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# Inherited Ventricular Arrhythmia in Zebrafish: Genetic Models and Phenotyping Tools


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## Contents



**Abstract** In the last years, the field of inheritable ventricular arrhythmia disease modelling has changed significantly with a push towards the use of novel cellular cardiomyocyte based models. However, there is a growing need for new in vivo

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models to study the disease pathology at the tissue and organ level. Zebrafish provide an excellent opportunity for in vivo modelling of inheritable ventricular arrhythmia syndromes due to the remarkable similarity between their cardiac electrophysiology and that of humans. Additionally, many state-of-the-art methods in gene editing and electrophysiological phenotyping are available for zebrafish research. In this review, we give a comprehensive overview of the published zebrafish genetic models for primary electrical disorders and arrhythmogenic cardiomyopathy. We summarise and discuss the strengths and weaknesses of the different technical approaches for the generation of genetically modified zebrafish disease models, as well as the electrophysiological approaches in zebrafish phenotyping. By providing this detailed overview, we aim to draw attention to the potential of the zebrafish model for studying arrhythmia syndromes at the organ level and as a platform for personalised medicine and drug testing.

**Keywords** Cardiac electrophysiology · Gene editing · Inheritable ventricular arrhythmia · Zebrafish

## 1 Introduction

The exploration of the genetic aetiology of inherited cardiac arrhythmia has recently encountered significant challenges, which require a revision of its concept. Many genes previously associated with inherited arrhythmia are currently under dispute (Adler et al. 2020; Hosseini et al. 2018), and there is increasing evidence that complex inheritance patterns might underlie its pathogenesis (Bezzina et al. 2013). Nevertheless, much remains to be discovered, as evidenced by the incomplete yield of genetic analyses in inherited arrhythmia syndromes, although this varies by condition with the highest yield observed for long QT syndrome (LQTS) (60–80%) (Ingles et al. 2020) and the lowest for Brugada syndrome (BrS) and short QT syndrome (SQTS) (20–30%) (Campuzano et al. 2018; Ingles et al. 2020).

The assignment of pathogenicity to genetic variants identified in established or candidate disease genes for inherited arrhythmia is particularly challenging. This process can be facilitated by functional characterisation in model systems which are able to recapitulate the complex genetic architecture of these disorders, and, at the same time, remain suitable for phenotyping the cardiac electrical and mechanical activity and morphology.

Non-cardiac cellular models (e.g. HEK-293 cells) are too limited to provide this complex paradigm. The recent utilisation of induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) was considered a major breakthrough, as it enabled the study of cardiomyocyte function within the patient's own genomic background (Shaheen et al. 2017). However, the generation of iPSC-CMs is expensive and time-consuming, and the cells often show a high degree of variability and immaturity (Shaheen et al. 2017). Importantly, iPSC-CMs remain an in vitro model, and can

therefore not be used for the assessment of cardiac pathophysiology in the body, where the heart is exposed to mechanical, hormonal and neuronal regulation.

Murine models have been implemented in the experimental study of several inherited arrhythmias (Huang 2017). However, the translation of findings from mouse to human is not always straightforward, as the cardiac electrophysiology substantially differs between these species. A murine heart is characterised by a faster heart rate, a shorter action potential (AP) duration, a lack of a distinct plateau phase (Kaese and Verheule 2012) and distinct ion channel compositions (Nerbonne et al. 2001).

The zebrafish (*Danio rerio*) is a widespread laboratory animal, which is frequently used for the assessment of functional and structural consequences of genetic variants. Despite a greater evolutionary distance, the zebrafish heart is a surprisingly suitable model for human cardiac electrophysiology. Although the zebrafish cardiac anatomy differs from humans because of a single atrium and ventricle, the zebrafish cardiac AP and electrocardiogram (ECG) resemble the human to a larger extent than the mouse heart does (Nemtsas et al. 2010). Zebrafish are also less expensive to house, produce a large number of offspring and are easier and quicker to genetically modify (Rafferty and Quinn 2018). These advantages make this experimental animal model a particularly attractive tool for high-throughput assays (e.g. phenotype based drug discovery) (MacRae and Peterson 2015) and personalised medicine research.

Numerous studies have examined the zebrafish cardiac electrophysiology (Abramochkin et al. 2018; Bovo et al. 2013; Brette et al. 2008; Nemtsas et al. 2010; Ravens 2018), as well as the potential of zebrafish for inherited cardiac arrhythmia modelling (Poon and Brand 2013; Verkerk and Remme 2012; Vornanen and Hassinen 2016). In our review, we provide a comprehensive overview of previously developed genetic zebrafish models of inherited arrhythmia syndromes, with an emphasis on LQTS, SQTS, catecholaminergic polymorphic ventricular tachycardia (CPVT), BrS and arrhythmogenic (right ventricular) cardiomyopathy (ARVC/AC(M)). We discuss the methods used for the generation and phenotyping of these models, as well as the strengths and weaknesses of these approaches.

## 2 Methods

The PubMed database was searched in February 2021 for articles which contained "zebrafish" in the title or abstract, as well as at least one of the following keywords: "primary electrical disorders", "PED", "cardiac arrhythmia", "channelopathy", "Brugada", "long QT syndrome", "LQTS", "catecholaminergic polymorphic ventricular tachycardia", "CPVT", "short QT syndrome", "SQTS", "arrhythmogenic right ventricular cardiomyopathy", "ARVC", "arrhythmogenic cardiomyopathy" or "ACM". The titles of all identified papers were screened for relevance and all titles which did not concern ventricular electrophysiology or ventricular arrhythmia were excluded. The abstracts and subsequently the full content of the remaining articles were screened for relevance to the included disease phenotypes and/or inherited

cardiac arrhythmia genes. Additional relevant articles were identified from the reference lists of the selected publications. Literature reviews and editorials were screened for relevant references, but only original research papers were included in the final selection.

## 3 Results

### 3.1 *The Current Status of Published Reports on Zebrafish Models of Inherited Arrhythmia*

The initial search strategy yielded 253 articles, which were filtered to a total of 32 after screening (Arnaout et al. 2007; Asimaki et al. 2014; Berchtold et al. 2016; Brodehl et al. 2019; Da'as et al. 2019; Giuliodori et al. 2018; Hassel et al. 2008; Heuser et al. 2006; Huttner et al. 2013; Jou et al. 2013; Juang et al. 2020; Kapoor et al. 2014; Koopman et al. 2021; Kopp et al. 2005; Langenbacher et al. 2005; Langenbacher et al. 2020; Langheinrich et al. 2003; Leong et al. 2013; Martin et al. 2009; Meder et al. 2011; Moriarty et al. 2012; Pott et al. 2018; Ramachandran et al. 2013; Rottbauer et al. 2001; Smeland et al. 2019; Sondergaard et al. 2015; Tanaka et al. 2019; Tessadori et al. 2018; Thorsen et al. 2017; van den Boogaard et al. 2012; Warren et al. 2001; Zhou et al. 2016). There was a trend towards an increased publication rate over the last 15 years. Most of these studies used zebrafish embryos whereas 11 studies (Arnaout et al. 2007; Asimaki et al. 2014; Brodehl et al. 2019; Hassel et al. 2008; Huttner et al. 2013; Juang et al. 2020; Koopman et al. 2021; Langenbacher et al. 2020; Meder et al. 2011; Smeland et al. 2019; Warren et al. 2001) included phenotyping of juvenile and/or adult zebrafish, which was performed either by patch-clamp analysis in isolated cardiomyocytes (Asimaki et al. 2014; Brodehl et al. 2019; Koopman et al. 2021; Smeland et al. 2019; Warren et al. 2001) or by an electrocardiogram assessment (ECG) (Arnaout et al. 2007; Hassel et al. 2008; Huttner et al. 2013; Juang et al. 2020; Langenbacher et al. 2020; Meder et al. 2011). LQTS was the most frequently studied condition, accounting for nine out of these 32 articles (28%, Fig. 1).

### 3.2 *Technical Approaches for the Generation of Genetically Modified Zebrafish Disease Models*

Both forward and reverse genetic approaches have been used for the generation of zebrafish models of inherited arrhythmia. Forward genetics aim at the discovery of the genetic basis for a known phenotype. In reverse genetics, a specific genetic change is generated to assess its phenotypical effects. One of the main hurdles in the application of both techniques in zebrafish is related to an additional duplication of

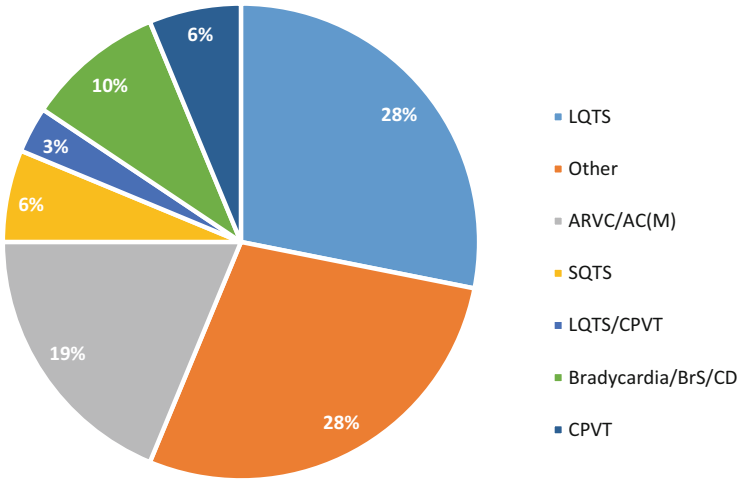


**Fig. 1** Percentage distribution of studied disorders (total number of studies: 32). LQTS: long QT syndrome, ARVC/AC(M): arrhythmogenic (right ventricular) cardiomyopathy, SQTS: short QT syndrome, BrS: Brugada syndrome, CD: conduction disease, CPVT: catecholaminergic polymorphic ventricular tachycardia. "Other" conditions included ABCC9-related Intellectual disability Myopathy Syndrome (AIMS) (Smeland et al. 2019), Cantu syndrome (Tessadori et al. 2018), studies on relevant cardiac arrhythmia genes with complex (Huttner et al. 2013; Koopman et al. 2021; Langenbacher et al. 2005, 2020; Rottbauer et al. 2001) or insufficiently characterised (Ramachandran et al. 2013) electrophysiological phenotypes and mutants whose causal mutation has not been fully characterised (Warren et al. 2001)

the zebrafish genome. As a consequence there are many human genes for which there are two orthologues in the zebrafish. If only a single copy of a gene is modified the phenotype can still be rescued due to compensation by its paralogue.

### 3.2.1 Forward Genetics

The first zebrafish models of inherited arrhythmia have been generated by large-scale forward mutagenesis screens (Table 1), where zebrafish were exposed to mutagens (e.g. ethylnitrosourea) and crossed out. Their offspring was screened for directly observable abnormalities of the heart rhythm (Rafferty and Quinn 2018). The causal genetic variants could subsequently be mapped and cloned (Rafferty and Quinn 2018).

The forward mutagenesis screens have led to the discovery of several mutants with a cardiac arrhythmia phenotype, such as the *island beat* (*isl*) (Rottbauer et al. 2001), *breakdance* (*bre*) (Kopp et al. 2005), *reggae* (*reg*) (Hassel et al. 2008), *hiphop* (*hip*) (Pott et al. 2018) and *grime* (Koopman et al. 2021) mutants (see Table 1 and "Genetic zebrafish models for inherited arrhythmias" for the description of the causal genes and phenotypes related to these mutations). Another mutant

**Table 1** Summary of the main techniques used for the generation of genetically modified zebrafish disease models

| Method | Advantages | Disadvantages | Cardiac arrhythmia disease models |
|---|---|---|---|
| Forward mutagenic screens | Hypothesis free<br>Possibility to study effect of specific mutations<br>Stable if not embryonically lethal | Not possible to target specific genes<br>Mapping required for identification of causal genes<br>Subtle phenotypes can be missed | *Isl* (*cacna1c*) (Rottbauer et al. 2001), *bre* (*kcnh6a*) (Kopp et al. 2005), *reg* (*kcnh6a*) (Hassel et al. 2008), *hip* (*atp1a1a.1*) (Pott et al. 2018), *slow mo* (unknown gene) (Warren et al. 2001), *grime* (*tmem161b*) (Koopman et al. 2021) |
| Morpholino knockdown | Phenotype observed in injected embryos<br>No mutagenesis required | Less representative of human mutation<br>Lack of genetic compensation<br>Transient<br>Off-target effects | *Kcnh6a* (Jou et al. 2013; Langheinrich et al. 2003; Tanaka et al. 2019), *slc4a3* (Thorsen et al. 2017), *cacna1c* (Ramachandran et al. 2013), *dsc2* (Heuser et al. 2006), *dspa and dspb* (Giuliodori et al. 2018), *plakoglobin1-a/jupa and plakoglobin-1b/jupb* (Martin et al. 2009), *pkp2* (Moriarty et al. 2012), *mog1* (Zhou et al. 2016), *atp1a1a.1* (Pott et al. 2018) |
| mRNA injections | Phenotype observed in injected embryos<br>No mutagenesis required<br>Possible to inject mRNA of human genes (e.g. to test specific variants)<br>Can be combined with morpholino to silence endogenous gene expression | Less physiological<br>Transient | *KCNH2/hERG* (Jou et al. 2013; Tanaka et al. 2019), *mog1* (Zhou et al. 2016), *SCL4A3* (Thorsen et al. 2017), *plakoglobin1-a/jupa and plakoglobin-1b/jupb* (Martin et al. 2009), *DSC2* (Heuser et al. 2006), *dspa and dspb* (Giuliodori et al. 2018), *kcnj2-12* (Leong et al. 2013), *CALM1* (Berchtold et al. 2016; Da'as et al. 2019; Sondergaard et al. 2015) |
| CRISPR/TALE knockout | Stable model<br>More representative of human disease<br>Relatively high mutagenic efficiency | Phenotype usually not visible in first generation<br>Off-target effects<br>Not possible to study effect of individual | CRISPR: a*bcc9* (Smeland et al. 2019), *gstm.3* (Juang et al. 2020), *tmem161b* (Koopman et al. 2021)<br>TALE: *mcu* |

(continued)

**Table 1** (continued)

| Method | Advantages | Disadvantages | Cardiac arrhythmia disease models |
|---|---|---|---|
| | | genetic variants (e.g. gain-of-function/ dominant negative) | (Langenbacher et al. 2020) |
| CRISPR knock-in | Stable model<br>Most representative of human disease<br>Possible to study effect of individual genetic variants (e.g. gain-of-function/dominant negative) | Low mutagenic efficiency<br>Phenotype not visible in first generation<br>Off-target effects | *Abcc9*, *kcnj8 and pln* (Tessadori et al. 2018) |
| Tol2 transposon | Stable model<br>Possible to introduce human genes/reporter genes<br>High mutagenic efficiency<br>Tissue/organ specific expression<br>Can be used for enhancer assay<br>Possible to study effect of individual genetic variants (e.g. gain-of-function/dominant negative) | Random insertion site<br>Does not alter endogenous genes | *SCN5A* (Huttner et al. 2013), *JUP* (Asimaki et al. 2014), *ILK* (Brodehl et al. 2019), enhancer assays (Kapoor et al. 2014; van den Boogaard et al. 2012) |

zebrafish line, called *slow mo* (Baker et al. 1997) was also discovered by this method, but the causal gene has not yet been identified.

Although mutagenesis screens have provided a useful identification method for essential genes involved in normal cardiac electrophysiology in zebrafish, some important genes were likely not identified due to compensation by the unaffected paralogue gene. Subtle phenotypes, which would require more extensive characterisation, could also easily be missed. Additionally, the random mutation process employed for forward genetics is less suitable for targeted translational studies. These disadvantages have paved the way for the application of reverse genetic methods in zebrafish for further modelling of inherited arrhythmia.

### 3.2.2 Reverse Genetics

Transient Zebrafish Models

In transient models, it is possible to temporarily alter gene products without directly affecting the genomic DNA. This can be achieved by reducing the expression of endogenous genes by morpholino injections and/or inducing the expression of exogenous gene products by mRNA injections (Table 1). Morpholinos are

oligomeric nucleotide analogues, which can reduce gene expression by binding complementary (pre)mRNA sequences leading to a gene knockdown. Morpholinos are able to either block ribosomal translation by binding the start codon or 5' untranslated region of an mRNA molecule or alter splicing by binding a splice-site and inhibiting pre-mRNA processing (Bill et al. 2009). The degree of translational inhibition varies among morpholino constructs (Kamachi et al. 2008) and can be quantified (e.g. by antibodies or RT-qPCR).

mRNA injections can be used to express zebrafish or human genes, which can be either wildtype or contain (potentially) pathogenic variants. This can be combined with morpholinos to simultaneously silence the endogenous zebrafish orthologue and assess the capacity of the injected mRNA to rescue the phenotype. However, this approach can be complicated by an inhibition of the translation of the injected mRNA by the morpholino, leading to an interference with the phenotype rescue (Joris et al. 2017).

Both morpholino knockdown and mRNA injections have the advantage of being easy to implement and providing a fast readout, as the phenotype can be observed already in the injected embryos. However, the knockdown effect is transient and can therefore only be assessed at the larval stage. Moreover, transient models are intrinsically less appropriate for the study of mutation-dependent pathomechanisms, as their phenotype is not induced by genomic alterations. Biased results have been reported, due to more pronounced phenotypes occurring by the use of morpholinos, compared to stable knockout (KO) mutants (Rossi et al. 2015). This effect appears to be related to the lack of engagement of genetic compensation, triggered by nonsense mediated decay, which is not activated by morpholinos (El-Brolosy et al. 2019). Additionally, off-target effects have been observed for morpholinos, which manifest as an unintentional reduction in the expression of non-targeted genes (Eisen and Smith 2008; Joris et al. 2017; Tessadori et al. 2020).

The use of morpholinos thus requires careful experimental design consideration. Additional validation can be performed by comparing the phenotype of morphants generated by morpholinos targeting different loci in the same gene and phenotype rescue experiments (Stainier et al. 2017). Nonetheless, a definite validation of a morpholino phenotype requires a comparison with a stable genetic KO model.

### Stable Models

The generation of stable genetic disease models has been greatly facilitated by the discovery of CRISPR-Cas9 (Jinek et al. 2012). This technique, derived from the bacterial defence system against bacteriophagic viruses, is based on the combination of a DNA cleaving enzyme (Cas9) and a customisable RNA sequence (guide RNA or gRNA), which limits the Cas9 activity to a pre-specified genomic locus (Fig. 2). These cleavage events are usually resolved by the error-prone endogenous cellular DNA repair process of non-homologous end joining, often resulting in insertions and deletions (indels) at the cleavage site (Fig. 2) (Adli 2018). The gRNA based genomic localisation mechanism is the main advantage of CRISPR compared to

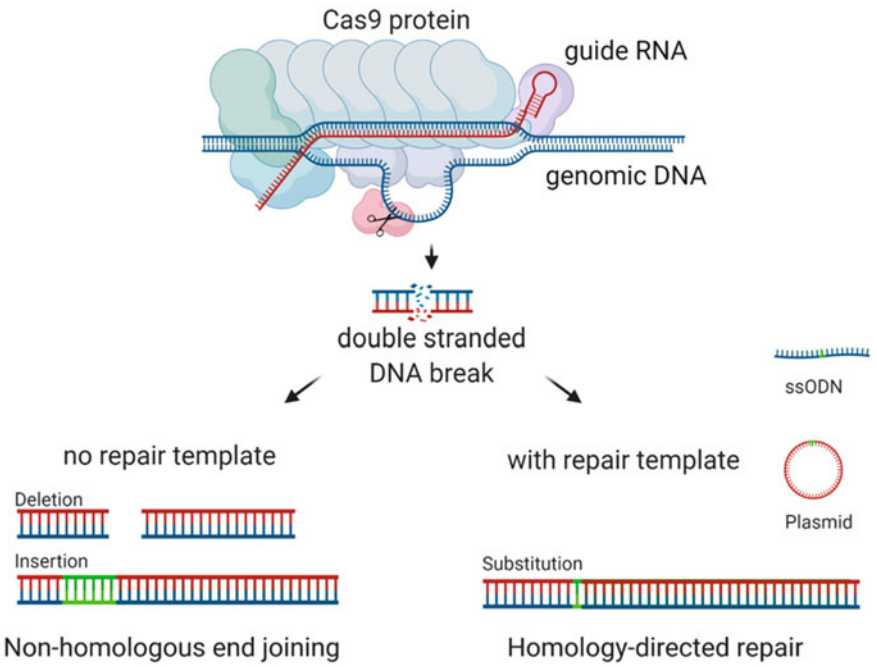


**Fig. 2** Principles and components of genomic modification by CRISPR-Cas9. ssODN: single-stranded oligo DNA nucleotides. Created with BioRender.com

previous approaches to genetic editing, such as transcription activator-like effectors (TALEs). These techniques rely on proteins for the direction of their cleavage activity, which renders them more challenging to customise, compared to CRISPR (Adli 2018).

When applied in zebrafish, Cas9 mRNA or protein, together with a gRNA, can be injected into fertilised zebrafish eggs at the one-cell stage (Albadri et al. 2017). The injected "founder" fish develop a variable ("mosaic") genomic sequence at the cleavage site due to different repair outcomes in individual cells during development. Some of the indel events will induce gene KO, either by disrupting the reading frame ("frameshift") in early exons or by altering DNA segments encoding essential protein components. The phenotypic effects of the KO can be difficult to assess in the founder generation due to mosaicism. When founder fish are raised to adulthood, potential germline transmitters can be bred to obtain offspring with a uniform genetic code. These stable mutants provide the opportunity to study the effect of the KO both at the larval and adult stage, as well as over multiple generations.

The generation of zebrafish CRISPR KO mutants is generally quite feasible (Table 1) (Varshney et al. 2015). Nonetheless, it is more time-consuming compared to gene knockdown and mRNA injections, as the phenotype is usually not observed in the injected embryos themselves. Some methods, such as injections with multiple gRNAs targeting different loci in the same gene (Wu et al. 2018) and/or injections

with an alternative form of gRNA (two-RNA component, crRNA:tracrRNA, duplex guide RNA) (Hoshijima et al. 2019), are being developed to compensate for this drawback by increasing the proportion of cells with gene KO in the founder generation.

The CRISPR/Cas9 technique is also capable of generating missense variants (knock-in, Table 1 and Fig. 2) by engaging another type of DNA repair process called homology-directed repair (Fig. 2) (Albadri et al. 2017). By providing an external repair template (usually either a plasmid or a single-stranded deoxynucleotide), it is possible to introduce specific base pair substitutions (Albadri et al. 2017; Boel et al. 2018; Prykhozhij et al. 2018; Tessadori et al. 2018). However, this method still remains challenging as its efficiency is rather low with knock-ins occurring in only up to 1–4% of targeted alleles (Boel et al. 2018).

Similar to morpholinos, off-target effects are also a risk with the use of CRISPR-Cas9. These manifest as DNA cleavage events, which occur at genomic sites other than the targeted region. CRISPR-Cas9 off-target effects can be mitigated by careful in silico design of the gRNA and breeding out the germline transmitters (Rafferty and Quinn 2018). The additional duplication of the zebrafish genome can also be a challenge for the application of CRISPR-Cas9. To study the function of a specific human gene, one often needs to generate mutations in both orthologues in the zebrafish.

### 3.2.3 Expression of Exogenous Genes

The Tol2 transposon method can stably insert exogenous genes into the zebrafish genome (Table 1) (Kawakami 2007). This method, based on autonomous mobile genetic elements identified in medaka fish, allows for the insertion of up to 11 kilo base pairs of DNA flanked by specific Tol2 sequences into random sites in the genome. Overall, up to 50–70% of the injected fish will produce transgenic offspring (Kawakami 2007). By coupling the transgene to a cell-type specific promoter, its expression can be limited to a certain tissue or organ. The Tol2 transposon method has been used to insert wildtype and mutant human genes (Asimaki et al. 2014; Brodehl et al. 2019; Huttner et al. 2013) and reporter proteins (van Opbergen et al. 2018) into the zebrafish genome (Table 1).

Apart from modelling functional alterations in protein-coding genes, zebrafish have also been used to characterise the function of putative enhancer sequences for cardiac arrhythmia genes (Kapoor et al. 2014; van den Boogaard et al. 2012). Enhancers are non-coding regulatory DNA sequences which interact with the activity of transcription factors to influence the expression of distally located genes (van den Boogaard et al. 2012). Enhancer assays make use of the Tol2 transposon method to insert putative enhancer sequences coupled to a promoter and a gene encoding a fluorescent protein. The regulatory effect of the enhancer sequence can be evaluated by monitoring the tissue expression of the fluorescent protein in the developing embryo. Similarly, zebrafish signalling pathway reporter lines are developed by inserting known pathway specific regulatory elements coupled to a

promoter and a gene encoding a fluorescent protein (Moro et al. 2013). These lines can be used to study factors which are expected to alter the activity of the pathway of interest (e.g. in a genetically altered zebrafish model for ARVC/AC(M) (Giuliodori et al. 2018)).

## *3.3 Electrophysiological Approaches in Zebrafish Phenotyping*

### 3.3.1 Methods to Assess the Electrophysiological Properties of the Zebrafish Heart

The characterisation of the zebrafish cardiac AP and its underlying currents has been performed by patch-clamp measurements on isolated cardiomyocytes and extracted hearts derived from adult (Abramochkin et al. 2018; Brette et al. 2008; Nemtsas et al. 2010; Zhang et al. 2011) and embryonic (Alday et al. 2014; Jou et al. 2010) zebrafish. Isolated cardiomyocytes can be derived by enzymatic digestion (typically with a mixture of collagenase and trypsin) of dissected adult (Abramochkin et al. 2018; Brette et al. 2008; Nemtsas et al. 2010) or embryonic (Alday et al. 2014) zebrafish hearts.

The electrophysiological characterisation of cardiomyocytes can be done by the patch-clamp method. In the clamp configuration, the recording electrode is brought into a close, high-resistance contact with the cell membrane (a seal), covering a patch of the membrane (Sakmann and Neher 1984). Several recording configurations are possible: a cell-attached, permeabilised and inside-out and outside-out isolated patches (Gurney 2000; Sakmann and Neher 1984). The whole-cell patch-clamp configuration is the most conventional mode, where the recording electrode forms a low resistance contact with the intracellular environment of the cell. Although this configuration disturbs the natural intracellular environment, it also allows to modify it. It has a high signal-to-noise resolution and is useful for characterisation of specific ion conductances.

The whole-cell configuration can be used for the voltage-clamp mode as well as the current-clamp mode (Wickenden 2014). In the current-clamp mode the current passing across the cell membrane is controlled to record the resulting changes in membrane voltage. This method measures the changes in the membrane potential and can be used for the characterisation of the zebrafish cardiac resting and action potentials. This can be assessed in zebrafish on both an isolated heart (Jou et al. 2010; Nemtsas et al. 2010) and single cardiomyocytes (Brette et al. 2008; Nemtsas et al. 2010; Rottbauer et al. 2001).

The voltage-clamp mode, where the voltage across the cell membrane is controlled and the resulting currents are recorded, is the way to assess membrane ion channels and electrogenic ion transporters. Importantly, due to technical limitations, it is not possible to control the voltage over a large membrane surface or over many electrically coupled cells (de Roos et al. 1996). This space phenomenon arises from

the resistance of the cytoplasm and the intercellular contacts, which induces a voltage drop over distance (de Roos et al. 1996). Because of this limitation, membrane voltage will no longer be uniformly distributed, and thus the cell, or several electrically coupled cells, cannot be efficiently voltage-clamped. This is especially important for the heart, where cardiomyocytes are tightly coupled electrically, creating an electrical syncytium. Therefore, voltage-clamp measurements of ionic current can only be performed in isolated cardiomyocytes or membrane patches (Fig. 3a) (Nemtsas et al. 2010; Skarsfeldt et al. 2018). In zebrafish, the voltage-clamp mode has been used to characterise the ionic currents of cardiomyocytes derived from enzymatic digestion of zebrafish hearts (Abramochkin et al. 2018; Alday et al. 2014; Brette et al. 2008; Nemtsas et al. 2010).

The membrane potential can also be assessed by a conventional sharp electrode impaled in the myocardium or isolated cardiomyocytes (Wickenden 2014). Although this impalement can be quite stressful for the cell, the method measures the average membrane potential of several neighbouring cells. Patch-clamp and sharp microelectrodes have been used for the phenotypical assessment of several inherited cardiac arrhythmias in zebrafish (Table 2) (Asimaki et al. 2014; Brodehl et al. 2019; Jou et al. 2013; Koopman et al. 2021; Rottbauer et al. 2001; Smeland et al. 2019; Warren et al. 2001). To date, these remain the most sensitive techniques for the characterisation of the cardiac membrane potential and specific ionic conductance. However, these methods are difficult to apply in vivo.

ECG recording is an important method, which provides information on the electrical properties of the entire heart and can be performed non-invasively in vivo (Milan et al. 2006). Indeed, ECG of adult (Hassel et al. 2008; Huttner et al. 2013; Langenbacher et al. 2020; Meder et al. 2011) and embryonic (Pott et al. 2018; Rottbauer et al. 2001; Tanaka et al. 2019; Thorsen et al. 2017) zebrafish are proven a popular and informative phenotyping tool in models for cardiac arrhythmia (Table 2). Similar to humans, the zebrafish ECG signal displays a P-wave, a QRS-complex and a T-wave (Fig. 3b). The ECG signal can expose arrhythmias as well as other electrophysiological or morphological abnormalities in the heart, e.g. QTc prolongation and hypertrophy. Because of their non-invasive nature, ECG recordings can be performed over a longer time span and are therefore useful for the detection of relatively rare arrhythmic events. ECG recordings in zebrafish require anaesthesia and the signal quality can be further improved by impaling needle electrodes or by opening the dermis and pericardial sac prior to the procedure in adult fish (Liu et al. 2016), although this intervention evidently increases the invasiveness of the procedure.

Important electrophysiological parameters, such as atrio-ventricular conduction delay and depolarisation and repolarisation abnormalities, can be derived from the ECG signal. ECG recordings show significant inter-experimental variability (Liu et al. 2016). For instance, measurements of the QTc interval in adult wildtype zebrafish can range from 250 ms to 600 ms between experiments (Liu et al. 2016). These differences can be attributed to several factors, including electrode placement, experimental temperature, nature or concentrations of anaesthetics and variable calculation methods for the QTc interval (such as the Bazett formula (Chablais

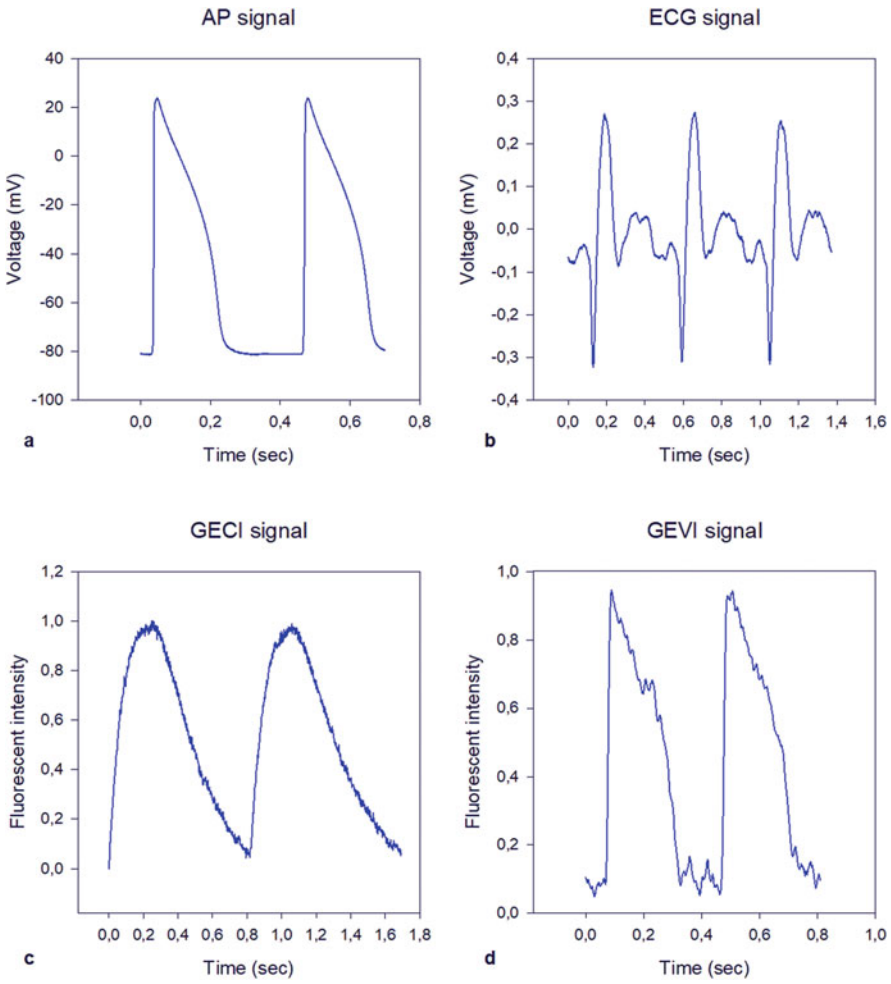


**Fig. 3** Overview of signals obtained by different electrophysiological phenotyping tools. (**a**) Patch-clamp recording of a cardiac action potential from a cardiomyocyte derived from the adult zebrafish heart. (**b**) Electrocardiogram (ECG) recorded from a zebrafish embryo at 3 days post fertilisation by surface glass electrode (Thorsen et al. 2017). (**c**) Fluorescent signal from the zebrafish embryo ventricle at 3 days post fertilisation representing a calcium transient, obtained by a genetically encoded calcium indicator (GECI) with a light sheet microscope; (**d**) Fluorescent signal from the ventricle of a zebrafish embryo at 3 days post fertilisation representing the cardiac action potential, obtained by a genetically encoded voltage indicator (GEVI) with a light sheet microscope

et al. 2011; Hassel et al. 2008; Tsai et al. 2011) or custom-made calculations (Arnaout et al. 2007; Milan et al. 2006)).

**Table 2** Summary of the main techniques for the electrophysiological characterisation of zebrafish

| Method | Advantages | Disadvantages | Examples of arrhythmia disease models |
|---|---|---|---|
| Patch-clamp | Extremely sensitive<br>Ionic current and membrane potential measurements possible<br>Embryo/larvae and adult | Labour intensive<br>Need for specialised equipment and expertise<br>Mostly single cell<br>Difficult to apply in vivo<br>Interference with intracellular environment | *KCNH2/hERG* (Jou et al. 2013), *isl* mutant (Rottbauer et al. 2001), *JUP* (Asimaki et al. 2014), *ILK* (Brodehl et al. 2019) |
| Electrocardiography (ECG) | In vivo<br>Minimally disruptive<br>Compatible with human phenotype<br>Overview of functioning of entire heart<br>Long recording time possible<br>Embryo/larvae and adult | Require anaesthesia<br>No direct measurement of action potential characteristics<br>Inter-experimental variability | *KCNH2/hERG* (Tanaka et al. 2019), *atp1a1a.1* (Pott et al. 2018), *slc4a3* (Thorsen et al. 2017), *cacna1c* (Rottbauer et al. 2001), *gstm.3* (Juang et al. 2020), *mcu* (Langenbacher et al. 2020) |
| (High speed) light microscopy video recordings | In vivo<br>Easy to perform<br>Limited technical requirements | Limited information (only atrial and ventricular heart rate and duration of systole/diastole)<br>Only feasible in embryos and optically translucent lines (e.g. Casper strain)<br>Embryo/larvae only | *CALM1* (Da'as et al. 2019; Sondergaard et al. 2015), *mog1* (Zhou et al. 2016), *kcnj2-12* (Leong et al. 2013), *bre* mutant (Kopp et al. 2005), *dsc2* (Heuser et al. 2006), *dspa and dspb* (Giuliodori et al. 2018), *plakoglobin1-a/jupa and plakoglobin-1b/jupb* (Martin et al. 2009), *pkp2* (Moriarty et al. 2012), *slc4a3* (Thorsen et al. 2017) |
| Calcium and voltage dyes | Some specialised technical requirements<br>Representation of most characteristics of cardiac action potentials (especially for voltage dyes)<br>Detection of localised action potential alterations<br>Possible to map | In vitro (explanted hearts)<br>Need to compensate for motion artefacts<br>Not cell/tissue specific<br>Increased background noise<br>Need for fluorescence microscopy set up<br>No information on | *Atp1a1a.1* (Pott et al. 2018), *bre* mutant (Meder et al. 2011), *reg* mutant (Hassel et al. 2008), *slc4a3* (Thorsen et al. 2017) |

(continued)

**Table 2** (continued)

| Method | Advantages | Disadvantages | Examples of arrhythmia disease models |
|---|---|---|---|
| | conduction pattern and speed<br>Embryo/larvae and adult | absolute membrane voltage or upstroke velocity<br>Less suitable for prolonged measurements (photobleaching and phototoxicity) | |
| Genetically encoded calcium and voltage indicators | In vivo<br>Cell-type specific<br>Stable transgenic transmission<br>Representation of most characteristics of cardiac action potentials (especially for GEVI)<br>Detection of localised action potential alterations<br>Possible to map conduction pattern and speed | Need to compensate for motion artefacts<br>Need for fluorescence microscopy set-up<br>No information on absolute membrane voltage or upstroke velocity<br>Less suitable for prolonged measurements (photobleaching and phototoxicity)<br>Need to insert indicator into the zebrafish genome<br>Embryo/larvae only | *Kcnh6a* (Arnaout et al. 2007) *tg*(cmlc2:gCaMP)$^{s878}$ line expressing a GECI) *Tmem161b*$^{uq4ks}$/tg (myl7:chimeric VSFP-butterfly CY) expressing a GEVI and *tmem161b*$^{uq4ks}$/tg (myl7:Gal4FF;UAS:GCaMP6f) line expressing a GECI (Koopman et al. 2021) |

*GEVI* genetically encoded voltage indicator, *GECI* genetically encoded calcium indicator

### 3.3.2 Imaging Techniques

The transparency of zebrafish larvae enables the observation of atrial and ventricular contractions under a light microscope, whether or not in combination with (high-speed) video recordings. Although this method only provides information on the heart rate and the atrio-ventricular synchrony, it has been the only method applied in several inherited arrhythmia studies, likely due to its ease of use and lack of technical requirements (Table 2) (Da'as et al. 2019; Kopp et al. 2005; Langheinrich et al. 2003; Leong et al. 2013; Sondergaard et al. 2015; Zhou et al. 2016). The obtained video recordings can be further translated to systole/diastole duration and ventricular morphology.

More options for imaging-based electrophysiological phenotyping are feasible by use of fluorescent microscopy. Calcium and voltage sensitive dyes are able to report changes in free cytosolic calcium concentration or membrane potential, respectively, as a fluorescent signal. The calcium sensitive Calcium Green dextran dye has been used previously to optically map embryonic zebrafish hearts in inherited arrhythmia models ex vivo (Table 2) (Hassel et al. 2008; Meder et al. 2011; Pott et al. 2018).

Other studies have described voltage mapping of ex vivo embryonic (Peal et al. 2011) and adult (Lin et al. 2014; Tessadori et al. 2012) hearts with the RH-237 dye (Lin et al. 2014) as well as the di-4 ANEPPS (aminonaphthylethenylpyridinium) dye (Peal et al. 2011; Tessadori et al. 2012). Importantly, due to different emission spectra, it is also possible to combine calcium and voltage dyes in a single experiment (Lin et al. 2015).

The main disadvantages of calcium and voltage sensitive dyes are the difficulty to use them in vivo and their lack of cell-specificity. The uptake of these dyes is not limited to excitable tissues and other surrounding cell types will also be loaded, thus generating significant background noise. To compensate for these drawbacks and enable cell-type specific in vivo optical mapping, genetically encoded calcium and voltage indicators (GECI and GEVI, respectively; Table 2) have been employed in zebrafish (Arnaout et al. 2007; Chi et al. 2008; Hou et al. 2014; Koopman et al. 2021; Tsutsui et al. 2010; van Opbergen et al. 2018; Weber et al. 2017).

Similar to dyes, GECI and GEVI are capable of reporting calcium and membrane voltage changes as a fluorescent signal (Fig. 3c–d). In practise, the genetic code for these indicators can be inserted into the genome of zebrafish. The myocardium-specific expression of the indicators is achieved by cardiac-specific promoters, such as the cardiac myosin light chain 2 (cmlc2 or myl7) promoter (Huttner et al. 2013). A recently developed zebrafish line with myocardium-specific expression of a GECI and a GEVI was used for the characterisation of drug-induced changes to the atrial and ventricular heart rate, activation pattern, conduction velocity and action potential duration (van Opbergen et al. 2018). By coupling GECI and GEVI with powerful microscopic techniques, such as light sheet imaging, it also becomes possible to optically map the electrical signals of the entire zebrafish heart (Weber et al. 2017). Although voltage dyes provide overall better brightness and kinetics than GEVI in vitro (Milosevic et al. 2020), due to the difficulty to load dyes intracellularly in vivo, GEVI outperform dyes in vivo (Mutoh et al. 2015).

The measurement of intracellular calcium concentrations by both dyes and genetically encoded indicators is a relatively well-established approach. The full optical characterisation of the cardiac membrane voltage has proven technically challenging. While calcium sensors are localised intracellularly, voltage sensors need to be incorporated in the cell membrane, and therefore tend to be dimmer. Moreover, some components of the membrane voltage signal, such as the AP rise, occur in a very short time span compared to the calcium transient. Voltage imaging thus requires both sensitive and high-speed imaging techniques and sensors with a fast response time. Despite these challenges, voltage imaging remains worthwhile as it provides a detailed characterisation of the cardiac AP. The development of GEVIs is a quickly evolving field and the newest GEVI are very promising due to their vastly improved brightness and kinetics (Shen et al. 2020).

Motion artefacts generated by the beating heart are an important hindrance to the use of both dyes and genetically encoded indicators. Currently, the most commonly used method to compensate for this problem relies on the inhibition of cardiac contraction by induction of electro-mechanical uncoupling. This can be done either pharmacologically, by exposing zebrafish embryos to the myosin ATPase activity

inhibitor blebbistatin (Jou et al. 2010) or the inhibitor of actin polymerisation cytochalasin D (Wang et al. 2013), or by blocking the expression of the *tnnt2* gene with the silent heart morpholino (Weber et al. 2017). Photobleaching is another challenge intrinsic to fluorescent imaging. This degradative process leads to an irreversible loss of the fluorescent intensity of a fluorophore upon each excitation. Fluorescent indicators susceptible to photobleaching are less suitable for prolonged imaging. Lastly, the application of fluorescent imaging can result in damage to cells and tissues (i.e. phototoxicity). The use of fluorescent indicators in vivo needs to be applied carefully with the minimal fluorescent intensity and exposure time required for the experiment.

## *3.4 Genetic Zebrafish Models for Inherited Arrhythmias*

### 3.4.1 Long QT Syndrome

KCNH2/hERG

Similar to humans, the rapidly activating outward rectifying potassium current ($I_{Kr}$) functions as the main repolarising current in zebrafish cardiomyocytes, although its kinetics are slightly different (Verkerk and Remme 2012). However, $I_{Kr}$ is not produced by the zebrafish *KCNH2/hERG* orthologue, but by the *kcnh6a* gene, which is primarily expressed in the central nervous system in humans (Vornanen and Hassinen 2016). Nonetheless, the zebrafish Kcnh6a protein shows 60% amino acid identity to the human KCNH2 and several studies have successfully phenotyped $I_{Kr}$-related LQTS (LQTS type 2) in zebrafish (Table 3) (Jou et al. 2013; Kopp et al. 2005; Langheinrich et al. 2003; Meder et al. 2011; Tanaka et al. 2019).

Forward mutagenesis screens have yielded the *bre* mutant with a p.(Ile59Ser) substitution in the *kcnh6a* gene. This *bre* mutation leads to a loss-of-function through an inhibition of protein trafficking to the cell membrane (Meder et al. 2011). Homozygous *bre* mutant zebrafish embryos have a normal morphology and show an intermittent dysregulation of atrio-ventricular synchrony with a single ventricular heart beat for each two atrial contractions (2:1 heart block) and a frequent embryonic lethality by 7 days post fertilisation (Kopp et al. 2005). The occasional adult survival of homozygous *bre* mutants suggests that both genetic and possibly environmental factors can influence the severity of the zebrafish arrhythmia phenotype. Similar variability in lethality is also observed in families with inherited cardiac arrhythmia (Cerrone et al. 2019).

This cardiac arrhythmia phenotype could be pharmacologically rescued by improving protein trafficking to the cell membrane by administration of cisapride (a prokinetic agent which was found to improve trafficking of mutant *KCNH2* channels) (Ficker et al. 2002) or chemical chaperones (Meder et al. 2011). Two additional pharmacological compounds, which modulate the *bre* phenotype, have been identified in a large chemical screen (2-MMB and the steroid flurandrenolide)

**Table 3** Summary of the existing models of inherited arrhythmia,

| Disorder | Gene | Genetic modification technique | Electrophysiological phenotyping technique | Electrophysiological phenotype | Reference |
|---|---|---|---|---|---|
| LQTS | *KCNH2* = *hERG* (human) *kcnh6* = *zerg* (zebrafish) | Forward mutagenesis screen (*bre* mutant, p.Ile59Ser substitution) | Light microscopy video recordings (embryo) | 2:1 heart block<br>Frequent embryonic lethality | (Kopp et al. 2005) |
| | | | Calcium dye in explanted hearts (embryo)<br>ECG (adult) | 2:1 heart block (embryo)<br>QT prolongation (adult) | (Meder et al. 2011) |
| | | Forward mutagenesis screen (p.Ile462Arg (S213) and p. Met521Lys (S290) substitutions)<br>Morpholino knockdown | Patch-clamp on explanted hearts + GECI (Tg(cmlc2: gCaMP)$^{s878}$) (embryo)<br>ECG (adult) | Homozygotes:<br>Silent ventricle and embryonic lethality (embryo)<br>Heterozygotes:<br>2:1 heart block, drug-induced APD prolongation (embryo)<br>QT prolongation (adult) | (Arnaout et al. 2007) |
| | | Morpholino knockdown | Light microscopy video recordings (embryo) | Irregular heart rate<br>2:1 heart block<br>Silent ventricle | (Langheinrich et al. 2003) |
| | | Morpholino knockdown + *KCNH2* mRNA overexpression | ECG (embryo) | 2:1 heart block<br>Ventricular asystole<br>QT-interval prolongation | (Tanaka et al. 2019) |
| | | | Light microscopy video recordings + patch-clamp (limited number for validation) (embryo) | 2:1 heart block<br>Prolonged APD | (Jou et al. 2013) |
| | *KCNJ2* (human) *kcnj2-12* (zebrafish) | *kcnj2-12* mRNA overexpression | Light microscopy video recordings (embryo) | Increased ratio of dead and malformed embryos | (Leong et al. 2013) |
| | Na+/K+-ATPase, *atpa1a.1* | Morpholino knockdown + forward mutagenesis screen (*hip* mutant) | Paced ECG + calcium dye (Calcium Green dextran) (embryo) | *Hip* mutants: irregular heart rate, prolonged QTc interval, partial heart block | (Pott et al. 2018) |

| | | | | | |
|---|---|---|---|---|---|
| | | | | Morpholino: morphological abnormalities and decreased systolic function (high dose), reduced and irregular heart rate block (low dose) | |
| | *NOS1AP* | Enhancer screen | – | – | (Kapoor et al. 2014) |
| SQTS | *KCNH2* = *hERG* (human)<br>*kcnh6* = *zerg* (zebrafish) | Forward mutagenesis screen (*reg* mutant) | Calcium dye (Calcium Green dextran) with pacing, sharp microelectrode (embryo)<br>ECG (adult) | Sino-atrial block, atrial fibrillation (embryo)<br>Shortened QTc interval (adult) | (Hassel et al. 2008) |
| | *SLC4A3* (AE3) | Morpholino knockdown | Light microscopy video recordings + ECG (embryo) | Shortened QT-interval | (Thorsen et al. 2017) |
| CPVT | *CALM1* | mRNA overexpression | Light microscopy video recordings and/or recordings of GFP-labeled Tg(myl7:GFP) hearts (embryo) | No phenotype or increased heart rate compared to controls (either at rest or induced by epinephrine) | (Da'as et al. 2019), (Berchtold et al. 2016), (Sondergaard et al. 2015) |
| ARVC/AC(M) | *DSC2*, *DSP*, *JUP*, *PKP2* | Morpholino knockdown + mRNA overexpression | Light microscopy video recordings (embryo) | Bradycardia, heart failure, abnormal cell–cell junctions | (Giuliodori et al. 2018), (Martin et al. 2009), (Moriarty et al. 2012), (Heuser et al. 2006) |
| | *JUP* | Tol2-mediated insertion of human wildtype and mutant *JUP* | Patch-clamp on isolated cardiomyocytes (adult) | Increased resting membrane potential, decreased maximal depolarisation rate, decreased sodium current density | (Asimaki et al. 2014) |
| | *ILK* | Tol2-mediated insertion of human wildtype and variant *ILK* | Light microscopy video recordings (embryo)<br>Patch-clamp on ex-vivo embryonic hearts | Decreased survival, no action potential abnormalities | (Brodehl et al. 2019) |

(continued)

**Table 3** (continued)

| Disorder | Gene | Genetic modification technique | Electrophysiological phenotyping technique | Electrophysiological phenotype | Reference |
|---|---|---|---|---|---|
| Other | *CACNA1C* | Forward mutagenesis screen (*isl* mutant) | Patch-clamp on isolated cardiomyocytes, ECG (embryo) | Abnormal ventricular morphology, no ventricular heartbeat, electrically unresponsive ventricles | (Rottbauer et al. 2001) |
| | | Morpholino knockdown | Light microscopy observation (embryo) | No ventricular heartbeat | (Ramachandran et al. 2013) |
| | *SCN5A* | Morpholino knockdown | Light microscopy observation (embryo) | Severe morphological cardiac defects | (Chopra et al. 2010) |
| | | Tol2-mediated insertion of human wildtype and mutant *SCN5A* +/− morpholino knockdown | Light microscopy video recordings, ECG (embryo)<br>ECG (adult) | Higher mortality, mild bradycardia, episodes of sinus pause (embryo)<br>Increase in the duration of the PR and QRS intervals (adult) | (Huttner et al. 2013) |
| | *SCN5A* + *SCN10A* | Enhancer screen | – | – | (van den Boogaard et al. 2012) |
| | *GSTM3* | CRISPR-Cas9 knockout | ECG (adult male) | Prolongation of PR and QRS interval after administration of flecaïnide<br>Inducibility of ventricular arrhythmia by flecaïnide or programmed extra-systolic stimulation | (Juang et al. 2020) |
| | *MOG1* | Morpholino knockdown + mRNA overexpression | Light microscopy video recordings (embryo) | Cardiac morphological defects, bradycardia (knockdown) or tachycardia (overexpression) | (Zhou et al. 2016) |
| | *Abcc9* | CRISPR-Cas9 knockout of *abcc9* gene | Light microscopy video recordings (embryo)<br>Patch-clamp (adult) | Morphological abnormalities, decreased mobility, heart failure (embryo)<br>Absent $K_{ATP}$ current (adult) | (Smeland et al. 2019) |

| | | | | | |
|---|---|---|---|---|---|
| | | CRISPR-Cas9 knock-in of *abcc9* and *kcnj8* genes | Light microscopy video recordings (embryo) | Enlarged hearts and an enhanced cardiac output | (Tessadori et al. 2018) |
| | *Ncx1h* | Forward mutagenesis screen (*tre* mutant)<br>Morpholino knock-down + mRNA overexpression | Light microscopy video recordings (embryo) | Atrial and ventricular fibrillation | (Langenbacher et al. 2005) |
| | *Mcu* | TALE knockout of *mcu* gene | ECG (adults) | Impaired cardiac function, abnormal QRS morphology, diminished R amplitude, episodes of sinus arrest | (Langenbacher et al. 2020) |
| | *Tmem161b* | *Grime* mutant (p.Cys466*), CRISPR knockout of *tmrm161b* gene | GEVI (tg(myl7:chimeric VSFP-butterfly CY)) and GECI (tg(myl7:gal4FF; UAS:GCaMP6f)) (homozygous embryos)<br>Patch-clamp (heterozygous adult) | Sino-atrial and atrio-ventricular abnormalities, faster and increased calcium release (homozygous embryos)<br>Early afterdepolarisations, abnormal AP duration (shorter APD20 but longer APD50 and APD90), increased $I_{Ks}$ and $I_{CaL}$ currents (heterozygous adults) | (Koopman et al. 2021) |

(Peal et al. 2011). The pharmacological effects of these drugs appear unrelated to trafficking (Peal et al. 2011). Flurandrenolide acts on the cardiomyocytes through the glucocorticoid receptor, but the exact mechanisms of action are unclear for both 2-MMB and flurandrenolide. *Bre* mutants that survive into adulthood display a prolonged QTc interval (Meder et al. 2011). Experimental pharmacological rescue of this defect in vivo illustrates the potential of zebrafish studies for research into personalised medical treatments.

Two other *kcnh6a* mutants, p.(Ile462Arg) and p.(Met521Lys) missense mutations (called *S213* and *S290*, respectively), were also discovered through forward mutagenesis screens. These mutant embryos displayed a more severe phenotype compared to the *bre* mutants, with silent ventricles and complete embryonic lethality in the homozygous state (Arnaout et al. 2007). Heterologous expression studies in Xenopus oocytes showed a complete lack of function for isolated mutant channels and dominant negative effects when co-expressed with the wildtype allele (Arnaout et al. 2007). Heterozygous mutant embryos showed a 2:1 heart block upon exposure to the $I_{Kr}$ blocking drug terfenadine, as well as a prolonged action potential duration at 90% of repolarisation (APD90), while adult heterozygotes had a prolonged QTc interval (Arnaout et al. 2007).

These findings from forward mutagenesis screens revealed a typical and easily observable feature of the LQTS phenotype in zebrafish, i.e. the 2:1 heart block. It is likely that this phenomenon is caused by increased ventricular refractoriness, rather than increased atrio-ventricular delay (Arnaout et al. 2007). The more severe phenotype observed in the *S213* and *S290* mutants compared to the *bre* mutants is an apt example of how allelic heterogeneity can act as a modifier of disease severity, which highlights the importance of allelic series variant modelling.

Several studies have also looked into the effect of morpholino knockdown of the *kcnh6a* gene, which resulted in a phenotype with an irregular heart rate, an atrio-ventricular block or a silent ventricle (Langheinrich et al. 2003). Co-injection of either the zebrafish *kcnh6a* (Arnaout et al. 2007) or the human *KCNH2* (Jou et al. 2013; Tanaka et al. 2019) mRNA could partially rescue normal cardiac function. This co-injection method was used to develop an in vivo assay to distinguish between benign and disease-causing *KCNH2* variants by examining the ability of *KCNH2* mRNA carrying these variants to rescue the LQTS phenotype (Jou et al. 2013). The zebrafish assay achieved a negative predictive value of 90% and a positive predictive value of 100% compared to heterologous expression (Jou et al. 2013). This assay has the advantage of being in vivo, easy to execute and high throughput, with a phenotype which could be observed directly under a light microscope in the injected embryos.

## KCNJ2

Mutations in the *KCNJ2* gene encoding the inward rectifier potassium channel 2 are the cause of LQTS type 7, also called Andersen-Tawil syndrome. A bioinformatical and transcriptional approach has identified the zebrafish *kcnj2-12* gene as the

zebrafish *KCNJ2* orthologue, although with a lower cardiac expression compared to human (Leong et al. 2013). Subsequent overexpression experiments with injections of human wildtype or mutant *KCNJ2* mRNA at identical concentrations showed an increased ratio of dead and malformed embryos in the mutant group. The cardiac phenotype was not thoroughly investigated, as heart rate was the only cardiac variable examined in this study (Table 3) (Leong et al. 2013).

### KCNQ1 and KCNE1

The expression of the slowly activating potassium current ($I_{Ks}$), generated by the protein products of the *KCNQ1* (alpha subunit) and *KCNE1/MINK* (beta subunit) genes, was difficult to confirm in adult zebrafish (Abramochkin et al. 2018) and is yet to be confirmed in zebrafish embryos. In zebrafish, there is pronounced reduction in the expression of the *KCNE1/MINK* beta subunit, which leads to a decreased current amplitude and altered kinetics with a rapid activation and decreased beta-adrenergic responsiveness (Abramochkin et al. 2018). The reduced expression of the beta subunit also alters the protein conformation of the channel complex and thereby its drug sensitivity. These differences make zebrafish a less attractive model for LQTS caused by *KCNQ1* and *KCNE1* mutations (i.e. LQTS type 1 and 5, respectively).

### Other LQTS Genes

Apart from the classical potassium channels, other genes that influence the length of the QTc interval have been studied in zebrafish (Kapoor et al. 2014; Pott et al. 2018). Forward mutagenesis screen revealed an LQTS phenotype in zebrafish embryos induced by missense mutations (*hip* mutants) in the *atpa1a.1* gene, encoding Na+/K +-ATPase (Table 3) (Pott et al. 2018). *Hip* mutants displayed a reduced and irregular heart rate, a prolonged QTc interval as well as a partial heart block. In contrast to the previously discovered complete loss-of-function *atpa1a.1* null alleles (*heart and mind* (Shu et al. 2003) and *small heart* (Yuan and Joseph 2004) forward screen mutants), the *hip* mutation showed no impact on cardiac morphology and systolic function, suggesting a partial, rather than complete, loss of function (Pott et al. 2018). Similarly, morpholino experiments have demonstrated an effect on the heart rate only at lower dosages and additional morphological defects with increasing injection dosages (Pott et al. 2018).

Zebrafish were also used as an in vivo screening tool for the enhancer activity of several non-coding variants in the *NOS1AP* gene locus with suspected QT-regulatory functions, which were identified by a genome-wide association (GWAS) study (Table 3) (Kapoor et al. 2014).

### 3.4.2 Short QT Syndrome

KCNH2/hERG

Similarly to LQTS, the first zebrafish SQTS mutant was discovered by means of a forward mutagenesis screen and concerned a p.(Leu499Pro) missense mutation in the *kcnh6a* gene (*reg* mutant, Table 3) (Hassel et al. 2008). This mutation resulted in a gain of function of the channel protein due to faulty channel inactivation. *Reg* mutant embryos displayed a phenotype with an intermittent loss of the heartbeat, which could be rescued or converted to an LQTS phenotype by injection of a morpholino targeted against *kcnh6a* or an $I_{Kr}$ blocking agent (terfenadine) (Hassel et al. 2008). Both homozygous and heterozygous adult *reg* mutants displayed shortened QTc intervals compared to wildtype controls (Hassel et al. 2008).

SLC4A3

Another zebrafish SQTS model involves the Solute Carrier Family 4 Member 3 (*SLC4A3*) gene, which encodes a $Cl^-$-$HCO3^-$-exchanger (AE3, Table 3) (Thorsen et al. 2017). This is the first anion membrane transporter which was shown to be involved in cardiac arrhythmia. Heterozygous loss-of-function mutations in *SLC4A3* were identified as a cause of SQTS by whole exome sequencing in an affected family and subsequent heterologous expression experiments (Thorsen et al. 2017). This effect was recapitulated by morpholino-induced knockdown in zebrafish embryos, which led to an elevated intracellular pH and a shortened QT-interval (Thorsen et al. 2017).

### 3.4.3 Catecholaminergic Polymorphic Ventricular Tachycardia

CALM1

Three studies have described the development and characterisation of calmodulin mRNA overexpression models (Table 3) (Berchtold et al. 2016; Da'as et al. 2019; Sondergaard et al. 2015). Human mutations in the *CALM1* gene are involved in both the CPVT phenotype (CPVT type 4) and the LQTS phenotype (LQTS type 14). Two studies found that zebrafish embryos injected with *CALM1* variants previously identified in patients with CPVT had a faster heart rate either at rest (Da'as et al. 2019) or upon exposure to epinephrine (Sondergaard et al. 2015). Additionally, the heart rate in the mutant group was irregular in comparison with controls (Da'as et al. 2019). In the third study, several *CALM1* variants associated with CPVT, LQTS and idiopathic ventricular fibrillation were tested, however only the mutation associated with LQTS gave rise to a phenotype with a reduced resting heart rate (Berchtold et al. 2016). As the electrophysiological phenotyping in all studies was limited to the

observation of the heart rate in transient overexpression models, further exploration is needed to expand on the zebrafish CPVT phenotype.

### 3.4.4 Arrhythmogenic Right Ventricular Cardiomyopathy

So far, four desmosomal genes implicated in ARVC/AC(M) (*DSC2*, *DSP*, *JUP* and *PKP2*) have been investigated in zebrafish by morpholino knockdown combined with mRNA overexpression (Table 3) (Giuliodori et al. 2018; Heuser et al. 2006; Martin et al. 2009; Moriarty et al. 2012). Knockdown for all studied genes led to a bradycardia phenotype, which was rescued by wildtype mRNA injections of the corresponding gene. When tested, injection of mutant mRNA did not lead to rescue and as such confirmed pathogenicity (Heuser et al. 2006). The morphant embryos showed signs of heart failure with diminished cardiac contractions, pericardial oedema and blood pooling. Electron microscopy confirmed abnormal cell–cell junctions in *dsc2* (Heuser et al. 2006), *dsp* (Giuliodori et al. 2018), *jup* (Martin et al. 2009) and *pkp2* (Moriarty et al. 2012) morphants. The studies on *dsp* and *jup* also examined signalling pathway alterations and uncovered abnormalities in the Wnt/β-catenin pathway (Giuliodori et al. 2018; Martin et al. 2009). These results await confirmation in genetic loss-of-function models.

The c.2057delTG mutation in the *JUP* gene is associated with an autosomal recessive form of ARVC/AC(M), with associated changes in the hair and skin (Naxos syndrome) (Asimaki et al. 2014). The effects of this mutation have been investigated in a zebrafish model with cardiomyocyte-specific overexpression of mutant human *JUP* generated with the Tol2 method (Asimaki et al. 2014). Zebrafish expressing this mutation displayed enlarged hearts with signs of heart failure by 4–6 weeks of age (Asimaki et al. 2014). Electrophysiological abnormalities were also detected with an elevated resting membrane potential, a decreased maximum rate of depolarisation (dV/dT max) and a reduction in sodium current density (Asimaki et al. 2014). This zebrafish model was also used in a chemical screen to identify a small molecule (SB216763 (SB2)), annotated as a GSK3β inhibitor, that rescues the disease phenotype (Asimaki et al. 2014). This pharmacological effect was later confirmed in a mouse model of ARVC/AC(M) (Chelko et al. 2016).

Finally, two variants (p.His33N and p.His77Tyr) in integrinlinked kinase (*ILK*), a non-desmosomal gene involved in cell–matrix interactions, were modelled in zebrafish by cardiomyocyte-specific overexpression of variant human *ILK* via the Tol2 method (Brodehl et al. 2019). Both variants were identified in families with ARVC/AC(M) (Brodehl et al. 2019). The p.His77Tyr embryos showed decreased survival after 5 days post fertilisation, with only 20% reaching adulthood (Brodehl et al. 2019). Although fractional shortening appeared to be mildly decreased in the p. His77Tyr line in the embryonic stage, no morphological abnormalities were detected on histology for both lines (at 3 and 12 days post fertilisation) (Brodehl et al. 2019). Patch-clamp studies on ex-vivo embryonic hearts displayed no abnormalities.

### 3.4.5 Other Conditions and Relevant Genes

CACNA1C

The *CACNA1C* gene encodes the alpha subunit of the cardiac L-type calcium channel, which is involved in several inherited cardiac arrhythmia conditions (LQTS, BrS and SQTS) (Bezzina et al. 2015). Similar to humans, the zebrafish AP plateau phase is maintained by calcium current. In zebrafish, this current is the product of both the L-type ($I_{CaL}$) and the T-type ($I_{CaT}$) calcium channels, while in healthy human adults, the T-type calcium current is only found in conductive tissue and the sino-atrial node (Haverinen et al. 2018; Zhang et al. 2011). The sarcolemmal calcium channels also play a role in the excitation-contraction coupling, as well as the action potential upstroke in zebrafish embryos (Alday et al. 2014; Bovo et al. 2013).

Forward mutagenesis screens have yielded zebrafish embryos with a silent ventricle and abnormal and uncoordinated contractions in the atrium, called the *isl* mutants (Table 3). This phenotype was mapped to homozygous nonsense mutations in the *cacna1c* gene (p.(Gln1077*) and p.(Leu1352*)) (Rottbauer et al. 2001). Similar observations were made in *cacna1c* knockdown morphants (Ramachandran et al. 2013). Voltage-clamp studies in zebrafish cardiomyocytes demonstrated an absence of the L-type calcium current in *isl* mutants (Rottbauer et al. 2001). Ventricles of *isl* mutants showed hypoplasia and were electrically unresponsive to external pacing (Rottbauer et al. 2001). Knockdown of *cacnb2.1*, the zebrafish orthologue of the main beta subunit of the cardiac L-type calcium channel in humans, resulted in cardiac developmental abnormalities (i.e. reduced cell proliferation and abnormal ventricular cell size and shape), bradycardia and heart failure (Chernyavskaya et al. 2012). These experiments clearly demonstrate that the L-type calcium channel is essential for normal cardiac development and AP generation in zebrafish.

SCN5A

The human *SCN5A* gene encodes the main cardiac sodium channel (Nav1.5). Mutations in *SCN5A* are encountered in BrS, cardiac conduction disorders, LQTS type 3 and dilated cardiomyopathy (Bezzina et al. 2015). In zebrafish, the cardiac sodium channel is encoded by two orthologues (*scn5Laa* and *scn5Lab*). The cardiac sodium current density is lower in zebrafish compared to humans, which likely contributes to the lower AP upstroke velocity observed in zebrafish (approximately 90 V/s in the adult zebrafish ventricle compared to 180 V/s in the human ventricle) (Brette et al. 2008; Nemtsas et al. 2010). The zebrafish *scn5Laa* and *scn5Lab* genes appear to play a role in embryonic cardiac differentiation by regulating early cardiac gene expression. Morphological abnormalities with a small heart size due to a reduced number of cardiomyocytes were observed upon morpholino knockdown

of either *scn5Laa* or *scn5Lab*, although these findings need to be confirmed in a stable KO model (Table 3) (Chopra et al. 2010).

The p.(Asp1275Asn) mutation in the *SCN5A* gene, leading to a loss-of-function of the Nav1.5 channel, was reported in multiple families with atrial dysrhythmia, conduction disturbances and dilated cardiomyopathy (Huttner et al. 2013). In order to study the effects of this mutation in zebrafish, stable transgenic lines with expression of either wildtype or mutant human *SCN5A* were generated by the Tol2 method (Table 3) (Huttner et al. 2013). Zebrafish expressing the p.(Asp1275Asn) *SCN5A* construct displayed a higher incidence of mortality, mild bradycardia and episodes of sinus pause, as well as an increased duration of the PR and QRS intervals, compared to non-transgenic controls and transgenic fish injected with the wildtype *SCN5A* construct (Huttner et al. 2013).

Morpholino knockdown of the endogenous *scn5Laa* and *scn5Lab* genes in these transgenic humanised fish led to severe morphological cardiac defects in approximately 79.8% of wildtype *SCN5A* and 86.9% of p.(Asp1275Asn) *SCN5A* embryos (Huttner et al. 2013). This only partial rescue seems to suggest that the endogenous zebrafish genes, rather than human *SCN5A*, remain the main contributors to the sodium current in humanised zebrafish. However, this effect has not yet been replicated in a stable genetic KO model and the lack of rescue could therefore still be attributed to morpholino off-target effects. Moreover, apart from *SCN5A* variant modelling, zebrafish have also been used to identify regulators of the *SCN5A* and *SCN10A* genes in an enhancer assay (Table 3) (van den Boogaard et al. 2012).

## GSTM3

A recent study aimed at the discovery of novel disease-causing genes by copy number variant analysis identified deletions of the *Glutathione S-transferase (GSTM3)* gene as a possible mechanism for BrS in a Taiwanese patient cohort (Juang et al. 2020). This gene is involved in cellular defence against oxidative stress. Adult male *gstm.3* KO zebrafish generated by CRISPR-Cas9 showed no ECG abnormalities at rest (Juang et al. 2020). When exposed to a sodium channel blocking agent (flecainide), both the heterozygous and homozygous *gstm.3* KO zebrafish displayed a pronounced prolongation in the PR and QRS intervals compared to wildtype zebrafish, as well as an increased incidence of ventricular arrhythmia.

The ventricular arrhythmia appeared to subside upon administration of the class I antiarrhythmic agent quinidine (Juang et al. 2020). Although quinidine acts on multiple signalling pathways in the heart, its therapeutic effect in BrS is mainly attributed to inhibition of the transient outward potassium current ($I_{to}$) (Zhou et al. 2010). However, this current is absent in zebrafish cardiomyocytes (Nemtsas et al. 2010). Thus the electrophysiological basis for the beneficial effect observed in *gstm.3* KO zebrafish is not clear. Moreover, both heterozygous and homozygous *gstm.3* KO zebrafish were more likely to develop ventricular arrhythmia when

induced by programmed extra-systolic stimulation compared to wildtype zebrafish (Juang et al. 2020).

### Nuclear Import Protein MOG1

The *MOG1* gene functions as a regulator of Ran, a small GTPase with an important role in transport through the nuclear pore complex (Zhou et al. 2016). Apart from its main function, *MOG1* has also been found to influence membrane trafficking of the Nav1.5 channel and at least one dominant negative mutation in *MOG1* has been associated with BrS (Zhou et al. 2016). In zebrafish embryos, *mog1* appears to have a role in the regulation of the heart rate and cardiac morphogenesis, as observed in morpholino-induced knockdown and mRNA overexpression experiments (Table 3) (Zhou et al. 2016). Overexpression experiments with human *MOG1* mRNA containing a variant associated with BrS led to a decreased heart rate. Unfortunately, more detailed reports on cardiac functioning in zebrafish *MOG1* models are not available, as the electrophysiological phenotyping in this study was limited to observations of the heart rate.

### $K_{ATP}$-Associated Genes

In humans, $K_{ATP}$ is composed of the pore-forming Kir6.x protein (encoded by *KCNJ8* and *KCNJ11* genes) and the sulfonylurea subunit (encoded by the *ABCC9* and *ABCC8* genes). Recently, two families with a loss-of-function splice site mutation in the *ABCC9* gene were identified. Homozygous carriers of this mutation displayed a phenotype with a spectrum of morphological, central nervous system and musculoskeletal abnormalities, as well as cardiac systolic dysfunction (*ABCC9*-related Intellectual disability Myopathy Syndrome, AIMS) (Smeland et al. 2019). *Abcc9* zebrafish KO models generated by CRISPR-Cas9 mimicked the human phenotype with morphological abnormalities, decreased mobility and decreased systolic function (Table 3) (Smeland et al. 2019). Gain-of-function mutations in $K_{ATP}$ genes have previously been associated with Cantú syndrome, which is characterised by hypertrichosis, distinctive facial features and cardiovascular abnormalities (Tessadori et al. 2018). Similar to the human phenotype, zebrafish knock-in models of Cantú syndrome mutations showed enlarged hearts and an enhanced cardiac output (Table 3) (Tessadori et al. 2018).

### Sodium-Calcium Exchanger (NCX) and Mitochondrial Calcium Uniporter (MCU)

The cardiac sodium-calcium exchanger, responsible for the extrusion of calcium from the cardiomyocyte after contraction, is encoded by several isoforms, of which *NCX1* shows the highest expression in the heart. The *ncx1h* gene has been identified

as the zebrafish *NCX1* orthologue. Both morpholino *ncx1h* knockdown models and forward screen derived loss-of-function *ncx1h* mutants (*tremblor* or *tre*) show ventricular and (even more pronounced) atrial fibrillation (Table 3) (Langenbacher et al. 2005). The phenotype could be rescued by injections with wildtype *ncx1h* mRNA.

In a follow-up study, the synthetic compound efsevin was found to lead to a rescue of the *tre* phenotype through modulation of the mitochondrial calcium uptake (Shimizu et al. 2015). These findings encouraged further research into the zebrafish cardiac calcium homeostasis, which led to the development of a zebrafish line with a TALE-based knockout of the mitochondrial calcium uniporter (*mcu*) (Langenbacher et al. 2020). Adult mutant zebrafish displayed an impaired cardiac function with ECG abnormalities (abnormal QRS morphology, diminished R amplitude and episodes of sinus arrest) (Langenbacher et al. 2020).

### Transmembrane Protein 161b (tmem161b)

The *grime* (*uq4ks*) mutant was discovered in a forward mutagenesis experiment designed for the identification of genes implicated in the regulation of cardiac rhythm (Koopman et al. 2021). This mutant line displayed skipped ventricular beats, irregular beats, a slower heart rate and lethality by 15 days post fertilisation (Koopman et al. 2021). This phenotype was mapped to a homozygous missense mutation leading to a premature stopcodon in the *tmem161b* gene (p.Cys466*) (Koopman et al. 2021). The mutation was further confirmed by replicating the phenotype in zebrafish embryos with compound heterozygosity for the p.Cys466* variant and a CRISPR generated knockout allele (Koopman et al. 2021).

Fluorescent imaging and patch-clamp experiments enabled a more thorough examination of the pathophysiology of the cardiac arrhythmia observed in *grime* mutants, as well as the mechanism of cardiac rhythm regulation by *tmem161b*. GEVI (tg(myl7:chimeric VSFP-butterfly CY zebrafish line) and GECI (tg(myl7:gal4FF; UAS:GCaMP6f zebrafish line) experiments in homozygous embryos revealed sino-atrial and atrio-ventricular abnormalities with a faster and increased calcium release (Koopman et al. 2021). Current-clamp experiments on cardiomyocytes derived from adult heterozygotes (homozygotes were not viable up to adulthood) showed an abnormal AP duration (shorter APD20 but longer APD50 and APD90) and early afterdepolarisations (Koopman et al. 2021). This effect was unravelled by voltage-clamp experiments which revealed increased $I_{Ks}$ and $I_{CaL}$ currents (Koopman et al. 2021).

## 4 Conclusion

Zebrafish cardiac disease modelling presents several challenges, both in the fields of genetic modelling (non-orthology, duplicated genes) and cardiac anatomy and electrophysiology (single atrium and ventricle, altered $I_{Ks}$ kinetics, absence of $I_{to}$). Nonetheless, zebrafish models have clearly proven successful in reproducing several human inherited cardiac arrhythmia phenotypes. There is a distinct phenotype in zebrafish models for LQTS, SQTS, AIMS and Cantú syndrome. The zebrafish models for CPVT and ARVC/AC(M) are also promising. However, caution is warranted in the interpretation of findings derived from transient morpholino models, as long as these results have not been confirmed with stable genetic mutants.

The lack of a clear phenotype in some inherited arrhythmia models can be attributed to the use of less in-depth phenotyping tools, e.g. light microscopy video recordings as an assessment of electrophysiological characteristics. However, more recent phenotyping tools, such as ECG and optical mapping with genetically encoded indicators, are very promising as they can be applied relatively easily in vivo and provide biologically relevant information on the electrical properties of the entire heart. We anticipate that future models will closely resemble human disease.

The use of CRISPR-Cas9 to model precise genetic modifications identified in patients will pave the way for research into specific variant based pathological mechanisms and personalised treatments. The use of these techniques will enable the field with detailed characterisation of the electrophysiological alterations in CPVT and ARVC/AC(M) disease models, and most likely demonstrate the zebrafish phenotype of yet unexplored inherited cardiac arrhythmias.

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