looping defects and cardiac edema (asterisks). Moreover, mutant embryos did not inflate their swim bladder (arrows), presumably due to a failure in gasping air because of craniofacial malformations (black arrow heads). (I-R) SPIM images of *hand2:EGFP;drl:mCherry*-expressing wildtype siblings and homozygous *bcl9*Δ29 embryos, ventral views, anterior to the top, imaged after viable heart-stopping BDM treatment. I, N depict
maximum-intensity projections and J,K,O,P close-ups of dotted square in I,N, while panels L,M,Q,R depict optical sections at AV canal level. Compared to siblings that form correctly looped hearts with AV canal valves and a BA (heart outlined with red dotted line, n=4, J-M), *bcl9Δ29* embryos show heart looping defects (N-R) and craniofacial malformations in both cartilage and head vascularization (n=8, N). (S-V) 6μm Z-confocal projections of sibling control (S) and homozygous *bcl9Δ29* (T) hearts at 5 dpf. Quantification of the ventricular (U) and BA area (V) in sibling hearts (n=16) compared to homozygous *bcl9Δ29* hearts (n=15) shows that the BA area is significantly smaller in the *bcl9Δ29* hearts (*P < 0.0221, unpaired t-test with Welch correction). Each data point represents the averaged ventricular or BA area from one heart; quantification derived from three independent experiments (N=3). ba, bulbus arteriosus; a, atrium; v, ventricle; av, atrioventricular canal. Scale bars B,C,F-H 500 μm, I-R 100 μm, S,T 20 μm.

**Figure 2:** *Pygo1/2* mutant zebrafish and mouse embryos develop cardiac malformations reminiscent of CHDs.

(A) Schematic of the tripartite complex comprised of beta-catenin, BCL9, and Pygo with their individual interaction domains, together tethered to a Wnt-responsive element by TCF/LEF. (B) Schematic representation of the zebrafish *pygo1* and *pygo2* genes with annotated NHD and PHD domains and the Cas9 cutting site to generate mutants. Gene locus represented as per genome annotation Zv10 with the main isoforms of both genes shown. See also description of Figure 1B. (C,D) CrispRVariants panel plot depictions of the germline alleles with a 5 bp or 1 bp deletion in *pygo1* and *pygo2*, respectively. Top shows genomic reference with the *pygo1Δ5*: g.2924_2928del and *pygo2Δ1*: g.3191del alleles shown below. Both alleles result in an out-of-frame deletion introducing a frameshift in the CDS. See also description of Figure 1C. (E,F) Brightfield images of live *pygo1Δ5;pygo2Δ1* double mutants reveal cardiac edema (asterisks) and craniofacial defects (arrow heads), and aberrant swim bladder inflation (arrows) as detected in *bcl9Δ29* mutants, lateral views, anterior to the left. (G-J) Haematoxylin/eosin stained transverse sections of the murine
heart at 10.5 (G,H) and 14.5 dpc (I,J). At 10.5 dpc the development of the heart and heart cushion was still largely normal in the mutants. At 14.5 dpc the mutants displayed markedly smaller and thinner valves (dashed outline) and compact ventricular myocardium (arrow head), highly dilated atria, and the atrial septum was missing (asterisks). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. (K-N) Gross anatomical view of heart and great vessels at 13.5/14.5 dpc as revealed by India ink injection. While normally the aorta (Ao) arises from the left ventricle (LV) and the pulmonary artery (Pa) from the right ventricle (RV), mutants showed a classic transposition of the great arteries (TGA, arrows) and hypoplastic Ao and Pa. (O,P) Schematic representation of the Bcl9/9l-Pygo1/2 interaction (O) and the molecular configuration of this interaction when the HD1 domain in Bcl9/9l is deleted in Bcl9/9l-∆HD1 mice (P); and heart sections at 14 dpc, stained with Haematoxylin/Eosin. The abrogation of this interaction leads to a delayed chamber septation (asterisks), hypoplastic myocardium (arrow head), and valve deficiency. Scale bar, 250 µm (E,F).

Figure 3: The BCL9-Pygo complex drives Wnt/β-catenin transcription during heart development.

(A) Schematic representation of the β-catenin transcriptional complex to outline the experimental strategy. Bcl9/9l simultaneously interacts with Pygo1/2 and β-catenin via the domains HD1 and HD2, respectively. (B) In Bcl9/9l-Δ1/Δ2 mice, no Bcl9/9l molecule can simultaneously bind Pygo1/2 and β-catenin; the deletion of the HD2 or HD1 domains abrogates Bcl9/9l binding to β-catenin (left panel) or Pygo1/2 (right panel), respectively. Bcl9/9l-Δ1/Δ2 embryos are never found after embryonic stage 14.5 dpc (table). (C-F) Haematoxylin/Eosin staining on heart sections of control (C) and Bcl9/9l-Δ1/Δ2 embryos (E) at 13.5 dpc. Bcl9/9l-Δ1/Δ2 embryos have pronounced heart defects, with thinned myocardium of the ventricular walls, malformations of the forming septum, the AV valves, and OFT. The expression of BATgal in vivo Wnt reporter in the OFT region is markedly reduced in Bcl9/9l-Δ1/Δ2 mutant (F) compared to control (D) hearts. RA, right atrium;
LA, left atrium; RV, right ventricle; LV, left ventricle. (G-L) *Axin2* expression in wildtype and *Bcl9/9l-Δ1/Δ2* OFT valves (G,J) and skin (H,K) at 13.5 dpc. Green boxes are drawn around transversal sections of individual valves of the OFT (G,J), and in skin sections from the limbs (H,K) for quantification of fluorescence intensity levels (I,L). *Axin2* expression is strongly reduced in the valves of *Bcl9/9l-Δ1/Δ2* embryos (J), while reduction in the skin is mild (K). The quantification of the ratio between *Axin2* expression in the valves and the skin in the different genotypes reveals a significant reduction of *Axin2* expression in *Bcl9/9l-Δ1/Δ2* valves (I,L, asterisk, p-value<0.05. (M-R) *In vivo* BATgal reporter expression in 13.5 dpc wildtype (M-O) and *Bcl9/9l-Δ1/Δ2* mutant (P-R) embryos. Mutant embryos have a slightly decreased reporter expression, but BATgal reporter expression is generally retained in most tissues. Reduced BATgal activity is observed in the craniofacial region (N,Q) and in the forelimbs (O,R). Loss of canonical Wnt signalling transcription in forelimbs is accompanied by severe developmental limb defects in *Bcl9/9l-Δ1/Δ2* embryos (compare O with R). (S-V) Fluorescent and brightfield images of *TCF-siam:Red bcl9Δ29* and wildtype zebrafish embryos, lateral (S,T) and ventral (U,V) views, anterior to the left. TCF reporter activity is specifically reduced in the cardiac OFT (arrow) and craniofacial apparatus (arrow heads), and altered in the atrio-ventricular valve (asterisks). Scale bars, 100 µm (C-F,G,H,J,K), 200 µm (S-V).

**Figure 4: Chemical manipulation of zebrafish heart development by Bcl9-β-catenin inhibitor LH-2-40.**

(A-B) Treatment with 10 µM LH-2-40 from 2-4 cell stage does not result in gastrulation defects, lateral views. (C-E) Brightfield images of 5 dpf old DMSO controls (C) and representative embryos treated with 10 µM LH-2-40 from 4-cell stage on (D-E) as assessed for phenotype classes quantified in (F-H), lateral views, anterior to the left. Phenotypes in LH-2-40 treated embryos become visible at 5 dpf, comparable to phenotypes observed in *bcl9Δ29* mutants. LH-2-40 treated embryos show a variable phenotype expressivity with mild to strong swim bladder inflation defects
(arrow heads $D,E$) linked with variable craniofacial defects (asterisks $D,E$, compare to control in $C$). (H-J) Similar dose-dependent phenotype penetrance and expressivity is observed after Bcl9 inhibition at 4-cells, shield, and 18 ss stage, suggesting that the observed phenotypes result from perturbed Bcl9 function in craniofacial and heart development after somitogenesis. Mild phenotypes are characterized by mild craniofacial and swim bladder inflation defects. Both phenotypes are more pronounced in the medium phenotype class (see $D$). Strong phenotypes are characterized by strong craniofacial defects, a complete failure to inflate the swim bladder (see $E$). (I-P) Fluorescent and brightfield images of $TCF:siamRed$ Bcl9-inhibited ($K,L,O,P$) and DMSO-treated ($I,J,M,N$) wildtype siblings at 3 and 5 dpf, lateral views, anterior to the left. $TCF$ reporter activity is severely reduced in the atrio-ventricular valve at 3 dpf (asterisks $I,K$) and altered in the craniofacial cartilage (arrow heads $I,K$). At 5 dpf, the $TCF$ reporter is aberrantly patterned in the atrio-ventricular valve (asterisks $M,O$) and fins (arrow heads $M,O$). Scale bars, 200 µm ($C-E,I-P$), 500 µm ($A,B$).

**Figure 5: The Bcl9-Pygo complex acts in neural crest and cardiac heart progenitor cells.**

(A) Schematic representation of the RNA-seq approach used for $bcl9^{\Delta 29}$ mutant zebrafish embryos. RNA extraction was performed on manually dissected anterior structures of 54 hpf embryos as indicated by the dashed box. (B) Comparing three independent biological replicates revealed 157 differentially expressed (74 up- and 83 downregulated) genes between wildtypes and $bcl9^{\Delta 29}$ mutants as represented in the volcano plot. Genes associated with cardiac development are highlighted. (C-F) Haematoxylin/Eosin staining of heart section of control ($C,E$) compared to $Wnt1-Cre; Pygo1/2-flox$ ($D$) and $Bcl9/9l-\Delta HD1/flox$ ($F$) 13.5 dpc siblings. (G-J) $Pax3$ expression in E10.5 embryos in the region of branchial arches: view of whole-mount RNA *in situ* hybridizations of control ($G$) compared to $Pygo1/2-KO$ ($I$) siblings. $H$ and $J$ are magnified insets of the regions marked with dashed squares in $G$ and $I$, respectively. (K-N) Haematoxylin/Eosin
staining of heart section of control (K) compared to Nkx2.5-Cre; Bcl9/9l-flox (L-N) 13.5 dpc siblings.

**Figure 6:** The Pygo-BCL9-β-catenin transcriptional complex controls the expression of a heart-specific genetic program.

(A) Schematic representation of the RNA-seq approach used for Pygo1/2-KO mutant mouse embryos. RNA extraction was performed on manually dissected branchial arches 3-6 and hearts of 10.5 dpc embryos as indicated by the dashed outline. (B) Volcano plot depicting the set of upregulated (23) and downregulated (43) genes in Pygo1/2 knockout (KO) mouse embryos with a fold change of <0.66 and >1.5, p<0.05 (n=4). (C) Gene Ontology (GO)-based categorization of a broader group consisting of the 500 most deregulated genes (based on p-value) indicates the biological processes affected. The total bar length indicates the number of genes that categorize to the indicated GO term, the red line indicates the expected number of genes in a random deep sequencing analysis, the color of the bar indicates the p-value for the gene enrichment in our analysis. (D) RT-qPCR validation of a subset of target genes. mRNA expression level was calculated based on Gapdh, and it is shown as arbitrary units (AU). One asterisk, p<0.05; two asterisks, p<0.01. (E-F) Whole-mount in situ hybridization (ISH) in 10.5 dpc control (E) and Pygo1/2-KO (F) mouse embryos shows tissue-specific (branchial arches) downregulation of the target gene Pitx2.

**Figure 7:** PYGO2 and β-catenin co-occupy regulatory regions of Wnt-target and heart-specific genes.

(A) Schematic representation of the ChIP-seq approach. Branchial arches 3-6 and hearts of 10.5 dpc wildtype embryos were dissected manually as indicated by the dashed outline. Immunoprecipitation was performed for β-catenin and PYGO2. (B) Venn diagram depicting the overlap between β-catenin and PYGO2 target genes. (C) Genomic distribution of β-catenin,
PYGO2 and overlapping peaks location indicates prominent binding to regulatory regions found within promoters or introns. (D) β-catenin and PYGO2 ChIP-seq peaks on the Wnt responsive elements of the prototypical target genes Axin2 (left panel) and Lef1 (right panel). Red columns indicate the position of previously described Wnt-responsive elements (WREs). (E) The binding motif of TCF/LEF transcription factors is present within β-catenin/PYGO2 overlapping peaks. (F) Gene Ontology (GO) analysis of common β-catenin and PYGO2 target genes reveals the direct regulation of processes associated with heart development. The total bar length indicates the number of genes that categorize to the indicated GO term, the red line indicates the expected number of genes in a random deep sequencing analysis, the color of the bar indicates the p-value for the gene enrichment in our analysis. (G) List of the 30 direct PYGO2/β-catenin target genes that have been previously implicated in heart development. (H) β-catenin and PYGO2 ChIP peaks (red columns) within the regulatory regions of genes involved in cardiac development and found downregulated upon Pygo2 loss (see also Fig. 6). ChIP-seq custom tracks are visualized in the Integrative Genomic Viewer (IGV) genome browser.
Supplementary Material

Supplemental Figure 1: MZbcl9l\(\Delta^4\) mutants are viable and fertile.

(A,B) Schematics showing the mutation induced in bcl9l\(\Delta^4\) mutants. Analogous to bcl9 mutants (see Figure 1B,C), we designed a sgRNA that induces mutagenesis between the HD1 and HD2 domain. Gene locus represented as per genome annotation Zv10 (A) with two isoforms that differ in the first coding exon and the untranslated regions (UTRs). The green dotted box represents a zoomed region of the gene locus, with the red line representing the location of the sgRNA used for mutagenesis; black boxes mark coding exons (CDS), white boxes mark UTRs, the blue boxes represent the part of the CDS that will contribute to HD1 and purple boxes to HD2. A 4 bp deletion at the Cas9 cutting side (B) leads to a frame-shift allele with a premature STOP codon before HD2. (C-G) Brightfield images of homozygous bcl9l\(\Delta^4\) mutants at different stages from gastrulation to adulthood, lateral views, anterior to the left. Maternal zygotic bcl9l mutants (MZbcl9l\(\Delta^4\)) mutants did not show any gastrulation defects (C,D). At 5 dpf, we could not detect any cardiac and craniofacial phenotypes (E,F) as observed in zygotic bcl9\(\Delta^{29}\) mutants (see Figure 1). (G) Representative image of a five-month old (5 mo) MZbcl9l\(\Delta^4\) F4 mutant obtained from a cross of two adult homozygous bcl9l\(\Delta^4\) mutants. Scale bars, 500 \(\mu\)m.

Supplemental Figure 2: bcl9 mutants feature normal early cardiac patterning, but variable cardiac phenotypes in bcl9\(\Delta^{29}\) mutants between 2-3 dpf.

(A-L) bcl9 mutants displayed unchanged nkx2.5 (A-D), myh6/amhc (E-H), and gata4 (I-L) expression at 24 hpf compared to wildtype siblings: lateral and dorsal views, anterior to the left. Scale bar, 250 \(\mu\)m. (M-O) Fluorescent and brightfield (inlets) images of drl:EGFP transgenic wildtype and bcl9\(\Delta^{29}\) mutant embryos at 72 hpf (M,N), lateral views, anterior to the left. The hearts of bcl9\(\Delta^{29}\) are patterned into atria and ventricle. The two chambers are slightly misaligned and the embryos develop cardiac edema (O). (O,P) Whole-mount ISH for vcana reveals expanded
expression around the atrio-ventricular canal and in the atrium (asterisks) suggesting a defect in valve formation, ventral views, anterior to the left. Scale bars, 200 µm (M,N), 500 µm (P,Q).

Diagram in O depicts mean with s.d. from representative clutch of embryos, genotype confirmed by PCR.

Supplemental Figure 3: Cardiac phenotypes of zebrafish bcl9Δ29 mutants.

(A-O) SPIM images of hand2:EGFP;drl:mCherry-expressing wildtype siblings and homozygous bcl9Δ29 embryos at 5 dpf; ventral views, anterior to the top, imaged after viable heart-stopping BDM treatment, two individual bcl9Δ29-mutant embryos are shown. A,F,K, bright field views to illustrate the absent swimbladder in mutants (sb, asterisks in F,K) and different expressivity of craniofacial defects (arrowhead, K); B-O maximum-intensity projection fluorescence close-ups of the heart (red dotted outlines in B,G,L); D,E,I,J,N,O depict optical sections at AV canal level. (P,Q) Confocal sections of sibling control (P) and homozygous bcl9Δ29 (Q) hearts, dissected and stained at 5 dpf to reveal the details of cardiac architecture. Also compare to Figure 2. ba, bulbus arteriosus; a, atrium; v ventricle; av, atrioventricular canal. Scale bars B-E,G-J,L-O 100 µm, S,T 20 µm.

Supplemental Figure 4: Craniofacial defects in bcl9 and pygo1/2 mutants.

(A-F) Alcian blue staining of the pharyngeal cartilage of 5 dpf wildtype, bcl9Δ29, and double homozygous pygo1Δ5;pygo2Δ1 embryos shown in ventral (A,C,E) and lateral (B,D,F) views, anterior to the left. bcl9 and pygo1/2 mutants have severe malformations of the pharyngeal apparatus with fusions defect of the ceratohyal (ch) and ceratobranchial 1 (cb1) arches and miss-shaped Meckel’s (m) and palatoquadrate (pq) cartilage. Scale bars, 100 µm. (G-L) SPIM-based brightfield imaging of wildtype-appearing siblings and homozygous pygo1Δ5;pygo2Δ1 embryos. Note absence of swim bladder (sb) and craniofacial defects in double-mutants (G,J). Ventral close-up of cardiac region (square in H,K, enlarged in I,L with red outline depicting the heart).
showing abnormal heart looping and smaller bulbus arteriosus region in homozygous mutants. (M) Quantification of phenotypes in four individual pygo1Δ5/wt x pygo2Δ1/wt crosses reveal defects in 7-18% of all embryos. Genotyping of phenotypic embryos revealed phenotype occurrence in homozygous pygo2Δ1 mutants in combination with homozygous or heterozygous pygo1Δ5 mutation. (N-P) MZpygo1Δ5 (N) and homozygous pygo2Δ1 (O) mutants, as well as mutants carrying a homozygous pygo1Δ5 combined with heterozygous pygo2Δ1 alleles (P) are viable and fertile; lateral views, anterior to the left.

Supplemental Figure 5: Pygo1/2-KO mouse embryos display severe valve and septal defects.

(A) Volume renderings generated from SPIM images of control (left panels) and Pygo1/2-KO (right panels) 13.5 dpc mouse heart embryos. The images show hearts from different perspectives. Additionally, an internal view is shown on the bottom of the panel. Dashed blues lines indicate the ario-ventricular septum. Dashed white lines indicate the opening, found only in mutant hearts due to septum malformations, between the cardiac chambers. (B) Virtual sections generated by SPIM imaging of 13.5 dpc mouse control (left panels) or Pygo1/2-KO (right panels) embryonic hearts. Dashed yellow lines mark atrio-ventricular valves. Reduction in valve leaflet thickness, resulting in aberrantly communicating chambers, is evident in the mutant.

Supplemental Figure 6: Bcl9/9l-ΔHD1 and Bcl9/9l-ΔHD1/ΔHD2 mouse embryos display heart defects recapitulating the Pygo1/2-KO phenotype.

(A-L) Haematoxylin/eosin stained transverse sections of the murine heart at 13.5 dpc. Compare control (A,B) with Bcl9/9l-ΔHD1 (C-F), and control (G,L) with Bcl9/9l-ΔHD1/-ΔHD2 mutant littermates (I-L). The reduction of the myocardium is indicated by arrowheads, while defective formation of the atrial septum by asterisks.
Supplementary Figure 7: Loss of the tripartite PYGO-BCL9-β-catenin complex formation leads to cartilage defects in the mouse.

(A-F) Alcian blue cartilage staining reveals several cartilage defects in Bcl9/9l-Δ1/Δ2 embryos, such as loss of digit formation (compare A,B with D,E) and rib bifurcations (C,D).

Supplemental Figure 8: Functional inhibition of Bcl9-β-catenin-interaction in bcl9Δ29 mutants and wildtypes leads to craniofacial, cardiac, and fin defects.

(A-E) Embryos obtained from a heterozygous bcl9Δ29 (bcl9Δ29/wt) incross were treated with 10 µM LH-2-40 from 4-cell stage on, lateral views, anterior to the left. Phenotype classes as described in Figure 4 were observed independent of the genotype of the embryo. (F) General phenotype penetrance is comparable to wildtype embryos treated with 10 µM LH-2-40 from 4-cell stage on (see Figure 4F). Nevertheless, the penetrance of strong phenotypes is increased in treated bcl9Δ29 crosses. (G,H) In addition to cardiac and craniofacial defects, Bcl9-inhibited embryos (wildtypes or bcl9Δ29 incrosses) are characterized by readily observable fin defects not observed in untreated bcl9Δ29 mutants (arrow heads H, compare to control in G).

Supplemental Figure 9: Total set of differentially expressed genes in bcl9Δ29 mutants compared to wildtype.

Volcano plot depicting the set of upregulated (74) and downregulated (83) genes in bcl9Δ29 mutant zebrafish embryos with an absolute value of the logFC above 1, p<0.12.

Supplementary Table S1: De-regulated genes in bcl9Δ29 zebrafish mutants with expression in cardiac, pharyngeal, and craniofacial precursors/derivatives as per ZFIN annotations and selected publications.
Supplementary Table S2: Examples of candidate genes deregulated in $bcl9^{\triangle29}$ zebrafish mutants and with known functions in heart and craniofacial development

Supplementary Table S3: Pygo1/2 loss-of-function leads to broad secondary gene expression changes.
CantuFelkerZimmerli_Fig3

A

Pygo1/2
NHD-N
β-catenin

B

Pygo1/2
NHD-N
β-catenin

stage (dpc)
embryo number

Bcl9/9l
Bcl9/9l

C

control

D

BATgal

E

BATgal

mean intensity

0.0
0.5
1.0
1.5

F

BATgal

mean intensity

0.0
0.5
1.0
1.5
2x

G

Axin2

H

skin

I

valve/skin

J

Axin2

K

skin

L

valve/skin

M

N

2x

O

BATgal

P

Q

R

S

TCF-siam:Red

T

TCF-siam:Red

U

wildtype

V

wildtype

bcl9\Delta29/\Delta29

wildtype
CantuFelkerZimmerli_Fig4

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- RNA extraction
- Genotyping
- Manual dissection at 54 hpf

B. 

- Scatter plot:
  - Log2 fold change vs. log10 p-value
  - Red dots: Wnt1-Cre; Pygo-flox
  - Blue dots: Pygo1/2 KO

C. 13.5 dpc control
D. 13.5 dpc Wnt1-Cre; Pygo-flox

E. 13.5 dpc control
F. 13.5 dpc Wnt1-Cre; Bcl9l−/−HD1/flox

G. 13.5 dpc control
H. 13.5 dpc Pygo1/2 KO

I. 13.5 dpc control
J. 13.5 dpc Pygo1/2 KO

K. 13.5 dpc control
L. 13.5 dpc Nkx2.5-Cre; Bcl9l-flox
M. 13.5 dpc Nkx2.5-Cre; Bcl9l-flox
N. 13.5 dpc Nkx2.5-Cre; Bcl9l-flox
A B D

CantuFelkerZimmerli_Fig6

A

- cranial neural crest
- cardiac neural crest
- heart

control vs Pygo1/2-KO

manual dissection

deep RNA-seq

RNA extraction

B

D

C

GO biological processes
Pygo1/2-KO vs control

skeletal system dev.
embryonic limb dev.
cardiac ventricle dev.

number of genes

p-value

1e−02
2e−02
3e−02
4e−02

mRNA expression (AU)

SIX2

Pitx2

Prx1

Msx1

E F

control
Pygo1/2-KO

Pitx2 mRNA ISH

* *
β-catenin and Pygo2 peak location

Overlapping peaks

Heart-specific target genes

Speg Flt2 Id3 Pou4f1 Sox11 Rara Six1 Six2 Nr2f2 Prkdc

Smarca4 Snai2 Twist1 Cited2 Hnmpu Trp53

Bcl9/9l Tcf/Lef HD2

β-catenin - Pygo2 common targets

skeletal system morphogenesis negative regulation of transcription in utero embryonic development heart morphogenesis heart development chromatin organization cardiac septum development

Gene associated with β-catenin peak location and Pygo2 peak location

Genes associated with β-catenin peak location

Genes associated with Pygo2 peak location

Embryonic and heart development

Overlapping peaks

p-value

4e-02 3e-02 2e-02 1e-02

Heart-specific target genes

Spea Flt2 Id3 Pou4f1 Sox11 Rara Six1 Six2 Nr2f2 Prkdc

Smarca4 Snai2 Twist1 Cited2 Hnmpu Trp53

Bcl9/9l Tcf/Lef HD2
**CantuFelkerZimmerli_Supplemental_Fig_S1**

**A**

*bcl9l, ENSDARG00000055054*

10 kb

Boxes indicate UTR and CDS. The Cas9 cut site is shown with a red box. The frameshift site is indicated by a red arrow.

**B**

*bcl9l^∆4/∆4: g.93611_93614del*

Reference sequence:

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ACGACCTGCGATTCCCTGACG
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Mutated sequence:

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ACGACCTGCGATTCCCTGACG
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Frameshift site:

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ACGACCTGCGATTCCCTGACG
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100 bp

**C** and **D**

5-6 hpf

**E** and **F**

5 dpf

**G**

5 mo
CantuFelkerZimmerli_Supplemental__Fig_S3

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**bcl9^{Δ29/WT} incross, 10 µM LH-2-40**

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**LH-2-40 @ 4-cell**

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- 15 µM n=90 N=3
- 10 µM n=73 N=3
- 1 µM n=70 N=3
- control n=72 N=3

5 dpf

G

H

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CantuFelkerZimmerli_Supplemental_Fig_S8
**Supplementary Table 1**

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## Supplementary Table 3

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