Beside the genetic components required to support newly identified CHD genes in trios and case/control study designs, **functional experiments** are essential to confirm the pathogenic effect of genes in animal models using knockout or knockdown experiments in mouse and zebrafish models. Where appropriate, the pathogenic effect of specific variants can also be investigated using cell-based assays such as luciferase activity experiments. Moreover, integrating exome and genome sequence data with gene expression data using RNA-Seq from fetal heart tissues at different developmental stages are likely to be a helpful tool to prioritize candidate genes. Integrating high-throughput genetics, functional genomics and cellular and animal modeling will require concerted effort and collaboration.

Appendix A

The following work was perfomed by Sebastian Gerety as part of chapter 3.

Methods: Functional experiments

Morpholino oligonucleotides (MO) were purchased from Gene Tools (Oregon, USA). One- to four-cell embryos were microinjected with 1.8 nl of morpholino diluted in water. The sequences of morpholinos used were zmym2 MO1: CTGAGTGTGGATGAATTACCAGATC, zmym2 MO2: ATTAAAATGACGTACTTCTTGCACA and tp53 GCGCCATTGCTTTGCAAGAATTG [521]. To eliminate off-target effects of morpholinos [522] we co-injected zmym2 MOs with tp53 MO.

The efficacy of the splice-blocking zmym2 MO1 was tested by RT-PCR. Embryos were injected with zmym2 MO1 or control MO, and grown until 24 hpf. RNA was extracted, and subject to RT-PCR with exonic primers spanning the targeted splice site, to detect correctly spliced mRNA. Additionally, to detect increased unspliced RNA, the above exonic primer was paired with a downstream intronic primer.
PRIMERS:
ZMYM2 MO1 Forward: CAAAAGTGGCGCTCTACCGTCTC
ZMYM2 MO1 Reverse exonic: GACGCCGATTGGGAGATCCATG

Results: Zebrafish morpholino knockout experiments

To assess whether *ZMYM2* has a role in heart development, my colleague, Sebastian Gretey, chose to perform loss of function experiments in the Zebrafish *Danio Rerio*. Their rapid, external development and a near-transparent body combined with rapid antisense oligo-mediated loss of function permits us to analyse gene function without the need for complex knockout technology.

Using the Ensembl browser, he first identified the zebrafish orthologue of *ZMYM2*, also called zmym2 (ENSDARG00000027353). The predicted zebrafish protein has a 50% amino acid identity with human *ZMYM2*, and shared synteny between the two species. Using the ENSEMBL predicted intron/exon structure of the zebrafish gene, Sebastian designed two antisense morpholinos, targeting the splice site at the end of the first and second coding exons. Injection of either of these morpholinos is predicted to cause intron retention, leading to premature truncation of the zmym2 transcript [523].

To determine if the morpholinos are effective at blocking splicing, he performed RT-PCR on injected embryos, which confirmed that zmym2 morpholino#1 injected embryos have an increase in unspliced mRNA and a decrease in correctly spliced mRNA across the target region (see Methods). These data confirm that zmym2 morpholino injection should decrease Zmym2 protein expression in the zebrafish embryo.

During heart morphogenesis in the zebrafish, a centrally aligned linear heart tube undergoes a lateral movement termed 'jogging', positioning it on the left side of the body by 24 hours post fertilisation (hpf) [524, 525]. Subsequent looping events in the second 24 hours of development results in an S-shaped heart structure resembling other vertebrate embryonic hearts, with ongoing blood flow. A number of genes implicated in ToF are linked to left-right asymmetry. To see whether the developing hearts in zmym2 morpholino injected embryos display any morphological defects, including aberrant jogging of the heart tube, or subsequent heart looping, both of which are strongly dependent on left-right asymmetry, he stained the heart tissue of zmym2 or control morpholino injected embryos by in situ hybridization with a CMLC2 RNA probe.

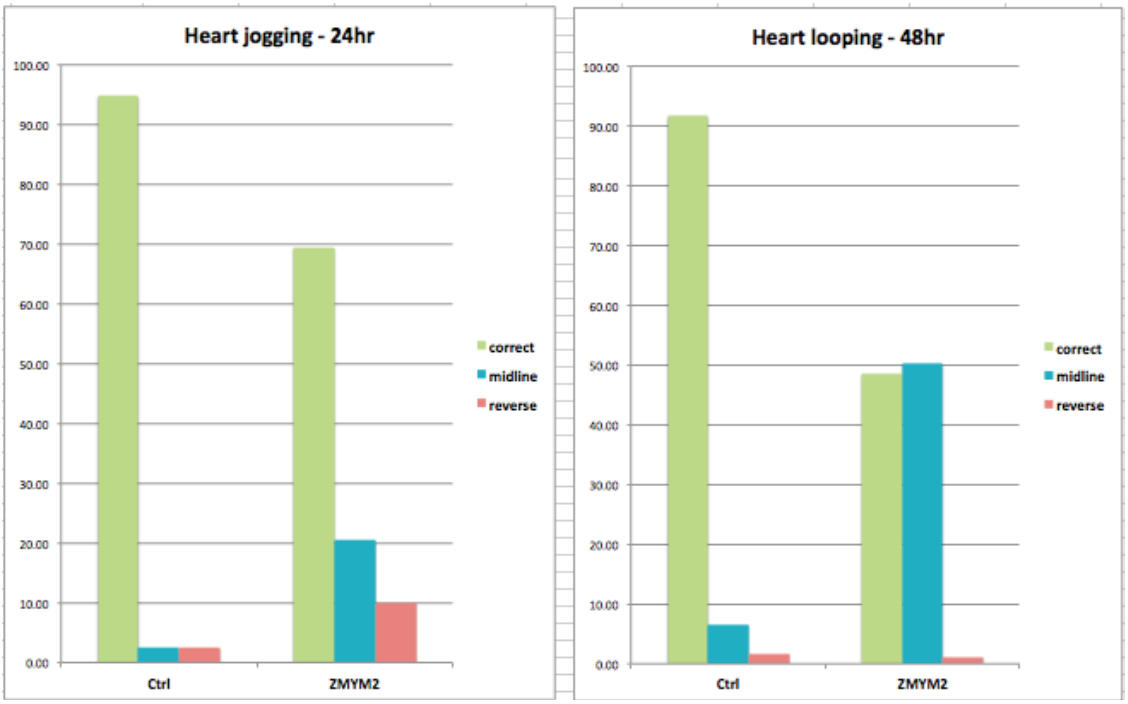

Figure A-1 Heart jogging and looping of the heart in the wild type and ZMYM2 morpholino injected embryos.

At 24 hpf, 94.9% of control injected embryos (n=91 embryos, 2 experiments) show a left jogging heart, while only 69.5% of zmym2 MO1 injected embryos show left jogging, with the remainder either central, or right jogging (n=70 embryos, 2 experiments). When analysed for heart looping at 48 hpf, 91.8% of control MO injected embryos (n=141, 3 experiments) showed correct looping, while only 48.5% of zmym2 MO1 injected embryos showed correct looping (Figure A-1). The remaining zmym2 MO1 injected embryos displayed a linear heart tube, in which looping had not occurred. The severity of the heart and other embryonic defects in zmym2 MO injected zebrfish results in dead or dying embryos by the fifth day post fertilisation.

Appendix B

The following work was perfomed by Sebastian Gerety as part of chapter 4.

Methods: *NR2F2* expression plasmids and luciferase constructs

My colleague, Sebastian Gerety, generated expression plasmids for *NR2F2* and its variants, the human wildtype *NR2F2* coding sequence was PCR amplified from a full length EST (Genbank acc.#BC042897), and cloned by Gibson assembly (New England Biolabs) into a CMV-driven pCS2-Cherry plasmid. To recreate the mutant forms of *NR2F2* (p.Lys70LysGln, p.Asp170Val, p.Asn205Ile, p.Glu251Asp, p.Ser341Tyr, and p.Ala412Ser), he amplified two PCR fragments overlapping each mutation, and cloned these as above. These expression constructs produce fusion proteins with fluorescent cherry domain [526] in order to monitor expression and localisation. To create the *NGFI-A* and *APOB* promoter driven Luciferase plasmids, he cloned synthetic DNA fragments for the rat *NGFI-A* upstream genomic region from -389 to +43 [527], and the human *APOB* upstream region from -139 to +121 [528], into a promoterless pGL3 Luciferase plasmid (Promega) by Gibson assembly (New England Biolabs).

Methods: Luciferase assays

HEK293T and HEPG2 cells were plated in 96-well plates, and transfected with 30ng of either *NGFI-A* or *APOB* luciferase plasmids, 0.75 ng of RL-TK renilla plasmid (Promega), and either 30ng of *NR2F2* expression plasmid (wildtype or variants) or 30ng of Cherry plasmid as a control. Two days after transfection, the cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System, according to the manufacturer's instructions (Promega). Each transfection was done in replicates (minimum three times) and the experiments were repeated 3-4 times. Luciferase readings were first normalized to the transfection control (renilla plasmid). Relative Response Ratios (Promega) were calculated based on negative and positive controls (cherry and *NR2F2* plasmid transfections), and outliers across all experiments were

identified by a median absolute deviation ratio >3. A t-test was performed to identify significant differences between variants and between promoters.

Results: Luciferase assays

Despite the availability of computational methods predicting the effect of missense variants on protein function, interpreting the significance of these mutations in human disease is notoriously difficult. My colleague Sebastian Gerety tested the consequence of the identified *NR2F2* variants in a functional assay. Nr2f2 is a transcriptional regulator, with both activating and repressive effects on target gene expression [512]. A number of *NR2F2* responsive genomic elements have been identified, which when placed upstream of a reporter gene can quantitate transcriptional regulator function of Nr2f2 variants [502, 527, 528]. Using the most widely employed element, the promoter region of the *NGFI-A* gene [527], to drive a luciferase reporter in HEK293 cells, he compared its level of activation by wildtype *NR2F2* with that of the patient-derived variants. Sebastian observed robust luciferase activation by wildtype Nr2f2, and equivalent levels of activity from variants p.Asp170Val and p.Ala412Ser. However, two variants (p.Glu251Asp and p.Ser341Tyr) show a significantly lower activity in this assay (20-24% reduction, $p<0.01$), while variants p.Lys70LysGln and p.Asn205Ile have an increased activity (13-15% increase, $p<0.03$) (Figure B-1).

As the function of nuclear receptors involves a complex interaction with other transcriptional coregulators, he hypothesized that the consequence of Nr2f2 mutations might be promoter context dependent. Sebastian therefore performed the luciferase assay on an alternative promoter fragment from the *APOB* gene, that has previously been shown to be bound by Nr2f2 and used for structure-function studies [528]. In agreement with our prediction, the activities of the variants on the APOB promoter in HEK293 cells were significantly different from those using the *NGFI-A* promoter (Figure B-1). Variants p.Asp170Val, p.Asn205Ile, p.Glu251Asp and p.Ser341Tyr all show strong reductions in transcriptional activity compared to wildtype Nr2f2 (26-52%